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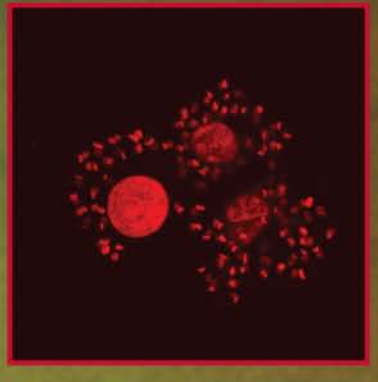

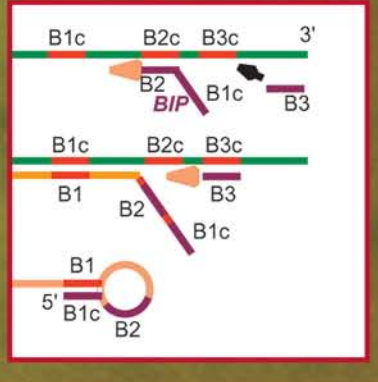

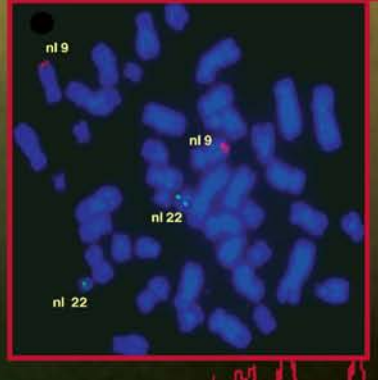

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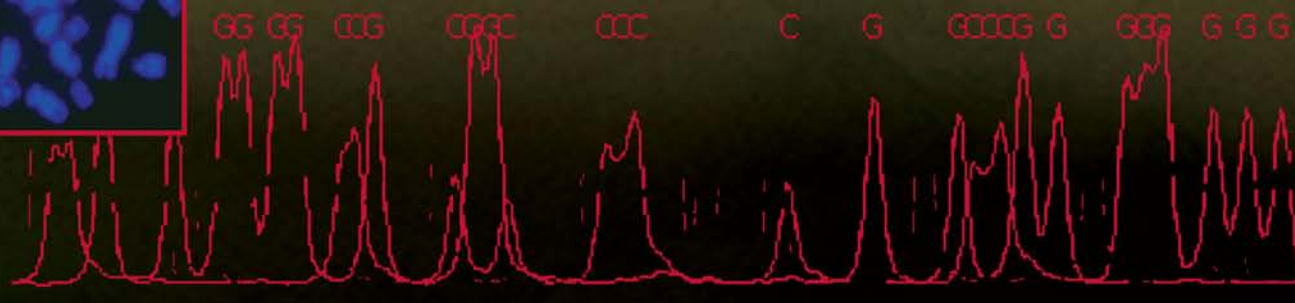

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edited by

Jürgen Fuchs
Department of Dermatology
Johann Wolfgang Goethe-Universität
Frankfurt, Germany

Maurizio Podda
Department of Dermatology
Johann Wolfgang Goethe-Universität
Frankfurt, Germany

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Florian and Paul

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Bimal Theophilus
Birmingham Children's Hospital NHS Trust, Birmingham, U.K.

Paul J. Van Diest
VU University Medical Center, Amsterdam, The Netherlands

Ishwar Chander Verma
Sir Ganga Ram Hospital, New Delhi, India

Akiyoshi Wada
RIKEN Genomic Sciences Center, Yokohama, Kanagawa, Japan

Victor W. Weedn
Carnegie Mellon University, Pittsburgh, Pennsylvania, U.S.A.

David S. Wilkinson
Virginia Commonwealth University, Richmond, Virginia, U.S.A.

Contributors

Ronald T. Acton / *University of Alabama at Birmingham, Birmingham, Alabama, U.S.A.*
Adewale Adeyinka / *Mayo Clinic and Mayo Foundation, Rochester, Minnesota, U.S.A.*
Farid E. Ahmed / *East Carolina University, Greenville, North Carolina, U.S.A.*
Joseph E. Alouf / *Institut Pasteur, Paris, France*
Özgül Alper / *Georgetown University Medical Center, Washington, D.C., U.S.A.*
Rhona M. Anderson / *MRC Radiation and Genome Stability Unit, Didcot, Harwell, Oxfordshire, U.K.*
M. Arock / *Faculty of Pharmacy, Paris, France*
Brian Austin / *Heriot-Watt University, Edinburgh, U.K.*
Ana Avellón-Calvo / *National Center for Microbiology, Madrid, Spain*
Adam Bagg / *Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.*
Deeksha Sarihyan Bali / *Duke University Medical Center, Durham, North Carolina, U.S.A.*
Carol Isaacson Barash / *Genetics, Ethics & Policy Consulting, Inc., Boston, Massachusetts, U.S.A.*
David E. Barton / *Our Lady's Hospital for the Sick, Dublin, Ireland*
James C. Barton / *Southern Iron Disorders Center, Birmingham, Alabama, U.S.A.*
Philip L. Beales / *Institute of Child Health (UCL), London, U.K.*
Cécile Bébéar / *Université Victor Segalen Bordeaux 2, Bordeaux, France*
Karsten Becker / *Westfälische Wilhelms-Universität Münster, Münster, Germany*
Clare E. Beesley / *University College London, London, U.K.*
Alexandra Benachi / *Hôpital Necker Enfants Malades, Paris, France*
Mark Benecke / *International Forensic Research & Consulting, Cologne, Germany*
Martin Bengtsson / *TATAA Biocenter AB, Göteborg, Sweden*
Diana E. Benn / *Kolling Institute of Medical Research, University of Sydney, Sydney, Australia*
Jörg Berg / *General Hospital Linz, Linz, Austria*
Annemarie Berger / *J.W. Goethe University, Frankfurt, Germany*
Michel G. Bergeron / *Universite Laval, Sainte-Foy, Quebec, Canada*
Claudia Biondi / *Universidad Nacional de Rosario, Rosario, Argentina*
Catherine Boileau / *Université Paris V, Hôpital Necker-Enfants Malades, Paris, France*
Maurice Boissinot / *Universite Laval, Sainte-Foy, Quebec, Canada*
Jörg Bojunga / *Saarland University Hospital, Homburg/Saar, Germany*
Serena Bonin / *International Centre for Genetic Engineering and Biotechnology (ICGEB) and University of Trieste, Trieste, Italy*
Gisèle Bonne / *INSERM U582, Paris, France*
Stéphane Bourgeois / *Université de Montréal, Montreal, Quebec, Canada*
Stephanie Bousart / *Evotec Technologies GmbH, Düsseldorf, Germany*
Antoinette A. T. P. Brink / *VU University Medical Center, Amsterdam, The Netherlands*
Michael S. Bronze / *University of Oklahoma, Oklahoma City, Oklahoma, U.S.A.*

Annette K. Broom / *The Western Australian Centre for Pathology and Medical Research and the University of Western Austrailia, Nedlands, Western Australia, Australia*

Natalia E. Broude / *Boston University, Boston, Massachusetts, U.S.A.*

W. Ted Brown / *New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.*

Ramon Brugada / *Baylor College of Medicine, Houston, Texas, U.S.A.*

Sylvia M. Bruisten / *GG&GD Municipal Health Service, Amsterdam, The Netherlands*

The-Hung Bui / *Karolinska Institutet/Karolinska Hospital, Stockholm, Sweden*

Stephen A. Bustin / *University of London, London, U.K.*

Neville J. Butcher / *University of Queensland, St. Lucia, Australia*

Catherine Caillaud / *AP-HP, Institut Cochin, Paris, France*

María J. Calasanz / *University of Navarra, Pamplona, Spain*

Martin J. Callaghan / *University of Oxford, Oxford, U.K.*

Paula M. Cannon / *Childrens Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, California, U.S.A.*

Antonio Cao / *Università degli Studi di Cagliari (UNICA), Cagliari, Sardinia, Italy*

Melody Caramins / *Royal Prince Alfred Hospital, Camperdown, Australia*

Lucie Carrier / *INSERM U582, Paris, France*

Cyril Carroll / *National University of Ireland, Galway, Ireland*

Barrie J. Carter / *Targeted Genetics Corporation, Seattle, Washington, U.S.A.*

Beatriz Carvalho / *VU University Medical Center, Amsterdam, The Netherlands*

Lilian Castilho / *Hemocentro Unicamp, Campinas, Brazil*

Kosum Chansiri / *Srinakharinwirot University, Bangkok, Thailand*

Karim Chemlal / *Institute of Tropical Medicine, Antwerp, Belgium*

Tian-Jian Chen / *University of South Alabama, Mobile, Alabama, U.S.A.*

Xiao-he Chen / *Southampton University Hospital, Southampton, U.K.*

Yuan-Tsong Chen / *Duke University Medical Center, Durham, North Carolina, U.S.A.*

Holger Christiansen / *University of Erlangen, Marburg, Germany*

Anna Cirulli / *Università di Bari, Bari, Italy*

John R. Clarke / *Imperial College of Science, Technology and Medicine, Paddington, London, U.K.*

Stuart C. Clarke / *Stobhill Hospital, Glasgow, Scotland*

Gwenaëlle Collod-Béroud / *Université Paris V, Hôpital Necker-Enfants Malades, Paris, France*

David N. Cooper / *University of Wales College of Medicine, Cardiff, U.K.*

Jean-Marc Costa / *American Hospital of Paris, Neuilly, France*

Lucio G. Costa / *University of Washington, Seattle, Washington, U.S.A.*

Carlos M. Cotorruelo / *Universidad Nacional de Rosario, Rosario, Argentina*

Paul Coucke / *Center for Medical Genetics, University Hospital, Gent, Belgium*

Yvonne E. Cruickshank / *University of Strathclyde, Glasgow, U.K.*

N. A. Cunliffe / *The University of Liverpool, Liverpool, U.K.*

Mary Jane Cunningham / *Houston Advanced Research Center (HARC), The Woodlands, Texas, U.S.A.*

Anna Maria D'Erchia / *Dipartimento di Biochimica e Biologia Moleculare, Università di Bari, Bari, Italy*

Savvas Damaskinos / *Biomedical Photometrics Inc., Waterloo, Ontario, Canada*

Dat D. Dao / *Houston Advanced Research Center (HARC), The Woodlands, Texas, U.S.A.*

Florian Daxboeck / *University of Vienna, Vienna, Austria*

Ian N. M. Day / *Southampton University Hospital, Southampton, U.K.*

Stephen Day / *Third Wave Technologies, Madison, Wisconsin, U.S.A.*

Dino A. De Angelis / *Memorial Sloan-Kettering Cancer Center, New York, New York, U.S.A.*
Phyllis Della-Latta / *Columbia University Medical Center, New York, New York, U.S.A.*
Vito G. DelVecchio / *Vital Probes, Inc., Mayfield, Pennsylvania, U.S.A.*
Vadim V. Demidov / *Boston University, Boston, Massachusetts, U.S.A.*
Anne De Paepe / *Center for Medical Genetics, University Hospital, Gent, Belgium*
Femke A. de Snoo / *Leiden University Medical Center, Leiden, The Netherlands*
Wolfgang Dietmaier / *University of Regensburg, Regensburg, Germany*
Arthur E. Dixon / *Biomedical Photometrics Inc., Waterloo, Ontario, Canada*
David H. Dockrell / *University of Sheffield Medical School, Sheffield, U.K.*
Gerry Dolan / *Nottingham Haemophilia Comprehensive Care Centre, Nottingham, U.K.*
Luciana Cresta de Barros Dolinsky / *Universidade Federal do Rio de Janeiro (UFRJ), and Universidade do Grande Rio (UNIGRANRIO), Rio de Janeiro, Brazil*
Friedrich Dorner / *Baxter BioScience, Vienna, Austria*
Jörg Dötsch / *University of Erlangen, Nürnberg, Germany*
Christian Drosten / *Bernhard-Nocht-Institute of Tropical Medicine, Hamburg, Germany*
Paul Eijk / *Vrije Universiteit Medical Centre, Amsterdam, The Netherlands*
Hermann Einsele / *Eberhard-Karls-Universität Tuebingen, Tuebingen, Germany*
Nabil El AiLa / *Institute of Tropical Medicine, Antwerp, Belgium*
Sameer Elsayed / *University of Calgary, Calgary, Alberta, Canada*
Deborah Elstein / *Shaare Zedek Medical Centre, Jerusalem, Israel*
Dwaine F. Emerich / *LCT BioPharma, Inc., Cranston, Rhode Island, U.S.A.*
M. Said Enayat / *The Birmingham Children's Hospital NHS Trust, Birmingham, U.K.*
Virginia Espina / *National Cancer Institute, Bethesda, Maryland, U.S.A.*
Andreas Essig / *University Hospital of Ulm, Ulm, Germany*
Maurice M. Exner / *Quest Diagnostics Nichols Institute, San Juan Capistrano, California, U.S.A.*
Yao-Shan Fan / *University of Miami School of Medicine, Miami, Florida, U.S.A.*
Gabriella A. Farcas / *University of Toronto, Toronto, Ontario, Canada*
Falko Fend / *Technical University Munich, Munich, Germany*
Peter C. H. Feng / *United States Food and Drug Administration, College Park, Maryland, U.S.A.*
F. Fenollar / *Unité des Rickettsies, Marseille, France*
Josef Finsterer / *Krankenanstalt Rudolfstiftung, Wien, Austria*
Derek J. Fisher / *University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A.*
M. Födinger / *Medical University of Vienna, Vienna, Austria*
Thorsten Forster / *University of Edinburgh Medical School, Edinburgh, U.K.*
Michel Franck / *Ecole Nationale Vétérinaire de Lyon, Marcy l'Etoile, France*
David John French / *LGC (Teddington) Ltd., Teddington, U.K.*
Monika Fritzer-Szekeres / *University of Vienna Medical School, Vienna, Austria*
Emilie Frobert / *Claude Bernard University-Hospices Civils de Lyon, Lyon, France*
Anthony A. Fryer / *Keele University Medical School, Staffordshire, U.K.*
Jürgen Fuchs / *J.W. Goethe University, Frankfurt, Germany*
Clement E. Furlong / *University of Washington, Seattle, Washington, U.S.A.*
Renzo Galanello / *Università degli Studi di Cagliari (UNICA), Cagliari, Sardinia, Italy*
Anna Gajko-Galicka / *Medical University of Bialystok, Bialystok, Poland*
Rosalind H. Ganderton / *Southampton University Hospital, Southampton, U.K.*
Alex Garvin / *AmberGen Inc., Boston, Massachusetts, U.S.A.*
Tom R. Gaunt / *Southampton University Hospital, Southampton, U.K.*

Peter Ghazal / *University of Edinburgh Medical School, Edinburgh, U.K.*
Thomas P. Gillis / *National Hansen's Disease Programs, Baton Rouge, Louisiana, U.S.A.*
Robert H. Gilman / *Johns Hopkins School of Public Health, Baltimore, Maryland, U.S.A.*
Philippe Gilot / *Institut National de la Recherche Agronomique (INRA), Nouzilly, France*
Lisa Giovannelli / *University of Florence, Florence, Italy*
Sadanand Gite / *AmberGen Inc., Boston, Massachusetts, U.S.A.*
Bert Gold / *National Cancer Institute, Frederick, Maryland, U.S.A.*
Willemijn M. Gommans / *Groningen University, Groningen, The Netherlands*
Patrick Gonin / *Généthon, Evry, France*
William M. Grady / *University of Washington Medical Center, Seattle, Washington, U.S.A.*
Ronald A. Greenfield / *University of Oklahoma, Oklahoma City, Oklahoma, U.S.A.*
Wayne W. Grody / *UCLA School of Medicine, Los Angeles, California, U.S.A.*
Michael A. Grotzer / *University Children's Hospital, Zürich, Switzerland*
Ginevra Guanti / *Università di Bari, Bari, Italy*
Dong-chuan Guo / *University of Texas-Houston Medical School, Houston, Texas, U.S.A.*
Rebecca A. Guy / *University of Toronto, Mississauga, Ontario, Canada*
Toshikazu Hada / *Hyogo College of Medicine, Nishinomiya, Hyogo, Japan*
Michelle Hadchouel / *Inserm U347, Le Kremlin Bicêtre, France*
Sinuhe Hahn / *University Women's Hospital, Basel, Switzerland*
Hidde J. Haisma / *Groningen University, Groningen, The Netherlands*
Juliane Halangk / *Charité, Berlin, Germany*
Val Hall / *University Hospital of Wales, Cardiff, U.K.*
Thomas Hankeln / *Johannes Gutenberg Universität Mainz, Mainz, Germany*
Nader Hanna / *University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.*
Timm C. Harder / *Landeslabor Schleswig-Holstein, Neumuenster, Germany*
C. Anthony Hart / *The University of Liverpool, Liverpool, Merseyside, U.K.*
Rachel L. Hart / *The University of Liverpool, Liverpool, U.K.*
Arndt Hartmann / *University of Basel, Basel, Switzerland*
Alexander C. Haushofer / *General Hospital of St. Pöelten, St. Pöelten, Lower Austria, Austria*
Nicholas K. Hayward / *Queensland Institute of Medical Research, Herston, Australia*
Qiushui He / *National Public Health Institute, Turku, Finland*
Holger Hebart / *Eberhard-Karls-Universität Tuebingen, Tuebingen, Germany*
Jannik Helweg-Larsen / *Hvidovre University Hospital, Hvidovre, Denmark*
Mario A. J. A. Hermsen / *University of Oviedo, Oviedo, Spain*
K. E. Herold / *University of Maryland, College Park, Maryland, U.S.A.*
Sam Hibbitts / *University of Wales College of Medicine, Cardiff, U.K.*
James A. Higgins / *United States Department of Agriculture–Agricultural Research Service, Beltsville, Maryland, U.S.A.*
A. S. High / *University of Leeds, Leeds, U.K.*
Marian Hill / *Nottingham Haemophilia Comprehensive Care Centre, Nottingham, U.K.*
Kenji Hirose / *National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan*
Martin Hofmann / *Institute of Virology and Immunoprophylaxis, Mittelhaeusern, Switzerland*
Wolf-Karsten Hofmann / *J. W. Goethe University, Frankfurt, Germany*
Elaine Holmes / *Imperial College London, London, U.K.*
Randall K. Holmes / *University of Colorado Health Sciences Center, Denver, Colorado, U.S.A.*
Wolfgang Holzgreve / *University Women's Hospital, Basel, Switzerland*

Christiane Honisch / *SEQUENOM Inc., San Diego, California, U.S.A.*

Erik Hopmans / *Vrije Universiteit Medical Centre, Amsterdam, The Netherlands*

Pierre Hopmeier / *Hospital Rudolfstiftung, Vienna, Austria*

Paul A. Horgen / *University of Toronto, Mississauga, Ontario, Canada*

Agnes Hotz-Wagenblatt / *Deutsches Krebsforschungszentrum, Heidelberg, Germany*

Bae-Li Hsi / *National Health Research Institutes, Taiwan, Republic of China*

M. Sofi Ibrahim / *The United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, U.S.A.*

Margareta Ieven / *University Hospital of Antwerp, Edegem, Belgium*

Magnus Ingelman-Sundberg / *Karolinska Institutet, Stockholm, Sweden*

Tim J. Inglis / *PathCentre, Perth, Western Australia, Australia*

Ken-ichiro Itoh / *National Institute of Infectious Diseases, Musashi-Murayama City, Tokyo, Japan*

Erik Iwarsson / *Karolinska Institutet/Karolinska Hospital, Stockholm, Sweden*

Louise Izatt / *Guy's Hospital, London, U.K.*

Alexandre Izmailov / *Visible Genetics, Inc., Toronto, Ontario, Canada*

K. K. Jain / *Jain PharmaBiotech, Basel, Switzerland*

John P. Jakupciak / *National Institute of Standards and Technology, Gaithersburg, Maryland, U.S.A.*

Syed M. Jalal / *Mayo Clinic and Mayo Foundation, Rochester, Minnesota, U.S.A.*

Edmund C. Jenkins / *New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.*

Enedina Jiménez-Cardoso / *Hospital Infantil de México Federico Gómez, D.F., Mexico*

Hans Joenje / *VU University Medical Center, Amsterdam, The Netherlands*

Kieran Jordan / *Teagasc, Moorepark, Fermoy, Co. Cork, Ireland*

Sangeeta Joshi / *Sir Ganga Ram Hospital, New Delhi, India*

Christian Jurinke / *SEQUENOM Inc., San Diego, California, U.S.A.*

Veli-Matti Kähäri / *University of Turku, Turku, Finland*

Vladislav Kahle / *Academy of Sciences of the Czech Republic, Brno, Czech Republic*

Kevin C. Kain / *University of Toronto, Toronto, Ontario, Canada*

Noor Kalsheker / *Queen's Medical Center, Nottingham, U.K.*

Shimeru Kamihira / *Nagasaki University, Nagasaki City, Japan*

Pornpimon Kanjanavas / *Srinakharinwirot University, Bangkok, Thailand*

Jonas Karlsson / *TATAA Biocenter AB, Göteborg, Sweden*

Soemini Kasanmoentalib / *Vrije Universiteit Medical Centre, Amsterdam, The Netherlands*

Andrew M. Kaz / *University of Washington Medical Center, Seattle, Washington, U.S.A.*

Dagmar I. Keller / *INSERM U582, Paris, France*

Dominic F. Kelly / *University of Oxford, Oxford, U.K.*

John M. Kelly / *London School of Hygiene and Tropical Medicine, London, U.K.*

Robert T. Kennedy / *University of Michigan, Ann Arbor, Michigan, U.S.A.*

Kagan Kerman / *Ege University, Bornova, Izmir, Turkey; Japan Advanced Institute of Science and Technology, Ishikawa, Japan*

Harald H. Kessler / *Medical University Graz, Graz, Austria*

Phaisan Khawsak / *Srinakharinwirot University, Bangkok, Thailand*

Sintawee Khuchareontaworn / *Srinakharinwirot University, Bangkok, Thailand*

C. William Kilpatrick / *University of Vermont, Burlington, Vermont, U.S.A.*

Christoph Klein / *Institute for Reference Materials and Measurements (IRMM), Geel, Belgium*

Samantha J. L. Knight / *University of Oxford, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Headington, U.K.*

Bruce R. Korf / *University of Alabama at Birmingham, Birmingham, Alabama, U.S.A.*
Hannu Korkeala / *University of Helsinki, Helsinki, Finland*
Maja Krajinovic / *Université de Montréal, Montreal, Quebec, Canada*
Thomas R. Kreil / *Baxter BioScience, Vienna, Austria*
Evelyne Kretzschmar / *Institute for Transfusion Medicine, Duisburg, Germany*
Guido Krupp / *artus GmbH, Hamburg, Germany*
Mikael Kubista / *TATAA Biocenter AB, Lundberg Laboratory, Göteborg, Sweden*
Alma Kuechler / *Klinik für Radiologie, Abteilung Strahlentherapie der FSU Jena, Jena, Germany*
Anver Kuliev / *Reproductive Genetics Institute, Chicago, Illinois, U.S.A.*
David J. Kwiatkowski / *Brigham and Women's Hospital, Boston, Massachusetts, U.S.A.*
Hae-Young Kwon / *Georgetown University Medical Center, Washington, D.C., U.S.A.*
Damian Labuda / *Université de Montréal, Montreal, Quebec, Canada*
Idoya Lahortiga / *University of Navarra, Pamplona, Spain*
Hans Henrik Larsen / *Hvidovre University Hospital, Hvidovre, Denmark*
Dong Soon Lee / *Seoul National University College of Medicine, Seoul, South Korea*
Mikael Leijon / *LightUp Technologies AB, Huddinge, Sweden*
Brynn Levy / *Mount Sinai School of Medicine, New York, New York, U.S.A.*
Laurie R. Lewis / *Houston Advanced Research Center (HARC), The Woodlands, Texas, U.S.A.*
Thomas Liehr / *Klinik für Radiologie, Abteilung Strahlentherapie der FSU Jena; Institut für Humangenetik und Anthropologie der FSU Jena, Jena, Germany*
Adrian Linacre / *University of Strathclyde, Glasgow, U.K.*
Luther E. Lindler / *Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland, U.S.A.*
John C. Lindon / *Imperial College London, London, U.K.*
Miia Lindström / *University of Helsinki, Helsinki, Finland*
Lance A. Liotta / *National Cancer Institute, Bethesda, Maryland, U.S.A.*
Chunyu Liu / *University of Chicago, Chicago, Illinois, U.S.A.*
Hsi Liu / *Centers for Disease Control and Prevention, Atlanta, Georgia, U.S.A.*
Weidong Liu / *Hyogo College of Medicine, Nishinomiya, Hyogo, Japan*
Juergen Loeffler / *Eberhard-Karls-Universität Tuebingen, Tuebingen, Germany*
Katherine Loens / *University of Antwerp, Wilrijk, Belgium*
Dietmar Lohmann / *Universtiätsklinikum Essen, Essen, Germany*
Bettina Lundgren / *Hvidovre University Hospital, Hvidovre, Denmark*
Ian M. MacDonald / *University of Alberta, Alberta, Canada*
John S. Mackenzie / *Curtin University of Technology, Shenton Park, Western Australia, Australia*
James N. Macpherson / *Salisbury District Hospital, Salisbury, Wiltshire, U.K.*
R. Mahnel / *Stiftung Deutsche Klinik für Diagnostik GmbH, Wiesbaden, Germany*
Johanna P. Mäkinen / *National Public Health Institute, Turku, Finland*
Fransiska Malfait / *Center for Medical Genetics, University Hospital, Gent, Belgium*
Helena Malmgren / *Karolinska Institutet/Karolinska Hospital, Stockholm, Sweden*
Hon Fong L. Mark / *Presbyterian Laboratory Services, Charlotte, North Carolina, U.S.A.*
Yvonne Mark / *F.I.T. Health Services, New York, New York, U.S.A.*
Deborah J. Marsh / *Kolling Institute of Medical Research, University of Sydney, Sydney, Australia*
T. Marth / *Stiftung Deutsche Klinik für Diagnostik GmbH, Wiesbaden, Germany*
Renée H. Martin / *University of Calgary, Calgary, Alberta, Canada*
Andrea Mast / *Third Wave Technologies, Madison, Wisconsin, U.S.A.*
Masafumi Matsuo / *Kobe University Graduate School of Medicine, Chuo, Kobe, Japan*

Gert Mayer / *Medical University Innsbruck, Innsbruck, Austria*
M. Mayerhofer / *Medical University of Vienna, Vienna, Austria*
Holgar Mayta / *Johns Hopkins School of Public Health, Baltimore, Maryland, U.S.A.*
Bruce A. McClane / *University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A.*
Heather E. McDermid / *University of Alberta, Edmonton, Alberta, Canada*
Shaun McKenzie / *University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.*
J. McLauchlin / *Health Protection Agency Food Safety Microbiology Laboratory, London, U.K.*
Donald P. McManus / *The Queensland Institute of Medical Research, Queensland, Australia*
Francis Mégraud / *Université Victor Segalen Bordeaux 2, Bordeaux, France*
Jens Mehlhose / *Robert Koch Institut, Berlin, Germany*
Chris J. L. M. Meijer / *VU University Medical Center, Amsterdam, The Netherlands*
Gerrit A. Meijer / *VU University Medical Center, Amsterdam, The Netherlands*
James D. Mellott / *The United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, U.S.A.*
Armelle Ménard / *Université Victor Segalen Bordeaux 2, Bordeaux, France*
Jussi Mertsola / *National Public Health Institute, Turku, Finland*
Jon F. Merz / *University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, U.S.A.*
Barbara Messner / *Klinikum vechts der Isar, Munich University of Technology, Munich, Germany*
Michèle Meunier-Rotival / *Inserm U347, Le Kremlin Bicêtre, France*
Hans Wilhelm Meyers / *Patent Attorneys von Kreisler Selting Werner, Köln, Germany*
Dianna M. Milewicz / *University of Texas-Houston Medical School, Houston, Texas, U.S.A.*
Rodney F. Minchin / *University of Queensland, St. Lucia, Australia*
Mara Mirasoli / *University of Bologna, Bologna, Italy*
Martina Mirlacher / *University of Basel, Basel, Switzerland*
Steven R. Monday / *United States Food and Drug Administration, College Park, Maryland, U.S.A.*
Florence Morfin / *Claude Bernard University-Hospices Civils de Lyon, Lyon, France*
Kevin Morgan / *Queen's Medical Center, Nottingham, U.K.*
Cesar V. Mujer / *Vital Probes, Inc., Mayfield, Pennsylvania, U.S.A.*
Heide Müller-Alouf / *Institut Pasteur, Paris, France*
David R. Murdoch / *Christchurch School of Medicine & Health Sciences, Christchurch, New Zealand*
Caroline Murphy / *Teagasc, Moorepark, Fermoy, Co. Cork, Ireland*
Maria A. Musarella / *SUNY Downstate Medical Center, Brooklyn, New York, U.S.A.*
Monica Musiani / *University of Bologna, Bologna, Italy*
Kentaro Nagamine / *Eiken Chemical Co., Ltd., Tochigi Pref., Japan*
Carlo Napolitano / *IRCCS, Fondazione S. Maugeri, Pavia, Italy*
Mari Nevas / *University of Helsinki, Helsinki, Finland*
Philip Ng / *Baylor College of Medicine, Houston, Texas, U.S.A.*
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Hans Helmut Niller / *Universität Regensburg, Regensburg, Germany*
Kerstin Nolkrantz / *University of Michigan, Ann Arbor, Michigan, U.S.A.*
Tsugunori Notomi / *Eiken Chemical Co., Ltd., Tochigi Pref., Japan*
Anders O. H. Nygren / *MRC-Holland Ltd., Amsterdam, The Netherlands*
Catherine D. O'Connell / *National Institute of Standards and Technology, Gaithersburg, Maryland, U.S.A.*
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Patrizia Pasini / *University of Bologna, Bologna, Italy*

M. Pazzagli / *University of Florence, Florence, Italy*

Franck Pellestor / *Institute of Human Genetics, Montpellier, France*

Sabine Pereyre / *Université Victor Segalen Bordeaux 2, Bordeaux, France*

Francesca Petrera / *International Centre for Genetic Engineering and Biotechnology (ICGEB), and University of Trieste, Trieste, Italy*

Sirichai Phantana / *Ministry of Public Health, Nontaburi, Thailand*

Heather Pierce / *University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.*

P. Pinzani / *University of Florence, Florence, Italy*

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Mario Plebani / *University Hospital of Padova, Padova, Italy*

Maurizio Podda / *J.W. Goethe University, Frankfurt, Germany*

Andrew J. Pollard / *University of Oxford, Oxford, U.K.*

Sven Poppert / *University Hospital of Ulm, Ulm, Germany*

Françoise Portaels / *Institute of Tropical Medicine, Antwerp, Belgium*

Janos Posfai / *New England Biolabs, Inc., Beverly, Massachusetts, U.S.A.*

Edoardo Pozio / *Istituto Superiore di Sanità, Rome, Italy*

Silvia G. Priori / *IRCCS, Foundazione S. Maugeri and University of Pavia, Pavia, Italy*

Mario C. O. Probst / *University of Regensburg, Regensburg, Germany*

Robert E. Pyatt / *Emory University, Atlanta, Georgia, U.S.A.*

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Wolfgang Rascher / *University of Erlangen, Nürnberg, Germany*

A. Rasooly / *United States Food and Drug Administration–Center for Devices and Radiological Health, Silver Spring, Maryland, U.S.A.*

Peter Rautenberg / *University Clinics Schleswig-Holstein, Campus Kiel, Kiel, Germany*

Udo Reischl / *University of Regensburg, Regensburg, Germany*

Carolyn Sue Richards / *Baylor College of Medicine, Houston, Texas, U.S.A.*

Leen Rigouts / *Institute of Tropical Medicine, Antwerp, Belgium*

Franziska Ringpfeil / *Jefferson Medical College, Philadelphia, Pennsylvania, U.S.A.*

G. Ripabelli / *University of Molise, Campobasso, Italy*

Marilyn C. Roberts / *University of Washington, Seattle, Washington, U.S.A.*

R. J. Roberts / *New England Biolabs, Inc., Beverly, Massachusetts, U.S.A.*

Robert Roberts / *Baylor College of Medicine, Houston, Texas, U.S.A.*

Aldo Roda / *University of Bologna, Bologna, Italy*

R. Stefan Ross / *University of Essen, Essen, Germany*

Kenneth Rothschild / *AmberGen Inc., Boston, Massachusetts, U.S.A.*

Marianne G. Rots / *Groningen University, Groningen, The Netherlands*

Lewis P. Rubin / *Women & Infants Hospital and Brown Medical School, Providence, Rhode Island, U.S.A.*

Marie Magdeleine Ruchoux / *Parc EuraSanté, Lille, France*

Michael Rudnicki / *Medical University Innsbruck, Innsbruck, Austria*

Carlo Sabbà / *Università di Bari, Bari, Italy*

Nagahiro Saijo / *National Cancer Center Hospital, Chuo-ku, Tokyo, Japan*

Marta Sánchez-Carbayo / *Memorial Sloan-Kettering Cancer Center, New York, New York, U.S.A.*

Yayoi Sato / *Chiba University, Chiba, Japan*

Guido Sauter / *University of Basel, Basel, Switzerland*

Elisabetta Sbisà / *Istituto di Tecnologie Biomediche Sezione di Bari CNR, Bari, Italy*

Hartmut Schmidt / *Charité, Berlin, Germany*

Kathrin D. Schmidt / *Eberhard-Karls-Universität Tuebingen, Tuebingen, Germany*

Jan P. Schouten / *MRC-Holland Ltd., Amsterdam, The Netherlands*

Hans Peter Schwarz / *Baxter BioScience, Vienna, Austria*

Fritz Schwarzmann / *Universität Regensburg, Regensburg, Germany*

Ikuo Sekine / *National Cancer Center Hospital, Chuo-ku, Tokyo, Japan*

N. Sergeev / *United States Food and Drug Administration–Center for Devices and Radiological Health, Silver Spring, Maryland, U.S.A.*

Isdore Chola Shamputa / *Institute of Tropical Medicine, Antwerp, Belgium*

Jay Shaw / *Nanogen, Inc., San Diego, California, U.S.A.*

Yosuke Shigematsu / *Fukui Medical University School of Nursing, Fukui, Japan*

Paul A. Sieving / *National Institute of Health, Rockville, Maryland, U.S.A.*

C. Sillaber / *Medical University of Vienna, Austria*

Ronald Simon / *University of Basel, Basel, Switzerland*

Durg V. Singh / *Institute of Life Sciences, Bhubaneswar, India*

Predrag Slijepcevic / *Brunel University, Middlesex, U.K.*

James G. Smedley, III / *University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A.*

David W. Smith / *The Western Australian Centre for Pathology and Medical Research and The University of Western Austrailia, Nedlands, Western Australia, Australia*

Heather J. Smith / *University of Manitoba, Winnipeg, Manitoba, Canada*

Peter J. F. Snijders / *VU University Medical Center, Amsterdam, The Netherlands*

Robert A. Sokolic / *National Cancer Institute, Bethesda, Maryland, U.S.A.*

K. Sotlar / *University of Tübingen, Tübingen, Germany*

Olivier A. E. Sparagano / *University of Newcastle, Newcastle, U.K.*

Ulrich Spengler / *artus Biotech USA, Inc., San Francisco, California, U.S.A.*

W. R. Sperr / *Medical University of Vienna, Austria*

Jennifer K. Spinler / *University of Colorado Health Sciences Center, Denver, Colorado, U.S.A.*

Eli Sprecher / *Rambam Medical Center, Haifa, Israel*

Glyn Stacey / *National Institute for Biological Standards and Control, Herts, U.K.*

Giorgio Stanta / *International Centre for Genetic Engineering and Biotechnology (ICGEB) and University of Trieste, Trieste, Italy*

Werner Steimer / *Klinikum vechts der Isar, Munich University of Technology, Munich, Germany*

Michael Steinert / *Universität Würzburg, Würzburg, Germany*

Evelyn Stelzl / *Medical University Graz, Graz, Austria*

Henrik Stender / *AdvanDx, Inc., Woburn, Massachusetts, U.S.A.*

Richard C. Strange / *Keele University Medical School, Staffordshire, U.K.*

Natalia D. Sumption / *MRC Radiation and Genome Stability Unit, Didcot, Harwell, Oxfordshire, U.K.*

Thomas Szekeres / *University of Vienna Medical School, Vienna, Austria*
Duan-Jun Tan / *Georgetown University Medical Center, Washington, D.C., U.S.A.*
Peerapan Tan-ariya / *Mahidol University, Nakorn Pathom, Thailand*
David Tarragó-Asensio / *National Center for Microbiology, Madrid, Spain*
Martin C. Taylor / *London School of Hygiene and Tropical Medicine, London, U.K.*
Mustafa Tekin / *Ankara University School of Medicine, Ankara, Turkey*
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Beatriz Marcheco Teruel / *National Center of Medical Genetics, Havana, Cuba*
Christopher G. Thanos / *Brown University, Providence, Rhode Island, U.S.A.*
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Dominic Thyagarajan / *Flinders University of South Australia, Bedford Park, Australia*
J.-M. Tiercy / *University Hospital, Geneva, Switzerland*
Ronald J. A. Trent / *Royal Prince Alfred Hospital, Camperdown, Australia*
Heiner Trobisch / *Institute for Transfusion Medicine, Duisburg, Germany*
Apollonia Tullo / *Istituto di Tecnologie Biomediche Sezione di Bari CNR, Bari, Italy*
Sumalee Tungpradabkul / *Mahidol University, Bangkok, Thailand*
Richard M. Twyman / *University of York, York, U.K.*
Jouni Uitto / *Jefferson Medical College, Philadelphia, Pennsylvania, U.S.A.*
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Meena Upadhyaya / *University of Wales College of Medicine, Cardiff, U.K.*
P. Valent / *Medical University of Vienna, Vienna, Austria*
Joannis E. Vamvakopoulos / *Biomedicum Helsinki, Helsinki, Finland*
Anne-Mieke Vandamme / *Rega Institute for Medical Research and University Hospitals, Leuven, Belgium*
Dirk van den Boom / *SEQUENOM Inc., San Diego, California, U.S.A.*
Adriaan J. C. van den Brule / *PAMM Institute, Eindhoven, The Netherlands*
Paul van den IJssel / *Vrije Universiteit Medical Centre, Amsterdam, The Netherlands*
Kristel Van Laethem / *Rega Institute for Medical Research and University Hospitals, Leuven, Belgium*
Milen Velinov / *New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.*
Simona Venturoli / *University of Bologna, Bologna, Italy*
Wenonah A. Vercoutere / *NASA Ames Research Center, Moffett Field, California, U.S.A.*
Jo-Anne Vergilio / *Brigham and Women's Hospital, Boston, Massachusetts, U.S.A.*
Yury Verlinsky / *Reproductive Genetics Institute, Chicago, Illinois, U.S.A.*
Fabrizio Vianello / *University of Padova, Padova, Italy*
Pia Vihinen / *Turku University Hospital, Turku, Finland*
Matti K. Viljanen / *National Public Health Institute, Turku, Finland*
Tamas Vincze / *New England Biolabs, Inc., Beverly, Massachusetts, U.S.A.*
Ernst Wagner / *Ludwig-Maximilians-Universität, Munich, Germany*
Gehua Wang / *Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba, Canada*
Haruo Watanabe / *National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan*
Chand Wattal / *Sir Ganga Ram Hospital, New Delhi, India*
Erin L. Weber / *Childrens Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, California, U.S.A.*
Elaine M. Weidenhammer / *Nanogen, Inc., San Diego, California, U.S.A.*
Marjan Marije Weiss / *VU University Medical Center, Amsterdam, The Netherlands*
Nele Wellinghausen / *University of Ulm, Ulm, Germany*

Qiyi Wen / *University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A.*

Gunar Westman / *TATAA Biocenter AB, Göteborg, Sweden*

Philip Lewis White / *University of Wales College of Medicine, Cardiff, U.K.*

Neil V. Whittock / *Peninsula Medical School, Exeter, U.K.*

Shane R. Wilkinson / *London School of Hygiene and Tropical Medicine, London, U.K.*

Diana L. Williams / *National Hansen's Disease Programs, Baton Rouge, Louisiana, U.S.A.*

Heiko Witt / *Charité, Berlin, Germany*

Hans Wolf / *Universität Regensburg, Regensburg, Germany*

Lee-Jun C. Wong / *Georgetown University Medical Center, Washington, D.C., U.S.A.*

Fann Wu / *Columbia University Medical Center, New York, New York, U.S.A.*

Josephine Wu / *Mount Sinai School of Medicine, New York, New York, U.S.A.*

Fei Ye / *Mount Sinai School of Medicine, New York, New York, U.S.A.*

Bauke Ylstra / *VU University Medical Center and Vrije Universiteit Medical Centre, Amsterdam, The Netherlands*

Bing Yu / *Royal Prince Alfred Hospital, Camperdown, Australia*

Marialuisa Zerbini / *University of Bologna, Bologna, Italy*

Stefan Zeuzem / *Saarland University Hospital, Homburg/Saar, Germany*

George G. Zhanel / *University of Manitoba, Winnipeg, Manitoba, Canada*

David Y. Zhang / *Mount Sinai School of Medicine, New York, New York, U.S.A.*

Wandi Zhang / *Mount Sinai School of Medicine, New York, New York, U.S.A.*

Nanbert Zhong / *New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.*

Ari Zimran / *Shaare Zedek Medical Centre, Jerusalem, Israel*

Rudolf Franz Zirwes / *Evotec Technologies GmbH, Düsseldorf, Germany*

Roberto T. Zori / *University of Florida, Gainesville, Florida, U.S.A.*

Neven Zoric / *TATAA Biocenter AB, Lundberg Laboratory and TATAA Biocenter AB, Göteborg, Sweden*

Isabel Zudaire / *University of Navarra, Pamplona, Spain*

Christian Michel Zwaan / *VU University Medical Center, Amsterdam, The Netherlands*

Contents

Preface

The human genome project has attracted a great deal of attention in recent years among the general public as well as the scientific community. Although it is likely to be a number of years before many of the expected benefits of the genomics revolution are realized, the impact of these scientific breakthroughs on medicine is likely to become apparent quickly and will transform the entire scene within a generation.

Recent advances in genomic and proteomic technologies open up new avenues in the diagnoses and management of human diseases, and the application of these techniques may initiate a paradigm shift in clinical medicine. Initially, polymerase chain reaction tests were performed only in highly specialized or research laboratories. Presently, routine molecular assays are widely performed in virtually all sections and specialties of the traditional clinical laboratory, including oncology, hematology, immunology, and blood transfusion. Evolving techniques of nucleic acid and protein microarrays may follow a similar path to the clinician in the hospital. In human genetics, molecular techniques have brought forth new procedures for increasingly specific, sensitive, fast, simple, reliable, automatable, and cost-effective diagnostic and predictive DNA analysis. All of these modern nucleic acid technologies have significantly simplified the routine assessment of patients in the genetics clinic. For instance, advances in molecular biology and in genomics have made possible the early identification of persons at increased risk of, e.g., a thrombophilic state, developing certain forms of cancer, or degenerating neurological disorders.

In clinical microbiology, procedures based on nucleic acid analysis offer a more rapid, simple, cost-effective, and universal approach to the identification of microorganisms. The ability to rapidly and unambiguously characterize microbial pathogens suspected of causing a disease can be critical to individual and public health. These molecular techniques are used increasingly in diagnostic laboratories to supplement or replace traditional identification methods, which are mostly based on microbial phenotypic characteristics. Molecular methods for the detection of anti-microbial resistance strains or virulence factors are in development, in field trial, or already in clinical use, and will supplement or replace traditional susceptibility and toxin testing. The expansion of this technology to smaller institutions, hospitals, and point-of-care testing will improve patient care and public health significantly.

Finally, genomics and proteomics will not only transform the diagnosis of diseases, but may radically influence the treatment and even the prevention of diseases in the future. In pharmacogenetics, the molecular analysis of the inherited nature of individual differences in drug metabolism, disposition, and effects will help to optimize drug therapy on the basis of each patient's genetic constitution. Such personalized medicine will allow more effective treatment of illness by using very specific diagnostics to provide actionable information to clinicians and patients, combined with the use of a new generation of targeted and highly effective medicines. Furthermore, identification of individual genetic susceptibility to infectious diseases may have an impact on prevention strategies. There is doubt that in the future, smart molecular diagnostic tools and the growing knowledge of genetic risk factors will lead to improved diagnoses and treatment for the patient and reduced health care costs to the society.

In view of these exciting developments, the *Encyclopedia of Medical Genomics and Proteomics* brings together the state-of-the-art knowledge and practical expertise of leading researchers and clinicians in the field, and provides a comprehensive overview on current medical applications of modern nucleic acid and protein technology. The *Encyclopedia* is a vehicle by which both scientists and the interested public can explore the most recent developments in today's genomic and proteomic medicine, and preview several of the foreseen applications of tomorrow. The subject areas generally include molecular methods and technologies in the diagnosis and management of infectious, neoplastic, and genetic diseases, including predictive genetic and pharmacogenetic testing, as well as tissue typing for transplantation medicine. Each entry includes descriptions and interpretations of state-of-the-art developments, concepts, and applications that will be useful to individuals encountering the field for the first time, or to experienced researchers updating or expanding their knowledge of medical applications of current genomic and proteomic technology. Key references direct the reader to timely and pertinent information sources.

It is the editors' hopes that the *Encyclopedia of Medical Genomics and Proteomics* will help health care providers, researchers, students, and nonprofessionals all better understand and participate in this remarkable emerging field. Its online edition (*www.dekker.com*) is updated regularly, to keep its information current.

The *Encyclopedia of Medical Genomics and Proteomics* resulted from the vision of Russell Dekker and a dedicated and creative Advisory Board of over 25 members, representing countries on all continents. The diligent efforts of this Board are deeply appreciated. We greatly thank the authors of more than 300 entries which, as recognized experts in their fields, lend their credibility and prestige to the *Encyclopedia*. In addition, we are indebted to the many reviewers whose constructive suggestions and insights materially enhanced the quality of individual entries and of the volume in total. We are especially grateful to the staff at Marcel Dekker, particularly Alison Cohen, for superb leadership in the handling of massive correspondence with the authors, reviewers, and the Editors.

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The Publisher as well as the Editors are committed to continual updating and refinement of what we believe is a valuable resource for transmitting knowledge and understanding about medical genomics and proteomics.

Jürgen Fuchs, Ph.D., M.D.
Maurizio Podda, M.D.

Accreditation of the Medical Laboratory—ISO 15189/ISO 17025

A

Mario Plebani
University-Hospital of Padova, Padova, Italy

INTRODUCTION

The availability of a new International Standard, ISO 15189:2003, specifically designed for medical laboratories, which contains requirements for both quality systems and competence, should stimulate the adoption of this standard and promote harmonization of accreditation programs at an international level.

The increasing demand for accountability, accessibility, professional excellence, customer satisfaction, and better cost management has underpinned the development of systems for quality assurance and improvement in healthcare, with an increasing focus on certification, accreditation, and several other forms of external review mechanisms. Moreover, many countries have introduced or are about to introduce regulations for quality assessment in laboratory services.

ACCREDITATION OF MEDICAL LABORATORIES

Medical laboratories seek recognition as being compliant with particular standards for a number of reasons. In some countries, accreditation is mandatory and in others it may be voluntary, but in any case it recognizes competence, facilitates exchanges of services, provides a valuable management tool, and ensures that the needs and requirements of all users are met. Moreover, it may be a precondition for a contract and an essential tool for reassuring laboratory professionals who aim to practice in accordance with accepted norms.[1] Table 1 shows the main benefits of accreditation for the medical laboratory, for the health-care system, and for patients.

The term ''accreditation,'' as applied to organizations rather than to specialty clinical training, reflects the origins of systematic assessment of hospitals against explicit standards and began in the United States in 1917.[2] According to the ISO/IEC Guide 2 definition, accreditation is ''a procedure by which an authoritative body gives formal recognition that a body or person is competent to carry out specific tasks.''[3] The distinction between certification and accreditation represented, in the past, a source of debate and concern. Certification activity is based upon standards such as ISO 9001:2000 which delineate the ''requirements for quality-management systems'' and are applicable to any activity. Accreditation systems are based on standards that, in addition to ''requirements for quality systems,'' take care of ''technical requirements'' that relate to achieving competence in all aspects of the specific activity, laboratory medicine in our case. Until the recent publication of the ISO 15189:2003 International Standard ''Medical laboratories—Particular requirements for quality and competence,'' there were no sector-specific ISO standards for quality management and technical competence in medical laboratories.[4] This is the reason why medical laboratories, in the past, had a choice between two separate and distinct lines for achieving recognition. The first line focuses on ''requirements for quality-management systems'' that are, by definition, applicable to any organization, and, generally, it is represented by the ISO 9000:2000 series.[5] The second line, with its origin in assessing the technical competence of a laboratory, is represented by ISO 17025:1999, a generic standard used in the accreditation of testing or calibration laboratory[6] (Fig. 1). Alternatively, other programs for laboratory accreditation are delivered by organization such as Clinical Pathology Accreditation (CPA-UK)[7] or College of Clinical Pathology (CAP) in the United States based on professional standards that basically aim to evaluate and improve technical competence. The availability of an International Standard specifically developed for medical laboratories should overpass any possible dispute and it should harmonize the laboratory approach to quality assessment and improvement. In fact, ISO 15189:2003 recognizes the importance of quality-management systems, as described in ISO 9000:2000, as well as technical requirements contained in ISO/IEC 17025:1999, but, in addition, it contains specific requirements for an organization, such as medical laboratories that, in addition to analytical competence, have to assure consultative and interpretative activities. The relationship between ISO 15189:2003, ISO/IEC 17025:1999, and ISO 9001:2000 is similar to that of a set of three ''Russian dolls'' (Fig. 2):

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Table 1 Benefits of accreditation

a)	*Benefits for the laboratory*
	– Improving quality of the work
	– Perfect documentation of the workflow
	– Total quality management
	– Education and increasing professional competence
	– Focus on patients' outcomes
	– Encouragement to develop interdepartmental cooperation
b)	*Benefits for the health-care system*
	– Improved quality of the system
	– Perfect documentation
	– Results comparability
	– Personnel and equipment specifications
	– Improved efficacy of laboratory services
	– Competition based on quality among different laboratories
c)	*Benefits for the patient*
	– Improving quality of the system
	– Results comparability
	– Transparency in information on laboratory quality
	– Improved safety and trust
	– Value for money

the inner doll being ISO 9001, the middle doll being ISO/IEC 17025, and the outer, ''all embracing doll,'' being ISO 15189.[8]

ISO 9001:2000 ''QUALITY MANAGEMENT SYSTEMS—REQUIREMENTS''

ISO 9001:2000 is one of a family of related quality-management system standards (ISO 9000:2000, ISO 9001:2000, ISO 9004:2000)[5,9,10] that specifies requirements for an organization wishing to establish an effective quality-management system that embraces the concept of continuous improvement and to demonstrate its ability to consistently provide a product that meets the users, needs. This International Standard, however, does not provide specifications for the report (product or service) itself, nor the requirements for assessing the technical competence of the medical laboratory to carry out the preexamination, examination, and postexamination processes necessary to produce the report. Therefore the individual laboratory has to select and implement standards and processes that aim to demonstrate competence and satisfy all users, without any guidance, at least without the guidance of standards recognized in the document itself.

ISO 17025:1999 GENERAL REQUIREMENTS FOR THE COMPETENCE OF TESTING AND CALIBRATION LABORATORIES

The introduction to this International Standard contains two statements of importance to those laboratories making the

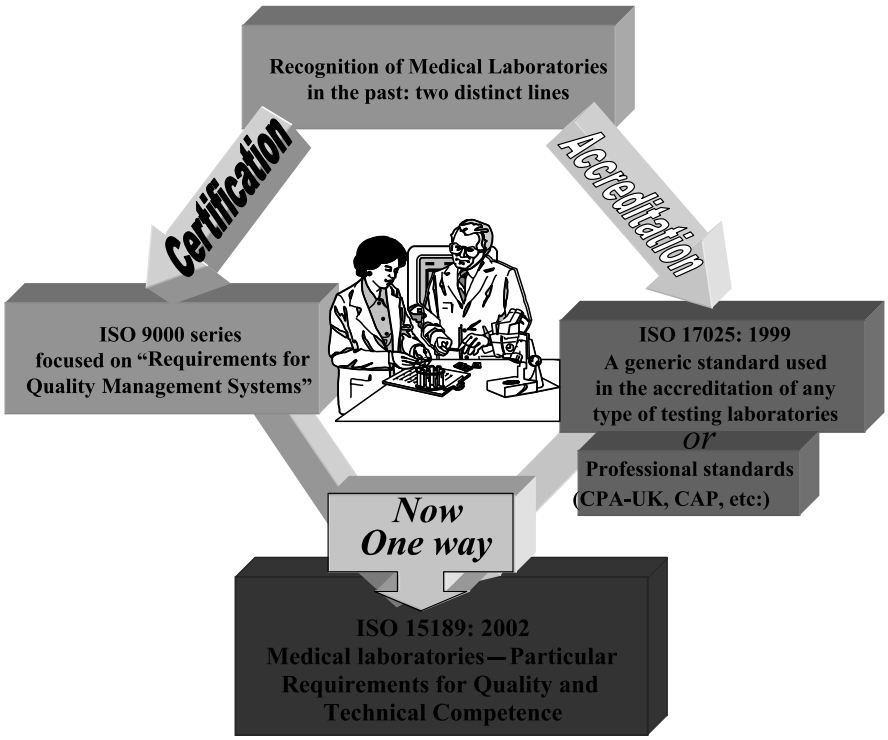

Fig. 1 Accreditation of medical laboratories: old and new standards.

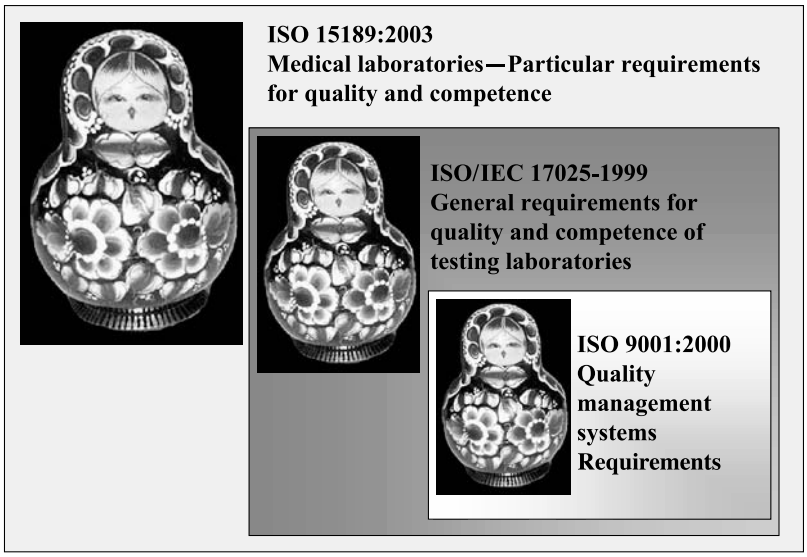

Fig. 2 Relationship between ISO 15189:2003, 180/IEC 17025:1999, and ISO 9001:2000. (*View this art in color at www.dekker.com.*)

choice between pursuing certification and/or accreditation. It states that testing and calibration laboratories that comply with ISO 17025:1999 will also operate in accordance with ISO 9001:2000, but goes on to remind the reader that certification against ISO 9001:2000 does not demonstrate the competence of the laboratory to produce technically valid data and results. The scope of this International Standard includes a number of key issues. In particular, it

1) specifies the general requirements for the competence to carry out tests and/or calibrations, including samples;
2) covers standard, nonstandard, and laboratory-developed methods;
3) is applicable to all testing and calibration laboratories regardless of the number of personnel or extent of the scope of its activities;
4) is for use by laboratories developing their quality, management, and technical systems that govern and improve their operations;
5) is for use by laboratory users, regulatory authorities, and accreditation bodies who wish to recognize the competence of an individual laboratory;
6) does not cover compliance with regulatory and safety requirements for the laboratory activities.

Limitations of ISO 17025:1999 are intrinsically related to the differences between testing/calibration and medical laboratories. Therefore aspects regarding preanalytical phase, which is extremely important for the interpretation and outcome of medical laboratory data, are practically not addressed at all in that document. With regard to the analytical phase, there is no mention in ISO 17025:1999 about requirements for internal quality control and external quality assessment, whereas in the postanalytical phase requirements for turnaround times, as well as for STAT and critical results, are not addressed.

PECULIARITIES OF ISO 15189:2003

ISO 151189:2003 recognizes the major particularities of medical laboratories in comparison to other chemical test laboratories. In contrast to calibration and testing laboratories, the methods applied in laboratory medicine are a compromise between available amount of sample material, turnaround time, test feasibility, and economical reality. Table 2 summarizes the principal differences regarding methods, sample size, validation of results, time span between sampling and the availability of a result (turnaround time), and measurement uncertainty.

Furthermore, there is an increasing relevance of ethical problems related to the use of laboratory samples for examination purposes other than those requested, to the need to obtain informed consent by the individual patient for some procedures, to the need to communicate results to the requesting physician or to other parties with the patient's consent, and the responsibility of the laboratory to ensure that results are correctly interpreted and applied in the patient's best interest.[11,12]

STRUCTURE AND CONTENTS OF ISO 15189:2003

The structure and contents of ISO 15189 follow that of any ISO document. In particular, it contains both normative and informative elements. The normative

Table 2 Main differences between ISO/IEC 17025 and ISO 15189

Issue	Medical laboratories	Test and calibration
1. Patient preparation	Important issue Essential for particular tests	Not relevant
2. Sample volume	Limited, mainly in particular patients (children, neonates, etc.) Methods are adapted to sample size	Sample size adapted to the method
3. Methods	In vitro diagnostics and developed ''in house''	Methods standardized according to national and international standards
4. Results	Results used for patient care and subjected to three (technical, biological, and nosological) validation levels	Pure analytical results and related measurement uncertainty
5. Measurement uncertainty	Related to analytical and biological quality specifications	Related to analytical quality specifications
6. Turnaround time	Extremely important	Not relevant
7. Clinical advice	Appropriateness of test request. Interpretation of results	Not relevant
8. Ethical problems	Increasingly important	Limited to ''state-of-the-art'' measurement

elements describe the scope of the standard and set out its provisions that can be expressed as requirements, recommendations, or statements.[2] Informative elements can be preliminary or supplementary. Preliminary elements are those that identify the document, introduce its content, and explain its background, its development, and its relationship with other documents. Supplementary elements provide information intended to assist with the understanding or use of the document. In the introduction, some essential concepts are explained. Firstly, it declares that laboratory services ''include arrangements for requisition, patient preparation, patient identification, collection of samples, transportation, storage, processing and examination of clinical samples, together with subsequent validation, interpretation, reporting and advice, in addition to the considerations of safety and ethics in medical laboratory work.'' A second important statement is that a second edition of the standard is anticipated, aimed at more closely aligning it with a second edition of ISO/IEC 17025 and with ISO 9001:2000. This emphasizes well the view of accreditation as a dynamic process and the nature of standards as ''a changing world.'' Scope, normative references, and terms and definition are described in

Table 3 Main clauses and primary subclauses of ISO 15189

4. Management requirements	5. Technical requirement
4.1 Organization and management	5.1 Personnel
4.2 Quality management system	5.2 Accommodation and environmental equipment
4.3 Document control	5.3 Laboratory equipment
4.4 Review of contracts	5.4 Preexamination procedures
4.5 Examination of referral laboratories	5.5 Examination procedures
4.6 External services and supplies	5.6 Assuring quality of examination procedures
4.7 Advisory services	5.7 Post-examination
4.8 Resolution of complaints	5.8 Reporting of results
4.9 Identification and control of nonconformities	
4.10 Correction action	
4.11 Preventive action	
4.12 Continual improvement	
4.13 Quality and technical records	
4.14 Internal audits	
4.15 Management review	

clauses 1, 2, and 3, respectively. The most interesting element in these clauses is represented by the definition of medical laboratories that, in turn, represent the structures to which the document is addressed. Medical laboratory is defined as ''laboratory for the biological, microbiological, immunological, chemical, immunohaematological, haematological, biophysical, cytological, pathological or other examination of materials derived from the human body for the purpose of providing information for the diagnosis, prevention and treatment of disease in, or assessment of the health of, human being, and which provide a consultant advisory service covering all aspects of laboratory investigations including the interpretation of results and advice on further appropriate investigation.'' It is clear therefore that ISO 15189 should represent the International Standard for the accreditation of all different types of medical laboratories that operate in the field of laboratory medicine. The main clauses and primary subclauses of ISO 15189 are shown in Table 3.

Very interesting informative elements are finally included in Annex B ''Recommendations for protection of laboratory information systems (LIS)'' and in Annex C ''Ethics in laboratory medicine.''

ACCREDITATION PROGRAMS

As well stated by David Burnett,[2] there are four essential elements in any accreditation system and they are described as:

1. The Standards with which a laboratory has to comply in order to gain accreditation.
2. The accreditation body which oversees the assessment and grants accreditation.
3. The assessors or inspectors who seek to establish compliance with the standards by conducting the assessment.
4. The user laboratory which is required to, or voluntarily seeks to, comply with the standards by being assessed.

An increasing consensus has been reached to recommend the adoption of ISO 15189 as the standard of choice for medical laboratories that seek to receive accreditation. Professional organizations (CPA) have immediately declared the acceptance of the new standards, whereas some accreditation bodies have been reluctant to adopt this standard, but at a recent General Assembly of the International Laboratory Accreditation Cooperation (http://www.ilac.org), endorsed by the European Cooperation for Accreditation (EA), the value of ISO 15189 as an alternative to ISO/IEC 17025 for laboratory accreditation has been established. A recent survey in Europe and elsewhere indicates than an overwhelming majority of accrediting bodies are choosing ISO 15189 as the International Standard against which to accredit medical laboratories.

Regarding the accreditation body, ISO 15189 states that ''...a laboratory seeking accreditation select an accreditation body which operates to appropriate standards and which takes into account the particular requirements of medical laboratories.'' Therefore it can be a specific accreditation body with formal links with existing national accreditation bodies, a subbranch of the national accreditation body, specifically directed to use ISO 15189 for the assessment of medical laboratories, or a professional organization (CPA in the United Kingdom, CCKLTest in the Netherlands, APL in Italy).

One of the major duties of the accreditation body is to take care of the education, training, retraining, and assessment of inspectors. Different types of inspectors exist: full-time ''peers,'' part-time ''peers,'' and external inspectors. The advantages and disadvantages of the different types of inspectors have been described. Taking into consideration the particular nature of ISO 15189, inspectors should assure the evaluation of technical competence in addition to assessment of the quality system.[13] Therefore, they have to demonstrate competence in laboratory medicine and, in particular, in all sectors of activity of the inspected laboratory.

CONCLUSION

The availability of a standard or set of requirements against which practice can be measured is essential in order to create a harmonization of practice in any field of endeavor, but in the case of medical laboratories, the need for harmonization is central to the health and welfare of the individual patient. Therefore the availability of ISO 15189 should promote the harmonization of different accreditation programs for medical laboratories through the adoption of this International Standard and a mutual recognition between the organizers of different accreditation programs.

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ACE Genotyping

Michael Rudnicki
Gert Mayer
Medical University Innsbruck, Innsbruck, Austria

INTRODUCTION

The renin–angiotensin system (RAS) is a cascade of enzymatic reactions that finally leads to the formation of angiotensin II (AngII). The protease renin, which is secreted by myoepithelial cells in renal afferent arterioles, cleaves the decapeptide angiotensin I (AngI) from the liver-derived polypeptide angiotensinogen (AGT). AngI serves as a substrate for the dipeptidase angiotensin-converting enzyme (ACE) which not only generates the octapeptide AngII but also degrades vasodilators such as bradykinin and kallidin. Because AngII is a potent vasoconstrictor and sodium-retaining hormone, the activation of ACE is followed by an increase in blood pressure. Therefore, ACE inhibitors are widely used in the treatment of hypertension. They effectively lower blood pressure, reduce cardiovascular events,[1] and reduce morbidity and mortality in congestive heart failure.[2] Furthermore, ACE inhibitors have proven superior to other antihypertensive drugs in protecting kidney function,[3,4] and they are beneficial for the kidney even when systemic blood pressure is normal.[5]

The human ACE gene is located on chromosome 17q23. Exons 4–11 (coding for the N-terminal domain) and 17–24 (coding for the C-terminal domain) of the ACE gene are highly similar, both in size and in sequence, implying that there has been a gene duplication event during evolution. The two forms of ACE are transcribed from the same gene using different promoters, located on exon 1 and on intron 12.

ACE GENE POLYMORPHISMS

The most widely studied polymorphism of the ACE gene is based on the presence (insertion, I) or absence (deletion, D) of a 287-base-pair element in intron 16.[6] Although the intraindividual plasma level of ACE is rather stable, the prominent interindividual variation has been linked to this I/D polymorphism,[6] which accounts for 47% of the phenotypic variance in healthy individuals. It has been shown that Caucasian subjects with ACE–DD genotype have the highest, subjects with ID genotype intermediate, and subjects with II genotype have the lowest concentrations. These findings have been confirmed in diverse populations including French centenarians,[7] Pima Indians,[8] and whites in the United States.[9] However, they could not be replicated for African-Americans, suggesting an important role for ethnic background.[9]

The ACE gene I/D polymorphism genotyping method originally published by Rigat et al.[6] results in a preferential amplification of the D allele with a possible mistyping of approximately 5% of ID heterozygotes as DD homozygotes.[10] One better technique is to use a second pair of primers, which are located inside the insertion-specific sequence as proposed by Lindpaintner et al.[10] Another improved method has been published by Evans et al.[11] and Ueda et al.[12] They used a third primer located in the insertion element that was added to the reaction cocktail together with the first set of primers. Addition of 5% DMSO and the application of a ''hot-start'' (i.e., initial heating at 95°C and using of wax platelets in the PCR tubes) procedure highly improved the specificity of the original protocol.

PHARMACOGENETIC IMPLICATIONS OF ACE GENOTYPING: STUDIES IN ESSENTIAL HYPERTENSION AND CARDIOVASCULAR DISEASE

In patients with essential hypertension, the ACE gene polymorphism and the response to treatment with ACE inhibitors was subject to research in several clinical trials (Table 1). Hingorani et al.[13] and Dudley et al.[14] could not describe any correlation between blood pressure response to an ACE inhibitor and ACE genotype in individuals with essential hypertension. Nakano et al.[15] reported that blood pressure response to a single dose of 50 mg captropril does not correlate with ACE genotype but rather with plasma renin activity. Stavroulakis et al.[16] showed that after treatment with fosinopril, blood pressure reduction was significantly greater in DD

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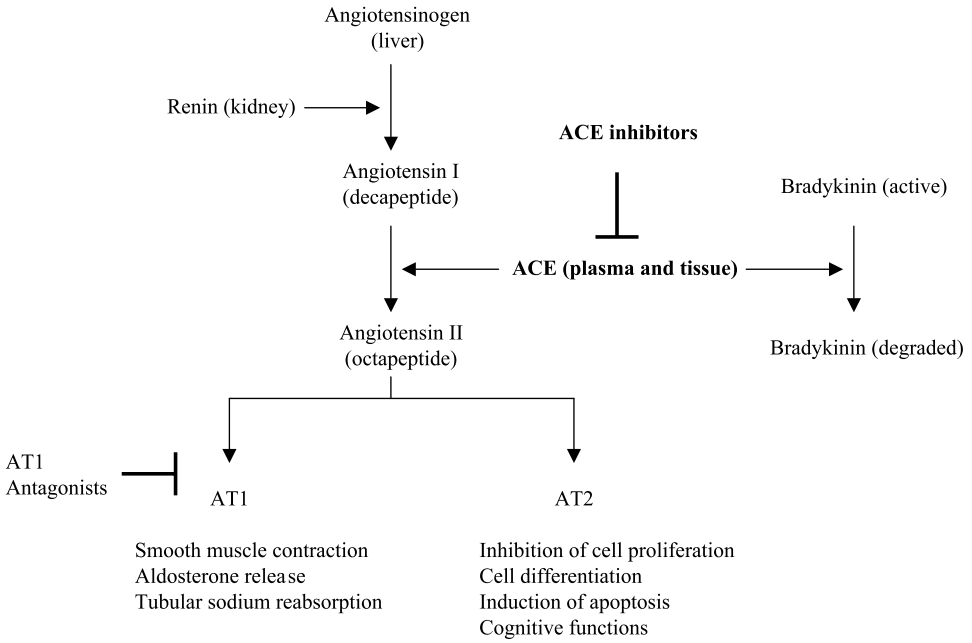

Fig. 1 The renin–angiotensin–aldosteron system (RAS) and the role of the angiotensin-converting enzyme (ACE) with targets for antihypertensive therapy (ACE inhibitors and AT1 antagonists). AT1 = angiotensin II type 1 receptor; AT2 = angiotensin II type 2 receptor.

patients compared to ID or II individuals. On the contrary, Ohmichi et al.[17] found that not only the reduction in diastolic blood pressure was higher in II than in ID or DD patients, but that the reduction in diastolic blood pressure was inversely correlated with plasma ACE activity as well. Sasaki et al.[18] found that regression of left ventricular hypertrophy and improvement in diastolic filling induced by ACE inhibitor therapy was more pronounced in the DD genotype group than in the ID or II group. O'Toole et al.[19] investigated the influence of the ACE genotype on the response to ACE inhibitors in patients with heart failure. They found a higher reduction in blood pressure in patients with the II genotype than in the other groups. Interestingly, this effect was only seen in patients treated with captopril (25 mg t.d.s.) but not in those treated with lisinopril (10 mg o.d.). In summary, the correlation between ACE I/D gene polymorphism and the response to treatment

Table 1 Studies in patients with essential hypertension evaluating the response to ACE inhibitor therapy in relation to ACE genotype

Reference	Study drug	Phenotype
Positive effect in II individuals		
[17]	Imidapril	Reduction in blood pressure
[19]	Lisinopril, captopril	Change in mean arterial pressure (captopril only)
Positive effect in DD individuals		
[16]	Fosinopril	Reduction in blood pressure
[18]	Enalapril	Regression of LVH and improvement in LVDF
No effect of ACE genotype		
[13]	n.s.	Blood pressure response
[14]	Atenolol, lisinopril, nifedipine SR	Blood pressure response
[15]	Captopril	Blood pressure response

LVH = left ventricular hypertrophy; LVDF = left ventricular diastolic filling; n.s. = not specified.

with ACE inhibitors in cardiovascular disorders remains highly controversial.

STUDIES IN RENAL DISEASE

The individual response to treatment with ACE inhibitors is highly variable.[20] Therefore, several studies have been conducted in which the efficacy of ACE inhibitor therapy was studied in relation to ACE genotype in patients with kidney diseases (Table 2). Yoshida et al.[21] studied the response to ACE inhibitor therapy in 21 Japanese patients with IgA-nephropathy, who were treated with lisinopril (10 mg/day). After 4 years of therapy, only patients with DD genotype showed a significant reduction in proteinuria. These results were confirmed by Moriyama et al.[22] in patients with various renal diseases and by Ha et al.[23] in patients with diabetic nephropathy. Those results are also consistent with a study performed by Perna et al.[24] Proteinuria, change of glomerular filtration rate, and progression to end-stage renal disease were effectively reduced by treatment with ramipril in patients with the DD genotype. Interestingly, ethnic background may play a very important role as mentioned before, as three of the four studies showing a more efficient reduction in proteinuria in DD individuals were performed in Asian patients.[21–23]

On the contrary, van Essen et al.[25] compared atenolol and enalapril in 81 Caucasian patients with nondiabetic renal disease. Patients with II and ID genotype showed a significantly higher reduction in urinary protein excretion than patients with DD genotype. These results are consistent with studies performed by Gonzalo et al.[26] and by Haas et al.[27] In the EURODIAB controlled trial of lisinopril in insulin-dependent diabetes mellitus (IDDM) (EUCLID),[5] Penno et al.[28] reported the highest increase in albumin excretion rate in II genotype patients when treated with placebo. However, when treated with lisinopril, patients with the II genotype showed the slowest increase in albuminuria. These results in patients with diabetic nephropathy were confirmed by Jacobsen et al.[29] In an observational follow-up study, patients with IDDM and diabetic nephropathy were investigated during captopril treatment (12.5–150 mg/day).[30] The treatment effect on reduction in proteinuria was similar in all genotype groups. Van der Kleij[31] studied the short-term renal response to ACE inhibition in 61 patients with nondiabetic renal disease and proteinuria >1 g/day. Therapy consisted of either enalapril or lisinopril (10–20 mg/day). The reduction of proteinuria by ACE inhibition was not significantly different between the genotype groups, as has also been shown by Burg et al.[32] in a prospective study and Björck et al.[33] in retrospectively analyzed data. In summary, as in the trials dealing with essential hypertension, the correlation between ACE I/D gene polymorphism and response to treatment with ACE inhibitors in patients with renal diseases remains highly controversial.

Table 2 Studies in patients with various renal diseases evaluating the response to ACE inhibitor therapy in relation to ACE genotype

Reference	Study drug	Phenotype
Improved effect of ACE inhibitor therapy in DD individuals		
[21]	Lisinopril	Proteinuria
[22]	n.s.	Proteinuria
[23]	n.s.	Proteinuria
[24]	Ramipril	Loss of GFR
Improved effect of ACE inhibitor therapy in II individuals		
[25]	Enalapril	Loss of GFR
[26]	Enalapril, Losartan	Proteinuria
[27]	Enalapril	Proteinuria
[28]	Lisinopril	Proteinuria
[29]	Captopril	Proteinuria
[30]	Captopril	Loss of GFR
No effect of ACE genotype on treatment response to ACE inhibitor therapy		
[31]	Enalapril, Lisinopril	Proteinuria
[32]	n.s.	Proteinuria
[33]	n.s.	Proteinuria

n.s. = not specified; GFR = glomerular filtration rate.

CONCLUSION

Several questions regarding the clinical utility of ACE genotyping remain unresolved. The clinical trials studying the correlation of ACE genotype and response to treatment with ACE inhibitors in patients with essential hypertension or kidney disease do not give a clear answer (Tables 1 and 2). Some of the studies have to be dealt with great caution because of small patient numbers and heterogeneous baseline characteristics. Even the choice of ACE inhibitor might have influenced the results of these clinical trials because it has been shown that ACE inhibitors differ in their affinity to ACE.[34] Finally, one has to consider if the ACE I/D polymorphism is the best of the hitherto described genetic markers for pharmacogenomic studies, as its clinical significance and its linkage to pathologies is still controversial.[35] Direct measurement of enzyme activity may not only allow a better estimation of individual risk

but provide a basis for more effective treatment with ACE inhibitors as well.

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Actinomyces spp.

Val Hall
University Hospital of Wales, Cardiff, U.K.

INTRODUCTION

Actinomyces species form part of the normal mucosal flora of humans and many animals and are opportunist pathogens involved in periodontal infections, soft tissue abscesses, or actinomycosis. Management of established actinomycosis usually demands surgical intervention and/or long-term aggressive antimicrobial therapy. However, clinical diagnosis and confirmation by microbiological culture and conventional identification methods are each notoriously problematical. In recent years, the application of molecular techniques has greatly improved the classification and identification of members of this genus. In the future, direct detection of *Actinomyces* spp. in clinical materials with specific DNA probes may overcome many of the traditional clinical diagnostic problems.

ACTINOMYCES SPECIES

General Description

Actinomyces spp. are facultatively anaerobic gram-positive bacilli that inhabit mucous membranes, particularly those of the oral cavity, of humans and animals.[1] Cell and colony morphologies, aerotolerance, and growth rates each vary considerably between species. At the time of writing, the genus comprises some 29 species, 17 of which have been classified since 1993 with the aid of modern phylogenetic techniques. Recent studies indicate heterogeneity within some species and the existence of additional, as yet undescribed species.

Causative Agents of Actinomycosis

The principal agents of human actinomycosis are *Actinomyces israelii*, *Actinomyces gerencseriae*, and the morphologically similar *Propionibacterium propionicum*. The cells of these species are filamentous, beaded, and branching gram-positive rods. The organisms in vivo form characteristic microcolonies with radiating filaments and clubbed ends composed of both bacterial and host materials and surrounded by neutrophils and foamy macrophages. In wound exudates, microcolonies may be visible to the naked eye as hard white or yellowish grains, commonly called ''sulphur granules.'' *Actinomyces* spp. are highly susceptible in vitro to a wide range of antimicrobial agents. However, within microcolonies, the organisms are protected from the actions of these and of host defenses, and this is thought to be a major pathogenicity factor. In the laboratory, the above-named species may require up to 10 days of anaerobic incubation on a serum-containing medium before characteristic white or beige ''breadcrumb'' or ''molar tooth'' colonies are visible. Colonies may be very gritty, pitting, and adherent to the agar. These growth characteristics and the almost universal presence of concomitant organisms, commonly members of the oral flora, can result in difficulties in laboratory isolation, identification, and susceptibility testing.

Species Associated with Intraoral Diseases

Actinomyces spp. are early colonizers of various niches within the oral cavity. Species commensal in the mouth include *A. israelii*, *A. gerencseriae*, *Actinomyces odontolyticus*, *Actinomyces meyeri*, *Actinomyces naeslundii/viscosus*, *Actinomyces georgiae* and, probably, *Actinomyces graevenitzii.* Some members of the genus, particularly those of the *A. naeslundii/viscosus* complex, are known to coaggregate with other bacteria and adhere to mammalian cells by means of fimbriae. Consequently, they play an important role in development of dental plaque. However, despite numerous studies, their roles in the highly complex processes of dental caries and periodontal diseases remain somewhat controversial. The recently described species *Actinomyces radicidentis* has been isolated, so far, only from infected root canals.

Other Species

The natural habitats, prevalence, and clinical significance of many recently described *Actinomyces* spp. have yet to be established. For the majority of these species, few isolates have been studied to date. However, it appears that species including *Actinomyces turicensis*, *Actinomyces radingae*, *Actinomyces europaeus*, *Actinomyces funkei*, *Actinomyces neuii*, and *Actinomyces urogenitalis* are principally involved in polymicrobial, superficial soft tissue infections in humans but do not cause actinomycosis. Natural habitats may include the genitourinary and gastrointestinal tracts and lipid-rich areas of the skin. The cell and colony morphologies of these species can be

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described as ''nondescript'' and may not resemble the well-known forms of the classical *Actinomyces* spp. Therefore, they may be easily overlooked in mixed cultures and, consequently, are probably underreported in clinical specimens.

CLINICAL FEATURES OF ACTINOMYCOSIS

Actinomycosis is a chronic suppurating disease, characterized by cavitating abscess formation and, in the advanced stages, by draining sinus tracts and considerable tissue damage. The disease affects principally the cervicofacial region. Thoracic, abdominal, and pelvic infections occur less frequently but may result in serious, occasionally life-threatening disease.[1] Onset is insidious, early symptoms may be vague, and clinical diagnosis is often difficult until overt signs appear. The medical literature abounds with cases of actinomycosis mistakenly diagnosed as tuberculosis or carcinoma, many of which were correctly diagnosed by histological methods only after surgery. Other sites of infection include the brain, lungs, and hepatic abscess. In cases of lacrimal canaliculitis, the tear duct becomes blocked by concretions of *Actinomyces* or *P. propionicum* and associated flora. Disease of the long bones or joints is rare but may be extremely debilitating, widely disseminated, and intractable. Carriage of *Actinomyces* spp. is strongly associated with intrauterine contraceptive devices (IUCDs) and may progress to pelvic infection.

PREVALENCE

In developed countries, advanced cases of cervicofacial actinomycosis are rare. The incidence of lesser infections and actinomycosis of other sites is difficult to ascertain but may be greatly underreported. The risk of pelvic actinomycosis arising from IUCD usage has remained controversial since the 1980s when the association of *Actinomyces* spp. was first discovered. However, carriage rates averaging 20% in millions of women using IUCDs and few reported cases of disease suggest that *Actinomyces* spp. are rare opportunist pathogens. Conversely, periodontal diseases are widespread in the developed world and *Actinomyces* spp. may play important roles in their development.

PATIENT MANAGEMENT

In simple, promptly diagnosed actinomycotic infections such as dental abscesses following surgery, a short course of antimicrobial therapy may be curative. *Actinomyces* spp. are highly susceptible to a wide range of agents and the pharmacokinetics of penicillin has made this the drug of choice. Various other agents may be used in penicillin-allergic patients, or where broad-spectrum cover for concomitant organisms is required. In advanced cases of actinomycosis, surgical debridement (ranging from drainage of abscesses to removal of major organs) together with long-term (for months or years) antimicrobial therapy and careful monitoring of signs and symptoms may be necessary to effect a cure. Relapses are not infrequent. In cases of lacrimal canaliculitis, manipulation or incision to remove the physical blockage and systemic antimicrobial therapy may be necessary.

Patients with IUCDs should be made aware of the symptoms of pelvic inflammatory disease and advised to consult their doctor if these arise. Asymptomatic patients should be monitored regularly and IUCDs replaced as recommended.

MOLECULAR CHARACTERIZATION

Classification

In common with many other genera, the classification of *Actinomyces* has been revolutionized by the application of 16S rRNA nucleotide sequence analysis. Early results were obtained by laborious reverse transcriptase methods.[2] However, rapid advances in DNA amplification, sequencing techniques, and data analysis software enabled the comprehensive phylogenetic analysis of the genus.[3] As a result, some former *Actinomyces* species have been transferred to other genera (e.g., *Arcanobacterium pyogenes*) and some novel genera (e.g., *Actinobaculum*, *Varibaculum*) and a plethora of novel species have been described. These include, from human sources, *A. radingae*, *A. turicensis*, *A. europaeus*, *A. graevenitzii*, *A. radicidentis*, *A. urogenitalis*, *A. funkei*, and *Actinomyces cardiffensis*. Additional novel species are indicated both from organisms cultivated from clinical materials[4] and from uncultivated clones.[5,6] Furthermore, these and other studies have demonstrated extensive genetic heterogeneity within strains currently assigned to the *A. naeslundii/viscosus* complex and, to a lesser extent, within *A. israelii*, *A. odontolyticus*, and some other species.

The robustness of current classification is ensured by the use of a polyphasic taxonomic approach, whereby phylogenetic divisions are supported by those derived from chemotaxonomic markers and phenotypic characteristics. Determination of cell wall sugars and amino acids, fatty acids, phospholipids, and menaquinones, and whole-cell protein profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been utilized. Collectively, these analyses indicate that the

genus *Actinomyces* represents at least three separate genera, as yet to be formally classified.[7]

Many clinicians and clinical microbiologists view taxonomic revisions as purely academic exercises—at best, irrelevant, and, at worst, confusing. However, a robust and discriminatory classification system, coupled with reliable means of identification, is a prerequisite for a meaningful analysis of prevalence, habitats, clinical associations, and potential pathogenicity of microorganisms. In addition, knowledge of genetic specificity and diversity within target species is essential for the development of DNA probes for direct detection of pathogens in clinical materials.

Identification

Identification by genotypic methods is particularly valuable for organisms such as *Actinomyces* spp., which are poorly differentiated in conventional biochemical or serological tests. Species-specific oligonucleotide probes targeting ribosomal DNA have been developed both for identification of cultivars and for direct detection, but their value has been limited by the high degrees of intraspecies genetic diversity occurring within *Actinomyces* spp.[2,8] Conversely, amplified rDNA restriction analysis [ARDRA or polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP)] has proved to be a highly discriminatory, practical, and cost-effective method for differentiation of *Actinomyces* and other facultatively anaerobic gram-positive bacilli to the species or subspecies levels. In two small studies, the restriction endonuclease *Mnl*I was recommended.[9,10] However, in studies of over 600 clinical and some veterinary isolates, banding patterns obtained from separate digestions by *Hae*III and *Hpa*II were analyzed.[11,12] Neither enzyme alone was sufficiently discriminatory, but, in combination, differentiated all currently recognized *Actinomyces*, *Arcanobacterium*, and *Actinobaculum* spp., some to subspecies level, and many *Propionibacterium*, *Lactobacillus*, *Bifidobacterium*, *Gardnerella*, *Mobiluncus*, and *Eubacterium* spp. Results correlated well with those obtained by conventional methods, but the latter lacked discrimination. Several recently described species (e.g., *A. funkei*, *A. urogenitalis*) were recognized as being distinct from existing species prior to their formal description by other workers, and a number of novel species, including *A. cardiffensis*, were detected. This method is now used routinely at the Anaerobe Reference Laboratory of the National Public Health Service for Wales for identification of nonsporing gram-positive bacilli referred from throughout the UK. Unfortunately, although only basic molecular expertise and equipment are necessary to perform this method, it remains impractical for use in the clinical laboratory for identification of occasional isolates.

Methods for PCR-RFLP and 16S rDNA Sequencing of *Actinomyces* Isolates

When an adequate and pure growth of the isolate has been cultured, genomic DNA may be extracted without the need for noxious chemicals or complex procedures. Boil a 1 μL loopful of the organism in 100 μL of Chelex resin (5%) for 8–10 min. Centrifuge at 13,000 rpm for 10 min. Use the supernate as target DNA. The almost complete 16S rRNA gene is amplified by PCR using ''universal'' primers pA (sequence AGAGTTTGATCCTGGCTCAG) and pH′ (AAGGAGGTGATCCAGCCGCA) in 45 μL of reaction mixture containing 1 U of *Taq* polymerase, each deoxynucleoside triphosphate at 200 mM, 20 pmol of each primer, 2.25 mM magnesium chloride, 10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100, and 5 μL of target DNA. For amplification, denature at 92°C for 2 min, anneal at 55°C for 1 min, and extend at 72°C for 1.5 min × 30 cycles with final extension for 5 min. Confirm the specific product of approximately 1600 bp by electrophoresis in 1% agarose using an appropriate molecular size marker. For DNA sequencing, purify the amplicon in a commercial PCR purification kit and proceed using a standard *Taq* dye-deoxy terminator sequencing kit and appropriate primers. For identification by PCR-RFLP, no purification step is necessary. Perform restriction endonuclease digestion by mixing 12.5 μL of PCR product with 1 μL of *Hae*III or *Hpa*II (10 U/μL) and 1.5 μL of matched incubation buffer. Mix and incubate at 37°C for 1.5–2 hr. Add 3 μL of loading buffer to the reaction mix and electrophorese 12 μL of sample in a 3.5% Metaphor agarose gel in TAE buffer with ethidium bromide (0.5 μg/mL) at 5 V/cm for 1.75 hr. Run appropriate size markers (e.g., 2 kb; Sigma, Poole, UK) at intervals alongside samples. Destain the gel in deionized water for 10 min and visualize under UV illumination. Analyze banding patterns by visual comparison with those of reference strains or, preferably, in a software package such as GelCompar (Applied Maths, Kortrijk, Belgium).[11]

Molecular Detection from Clinical Material

To date, direct detection of *Actinomyces* spp. has been applied principally to investigations of their roles in intraoral diseases. In a study of the microbial composition of supragingival and subgingival plaques in subjects with adult periodontitis, Ximénez-Fyvie et al.[13] used whole genomic DNA probes and checkerboard DNA–DNA hybridization as described by Socransky et al.[14] to detect the presence and levels of 40 bacterial taxa including

A. israelii, *A. gerencseriae*, *A. naeslundii* genospecies 1 and 2, and *A. odontolyticus*. *Actinomyces* spp. were the most prevalent of the 40 taxa in both supragingival and subgingival habitats, and higher counts were detected in supragingival sites.

Extensive studies designed to detect both cultivable and noncultivable bacteria, including novel taxa, have utilized PCR with all-bacterial or selective primers, followed by cloning in *Escherichia coli* and sequencing of clones. Together with checkerboard DNA–DNA hybridization for selected taxa, this method has been used to determine species identity for the investigation of bacterial diversity in subgingival plaque[5] and tongue dorsa[6] and species associated with childhood caries.[15] Among the large numbers of novel phylotypes detected in these studies, several *Actinomyces* spp.-like clones were recognized. *A. naeslundii* and *A. naeslundii*-like clones and *A. gerencseriae* were detected in cases of refractory periodontitis and in healthy subjects. *A. israelii* and *A. georgiae* were detected in healthy subjects and *A. odontolyticus* was associated with refractory periodontitis. In the study of childhood caries, it was concluded that *A. gerencseriae* and other *Actinomyces* spp. may play an important role in caries initiation.

The wide genetic diversity apparent within *Actinomyces* spp. and particularly in the *A. naeslundii/viscosus* complex has hindered the development of specific riboprobes for detection of pathogenic *Actinomyces* in clinical specimens. Furthermore, such diversity indicates that where investigations of pathogenicity factors, adherence, etc. are based on only one or a small number of strains, results should be treated with caution as these may not be representative of the whole population.

To overcome problems of low sensitivity of specific riboprobes, Kiyama et al.[16] targeted a 756-bp *Actinomyces*-specific fragment of the sialidase gene. As sialidase activity facilitates adherence to buccal epithelial and other mammalian cells, the presence of this gene is relevant to bacteria associated with periodontal disease. The specific DNA fragment was amplified by PCR prior to hybridization with a digoxigenin-labeled probe. Universal detection of a 625-bp highly conserved region of the 16S rRNA gene acted as a control of the method, and it was found to be highly sensitive and specific for the members of the *A. viscosus/naeslundii* complex examined.

CONCLUSION

The application of modern genotypic methods has greatly clarified the phylogenetic status of members of the genus *Actinomyces* and has led to the recognition of many additional species and subspecies. Identification of *Actinomyces* spp. by PCR-RFLP with endonucleases *Hae*III and *Hpa*II has proved to be a highly discriminatory method, far superior to conventional biochemical tests. PCR-RFLP is practical for testing small batches of clinical isolates in the reference laboratory setting. Resulting data will further aid the establishment of the natural habitats, prevalence, and clinical significance of *Actinomyces* spp. Meanwhile, in the field of oral microbiological research, the use of cloning, sequencing, and DNA–DNA hybridization continues to increase the knowledge of the bacterial components of specific oral niches and to aid in the elucidation of the complex etiology of periodontal diseases.

The laboratory cultivation of *Actinomyces* spp. from clinical material remains problematical. The direct detection of the causative agents of actinomycosis, ideally from histological sections as well as microbiological specimens, could greatly improve early diagnosis and patient management. At present, success in this has been limited, principally due to intraspecies genetic heterogeneity. Clearly, this is an area that warrants further research and development.

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Acute Myeloid Leukemia (AML)—Cytogenetic Detection of Selected Recurrent Chromosomal Abnormalities

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Hon Fong L. Mark
Presbyterian Laboratory Services, Charlotte, North Carolina, U.S.A.

Robert A. Sokolic
National Cancer Institute, Bethesda, Maryland, U.S.A.

Yvonne Mark
F.I.T. Health Services, New York, New York, U.S.A.

INTRODUCTION

Leukemia is a neoplastic proliferation of hematopoietic cells. Leukemia can be classified as either myelocytic (myeloid) or lymphocytic (lymphoid) depending on the lineage of the leukemic cells. Leukemia can also be either chronic or acute, depending on the clinical course and on the predominant maturational stage of the malignant clone. In this article, we will focus our discussion on acute myelocytic (myeloid) leukemia (AML), the most common acute leukemia found in adults.

Until recently, the leukemias have been classified using a system proposed by the French–American–British (FAB) Cooperative Group. Under this system, eight subgroups (M0 to M7) have been defined for AML and three subgroups have been designated for the acute lymphocytic leukemias (ALL). This classification also includes the myelodysplastic syndromes (MDS) and the myeloproliferative diseases (MPD). The former category includes the following five subgroups: refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEBT), and chronic myelomonocytic leukemia (CMMoL). The MPDs include chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis with myeloid metaplasia (MMM). The above classification was established on the basis of morphological, cytochemical, and immunological parameters. Several of the above disease entities are more or less associated with specific chromosomal rearrangements, selected examples of which are given in Fig. 1, courtesy of Dr. J. Anastasi.

Although the FAB system has served as the gold standard for classification of malignant hematological diseases for a number of years, cancer centers are increasingly adopting the system proposed by the World Health Organization (WHO), as further discussed below.

THE WHO CLASSIFICATION OF LEUKEMIAS

Harris et al.[1] reported the WHO classification of neoplastic diseases of the hematopoietic and lymphoid tissues in 1999. This system modifies and incorporates the Revised European–American Lymphoma (REAL) classification[2] and extends the principles underlying that schema to the classification of myeloid diseases. Like the REAL system, the WHO system attempts to classify neoplastic lymphohematopoietic diseases into discrete entities with unique clinico-biological features. In this way, the WHO classification is a conceptual improvement over the FAB classification,[3–6] which primarily relied on morphological criteria.

To divide the lymphohematopoietic neoplasms into clinically relevant and biologically discrete entities, multiple diagnostic modalities are employed in the WHO system, including morphology, immunophenotype, clinical history, and cytogenetic abnormalities. With respect to cytogenetic and fluorescent in situ hybridization (FISH) evaluations of AML, the WHO classification has significantly upgraded the role of these modalities in diagnosis, prognosis, and relevance to treatment decisions. Within the acute leukemias, the major WHO categories include therapy-related AML-MDS, AML with multilineage dysplasia, and AMLs with recurrent cytogenetic translocations in addition to AML not otherwise categorized.[1] Thus cytogenetic data define one major group of AMLs. The delineation of specific cytogenetic diseases owes much to studies showing that cytogenetics could predict response to therapy[7–11] and therefore could be used to make treatment decisions.[7,12]

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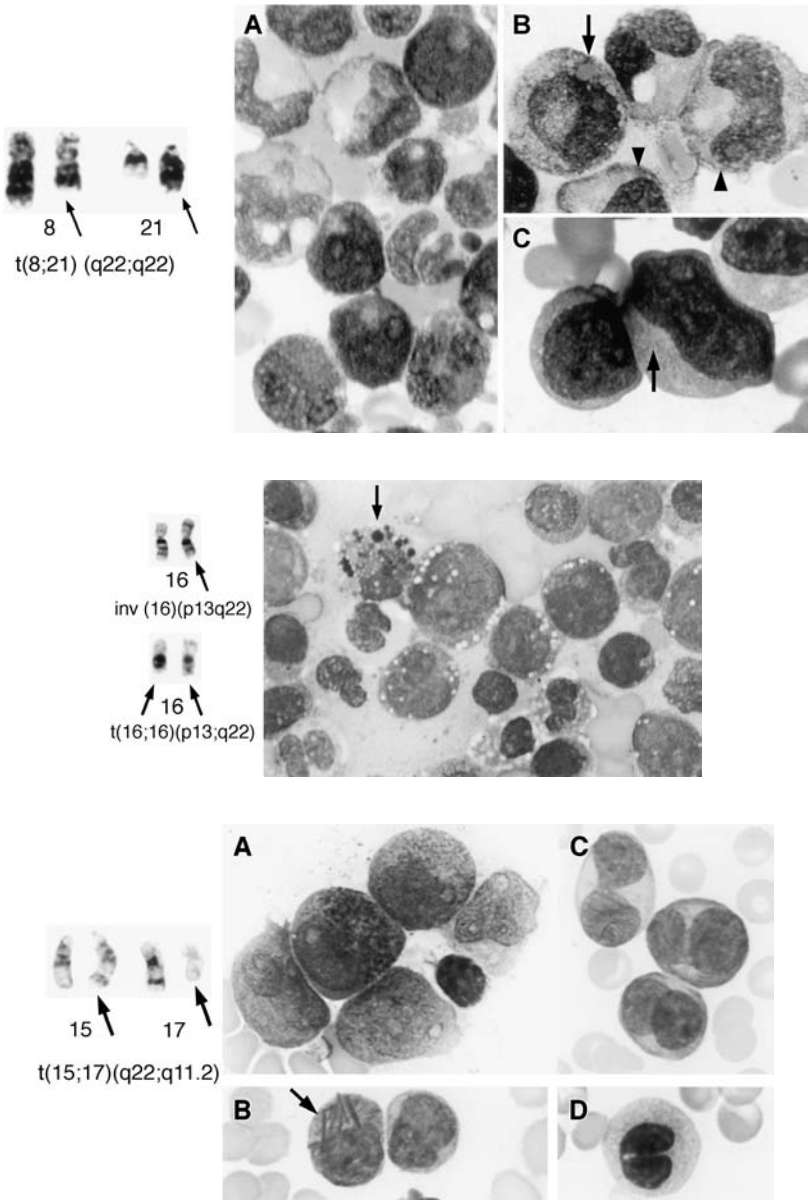

Fig. 1 Selected examples of specific chromosomal rearrangements associated with different AML subtypes. (From Ref. [18].)

CONVENTIONAL CYTOGENETICS IN DETECTING RECURRING CHROMOSOMAL ABNORMALITIES IN HEMATOPOIETIC DISORDERS

Conventional cytogenetic analysis using G-banding is a powerful technique that enables one to gain a complete picture of the human genome at a glance (Fig. 2). Bone marrow is the tissue of choice for conventional cytogenetic studies of acquired chromosomal abnormalities in most hematological conditions. Nevertheless, a stimulated culture of peripheral blood is sometimes performed besides an unstimulated culture of the bone marrow to ascertain whether an observed chromosomal abnormality, when present in all sampled cells, is constitutional in origin.[13,14]

The first consistent acquired chromosomal abnormality that was associated with a single cancer type, CML, was described by Nowell and Hungerford.[15] The marker chromosome, named the Philadelphia (Ph^1) chromosome, in honor of the city where it was discovered, was later found by Rowley[16] to be a reciprocal translocation between chromosomes 9 and 22 using the G-banding technique. This translocation, in which the c-abl oncogene

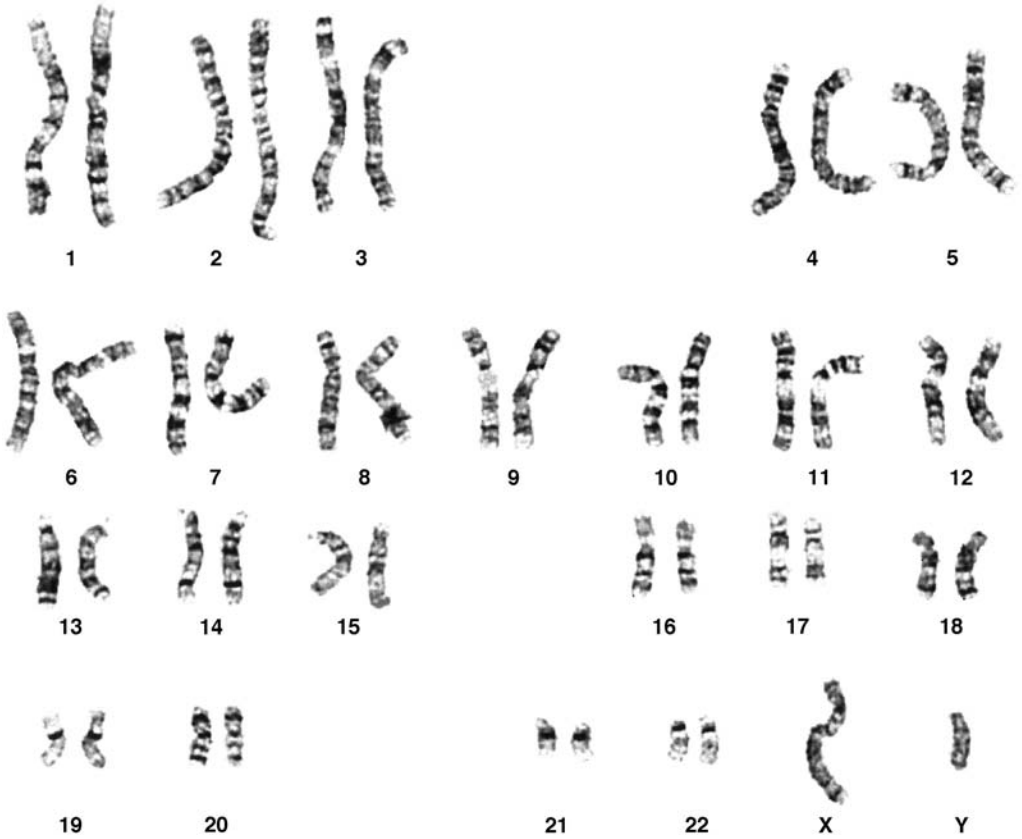

Fig. 2 A normal G-banded male diploid karyotype. [Courtesy of Cytogenetics Department (Dr. H.F.L. Mark, Director), Presbyterian Laboratory Services, Charlotte, North Carolina.]

on 9q34 is fused to the breakpoint cluster region (bcr) locus on chromosome 22, results in a hybrid gene. Subsequently, this gene produces a fusion protein with increased tyrosine kinase activity and eventually leads to myeloid cell transformation. Figure 3 is a simplified illustration of the structural chromosomal rearrangement giving rise to the Philadelphia translocation. It is given special attention here because this reciprocal translocation mechanism was subsequently found to be a common theme: Many of the other structural chromosomal abnormalities found in acute myelocytic leukemia can be explained by the same or similar paradigm. Because of space limitations, the detailed breakage and reunion events leading to the common translocations encountered in the various AML subtypes will not be repeated here. Instead, the reader is referred to the figures provided in the products (probes) section of the Vysis website,[17] as well as in a review by Anastasi and Roulston.[18] Selected examples of the common chromosomal translocations encountered in AML are given in Fig. 1.

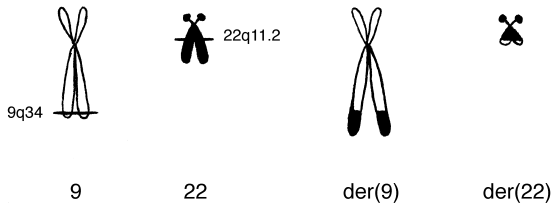

Fig. 3 A simplified illustration of the structural chromosomal rearrangement giving rise to the Philadelphia translocation. Der(22) denotes the Philadelphia chromosome. Chromosomes are not necessarily drawn to scale.

Many patients with AML have characteristic recurring chromosomal abnormalities that were detectable in metaphase preparations by conventional cytogenetics, even before the advent of fluorescent (or fluorescence) in situ hybridization (FISH). FISH is a powerful technique for detecting both numerical and structural cytogenetic abnormalities in nondividing (interphase) as well as dividing (metaphase) cells. On the other hand, conventional cytogenetics can only be performed when the mitotic index is within acceptable limits (i.e., when there is a sufficient number of metaphases to complete a meaningful analysis). Another advantage of FISH is that a large number of cells can readily be scored using the

technique, whereas metaphase cytogenetic analysis is cumbersome and labor intensive when analyzing beyond the standard number of cells.[13,19–22]

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

FISH is a molecular cytogenetic technique that enables the detection of a probe that binds to homologous sequences on a chromosome that is fixed on a glass slide. FISH can be performed on a variety of specimen types such as peripheral blood, bone marrow, and pathological sections.[23,24] Probes used for FISH for cancer applications are most commonly locus-specific DNA probes or alpha-satellite centromere enumeration probes, or a combination of both. The different types of FISH probes and their applications are extensively discussed elsewhere.[25–27]

The technical steps involved in performing FISH are similar to nonfluorescent in situ hybridization techniques. Figure 4 depicts a simplified FISH procedure used in many clinical and research cytogenetic laboratories, whereas Fig. 5 is a simplified schematic representation of the detection of the Philadelphia translocation using FISH. This translocation, characteristic of CML, is used here as an example to illustrate the utility of FISH. Variations of the same basic paradigm enable the detection of most structural chromosomal rearrangements commonly found in AML and other hematopoietic disorders. Other details associated with the principles and techniques of FISH were described by Blancato and Haddad.[25] Research and clinical applications of FISH in cancer cytogenetics include detection of numerical chromosomal abnormalities (aneuploidies), structural chromosomal abnormalities such as translocations, inversions, deletions, duplications, isochromosomes, derivative chromosomes, dicentrics, rings, acentrics, double minutes, amplifications and marker chromosome identification.[28–30] FISH is especially useful in studying a large number of nondividing cells for the determination of clonality, particularly in suboptimal cancer preparations where the mitotic index is low.[31] Additional applications have been summarized elsewhere.[27] Examples of clinical applications of FISH to detect chromosomal abnormalities in AML are further discussed below.

CLINICAL UTILITY OF FISH DETECTION OF RECURRING NUMERICAL AND STRUCTURAL CHROMOSOMAL ABNORMALITIES IN ACUTE MYELOID LEUKEMIA

The convention established by the Fourth International Workshop on Chromosomes in Leukemia[32] serves as the basis for the definition of a clonal abnormality. In clinical cytogenetics, the presence of two or more trisomic or structurally rearranged cells, or three or more monosomic cells, is considered clonal.[4] Following this criterion, a number of recurring clonal cytogenetic abnormalities have been identified in AML based on G-banded studies alone. Selected chromosomal abnormalities found in AML are given in Table 1. The reader is referred to Refs. [6,18] for details and more in-depth discussions.

There are three areas in which FISH is particularly useful in the management of myeloid disease. Perhaps most importantly, FISH is an important part of the diagnostic workup of myeloid malignancy. As noted above, the presence or absence of specific recurrent cytogenetic abnormalities has a major impact on the treatment of AML. Cytogenetically occult lesions, detectable only by FISH, are known to occur,[33] and these could potentially dictate the difference between hematopoietic stem cell transplantation and simple consolidation chemotherapy as postinduction treatment. Moreover, the choice of postinduction chemotherapy is to some extent influenced by cytogenetics, because high-dose cytarabine has been shown to be most effective in the setting of core-binding factor mutations, detected cytogenetically as t(8;21) and inv(16)/t(16;16) chromosome rearrangements.[34] In these diseases, high-dose cytarabine is more strongly indicated than in other types of AML.[35]

Because FISH and traditional cytogenetic banding techniques can have discrepant results,[24,33,36,37] it is important to consider both techniques in the initial cytogenetic assessment of AML. Consideration of the strengths and weaknesses of both cytogenetic techniques (FISH and conventional G-banding) makes it clear how these two assays are complementary in the diagnosis of AML. While FISH has the advantage of being able to evaluate many more cells than conventional cytogenetics, it has the requirement of a prior determination of suspected cytogenetic abnormalities for which one will probe. Because the number of reported cytogenetic abnormalities in AML is large, one cannot search for all such abnormalities at once. In practice, specific FISH probes can be used when the clinical situation and/or morphological criteria suggest specific abnormalities. For example, an FAB diagnosis of acute monocytic leukemia with bone marrow eosinophilia should prompt one to FISH for chromosomes 8, 21, and 16. Acute myeloid leukemia arising after an antecedent hematological disorder should be evaluated with FISH for chromosomes 5, 7, and 8.[37]

FISH is equally important in follow-up of patients treated with either transplant or chemotherapy.[38] For patients relapsing from standard dose consolidation chemotherapy, high-dose antineoplastic therapy with

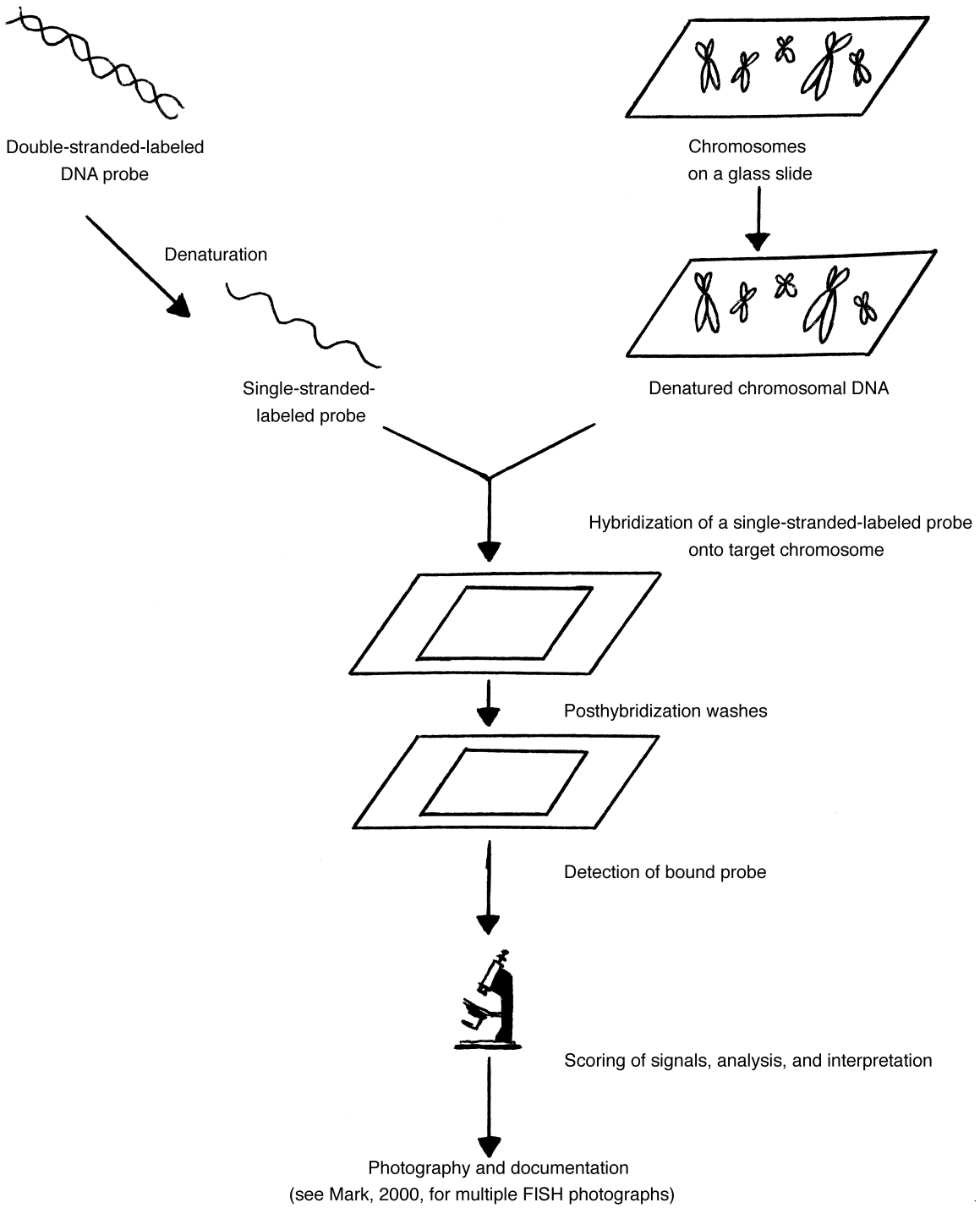

Fig. 4 A simplified illustration of the FISH protocol. (From Ref. [25].)

allogeneic hematopoietic stem cell transplantation is the only known curative modality. Because transplantation is most successful when the disease burden is low, and because FISH is, in general, a more sensitive test than standard cytogenetics, FISH should be a standard part of posttreatment monitoring in all patients with a history of AML with known cytogenetic abnormalities.

In the posttransplant setting, donor lymphocyte infusion (DLI) can be considered to treat relapsed disease. Although this therapy is most useful in CML, it has been

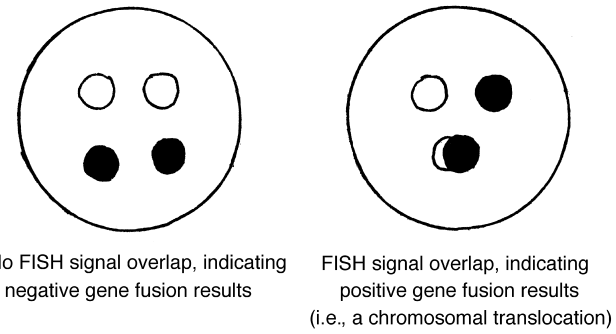

Fig. 5 A simplified schematic representation of the detection of a fusion gene resulting from a translocation using FISH. Variations of the same basic paradigm enable the detection of most structural chromosomal rearrangements commonly found in AML and other hematopoietic disorders.

used in AML as well, wherein, again, treatment with the smallest possible disease burden is felt to be the most likely to succeed. As for postchemotherapy follow-up, cytogenetics and FISH should be standard posttransplant follow-up for all patients with a history of AML with known cytogenetic abnormalities.

There is an additional use for FISH in the posttransplant setting. Because a goal of hematopoietic stem cell transplantation is to completely replace the host's diseased marrow with donor marrow, it is important to demonstrate that this has in fact happened. Donor–host chimerism can be followed by several methods, some of which are labor intensive. In the case in which the donor and host are of opposite sexes, FISH for the X and Y chromosomes can be used to efficiently monitor for persistence, recurrence, or disappearance of host hematopoiesis. This becomes particularly important with the recent development of nonmyeloablative preparative regimens and reduced intensity transplants. Using these strategies, conversion to complete donor chimerism can take up to several months, and DLI may be given simply for persistent host chimerism. In such a clinical setting, an accurate assessment of the levels of donor and host chimerism is crucial and is most simply performed, if the donor and host are sex-mismatched, by FISH.

Finally, one word of caution needs to be mentioned. FISH alone, while more sensitive than conventional cytogenetics, is not a replacement for the standard assay. A clinical error is to order FISH for known chromosomal abnormalities without conventional cytogenetics. This

Table 1 Selected recurrent chromosomal abnormalities found in AML

Selected chromosomal abnormalities	Selected genetic loci	Prognostic significance
inv(3)(q21q26), t(3;3)(q21;q26), ins(3;3)(q26;q21q26)		Intermediate
Trisomy 4		Intermediate
Monosomy 5 or del(5q)		Poor[a]
t(6;9)(p23;q34)		Intermediate
t(6;11)(q27;q23)		Intermediate
Monosomy 7 or del(7q)		Poor
Trisomy 8		Poor
t(8;21)(q22;q22)	AML1/ETO	Good
Trisomy 9		Intermediate
t(9;11)(p22;q23)		Intermediate
Trisomy 10		Intermediate
Trisomy 11		Intermediate
11q23 rearrangements	MLL	Intermediate
Trisomy 13		Intermediate
t(15;17)(q22;q11.2)	PML/RARA	Good
inv(16)(p13q22), t(16;16)(p13;q22), del(16)(q22)	CBFB/MYH11	Good
del(20q)		Intermediate
Trisomy 21		Intermediate
Trisomy 22		Intermediate
Trisomy X		Intermediate
− Y		Intermediate
No chromosomal abnormalities (normal cytogenetics)		Intermediate
Complex karyotype		Poor

Selected translocations in AML amenable to FISH detection are given in Fig. 1.
[a]Should not be confused with the so-called ''5q-'' chromosome. The latter abnormality, when part of the ''5q-'' syndrome, carries a good prognosis.

will, of course, fail to detect potentially new diagnostic chromosomal abnormalities, thus missing the opportunity for early intervention in the setting of recurrent disease prior to hematological relapse.

CONCLUSION

Cytogenetic analysis of chromosomes in cancer has been exploited as a powerful diagnostic tool, as a means of gaining prognostic information, and as a means of identifying markers for the malignant clone useful in follow-up.[18] Despite the fact that the exact pathogenetic significance of many chromosomal abnormalities is still unknown and that many questions still remain unanswered although clinical cytogenetics has become a formally recognized scientific and medical specialty, cancer cytogenetics has become established as a routine part of the management of patients with leukemia, from diagnosis and prognosis, through treatment, remission, relapse, transplant, and posttransplantation care. In the not-too-distant future, an exact identification of breakpoints and gene loci involved in most, if not all, structural chromosomal abnormalities implicated in cancer should be possible. This will lead to the development and application of more precisely targeted antineoplastic therapy predicated by the results of FISH.

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Adeno-Associated Viral Vectors

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Barrie J. Carter
Targeted Genetics Corporation, Seattle, Washington, U.S.A.

INTRODUCTION

Adeno-associated virus (AAV) vectors have several advantageous features as gene delivery vehicles. Adeno-associated virus does not cause disease or malignancy in any animal species. Adeno-associated virus vectors do not contain viral genes that could elicit undesirable cellular immune responses and appear not to induce inflammatory responses. Adeno-associated virus vectors can mediate long-term gene expression when administered in vivo. They may be best suited for infrequent delivery so that any potential host antibody response to the AAV capsid protein may be less inhibitory. An AAV vector has progressed to phase II trials in cystic fibrosis patients and other AAV vectors have now entered phase I trials for several other clinical indications. The earlier work on AAV vectors and all the current clinical trials used AAV serotype 2 as a prototype. Expanded understanding of AAV biology and other serotypes likely will lead to many more clinical applications of AAV gene therapies in the next several years. Several extensive reviews of the literature are available.

ADENO-ASSOCIATED VIRUS

Adeno-associated virus is a small, DNA-containing parvovirus that has been isolated from humans and other animal species.[1–4] Several serotypes of AAV have been isolated from humans and nonhuman primates.[1,4] Adeno-associated virus is defective and replicates only in host cell nuclei when certain functions are provided by a coinfecting helper adenovirus (Ad) or herpesvirus.[1,2] The mechanism of the helper function is not clearly defined but only a limited set of adenovirus genes, E1, E2A, E4, and the VA RNA are required.[1] Adeno-associated virus has a broad host range but the efficiency of transduction with AAV vectors of different serotypes varies because of differences in cell receptors utilized for entry, cellular trafficking of AAV particles, and uncoating of the viral genome in the cell nucleus. For AAV to replicate, or to function as a gene delivery vehicle, the uncoated single-stranded DNA genome must be converted to a double-stranded molecule to permit transcription and gene expression. Adeno-associated virus infection of cultured cell lines in the absence of helper functions results in persistence of the viral genome as a latent provirus which exhibits a high preference for integration at a specific region on human chromosome 19, but the efficiency and specificity of this process are mediated by the AAV *rep* gene.[5] Adeno-associated virus vectors have no *rep* gene and do not integrate efficiently.[1]

Adeno-associated virus particles are stable to heat and withstand robust purification procedures. Each particle has a protein coat and contains one linear, single-stranded DNA genome, but equal numbers of AAV particles contain a strand of either ''plus'' or ''minus'' complementary sense, and both types of particle are equally infectious. The AAV2 DNA[1] has one copy of the 145-nucleotide-long inverted terminal repeat (ITR) at each end enclosing two open reading frames for the *rep* gene and *cap* gene, respectively. The ITR sequences provide *cis*-acting functions for replication and encapsidation. The family of four rep proteins, Rep78, Rep68, Rep52, and Rep40, and the family of the three capsid proteins, VP1, VP2, and VP3, provide *trans*-acting functions for replication and encapsidation. In the presence of a helper such as adenovirus[1,2] the AAV single-strand genome is converted to a duplex replicating form (RF) then amplified to a large pool of progeny RF molecules that are precursors to encapsidated progeny genomes. In a single growth cycle of several days, AAV may yield in excess of 100,000 particles per cell. This large burst size is important for high specific productivity in vector production.

Design and Production of AAV Vectors

Adeno-associated virus vector production was enabled by the molecular cloning of double-strand AAV DNA into bacterial plasmids followed by transfection into helper virus-infected mammalian cells.[1,6] This results in replication of the AAV genome free of any plasmid sequence to yield AAV particles. For AAV vector construction, the *cis*-acting ITR sequences must be retained and the unique sequence is replaced with foreign DNA.[1,6] There is a limit of about 5 kb of DNA that can be packaged in an AAV vector particle. Production of AAV vectors in host cells can be accomplished entirely by DNA transfection-based methods or by cell-based systems

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that do not require DNA transfection.[1,6] Both approaches can give a specific productivity in excess of 10^4 vector particles per cell, but the cell-based systems may be more amenable to scale-up for commercial production.

In DNA transfection-based systems, human 293 cells are transfected with DNA plasmids containing the AAV vector cassette, the rep and cap genes, and the adenovirus E2A, E4, and VA genes. Alternatively, stable producer cell lines that contain the rep and cap complementing genes and the vector genome are infected with Ad. Producer cell lines are readily scalable AAV, but a new producer cell line must be generated for each individual AAV vector. A third approach uses a packaging cell line containing a rep–cap gene cassette that is then coinfected with an Ad/AAV hybrid virus, which is an E1 gene-deleted Ad containing the AAV-ITR vector cassette, and Ad to provide E1. After infection, the rep–cap genes, the AAV-ITR cassette is amplified and packaged into AAV particles. The same packaging cell line can be used for production of different AAV vectors simply by changing the Ad/AAV hybrid virus. Variations of these methods use herpes simplex virus (HSV) in the production of AAV vectors by utilizing a hybrid virus in which the AAV *rep–cap* genes were inserted into the HSV genome. Insect cells may be used with baculovirus vectors containing rep–cap gene cassettes or the AAV-ITR vector cassette to produce AAV vectors. Adeno-associated virus vector purification is generally accomplished by chromatographic methods, including ion exchange and affinity resins.[1] Adeno-associated virus vector amounts and concentrations are measured by the number of vector genomes that are encapsidated, and thus protected from digestion by DNAse, and are expressed in units of DNAse-resistant particles (DRP).

AAV Vector Genome Metabolism

A complex pathway of events[1] impact the function of AAV vectors such as the availability of specific cell-surface receptors, cellular trafficking, and potential diversion of AAV particles into a ubiquitin-mediated proteosome degradation pathway, and delay in uncoating in the cell nucleus. The uncoated single-strand genome must be converted to a duplex molecule. Following this, a succession of additional events result in the vector genome being converted into larger, mostly circular concatemeric molecules.

Single-strand to double-strand conversion step may be rate-limiting, but this can be overcome by using self-complementary vector genomes that are not more than half the size of the AAV genome. These genomes are packaged as a dimeric molecule equivalent to a RF formed during vector genome replication. Upon uncoating, these molecules reassociate (''snap back'') to duplex molecules with pseudo zero-order kinetics.[7] The ability of AAV vectors to form concatemers also extends the capacity of AAV vectors in a ''dual-vector'' system. Expression cassettes up to 9 kb in size can be divided between two AAV vectors, and coinfection forms concatemers to generate an intact expression cassette.[8]

Wild-type AAV, in cell culture, integrates into human chromosome 19, but this process requires the rep gene which is not present in AAV vectors. In contrast, a variety of animal models show that, in the absence of selective pressure, AAV vectors persist predominantly as circular concatemers. The circularization appears to involve recombination between ITRs.[9] The majority of these concatemers are episomal and integrated copies of vector in organs such as liver or muscle may be rare.[10,11] The relative rarity of integration is of importance in assessing the safety profile of AAV vectors for clinical applications.

In Vivo Targets for AAV Vectors

The preferred in vivo targets for AAV vectors are cells that are nondividing or turnover relatively slowly. Early studies using AAV2 vectors demonstrated transgene expression in organs such as lung, muscle, liver, brain, and retina[12–14] with persistence for months to years. The relative hierarchy of transduction efficiencies by individual serotypes is not fully clarified, but several other serotypes show enhanced efficiency in various organs.[1] Additional efforts are underway to modify the AAV capsid sequences to alter or enhance targeting of specific cells.[15]

Clinical Applications

Administration of AAV vectors does not appear to induce significant innate or proinflammatory responses, and in the absence of any viral genes cellular immune responses against the viral components are not readily evoked. Adeno-associated virus vectors may be used mainly for clinical applications requiring only infrequent delivery, but potential humoral immune responses against the viral capsid, either preexisting in the human population or induced by vector administration, must be considered.[1,4] It will require studies in humans to determine how neutralizing antibody responses to AAV vector capsids might impact applications of AAV vectors. There have been few, if any, documented reports or indications of toxicity mediated by AAV vectors in animal studies or human clinical trials.

The toxicity of an AAV vector has been extensively tested for a vector expressing the cystic fibrosis transmembrane regulator (CFTR) protein following delivery of these AAV–CFTR vector particles directly to the lung in rabbits and nonhuman primates.[16] The favorable

safety profile of AAV-CFTR in the preclinical studies was predictive of a similar safety profile observed in the clinical trials. Adeno-associated virus–cystic fibrosis transmembrane regulator was the first AAV vector introduced into clinical trials and it has been tested in a series of trials in CF patients by delivery to the lung, the nasal epithelium, and the maxillary sinus, and has now completed an initial phase II trial in the lung. The phase II multidose, double-blinded, placebo-controlled, and randomized trial was conducted in 37 CF patients with mild CF disease.[17] Patients received three doses (10^{13} DRP per dose) at monthly intervals, administered by inhaled aerosol. Multidose administration was safe and well tolerated, and met the primary endpoint of safety of repeated delivery. There was a significant treatment-related effect of positive increase in pulmonary function as measured by FEV1 at 30 days and a trend to a decrease in the proinflammatory cytokine IL-8 in the lung which was significant at 14 days.

A clinical study of intramuscular injection of an AAV2 vector expressing human FIX in adults with severe hemophilia B was recently completed and showed a low-level expression of FIX in transduced muscle and some possible modest changes in clinical endpoints including circulating levels of FIX and reduced frequency of factor IX protein self-administration by two patients.[18] A second phase I clinical trial with the same AAV2-FIX vector has been initiated in hemophilia B patients, but in this trial the vector is administered via hepatic artery injection to target the liver which may be the natural source of FIX production.

Several clinical trials of AAV vectors in the central nervous system have begun. Patients suffering from Parkinson's disease (PD) are being administered, by coinjection into the subthalamic nucleus (STN) region of the brain, two different AAV2 vectors each expressing one of the isoforms of glutamic acid decarboxylase.[19] Canavan disease is a childhood leukodystrophy that results from an autosomal recessive mutation in the gene for aspartoacyclase (ASPA) that causes accumulation of the toxic metabolite *N*-acetyl-aspartate. A clinical trial is underway in patients aged between 3 months and 6 years who are being administered an AAV2–ASPA vector in up to six sites in the frontal, varietal, and occipital regions of the brain.[20]

Hereditary emphysema results from mutations in the gene for the protease alpha-1-anti-trypsin (α1AT), and a clinical trial of intramuscular administration of AAV2–α1AT vector in patients with this disease has begun.[21] Rheumatoid arthritis (RA) is a chronic autoimmune disorder that causes significant disability including inflammatory joint disease, and TNF-α is a clinically validated target. In an induced arthritis model in Lewis rats, administration of an AAV vector encoding soluble rat TNF receptor:fc fusion gene, either systemically (intramuscular), or locally (intraarticular), resulted in profound suppression of arthritis.[22] Based upon this, a phase I double-blind, placebo-controlled dose escalation trial has begun in patients with rheumatoid arthritis to assess the safety of intraarticular injection of an AAV2 vector expressing a soluble human TNF receptor:fc fusion gene. The long-term durability of transgene expression in the muscle suggested that AAV vectors may have utility as vaccines.[23] An AAV2 vector expressing HIV genes, administered by intramuscular injection, is currently being tested in phase I clinical trials in seronegative subjects in Europe.

CONCLUSION

There is increasingly rapid progress in developing therapeutic applications of AAV vectors. The early clinical testing of AAV vectors for the treatment of CF was extremely important in initiating the regulatory environment for AAV vectors and demonstrating the inherent good safety profile of AAV vectors. As more groups have extended investigations to additional in vivo models, the potential utility of AAV vectors as therapeutic gene delivery vehicles has gained more widespread interest. The development of more sophisticated production systems for AAV vectors has enhanced both the quantity and quality of vectors that can be produced. Additional work to understand the intracellular metabolism of AAV vectors and modify the transduction efficiency by judicious choice of serotype or by modification of the capsid structure will expand the use and potency of AAV vectors. It is likely that in the next few years there will be a significant increase in clinical testing of AAV vectors for additional therapeutic applications.

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Adenoviral Gene Therapy

A

Willemijn M. Gommans
Hidde J. Haisma
Marianne G. Rots
Groningen University, Groningen, The Netherlands

INTRODUCTION

Over the last decade gene therapy has evolved as a promising method for the treatment of many diseases. To deliver the genetic material into cells, a viral vector can be utilized. One of the most efficient vectors available at this moment is the adenovirus. Incorporation of specific transgenes into the genome of the adenovirus ensures that the genetic material will be delivered into the nucleus after viral infection. One multigenetic disease which receives much attention in the research area is cancer. Many attempts are made to tackle this still largely incurable disease with gene therapy. The adenovirus may serve as a vector to deliver toxic genes to the malignant cells, but, on the other hand, this virus can also be used as a tool by itself to kill these cells. In the latter approach, the virus may replicate specifically in tumor cells, causing cell lysis and release of new virus progeny.

THE ADENOVIRUS AS GENE THERAPY VECTOR

Our understanding about the biology of the adenovirus has increased enormously in the last decades, and today this virus is commonly used as a vector for gene transfer.[1] This knowledge about the adenovirus has led to a fast progression in the development of new gene therapeutic agents. The adenovirus is known for its efficient infection of dividing and nondividing cells, resulting in an efficient delivery of therapeutic genes to the site of action. Other advantages for the use of adenoviral vectors are the possibility to produce these viruses in very high titers and that they are relatively harmless.

To increase the safety measurements of adenoviral vectors in gene therapy, most adenoviral vectors used are unable to replicate. This elimination of replication is caused by the deletion of the E1A and E1B region of the adenoviral genome. This region is necessary for the adenovirus to replicate. The E3 region of the adenoviral genome may also be deleted to increase the space in the genome available for the insertion of transgenes up to 7.5 kb. The E3 region is not required for replication of the adenovirus, but includes genes that are able to interfere with the defense system of the host cell after infection.

Currently, the realization that clinical applications of adenoviral vectors encounter limitations becomes more and more clear. Although adenoviruses are among the most efficient vectors used in gene therapy, they are not always efficient enough. This is especially true for cancer gene therapy, where it is necessary to kill every tumor cell, otherwise the malignant tissue will reoccur. One intratumoral injected dose of adenoviral vectors will infect maximally 10% of the tumor cells, thus the therapeutic effect will be limited. Another major limitation of adenoviral vectors is the biodistribution of the adenovirus. When administrated intravenously, the majority of the virus ends up in the liver, where it can cause severe liver toxicity. Therefore a large proportion of research is conducted on the untargeting of the liver.

There are two main routes to untarget the liver and to achieve the specific gene therapeutic effect in particular sites of the body. The first one is transductional retargeting and the second one is transcriptional retargeting. Transductional retargeting changes the cell entry route of the virus, whereas transcriptional retargeting restricts the expression of genes to the target cells (Fig. 1).

RETARGETING OF ADENOVIRAL VECTORS

The adenovirus contains a protein capsid with protruding fibers. These fiber proteins form a trimer and the C-terminal region of these fibers is called the fiber knob. The fiber knob recognizes and binds to the host cell via the coxsackievirus–adenovirus receptor (CAR), which is present on many cell types. Upon binding to CAR, secondary interactions between the adenovirus and cell surface receptors cause the internalization of the adenovirus. By shielding or modifying the fiber of the adenovirus, the native tropism can be altered. One possibility is to construct bispecific antibodies that on one side bind to the adenoviral fiber knob and on the other side will bind to an antigen present on target cells. This way, the adenovirus will be retargeted to cells that express this specific antigen. A second possibility is to construct

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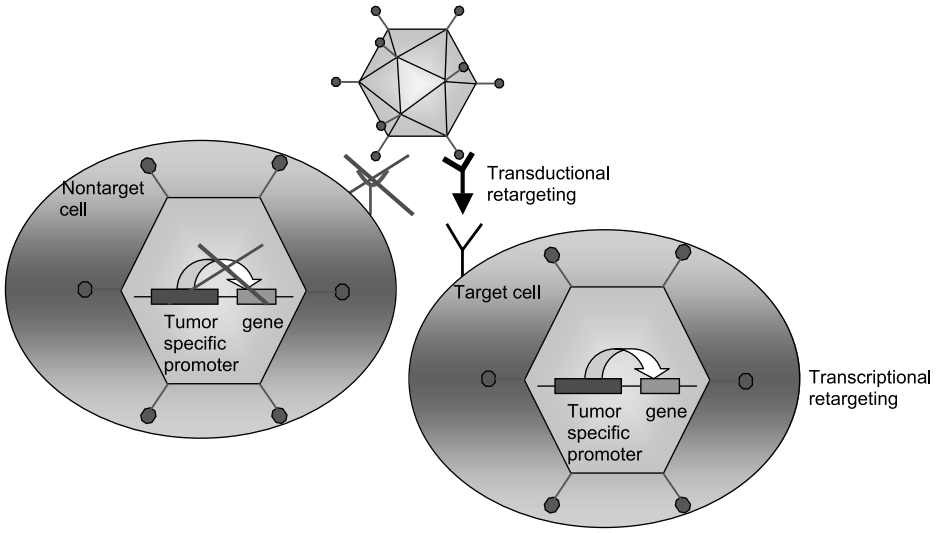

Fig. 1 To achieve transgene expression specifically in the tissue of interest it is possible to either change the homing of the virus (transductional retargeting) or to alter the expression profile of the transgene (transcriptional retargeting). (*View this art in color at www.dekker.com.*)

an adenobody, fusing an antibody directed against the fiber knob with a ligand for a receptor present on the cell type of interest. A third possibility to modify the tropism of the adenovirus is to genetically alter the fiber knob itself. It is possible to genetically insert sequences to be expressed within the fiber knob that are recognized by a certain receptor present on the cell type of interest without modifying the interactions within the trimer itself. However, it has to be noted that although interactions between the CAR receptor and the adenoviral fiber knob play an important role in cell uptake in vitro, these interactions might not play a crucial role in vivo.[2] Adenoviral particles containing a mutation within the fiber knob which abolished binding to the CAR receptor were systemically administrated in nonhuman primates. Surprisingly, the biodistribution of this ablated adenovirus was similar compared to the nonmodified adenovirus. This study showed that other receptors such as the heparan sulfate glycosaminoglycans (HSG) might play an important role in liver uptake. The construction of an adenovirus which is ablated for interactions between the virus and the HSG receptors can be a promising way to untarget the liver. Another approach to prevent uptake by the liver is PEG-ylation of the adenovirus.[3] Coating of the adenovirus by polyethylene glycol (PEG) will shield the adenovirus and prevent uptake by the liver. It is feasible to bind a homing device onto the PEG. This way the PEG-ylated adenovirus will be directed to the site of interest.[3]

Besides transductional retargeting of the adenovirus to the tissue or cell type of interest, it is also possible to transcriptionally retarget the adenoviral vector. Transcriptional retargeting is the modification of the transgene expression profile to increase the specificity of gene transcription towards a certain tissue or cell type. Such increased specificity can be obtained by placing the gene of interest under control of a tumor- or tissue-specific promoter. This way, the gene of interest will only be transcribed in cells where the promoter is active.

ONCOLYTIC ADENOVIRUSES

In the battle against cancer it is very important to kill every tumor cell present; otherwise, the remaining malignant cells will continue to grow and form new tumors. Although adenoviral vectors are one of the most efficient vectors used, one injected dose is thought to infect maximally 10% of the tumor cells. Thus it is necessary to increase the efficiency of adenoviral infection. An approach to increase this efficiency is to allow the virus to replicate, thereby increasing the amount of infectious particles. Replication of the adenovirus inside these cells will eventually lead to cell lysis, thereby releasing newly formed virions within the tumor mass. The subsequent viral generations will continue this cycle of infection, replication, and cell killing, thereby killing more and more tumor cells, resulting in the elimination of the tumor mass. Obviously, adenoviral replication should be confined to the tumor cells. Adenoviruses that can only replicate inside malignant cells are termed conditionally replicating adenoviruses (CRAds).[4]

Two different approaches can be distinguished in constructing CRAds, generating either type I or type II CRAds. Type I CRAds contain a specific deletion in the adenoviral genome based on genes that are differentially expressed between tumor and normal cells. Due to this mutation normal cells are not permissive to adenoviral replication. In tumor cells this host cell defense mechanism is deregulated, thereby creating the perfect environment for the virus to replicate.

A well-known example of a type I CRAd is the adenovirus Onyx-015, also known as *dl*1520.[5] This virus contains a deletion in the adenoviral E1B-region. As mentioned before, the E1A and E1B regions are important for the replication of the adenovirus. The adenoviral E1B 55-kDa gene codes for a protein that inhibits p53 function which is an essential step in viral replication. Onyx-015 is deleted for E1B 55 kDa, and thus unable to inhibit p53. Therefore in normal cells, where p53 is functionally present, Onyx-015 will not be able to replicate as p53 cannot be inhibited by this virus. However, in most tumor cells the function of p53 is already disturbed and the ability of the virus to inactivate p53 is not required anymore. This causes selective adenoviral replication in p53-deficient tumor cells. At this moment, phase I, II, and III clinical trails have been conducted with limited success.

Type II CRAds are adenoviruses where the expression of genes essential for adenoviral replication are under the control of a tumor-specific promoter. When this promoter is active, expression of this gene will result in replication of the virus. These promoters should mainly be active in tumor cells to ensure that replication predominantly occurs inside the malignant tissue. An example of a tissue-specific promoter is the prostate-specific antigen (PSA) promoter which is highly active in PSA-producing prostate cells and shows limited activity in other tissues. Placing a gene essential for replication directly under the control of the PSA promoter directs adenoviral replication primarily to prostate cells that express PSA.[6] Thus this adenovirus will replicate inside prostate (tumor) tissue while sparing the other tissues. At the moment, phase I and II trials are being conducted. Another example of a tumor-specific promoter is the telomerase promoter, which is active in more than 80% of all tumors. An adenovirus which has the expression of a replication essential gene under control of the telomerase promoter can therefore replicate in a broad range of tumor types. Other examples of tissue- or tumor-specific promoters are the epithelial gycoprotein-2 promoter, which is active in most epithelial-derived cancers making it a good candidate for the treatment of many tumor types, and the tyrosinase promoter, which is highly active in melanoma cells.

Although the theoretical idea behind type I and type II CRAds is very attractive, some drawbacks exist. In the case of an adenovirus where the expression of the replication essential gene E1 was restricted to melanoma cells using the tyrosinase promoter, replication was shown to be specific for melanoma cells at low infectious units. However, at a higher dosage replication was also demonstrated in nonmelanoma cells.[7] This loss of specificity might be due to the presence of adenoviral promoter-like sequences upstream of the tyrosinase promoter. These sequences may interfere with the specific regulation of the tyrosinase promoter, causing activation of transcription in cells which lack actual tyrosinase promoter activity. Another possibility is the presence of E1-like proteins inside the cell, compensating for the lack of adenoviral E1 expression in nonmelanoma cells.

DOUBLE-CONTROLLED CONDITIONALLY REPLICATING ADENOVIRUSES

After the systemic administration of adenoviral vectors, most of the virus ends up in the liver. When adenoviral replication is not strictly restricted to certain cell types or tissues, severe liver damage might occur due to adenoviral replication and consequential lysis of the liver cells. Because of this importance to restrict adenoviral replication, solutions have to be found for the observed aspecific replication of CRAds. One solution is a double-controlled conditionally replicating adenovirus (dcCRAd).[8] In a dcCRAd not one but two replication essential genes are controlled by two tumor-specific promoters. For example, both the adenoviral E1A and E1B genes have been placed under the control of two different tumor-specific promoters. Also, studies have already been conducted with both the E1A and the E4 gene under control of two different tumor-specific promoters.[9] E1A, E1B, and E4 are all examples of adenoviral genes that are essential for replication of the adenovirus. The approach of a dcCRAd preferentially includes a more general promoter. For example, the telomerase promoter which has shown activity in more than 80% of all tumors and which is inactive in liver cells. The telomerase promoter might work in concert with a tissue-specific promoter such as the tyrosinase promoter to restrict the expression of two genes essential for adenoviral replication. Only in melanoma cells which contain both telomerase and tyrosinase activity will replication occur.

Another approach for designing a dcCRAd is to combine a type I and a type II CRAd. On one hand, these adenoviruses contain a deletion in the adenoviral genome to prevent replication in cells without a disturbed cell cycle. On the other hand, these viruses also contain a tumor-specific promoter to restrict the expression of adenoviral genes essential for replication to cells where this promoter is active.

CONCLUSION

Adenoviruses can be utilized as a vector for the delivery of genetic material into cells. They possess many valuable characteristics, making them good candidates as gene therapy vectors. However, a major obstacle for clinical applications of adenoviral gene therapy vectors is the biodistribution of the virus. When administrated systemically, over 90% of the administrated adenovirus ends up in the liver where it can cause liver toxicity. Two different approaches are being pursued to deal with this problem, referred to as either transductional or transcriptional retargeting.

Transductional retargeting is focused on preventing the liver uptake. Either by modifying or shielding the fiber knob, thereby inhibiting binding to the CAR receptor, or by shielding the complete adenovirus by means of PEGylation. The next step is the addition of a homing device for retargeting to the tissue or cell type of interest.

Transcriptional retargeting does not prevent liver uptake, but gene expression will only occur in the target tissue or cell type. This can be accomplished by placing the transgene under the control of a tumor- or tissue-specific promoter. When genes essential for adenoviral replication are placed under control of such a promoter selective replication will occur, thereby enhancing the efficiency of infection.

By combining these two fields of expertise it is possible to construct an adenovirus that, on one hand, circumvents the liver because it is transductional retargeted and homes to the tissue of interest. On the other hand, this adenovirus will selectively express the transgene in the targeted tissue.[10] This means that specificity is increased and therefore it is possible to systemically administrate a higher dosage of the adenovirus, increasing the efficiency of adenoviral gene therapy. It is also feasible to transductionally retarget CRAds. In this case more infectious viral particles will reach the tumor environment, increasing the initial infectious dose and subsequent infection efficiency.

Although much progress is made in the transductional and transcriptional retargeting of adenoviral vectors, there are still hurdles to overcome. Success in the clinic using CRAds is not convincing. One problem might be that tumors not only consist of tumor cells but also of connective tissue impairing the spread of the adenovirus throughout the tumor tissue. However, the combination of adenoviral vectors and chemotherapy seems to result in synergistic antitumor effects. Compared to other nonviral and viral vectors, the adenovirus is among the most efficient vectors currently used and is relatively harmless. Progress is made very rapidly and the use of adenoviruses in the clinic is a promising method for the treatment of cancer.

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Alagille Syndrome

Michelle Hadchouel
Michèle Meunier-Rotival
Inserm U347, Le Kremlin Bicêtre, France

INTRODUCTION

Alagille syndrome (AGS; Mendelian Inheritance in Man 118450) was described more than 30 years ago as a genetic entity characterized by five major features: chronic cholestasis owing to paucity of interlobular bile ducts, peripheral pulmonary stenosis, butterfly-like vertebrae, posterior embryotoxon, and peculiar facies. Alagille syndrome is inherited in an autosomal dominant manner. The high variability of phenotypic findings led rapidly to a modification of the definition. It is widely accepted that individuals presenting with three of the five main features are considered to have AGS. As initial descriptions, minor less-frequent features were added to the list of criteria including other manifestations involving previously identified targets such as the vascular system, the bones, and the eye, and those involving previously unspecified organs or tissues such as the ear, the pancreas, the kidney, and the intestine. The disease-causing gene is Jagged1 that encodes a protein belonging to the family of Notch ligands. There are now more than 226 described intragenic mutations of the *JAGGED1* (*JAG1*) gene, none corresponding to the intracellular part of the protein. *JAG1* mutations are identified in roughly 70% of patients meeting criteria for AGS leaving 30% of patients with no detectable alteration of the coding sequence. As most often in human disease there is no genotype/phenotype correlation.

The expression pattern of *JAG1* had enabled the manifestations already observed to be explained and to open a field for investigations to evidence other system involvement. The role of the Notch signaling pathway during normal liver and bile duct development is still unclear and the mechanism of action of the mutated *JAG1* gene remains to be elucidated.[5] Haploinsufficiency is supported by the entire deletion of *JAG1* in few patients, but a dominant negative effect of a truncated JAG1 protein cannot be excluded.

CLINICAL DESCRIPTION

The syndrome was initially described as the association of five major features: paucity of interlobular bile ducts, peripheral pulmonary artery stenosis, butterfly-like vertebral arch defect, posterior embryotoxon, and a peculiar facies.[1] However, minor features such as renal abnormalities, skeletal defect, high-pitched voice, and mental and growth retardation were also described. In addition, some patients occasionally present with clinical features including other arterial stenosis, small bowel atresia, deafness, and diabetes.[2]

Because of the variable expressivity of the syndrome, individuals presenting with three of the five main features are considered to have AGS.[1] All associations are observed and liver or heart manifestations may be absent. Liver and heart involvement are present in 95% of the patients, posterior embryotoxon in 80%, and vertebral arch defect in 65%.[2]

Hepatic Manifestations

In a series of 163 children with liver involvement, 132 presented with neonatal cholestatic jaundice, and among the 31 patients who did not present with neonatal jaundice 5 became jaundiced later.[3] Neonatal cholestasis may be complete with acholic stools. The evolution is characterized by persistent or intermittent icterus, but usually biological cholestasis persists. Pruritus, hypercholesterolemia, xanthomas, and fat-soluble vitamin malabsorption are nonspecific consequences of the cholestasis. In some patients, unexplained attenuation or disappearance of pruritus and xanthomas occurs along with decrease in cholesterol level. Bile duct paucity is usually demonstrated by liver biopsy. This biopsy could be unnecessary in patients with complete syndrome.[4]

Cardiovascular Manifestations

Peripheral pulmonic stenosis is the characteristic cardiovascular abnormality of AGS, but complex intracardiac disease especially tetralogy of Fallot is not uncommon. The incidence of more diffuse vascular disease, including aortic coarctation or stenosis of other main arteries, is probably underestimated.[5]

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Vertebral Abnormalities

Although vertebral abnormalities have no functional consequences, they are important because they are useful for diagnosis. They consist of a defect of fusion between the two anlagen of the vertebral body giving the aspect of butterfly-like vertebrae. They are mainly observed at the dorsal level.

Ocular Manifestations

The most common finding in AGS patients is posterior embryotoxon, which is the more minor form of eye anterior chamber abnormality. Posterior embryotoxon has no consequence on visual acuity and is present in 10–15% of normal individuals. All the spectrum of eye anterior chamber abnormalities may be observed including glaucoma. Retinal changes such as pigmentary retinitis were formerly ascribed to vitamin deficiency, but progressive blindness may occur even with normal vitamin level.[6]

Peculiar Facies

Although the specificity of peculiar facies has been a matter of debate, there is a facial resemblance among the patients who have a prominent forehead, deep-set eyes, mild hypertelorism, a straight nose, and a small pointed chin without true dysmorphy.

Renal Manifestations

Renal abnormalities are observed approximately in 60% of AGS patients and could be considered as a sixth major feature. Tubular acidosis is frequent during infancy and usually disappears, but renal failure may occur in adulthood. Abnormalities of renal development such as renal cysts are common.[3,7–9]

DIFFERENTIAL DIAGNOSIS

The diagnosis of Alagille syndrome is primarily based on the presence of at least three out of the five main features. The differential diagnosis of each feature of the syndrome will result in a very long and tedious list. Association of butterfly vertebra and cardiac defects has been reported in patients with deletions of 22q11. Atrial septal defect associated with eye anterior chamber anomaly may be observed in Turner syndrome.

The main issue of differential diagnosis concerns diagnosis of neonatal cholestasis. The incidence of neonatal cholestasis is thought to be 1 in 2000 live births; the incidence of extrahepatic biliary atresia is between 1 in 8000, and 1 in 15,000 live births; and AGS incidence is probably between 1 in 70,000, and 1 in 100,000 live births. Complete and permanent acholic stools lasting more than 7 days suggest extrahepatic biliary atresia but may be observed in AGS. Even if biliary hypoplasia has been evidenced by microscopic examination of the biliary remnant of AGS patients, true atresia has never been observed. Moreover, it has been suggested that prognosis is worse in AGS patients who had undergone hepatoportoenterostosmy. Therefore it is important to look for extrahepatic features of AGS before surgery; conversely, cardiac abnormalities may be associated with biliary atresia, and needle liver biopsy is helpful in such situations where timing for surgery is crucial for prognosis of extrahepatic biliary atresia.[4]

MANAGEMENT AND PROGNOSIS

Most of the time the diagnosis is suspected in an infant with heart defect or cholestasis.

The diagnosis relies on:

- Clinical examination: facies, icterus, xanthomas, cardiac murmur, and liver enlargement.
- Chest radiographs: butterfly-vertebrae.
- Ophthalmologic examination: embryotoxon and other anterior chamber anomalies, retinitis.

Further evaluation includes:

Renal ultrasound examination and function tests.
Ear examination and audiogram.

There is no specific treatment, and except for the presence of complex congenital heart diseases the presence or severity of a clinical feature carries no predictive value.

Complex cardiac disease may require surgery; pulmonary stenosis is usually well tolerated and does not progress. When liver involvement is present, careful nutritional management is mandatory especially if liver transplantation is indicated. Supplementary lipid-soluble vitamins must be given regularly either orally or by intramuscular injections. Relief of pruritus is a major problem; ursodeoxycholic acid may be used but rifampicin and opioid-antagonists are more effective. The prognosis of liver disease is worse in children who present with neonatal cholestatic jaundice. However, severe liver complications are possible even after late onset of liver disease, demanding follow-up throughout life. Liver transplantation is eventually necessary in roughly 30% of patients.[3,8,9]

Spontaneous intracranial bleeding is a widely recognized complication, and AGS patients are at special risk for bleeding, either spontaneous or during surgery or other medical procedures. This should be taken into account before deciding on an invasive procedure.[10]

MOLECULAR GENETICS

The autosomal mode of transmission with reduced penetrance suggested by analysis of a limited number of families was confirmed by a segregation analysis. The proportion of sporadic cases when both parents are clinically normal was estimated to be 50%. These findings and the variability of expression of disease within families make genetic counseling difficult until the identification of *JAG1* mutations.[14]

JAG1 Is So Far the Only Responsible Gene

Identification of deletions of the short arm of chromosome 20 allows assigning AGS locus to 20p12. *JAG1* gene was mapped to this region, and mutations in the coding sequence were identified in AGS patients.[11,12]

Catalogue of the Mutations of the *JAG1* Gene

Most of the published data were obtain by SSCP followed by sequencing of exons with modified profile. There are now more than 226 described intragenic mutations of the *JAGGED1* gene, none corresponding to the intracellular part of the protein and there is no clustering or ''hotspots.''[13] Most of the mutations (66%) introduce premature stop codon, but all kinds of mutations including total gene deletion (5%), missense (14%), and splicing (15%) have been observed.[14] A number of polymorphisms have been described and in the absence of a functional study, the distinction between missense mutations and polymorphism relies on the usual criteria. Most of the missense mutations are in the EGF-like repeats, the cysteine-rich region, or affect amino acids conserved in JAG1. *JAG1* mutations are identified in roughly 70% of patients meeting the criteria for AGS, leaving 30% of patients with no detectable alteration of the coding sequence.[14,15]

Transmission and Phenotype–Genotype Correlation

Two thirds of the *JAG1* mutations are de novo. No phenotypic difference is observed between patients with or without visible rearrangement in 20p12, neither between patients with and without an identified mutation in *JAG1*. Furthermore, the number and the severity of the features vary among patients carrying the same mutations even within the same family. A significant frequency of mosaicism for *JAG1* mutations has been reported.[13]

Normal and Abnormal *JAG1* Gene Product

JAG1 gene encodes a transmembrane protein belonging to the family of Notch ligands. Notch signaling pathway seems to control the ability of a broad spectrum of precursor cells to progress to a more differentiated state both during development and oncogenesis.[16,17] Analysis of JAG1 expression during human development demonstrates that renal manifestations and other miscellaneous features previously described were not coincidental.[18]

The mechanism causing AGS may involve haploinsufficiency or a dominant negative effect. Most of the mutations (70%) introduce premature stop codon, which is expected to result in truncated secreted proteins. Such truncated Notch ligands in Drosophila have a dominant negative effect.[19] However, corresponding mRNA may be degraded by nonsense mediated RNA decay, resulting in a haploinsufficiency state comparable to the entire deletion of the gene.[20]

JAG1 mutations are identified in 70% of AGS patients; although alteration outside the coding sequence is still possible, the involvement of other components of Notch signaling pathway either as another disease-causing gene or as a modifier is very likely. This hypothesis is supported by recent demonstration that hypomorphic allele of Notch2 may be both responsible for bile duct paucity and acts as a genetic modifier of Jag1 haploinsufficiency in a mice model.[21]

GENETIC COUNSELING AND PRENATAL TESTING

There is a general agreement for considering that genetic testing is not necessary for the diagnosis of AGS which relies mainly on clinical examination. But genetic testing of the proband is mandatory before genetic counseling. This testing includes karyotyping and fluorescent in situ hybridization (FISH) looking for deletion, and if negative sequencing of *JAG1* gene coding sequence exists.

Genetic Counseling

Risk for the sibs of a proband

If a mutation of JAG1 is present, prenatal genetic testing is available, and testing of the parents will allow to determine whether the mutation is transmitted or de novo.

If the mutation is transmitted, the risk in sibs is 50%.
If the mutation is de novo, the risk for sibs is not null because of possible germline mosaicism (estimated to be 8% in a series) but no figure could be precise.

If there is no mutation, clinical examination of the parents including liver function tests, cardiac evaluation, chest radiograph, and ophthalmologic examination must be undertaken. The presence of AGS peculiar facies in the parents must be considered with caution if isolated.

If one parent is considered as affected, the risk for sibs is 50%.
If there is no affected parent, determined through genetic counseling as mentioned above, the risk for sibs is not null but difficult to assess.

Risk for offspring of a proband

The offspring of an AGS patient has a 50% chance of inheriting the syndrome.

Prenatal testing

Prenatal molecular testing, if available, can be performed on cells obtained by chorionic villous sampling at 10–12 weeks of gestation or amniocentesis at 16–18 weeks of gestation.

Fetal ultrasound examination may show cardiac defects and kidney abnormalities.

In all cases, due to high variable expressivity, it is impossible to predict the number and the severity of the features in affected sib or offspring of a proband.

CONCLUSION

Alagille syndrome was initially described as the association of five major features: paucity of interlobular bile ducts, peripheral pulmonary artery stenosis, butterfly-like vertebral arch defect, posterior embryotoxon, and a peculiar facies. Because of the variable expressivity of the syndrome, individuals presenting with three of the five main features are considered to have AGS. There is a general agreement for considering that genetic testing is not necessary for the diagnosis of AGS which relies mainly on clinical examination. But genetic testing of the proband is mandatory before genetic counseling. This testing includes karyotyping and FISH looking for deletion, and if negative sequencing of *JAG1* gene coding sequence exists. In all cases, due to high variable expressivity, it is impossible to predict the number and the severity of the features in affected sib or offspring of a proband. *JAG1* mutations are identified in 70% of AGS patients, although alteration outside the coding sequence is still possible, involvement of other components of Notch signaling pathway either as another disease-causing gene or as a modifier is very likely and is under study.

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Allele-Specific Oligonucleotide Hybridization

Damian Labuda
Maja Krajinovic
Stéphane Bourgeois
Université de Montréal, Montreal, Quebec, Canada

INTRODUCTION

Allele-specific oligonucleotide (ASO) hybridization was first applied to probe genomic DNA samples for the presence of point mutations causing sickle cell anemia and α_1-antitrypsin deficiency. In both cases, the authors used nonadecanucleotides (19-mers) as ASO probes. Although one probe was complementary to the normal allele, the second one matched the mutant allele. Because a mismatched base pair decreases the stability of a nucleic acid duplex, appropriate adjustment of the hybridization and washing temperatures permitted discrimination between the normal, mutant, and heterozygous genotype. With genomic DNA samples, this technique could have been used only for the genotyping of single copy genes. Once the polymerase chain reaction (PCR) has been introduced, this approach could be adapted to almost any genomic fragment by using diverse sources of amplifiable DNA.

Single nucleotide polymorphisms as well as small insertions or deletions (for simplicity, collectively referred to as SNPs) are the greatest source of genomic variability. Most mutations causing hereditary disorders belong to this category as well. Because of their functional impact and/or their usefulness as markers in mapping and association studies, SNPs are frequently typed in the context of population genetic surveys as well as in diagnostic and/or genetic epidemiological analyses. The wide applicability of ASO hybridization (especially in its combination with PCR), the absence of need for costly investments, and straightforward laboratory implementation made this technique very popular as a routine genotyping tool.

EXPERIMENTAL

Genotyping by ASO applies to previously ascertained polymorphisms and/or mutations and is not intended to discover new DNA variants. Through PCR amplification, one aims at a single polymorphic site, but it is also customary to target a number of variant sites residing within a longer amplifiable DNA segment[1] or even those scattered on unrelated genomic fragments that can be simultaneously amplified in a multiplex PCR.[2]

Following PCR, amplicons are immobilized on a solid support by dot blotting and ultraviolet (UV) fixation,[3] usually in duplicates to allow parallel probing of both alleles with the corresponding ASO probes. Typical result of ASO hybridization is illustrated in Fig. 1. SNPs are usually biallelic; even if they are not, the same detection principles apply to triallelic sites or small haplotypes composed of two polymorphic sites, one next to another, except that more than two probes could then be needed. Usually ASO probes are designed as 15-mers to 20-mers, with the polymorphic site in the middle of the sequence and not less than three nucleotides from the end. The shorter is the oligonucleotide probe, the more pronounced is the effect of mispairing at the polymorphic site, thus facilitating discrimination between the alleles. However, an overall duplex stability counts as well; too short a probe could render a signal of a fully matched complex unstable and compromise its detection. In our hands, 15-mer ASO probes worked well, whereas more than 20-mers were also recommended by others.

In a classical ASO protocol, great emphasis is made on the choice of the hybridization temperature to discriminate between fully matched and mismatched probes.[5] Hybridization is carried out a few degrees below the melting temperature (T_m) of the fully matched probe. In practice, the specificity (i.e., discrimination between the alleles) is achieved through posthybridization washes that selectively eliminate the mismatched, less stable probe from the target. The washing temperature, time, and salt concentration are experimentally determined and are typically ''personalized'' in each laboratory. In an alternative dynamic ASO protocol,[6] rather than using constant temperature, the hybridization step covers a temperature gradient, from above the melting point down to room temperature (75–30°C), such that each probe has an opportunity to find its optimal hybridization conditions. To prevent the formation of the mismatched complex between the labelled probe and the opposite allele, the unlabelled opposite oligonucleotide is added in 15-fold or greater excess to the hybridization mixture. Such blocking of the mismatched binding by using the opposite allele probe as a competitor has been used before.[7,8] It appears that the dynamic ASO makes full use of the presence of the competitor. The limitation in

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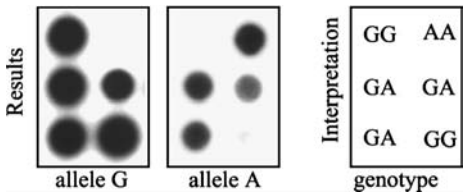

Fig. 1 ASO hybridization of G/A polymorphism at position 80 in RFC1 (reduced folate carrier) described earlier by Laverdiere et al.[4] The PCR product of 207-bp encompassing $G_{80}A$ polymorphic sites was transferred in duplicate to a HYBOND N^+ (Amersham Pharmacia Biotech) membrane. Identical twin membranes were hybridized with the ASO probe specific for the G_{80} (5′ gcacacgaggCgccg) or A_{80} (5′ gcacacgaggtgccg). Hybridization with ASO-specific probe was carried out at 45°C in a ×20 excess of the nonlabeled probe for the other variant allele.

the probe design is that both of them have to be from the same strand, ideally of the same sequence and differing only at the polymorphic site. However, the ASO for one allele serves, at the same time, as the competitor for the second allele, and vice versa. Following hybridization, dot blots are removed from the solution, rinsed at room temperature to wash out excess nonbound radioactivity, and exposed. No additional specificity-increasing washes are required. The most important advantage of dynamic ASO is that the same hybridization conditions work for most of the systems. As a consequence, multiple hybridizations can be simultaneously carried out in the same hybridization oven, disregarding different T_m values of the ASO probes used.

Suggested Protocols

Dot blotting

Twenty microliters of post-PCR mixture is denatured by the addition of 180 μL of 0.4 M NaOH, 20 mM EDTA, which may include 4 μL of 0.02% Orange II (optional spotting dye used to trace the blotting accuracy). Equal portions of the resulting solution are spotted in parallel on two previously water-wet Hybond N^+ nylon membranes (Amersham Pharmacia Biotech), thus creating two exact dot blot replicates of the analyzed DNA segments. Manifold is usually used to transfer DNA onto the membrane but direct spotting can also be used. DNA is then fixed on the membranes by UV irradiation (e.g., using light of 254 nm at 120,000 μJ/cm^2 in Stratalinker 1800; Stratagene) and the membrane is subsequently washed to remove NaOH and excess salt.

Hybridization

Membranes are prehybridized at 75°C for 30 min (Boekel Big Shot hybridization oven) in 25 mL of 1× SSPE [150 mM NaCl, 10 mM NaH_2PO, 1.1 mM EDTA, pH 7.4, 0.75 M NaCl, 70 mM Tris/HCl, pH 7.4, containing 1% sodium dodecyl sulfate (SDS) and 200 μg/mL heparin]. ASO probe (15-mer) is 5′-labeled using γ-[^{32}P]-ATP (6000 Ci/mmol; Amersham Pharmacia Biotech) and T4 kinase (Gibco BRL). Usually the volume of hybridization mixture is increased by 5 mL for each additional membrane. The ASO probe is added to the hybridization mixture to a final concentration of 50 pM accompanied with the opposite allele competitor at 750 pM. Hybridization is then carried out at a decreasing temperature by letting the oven cool down to 30°C and leaving 30 min for equilibration at this temperature. After two brief washings in 2× SSPE containing 1% SDS at room temperature, the membranes are exposed at −80°C with intensifying screens or read by Phosphoimager. Most of the polymorphisms do not require any special adjustment of this protocol and work outright. If the background appears too high and simple shortening of the time of exposure does not help, it can be eliminated: 1) by increasing the competitor/probe ratio; 2) by increasing the probe concentration (keeping the same competitor/probe ratio); or 3) by decreasing the amount of dot-blotted target DNA. Rarely, design of new ASO probes is required.

TECHNICAL COMMENTS

Probe Design

1. It is known that both strands of the DNA are not necessarily equivalent as probes in hybridization. This effect was observed with longer probes[9] but also applies to short oligonucleotides. Purine-rich oligonucleotides have a tendency to be better binders to a longer template than their pyrimidine-rich equivalents.[10] The former would maintain a more rigid structure, stabilized by stacking interactions because of the presence of purines, thus favoring template binding. The latter that are more coiled need additional energy to adopt helical extended structure consistent with double-stranded duplex formation. Note that pyrimidine-rich complement within a longer template is stabilized by stacking with its nearest neighbors from 5′ and 3′ sides through a dangling-ends effect, such that it is less coiled than its isolated/naked oligonucleotide complement. In conclusion, choosing (+) rather than (−) strand for ASO design

may modify the kinetics of duplex formation and its stability, and can be used to improve the test.
2. Although the rule of thumb is to place the probed polymorphic site in the middle of ASO, it is advisable to readjust its position if both flanking sequences differ in stability because of uneven composition in (G+C)/(A+T) and/or unequal redistribution of pyrimidines and purines.

Hybridization Conditions

1. Hybridization of short oligonucleotides is usually very fast, requiring milliseconds rather than minutes to occur. Hybridization times prescribed by the protocol reflect the time needed to equilibrate the temperature and concentrations and to assure the uniform contact of hybridization solution with the membrane.
2. Note that for a 500-nucleotide-long amplification, 100 ng of PCR product, typically found in a 10 μL reaction aliquot, corresponds to 0.32 pmol. When applied per single spot, it leads to about 30 pmol of the target DNA on a single 96-spot membrane. Therefore the probe at 50 pM concentration or 1.25 pmol/25 mL is in shortage as compared to the target. Thus, increasing the number of membranes may require an increase in probe concentration, or decrease in the concentration of spotted PCR products to keep background low and signal-to-noise high.

CONCLUSION

First ASO application in sickle cell anemia[12] and α_1-antitrypsin deficiency[13] were subsequently expanded, since the advent of PCR, to a variety of systems.[14] It was used to analyze disease-causing mutations,[15] study the mechanism of carcinogenesis,[16] or distinguish different haplotypes of the major histocompatibility locus.[17] ASO hybridization also found large application in studies of candidate susceptibility loci in complex disorders,[18,19] as well as in investigations of genetic variants predisposing individuals to adverse drug reaction.[1] ASO takes advantage of the difference in stability between the fully complementary and the mismatched DNA duplex. This difference is used to distinguish between allelic states differing as little as by a single nucleotide. Typically, two oligonucleotide 15-mer ASO probes are designed, each complementary to one of the alleles. In a classical protocol, hybridization is carried out at a temperature favoring the formation of a fully matched duplex, whereas the binding specificity is essentially obtained through stringent post-hybridization washes. In a dynamic ASO protocol, hybridization starts well above the melting point and a decreasing temperature gradient is applied. The labelled probe ''finds'' its target during the cooling of the hybridization mixture. Formation of the mismatched complex is prevented by the presence of the competitor probe that is fully complementary to the opposite allele. ASO hybridization finds application in diagnostic and genetic epidemiological surveys, is reliable, and is easy to implement.

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Alpha-1-Antitrypsin Deficiency

Noor Kalsheker
Kevin Morgan
Queen's Medical Center, Nottingham, U.K.

INTRODUCTION

Alpha-1-antitrypsin (AAT), also referred to as SERPIN A1, is the most abundant serine protease inhibitor (PI; serpin) in human plasma. It is a glycoprotein with broad inhibitory capacity against a variety of serine proteases and its main physiological target is neutrophil elastase. The three-dimensional structure of AAT and other serpins has provided major insights into the mechanism of inhibition of cognate proteases. AAT is a globular protein with an exposed reactive center loop (RCL). The protease docks on to the RCL, and cleavage of a methionine–serine bond at positions 358–359 in the loop results in a major conformational change. The loop inserts into a space in the A-sheet as a strand, and the protease then moves to the opposite pole of the molecule. During this translocation process, the catalytic site of the serine protease is distorted so that it is rendered inactive (Fig. 1). The PI complex is then rapidly removed from the circulation. This is a very efficient mechanism for controlling serine protease activity.

GENETIC VARIATION

The *AAT* gene is highly polymorphic. Historically, variants were characterized by gel electrophoresis of serum and initially divided into three groups according to their migration [medium (M), fast (F), and slow (S)], and, conventionally, these variants are preceded by PI. With advances in electrophoresis technology, further variants were identified based on migration characteristics in a pH 4–5 isoelectric focusing (IEF) gel. Anodal variants were assigned letters from the beginning of the alphabet. Several bands are observed on IEF, resulting from the degree of glycosylation. In a pH gradient of 4–5, AAT variants migrate as two major bands designated 4 and 6, and three minor bands 2, 7, and 8.

The migration of these bands is altered by amino acid substitutions (Fig. 2). At least four common M subtypes have been identified (designated M1–M4), with two other rare M subtypes (M5 and M6) and two common deficiency variants (designated S and Z) also arising from single-base substitutions. Other rare deficiency variants are characterized by a combination of the letter of the most closely migrating variant, and the origin of the place of the oldest living carrier. Examples of some of the variants in which the mutations have been characterized are shown in Table 1.

A mean normal plasma concentration of less than 11 μM is classified as deficient, and concentrations for the different common variants are typically in the range of 20–53 μM for MM, 18–52 μM for MS, 24–48 μM for SS, 15–42 μM for MZ, 10–23 μM for SZ, and 3.4–7.0 μM for ZZ.[2] The range of concentrations for PI ZZ falls well below this threshold, as does the very lower end of SZ, and both contribute as risk factors for disease. PI Null variants are rare, and there is considerable heterogeneity of mutations that cause them (Table 1).

THE *AAT* GENE

The 12-kb gene is located on the long arm of chromosome 14 in a cluster of other serpins, and two major promoters have been identified: one for hepatocytes,[3] the cell type accounting for most of the circulating *AAT*, and the other, which is effective in other tissues such as monocytes and lung alveolar epithelial cells.[4] The gene is made up of four coding exons and three noncoding exons, and, during mRNA expression, the noncoding exons are differentially spliced. A diagrammatic representation of the gene is shown in Fig. 3.

AAT GENE REGULATION

AAT is an acute-phase protein, and plasma concentrations can increase two- to threefold during inflammation. These increases are mainly mediated by the cytokines interleukin-6 and oncostatin M.[5] In hepatocytes, basal expression is regulated by the hepatocyte 5′ promoter, but during the acute-phase response, a 3′ enhancer plays a major role in modulating the cytokine-induced response.[6]

AAT DEFICIENCY AND DISEASE

Homozygous Z individuals are predisposed to developing childhood cirrhosis[7] and pulmonary emphysema in early

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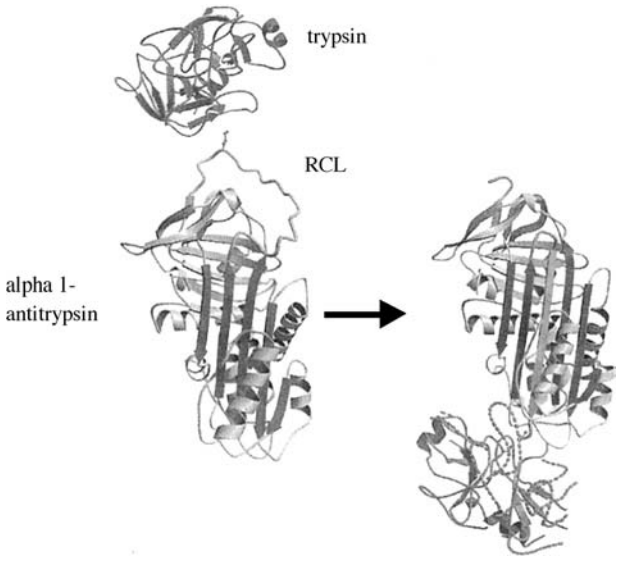

Fig. 1 Interaction of the protease trypsin with AAT. Trypsin docks on to the exposed RCL, cleaves the RCL, and the loop inserts into a sheet. The protease is then translocated to the opposite pole of the molecule and the active site of the protease is distorted. (See Ref. [1].)

adult life, particularly in cigarette smokers.[8] The Z variant is prevalent in northern Europeans, with a frequency of about 1.5% in Sweden and Norway, and greater than 2% in Denmark, Latvia, and Estonia.[9] It is extremely rare in Asian and African populations. The Z protein is synthesized normally, but tends to polymerize in hepatocytes. The polymerization results from an intermolecular interaction in which one RCL inserts into a space in the A-sheet. The Z mutation favors polymerization and the accumulated aggregates cause damage to the hepatocytes, which can result in childhood cirrhosis.[10] Only about 3% of PI Z individuals develop severe liver disease that often requires liver transplantation—the only effective treatment for them.[11] In a prospective screening study carried out in 200,000 newborns in Sweden, 120 children with PI ZZ were followed up.[12] Eighteen percent of the PI Z children developed clinically recognizable liver abnormalities, 7.3% had prolonged obstructive jaundice with marked evidence of liver disease, 4.1% had prolonged jaundice with mild liver disease, and 6.4% had other abnormalities suggestive of liver disease such as hepatomegaly, splenomegaly, or unexplained failure to thrive. The most common presentation is the ''neonatal hepatitis syndrome,'' which is characterized by conjugated hyperbilirubinemia and raised serum transminases. Features of cholestasis generally appear between 4 days and 2 months of age, and can persist for up to 8 months. About 14–29% of individuals with neonatal hepatitis syndrome have been found to have AAT deficiency. Most of these cases resolve without apparent long-term consequences.

The association with pulmonary emphysema is related to cigarette smoking, highlighting an important interaction between genetic and environmental factors. This is thought to arise from the action of neutrophil elastase, a major protease of neutrophils released during an inflammatory state. Deficiency of AAT, its major inhibitor,

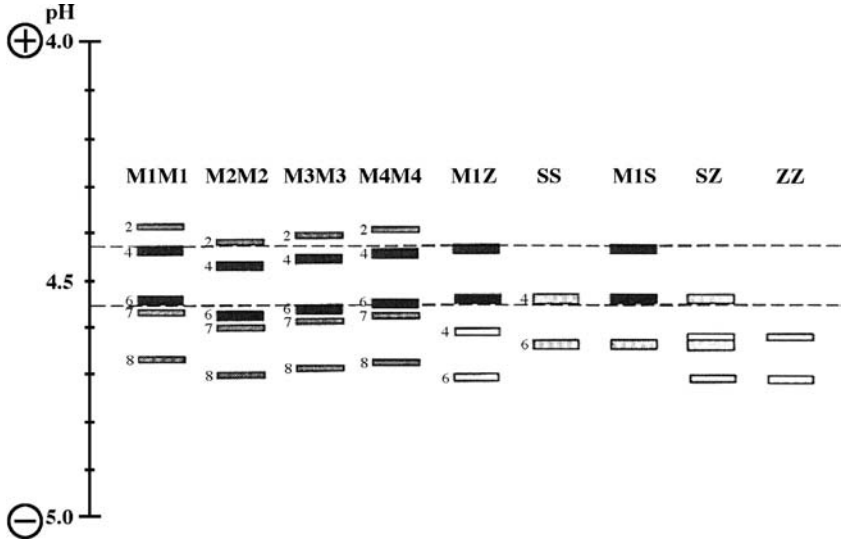

Fig. 2 AAT PI types separated according to their isoelectric point. A pH gradient of 4–5 is used and the variants migrate as two major bands, designated 4 and 6, and minor bands 2, 7, and 8. Variation in migration results from amino acid substitutions. The Z variant migrates toward the cathode and the S variant has an intermediate position to M and Z.

Table 1 Normal and deficiency variants of AAT

Allele	Base allele	Exon	Mutation
Normal AAT alleles			
M1 (Ala 213)			
M1 (Val 213)	M1 (Ala 213)	III	Ala 213 G*C*G 6 Val G*T*G
M3	M1 (Val 213)	V	Glu 376 GA*A* 6 Asp GA*C*
M2	M3	II	*Arg 101 CGT* 6 His CAT
M4	M1 (Val 213)	II	*Arg 101 CGT* 6 His CAT
Deficiency alleles			
Z	M1 (Ala 213)	V	Glu 342 *G*AG 6 Lys *A*AG
S	M1 (Val 213)	III	Glu 264 GAA 6 ValG*T*A
M heerleen	M1 (Ala 213)	V	Pro 369 C*C*C 6 Leu *T*C
M malton	M2	II	Phe 52 *TTC* 6 delete
M Palermo	M1 (Val 213)	II	Phe 51 *TT*C 6 delete
S iiyama	M1 (Val 213)	II	Phe 53 T*T*T 6 Ser T*C*C
Null alleles			
QO bellingham	M1 (Val 213)	III	Lys 217 *A*AG 6 stop 217 *T*AG
QO hongkong	M2	IV	Leu 318 *TC* 6 5′ shift 6 stop 334 TAA

The mutations shown are not an exhaustive list of variants, but represent the more common ones described.

results in progressive lung damage, because elastase has the propensity to destroy the connective tissues of the lung, causing destruction of the normal architecture of air spaces, thus reducing the surface area for gaseous exchange. Indeed, it was this association that led to the identification of AAT deficiency as a genetic disease. The methionine residue in the RCL is susceptible to oxidation, resulting in methionine sulphoxide, and the latter is a poor inhibitor[13] of neutrophil elastase, with a potency of 1/2000 that of the native inhibitor. The oxidation is more likely to occur during inflammation, and this may exacerbate genetic deficiency.

Pulmonary function is well preserved in early adult life in nonsmoking PI ZZ individuals. Pulmonary function declines naturally with age, but this rate of decline is markedly accelerated in PI ZZ individuals. Chronic obstructive pulmonary disease (COPD) in AAT deficiency generally occurs in the age range of 25–40 years, although not all PI ZZ individuals develop COPD. Larsson[14] estimated that smoking shortens median survival by 23 years.

A number of other disease associations have been described, although some of these are controversial. There is a well-recognized association with panniculitis, which responds to AAT replacement therapy. Other associations include arterial aneurysms, pancreatitis, and renal disease.

AAT REPLACEMENT THERAPY

The potential to treat lung disease by replacement to augment the lung antiprotease protective screen is available either by the intravenous route or by inhalation

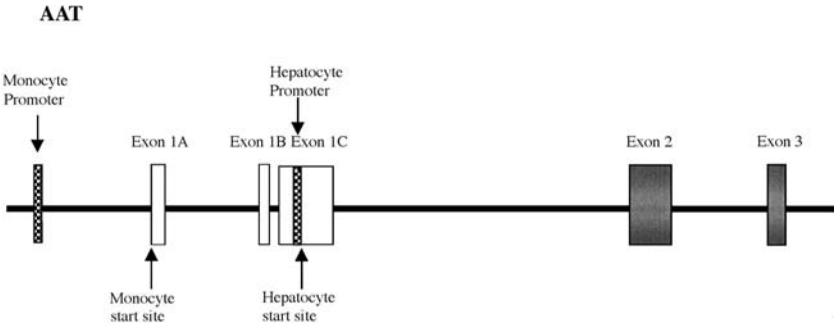

Fig. 3 A diagrammatic representation of the structure of the *AAT* gene. The coding exons are dark-shaded; untranslated exons are in lighter shade; and the location of tissue-specific promoters and a 3′ enhancer is also shown.

through the nasal route. Early studies (by Gadek et al.[15]) demonstrated that intravenous administration of AAT was feasible and biochemically effective. Studies have shown that aerosol administration too is effective.[16]

Although these studies demonstrate biochemical efficacy, it is not yet known whether they influence the outcome of the chronic respiratory disease associated with AAT deficiency. In a large study undertaken in the United States, 1129 subjects with severe AAT deficiency were monitored for 3.5–7 years. The 5-year mortality rate was 19%. The mortality rate was lower in those receiving augmentation therapy,[17] suggesting that long-term replacement therapy may be beneficial. There are anecdotal reports of successful treatment of panniculitis arising from AAT deficiency.

LABORATORY DIAGNOSIS OF AAT DEFICIENCY

Serum Quantification

Immunological methods are used to determine serum AAT concentrations, and serum standards are used to determine the concentration of the antigen in an unknown sample. Neonates have significantly lower serum concentrations compared to adults. In one study, the mean concentration observed in neonates for PI M was 23.8 ± 5.5 μM, compared with the PI M adults, where the concentration was 32.7 ± 8.7 μM.[18] A concentration of less than 11 μM can be used as a cutoff for deficiency states, and provides a useful screening test. It is important to ensure that these concentrations are observed in the basal state in the absence of an acute-phase reaction. It is therefore useful to measure concurrently serum C-reactive protein (CRP), one of the most sensitive markers for the acute-phase response, to exclude an acute-phase reaction. The value of screening newborns and the general population for deficiency remains controversial.

IEF and Genetic Testing

Many laboratories still use IEF to determine protein type.[19] This requires considerable expertise for the interpretation of banding patterns and relies on sample stability for reliable interpretation. The resolution can be improved with ultrathin polyacrylamide gels[20] and sensitivity can be improved by immunoblotting. Many laboratories use DNA-based methods involving polymerase chain reaction and either allele-specific amplification, for example, or the introduction of a restriction enzyme site at the location of the Z variant.[21,22] The DNA-based assays are designed to look for the common deficiency variants, S and Z, and will not detect rare variants. This is a limitation of the tests, as rare variants may be falsely reported as normal, and the use of serum AAT measurements minimizes this likelihood, but does not completely exclude it. DNA-based tests do offer a robust and reliable method for detecting the common S and Z alleles.

CONCLUSION

Much progress has been made in understanding the pathophysiology of AAT deficiency-related disease and its natural history. These developments provide new opportunities for developing specific treatments and may, in the future, result in more widespread screening for deficiency states if early intervention is shown to be effective.

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Alpha-Thalassemia

Renzo Galanello
Antonio Cao
Università degli Studi di Cagliari (UNICA), Cagliari, Sardinia, Italy

INTRODUCTION

Alpha-thalassemias are genetic defects, very common among some populations, characterized by the reduction (alpha$^+$) or complete absence (alpha$^\circ$) of alpha-globin chain production. A large number of alpha-thalassemia alleles have been described and their interaction results in a wide spectrum of hematological and clinical phenotypes, ranging from the silent carrier state to the very severe condition of hemoglobin Bart's hydrops fetalis syndrome, which is lethal in utero or soon after birth.

PREVALENCE AND DISTRIBUTION

Alpha$^+$-thalassemia is commonly found in Southeast Asia, China, Africa, reaching a very high prevalence, about 80%, in Papua (New Guinea).[1] In Mediterranean and Middle Eastern countries alpha$^+$-thalassemia carrier state has an incidence of 5–10% (up to 30% in Sardinia).[2] Alpha$^\circ$-thalassemia is found mainly in Southeast Asia; where, as a consequence, the derived clinical relevant genotypes (HbH disease and Hb Bart's hydrops fetalis) are also common. In Mediterranean and African population alpha$^\circ$-thalassemia is rare.

CLINICAL ASPECTS

Four clinical conditions of increased severity have been described (Table 1). The *silent carrier state* results from the presence of a single alpha-globin gene defect (−alpha/alpha alpha) and is characterized in the newborn by a very mild increased percentage (1–2%) of Hb Bart's, a tetramer of globin chains (gamma$_4$), whereas in the adult may be completely silent or associated with a moderate microcytosis and hypochromia with normal HbA_2.

Subjects with two residual functional alpha genes, either in *cis* (− −/alpha alpha) or in *trans* (−alpha/−alpha) clearly show the *alpha-thalassemia carrier state*, characterized in the newborn by a moderate increase (5–6%) of Hb Bart's, and in the adult by thalassemia-like red blood cell indices with normal HbA_2 and HbF.

HbH disease is a clinical condition resulting from the presence of a single functional alpha globin gene (− −/−alpha) or (− −/alpha$^{\text{nondeletion}}$ alpha). As a consequence there is a relative excess of beta globin chains, which form a beta$_4$ tetramer (HbH). HbH is unstable and precipitates inside the red cells and to some extent in erythroid precursors resulting in premature erythrocyte destruction. The syndrome of HbH disease shows a wide variability in clinical and hematological severity. The most significant features are microcytic and hypochromic hemolytic anemia, hepatosplenomegaly, and jaundice. Subjects with HbH disease may have acute episodes of hemolysis, requiring occasional blood transfusions, in response to oxidant drugs, fever, and infections.

The severity of HbH disease correlates with the degree of alpha chain deficiency. Therefore the more severe phenotypes are associated with interactions involving nondeletion alpha-thalassemia defects of the dominant alpha$_2$ gene including − −/alpha C$^{\text{constant}}$ S$^{\text{pring}}$ alpha, − −/alpha$^{\text{NcoI}}$ alpha , − −/alpha$^{\text{HphI}}$ alpha.[3] In general, patients with HbH disease can survive without any treatment. However, folic acid supplementation is recommended by some clinicians. Oxidant drugs should be avoided because of the risk of hemolytic crisis. In the presence of hypersplenism splenectomy may be performed, but the potential complications of severe life-threatening venous thrombosis should be considered. Two peculiar types of HbH disease have been reported. One is acquired, associated with myelodisplasia, and characterized by the presence of classical HbH inclusion bodies in red blood cells, often detectable levels of HbH and a severe microcytic and hypochromic anemia. The structural analysis of the alpha globin genes and of their flanking regions are normal, and a downregulation of the alpha genes by mutation in trans-acting genes has been proposed. The other is the alpha-thalassemia associated with mental retardation syndromes, which includes two different forms.[4] The first is characterized by a relatively mild mental retardation and variable facial and skeletal abnormalities. This form, known as ATR 16 syndrome, is due to extended deletions (1 to 2 Mb) of the short arm of chromosome 16. A second group of patients has a complex phenotype with quite uniform clinical features (hypertelorism, flat nasal bridge, triangular upturned nose, wide

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Table 1 Clinical phenotypes and corresponding genotypes of alpha-thalassemia

Clinical phenotype		Genotype
Silent carrier	Normal or mild microcytosis	−alpha/alpha alpha
Alpha thalassemia trait	Microcytosis hypochromia	−alpha/−alpha −−/alpha alpha alphand alpha/alpha alpha
HbH disease	Moderate (rarely severe) microcytic hypochromic haemolytic anemia, hepotosplenomegaly, mild jaundice	−−/−alpha −−/alphand alpha
Hb Bart's hydrops fetalis syndrome	Very severe anemia oedema, hepatosplenomegaly, death in utero or soon after birth	−−/−−

nd = nondeletion.

mouth, genital abnormalities) and severe mental retardation. These patients did not show structural changes of the alpha cluster and the transmission is X-linked (ATR-X syndrome). Mutations in an X-encoded gene, the XH2gene, a member of the SNF2 family of helicase/ATPases. It has been suggested that this gene is involved in a wide variety of cellular processes, such as transcriptional regulation, recombination, replication, and DNA repair.[4]

Hb Bart's hydrops fetalis syndrome is the most severe form of alpha-thalassemia. In most of the cases it is associated with the absent function of all four alpha globin genes (−−/−−). The affected fetus cannot produce any alpha globin to make HbF (alpha$_2$gamma$_2$) or HbA (alpha$_2$beta$_2$). Fetal blood contains only Hb Bart's (gamma$_4$) and small amounts of hemoglobins Portland (zeta$_2$gamma$_2$ and zeta$_2$beta$_2$). The resulting clinical features are those of severe anemia leading to asphyxia, hydrops fetalis, and stillbirth or neonatal death.[5] Other congenital defects have been reported, including cardiac skeletal and urogenital abnormalities. Maternal complications during pregnancy have been reported, including mild preeclampsia (hypertension and fluid retention with or without proteinuria), poly-oligo-hydramnios (increased or reduced accumulation of amniotic fluid, respectively). At present there is no effective treatment for Hb Bart's hydrops fetalis syndrome. Attempts of intrauterine transfusions or in utero hematopoietic stem cell transplantation have been unsuccessfully performed.

DIAGNOSIS

Identification of alpha-thalassemia carriers is difficult because they do not have typical changes in HbA2 or HbF. Carriers with −alpha/alpha/alpha and −−/alpha alpha genotypes have always reduced MCV and MCH, while −alpha/alpha alpha carriers may have normal red cell indices or only slightly reduced MCV and MCH. The hemoglobin pattern is normal.

Reliable diagnosis of the alpha-thalassemia carrier states can only be achieved by DNA analysis with PCR-based methods or globin chain synthesis analysis that shows an alpha/beta ratio lower than 0.9 (see below ''Molecular Diagnosis of Alpha-Thalassemia'').[6] Identification of alpha°-thalassamia carriers is important for the prevention of Hb Bart's hydrops fetalis syndrome. In the newborn the electrophoretic detection of the fast moving band of Hb Bart's is an indication of the presence of alpha-thalassemia. However, in some carriers Hb Bart's may be not detected.

HbH is easily detected as a fast-moving band by cellulose acetate electrophoresis. Another simple and very sensitive test consists in the detection of inclusion bodies in the red blood cells which can be generated by incubating peripheral blood with supravital stains.[6] Determination of alpha globin genotype by molecular methods is useful for prognosis of HbH disease, as the nondeletion forms are more severe than the deletion forms.[3] In the neonatal period subjects with HbH disease genotype can be detected by hemoglobin electrophoresis because they have elevated levels (−25%) of Hb Bart's. Hb Bart's hydrops fetalis syndrome can be prenatally identified by DNA analysis, fetal blood analysis, or using ultrasound (cardiothoracic ratio greater than 0.05).[6,7]

MOLECULAR GENETICS

The human alpha globin genes are duplicated and mapped in the telomeric region of chromosome 16 (16p 13.3), in a cluster containing an embryonic zeta$_2$, three pseudogenes (pseudo zeta$_1$, pseudo alpha$_1$, pseudo alpha$_2$) and one gene (theta$_1$) of unknown function (Fig. 1).[8] The alpha complex is arranged in the order of its expression during the development. There is a remarkable homology

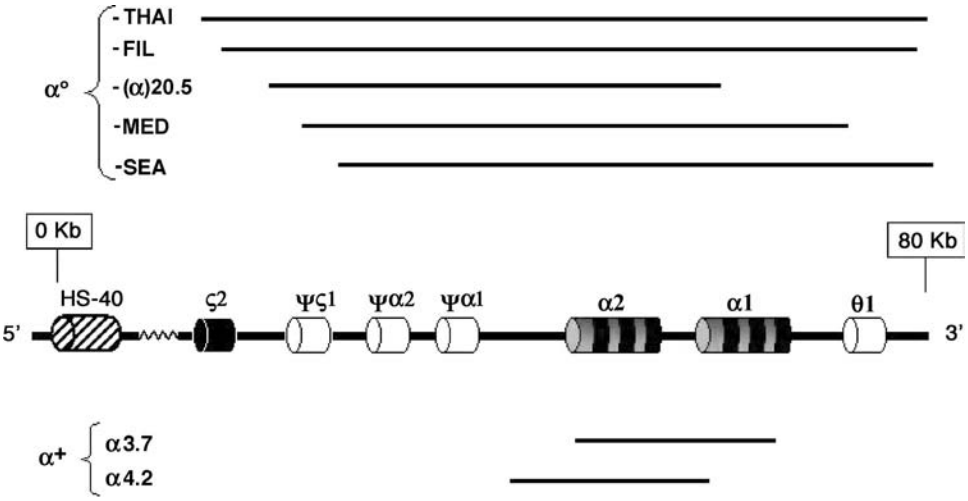

Fig. 1 Structure of alpha globin gene cluster and most common deletion alpha-thalassemia defects. (*View this art in color at www.dekker.com.*)

between $alpha_2$ and $alpha_1$ gene maintained during evolution through repeated rounds of gene conversion.[9] The level of transcription of the two alpha genes differs, encoding the $alpha_2$ gene two to three times more alpha globin than $alpha_1$ gene.[10] The different expression of the two alpha genes has implications for the amount of hemoglobin variant present in carriers of $alpha_1$ or $alpha_2$ globin mutations, and for the pathophysiology of the deletional and nondeletional forms of alpha-thalassemia. The presence and integrity of a DNA region, known as HS40, located 40 kb upstream of the alpha globin gene cluster are critical for the expression of alpha globin genes.[11] Deletions of this regulatory element produce alpha-thalassemia, although both alpha globin genes on the chromosome are intact. A deletion, removing the alpha 1 globin and the theta-globin genes, that results in the transcription of an antisense RNA, leading to silencing of $alpha_2$ gene and in the methylation of its CpG island, has been recently reported as a novel mechanism of human genetic disease.[12]

Deletion Alpha-Thalassemia

Alpha-thalassemia is caused most frequently by deletions of DNA that involve one or both alpha globin genes. The most common deletions remove a single alpha globin gene, resulting in the mild $alpha^+$-thalassemia phenotype ($-$alpha/alpha alpha). These deletions are produced by unequal crossing over within two highly homologous regions, extending for about 4 kb, that contain $alpha_1$ and $alpha_2$ gene.[13] Reciprocal recombination between Z boxes results in a chromosome with a 3.7-kb deletion containing only one alpha gene ($-alpha^{3.7}$), whereas recombination between X boxes produces a 4.2-kb deletion ($-alpha^{4.2}$) (Fig. 1). As a consequence of these recombinational events, chromosomes containing three alpha globin genes are produced.[14] The $-alpha^{3.7}$ and $-alpha^{4.2}$ deletions are the most common $alpha^+$-thalassemia determinants. Other rare deletions totally or partially remove one of the two alpha globin genes.

Extended deletions, from 100 to over 250 kb, removing all or part of the cluster including both alpha globin genes and sometimes the embryonic $zeta_2$ gene, result in $alpha^\circ$-thalassemia and hence no alpha chain synthesis occurs. Illegitimate recombination, reciprocal translocation, and truncation of chromosome 16 are the molecular mechanisms responsible for these deletions. More than 15 different $alpha^\circ$-thalassemia deletions have been described, the most common being the Southeast Asian, Filipino, and Mediterranean types (Fig. 1). Two deletions [$-(alpha)^{5.2}$ and $-(alpha)^{20\text{-}5}$] removing the alpha -2 and partially the $alpha_1$ globin gene also result in $alpha^\circ$-thalassemia.

Several different deletions involving the HS-40 regulatory region but leaving both alpha genes intact have been reported and all result in $alpha^\circ$-thalassemia.[14]

Nondeletion Alpha-Thalassemia

Less frequently, alpha-thalassemia is determined by nondeletion defects, which include single nucleotide substitutions or oligonucleotide deletions/insertions in regions critical for alpha globin gene expression.[14] Several molecular mechanisms (abnormalities of RNA splicing and of initiation of mRNA translation, frameshift and nonsense mutations, in-frame deletions, and chain termination mutations) have been described, the majority

occurring in the alpha$_2$ gene and producing alpha$^+$-thalassemia. Hb Constant Spring, the most common nondeletion defect that is present in Southeast Asian population, results from a mutation in the stop codon of alpha$_2$ globin gene (TAA→CAA, stop→Glu). The change of the stop codon to one amino acid allows mRNA translation to continue to the next in-phase stop codon located within the polyadenilation signal. The result is one alpha chain variant (Hb Constant Spring) elongated by 31 amino acids which is produced in a very low amount (~1%). The instability of the mRNA due to disruption of the untranslated region may be the reason for the reduced production of Hb Constant Spring.[15]

As for beta globin gene, mutations of alpha genes which result in the production of hyperunstable globin variants, such as Hb Quong Sze, (alpha 109 Leu→Pro) Hb Heraklion (alpha 137 pro→0), and Hb Agrinio (alpha 29 Leu→Pro), that are unable to assemble in stable tetramers and are thus rapidly degraded, might produce the phenotype of alpha-thalassemia.[14]

PRENATAL DIAGNOSIS

Prenatal diagnosis is always indicated for the prevention of Hb Bart's hydrops fetalis syndrome for the severity of the disease and even to avoid severe maternal complications during pregnancy.

HbH disease is not considered to be among those hemoglobinopathies targeted for prevention.[1]

MOLECULAR DIAGNOSIS OF ALPHA-THALASSEMIA

The presence of alpha-thalassemia, suspected on the basis of hematological and/or clinical findings [i.e., microcytosis with normal HbA_2 and F and normal serum iron status in carriers; microcytic and hypocromic hemolytic anemia with RBC inclusion bodies and a fast-moving band (HbH) at the hemoglobin electrophoresis; presence of Hb Bart's in the newborn], should be confirmed, if requested, by globin gene synthesis or even better by globin gene DNA analysis.

The exact definition of the genotype with molecular methods is relevant for genetic counseling in carriers and for prognosis in HbH disease patients. In the last few years several approaches based on polymerase chain reaction (PCR) have been developed for the detection of the most common deletional and nondeletional types of alpha-thalassemia (for references, see Ref. [16]).

The common deletion alpha$^\circ$- or alpha$^+$-thalassemias are detected using two primers (specific for each deletion type), which flank the deletion breakpoints. Amplification occurs only in the presence of the deletion, whereas it does not occur in normal subjects because the two primers are separated by too great a distance. This approach is known as GAP-PCR. As a control, DNA from a normal subject is simultaneously amplified using one of the primers flanking the breakpoint and a primer homologous to a DNA region deleted by the mutation. The less common deletion alleles have to be identified by Southern blot analysis.

Nondeletional forms of alpha thalassemia are detected by selective amplification of alpha$_1$ and alpha$_2$ genes, followed by restriction enzyme analysis, when the mutation creates or abolishes a cleavage site [i.e., *Nco*I for T→G alpha$_2$ initiation codon mutation, *Hph*I for the −5 bp (donor) alpha$_2$ IVSI-1 deletion, *Mse*I for Hb Constant Spring] or by Dot Blot analysis or ARMS with specific oligo-probes. A strategy for the diagnosis of all the known point mutations, which involves the combined application of denaturing gradient gel electrophoresis (DGGE) and single-strand conformational analysis (SSCA), followed by direct DNA sequencing, has been reported.[17]

Multiplex PCR assays including up to seven alpha-thalassemia deletional alleles or common Southeast Asian nondeletional alleles have been recently described and seem to give robust and reproducible results.[18–20]

CONCLUSION

Alpha-thalassemias are common hemoglobinopathies, very heterogeneous at clinical and molecular level. Four different clinical conditions of increased severity have been described: the silent carrier state, alpha-thalassemia trait, HbH disease, and Hb Bart's hydrops fetalis syndrome. HbH disease is a moderate, very rarely severe, microcytic, and hypochromic hemolytic anemia, whereas Hb Bart's hydrops fetalis syndrome is a condition not compatible with postnatal life. Deletion defects are more common than nondeletion defects. The carrier state is suspected by hematological methods, but the precise identification is possible with several PCR-based procedures or globin chain synthesis analysis. Prenatal diagnosis is only indicated for Hb Bart's hydrops fetalis syndrome.

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Alzheimer's Disease, Familial, Early Onset

Beatriz Marcheco Teruel
National Center of Medical Genetics, Havana, Cuba

INTRODUCTION

Alzheimer's disease (AD; OMIM no. 104300) is a progressive and degenerative disorder that attacks the brain, which was first described by Alois Alzheimer.[1] Alzheimer's disease is the most common type of dementia and is a major cause of death and disability in developed countries. The main symptoms of the disease are memory loss, cognitive impairment, deterioration of motor skills, and withdrawal from social contact. The clinical diagnosis can be difficult to establish and it is only confirmed by postmortem microscopic examination of the brain. The typical features of the neuropathological examination are: cerebral cortical atrophy, the presence of amyloid plaques in the extracellular space composed mainly of β-amyloid (Aβ) peptide, deposition of amyloid in the wall of blood vessels, and the presence of intraneuronal neurofibrillary tangles, consisting of hyperphosphorylated microtubule-associated protein tau.

Alzheimer's disease can be *familial* or *sporadic* and its complex etiology comprises both *genetic* and *environmental* factors. In most cases, the symptoms of the disease appear after 65 years of age (late-onset form); however, around 5% of all AD cases have an early onset (i.e., before age 65 years).[2,3] Sixty-one percent of patients with early-onset AD had a positive family history and 13% had affected individuals in at least three generations. A prevalence of early-onset AD of 41.2 per 100,000 for the population at risk (i.e., persons aged 40–59 years) has been reported.[4] The disease has severe emotional and financial consequences for individuals, families, and society.

CLINICAL DESCRIPTION

The early-onset form of AD generally runs in families with multiple affected individuals and it is mostly inherited as an autosomal dominant trait. The disease usually begins in the 40s or early 50s with a relatively rapid progression.

Alzheimer's disease is characterized by gradual loss of memory, decline in other cognitive functions, and decrease in functional capacity. Other common symptoms include confusion, poor judgment, language disturbance, agitation, withdrawal, and hallucinations. Some patients may develop seizures, Parkinsonian features, increased muscle tone, myoclonus, incontinence, and mutism.[5,6] Survival disease is variable in patients with AD, and they usually die of infections, with death occurring approximately 10 years after the onset of symptoms.

The clinical diagnosis of AD is based on criteria defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R, American Psychiatric Association 1987) and the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA).[7] The NINCDS-ADRDA criteria provide guidelines for a clinical diagnosis of probable and possible AD. A diagnosis of definite AD can be confirmed by neuropathological examination of the brain tissue either from biopsy or autopsy material. The criteria of the Neuropathology Task Force of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD)[8] have been commonly used to set the neuropathologic diagnosis of AD.

Differential diagnosis could be established in the first instance between the familial and sporadic forms of the disease over the basis of the presence of a positive family history and by taking into account that familial and sporadic cases appear (clinically and pathologically) to have the same phenotype. Other genetic causes of early-onset dementia as well as prion disease, fronto-temporal dementia, and CADASIL should be considered.

MANAGEMENT

There is currently no cure or prevention for AD because its basis are still not well understood. Pharmacological agents and psychosocial intervention may help to manage each symptom and provide temporary improvement in cognitive functioning for some patients. Cholinesterase inhibitors such as Tacrine (with hepatotoxic effects), Aricept, Exelon, and Remynil are the most used. Nonsteroidal antiinflammatory drugs (NSAIDs), estrogen replacement therapy (ERT), and lipid-lowering agents are also being investigated.

Effective management of AD also comprises educational information, counselling, and emotional support for patients, caregivers, and other family members.

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MOLECULAR GENETICS

Studies focusing on large families with the rare, early-onset, autosomal dominant form of the disease led to the discovery that mutations in the amyloid precursor protein (*APP*)[9] Presenilin 1 (*PSEN1*),[10,11] and Presenilin 2 (*PSEN2*)[12] genes cause AD.

Although these autosomal dominant families represent only a small percentage of the total AD cases, functional studies of the effects of the identified mutations have become an important way to dissect the causes and underlying disease mechanisms leading to AD. Mutations in all three genes produce an increase of plasma amyloid β (Aβ42),[13] which forms the core of neuritic plaques found in brains of people with AD.

The *APP* gene in chromosome 21 (region 21q11.2–q21.1) was the first gene to be associated with early-onset familial AD (AD1).[14] The original clue came from the observation that individuals with Down syndrome, who have chromosome 21 trisomy, invariably develop the clinical and pathological features of AD if they live over 30 years.[15]

The *APP* gene has 19 exons and encodes the amyloid precursor protein (Aβ) of 695–770 amino acids, which is proteolytically cleaved to form Aβ, the major component of amyloid plaques in the brain tissue. All *APP* mutations (16 mutations) occurs in exons 16 and 17.

AD1 accounts for no more than 10–15% of early-onset familial AD.[4] The mean age of AD1 onset is around the early 50s, with a range from 43 to 62 years.[3]

Mutations in *PSEN1* (AD3) located in chromosome 14 (region 14q24.3) are responsible for 30–70% of the AD cases with familial early onset. *PSEN1* gene contains 10 protein-coding exons and is predicted to encode a serpentine protein (467 amino acids) with 7–10 transmembrane domains. To date, at least 130 different mutations have been described in the *PSEN1* gene in more than 200 unrelated families (Fig. 1). Most mutations lie in exon 7 (28 mutations), exon 5 (27 mutations), and exon 8 (23 mutations). The majority of these are missense mutations giving rise to the substitution of a single amino acid (AD mutation database; http://molgen-ww.uia.ac.be/admutations/).

It has been speculated that most AD-related mutations result in a gain of function. Mutations in *PSEN1* alter the processing of βAPP by preferentially favoring the production of potentially toxic long-tailed Aβ peptides ending at residue 42 or 43.[16]

PSEN1 mutations are associated with the earliest age of onset (29–62 years) of the three early-onset form genes.[3] It has been described that families of different ethnic backgrounds with the same mutation also exhibit similar ages of onset.

The *PSEN2* gene (AD4), a rare cause of early-onset AD (2% of all early-onset familial AD), is located on chromosome 1 (region 1q31–q42) and it was found using sequence homology strategy to the PSEN1. The nine mutations in the *PSEN2* gene resulting in AD have been identified in 15 families with a broad range of onset age (40–88 years).[3,12,18,19]

The high similarity in clinical and neuropathological features between the early-onset familial and ''sporadic'' late-onset AD suggests that similar pathophysiological factors are involved.

In the near future, it is probable that other AD genes for early-onset familial AD will be identified because familial pedigrees with several affected members with the early-onset autosomal dominant form and not known mutations in the previously reported genes have been described.[20]

MOLECULAR GENETIC TESTING

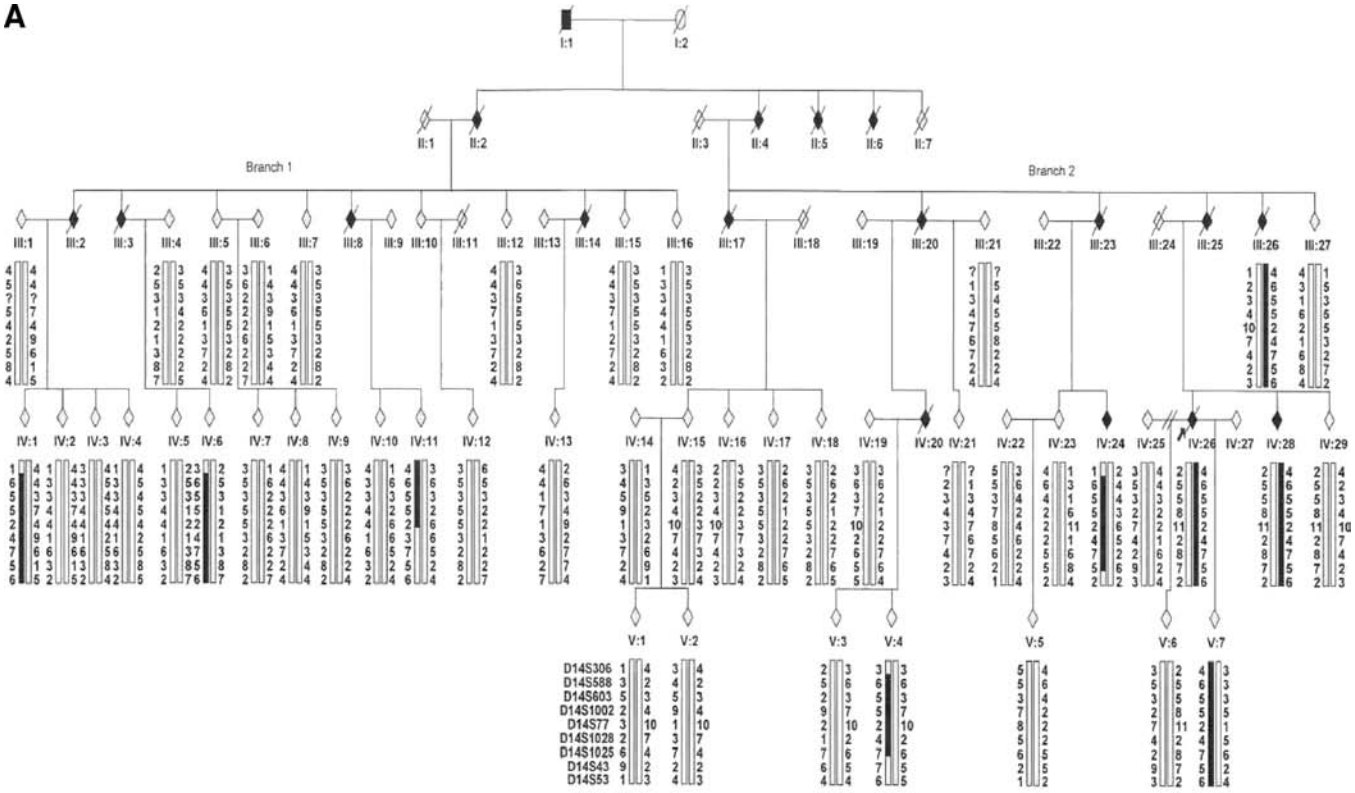
Currently, molecular diagnostic tests are available for AD. With regard to PSEN1 (AD3), it is possible to perform mutation analysis, sequence analysis, and mutation scanning on a clinical basis. For identification of a *PSEN1* mutation, a history of early-onset AD and another family member (principally a first-degree relative) also with early-onset AD is relevant.

The *PSEN2* gene is well characterized, but only nine mutations have been found; most of them are point mutations in the coding region, causing an amino acid change. On one of these mutations (a substitution of an isoleucine for an asparagine at codon 141), the DNA sequence change (AAC vs. ATC) creates a Sau 3a restriction enzyme cleavage site, whose mutation is easy to detect. For PSEN2, sequence analysis and mutation scanning are available on a clinical basis.

Forty-three families with 16 APP (AD1) mutations have been defined. It is probable that because of the limited number of affected individuals, the molecular genetic testing is available only on a research basis by direct DNA study.

GENETIC COUNSELING

In general, genetic testing for AD, diagnosis or disease prediction, is a controversial point and is presently recommended in rare, early-onset familial cases with presumable autosomal dominant inheritance only.[21] Such a testing is not useful in predicting age of onset and severity of symptoms.



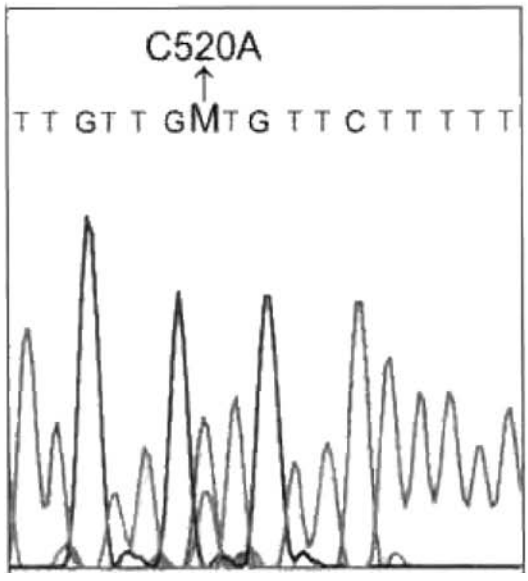


Fig. 1 (A) Pedigree of a large Cuban family with early-onset familial AD. Haplotypes corresponding to the chromosome 14q24.3 region are shown. Filled (black) symbols represent demented individuals. Gender and age are not revealed for reasons of confidentiality. Filled (black) bars indicate disease haplotype. (B) Direct sequencing revealed a heterozygous base change C A (C520A) in exon 6, which is predicted to cause an amino acid change from leucine to methionine in the transmembrana 3 domain (TMIII) of the Presenilin 1 protein. (From Ref. [17].) (*View this art in color at www.dekker.com.*)

Most individuals diagnosed with AD have had an affected parent or at least a second-degree relative with early onset of the disease.

The offspring of an affected individual has a 50% risk of having inherited the altered gene; such a chance is much lower when the parents of probands are clinically unaffected.

Predictive Testing

Many relatives whose family members suffer from the disease wonder about their personal risk of developing AD, but as for Huntington's disease, no prevention or efficient cure is available for AD and predictive testing of AD will become a profound dilemma for individuals at high risk for the disorder. The consequences of providing potentially devastating information to the individual and the family should be weighed against the principles of autonomy and self-determination of the family members.

Prenatal Diagnosis

For AD mutations, if the disease-causing allele of an affected family member has been previously identified, it is possible to carry out prenatal diagnosis by analysis of DNA extracted from fetal cells obtained by chorionic villus sampling or amniocentesis at 10–12 weeks of pregnancy or 16–18 weeks, respectively. In general, request for prenatal testing for adult-onset diseases is not common and many factors affect the uptake of prenatal testing for single gene conditions such as age of onset, severity of the disease, certainty of the test, and, especially, possibility of treatment. The decision is also influenced by legal and social rules of different countries and societies.

CONCLUSION

This review has focused on some of the most important aspects in understanding the occurrence of early-onset familial AD. Genetic factors are of established importance and are being intensively studied. If we are able to elucidate all the mechanisms leading to AD at the molecular level, then it will be possible to develop strategies for prevention and effective treatments either through targeted drugs or other environmental interventions. It will make be possible to improve the use of DNA testing to confirm a diagnosis of AD, or to make predictive statements for an individual about the particular risk of developing AD later in life.

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Aminoglycoside-Induced Deafness

Mustafa Tekin
Ankara University School of Medicine, Ankara, Turkey

INTRODUCTION

Our understanding of the genetic basis of aminoglycoside-induced deafness (AID) has greatly improved during the past 15 years. The discovery of the first mitochondrial mutation (A1555G) in 1993, leading to susceptibility to aminoglycoside ototoxicity, was the most remarkable success in this area. Many individuals from different parts of the world have since been identified with this mutation, suggesting that the number of people at risk for ototoxicity may be considerably high, especially in some populations. Two other mutations in the mitochondrial 12S ribosomal RNA (rRNA) gene, C1494T and 961delTinsC(n), have been implicated to cause AID.

OVERVIEW

Aminoglycoside antibiotics are used for the treatment of aerobic gram-negative—and, less commonly, gram-positive—bacterial infections. Streptomycin, the first aminoglycoside introduced in the 1940s, is still in use as first-line antituberculosis drug. Gentamycin, tobramycin, and amikacin are more commonly used in developed countries.[1,2]

The ototoxicity associated with the use of aminoglycosides was recognized soon after they became available, for which a loss of 15 dB or more at any frequency in one or both ears is a commonly used description.[2]

Aminoglycosides can cause deafness in almost all mammalian species when given in massive doses. However, individuals who experienced hearing loss following short-term aminoglycoside use at regular doses, sometimes with a family history of AID, were indicative of a genetic mechanism. It was first suggested in 1989 that the genetic defect might be in the mitochondrial DNA based on the reported pedigrees of AID.[3] It was later clearly demonstrated that the inheritance pattern was matrilineal[4] in a district of China where 167 of 763 (22%) deaf individuals had a history of aminoglycoside exposure. Three Chinese families with histories of AID and an Arab–Israeli family with maternally inherited nonsyndromic deafness (not associated with aminoglycoside exposure) were used to discover the genetic defect in the mitochondrial DNA.[1] As a result of this effort, an A→G transition at position 1555 of the 12S rRNA was identified.[1]

A1555G MUTATION IN THE 12S rRNA GENE

A1555G mutation can induce hearing loss in the presence or absence of exposure to aminoglycoside antibiotics. It was homoplasmic in almost all of the reported cases, although a low level of heteroplasmy has been reported.

There are currently available polymerase chain reaction (PCR)-based screening tests for this mutation. Following are the details of a simple PCR-restriction fragment length polymorphism (RFLP) test:[1]

Primers

Forward 5′-CCGCCATCTTCAGCAAACCCT-3′.
Reverse 5′-TGAAGTCTTAGCATGTACTGCTCG-3′.

Following amplification, PCR products are digested with the *Alw*26I restriction enzyme, of which one cleavage site is abolished when A1555G is present.

Biochemical and Molecular Evidence for the Pathogenicity of A1555G

Because aminoglycosides bind the small rRNA and exert their effect by inhibiting or inducing errors in protein synthesis, it is not surprising that mutations in the corresponding 12S rRNA in humans are responsible for AID.[1] Aminoglycosides bind at, or near, two regions of the *Escherichia coli* small rRNA and these regions, brought together in the secondary and tertiary structures, surround the decoding site of the ribosome (Fig. 1A).

The A1555G mutation introduces a new base pair at a region of the human 12S rRNA (Fig. 1B and C). Disruption of the base pairing at the corresponding region in *E. coli* results in resistance to aminoglycosides.[1] It was experimentally shown that the A1555G RNA analog stoichiometrically binds aminoglycosides with high affinity, whereas the wild type construct does not.[5]

In vitro experiments have confirmed the role of A1555G in AID.[6–8] Lymphoblastoid cell lines obtained from individuals of the original Arab–Israeli family

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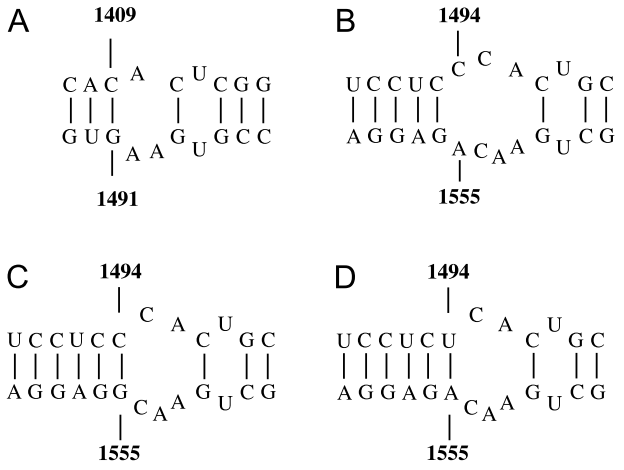

Fig. 1 Wild-type aminoglycoside binding site in *E. coli* 16S rRNA (A) and human 12S rRNA (B) (numbers are in *E. coli* numbering system in A). Positions of the A1555G (C) and C1494T (D) mutations.

showed a lower growth rate, mitochondrial protein synthesis, total oxygen consumption, and respiratory chain complexes-dependent respiration compared with control cell lines.[6] The severity of mitochondrial dysfunction in the mutant cell lines was correlated with the presence or absence of hearing loss in persons carrying the mutation.[6] Exposure of the cells to high concentrations of aminoglycosides significantly decreased the rates of growth and translation.[6,7] When the mitochondria with A1555G was transferred into human mtDNA-less cells, the transformant cells exhibited significantly lower growth rate, mitochondrial protein synthesis, and substrate-dependent respiration compared with control cells. These decreases were very similar in deaf and hearing members of the family with the mutation, suggesting that the nuclear gene(s) plays an important role(s) in deafness phenotype.[8]

Epidemiology

Because A1555G can be found in persons with completely normal hearing, AID, progressive and late-onset high-frequency hearing loss, and congenital- or prelingual-onset severe to profound deafness without aminoglycoside exposure, it is difficult to obtain its true frequency in a given population. Although A1555G was originally described and frequently found in patients coming from families in which other relatives also have AID, sporadic cases with or without AID,[9,10] patients coming from families with hearing loss showing maternal inheritance, or one of the forms of Mendelian inheritance without AID is reported.[11,12]

The proportion of cases with mutation among patients with AID has ranged from 3%[9] to 44%[13,14] in different populations with different ascertainment strategies (Table 1). If there is a family history of AID[14] or maternal inheritance in the pedigree,[11] the possibility of finding this mutation increases. A number of studies have demonstrated that A1555G is associated with different mitochondrial haplotypes,[11,25] although founder effects have also been identified in some families,[25] implying that this mutation has arisen several times in the past and is not limited to a certain ethnic group.

It is important, yet difficult, to find the frequency of A1555G in the hearing population. Based on their results of screening in matrilineal pedigrees, Lehtonen et al.[16] estimated that the minimum frequency of A1555G was 4.7/100,000 in northern Finland. Tang et al.[24] studied anonymized 1173 blood samples obtained for newborn screening in Texas and identified one positive result (9/10,000). Similarly, in a study from New Zealand,[26] A1555G was found in 1 of 206 unselected samples. However, it should be noted that the A1555G mutation was not found in approximately 1500 control samples studied as control in different populations.[1,11,17,21]

Phenotype Associated with the A1555G Mutation

Hearing loss usually manifests itself within 1–3 months following—sometimes even one dose of—aminoglycoside use.[27] The onset and severity of hearing loss are unrelated to age at the time of exposure. The hearing loss is sensorineural (originating from the cochlear lesion) and, in most cases, is symmetrical—almost always involving the high frequencies.[27] The vestibular system appears not to be involved.

A significant portion of individuals with A1555G has normal hearing for their entire life in case they have not been exposed to aminoglycosides. In the absence of aminoglycoside exposure, the probability of an individual possessing the mtDNA A1555G mutation developing hearing loss has been estimated to be 40% by the age of 30 years, and 60% by the age of 60 years.[11]

Genetic Modifiers Implicated in A1555G-Related Deafness

The original Arab–Israeli family and additional families with A1555G without AID suggested the presence of a nuclear modifier gene. Extensive search using different linkage strategies yielded a region on chromosome 8, around the microsatellite marker D8S277.[28]

The common deafness gene *GJB2* and mitochondrial A7444G mutations have been reported to act as modifiers for A1555G in some studies,[21,29] although further

Table 1 Summary of published studies on the frequency of A1555G in different populations (country names in alphabetical order)

Country	Numbers of A1555G-detected/ screened families	Numbers of A1555G-detected/ AID	Numbers of A1555G-detected/ AID	Characteristics of the screened population	Control group	Reference
China	1/36 (3%)	1/36	—	Sporadic cases with AID	—	[9]
China	15/34 (44%)	15/34	15/34	Familial cases with AID	—	[14]
Denmark	2/85 (2.4%)	—	—	Varying degrees of hearing loss	—	[15]
Finland	2/117 (1.7%)	—	2/117	Families with MID	—	[16]
Germany	1/139 (0.7%)	—	—	Severe to profound congenital deafness	0/160	[17]
Hungary	0/56	—	—	Sporadic cases with severe to profound deafness	0/224	[17]
Indonesia	4/75 (5.3%)	NA	NA	70% profound; 24% severe; 3% moderate to severe; 3% moderate hearing loss	0/100	[18]
Italy	0/80	—	0/16	Moderate to severe hearing loss	—	[19]
Japan	7/138 (5.1%)	2/6	4/8 (without AID)	10% prelingual; 86% postlingual; 4% AID/47% sporadic; 53% familial	—	[20]
Japan	11/319 (3.4%)	7/21	—	18% profound; 10% severe; 34% moderate; 38% mild/92% sporadic; 8% familial	—	[10]
Japan	14/140 (10%)	13/22	—	Profound deafness	—	[10]
Mongolia	37/480 (7.7%)	NA	NA	Severe to profound deafness	0/389	[21]
Poland	3/125 (2.4%)	—	—	Severe to profound congenital deafness	0/89	[17]
Spain	42/209 (20%)	NA	28 (at least seven did not have AID)/30	(Approximately 50% congenital, 50% late-onset in authors' earlier report) 86% familial; 14% sporadic	0/200	[11,12]
Turkey	3/168 (1.8%)	—	—	Prelingual severe to profound deafness/57% familial; 43% sporadic	—	[22]
UK	0/202	0/1	0/10 (possible)	46% familial; 54% sporadic	—	[23]
US	7/41 (17%)	7/41	—	All probands with AID; four had familial AID	—	[13]

MID = Maternally inherited deafness; NA = not available.

studies failed to confirm these results in other populations.[12]

C1494T MUTATION IN THE 12S rRNA GENE

The homoplasmic C1494T mutation was recently identified in a large Chinese family with AID and maternal inheritance.[14] Twenty of 39 matrilineal relatives, who are supposed to carry the mutation, exhibited hearing loss. Clinical data have shown that the treatment with aminoglycosides can induce or worsen deafness in matrilineal relatives. The mutation occurs at a position where the A at position 1555 can make a pair with the mutant base (Fig. 1D). Additional data indicated that the cell lines derived from carriers showed a lower rate of total oxygen consumption and a significant increase in doubling time with the high concentration of aminoglycosides. Nuclear background probably plays a role in the deafness phenotype and AID with the C1494T mutation.[14]

OTHER MUTATIONS IN THE 12S rRNA GENE

Deletion of a T at position 961 and insertion of varying numbers of G were observed in a patient during a search in the mtDNA in 35 sporadic Chinese cases with AID.[30] The same mutation was not detected in 799 control samples.[30] This mutation was later found in an Italian family, in which five maternal relatives became deaf after aminoglycoside exposure.[31] However, in a recent study, 961delTinsC(n) was detected in 7 of 1173 anonymized blood samples in the United States.[24] This result suggests that either the role of this mutation is questionable in AID, or the population at risk for AID is extremely high in the United States.

The T1095C mutation was found in two Italian families.[19,32] In one family, the proband had Parkinson's disease, neuropathy, and a history of AID.[32] The second family with matrilineal inheritance of hearing loss included two maternal relatives of the proband who had histories of AID.[19] More data are needed to draw a firm conclusion about the role of this mutation in AID.

CONCLUSION

The multinational occurrence of the A1555G mutation, with high frequencies in some countries, makes the AID associated with this genetic change a public health problem. Until more precise data are available on the frequencies of this mutation and mechanisms of its action, in coordination with the nuclear or mitochondrial genome, a detailed family history for deafness, especially aminoglycoside-related deafness, will help decrease the number of cases with AID. Currently available PCR-based tests may be used when an aminoglycoside treatment is necessary. Using widely available, precise, less expensive, and rapid molecular screening tests for already known mutations in population screening is open for discussion. Identification of other genetic changes will certainly help create better strategies for large-scale screening.

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Amplification Refractory Mutation System PCR

Joannis E. Vamvakopoulos
Biomedicum Helsinki, Helsinki, Finland

INTRODUCTION

Several technologies for probing genetic variation have emerged in the past 20 years, many of which incorporate in vitro enzymatic DNA amplification by the polymerase chain reaction (PCR) in its modern, *Taq*-polymerase-based incarnation. Among them, amplification refractory mutation system (ARMS)-PCR stands out for its speed, simplicity, versatility, and low cost. Since its concurrent inception by six separate groups working independently,[1–6] these features of ARMS-PCR have established it as arguably the single, most popular genotyping technique. Evidence of this abounds in the scientific literature despite some confusing nomenclature issues that persist today (Table 1). The popularity of ARMS-PCR is also apparent by its continuous development over the years (Table 2). Today, ARMS-PCR embraces all formats, from single tube to array, and all conceivable applications, from SNP to universal genotyping.

AN OVERVIEW OF ARMS-PCR: FROM PRINCIPLES TO PRACTICE

Principle and Formats of ARMS-PCR

Accurate genotyping requires stringent allelic discrimination. ARMS-PCR relies on 3′-end primer mismatching to achieve a high degree of allelic specificity. As shown in Fig. 1, the original two-tube incarnation of ARMS-PCR utilizes five primers—two of these are allele-specific and complementary to the polymorphic site at their 3′ terminus, while a common companion primer serves to yield an allele-specific amplification product. Because *Taq* DNA polymerase lacks $3' \rightarrow 5'$ exonuclease activity,[7] allelic specificity is conferred by its inability to efficiently extend 3′ end-mismatched primers. Because most known SNPs are biallelic, two reactions (each containing either one of the allele-specific primers) are required for definitive genotype assignment. An additional pair of irrelevant, fully matched primers, yielding an amplicon of different size serves as internal control to flag false-negative reactions (i.e., those resulting from amplification failure).

Single-Tube ARMS-PCR

Table 2 lists key features of ARMS-PCR and those of numerous subsequent modifications. The initial crucial limitation of having to perform two PCR reactions to establish a single biallelic genotype, along with the associated requirement for an internal control reaction, were soon overcome with the development of various single-tube formats. Here both allele-specific primers are present in the same reaction and allelic discrimination is achieved by either varying the length of these primers[8–10] or by engineering them to amplify on opposite directions and produce amplicons of different lengths.[11–13]

Two-Reaction ARMS-PCR

While single-tube ARMS-PCR arguably represents a more refined, cost-effective, and high-throughput format, it also requires additional expertise, particularly in primer design. It is perhaps for this reason that conventional two-tube ARMS-PCR continued to evolve and has remained popular to date. Substantial modifications have been described that aimed at increasing both assay sensitivity[14] and specificity.[15,16] The concept of multiplex universal genotyping (MUG;[17]), which renders ARMS-PCR applicable to any type of genomic variation and particularly useful for defining mixed (i.e., single-nucleotide and length) polymorphic haplotypes, is the latest addition to an expanding collection of conventional ARMS-PCR formats.

Microarray ARMS-PCR

The advent of microarray technology promises to deliver rapid, high-throughput genotyping through the development of solid-phase, miniaturized assay formats. The forerunner of microarray ARMS-PCR came in the form of allele-specific primer extension, where arrayed allele-specific primers are queried with multiplex preamplified template DNA containing the polymorphisms of interest in the presence of reverse transcriptase and a mixture of ribonucleotides, one of which is fluorescently labeled. Practical difficulties in performing solid-phase PCR, as

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Table 1 Alternative designations for ARMS-PCR

Method designation	Reference
Mutation-specific PCR	[1]
Amplification refractory mutation system (ARMS)	[2]
Allele-specific PCR (AS-PCR)	[3–5]
PCR amplification of specific alleles (PASA)	[6]
PCR with sequence-specific primers (PCR-SSP)	[39]

well as the markedly higher signal-to-noise ratio achieved with RT, were the principal reasons for its use here instead of DNA polymerase. These limitations were recently overcome with the development of a two-phase, on-chip PCR platform that achieves simultaneous multiplex amplification of genomic DNA and immobilized allele-specific primer extension using *Taq* DNA polymerase.[18,19]

Design and Optimization of ARMS-PCR Assays

With its numerous formats, ARMS-PCR offers unparalleled flexibility in the design of genotyping assays. Major initial considerations guiding the design process itself include the number and type of genetic variation(s) under study; available equipment, personnel, and expertise in primer design; desired sample throughput; and cost. Once a suitable ARMS-PCR format has been selected, the issue of allele-specific amplicon detection should be separately addressed (reviewed elsewhere in this volume). The design process is complete following customization of the assay to the polymorphisms of interest (primer design), optimization of PCR conditions, and troubleshooting of any problems.

Optimization and Troubleshooting

For all its merits, ARMS-PCR is subject to the same limitations that apply to conventional PCR. Problems such as mispriming and primer dimer formation can be minimized by using suitable software for basic primer design. Complications in basic primer design arising from polymorphisms lying close to the SNP of interest can be overcome by strategically incorporating deoxyinosine (dI) residues into the primer sequence.[1]

Since the original description of ARMS-PCR, several authors have shown that primer 3′-terminal mismatch discrimination with DNA polymerases is not absolute; extension of such mismatches does occur, albeit at a much lower rate than that of a perfectly matched primer. Extension efficiency depends on several factors, primarily the nature of the mismatch[4,20–24] as well as its sequence context,[23] template abundance,[25] and dNTP concentration.[1,21] DNA-dependent DNA polymerases generally achieve better allelic discrimination than reverse transcriptases.[21,22]

While optimization of the PCR reaction itself is typically straightforward, the potential for discrimination failure occasionally necessitates troubleshooting of ARMS-PCR. Initial measures should focus on readily modifiable reaction components and might include reducing the number of amplification cycles,[24] the amount of template DNA,[25] dNTP concentration,[1,8] or performing the reaction under rapid-cycle conditions.[26] In rare instances where these measures would fail or prove difficult to implement (e.g., when running multiplex reactions or pooled DNA),

Table 2 Comparison of different platforms for ARMS-PCR

Method designation	Format	Special features	Reference
ARMS-PCR (AS-PCR; PASA)	Two tube	—	[1–6]
ADPL AS-PCR	Single tube	Seminested design	[8]
PAMSA	Single tube	—	[9]
Asymmetric AS-PCR	Two tube	Seminested design	[14]
Mutagenically separated AS-PCR	Single tube	Intraprimer mismatches	[10]
Competitive blocker AS-PCR	Two tube	CB primers	[15]
Gap-ligase chain reaction	Swo tube	*Taq* ligase	[16]
Bidirectional PASA	Single tube	Tailed AS primers	[11]
PCR-CTPP	Single tube	Tailless primers	[12]
Tetra-primer ARMS-PCR	Single tube	Long, tailless primers; asymmetric design	[13]
Microarray ARMS-PCR	Array	Solid-phase platform	[18,19]
MUG-PCR	Two tube	Universal genotyping platform	[17]

Abbreviations: ADPL = allele discrimination by primer length; PAMSA = PCR amplification of multiple specific alleles; CB = competitive blocker; CTPP = confronting two-pair primer; MUG = multiplex universal genotyping.

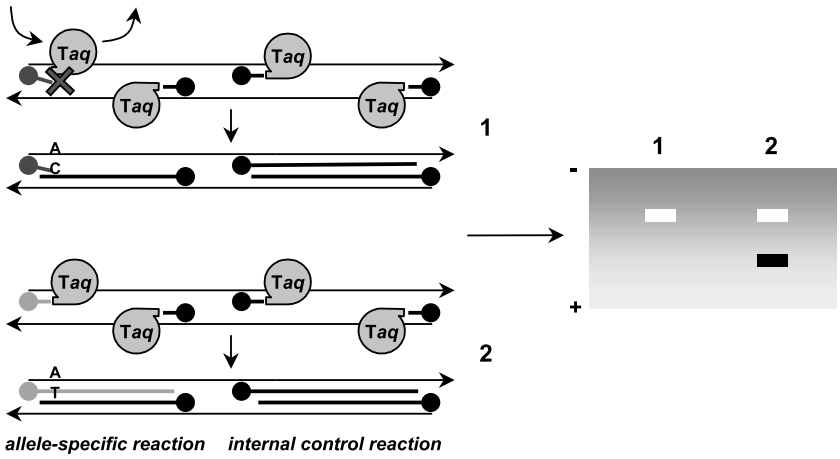

Fig. 1 Principle of ARMS-PCR (see text for details). (From Vamvakopoulos, J.E., 2001. A potential role for interleukin-1 in chronic allograft rejection. PhD thesis. University of Cambridge, U.K.) (*View this art in color at www.dekker.com.*)

further action should involve modification of the allele-specific primers[20,22,25,27–29] or use of depository oligos.[30]

ARMS-PCR relying on intercalating fluorescent dyes for amplicon detection may require additional trouble-shooting aimed at minimizing nonspecific fluorescence from primers and primer dimers. This issue becomes crucial for applications demanding optimal sensitivity, such as genotyping of DNA pools and monitoring minimal residual disease or the emergence of drug-resistant infectious strains in clinical samples. Primer design measures for minimizing primer dimer formation have been described;[31] alternatively, optimal signal-to-noise ratio in these instances can be achieved by simply raising the reaction pH to 11.7 before amplicon detection.[32]

APPLICATIONS OF ARMS-PCR: GENOTYPING AND BEYOND

The versatility and success of ARMS-PCR as a methodological tool can best be judged by its uses. Here I summarize some of the most important, diagnostically oriented applications of this technique.

Clinical Genetics and Cancer Management

ARMS-PCR was originally conceived mainly for the purpose of detecting cancer-related mutations[1] and for studying the genetic basis of heritable disorders.[2–6] In this respect the field of clinical genetics was quick to explore the potential of this technique: rapid, PCR-based assays for several genetic diseases where the causative mutation was known were developed soon after the first description of ARMS-PCR.[33,34] Furthermore, the power of ARMS-PCR to detect mutations with unparalleled sensitivity has revolutionized two key aspects of cancer management, namely molecular diagnosis[35] and the monitoring of minimal residual disease (MRD).[36,37]

Tissue Typing and Cell Therapeutics

HLA typing was one of the very first applications of ARMS-PCR and, in retrospect, the impact of this technique on histocompatibility testing has been momentous. Fugger et al.[38] pioneered the application of ARMS-PCR in this field, while Olerup, Zetterquist, and co-workers are credited with much of the work leading to the routine clinical application of this technique.[39,40] Today, ARMS-PCR is the bedrock of HLA typing. With new HLA specificities being integrated as they are discovered,[41] this situation looks set to persist long into the future.

Also of clinical relevance is the application of ARMS-PCR to the functional and quantitative study of cellular chimerism following cell or solid organ transplantation. Accurate quantitative measurement of cellular chimerism by ARMS-PCR is now feasible[42,43] and should greatly facilitate further advances in this field.

Studies of Interindividual Genetic Variation

The fact that ARMS-PCR is well suited to studying single nucleotide polymorphisms (SNPs), which account for over 80% of interindividual genetic variation, makes it

an ideal tool for related functional and epidemiological studies. Besides conventional, biallelic SNPs, ARMS-PCR is easily adaptable to the study of triallelic SNPs,[44] small insertions, and deletions.[45] Where marker informativeness becomes crucial (e.g., in mapping disease traits), this technique can also accommodate length polymorphisms alongside SNPs;[17] in that same context, it is also unique in allowing direct haplotyping of genetic markers.[17,46,47] Last but not least, the high sensitivity of this method makes it ideal for genotyping of pooled DNA samples.[48,49]

CONCLUSION

Fifteen years after its original description, ARMS-PCR remains an immensely popular and evolving technique. Rapid, reliable, versatile, and accessible to even the smallest laboratory, it is the definitive ''poor man's genotyping method.'' Yet, advanced design features and the integration of new technologies have propelled it to the forefront of large-scale genomic research. Until inexpensive, on-the-fly whole-genome sequencing becomes feasible, this technique is here to stay.

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Amplified Fragment Length Polymorphism (AFLP)—Application for DNA Fingerprinting

G. Ripabelli
University of Molise, Campobasso, Italy

J. McLauchlin
Health Protection Agency Food Safety Microbiology Laboratory, London, U.K.

INTRODUCTION

The analysis of nucleic acid markers that reflects similarities in DNA or RNA sequences (DNA or RNA fingerprinting) has become widespread, and has many uses, including identifying progenitors, siblings or paternity, establishing identity of target recovered from different locations, assessing population structure including clonality or degree of recombination, predicting phenotypes, etc. Factors influencing the choice of a fingerprinting technique include the type of application, the target organism under investigation (animal, plant, microorganism), the source of target material available, the resources and skill base to perform tests, and the availability of data for comparative analysis. Ideally, the method should be cheap, rapid, reproducible, discriminatory, independent of other methods, and applicable to all types of samples including to targets without prior sequence or genetic mapping information. Moreover, the method should be amenable to automation, easy to interpret and standardize between different testing sites, capable of comparisons with different methods, and require minimal molecular skills from the operators and those interpreting the data. Currently, no single technique is able to meet all of these requirements.

Initially, the most common DNA fingerprinting techniques were based on restriction fragment length polymorphism, some with subsequent Southern blotting-based analysis. However, polymerase chain reaction (PCR)-based procedures have become more common, one of which is amplified fragment length polymorphism (AFLP) analysis. AFLP has advantages over other similar fingerprinting techniques in terms of reproducibility, discrimination, labor intensiveness, and speed. AFLP is easier to perform and requires simpler and less expensive equipment than pulsed-field gel electrophoresis, which is widely considered as the ''gold standard'' for molecular epidemiological studies of pathogenic microorganisms. Because of the high stringency of the PCR procedure, AFLP is inherently more reproducible than randomly amplified polymorphic DNA analysis. It does not require Southern blotting followed by probing with labeled nucleotides such as is required for ribotyping or insertion sequence typing (IST). Most importantly, AFLP markers have a high multiplex ratio (the number of different genetic loci simultaneously analyzed per experiment) that allows characterization of, at least in part, the entire genome. In contrast, assays characterizing single loci have a multiplex ratio of 1.

AFLP ANALYSIS

AFLP is a PCR-based technique able to provide high-throughput DNA fingerprinting for rapid and accurate screening of genetic diversity, which was correctly originally described as able to ''bridge the gap between genetic and physical maps.''[1] The method comprises of a restriction-enzymatic digestion of genomic DNA followed by ligation of double-stranded oligonucleotide adapters that are complimentary to the restriction sites at the ends of the DNA fragments. The ligated ends are designed not to regenerate the restriction sites, and because the adapters are not phosphorylated, adapter-to-adapter ligation is prevented. Because the adaptors are of known DNA sequences, subsequent PCR amplification under high stringency is performed using primers that have exact complementarity with the template DNA generated by the adaptors. Patterns of amplified product differing in size constitute the final output of the process (Fig. 1). Despite the name, and despite its similarity with restriction fragment length polymorphism, AFLP detects the presence or absence of restriction fragments rather than their length polymorphism.

DNA Extraction

The starting DNA is crucial for successful AFLP analysis. This should ideally be of high molecular weight and as free as possible from contamination by other biomolecules. DNA extraction methods (e.g., those based on

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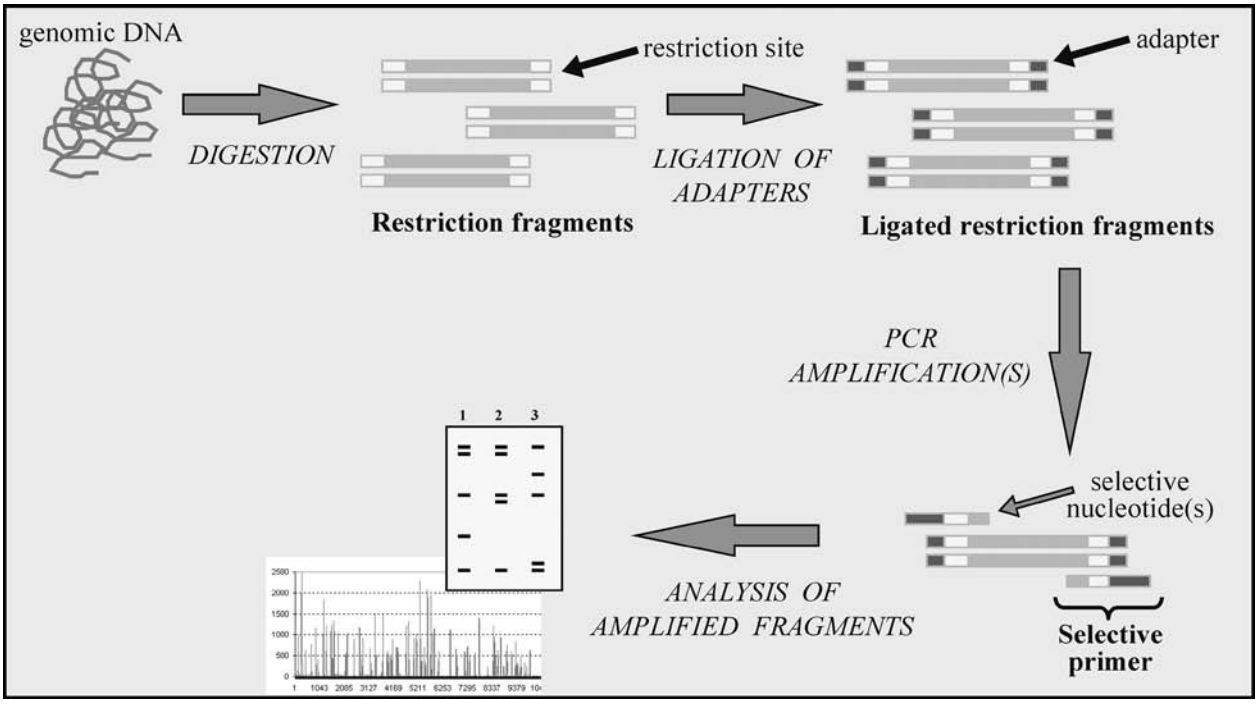

Fig. 1 Amplified fragment length polymorphism procedure. (*View this art in color at www.dekker.com.*)

phenol chloroform, guanidinium thiocyanate, and cetyltrimethyl-ammonium bromide) suitable for AFLP analysis are widely available. Because the PCR primers are not based on specific sequences, target DNA must be free from nucleic acids from other sources and hence, this method is problematic for analysis of microorganisms that cannot be grown in axenic culture or for target DNA that cannot be separated from DNA derived from extraneous sources.

Restriction Digestion and Ligation

Optimal restriction digestion is fundamental for correct AFLP patterns analysis, and the original methods used a combination of two enzymes with rare and frequent cutting sites. Hence, the fragments generated contain a frequent restriction site sequence at one end, plus a rare site sequence at the other. The rationale for this choice, particularly for functional analysis of genomes with very high complexity (e.g., for plant genomes), is that only fragments with combined rare and frequent restriction sites will be amplified, thus limiting the number of selective nucleotides in primers used for subsequent amplifications.[1] Alternative methods using only a single enzyme (sAFLP) are also available.

The availability of many different restriction enzymes with corresponding adapters and primer combinations provides a great deal of flexibility and ''tuning'' of the number of AFLP fragments generated. Theoretically, any restriction enzyme, alone or in combination, could be used to generate DNA profiles, irrespective of the complexity and utility required. However, rarely cutting enzymes will generate few fragments, thus reducing the probability for polymorphism detection, and frequently cutting enzymes are unlikely to generate fingerprints appropriate for interpretation.

Primer Selection and PCR

Because the adaptors are of known DNA sequence, subsequent PCR amplification of all DNA fragments can be achieved using primers that have exact complementarity with the adaptor sequences. Alternatively, the 3′ ends of the PCR primers can include one or more additional ''selective'' nucleotides, thus allowing amplification of a subset of the adapted–restricted fragments; it is usually necessary to test primers with up to three selective nucleotides to obtain satisfactory patterns.[2]

Pattern Analysis and Evaluation of Assays

Depending on the methods used to perform AFLP assay, the amplified fragments can be visualized on denaturing polyacrylamide gels (dPAGE) through autoradiography,[1]

on agarose gel with ethidium bromide staining and UV transillumination,[3] or by using fluorescently labeled primers through a polyacrylamide matrix in an automated sequencer.[4] If comparisons of simple patterns of relatively low discrimination for small numbers of samples are required, these can be adequately achieved by visual inspection. However, once more complex AFLP patterns are analyzed together with the need for intralaboratory and interlaboratory analysis, the application of bioinformatics is essential for analysis: the high reproducibility of AFLP supports the generation of electronic databases.[5] A systematic review of final pattern analysis and its evaluation are outside the scope of this article; however, the fundamental aspects of DNA fingerprinting analysis as developed for other systems[6] must be applied. In addition, the assessment of reproducibility (the repeatability of test results), typability (proportion of targets in a population that can be analyzed), and discrimination (number of different types identified) for each individual system must be evaluated, usually with a degree of empiricism.

APPLICATIONS OF AFLP ANALYSIS

AFLP has been successfully applied to DNA fingerprinting of a very wide variety of biological sample including plants, animals, and both eukaryotic and prokaryotic microorganisms and viruses.[1,7] In plant studies, it has been useful to estimate genetic diversity[8–10] and to resolve phylogenetic relationships.[10–12] Because AFLP markers cover the entire chromosomes, this technique can be successfully applied for breeding analysis including for variety identification, germoplasm management, indirect selection of agriculturally important traits, and backcross breeding.[13] Identification of markers closely linked to resistance genes,[14] construction of plant genetic maps,[15,16] and quantitative trait loci analysis (i.e., QLT analysis is that of traits that do not fall into discrete classes, and are controlled by multiple independently Mendelianly segregating genes) have been achieved by AFLP.

Applications of AFLP to animal genetics and linkage analysis have not been as widespread as in plant genetics, probably because of availability of dense microsatellite maps.[13] However, AFLP analysis has been used to investigate genetic diversity among domestic cattle,[17,18] and for human genome fingerprinting resulting in an efficient and rapid approach for identifying and scoring multiple variants.[19] AFLP-based human mRNA fingerprinting,[20] where the target is cDNA rather than genomic DNA, had been used for analysis of expression of genes involved in cancer development[21] and to evaluate changes in gene expression after exposure to xenobiotics.[22]

AFLP is well suited to the study of eukaryotic parasitic,[23–25] fungi,[26,27] viruses, and bacteria.[1] The power of the technique for comparative genomics is illustrated by the study of Arnold et al.,[28] which showed that AFLP generated >90% of the fragments predicted from the *Escherichia coli* K12 genome. The technique has proven especially useful for analysis of bacteria pathogenic to humans and other animals, and the authors' experience has shown AFLP to be highly suitable for public health microbiology and molecular epidemiological analysis.[29–33] The robustness of the system is illustrated by the successful interlaboratory comparison of AFLP patterns via the internet to identify strains of the bacterium *Legionella pneumophila*, the causative agent of Legionnaires' disease[34] (http://www.ewgli.org/).

CONCLUSION

In summary, PCR-based DNA fingerprinting techniques are becoming increasingly used for a wide range of organisms, and AFLP analysis has considerable advantages for certain applications. The method is robust, relatively easy to perform, sufficiently reproducible to provide the basis for reliable intralaboratory and interlaboratory studies, and can be easily modified (tuned) to provide the desired level of discrimination. AFLP markers have a high multiplex ratio (the number of different genetic loci that may be simultaneously analyzed per experiment), which is of considerable advantage because this allows analysis of, at least in part, the entire genome rather than single locations.[35,36] AFLP has been successfully applied to fingerprint DNA material from a very wide variety of biological sample including plants, animals, viruses, and both eukaryotic and prokaryotic microorganisms.[1,7,37,38] Applications have been particularly successful in the areas of analysis of plant genomes and those from pathogenic bacteria for public health microbiology and molecular epidemiology. Until very high throughput multiple-sequence analysis of whole genomes becomes technically feasible on a wide and rapid scale, AFLP should be seriously considered as the method of choice to investigate genome polymorphisms.

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Angelman Syndrome

Milen Velinov
Edmund C. Jenkins
New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

INTRODUCTION

In 1965 Harry Angelman first reported on three patients with severe mental retardation, who had ''flat heads, jerky movements, protruding tongues and bouts of laughter'' that ''gave them specific resemblance to puppets.'' This was the first report on a devastating condition that was for some time referred to as the ''happy puppet syndrome'' but is currently known as Angelman syndrome (AS).

Angelman syndrome is an example of imprinted gene abnormality in humans. The gene, the function of which is deficient in at least 85% of AS cases, E6-AP-ubiquitine-ligase (UBE3A) shows brain-specific imprinting in that only its maternal allele is active in the brain. Four known molecular mechanisms can lead to UBE3A deficiency. For the remaining 15% of patients the underlying mechanism is unknown. Although the recurrence risk for subsequent pregnancies in most affected families is low, in some cases it can reach 50%. As no single laboratory test can identify all AS cases and subtypes, using a sequential diagnostic testing algorithm is suggested.

OVERVIEW

Prevalence

The reported AS prevalence is in the range of 1 in 10,000–20,000.[1,2]

Clinical Features

Angelman syndrome patients[3] have global developmental delay, usually first noticed at 6–12 months of life. Most patients never develop speech, but some may have minimal use of words. All have frequent inappropriate laughter or smiling and significant motor delay—they begin walking at an average age of 4–5 years. Typical neurological manifestations are ataxia and tremulous movements. Most, but not all, have microcephaly. Most develop seizures before 3 years of age and have characteristic abnormal EEG patterns.[4] Features that are present in most cases include characteristic appearance with flat occiput, protruding tongue, prognatia, wide smiling mouth, as well as hypopigmentation and strabismus (Fig. 1). Sleep disturbances and fascination with water can also be present. Clinical diagnostic criteria for AS have been developed by a panel of professionals experienced in the care for AS.[6]

MOLECULAR BASIS AND IMPLICATIONS FOR GENETIC COUNSELING

Angelman syndrome is divided into five major types:

Type I. De novo interstitial deletions of region 15q11q13 are found in about 70% of all AS cases (Table 1).[7] Similar deletions have been reported in association with Prader–Willi syndrome (PWS), a disorder with different phenotype (see Prader–Willi syndrome). The deleted chromosomal area in AS is of maternal origin in contrast to PWS where the origin of the deletions is paternal.[8] These findings suggested the presence of imprinted genes in the PWS/AS region. The common PWS/AS deletion (see also Prader–Willi syndrome) spans about 4 Mb and is routinely diagnosed by FISH analysis.[9] In cases with the common deletion (Type I a) the recurrence risk is lower than 1%.[10] In less than 1% of all AS cases, referred to as Type I b, chromosomal abnormalities are observed in nonaffected parents. Two types of families have been reported: 1) an unbalanced translocation in the affected individual with a balanced translocation in his/her mother. In one such family there were affected children with both AS and PWS depending on the transmitting parent;[11] 2) familial interstitial deletion that is present in the patient, his unaffected mother, and possibly the unaffected grandfather.[12] Such deletions are smaller and do not include the region for PWS—when transmitted from the grandfather to the mother they do not lead to PWS. In Type I b families the estimated recurrence risk is up to 50%.

Type II. Uniparental disomy 15 (UPD-15). About 2–3% of AS cases are the result of paternal UPD-15[13]—inheritance of both chromosomes 15 from the father. It

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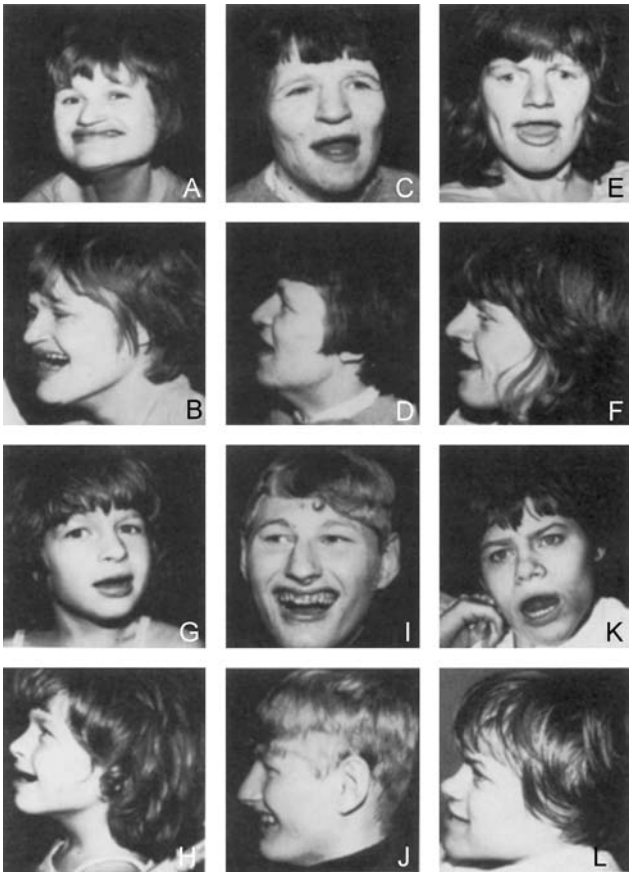

Fig. 1 Photographs of six 11–33-year-old patients with Angelman syndrome. (From Ref. [5].)

seems that most paternal UPD-15 errors occur during postzygotic divisions.[14] Increased parental age is documented in UPD cases.[15]

Although the recurrence risk for UPD (Type II a) is lower than 1%, cases of AS with paternal UPD-15 have been reported in association with familial translocations involving chromosome 15[16] (Type II b). In such cases the recurrence risk may be increased, but the available information is limited.

Type III. Imprinting defects. Genomic imprinting in the PWS/AS region is associated with specific DNA methylation patterns reflecting the chromosome's parental origin (see ''Laboratory Testing''). The methylation patterns are abnormal in both deletion and UPD–AS cases. About 5% of AS patients do not have FISH-identifiable deletions or UPD, but have abnormal methylation patterns lacking methylated (maternally specific) DNA sequences despite the established biparental inheritance. In some cases this imprinting defect is associated with smaller deletions in the region, involving the 5′ end of the gene SNRPN.[17] This chromosomal locus referred to as an imprinting center (IC) is involved in the imprinting switch process during gametogenesis, which allows the ''resetting'' of the DNA methylation patterns in the gametes to reflect their parental origin. Two physically separated control centers (minimal regions) were identified within the IC region. An 880-bp sequence is believed to be involved in the paternal-to-maternal imprint switch, and when deleted leads to AS. A 4.3-kb sequence is deleted in PWS families with IC mutations and appears to be involved in the maternal-to-paternal imprint switch.[17] Imprinting center deletions are found in about half of the imprinting defect cases. In families with IC deletions (Type III a) the mothers are unaffected carriers of the deletion and have a 50% chance to have another child with AS.[10] In the imprinting defect cases with no identifiable IC deletions (Type III b) the recurrence risk seems to be low as recurrence has not been observed, but the existing data are limited. It seems likely that Type III b abnormalities occur sporadically and are postzygotic.[18] Such abnormalities were recently reported in association with intracellular sperm injection procedures.[19]

Type IV. UBE3A mutations. At least 20% of the AS patients have normal methylation patterns. In up to 50% of those cases single-gene mutations of the maternally derived allele of the gene UBE3A, which is located within the AS/PWS chromosomal region, were identified.[20,21] Mutations of UBE3A can produce the entire AS phenotype in contrast to PWS, where deficiencies of more than one gene contribute to the disease phenotype. UBE3A gene mutations are found more frequently in familial than in sporadic cases.[20,21] In familial cases the mother of the affected individual carries a silent mutation and the risk for subsequent pregnancies is 50%. Cases of gonadal mosaicism for UBE3A mutations that may lead to recurrence have been reported in significant proportion of mothers.[21] Such findings prompt caution while providing genetic counseling for families in which the mutation is not identified in the mother, as the recurrence risk may not be lower than 1%.[22]

Type V. Unknown. There is no confirmatory laboratory test for the last group of AS patients. Their methylation testing and UBE3A mutation screening are normal. Special effort should be made in this group of patients to rule out different etiologies with similar phenotypes.[23]

UBE3A DEFICIENCY

The UBE3A gene encodes E6-AP ubiquitin protein ligase, a protein that takes part of the ubiquitin–proteasome

Table 1 Molecular types of AS

Type	Mechanism	Laboratory testing	Proportion of all (%)	Recurrence risk	Phenotype
Ia	De novo Del 15q11q13	MT[a]+ FISH+	70%	<1%	Severe
Ib	Inherited chromosomal abnormalities	Chromosome analysis–abnormal MT+	<1%	Up to 50%	Unclear
IIa	Paternal uniparental disomy	MT+, FISH−, UPD study+	2–3%	<1%	Mild
IIb	Uniparental disomy and chromosomal aberration	Chromosome analysis–abnormal, MT+, UPD study+	<1%	? Increased, but limited data	Unclear
IIIa	Imprinting defect with IC deletion	MT+, FISH−, UPD−, IC del+	2%	Up to 50%	Intermediate
IIIb	Imprinting defect without IC deletion	MT+, FISH−, UPD−, IC del−	2%	<1%	Intermediate
IVa	UBE3A mutation, mother is carrier	MT−, UBE3A mutation+ (mother+)	2–5%	50%	Intermediate
IVb	UBE3A mutation, mother not a carrier	MT−, UBE3A mutation+ (mother−)	2–5%	? Higher than in general population	Intermediate
V	Unknown defect	All tests negative	15%	Unclear	Unclear

[a]MT = DNA methylation testing.

proteolytic pathway where the proteolysis occurs after conjugation to ubiquitin.[24] This gene is imprinted in the brain where only its maternal allele is functional.[25] A mouse model of AS was developed by generating a UBE3A null mutation. The affected mice show cognitive impairments, inducible seizures, and deficit in long-term potentiation.[26] Still at this time a direct link between the UBE3A deficiency and the clinical features of AS has not been positively established.

Phenotype–Genotype Correlations

The most severe AS phenotype is observed in Type I (deletion) patients. The clinical severity in this group is believed to be due to additional deleted genes in this region. For example, the severity of seizure disorder may be due to the deletion of the GABA receptor gene GABRB3, which is located in the deleted area;[27] the hypopigmentation is linked to the deleted P gene in this area.[28] The UPD patients form the other end of the clinical spectrum with the mildest phenotypes. The severity in patients with imprinting defects and UBE3A mutations seems to fall between the deletion and UPD categories.

Diagnostic Testing

The diagnostic tests bellow are listed in order of their application for diagnostic evaluation (Table 1 and Fig. 2):

- *G-banded chromosome analysis* should be undertaken in every individual with suspected AS for two important reasons: 1) to rule out familial chromosomal abnormalities, associated with increased recurrence risk; 2) to rule out a non-AS chromosomal abnormality that may mimic the AS phenotype.[23]
- *DNA methylation testing* is done as an initial evaluation for all patients with suspected AS and will identify up to 80% of the AS cases: the ones with deletion, UPD, and imprinting defect.[29] DNA methylation is a genomic process that mediates epigenetic regulations of gene expression. It involves the cytosines in the CpG islands and is generally associated with down regulation of gene expression. A CpG area at the 5′ end of the SNRPN gene in the proximal 15q region is methylated if maternally derived and not methylated if paternally derived. This gene region is used to look for DNA methylation abnormalities in AS or PWS. In AS cases of deletions, paternal UPD-15, and imprinting defects the DNA methylation patterns are abnormal reflecting the deficiency of the maternally specific (methylated) 15q region. Diagnostic DNA methylation testing is traditionally done using a Southern-blotting protocol, based on the use of methylation-sensitive restriction enzymes.[17] More recently, PCR-based methylation tests have been developed based on methylation-specific DNA sequence changes introduced by sodium bisulfite modification.[29] Such tests require minimal amounts of DNA and have been used to retest archived patient cells after negative FISH analyses.[30]
- *FISH analysis* using commercially available probes specific for the SNRPN locus is done to look for 15q11q13 deletions after a positive DNA methylation testing,[9] (Fig. 2).
- *UPD analysis* is done in cases of abnormal DNA methylation and normal FISH analysis. This testing protocol uses DNA polymorphisms to trace the inheritance of chromosome 15. DNA specimens from both parents are necessary.[31]
- *IC mutation screening*. In methylation positive, FISH, and UPD-negative cases a search for IC deletion can be done,[17] but is not currently available for routine use.
- *UBE3A mutation screening*. In cases of normal DNA-methylation patterns, the patient phenotype needs reevaluation in order to decide whether UBE3A testing is warranted. If typical AS features are observed UBE3A mutation screening can be considered.[20,21]

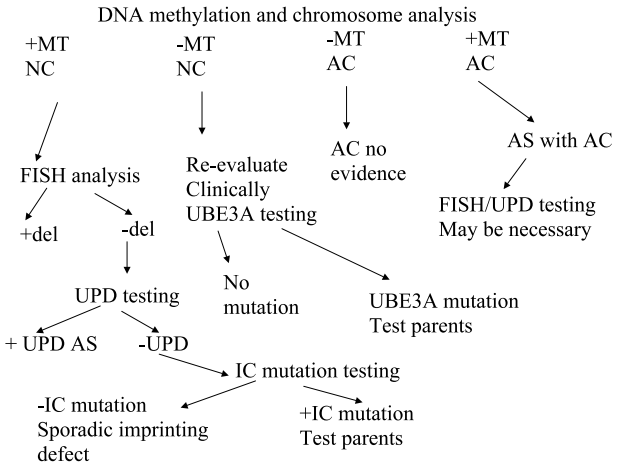

Fig. 2 Algorithm for the diagnostic evaluations for AS. Abbreviations: MT=DNA methylation testing; + positive; − negative; NC=normal chromosome analysis; AC=abnormal chromosome analysis; UPD=uniparental disomy; IC=imprinting center.

CONCLUSION

Angelman syndrome is a devastating condition presenting severe developmental delay, epilepsy, neurological, and

behavioral abnormalities. All various identifiable molecular mechanisms by which the disorder may occur ultimately lead to a deficiency of the UBE3A gene that is involved in ubiquitin-mediated proteolysis. Future research on AS may involve identifying the exact mechanism by which UBE3A deficiency leads to the characteristic clinical manifestations; developing new specific management strategies; establishing the exact molecular basis for the AS patients with negative laboratory testing; studying the possible connection between intracellular sperm injection and the occurrence of sporadic imprinting abnormalities.

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Arylamine *N*-Acetyltransferase Gene Polymorphism

A

Neville J. Butcher
Rodney F. Minchin
University of Queensland, St. Lucia, Australia

INTRODUCTION

Acetylation catalyzed by the arylamine *N*-acetyltransferases (NATs; EC 2.3.1.5) is a major biotransformation pathway for arylamine and hydrazine drugs, as well as many carcinogens that we are exposed to on a daily basis found in foodstuffs, cigarette smoke, and the environment. These compounds can either be detoxified (*N*-acetylation) by NATs and eliminated from the body, or bioactivated (*O*-acetylation) to metabolites that have the potential to cause toxicity such as cancer. As a result, NAT levels in the body have clinical importance with regard to drug effect and individual susceptibility to toxicity. In humans, these reactions are catalyzed by two closely related, highly polymorphic enzymes (NAT1 and NAT2), which differ in their substrate specificity and tissue distribution. This article focuses on recent advances in the molecular genetics of the human NATs and their clinical implications.

GENOMIC ORGANIZATION

There are three human NAT genes. Two encode functional proteins and are designated *NAT1* and *NAT2*, and the third is a pseudogene (*NATP1*) that encodes a truncated nonfunctional protein. All are located on the short arm of chromosome 8 and have been mapped to 8p21.3–23.1, a region commonly deleted in human cancers. Both functional NATs are encoded by single intronless exons and the protein-coding regions share an 87% nucleotide homology and are 870 bp in length.

PROTEIN CHARACTERISTICS

Human NAT1 and NAT2 both have an approximate molecular mass of 33 kDa and each consists of 290 amino acids, sharing an 81% homology. NAT1 appears to be ubiquitously expressed, whereas NAT2 expression is restricted to the liver and gut. The active site of NAT enzymes contains the catalytic triad cysteine–histidine–aspartate,[1] which is similar to that of the cysteine protease superfamily of proteins.

SUBSTRATE SPECIFICITY

NAT1 and NAT2 have different but overlapping substrate specificities, although no substrate is exclusively acetylated by one isozyme or the other, and no clear structural motif that determines substrate specificity for the different isoforms has been identified. Substrates preferentially *N*-acetylated by human NAT1 are *p*-aminobenzoic acid, *p*-aminosalicylic acid, sulfamethoxazole, and the folate catabolite *p*-aminobenzoyl glutamate. Substrates primarily *N*-acetylated by human NAT2 include a number of sulfonamides (sulfamethazine, sulfapyridine, sulfadiazine, sulfameridine, and sulfadoxine), isoniazid, aminoglutethimide, amonafide, procainamide, dapsone, dipyrone, endralazine, hydralazine, prizidilol, batracylin, and metabolites of clonazepam and caffeine. Some compounds, such as the carcinogenic aromatic amines 2-aminofluorene, benzidine, 4-aminobiphenyl, 4,4-dichloroaniline and 2-naphthylamine, and the food-derived heterocyclic amines 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and 2-amino-3,4-dimethyl-imidazo[4,5-*f*]quinoxaline, are *N*-acetylated to varying degrees by both human NAT1 and NAT2. Generally, *O*-acetylation of the *N*-hydroxy metabolites of carbocyclic arylamines is catalyzed selectively by NAT1, whereas NAT2 *O*-acetylates *N*-hydroxy metabolites of the dietary heterocyclic amine carcinogens.

NAT POLYMORPHISM

An international committee has been established to oversee the nomenclature of the NATs[2] and a Web site that provides information about the naming of existing and new alleles for all species can be found at http://www.louisville.edu/medschool/pharmacology/NAT.html.

NAT2 Alleles

Since the human *NAT2* locus was established as the site of the classical acetylation polymorphism, the study of *NAT2* allelic variation has been an area of intense investigation. To date, 36 different *NAT2* alleles have been detected in human populations.[3] Each of the variant alleles is composed of between one and four nucleotide substitutions,

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of which 16 have been identified, located in the protein-coding region of the gene. The correlation between NAT2 genotype and phenotype is well established. Moreover, there is a gene-dosage effect. Individuals who are homozygous for slow *NAT2* alleles have a slow acetylator phenotype, individuals heterozygous for slow *NAT2* alleles have an intermediate acetylator phenotype, and individuals who lack slow *NAT2* alleles have a rapid acetylator phenotype.

Molecular mechanisms of NAT2 slow acetylation

The molecular mechanisms responsible for the production of the NAT2 slow acetylator phenotype are not well understood. Initial studies in liver tissue showed that the slow phenotype was due to a marked decrease in NAT2 protein content. In bacterial[4] and yeast[5] expression systems, some base changes caused slow acetylation by producing an unstable protein, whereas others caused slow acetylation by a reduction in NAT2 protein expression.

NAT2 allele frequency

In Caucasian and African populations, the frequency of the slow acetylation phenotype varies between 40% and 70%, whereas that of Asian populations, such as Japanese, Chinese, Korean, and Thai, range from 10% to 30%.[6] This difference reflects slow acetylator allele frequency. Caucasian and African populations have high frequencies of *NAT2*5* alleles (>28%) and low frequencies of *NAT2*7* alleles (<5%), whereas Asian populations have low incidences of *NAT2*5* alleles (<7%) and higher incidences of *NAT2*7* alleles (>10%). Also, *NAT2*14* alleles are almost absent from Caucasian and Asian populations (<1%), but are present in African populations at comparably higher frequencies (>8%).

NAT1 Alleles

Historically, NAT1 was thought to be genetically invariant. However, wide interindividual variability in NAT1 activities was suggestive of a genetic polymorphism. The first reported allelic variation at the *NAT1* locus was in 1993[7] and marked the beginning of a systematic survey of *NAT1* genotypes. To date, 26 different *NAT1* alleles have been detected in human populations.[3] However, only a small number of these alter phenotype.

A genotype/phenotype relationship involving the slow acetylator alleles *NAT1*14*, *NAT1*15*, *NAT1*17*, and *NAT1*22* in human blood cells exists. Individuals heterozygous for these alleles have approximately half the activity of individuals who are wild-type (*NAT1*4*). Similar to NAT2 in the liver, low activity was caused by a parallel decrease in NAT1 protein content.[8] A gene-dosage effect similar to that seen for NAT2 alleles also exists for the low activity *NAT1* alleles. The *NAT1*10* allele has been associated with increased activity in bladder and colon tissue, compared to wild-type.[9] In addition, higher levels of DNA adducts have been detected in bladder tissue from *NAT1*10* heterozygotes. The functional significance of this allele is controversial, as several recent studies do not support the idea that *NAT1*10* is associated with elevated NAT1 activity.[10–14]

Molecular mechanisms of altered NAT1 acetylation

The molecular mechanism for slow acetylation caused by the *NAT1*14*, *NAT1*15*, *NAT1*17*, and *NAT1*22* alleles has been recently elucidated. These alleles produce mutant proteins that are rapidly polyubiquitinated and targeted for degradation by the 26S proteasome. Moreover, degradation correlates with the nonacetylated state of the mutant proteins.[8] It is likely that this same mechanism exists for the slow NAT2 acetylator phenotype. *NAT1*10* contains nucleotide substitutions located in the 3′-untranslated region that alter the consensus polyadenylation signal (AA**T**AAA → AAA**A**AA), leading to the suggestion that increased activity may be due to enhanced mRNA stability.

NAT1 allele frequency

The frequency of slow acetylator alleles for NAT1 is low. The most common low activity allele, *NAT1*14*, has been identified in Caucasian populations ranging from 1.3% to 3.7%. Interestingly, a much higher frequency of the *NAT1*14* allele (25%) was reported for a Lebanese population. This indicates that NAT1, like NAT2, shows considerable interethnic variability.

NAT AND DISEASE

The role of NATs in human disease has recently been extensively reviewed.[3] The following is a summation of findings to date. The association between acetylator status and the risk of various diseases has been extensively reported. Altered risk with either the slow or rapid phenotype has been observed for bladder, colon and breast cancer, systemic lupus erythematosis, diabetes, age-related cataracts, Gilbert's disease, Parkinson's disease, and Alzheimer's disease. These associations imply a role for environmental factors that are metabolized by the NATs, in particular NAT2, in each disorder. However,

identifying those factors has remained elusive. Humans are exposed to many toxic NAT substrates including the food-derived heterocyclics present in the diet as well as arylamines such as 4-aminobiphenyl and β-naphthylamine present in tobacco smoke. Moreover, occupational exposure to arylamine carcinogens such as benzidine has also been reported.

Because of the role of acetylation in the metabolic activation and detoxification of arylamine and heterocyclic carcinogens, acetylator status and cancer risk have been widely investigated. Unlike the relatively rare but highly penetrant genes involved in familial cancers, those genes responsible for metabolic polymorphisms have low penetrance and cause only a moderate increase in cancer risk. Nevertheless, their widespread occurrence in the general population suggests they are a significant contributor to individual risk. However, few diseases have consistently demonstrated a relationship between phenotype and risk. For example, several studies have implicated the rapid phenotype as an increased risk factor for colon cancer,[15–17] whereas others have been unable to confirm this finding.[18–20] Geographical differences, ethnicity, lack of study power, dietary differences, and differences in other risk factors between study groups have been suggested as reasons for variable results from independent laboratories. Recent reports suggesting that NAT activity may be altered by environmental factors and substrate-dependent downregulation may also explain why inconsistent associations have been seen.

When acetylator phenotype has been linked to carcinogen exposure, more consistent results have been reported. For example, the rapid phenotype has emerged as a strong risk factor for colorectal cancer in those individuals who have a higher exposure to the food-derived heterocyclic amines. These observations provide strong circumstantial evidence that the heterocyclic amines have an important role in colorectal cancer, and extensive animal studies support this. They also illustrate the importance of establishing associations between genetic polymorphisms and disease risk. From such studies, it should be possible to pursue the causative agent(s) of the disease where no obvious candidate agent is evident.

Recently, the NAT2 acetylator phenotype has been linked to increased risk associated with neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. For late-onset Alzheimer's disease, an odds ratio of 3.0 (95% CI 1.3–7.3) has been reported for the rapid phenotype in non-apoE epsilon carriers.[21] By contrast, the slow phenotype appears to increase the risk of Parkinson's disease with an odds ratio of 3.58 (95% CI 1.96–6.56).[22] Although these results need to be confirmed with larger epidemiological studies, they point to environmental factors that are substrates for the NATs having a role in the onset of these diseases. Alternatively, the different alleles for NAT2 that produce the rapid or slow phenotype may cosegregate with unrelated genes that are the causative agent for the different neurodegenerative diseases.

NAT AND DRUG RESPONSE

The genetic polymorphism in *N*-acetyltransferase activity was first discovered in patients treated with isoniazid for tuberculosis. This drug is primarily excreted following acetylation catalyzed by NAT2. Many other therapeutic

Table 1 Effect of acetylator status on drug response and toxicity

Drug	Phenotype	Effect	Reference
Dapsone	Slow	Neurotoxicity	[24,25]
Sulfamethoxazole	Slow	Hypersensitivity	[26]
Hydralazine	Slow	Systemic lupus erythematosus	[27]
	Rapid	Decreased therapeutic effect	[23]
Isoniazid	Slow	Interaction with phenytoin	[28]
	Slow	Interaction with rifampicin	[29]
Cotrimoxazole	Slow	Various adverse reactions	[30]
Sulfasalazine	Slow	Various toxicities	[31]
	Slow	Hepatotoxicity	[32]
	Slow	Nausea/vomiting	[33]
Amonafide	Rapid	Leukopenia	[34,35]
Procainamide	Slow	Systemic lupus erythematosus	[36,37]
Phenelzine	Rapid	Decreased therapeutic effect	[38]
p-Phenylenediamine	Slow	Contact dermatitis	[39]

Source: Adapted from Ref. [3].

agents are now known to be polymorphically acetylated in humans. However, the incidence of failed or less effective clinical response as a consequence of acetylation polymorphism is uncommon. This is because most drugs that are metabolized by the NATs have a wide therapeutic window or because acetylation is a minor metabolic pathway. An exception is hydralazine. Early studies showed that the antihypertensive activity of hydralazine was less in rapid acetylators and that a 40% higher dose was necessary for a similar therapeutic effect compared with slow acetylators.[23] This difference appeared to be due to a change in the bioavailability of the drug, which decreased from 33% in slow acetylators to less than 10% in rapid acetylators.

A more common consequence of the polymorphic acetylation of therapeutic agents is an increase in the frequency and severity of side effects associated with either the rapid or slow phenotype (Table 1). These adverse effects often arise as the result of a shift in the metabolic pathways responsible for the activation and detoxification of the drug. This is best illustrated by the amine-containing sulfonamides, such as sulfamethoxazole, that undergo hydroxylation to a reactive *N*-hydroxy metabolite capable of covalently binding to macromolecules and giving rise to idiosyncratic adverse reactions. These drugs can also be acetylated by NAT2 to nonreactive *N*-acetyl metabolites. In slow acetylators, a higher proportion of the drug is *N*-hydroxylated and, consequently, these individuals are at a greater risk of sulfonamide-induced toxicity. However, the incidence of severe adverse side effects to sulfonamides is much less than the incidence of the slow acetylator phenotype suggesting that other factors predispose individuals to idiosyncratic adverse reactions.

Risk of developing side effects, such as neurotoxicity or hemolytic anemia, to dapsone therapy is very similar to that described for the sulfonamides. The most severe incidence of toxicity occurred in individuals with a slow acetylator phenotype who are rapid hydroxylators, which is consistent with the role each pathway has in the activation and detoxification of the drug.

While slow acetylators are at a greater risk of toxicity from sulfonamides and dapsone, other therapeutic agents exhibit increased incidence of adverse reactions in rapid acetylators. Amonafide is a novel arylamine that has previously been in clinical trials for the treatment of various cancers. It undergoes *N*-acetylation to an active metabolite that contributes to systemic toxicity. Several studies have shown that myelosuppression is greater in rapid acetylators (white blood cell nadirs of 500/μL) than in slow acetylators (white blood cell nadirs of 3400/μL) following a standard dose of 300 mg/m^2 daily for 5 days.[35] This has led to different recommended doses for the two groups.[34]

CONCLUSION

Although considerable allelic variation exists for both *NAT1* and *NAT2*, our understanding of the molecular mechanisms and functional significance of many of these alleles, particularly for NAT1, is still limited. Much of the research in the area of NATs has involved identifying relationships between allele frequencies and disease, particularly different forms of cancers. Although several studies have reported associations between different *NAT* alleles and various cancers, other studies have failed to do so. While these inconsistencies may be due to several factors, such as differences in exposure to arylamine carcinogens, it may well be that genotype does not necessarily accurately reflect phenotype.

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Astrovirus

C. Anthony Hart
Rachel L. Hart
The University of Liverpool, Liverpool, U.K.

INTRODUCTION

Astroviruses cause diarrheal disease in a number of different animal species (humans, pigs, sheep, turkeys) and hepatitis in ducklings. Astroviridae are a family of small (27–30 nm), round, unenveloped viruses with a characteristic star-shape on their capsid surface. Their genomes are single-stranded linear positive sense RNA of 6.8 to 7.9 kb. The genome encodes three open reading frames (ORF). ORF1a encodes a serine protease; ORF1b, an RNA-dependent RNA polymerase; and ORF2, the viral capsid protein. There are currently eight known human astrovirus serotypes. All cause diarrheal disease predominantly in infants which is usually mild. Nosocomial outbreaks and outbreaks of infection in adults can occur.

THE VIRUS

The family Astroviridae encompasses small nonenveloped viruses that infect a variety of animal species. They were originally classified among the small round structured viruses (SRSV) causing diarrhea in humans. They are round with icosahedral symmetry and 27–30 nm in diameter. They were first described by Madeley and Cosgrove[1] as a potential cause of diarrheal disease in human infants and named for the characteristic five- or six-pointed star (astron is Greek for a star), visible on the capsid surface by negative stain electron microscopy (Fig. 1). There are small surface projections consisting of 30 dimeric spikes protruding some 50 Å from the virus surface. In 1981, serial passage of human astrovirus was achieved in primary human embryo kidney (HEK) cells by Lee and Kurtz,[2] but astrovirus is more conveniently cultured in CaCo-2 cells. However, trypsin must be included in the culture medium.[3] Astrovirus particles are stable at pH 3, but disassemble at pH 10.5 and are resistant to chloroform, detergents (nonionic or ionic), and lipid solvents such as ether. At 60°C astrovirus retains infectivity for 5 but not 10 min. It is stable for years at −70°C but is disrupted by repeated freeze–thaw cycles.[4] Human astroviruses (HuAst) survived for 5–6 days when dried at 20°C in fecal material onto porous or nonporous material. Survival was significantly longer at 4°C. Its survival was equivalent to that of adenoviruses but less good than that of rotaviruses.[5]

The genome is positive sense single-stranded linear unsegmented RNA and 6.8 to 7.9 kb long.[6] It has a poly-A tail at the 3′ end. During infection both genomic (6.8 kb) and subgenomic (2.4 kb) RNA are produced. The genome encodes three ORFs. At the 5′ end of the genome are encoded ORF1a and ORF1b. The third ORF (OFR-2) is found at the 3′ end as is the subgenomic fragment in infected cells. ORF1a and ORF1b encode nonstructural proteins whereas ORF2 encodes the capsid proteins. At the 5′ end there is an untranslated region of 80–85 nucleotides depending on the astrovirus serotype. ORF1a varies in size from 2763 to 2784 nucleotides also depending upon the serotype. There is an area of overlap between ORF1a and ORF1b of between 61 nt (for HuAst serotype 3) and 73 nt (for serotypes 1 and 2). Similarly, ORF1b varies in length from 1548 to 1560 nt depending upon serotype. ORF1a encodes a putative protein (nsp1a) of 920 to 935 amino acids in length.[6] It contains a serine protease motif similar to that seen in other positive sense RNA viruses such as feline calicivirus (FCV). However, a major difference between it and FCV is the substitution of a serine for a cysteine residue at the third catalytic amino acid residue.[7] Downstream of the protease motif is a nuclear localization signal which directs ORF1a protein to the nucleus of infected cells as has been determined by immunofluorescence and immunoprecipitation.[8,9] The 920-amino acid nsp-1a is itself proteolytically cleaved into smaller proteins and in particular, cleavage around amino acid 410 appears to be mediated by the polypeptide itself.[10] ORF1b encodes a polypeptide containing 515–519 amino acids and contains motifs suggestive of an RNA-dependent RNA polymerase most closely resembling those of plant viruses, bymovirus, and potyvirus.[11] There is an overlap between the end of ORF1a and the beginning of ORF1b which is highly conserved. This suggests there is a ribosomal frameshift mechanism involved in the translation of the polypeptides.

ORF2 encodes a viral structural protein varying in length from 782 to 794 amino acids depending on serotype.[6] The polypeptide is conserved for the first

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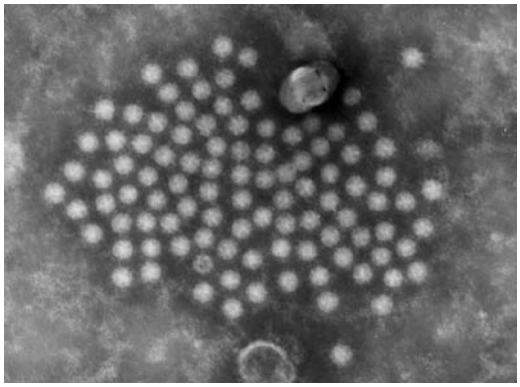

Fig. 1 Negative stain electron micrograph of astrovirus in feces.

415 N-terminal amino acids but shows variability thereafter. The polypeptide is cleaved proteolytically to 20–40-kDa structural proteins, and, for example, the 26-kDa structural protein expresses a neutralization epitope.

Currently, eight serotypes of human astrovirus are recognized (HuAst 1–8) based upon epitopes on ORF2. It is also possible to genotype HuAst by examination of sequence data of ORF1a, ORF1b, and the 5′ end of ORF2,[12] and genotypes equivalent to the eight serotypes have been defined. Recently, evidence for a novel recombinant HuAst has been uncovered.[13] There is no cross-reactivity between the HuAst serotypes, and immunity to one does not give immunity to the others. Astroviruses have been detected in diarrheic animals and as a cause of hepatitis in ducks. Phylogenetic analyses of a large collection of sequences from a variety of astroviruses showed that all the HuAst clustered together separate from the nonhuman strains; however, the branching order of the astroviruses was the opposite of their host species indicating the possibility of cross-species transmission.[14]

PATHOGENESIS AND IMMUNITY

HuAst are transmitted by the feco-oral route,[15] either directly person-to-person or indirectly via contaminated food or water. During infection, between 10^8 and 10^{13} genome equivalents are shed per gram of feces.[16] The infectious dose is not known but thought to be low. Little is known of how HuAst cause diarrheal disease. However, in experimental infection in gnotobiotic lambs, ovine astrovirus caused infection of mature enterocytes in the upper two thirds of the villi resulting eventually in transient villous flattening and crypt hypertrophy.[17] In turkeys, astrovirus causes diarrhea in the absence of inflammation and of enterocyte death,[18] but intestinal maltase activity decreases suggesting an osmotic mechanism for diarrhea.[19]

The major determinants of astrovirus immunity are as yet unclear. Serum antibody does seem to correlate with immunity in volunteer studies,[15] and specific T cells that could recognize astrovirus antigen have been found in the adult lamina propria.[20]

There seems to be an association between HIV infection and astrovirus infection and diarrhea.[21,22] Similar associations between astrovirus infection and other forms of immunodeficiency have been described.[6]

EPIDEMIOLOGY AND CLINICAL FEATURES

HuAst have been detected in all countries where they have been sought and thus have a worldwide distribution. In temperate countries, HuAst infection peaks in winter usually 4–6 weeks prior to the rotavirus peaks.[23] In general, HuAst diarrhea is predominantly a disease of infants with an age-specific incidence of 0.38 for those under 6 months and 0.40 for those aged 6–12 months.[24] However, further episodes of infection with different astrovirus serotypes do occur subsequently. These are usually milder than the first infection. Astrovirus is the second or third most important cause of diarrheal disease in children being responsible for 5–10% of cases in hospital-based studies and for 10–25% of cases in community-based studies.[25] Globally, serotype 1 is most frequently detected.[22,26–28] Recently, HuAst serotype 3 has been associated with higher stool viral levels and increased incidence of persistent diarrhea.[16] Outbreaks of astrovirus infection have been described involving the rarer serotypes.[29]

The incubation period was 3–4 days in adult volunteers but in children in Egypt was 24–36 hr.[24] The illness which consists of acute watery diarrhea with or without nausea and vomiting is usually milder than that due to rotavirus. It lasts on average 5–6 days.[30]

DIAGNOSIS

Astroviruses were first detected by negative stain electron microscopy (Fig. 1), but the characteristic star-shaped capsid is found in only 10% of the viral particles in fecal samples. It is now clear that electron microscopy is less sensitive than antigen detection or RT-PCR.

An antigen-capture ELISA was developed in 1990.[31] The lower limits of detection sensitivity of such an ELISA are estimated to be 10^5 to 10^6 particles per gram. A commercial kit version of this is available which can

Table 1 Primers for RT-PCR detection and genotyping Astrovirus

Primer	Region	Nucleotides	Sense	Sequence (5′–3′)
Mon 340	ORF1a	1180–1202	+	CGT CAT TAT TTA TAT CAT ACT
Mon 348	ORF1a	1469–1448	–	ACA TGT GCT GCT GTT ACT ATG
Mon 269	ORF2	4526–4545	+	CAA CTC AGG AAA CAG GGT GT
Mon 270	ORF2	4974–4955	–	TCA GAT GCA TTG TCA TTG GT
Mon 245	ORF2	4934–4953	+	TTA GTG AGC CAC CAG CCA TC
Mon 244	ORF2	4560–4541	–	GGT GTC ACA GGA CCA AAA CC

detect each of the eight astrovirus serotypes (IDEIA Astrovirus, Dako Diagnostics, United Kingdom), and this has been shown to have a sensitivity of 100% and specificity of 98.6%.[31]

Detection of virus genome by RT-PCR is estimated to detect as few as 10 to 100 astrovirus particles.[32] A number of different targets have been used. One strategy employs primers (Mon244 and Mon245) which amplify a 413-nt fragment of ORF2 (Table 1), and if this is negative, primers Mon340/348 which amplify a 289-nt fragment of ORF1a are used.[22,29,33] For genotyping, fragments of ORF1a, ORF1b, and ORF2 are amplified by RT-PCR and sequenced.[29] A novel hanging drop single-tube nested RT-PCR method has recently been devised.[34] Recombinant antigens have been used to determine the seroprevalence of astrovirus infections.[35] This is of little value for acute diagnosis.

MANAGEMENT AND PREVENTION

Astrovirus gastroenteritis is usually mild, but if the patient is dehydrated, appropriate rehydration therapy should be given. In hospital, appropriate infection control measures must be used to prevent nosocomial spread. Methanol 90% v/v is a better disinfectant than ethanol.[36] There are no vaccines available for preventing astrovirus infection in humans.

CONCLUSION

Astrovirus is the second or third commonest cause of infantile gastroenteritis. There are eight serotypes with little cross-immunity between serotypes. Outbreaks of infection can occur in children and adults. The positive sense single-stranded RNA genome encodes three open reading frames: ORF1a (nonstructural: protease), ORF1b (RNA polymerase), and ORF2 (capsid protein).

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Automated DNA Sequencing

Alexandre Izmailov
Visible Genetics, Inc., Toronto, Ontario, Canada

INTRODUCTION

DNA sequencing is an important tool for molecular biology, genetic studies, pharmacogenomics, and other areas of fundamental and applied science where information is required about unknown DNA, mutations, DNA fragment size, for DNA fingerprinting or prediction of drug resistance of viruses. Originally suggested, and widely used, manual sequencers cannot satisfy a growing demand for throughput increase, high reliability, and ease of use. This necessitates the development of automated DNA sequencers. Automation may include data collection and analysis, or as in the case of fully automated sequencers, also sample loading and sieving matrix replacement. The output of automated DNA sequencers is a base calling report, which represents the sequence of the nucleotides in the fragment of a DNA molecule. Different applications may require specific features of a DNA sequencer, but base calling accuracy and read length remain major figures of merit for all sequencers. Clinical applications and research, which involves large volumes of data, require high throughput and a high level of automation. However, automated DNA sequencers targeted to research laboratories must also provide flexibility in their features. Careful assessment of the user requirements allows an optimum selection of DNA sequencer from the wide variety of existing instruments.

SEQUENCING CHEMISTRIES

The goal of DNA sequencing is to obtain information about the sequence of nucleotide bases—adenine (A), guanine (G), cytosine (C), and thymine (T)—which constitutes a particular DNA molecule. In order to obtain this information, the sample under investigation is modified in DNA sequencing reactions, which can be based on one of two approaches: enzymatic synthesis of DNA fragments with chain termination by dideoxynucleotides ddNTP (terminators)[1] or by chemical degradation method.[2] The first method is faster, easier to implement, and it is currently the method of choice. Terminators (ddNTP) lack a 3′OH group that is necessary for the extension of DNA's sugar phosphate backbone. Thus the DNA chain cannot be extended beyond the incorporated ddNTP. A mixture of all four dNTPs and one ddNTP is used in each reaction. For example, in a T-terminated reaction a ddTTP is added to a mixture of dNTPs. This allows elongation of the DNA chain until ddTTP is occasionally attached to the growing molecule, which stops the reaction. This process is random and as a result, fragments of different lengths are produced, each terminated at a T-position, for all T's from the primer to the end of the sequence. Four reactions are required for complete description of the DNA sequence, each representing an A-, C-, G-, or T-terminated ladder. In order to enable observation of DNA bands, the fragments are labeled with a tag (radioactive or fluorescent) attached to either the primer (primer chemistry) or ddNTPs (terminator chemistry).

STRUCTURE OF A DNA SEQUENCER

A DNA sequencer is composed of an electrophoresis system, which includes a sieving matrix (e.g., polyacrylamide gel), buffer, a high-voltage power supply, and a detection system. When a high voltage (1000 V to 10 kV) is applied to the gel, an electric field forces negatively charged fragments of DNA molecules to move through the mesh created by polymer molecules constituting the gel. The difference in the friction for the DNA fragments of different sizes leads to their spatial separation. Groups of molecules of the same size propagate through the gel as a set of confined bands with the width defined by such factors as diffusion, injection conditions, sample volume, field, and temperature gradients, etc.

Manual and automated instruments, in their principle of operation, mainly differ by the method of detection. In manual sequencers the electrophoretic run is stopped as soon as the first base of interest (typically a primer) reaches the end of the gel. Then, information about DNA sequence is obtained by determining the relative position of the bands in the gel. In automated sequencers, the detection system records variations of the output intensity over time at a fixed location in the gel. The difference in the migration times of various DNA chain lengths results in a set of electrophoretic peaks.

Automated DNA sequencers can be divided into three major categories based on the method used for

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sieving matrix support: slab gel, capillary, and microarray sequencers.

SIEVING MATRIX

A polymer most frequently used as a sieving matrix in automatic DNA sequencers is polyacrylamide. It can be used in linear or cross-linked form. The porosity of the gel is defined by the concentration of acrylamide and cross-linking agent (typically *N*,*N*′-methylene-*bis*-acrylamide). The concentration of acrylamide may vary from 3% to 10%.[3] The concentration of a cross-linker is ~3% to 5% of the total acrylamide concentration. An increase in the acrylamide concentration suggests a higher resolution of the DNA bands and allows calling more bases, but this leads to slower runs. Linear polyacrylamide (LPA), which is more fluid, is typically used in capillary electrophoresis (CE) where the sieving matrix is replaced after each run so the capillary can be reused. The last requirement is a result of the high cost of the capillary arrays and complexity of alignment required after their replacement. Other types of polymers used in CE include hydroxyethyl cellulose (HEC), poly(ethylenoxyde) (PEO), polyvinyl pyrrolidone (PVP), and others.[4]

One of the major figures of merits in the DNA sequencers is the resolution of the DNA bands. A reduction in the width of the DNA band improves resolution, allowing base calling of longer DNA chains. Resolution depends also on the distance which DNA molecules migrate in a sieving matrix. Separation lengths of 10 to 50 cm are typical but they may be longer for higher resolution. However, there is a trade-off between increase in resolution and run time: for a given voltage, velocity of DNA molecules in the gel is inversely proportional to the separating length, L, resulting in a migration time proportional to L^2.

For analysis of single-stranded DNA, denaturants such as urea (concentration 4–8 M) or formamide (up to 10%) are added to a gel. Additionally, the electrophoretic runs are conducted at elevated temperature (40–70 °C), which helps to prevent self-hybridization.

METHODS OF DETECTION

Radiolabeling

DNA molecules separated by the sieving matrix need to be detected. In the manual sequencers an autoradiographic method is used[3] where either a primer or terminators contain a radioisotope (typically ^{32}P or ^{35}S). After completion of the electrophoretic run, the gel is dried and placed in contact with an X-ray film. Exposed film is developed and manually analyzed. Lack of automation in the data analysis and risk associated with using radioactive materials are the most serious drawbacks of this method. Also, the resolution at high base numbers in manual sequencers is lower than that in automatic sequencers because of the much shorter migration distance.

Laser-Induced Fluorescence (LIF)

Laser-induced fluorescence is a standard detection method in a majority of the automated sequencers. This method is extremely sensitive, allows automation of base calling, avoids using radioactive materials, and promotes a throughput increase by using several spectrally distinct labels simultaneously. Laser-induced fluorescence is based on the excitation of a dye label attached to a DNA molecule by laser light. Resulting fluorescence is collected and converted to an electrical signal by a photosensitive element [photomultiplier tube (PMT), photodiode, or charge-coupled device (CCD) element]. The output signal (trace) represents a series of peaks each corresponding to a DNA chain of a certain size. One trace provides information about one of the four bases (A, C, G, or T). Therefore, in order to obtain information about a complete sequence, four traces are required. This is achieved either by using four physically separated lanes (or capillaries) or by running four differently labeled samples in a single lane.

If a single laser is used in the sequencer, different parts of the gel or different capillaries are illuminated through a scanning objective lens. Alternatively, a sheath cell[5] can be used with simultaneous illumination of several DNA traces. Some DNA sequencers contain several lasers either with different wavelengths improving excitation[6] or with the same wavelength providing excitation of multiple samples without using moving parts.[7]

Collected fluorescence is converted into digital form and processed by a computer. Software packages used in different sequencers vary in their functionality and efficiency, but all of them involve the following common steps: noise reduction, base line subtraction, color cross-talk compensation, trace alignment, and peak recognition. A typical example of the resulting base calling is shown in Fig. 1.

Dye Labeling

Dye labeling is typically used for sample identification in the automatic DNA sequencers. The properties of a dye shall satisfy the following major requirements: 1) Dye structure shall allow attachment to a primer or ddNTP with a reasonable yield. 2) Absorption spectrum shall match the laser wavelength. 3) Extinction coefficient and quantum yield shall be high enough to provide acceptable sensitivity. 4) Dyes shall be chemically and thermally

stable in order to withstand cycle sequencing conditions 5) Dyes shall be photo stable. 6) Mobility of the DNA fragments labeled with different dyes shall be equalized, which can be achieved by optimization of the labeling process.[8]

Both emission and absorption spectra of the organic dyes employed in DNA labeling are broad (typically 20–50 nm). This relaxes the requirement for spectral content of the excitation light.

Overlapping absorption spectra of several dyes used for labeling allows excitation of all these dyes with a single laser source. However, efficiency of excitation drops off quickly as the laser wavelength deviates from the absorption maximum of the dye and results in a significant drop in sensitivity. To avoid these losses, energy transfer (ET) dyes were introduced.[9]

In the case of multicolor sequencers (when two or more dyes are used for DNA labeling), collected radiation must be spectrally separated by means of a diffractive element or a set of interference filters and is registered by separate PMTs or by a CCD. Alternatively, a single PMT can be used with a set of replaceable filters (a ''color wheel''). A more sophisticated detection method used for sensitivity increase is based on single-photon detection.[10]

A complication arises because the emission spectra of the dyes used for labeling different samples overlap and color cross-talk correction of the collected data is required. Improper compensation of the cross-talk results in false peaks in the processed data.

Both visible and near IR dyes can be used for DNA labeling in automated DNA sequencers. The advantage of using red and near IR dyes is lower background

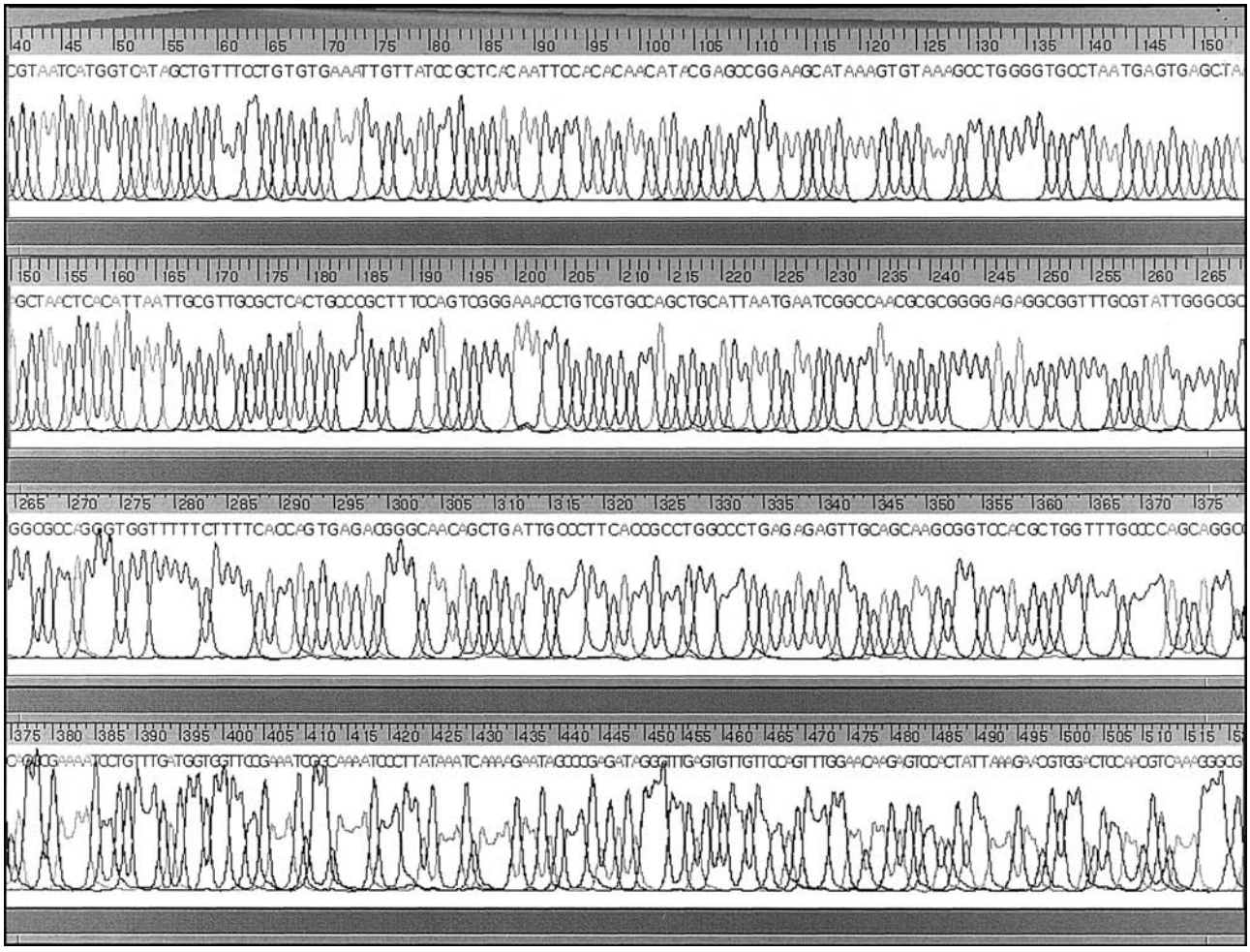

Fig. 1 A typical result of base calling obtained with an automated DNA sequencer. The Ml3 sequencing by ultrathin slab gel electrophoresis. The signal obtained with the Long-Read Tower™ sequencer has been base called with GeneObjects software. (From Ref. [7].) The accuracy of base calling is 98.5 % up to 500 bases. Similar results are typically produced with other types of automated DNA sequencers, including CE instruments.

fluorescence and decreased laser light scattering at longer wavelengths. This results in better sensitivity. IR dyes can be excited by laser diodes (LD) resulting in a reduction of instrument design complexity.[6,7]

Dye labels can be covalently linked either to a primer or to each of four types of ddNTPs.[11] In the last case, all four sequencing reactions can be carried out in one tube instead of the four used in primer-labeled chemistry. However, dye-labeled terminators may introduce significant variability in the peak intensity resulting in decreased base calling accuracy and reliability. For clinical applications dye primer chemistry may be preferred because of the higher reliability of the results. Terminator chemistry provides more flexibility, which may be advantageous for the research laboratories.

In some automated sequencers less than four colors are used for DNA labeling. For example, a two-dye approach is used in the Long-Read Tower™ and LICOR sequencers. The sample is prepared by running two sequencing reactions in the same tube with differently labeled forward and reverse primers. This approach enables the increase of the read length, accuracy, and reliability of base calling.

TYPES OF DNA SEQUENCERS

Slab Gel Sequencers

Initially, automated DNA sequencers were based on slab gel electrophoresis with low throughput and automation limited to data collection and analysis. Gel filling and sample loading are done manually. The support glass plates require cleaning after each run. Poor dissipation of heat generated in a slab gel through relatively thick glass plates limits the electric field strength and prevents short run times. Both of these problems are eliminated in the disposable ultrathin slab gels (50 μm thick), which can withstand higher electric field strength, E (100 V/cm), resulting in shorter run times.[7]

Capillary Electrophoresis Sequencers

Capillary electrophoresis (CE) DNA sequencers were introduced to increase analysis throughput. This is a direct result of high electric fields (100–250 V/cm) and multiple capillaries (up to 384 capillaries). Both sieving matrix replacement and sample loading can be automated. The small diameter of capillaries (internal diameter is 50–100 μm) allows for good heat dissipation. Capillaries having smaller diameters (2–10 μm) are not practical as they do not provide enough signal intensity, are difficult to fill with acrylamide, and do not provide reproducible performance.[12] The ability to use replaceable sieving matrices makes CE systems practical as repetitive runs are possible with the same capillary up to 200 times or more.

Electra-kinetic sample loading allows high automation of CE sequencers. But optimization of loading conditions and sample purification is required. These are critical for reducing variability of peak intensity, suppressing decay in gel current and bubble formation, increasing capillary lifetime. Salt concentration, ratio of labeled DNA to unlabeled DNA, and total volume of loaded sample all affect the success of CE analysis.

Typical read length in CE DNA sequencers is 400–600 bases in several hours, but can be as high as 1100 bases.[13] Run conditions can be optimized for faster runs, if necessary, by reducing separation lengths, decreasing polymer concentration, increasing voltage or/and gel temperature.

Examples of commercial automated DNA sequencers are shown in Table 1.

A variety of automated DNA sequencers is available, providing similar performance characteristics but

Table 1 Automated DNA sequencers

Model	Read length (nt)	Number of lanes	Run time (hr)	Type
Al31 3731377	850	48/64/96	10	Slab
LI-COR 4300 L	1000–1200	48/64	10	Slab
LI-COR 4300 S/ L	800		6	
	500		1.5–3	
Long-Read Tower™	300/500/700	16	0.51/14	Ultrathin slab
ABI 310	500–600	1	3	Capillary
ABI 3700	550	96	5	Capillary
ABI 3730 (xl)	550–1100	48/96	0.5–2	Capillary
CEQ™ 2000	500	8	2	Capillary
CEQ™ 8000	700	8	2	Capillary
MegaBACE™ 1000	550	96	2	Capillary
MegaBACE™ 4000	550	384	2	Capillary

Source: From Refs. [6,14–17].

differing in throughput, flexibility, complexity, and cost. Although generally higher throughput is desirable (especially for clinical labs), for some applications the major requirement may be short run time, flexibility of characteristics, or low cost of analysis. In this case smaller DNA sequencers may be advantageous.

Microarray Sequencers

Capillary microarray and microfabricated sequencers are still under development and promise a significant size reduction of automated sequencers providing high throughput and base calling accuracy.[18,19] A 96-channel radial capillary array was demonstrated in Ref. [20]. The microchannel plate provides high resolution due to the use of tapered turns that provide an effective separation length of 15.9 cm on a compact 150-mm-diameter wafer. An average read length of 430 bases was obtained on this device. In general, miniaturization typically leads to a reduction of separation distance causing poor resolution, insufficient read length, low base calling accuracy, all of which currently limit the application of these devices.

CONCLUSION

Automated DNA sequencers provide important information about DNA structure. Plurality of existing instruments can satisfy a variety of requirements, which may arise in a particular experiment. Although modern automated DNA sequencers are well developed, continuous improvement of their structure, components, and run conditions is still ongoing. The development of new approaches is focused on throughput increase, reduction of sequencer size, and improvement of accuracy, reliability, and read length.

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Automated Nucleic Acid Extraction

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Juergen Loeffler
Kathrin D. Schmidt
Holger Hebart
Hermann Einsele
Eberhard-Karls-Universität Tuebingen, Tuebingen, Germany

INTRODUCTION

In the past, the extraction and purification of nucleic acids had been a complicated, time-, and labor-intensive process. Manual extraction of nucleic acids requires repeated centrifugation steps followed by removal of supernatants and, depending on the type of specimen, additional mechanical treatment, e.g., for the lysis of blood cells, organ biopsies, or bacterial and fungal cell walls. In addition, many extraction methods suffer from the presence of strong inhibitors that are present in different clinical specimens, such as sera and stool, and may lead to false-negative results. Furthermore, many manual protocols rely on the use of toxic chemicals, such as guanidinium thiocyanate or phenol–chloroform.

Robotic workstations for the extraction and purification of nucleic acids should fulfill a true walk-away automation. The technology should allow a high throughput of samples; the yield, purity, reproducibility, and scalability of the RNA and DNA as well as the speed, accuracy, and reliability of the assay should be maximal, whereas the risk of cross-contamination must be minimal.

Here we give an overview of commercially available workstations for an automated extraction of nucleic acids.

BioRobot WORKSTATIONS

The BioRobot EZ1 workstation (Qiagen, Hilden, Germany) purifies nucleic acids from many clinically relevant specimens; one to six samples can be processed within 15 min of time. This rapid purification is feasible using a magnetic particle technology: nucleic acids in sample lysates are isolated in one step through their binding to the silica surface of magnetic particles. The automated protocols for nucleic acid purification are provided on special preprogrammed cards.

The BioRobot M48/M96 workstations run the identical magnetic particle technology, but the pipet tips function as reaction chambers. However, up to 48/96 samples can be processed per run in increments of six samples. The pipettor head contains six syringe pumps, which operate simultaneously to allow aspiration or dispensing of volumes between 25 and 1000 μL. The workstation uses UV light to decontaminate surfaces in-between runs.

Finally, the BioRobot 9604 workstation allows automated nucleic acid purification for up to 2×96 samples in parallel.

Xu et al.[1] describe an automated assay for the extraction of HIV RNA by using the BioRobot 9604. They conclude that this procedure yields comparable results to a manual RNA extraction method with improved precision. The assay was found to be linear down to 50 copies.

For the extraction of hepatitis C virus RNA, it was shown that it is possible to achieve a detection level of 12.8 IU/mL (95% confidence). Cross-contamination studies have confirmed that the use of the BioRobot 9604 does not pose a detectable contamination risk.[2]

For hepatitis B virus detection, Mitsunaga et al. describe that it was possible to quantify DNA in all samples extracted by the BioRobot 9604 which contained more than 500 genome equivalents/mL. Extraction of 96 samples could be completed within 2 hr.[3]

COBAS AmpliPrep

The COBAS AmpliPrep robotic workstation (Roche Diagnostics, Mannheim, Germany) is a fully automated system which isolates DNA and RNA targets. It is based on silico-coated glass particles which bind the extracted nucleic acids. Internal controls allow target recovery monitoring. Up to 72 samples (three racks of 24 specimens each) can be loaded on the system simultaneously. Reagents are delivered in sealed, bar-coded, ready-to-use cassettes. The pipetting integrity check ensures the accurate pipetting of samples and reagents, and a clot detection reduces the risk of incorrect results. Besides kits for the extraction of generic DNA, introductory kits for hepatitis C virus and human immunodeficiency virus type 1 detection can be acquired. This product is not available in the United States.

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In a preliminary study, the COBAS AmpliPrep was evaluated for the preparation of human immunodeficiency virus RNA and was compared to a manual sample preparation protocol.[4] The authors assessed the reproducibility by analyzing 584 plasma samples from infected patients. They achieved an interassay coefficient of variation between 39.4% and 48.4% and an intraassay coefficient of variation from 6.2% to 58.0%. The mean viral load of 152 samples, which were found to be in the linear range of both tests, was 3.7 log(10) HIV copies/mL by the COBAS AmpliPrep compared to 3.73 log(10) copies/mL by the manual test (no significant difference). The authors concluded that the COBAS AmpliPrep assay is reproducible and sensitive.

Miyachi et al.[5] evaluated the COBAS AmpliPrep protocol for hepatitis C virus RNA extraction. Assay linearity was observed to range between 500 and 850,000 IU/mL by using serial dilutions. Comparison of the COBAS AmpliPrep test results to those obtained by a manual extraction method showed a good correlation [$R(2)=0.972$, $n=86$]. When known PCR inhibitors such as heparin, dextran sulfate, hemoglobin, and bilirubin were added to the samples prior to extraction, the automated assay successfully eliminated the inhibitory effects.

KingFisher mL

The KingFisher technology (Thermo Electron, Waltham, USA) uses shifting of magnetic particles through the different phases of the purification process. Volumes from 50 to 1000 μL can be processed, and because of the 3×5 fixed magnetic rods and the 3×5 separate disposable tube strips, a maximum capacity of 15 samples per run can be achieved. Besides RNA and genomic DNA, the KingFisher instrument enables the isolation of proteins from a variety of starting volumes.

Saukkoriipi et al.[6] compared the KingFisher instrument to a manual DNA extraction method for the purification of *Streptococcus pneumoniae* DNA from 50 nasopharyngeal swab samples. All pneumococcal culture-positive samples (44%) were PCR-positive regardless of the extraction method. Additionally, from the culture-negative specimens, 71% of the manually extracted and 82% of the samples purified with the KingFisher instrument were PCR-positive.

m1000

The m1000 sample preparation platform (Abbott Laboratories, Abbott Park, USA) uses a magnetic particle technology for the extraction and purification of RNA and DNA. It provides a fully automated sample preparation and is capable of processing 48 samples within 2 hr for a sample volume of 1 mL. Analyte-specific reagents (ASR) may be used for the preparation of samples for hepatitis C virus analysis as well as for the extraction of human DNA for HLA typing. The robot contains liquid-level sensing and a clot detection system. Standard primary bar-coded tubes are used.

Silva et al.[7] evaluated the performance of the m1000 instrument for the extraction of genomic human DNA from frozen blood specimens followed by HLA-DRB sequencing-based typing. Their results showed that the DNA yield was consistently high with an average concentration of 20 ng/μL (from 0.2 mL blood). The average (260:280 nm) ratio was 1.7, and the purified DNA was free of inhibitors; all DNA samples could successfully be amplified and sequenced.

MagNA Pure LC INSTRUMENT

Magnetism is the underlying principle of the automated nucleic acid isolation performed by the MagNA Pure system (Roche Diagnostics, Mannheim, Germany). The instrument has a completely closed housing as well as an automatic clot and tip loss detection. No filtration, centrifugation, or vacuum pumps are necessary, which minimizes the risk of cross-contamination. Up to 32 nucleic acid isolations (the eight-nozzle pipette head allows a variable number of samples from 1 to 32 per run) can be performed within one run. Extraction of human, viral, bacterial, and fungal DNA, RNA, and mRNA is possible. The protocol is applicable to a broad variety of samples such as blood, cultured cells, biopsies, sputum, urine, stool, plant tissue, and food products. After the extraction procedure, isolated nucleic acids and PCR mixes can be pipetted automatically into PCR tubes, 96-well plates, LightCycler capillaries (Roche Diagnostics), or COBAS A-Rings (Roche Diagnostics). Sample information can be entered manually or via a barcode reader. Samples are loaded into nuclease-free sample cartridges with a capacity of 4×8 wells. The initial sample volume is 20–1000 μL (or up to 5×10^6 cells or 10 mg of homogenized tissue); the dispensable volume is 5–1000 μL with a 2–3% variance.

For the isolation of DNA or total nucleic acids, the sample material is initially incubated with a lysis buffer and proteinase K, and then magnetic glass particles are added. Nucleic acids bind to the surface of the magnetic glass beads and are thus separated from the sample remnants. For the extraction of mRNA, DNA is removed by DNase digestion. Streptavidin-coated magnetic beads and biotin-labeled oligo (dT) nucleotides are added to the lysed sample. The mRNA binds specifically to the glass beads through the oligo (dT) nucleotides.

In addition, since 2003, a smaller version named MagNA Pure Compact System is available. This system

allows the extraction of nucleic acids from eight samples in parallel using bar-coded prefilled reagent cartridges and assembled tip trays.

Comparison of the MagNA Pure and a manual extraction protocol (phenol/chloroform) showed equivalent detection sensitivities for the extraction of *Borrelia burgdorferi* DNA from a variety of specimens such as urine, blood, and cerebral spinal fluid. All 80 positive samples (as determined by an independent method) were also tested positive by MagNA Pure extraction.[8]

Wolk et al.[9] describe a protocol for the extraction of DNA from *Encephalitozoon* species. These species cause microsporidiosis, a disease of which the prevalence is likely to be underestimated because of the labor-intensive, insensitive, and nonspecific conventional methods used for the diagnosis of the spores in feces. The detection limit of the assay was 100–10,000 × lower compared to microscopy with trichrome stain.

Our group[10] demonstrated that the MagNA Pure technique provides rapid automated DNA isolation from numerous pathogenic fungi revealing high sensitivity and purity. Although the fungal cell wall is highly resistant to mechanical, chemical, and enzymatical treatment, we could achieve a sensitivity of 1 colony forming unit/mL blood for *Candida albicans*.

In addition, there is a need for simple and sensitive assays for mRNA detection, e.g., to assess innate and adaptive immune responses[11] or to determine different subpopulations of cells.[12] The MagNA Pure protocol allowed extraction of mRNA from 32 samples in parallel within 1.5 hr, compared to at least 3 hr using manual methods. The amount (3–6 μg RNA from 10^6 PBMC) and purity (260:280 nm ratio in spectral photometer 1.8–1.9) of RNA were comparable or higher than those achieved by manual extraction.[12]

Finally, Williams et al. compared the BioRobot 9604 workstation to the MagNA Pure instrument, extracting genomic DNA from 106 blood samples followed by genotyping for factor V Leiden.[13] The comparison showed that both methods were similar in DNA yields (8.1 ± 2.3 μg by MagNA Pure, 7.1 ± 2.3 μg by BioRobot), although the DNA purity was higher in samples extracted by MagNA Pure (ratio 1.9 ± 0.11 vs. 1.6 ± 0.24). The extraction failure rate was 0.7% by MagNA Pure and 2.0% by BioRobot. Furthermore, with both assays the analysis of DNA extracted from sodium citrate and heparin anticoagulated blood samples was feasible. Finally, five heterozygous samples were analyzed in two separate runs. Statistical analysis using an *F*-test indicated that the ΔT_ms for the MagNA Pure were more consistent ($p < 0.001$) which may be due to differences in sample purity.

NucliSens EXTRACTOR

This instrument (bioMérieux, Marcy 1'Etoile, France) is able to extract DNA and RNA, and has been designed for a highly variable initial sample volume from 10 up to 2000 μL. The concentration factor can be up to 55-fold, e.g., a 2000-μL sample volume input can be eluted in 35 μL. The closed system minimizes carryover and environmental template contamination. Plasma, serum, whole blood as well as urine, semen, feces, and sputum can be analyzed within 45 min per run, and up to 10 samples can be processed within one run.

The extraction protocol is based on a method described by Boom et al.[14] After lysis of the samples and addition of silica, nucleic acids bound to the silica are captured on a filter by applying air pressure on the closed sample

Table 1 Comparison of eight different workstations for automated nucleic acid extraction concerning assay duration, maximal number of samples, processing volume, and price

Workstation	Duration per run (maximal sample load)	Maximal number of samples per run	Processing volume	Approximate price of workstation
EZ1 (Qiagen)	15 min	6 samples	25–1000 μL[a]	U.S.$35,000
BioRobot 9604 (Qiagen)	2 hr	2 × 96 samples		U.S.$100,000
COBAS AmpliPrep (Roche)	3 hr	3 × 24 samples	200–1000 μL	U.S.$50,000
KingFisher mL (Thermo Electron)	45 min	15 samples	50–1000 μL	U.S.$15,000
m1000 (Abbott)	2 hr	48 samples	200 or 1000 μL	U.S.$110,000
MagNApure LC (Roche)	1.5 hr	32 samples	20–1000 μL	U.S.$90,000
MagNAPure Compact (Roche)	30 min	8 samples	100–1000 μl	U.S.$35,000
NucliSens Extractor (bioMérieux)	45 min	10 samples	10–2000 μL	n.a.

n.a. = not available.
[a]Depending on the workstation.

chamber. The silica particles remain on top of the filter. Through a hollow air-pen, multiple wash cycles are performed. After drying of the silica at 56°C, the nucleic acids are eluted into an elution tube by means of air pressure.

Jongerius et al.[15] used this technology for the extraction of hepatitis C virus RNA. They found a cutoff value of 16 genome equivalents (geq)/mL with a 100% hit rate, demonstrating a high sensitivity of this assay for the extraction of RNA.

Comparison of the BioRobot 9604 to the NucliSens Extractor for the isolation of hepatitis C virus RNA showed a lower detection limit of 82 vs. 12 geq/mL.[16]

CONCLUSION

Current manual methods for the extraction of nucleic acids are suitable for low-throughput analysis.[17] However, these methods are unable to cope with the increasing demand for molecular diagnosis of infectious diseases and genetic analysis, necessitating the development of new, fully automated, and reliable methods. All automated workstations described reveal high yield, purity, reproducibility, scalability, accuracy, and reliability for the extraction of nucleic acids (Table 1). However, as different laboratories may claim unique performances of a workstation, the individual choice of an instrument should depend on the type and volume of samples to be analyzed and on the throughput of samples per day.

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Barriers to Integrating New Genetic Knowledge into Daily Medical Practice

B

Carol Isaacson Barash
Genetics, Ethics & Policy Consulting, Inc., Boston, Massachusetts, U.S.A.

INTRODUCTION

The expectation that diagnostic genomics and proteomics, along with toxicogenomics, chemicalgenomics, and BIO IT, will revolutionize health care is big and widely held. New knowledge emerging from these fields promises an understanding of gene function thorough enough to enable the mitigation of adverse effects of genetic risks, therapies that are more effective and safer, and the opportunity to ameliorate health risk factors in the context of environmental and socioeconomic risk factors. In other words, they promise a deeper understanding of disease and its causes, and with it strategies to promote health, prevent disease, and lower overall mortality and morbidity. Achieving these ends requires translating this new genomic knowledge into daily medical practice. Adoption of genomic innovations is, however, not an automatic given or foregone conclusion. Rather, it is a formidable challenge. The expected benefits of diagnostic genomics and proteomics cannot be realized unless practitioners accurately understand new genetic knowledge, apply that understanding appropriately in daily practice, and work with patients to ensure that they can make voluntary informed choices about their care. Adoption (or successful incorporation of innovation into practice) of genomics innovations (diagnostic genomics, proteomics, or toxicogenomics) requires ensuring that practitioners spontaneously think about the possibility of genetic involvement, when appropriate, and pursue that possibility accurately and efficiently.

THE CHALLENGE OF INTEGRATING GENOMIC INNOVATION

It is commonly believed that innovations succeed on their own merit. Those without real merit fail in the market place; those with superior merit succeed simply on the basis of their superiority over existing alternatives. *However*, experience shows otherwise. Technological capability and superior engineering do not, in fact, guarantee acceptance (Table 1). Great innovations themselves are often insufficient to ensure usability, effectiveness, or user satisfaction. In other words, good technology does not equal adoption. Why? (Fig. 1) Because factors integral to users' experiences, such as intuitive approaches to problem solving, environmental factors or underlying beliefs, values or attitudes, play an integral role in adoption. More specifically, these factors can facilitate or thwart integration of new knowledge and thus bar acceptance. For example, the problem-solving logic inherent in IT tools may well serve a developer's needs, but prove incoherent with end users' intuitive problem-solving processes, thereby failing to be a useful solution for the employee.[1]

Similarly, physician lag in adopting innovation is well known. The history of medical practice, in particular, is replete with examples of un- and underutilized innovations, whether new IT solutions, devices, diagnostics, clinical guidelines, or therapeutics.[2–5] This history suggests the very real possibility that genetics innovations will not be readily or accurately integrated into daily practice without an identification of and remedy to barriers (Table 2).

A central goal of genetics educational initiatives, indeed, has been to promote adoption and accomplish the primary aim of effecting a change in medical practice. The desired behavioral change is to improve physician decision making about potential genetic involvement in the face of uncertainty. Existing educational programs have increased clinician *awareness* of advances in molecular medicine.[6] Clinical competency in problem solving about potential involvement depends on more than mere awareness[7] (Table 3). Despite the high quality of many current genetics education curriculum, education assessments confirm that practitioners remain unconvinced about the relevance of genetics advances to their practices as well as *uneasy about how to integrate* this new information into point-of-care service.[8–10] Education alone, then, has been less than fully successful and changing clinician behavior so that practice reflects the appropriate incorporation of innovation. The shortcomings are due far less to the quality and delivery of educational programs than they are to a lack of understanding of practitioner diagnostic problem-solving strategies, and both the environmental and internal factors (i.e., cognitive, attitudinal, and normative factors) that influence those strategies. Researchers in continuing medical

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Table 1 Predicting innovation

1943	IBM, Chairman (Thomas Watson)	''I think there is a world market for about 5 computers''
1977	Digital Equipment Co., President (Ken Olson)	''There is no reason for any individual to have a computer in their home''
1981	Microsoft, CEO (Bill Gates)	''640K [of memory] ought to be enough for anybody''

education show that such factors, hidden within the day to day of medical practice, nonetheless can pose significant obstacles to acceptance.[11–14]

Cognitive barriers represent a particularly difficult hurdle, primarily because they are embedded in clinical problem-solving strategies that become deeply engrained over time in one's practice.[15] For example, clinicians rarely use formal probabilistic modes as they engage in diagnostic reasoning. Instead, they use induction and heuristics as problem-solving tactics because heuristics[16] (i.e., rules of thumb or shortcuts), in particular, drastically reduce the number of steps needed in a search for solutions to a problem, or a diagnosis. Heuristics, reasoning shortcuts, and other thinking habits aid physicians in accurately and efficiently solving clinical conundrums, making diagnoses, or determining medical decisions. Although these tactics are neither precise indicators of prevalence nor other probability associations, heuristics are convenient and frequently correct. As such they serve a practitioner well, given time, and other practice, constraints. Furthermore, their accuracy reinforces their continued use. In fact, they are typically learned in medical school, refined to one's idiosyncratic ways of framing and solving diagnostic puzzles and continually reinforced in daily practice. As such, they become more ingrained in a clinician's intuitive diagnostic approaches.[17]

Fig. 1 Innovativeness doesn't ensure adoption. (*View this art in color at www.dekker.com.*)

The ''availability'' heuristic is used to judge frequency and probability but relies on the cases brought to mind as a basis for judgment. As such, it can impede focus on case specifics. The ''representative'' heuristic assumes that a present case so closely resembles a well-defined case that the probability of the disease occurring in this case is that of the well-defined case. Resemblances between a patient's features and a classic disease pattern can produce exaggerated estimates of the likelihood that present disease as in fact the classic one. In other words, the class of things retrieved most easily leads subjects to believe that *that* class is more frequent than those classes less easily retrievable. As such this tactic can result in failure to consider either prior probability or factors that limit predictive accuracy of thought generated.

Applied to genetic issues, however, these techniques are less useful and may even contribute to failure to detect genetic involvement or appropriately consider genetic influences, thereby resulting in misdiagnosis or mistreatment. Specific characteristics of genetics thwart the clinical utility of these clinical reasoning strategies that are designed to identify observable pathology (phenotype), determine a proximate cause (diagnosis), and prescribe appropriate treatment and management. In particular, scientists determined that one gene can affect more than one trait (pleiotropy), that any single trait can be affected by more than one gene, and that the majority of traits are affected by environmental factors as well as by other genes. Identifying the cause of a clinical symptom (or trait) is then far more complicated than identifying a symptom or determining that a number of symptoms indicate the presence of disease. Furthermore, determining the clinical significance of any genetic findings (that is, what do the genetic findings mean in the context of the patient's biochemical, hormonal and other biological processes) is more complicated than many general medical diagnoses, such as detecting the presence or absence of an otitis media or strep throat. The utility of heuristics and clinical reasoning shortcuts in the context of complicated genetic involvement is thus compromised.[18–20]

Constructing, understanding and problem solving are cognitively similar activities. Expertise in clinical problem solving has been shown to consist of highly automated, yet highly accurate perceptual and cognitive processes. For example, expert behavior is characterized by rapid recognition of key aspects of problems. Experts

Table 2 Indicators of physician receptivity to genomics innovations

''I've taken CME in the new genetics, but I shy away from it. It takes lots of time, is changing so fast and is so complicated. But, I have the working consciousness of a geneticist. I look for things.''	(Primary Care Physician recently trained in genetics advances)
''Several doctors (at a bioethics conference) admitted they had never heard the pharmacogenomics until the conference and weren't quite sure what they would do with a genetic test if they had one.''	(AMA News, Sept. 2000)
''Teachers open the door, but you must enter by yourself.''	(Chinese Proverb)

in any domain, for example in primary-care delivery, are known to use more elaborate but more flexible schemas for conceptualizing, understanding, and reasoning through problems, as well as domain-specific conceptual and procedural knowledge. These skills enable clinicians to identify critical cues within data presented and reason using data-based hypothesis generation, both of which are considered marks of expertise.[21]

Research into classical genetics problem solving shows that domain-specific and procedural knowledge is crucial to successful problem solving.[22] Knowledge-based and procedural errors in reasoning typically include knowledge deficits, factual errors, recall failure, fallacies of inference, fallacies of availability, metajudgments about the importance of pieces of information, and misapplied knowledge. Research into the effectiveness of genetics education programs indicates that clinicians have displayed a proclivity for confirming genotype solely on the basis of phenotypes ascertained through physical exam. Family history characteristics, which could trigger suspicion of possible heritable involvement, were not spontaneously considered by clinicians.[23] This tactic, which is a very narrow approach to genetics thinking, represents problematic genetic reasoning competency. Clinicians are trained to diagnose on the basis of observed phenomenon, (phenotype) and biochemical tests indicated what has most likely caused the physical finding. This approach, however, motivates practitioners to consider genetic involvement from the physical (phenotype) to the internal (genotype) and therein lies a major potential for error in clinical judgment. Research further indicates that clinicians with recent training in advances in genetics did not perform as well as clinicians lacking specific genetics training but known to be outstanding clinical problem solvers.[24]

Table 3 Genetics' barriers to adoption

Complicates typical strategies to ensure innovation adoption
Challenges the efficiency and efficacy of the usual clinical reasoning strategies involved in medical problem solving
Ethical concerns increase the stakes in using genetics in practice

Assimilation of new genetic knowledge occurs when the possibility of genetics involvement is continually brought to mind, entertained, pursued, and rigorously tested for validation or falsification. Deriving genetic suspicions solely from phenotypic characteristics does not achieve the desired clinical competency in identifying or making medical decisions about potential genetic involvement because pleiotropy, phenotypic variability, and other aforementioned characteristics of genetics introduce considerable room for error in clinical judgment. Physicians will become clinically competent genetic thinkers when their clinical thinking attends to the particular characteristics of genetics.

In particular, clinicians will display competence in genomic medicine when they apply more substantive genomic knowledge, a higher level of inquisitiveness, a broader range of diagnostic hypotheses and interpretations of clinical findings, as well as a more methodical manner of testing candidate hypotheses. A lower threshold for considering possible genetic involvement and consulting a geneticist to validate a suspicion will also serve clinicians well. In addition, family history gathering focused on identifying possible inheritance patterns and recurrence risk will serve clinicians well in refining possible genetic involvement. Reliance on textbook definitions and diagnostic criteria may contribute to a failure to appreciate phenotypic variability. Furthermore, as research suggests that clinicians view a ''genetic'' diagnosis as unfortunate and associated with dire medical and social consequences, they will continue to be reluctant to confer a genetic diagnosis. As knowledge increases and positive actions are proven to mitigate genetic risks, and the ill effects of a genetic diagnosis, clinicians are likely to shed this reluctance.

CONCLUSION

Genomics, as we now understand it, has, and will continue to have, a major role in health care as each of us carries roughly five to six mutations capable of causing significant disease. The major causes of death in the United States are now known to involve genetic influences. The

full extent of those influences, including the interactions between genes and genes, and genes and environments, is not yet known. Increasingly, we are learning that genetic mutations influence both the development and expression of disease. We expect that as these influences and interactions become better known we will be able to more precisely understand disease development and expression and so better able to target and manage root causes and effects. Furthermore, as our knowledge increases, we are likely to learn that some traits, which cause disease in certain environments, confer protection against disease development in other environments. Such traits are likely to be similar to sickle cell trait in contributing to sickle cell disease in particular contexts but affording immunity to malaria in other contexts.

Identifying genetic influences and understanding what they mean in the context of a patient's other risk factors is further complicated by features of genetics and heritability that complicate clinicians' usual approaches to categorizing symptoms into candidate diagnoses. Unless or until clinicians' medical decision-making processes trigger suspicion that a genetics factor may be present and pursue a thorough testing of that genetics hypothesis, ''learning'' about advances in human genetics will not be fully integrated into daily practice, and patients will not realize the benefits of genomic medicine.

Medical education research shows that knowledge, skill, and motivation, while necessary to physician acceptance, are, nonetheless, insufficient to bring about performance change.[25] This is largely because the practice of medicine, while anchored in the basic sciences, is itself an applied science but importantly also a normative activity. In other words, it is not so much what the physician knows, can do, or wants to do as much as what the physician *actually does* to (and for) patients that is of primary importance. What physicians do in practice has implications for their ability to incorporate innovation into daily activities; those daily activities, and habits, give rise to experience that in turn is a powerful determinant of behavior. Medical problem-solving literature, for example, supports this contention, indicating that what physicians actually do in practice is more related to their previous clinical experience than to their fund of medical knowledge.[26] Further, experts have shown that by understanding what needs to be done, how it is to be done, with awareness of potential barriers to successful implementation, physician learners are primed to apply new knowledge (or technological innovation) to a particular problem. Integrating innovation into practice gives rise to a new experience and, ultimately, it is the new experience that gives rise to the potential for learning.

The nature of genetics poses particularly difficult challenges to integration into daily medical practice. Continuing medical education courses, as well as strategies for adopting innovation, often fail to attend to key elements of clinical experience, and in so doing fail to address key parts of an individual's learning process and the relationship between those parts. The end result is the failure of education to effect the desired performance change. In regard to adoption of genetics innovation, unless or until educators know more about clinicians' thinking habits, which are used to assimilate new knowledge, much new knowledge may go underutilized, and potential patient benefits go unrealized. Identifying features of clinical reasoning that promote and thwart accurate and efficient medical decision making about potential genetic involvement can inform the development of future genetics education as well as specific strategies for changing physician behavior. Acceptance can then keep pace with genomic innovation.

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Basal Cell Nevus Syndrome

A. S. High
University of Leeds, Leeds, U.K.

INTRODUCTION

This disorder, caused by mutations in the human homologue of the *Drosophila* ''patched'' gene (OMIM 601309), is inherited in an autosomal dominant manner and shows high penetrance and variable expressivity. It produces a bewildering array of clinical appearances including the following: *Face*: broad facies; frontal and biparietal bossing; mild mandibular prognathism; odontogenic keratocysts of jaws. *Eyes*: strabismus; lateral displacement of the inner canthi; hypertelorism; subconjunctival epithelial cysts; iris coloboma; glaucoma. *Nose*: broad nasal root. *Mouth*: cleft lip; cleft palate. *Cardiovascular*: cardiac fibroma. *Respiratory*: congenital lung cyst. *Abdomen*: lymphomesenteric cysts, often calcified; hamartomatous stomach polyps. *Genitourinary*: ovarian fibromata; ovarian carcinoma. *Skeletal*: bifid ribs; synostotic ribs; hypoplastic ribs; scoliosis; kyphoscoliosis; abnormal cervical vertebrae; brachydactyly. *Skin*: basal cell nevi; basal cell carcinoma (BCC); pits of palms and soles. *Neurological*: mental retardation; desmoplastic medulloblastoma. Other rare lesions are also reported.

It is associated with a paternal age effect and abnormal sensitivity to therapeutic radiation. Diagnosis using clinical phenotype or genotype is still problematic and there is no universally accepted ''gold standard.''

DIFFERENTIAL DIAGNOSIS

Most clinicians still rely on a set of criteria originally formulated by Kimonis et al.[1] Table 1 shows a current list of qualifying criteria. It is worth noting that a number of other related but apparently distinct disorders are recognized. Rombo syndrome[2] is a familial disorder with vermiculate atrophoderma, milia, hypotrichosis, trichoepitheliomas, basal cell carcinomas, and peripheral vasodilation with cyanosis, whereas Bazex–Dupré–Christol syndrome[3] combines major characteristics of basal cell nevus syndrome (BCNS) with follicular atrophoderma, hypotrichosis, hypohydrosis, and minor skin defects. Occasionally, acquired BCCs develop after contact with arsenic and exposure to radiation, and even burns, scars, vaccinations, or tattoos are rare contributing factors.

BASAL CELL NEVUS SYNDROME

In basal cell nevus syndrome (BCNS) there is an increased frequency of neoplasms, the result of excessive activation of segments of the Hedgehog pathway.

Patched is a target gene in the Hedgehog signalling network. *Patched* (*Ptch1*) encodes a transmembrane protein that acts as a negative regulator of *hedgehog* (*Hh*) signalling (Fig. 1). [Note: There is a general move toward abandoning the acronyms *Ptc* and *PTC* in favor of *Ptch* and *PTCH*, thereby avoiding confusion with phenylthiocarbamide tasting (PTC; OMIM 171200)]. The 1500-amino acid glycoprotein has 12 hydrophobic, membrane-spanning domains, intracellular amino- and carboxy-terminal regions, and two large hydrophilic extracellular loops where Hh ligand binding occurs.

Ptch has dual roles in sequestering and transducing Hh. When the second large extracellular loop, essential for ligand binding, is deleted by a *Ptch1* mutation, Hh binding to Ptch cannot occur, but repression of Smoothened (Smo) by Ptch is unaffected. When a C-terminal truncation is caused by a *Ptch1* mutation, Ptch can no longer repress Smo, but Hh binding to Ptch is unaffected.

In the Hedgehog signalling network, mutations result in various phenotypes, including, among others, holoprosencephaly, BCNS, Pallister–Hall syndrome, Greig cephalopolysyndactyly, Rubinstein–Taybi syndrome, isolated BCCs, and desmoplastic medulloblastoma.

Human *PTCH1*, a tumor suppressor gene, maps to 9q22.3. Most mutations in *PTCH1* result in protein truncation. However, four rare *PTCH* missense mutations have resulted in holoprosencephaly—two in the extracellular loops required for SHH binding and two in the intracellular loops that may be involved in PTCH–SMO interaction.[4]

Four features of Hedgehog signalling are noteworthy. First, autoprocessing generates an active Hedgehog ligand with a C-terminal cholesterol moiety. Then, palmitoylation results in an N-terminal palmitate. The active Hedgehog ligand therefore becomes double lipid modified. Second, most membrane-bound receptors activate downstream signalling on ligand binding. However, Ptch is *repressed* by its Hedgehog ligand, freeing Smoothened for downstream signalling.

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Table 1 Diagnostic criteria for BCNS

Diagnosis of BCNS made in the presence of two major, or one major and two minor criteria
Major criteria
1. >2 BCCs or one at <20 years old
2. Histologically confirmed odontogenic keratocysts of jaw
3. ≥3 palmar or plantar pits
4. Bilamellar calcification of falx cerebri
5. Bifid, fused, or markedly splayed ribs
6. First-degree relative with BCNS syndrome
7. Desmoplastic medulloblastoma
Minor criteria
Any two of the following features:
1. Macrocephaly determined after adjustment for height
2. Congenital malformations: cleft lip or palate, frontal bossing, ''coarse face,'' moderate or severe hypertelorism
3. Other skeletal abnormalities: Sprengel deformity, marked pectus deformity, marked syndactyly of digits
4. Radiological abnormalities: bridging of the sella turçica vertebral anomalies such as hemivertebrae, fusion or elongation of the vertebral bodies, modeling defects of the hands and feet, or flame-shaped lucencies of hands or feet
5. Ovarian fibroma

Third, Ptch and Smoothened may shuttle oppositely between plasma membrane and endocytic vesicles in response to Hedgehog ligand. They may not interact physically, Ptch working catalytically rather than stoichiometrically.[5] In contrast, Incardona et al.[6] suggested that Ptch and Smoothened colocalize in the absence of Hedgehog and both proteins endocytose on ligand binding, after which Smoothened subsequently segregates from the Ptch–Hedgehog complex.

Fourth, the network has a bifunctional transcription regulator, Cubitus interruptus. In the absence of Hedgehog ligand, a truncated transcription repressor is generated that binds target genes and blocks transcription. With Hedgehog ligand, a full-length transcriptional activator binds target genes and up-regulates transcription.

Hedgehog is proficient at both short- and long-range signalling. It is tightly regulated on many levels. Cholesterol-modified Hedgehog, which should be anchored to the cell membrane, can be further modified at the lipid rafts where it multimerizes and becomes soluble and diffusible.[7] Dispatched (OMIM 607502; another 12-pass transmembrane protein) functions to release multimeric Hedgehog, making it available for long-range signalling.

The up-regulation of Ptch expression, resulting in Ptch protein at the cell membrane, sequesters Hedgehog and limits its spread beyond the cells in which it is produced. Thus, a balance is created by the antagonism of Hedgehog and Ptch, whose relative concentrations alternate with regard to each other. Many other factors including Megalin, Rab23, Hip, GAS1, PKA, GSK3, CK1, Slimb, SAP18, and CBP are essential for the Hedgehog signalling network (see review in Ref. [8]).

Besides Ptch, Megalin can also bind Hedgehog with high affinity.[9] Megalin-mediated endocytosis of multimeric Hedgehog may perhaps regulate its availability to Ptch by competing with Ptch to limit the levels of multimeric Hedgehog. Alternatively, Megalin may deliver multimeric Hedgehog to vesicular pools of Ptch.

Hedgehog binding to Ptch alleviates Ptch-mediated repression of Smo by a conformational change that frees Smo for downstream signalling. Kalderon[10] reviewed four models for the regulation of Smo by Ptch and Hh. The classical model has already been discussed. A second model postulates that Hh activates Smo by causing dissociation of the Ptch/Smo complex. In a third model, Ptch inhibits Smo through a diffusible intermediate and Hh binding to Ptch alters the activity of the intermediate, allowing Smo to become activated. In a fourth model, Ptch acts catalytically via a small molecule to suppress Smo, and on Hh binding to Ptch, Smo is activated by becoming dissociated from Ptch and the small molecule.

Intracellular localization of Ptch was demonstrated by Capdevila et al.,[11] who induced a mutation in *Shibire* [OMIM 602377] that affects endocytosis. Incardona

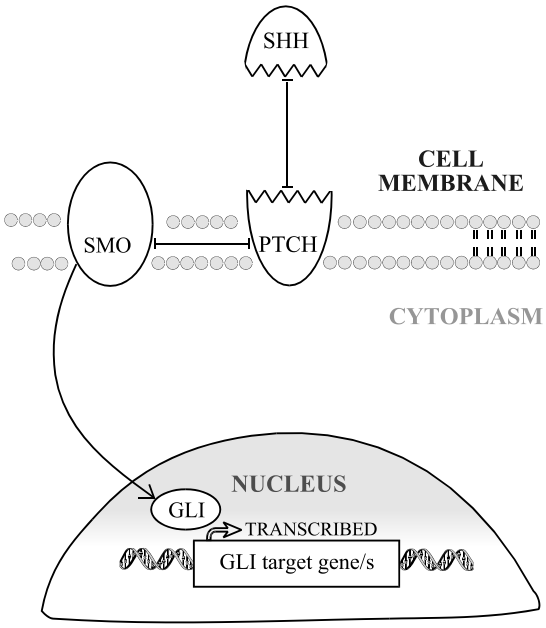

Fig. 1 Sonic Hedgehog pathway. SHH acts on the receptor complex of Ptch and Smo to inhibit repression of Smo by Ptch. Smo then signal activates gli intracellularly, resulting in downstream transcription of target genes. (*View this art in color at www.dekker.com.*)

et al.,[12] noting that M-Shh-N and Ptch colocalize in subcellular vesicles, suggested that internalization of M-Shh-N is mediated by Ptch. At present it is unclear whether Ptch eventually separates from M-Shh-N, with M-Shh-N being directed to lysosomes for degradation and Ptch returning to cell surface. It has been suggested that Ptch and Smo shuttle oppositely between the plasma membrane and cytoplasmic vesicles in response to M-Hh-N ligand.

In the absence of M-Hh-N binding, Ptch may be found principally at the cell membrane. At the same time, Smo may occupy intracellular vesicles. The binding of M-Hh-N to Ptch triggers a dynamin-mediated internalization into endosomes[13] and this results, indirectly, in the migration of vesicular Smo to the cell membrane.

Caveolae and caveolin-1 protein, which coats caveolae, may play a role in M-Hh-N uptake by Ptch.[14] However, only Ptch interacts specifically with caveolin-1 whereas Smo does not.

Ptch mediates the posttranslational modification of the C-terminal domain of Smo. Modification may include potential enzymatic candidates such as a phosphatase, Protein kinase A, or cyclin Bl that alter the phosphorylation status of Smo, affecting its stability, activity, and subcellular localization.[15] When Smo moves to the cell surface, hyperphosphorylation takes place, a putative Smo kinase increasing its activity. How closely linked phosphorylation of Smo is to its transport to the cell surface is not known at present. Unstimulated Ptch may promote dephosphorylation of Smo, a putative Smo phosphorylase decreasing Smo's activity.

Denef et al.[16] provided some evidence that Ptch and Smo do not interact physically in transducing M-Hh-N signals. They suggested that the regulatory interaction between Ptch and Smo need not be stoichiometric. It has been found[5] that Ptch and Smo are not significantly associated together within responding cells. They demonstrated that Ptch, unbounded by M-Hh-N, acts substoichiometrically to suppress activity of Smo. Extremely low levels of Ptch were sufficient to substantially reduce Smo activity. For example, a Ptch:Smo ratio of 1:45 suppressed nearly 80% of Smo activity. They suggested that in the absence of M-Hh-N, Ptch may translocate a small molecule across the lipid bilayer that regulates the activity state of Smo.

In contrast, Incardona et al.[6] provided some evidence that supported a model in which colocalization of Ptch and Smo occurs in the absence of M-Shh-N. On M-Shh-N binding to Ptch, both Ptch and Smo may undergo simultaneous transport into the endocytic pathway where Smo is subsequently segregated from the Ptch/M-Shh-N complex, which is destined for lysosomal degradation. Possibly, late endosomal sorting may control the generation of active Smo.

Whether Ptch eventually separates from M-Shh-N, with M-Shh-N being directed to lysosomes for degradation and with Ptch returning to the cell surface, is unclear at present.

To date, NBCS has not been reported with mutations in *PTCH2*, *SHH*, *SMO*, or *GUI*, but there are reasons for believing that such mutations may be found. *PTCH2* has been reported with a mutation in a medulloblastoma, whereas medulloblastoma is a feature of NBCS in about 3–5% of cases. A disputed mutation in *SHH* has been reported in one isolated BCC. Furthermore, BCCs and skeletal malformations are reported in hypersonic transgenic mice. Mutations in SMO have been reported in isolated basal cell carcinomas, and expression of *GLIl* has been found in almost all isolated BCCs studied.

Target genes for this disorder include *Ptch* (*PTCH*), the *Wnt* (*WNT*) family, and the *bone morphogenetic proteins* (*BMPs*) of the TGFβ superfamily. These are essential for normal embryonic development and differentiation of many adult tissues. Experiments in mice, chicks, and flies have shown that modification of genes or exposure to teratogens causes interesting and similar changes to reported human phenotypes. For example,

Ptch +/− in mice results in skeletal defects and neoplasms, but changes can be inhibited by cyclopamine. [This plant steroidal alkaloid induces cyclopia in vertebrate embryos and has been shown to act by inhibiting the cellular response to the Shh signal (see below)].

Ptch −/− in mice is lethal, producing overgrown and open neural tubes.

$Ptch^{1130X}$ in flies causes C-terminal truncation and abolishes Smo repression, but not Hh binding.

$Ptch^{\Delta loop2}$ in chicks cannot bind Shh ligand.

$Ptch^{sterol\ sensor}$ mutations in flies abolishes Smo repression, but not HH binding.

Taipale et al.[17] showed that cyclopamine or synthetic derivatives with improved potency block activation of the Hh response pathway and abnormal cell growth associated with both types of oncogenic mutation. The substance was discovered through epidemiological investigations of cyclopia in sheep in the Western United States. Cyclopia was found to result when pregnant females ate the lily *Veratrum californicum*. The ''active'' ingredient was named ''cyclopamine.'' Although cyclopamine has no apparent effect on adult animals, it consistently leads to severe holoprosencephaly in developing embryos. Because this condition can be caused by either mutations in human *hedgehog* or exposure to cyclopamine, it was proposed that cyclopamine suppresses Hedgehog. Taipale et al.[17] demonstrated that cyclopamine acts by affecting the balance between active and inactive forms of

Smoothened and may therefore have a role in treating BCCs and BCNS.

As epidemiological studies have shown, adult sheep tolerate cyclopamine well, and the results are encouraging. They do not directly address the question of whether switching off the Hedgehog pathway will cure basal cell carcinomas or prevent BCNS. BCCs, like most other human cancers, have mutations in many genes, and it is probably the accumulation of mutations that results in malignancy. Perhaps the Hedgehog pathway must be activated to kick-start tumor development, but may not need to be kept activated to maintain a fully malignant state. Indeed, in naturally occurring BCCs, shutting off this pathway might be too little, too late! Treating basal cell carcinomas with cyclopamine might be expected to suppress the development of neoplastic cells and perhaps limit growth, but not automatically kill them. There is some early evidence that tumor cells that have been suppressed in this way might undergo programmed cell death or be eradicated by the body's natural defenses. If not, then tumors treated with cyclopamine may eventually reappear when the drug is stopped.

MUTATION DETECTION

Blood samples are taken for straightforward DNA sequencing to assess the exact PTC gene mutation carried, but novel approaches including a protein truncation assay are being increasingly used to improve the yield of mutation detection. Standard sequencing detects minor changes in DNA [e.g., single-strand conformation polymorphism (SSCP) analysis, heteroduplex analysis, and denaturing gradient gel electrophoresis (DGGE) analysis], including those that are not pathogenic (polymorphisms) or are of questionable pathogenicity (missense mutations). The protein truncation test[18] specifically uncovers certain disease-causing mutations, namely, those causing premature translation termination. Initially developed for use with Duchenne muscular dystrophy, PTT is now used for screening many different disease genes and is proving useful in BCNS diagnosis.

TREATMENT

Treatment of BCCs includes surgical excision, curettage and electrodesiccation, cryosurgery, Mohs' micrographic surgery, and ultrapulsed CO_2 laser treatment. These methods, although effective, have economic, physical, and psychological implications, primarily because of the risk of scarring and recurrence. New, more inclusive selection criteria for surgery, combined with more effective neoadjuvant chemotherapy based on 5-fluoruracil and topically applied 5% imiquimod, are proving useful in reducing scarring. Radiotherapy is now avoided completely.

CONCLUSION

There is, however, much cause for optimism in this field. Fortunately, there is a good mouse model for BCNS that will help answer questions about the effects of cyclopamine on tumors in vivo. If this, or a related compound, progresses to clinical trials, investigators should not forget its source, and to avoid women of childbearing age in any study! However, the drive to understand the biology and behavior of BCCs—the world's commonest human cancer—will ultimately lead to spin-offs that will undoubtedly help NBCS sufferers.

The routine clinical implementation of genomics-based diagnosis and outcome predictors, as reported with desmoplastic medulloblastoma and *PTCH*[19] must await confirmation in independent data sets, and models may need to be modified as diagnostic and treatment regimens evolve.

For patients predicted to have a favorable outcome, efforts to minimize toxicity of therapy might be indicated, whereas for those predicted not to respond to standard therapy, earlier treatment with experimental regimens might be considered. Genomic technologies have the potential to advance treatment planning beyond the empirical, toward a more molecularly defined, individualized approach.

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Bead-Based Technology in Nucleic Acid Testing

B

Mario C. O. Probst
University of Regensburg, Regensburg, Germany

INTRODUCTION

Further development and improvement of molecular diagnosis depends on the development of robust, automatable, and cost-efficient technologies. In addition to conventional methods, a variety of homogeneous amplification assays that are based on fluorescence are available and facilitate detection of amplicons and characterization of mutations, single nucleotide polymorphisms (SNPs), and other sequence variations in DNA. Especially, SNPs have recently gained great attention, being markers for susceptibility to disease or response to therapeutic drugs.

To serve the increasing request for the simultaneous determination of larger numbers of parameters, DNA arrays (DNA chips) of different densities are available that allow high throughput and multiplex sequence and gene expression analysis. They require dedicated automation that may not be available in routine laboratories and their capabilities exceed by far what is currently required for diagnostic purposes. These conventional DNA and oligonucleotide arrays consist of a variable number of spatially arranged spots with nucleic acids on solid supports. The production of such arrays in small numbers for specific questions may be time consuming and costly. In addition, high-density arrays may not be necessary for many diagnostic purposes.

An interesting alternative to DNA chips is bead-based technology. The main advantage of this technology is that different species of beads are coated with the desired oligonucleotides prior to mixing the distinct populations together. A so-called suspension array for multiple analyses can be generated and modified according to the individual needs. Such suspension arrays can be supplemented with additional bead populations any time.

TECHNOLOGY

Beads are homogenous microspheres produced from polymer by special synthesis techniques. The most common material used is polystyrene and available bead sizes range from 0.4 to 200 μm. In order to function as carriers for binding interactions, beads have to be ''sensitized'' by attaching biological ligands such as antibodies or single-stranded nucleic acids to their surface. The first bead-based assay was reported in 1956, when Singer and Plotz introduced their rheumatoid factor agglutination test.[1] Later, beads were introduced in flow cytometry, which is a powerful combination, because here, like cells, several hundreds of beads can be discriminated by fluorescence intensity and spectral diversity in a couple of seconds. As a large number of each distinct species are measured, a mean signal with high precision is obtained. The increasing request for the simultaneous determination of complex parameter profiles in small sample volumes brought up the idea of multiplexing with microspheres as carriers for tests and assays, which can be analyzed and discriminated by flow cytometry. First, attempts to use microspheres of different size have been made, then beads were impregnated with fluorescent dyes, to make them spectrally distinctable. The maximum number of such populations depends on the availability of fluorescent dyes, the technologies to impregnate the beads, and the number of suitable fluorescent channels available in a flow cytometer. By color-coding beads with a blend of different amounts of only two fluorescent dyes, a set of 100 distinct bead populations can be generated (Fig. 1), which are commercially available as LabMAP Beads (Luminex Corporation, Austin, TX). Numerous companies have licensed the LabMAP technology as platform for the development of their bead-based assays especially in immunology and microbiology. Now that the conquest of bead-based assays over the traditional microtiter plate ELISA has begun, beads have been discovered as carriers for nucleic acid testing. Here, like antigens or antibodies, oligonucleotides of interest can be attached to beads using simple chemical reactions. Thus each bead population corresponds to a specific oligonucleotide. In a suspension hybridization, amplicons that carry another fluorescence can be immobilized to the beads by hybridization. Analysis is performed in the flow cytometer, where this additional fluorescence, that was brought to the bead by successful hybridization, is also detected. A conventional flow cytometer with three fluorescence channels measures two dyes that specify the bead population and a reporter dye that specifies hybridization events. By using more sophisticated flow cytometers with additional fluorescent channels (e.g., FACSCalibur, BD Biosciences, San Diego, CA), it would be possible to use more dyes for

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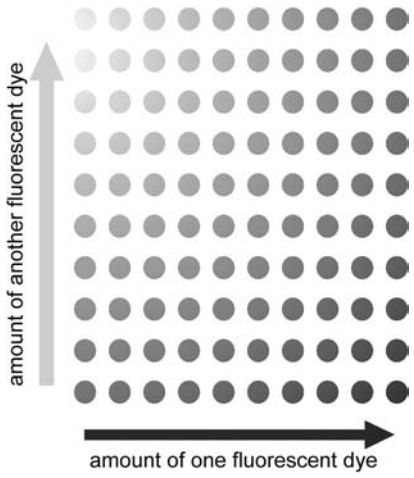

Fig. 1 By impregnating beads with a blend of two fluorescent dyes, a set of 100 color-coded species can be generated, which can be discriminated in a flow cytometer.

color-coding of beads or more colors for the test reaction. However, flow cytometers are expensive and their capabilities exceed what is required for bead-based analysis. With the Luminex100 (Luminex), a low-cost flow cytometer is available that is specifically adapted to the measurement of color-coded beads.[2,3]

ASSAY FORMATS

Direct Hybridization

Several formats of wet-ware were adopted to bead-based nucleic acid testing. The simplest method is the direct hybridization of labeled nucleic acids to beads carrying a sequence-specific oligonucleotide.

An assay for gene expression analysis using color-coded microspheres was developed using this technique. In Ref. [4], labeled cRNA was generated which was hybridized to a set of 20 bead populations carrying specific capture sequences of 25 bp for 20 *Arabidopsis* genes. The obtained expression profiles were similar to those obtained by Affymetrix GeneChip Analysis.[4]

In addition to expression profiling, sequence-specific hybridization of labeled PCR product to microspheres has been performed. A sensitive multiplexed bead assay for the detection of three viral nucleic acids (HIV, HSV, and HCV) was developed. Here labeled primers were used in the amplification of the relevant viral nucleic acids, which were hybridized to sequence-specific oligonucleotides bound to the beads.[5]

A third test applying the format of direct hybridization was a screening test for mutations in the CFTR gene, which can cause cystic fibrosis. A set of 10 bead populations, each carrying a sequence-specific oligonucleotide for 10 common mutations, and 10 bead populations, each carrying the according wild-type sequence, were used. The relevant DNA segments were amplified by PCR using labeled primers prior to hybridization to the beads.[6]

Competitive Hybridization

As hybridization of longer PCR products to microspheres results in significant cross-talk and decreased sensitivity, a different assay format, the competitive hybridization, was applied. With this technique, allele-specific, fluorescently labeled oligonucleotides (reporter oligonucleotides) are hybridized to the target amplicon. Afterwards, microspheres coated with oligonucleotides complementary to the reporter oligonucleotides are added and capture the remaining nonhybridized oligonucleotides. A decrease in fluorescence intensity compared to the negative control (inhibition of fluorescence) will report a positive test result. This assay format was used, for example, for a multiplexed hybridization assay to perform HLA-DQA1 tissue typing of PCR-amplified human genomic DNA.[7]

Zip Code Techniques

The simultaneous and efficient hybridization of oligonucleotides with different lengths and GC contents is still a problem, even when using special hybridization buffers. In addition, the allelic discrimination of single nucleotide polymorphisms by hybridization is problematic because of the similarity of the sequences. To this end, the idea of using so-called zip codes was brought to light. Here, probes for allelic discrimination carry a 5′-dangling end with an artificial unique DNA sequence for hybridization to beads subsequent to allelic discrimination. These zip codes are designed with identical length and GC content and limited cross-talk in simultaneous hybridization to their complementary sequence bound to the bead. An additional advantage is that the development of novel assays no longer requires production of differently coated beads. With this assay format, three different techniques have been evaluated: the oligo ligation assay (OLA), minisequencing (also called single-base chain extension, SBCE), and the allele-specific primer extension (ASPE).

In the OLA, two adjacent hybridized oligonucleotides are enzymatically ligated to each other using DNA ligase. This reaction only takes place when the nucleotides next to the ligation position are fully complementary. The first (capture) oligonucleotide is designed to hybridize to the target amplicon with its 3′ base complementary to the polymorphic base. It also carries a 5′-dangling end with the zip code. The second (reporter) oligonucleotide

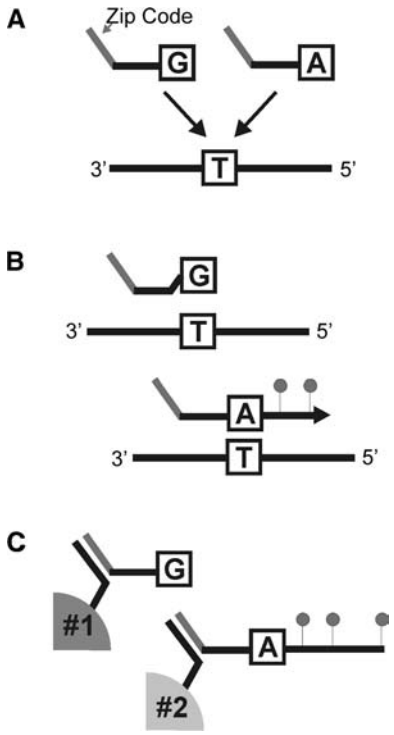

Fig. 2 Principle of the ASPE reaction. A) For each allele, one primer is designed carrying a 5′-dangling end with a unique zip code sequence. B) Only a primer hybridized with a perfect match at the 3′ end will be elongated and labels will be incorporated. C) Subsequent to allelic discrimination, primers are captured by color-coded beads carrying complementary zip code sequences.

hybridizes to the target sequence just adjacent to the 3′ end of the capture probe and carries a fluorescent tag at its 3′ end. If the capture probe matches exactly the SNP query position, the probes are ligated to each other, bringing a fluorescent tag to the capture oligonucleotide, which is detected by flow cytometry subsequent to hybridization to the bead populations. Two detection probes with different zip codes are required to cover each of the two possible alleles. This method has been successfully applied in the analysis of nine SNP markers located near the ApoE locus on chromosome 19.[8]

In OLA, one additional labeled reporter oligonucleotide is required for each variation. In minisequencing, only one primer for each allele is necessary, which is extended by one single-labeled nucleotide terminator. The SBCE primer, carrying a zip code at its 5′-dangling end, hybridizes to the target amplicon with its 3′-end one base upstream of the SNP query position. The primer is elongated by DNA polymerase using labeled dideoxynucleotide triphosphates (lacking a 3′ hydroxyl group), so that only a single base—complementary to the polymorphic base being analyzed—is incorporated. The disadvantage of the SBCE format is that up to four different labels are required in multiplexing assays to cover each ddNTP, and hence a sophisticated flow cytometer with six fluorescence channels would be necessary. The alternative is to use a separate reaction for each type of terminator nucleotide. Then the analysis can be performed on a standard flow cytometer, but up to four different reactions are required in a multiplex assay to cover all possible nucleotides. This method has been successfully performed with 55 randomly selected SNPs near the ApoE locus.[9]

In allele-specific primer extension only one reaction is required for genotyping. Here, like in OLA, one oligonucleotide is designed for each allele to hybridize to the target amplicon with its 3′ base complementary to the polymorphic base and a 5′-dangling end with a unique zip code. Standard deoxynucleotides are used, with one type of them (e.g., dCTP) being labeled. A DNA polymerase lacking 3′–5′ exonuclease activity is used to elongate primers with a perfect match at their 3′ end. During elongation, dependent on the sequence, several labels are incorporated which increases sensitivity compared to formats incorporating only one label (like OLA or SBCE). After the genotyping reaction, the zip-coded primers are hybridized to color-coded beads (Fig. 2). Only one fluorescent channel is required for detection of each possible genotype and no labeled oligonucleotides are required, which are expensive. This format seems to be the most suited and cost-efficient method for bead-based nucleic acid testing of SNPs and mutations. For evaluation, 20 randomly selected SNPs have successfully been analyzed in 633 probands applying this method.[10]

A set of 100 color-coded beads carrying unique zip codes (FlexMAP Beads) is available as platform for the development of bead-based nucleic acid tests (Luminex). Several commercially available kits (Tm Bioscience, Toronto, Canada) have been developed with this platform, applying the ASPE format, including multiplex tests for screening of sequence variations in genes relevant for blood coagulation (Factor V, Prothrombin, MTHFR), drug metabolism (CYP450-2D6), and cystic fibrosis (CFTR).[3,11]

CONCLUSION

At this time, bead-based nucleic acid testing might not be able to compete with high-density DNA arrays in gene expression profiling and with the determination of more precise expression levels using homogeneous real-time PCR methods. However, it has been shown that bead-based assays produce accurate results in low-density gene expression profiling and sequence detection. In terms of the analysis of sequence variations, especially genotyping

of single nucleotide polymorphisms and point-mutations, bead-based technology provides a real alternative to conventional methods because of its flexibility and high multiplexing capability. The individual combination and the simultaneous determination of complete parameter profiles might be more cost-efficient than conventional single-analysis methods and so could find their way into laboratory diagnostics. Flow cytometry is a robust and automatable platform for bead-based testing. A large set of commercially available zip code beads (Luminex FlexMAP beads) provides an ideal condition for the development of new bead-based nucleic acid tests. With allele-specific primer extension (ASPE), an optimal wetware has been established, allowing high specificity, increased sensitivity, and thus requiring a less sophisticated platform at the same time.

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Becker Muscular Dystrophy

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Masafumi Matsuo
Kobe University Graduate School of Medicine, Chuo, Kobe, Japan

INTRODUCTION

Becker muscular dystrophy (BMD) is an X-linked recessive inherited disease with a worldwide incidence of 1 in 35,000 male births. Becker muscular dystrophy is characterized by progressive muscle wasting but is distinguished by delayed onset, later dependence on wheelchair support, and longer life span from Duchenne muscular dystrophy (DMD) that follows severe progressive muscle wasting.

Dystrophin, the gene defective in not only BMD but also DMD, was isolated in 1986. Since then, genetic diagnosis of BMD has been done leading to better understanding of the disease process, and the difference between DMD and BMD can be explained at the molecular level by the reading frame rule.

CLINICAL DIAGNOSIS

Becker muscular dystrophy patients show normal growth and development in their early childhood. In BMD, affected men start to show disturbance of walking due to muscle weakness at 20s or over. He maintains to walk but his muscle strength gradually decreases. Dilated cardiomyopathy is sometimes an initial clinical sign for the diagnosis of BMD. The muscle weakness involved in BMD follows a mild downward course with patients living near normal lives.[1]

Serum creatine kinase (CK) is markedly increased. This marked elevation of serum CK is the most important hallmark for the diagnosis of BMD, but the level of elevation of serum CK is not so high compared to DMD. During the asymptomatic period, elevation of serum CK is the sole sign for BMD. Some BMD patients are identified accidentally because of elevations of AST or ALT which are commonly examined for liver function as serum CK elevation is accompanied with elevations of AST and ALT.

Becker muscular dystrophy should be included in the differential diagnosis of moderately elevated serum CK in males. Pathological examination of biopsied muscle consolidates the diagnosis of BMD.

GENE DIAGNOSIS

Becker muscular dystrophy is caused by mutations of the dystrophin gene that is also mutated in DMD.[2] Therefore, both DMD and BMD are sometimes called as dystrophinopathy. Furthermore, not only types but also locations of mutations identified in both BMD and DMD are quite similar.[3] For gene diagnosis of BMD both multiplex PCR and Southern blot analysis have been employed as in the genetic diagnosis of DMD.[4–6] Nearly two-thirds of mutations identified on the dystrophin gene are deletions or duplications occupying a single or multiple exons. Every mutation identified in BMD cases would be examined based on the following reading frame rule.[3] In the rest of the BMD cases it is rather difficult to identify the responsible mutations on the dystrophin gene as a single nucleotide change is supposed to be present. However, some point mutations that induced exon skipping have been reported.[7,8]

READING-FRAME RULE OF DMD/BMD

Although both DMD and BMD patients have been shown to have deletion or duplication mutations of the dystrophin gene, the extent of the deletion does not always correlate with the severity of the disease: some BMD patients with mild symptoms have deletions encompassing numerous exons, whereas some DMD patients with severe symptoms lack only a few exons. In some cases, the long deletions resulting in BMD and the short deletions resulting in DMD may even overlap. The reading-frame rule explains the difference between DMD and BMD as follows: in DMD the translational reading frame of the dystrophin mRNA is shifted after a deletion or duplication mutation whereas it is maintained in BMD.[3] According to the reading-frame rule, BMD patients with long deletions are able to produce dystrophin mRNA that would still direct the production of an internally truncated semifunctional protein. Shorter deletions harbored by severe DMD patients, on the other hand, would bring together exons that, when spliced, would change the translational reading frame in the mRNA, such that a

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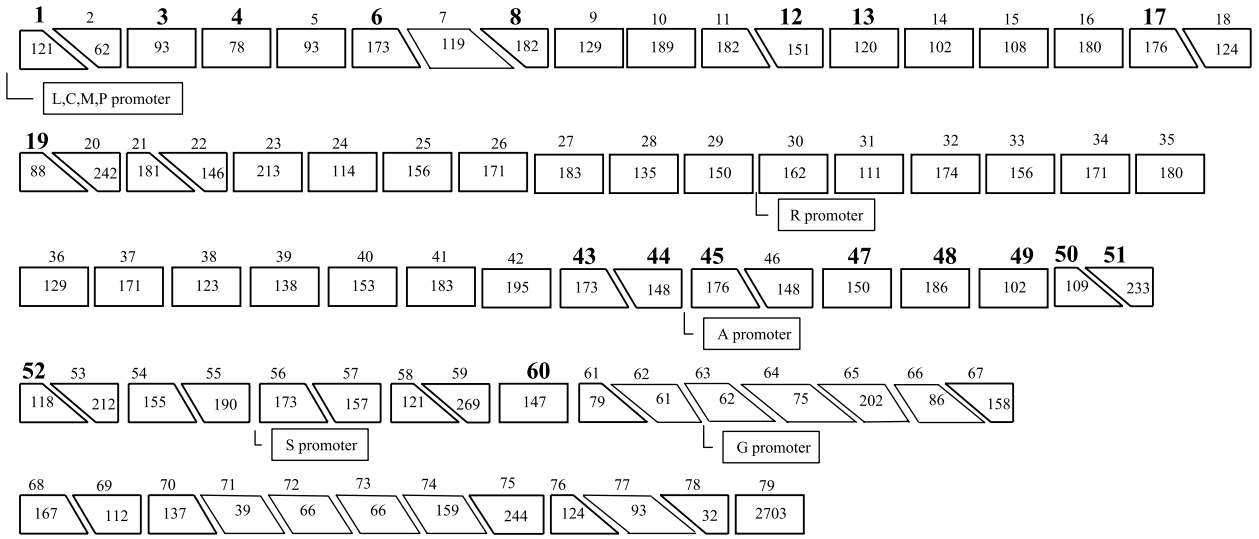

Fig. 1 Schematic description of the exon structure of the dystrophin gene. The dystrophin gene consisting of 79 exons (boxes) and at least eight alternative promoters (boxes under the lined boxes) is schematically described. Numbers over the box indicate exon number, the bold numbers being the exons that are examined by multiplex PCR. Quadrilaterals and parallelograms indicate in-frame exons (type 0 exons). Trapezoids indicate out-of-frame exons (type 1 or type 2 exons).

premature stop codon is created. This rule predicts that milder BMD patients would produce a smaller semifunctional protein whereas DMD patients would either produce a severely truncated dystrophin lacking the entire C-terminal region or would not produce dystrophin at all.

Subsequent gene analyses have shown that over 90% of the deletion–duplication mutations that cause BMD maintain the dystrophin mRNA reading frame whereas those causing DMD are frameshifts.[9] Accordingly, point mutations identified in DMD are nonsense mutations[10] except in rare DMD cases with missense mutations.[11,12]

Considering that molecular therapy for DMD to change the reading frame from out-of-frame to in-frame has been proposed,[13,14] it is important to see the resulting translational reading frame of dystrophin mRNA after the identification of a deletion or duplication mutation. Exons of the dystrophin gene are classified into three types according to the number of nucleotides encoded in the exon (Fig. 1): 1) in-frame exon that encodes nucleotides of multiples of 3 (type 0 exon); 2) two out-of-frame exons that have nucleotides of multiples of 3+1 or 2 (type 1 exon or type 2 exon, respectively). Among the 79 exons, 40, 18, and 21 exons are classified into types 0, 1, and 2 exons, respectively. In cases with deletion/duplication of the dystrophin gene the reading frame can be determined as described in Fig. 1. Cases having a deletion of a type 2 exon, e.g., exon 45, should be DMD based on the reading frame rule. Although gene diagnosis of DMD/BMD has been conducted, not all DMD/BMD cases have been examined for its reading frame.

In other types of mutations, nonsense mutations are expected to be identified in DMD. However, nonsense mutation that should result in DMD phenotype has been identified in BMD cases,[8,15] where exon skipping is shown as a mechanism that modified clinical phenotype. Furthermore, BMD has been shown to have a nonsense mutation in in-frame exons.[16–18] Detailed analysis of genotype–phenotype correlation would lead a better understanding of molecular mechanism of dystrophinopathy.

PATHOLOGICAL DIAGNOSIS

The pathological examination of biopsied skeletal muscle confirms the diagnosis of BMD. Immunohistochemical analyses of normal muscle demonstrate that dystrophin is present along with muscle cell membranes. Muscle from BMD patients contains reduced amounts of dystrophin that is stained discontinuously and patchy along the muscle cell membranes.[19] Western blot analysis using dystrophin antibody reveals a band corresponding to 427 kDa, close to the predicted size of dystrophin, in extracts of normal muscle tissue. Shorter or lower amount of dystrophin is detected in muscle extracts from patients with BMD.

Dystrophin contains 3685 amino acids organized in four domains: N-terminal actin binding, triple helical rod, cystein-rich, and C-terminal domains. The internally truncated dystrophin identified in BMD maintains both N-terminal and C-terminal domains, but lacks some of the 24 repeat sequences of triple helical rod domain. Therefore, dystrophin is stained when antibody recognizing either N-terminal or C-terminal domains is used, but in

some cases no dystrophin is stained as in DMD when antibody recognizing rod domain is employed.[20]

TREATMENT

For BMD patients, supportive therapies such as rehabilitation or ventilator support are clinically employed, but no effective way to improve the clinical course is available. Gene therapy has been considered a cure for BMD but no clinically applicable way has been established.

CONCLUSION

Becker muscular dystrophy is a mild muscle wasting disease and characterized by dystrophin abnormality in skeletal muscle. Currently, no effective treatment is available although a molecular understanding of BMD developed well.

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Beta-Thalassemias

Antonio Cao
Renzo Galanello
Università degli Studi di Cagliari (UNICA), Cagliari, Sardinia, Italy

INTRODUCTION

The β-thalassemias are a markedly heterogeneous group of autosomal recessive disorders resulting from reduced (β^+) or absent (β°) production of the β-globin chains. The resulting shortage of β-globin determines an excess of unassembled α-globin chains that precipitate on the red blood cell membranes causing premature destruction by apoptosis of the erythroid precursors and consequently markedly ineffective erythropoiesis.

PREVALENCE AND DISTRIBUTION

The β-thalassemias are prevalent in populations in the Mediterranean area, Middle East, Transcaucasus, Central Asia, Indian subcontinent, and the Far East. The highest incidences are reported in Cyprus (14%), Sardinia (12%), and Southeast Asia. However, because of population migration and partly to the slave trade, β-thalassemia is now common also in Northern Europe, North and South America, the Caribbean, and Australia.[1]

CLINICAL ASPECTS

At the clinical–hematological level, the severity of the β-thalassemias depends on the extent of α-chain/non-α-chain (β+γ) imbalance. Clinically, we distinguish four conditions of increasing severity, i.e., silent β-thalassemia, the β-thalassemia carrier state, thalassemia intermedia, and thalassemia major. Silent β-thalassemia is produced by heterozygosity for extremely mild β-thalassemia mutations. This develops a clinical, hematologically silent phenotype and can only be recognized by a moderate imbalance of the α/non-α-chain synthesis. The β-thalassemia carrier state results from heterozygosity of typical β-thalassemia mutations and is clinically asymptomatic and hematologically defined by microcytosis (low MCV), hypochromia (low MCH), increased percentage of the minor fraction of adult Hb, namely, HbA_2, and mild unbalance of the α/non-α-chain synthesis. Thalassemia intermedia most commonly results from homozygosity for β-thalassemia and is clinically characterized by microcytic anemia of variable severity, for which regular transfusions are not required. Thalassemia major, the most severe form, also results from homozygosity for β-thalassemia and is characterized by a severe transfusion-dependent anemia.[2]

From the genetic point of view, β-thalassemia can be classified as a simple β-thalassemia, where only the β-globin gene is affected, and a complex β-thalassemia one in which also the γ and δ genes are involved.

Thalassemia Major

The clinical picture of β-thalassemia major is characterized by presentation with pallor and spleen and liver enlargement at 6–24 months of age. Typical skeletal changes resulting from expansion of the bone marrow are evident only in patients in whom transfusions were initiated late or are undertransfused. At the age of 10–11 years, regularly transfused thalassemia major patients are at risk of developing severe complications related to iron overload. These complications include growth retardation, failure of sexual maturation, dilated myocardiopathy, liver fibrosis or cirrhosis, diabetes mellitus, and insufficiency of parathyroid, thyroid, and pituitary glands. Other complications are hypersplenism, B or C virus chronic hepatitis, HIV infection, venous thrombosis, and osteoporosis. Survival of well-transfused patients treated with appropriate chelation extends beyond the third decade.[2,3]

Thalassemia Intermedia

Clinical features are pallor, jaundice, cholelithiasis, liver and spleen enlargement, moderate to severe skeletal changes, leg ulcers, extramedullary masses of hyperplastic bone marrow, and tendency to develop osteopenia, osteoporosis, and thrombotic complications. Compared to thalassemia major, iron overload occurs later and is a result of increased intestinal iron absorption caused by ineffective erythropoiesis.

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Diagnosis

Besides the picture described above, diagnosis of both β-thalassemia major and β-thalassemia intermedia is based on the following hematological features:

1. Typical peripheral blood smear showing microcytosis, hypochromia, anisocytosis, poikilocytosis, and nucleated red blood cells.
2. Hb pattern (by cellulose acetate electrophoresis or HPLC), characterized by HbF, and trace of HbA_2 (β°-thalassemia) or 10–30% HbA, 70–90 HbF, and trace of HbA_2 (β^+-thalassemia).
3. Complete absence (β°-thalassemia) or extreme reduction (β^+-thalassemia) of β-chains by in vitro synthesis of radioactive-labeled globin chains.[2,3]

Management of Thalassemia Major

Patients affected by thalassemia major are treated with regular blood transfusions and iron chelation therapy with desferrioxamine B (DFO). Life expectancy with this treatment extends to the third decade. The alternative oral iron chelator deferiprone (L1) is indicated only in patients with proven allergy or toxicity from DFO. Alternative chelation strategies and drugs, including the combination of deferiprone and DFO or ICL670 alone, are under investigation.

Bone marrow transplantation (BMT) or cord blood transplantation from HLA-identical siblings represents an alternative to traditional transfusion and chelation therapy. In patients without liver fibrosis and low level of iron accumulation, the disease-free survival is over 90%. However, BMT from HLA-identical but unrelated donors has a lower (60%) disease-free survival.[4–6]

Management of Thalassemia Intermedia

The treatment of patients with thalassemia intermedia is symptomatic and based on splenectomy, folic acid supplementation, and chelation therapy. The treatment of extramedullary masses is based on radiotherapy, transfusions, or hydroxyurea.

Other Therapies

Attempts to increase HbF production through the administration of a variety of agents have produced variable but not substantial effects. These agents have included 5-azacytidine, erythropoietin, butyrate compounds, and hydroxyurea.

The possibility of correction of the molecular defect in hematopoietic stem cells by transfer of a normal gene via a suitable vector or by homologous recombination is being actively investigated.

Encouraging results have been obtained recently with the lentivirus mediated transfer of a normal human γ- or β-globin gene.[7]

MOLECULAR GENETICS

β-Thalassemias are very heterogeneous at the molecular level. More than 200 different disease-causing mutations have been identified so far (Table 1).[2,3,8] The large majority of mutations are simple single nucleotide substitutions or deletion/insertions of one nucleotide or oligonucleotides leading to frameshift. The β°-thalassemias are rarely a result of a mechanism of gross gene deletion involving the β-globin gene or the LCR. The β°-thalassemias result from nonsense mutation, frameshift, or splicing mutations involving the invariant dinucleotides. The β^+-thalassemia mutations are produced by mutations of the promoter area (CACCC or TATA box), the polyadenylation signal, conserved sequences in the 5′ or 3′ untranslated regions, or by splicing abnormalities resulting from mutation of the consensus sequences around the GT/AG invariant dinucleotides. An interesting category of β^+-thalassemias is that associated with a consistent residual output of β-globin chains or constantly associated with a high γ-chain production rate, thereby determining a mild phenotype (Table 2). The most common mild mutations are β+ IVS1 nt 6 (T→C), which is found in the Mediterranean area, and β codon 26 (G→A), which gives rise to HbE that prevails in Southeast Asia. Finally, silent alleles result from mutations of the distal CACCC box and from conserved sequences in the 5′ untranslated region, in the polyadenylation signal, or within consensus sequences affecting the splicing process (Table 3). The complex β-thalassemias (δβ or γδβ-thalassemias) result from deletion of a variable extent of the β-globin gene cluster.

Several nucleotide substitutions (polymorphisms) in the β-globin cluster have been found not randomly associated, but grouped in subsets (in linkage), called haplotypes. In each population, different specific β-Thalassemia mutations are associated with different haplotypes. However, the same mutation can be contained in different haplotypes.

For example, in Mediterranean countries, most of the patients with haplotype I have the mutation IVS1–110 G→A. More rarely, the frameshift mutation at codon 6 (-A) and the β° 39 C→T mutation are also associated with haplotype I.

Table 1 Most common β-thalassemia mutations in populations at risk

Mutations	Percentage of patients (%)	Population
−87 C→G IVS1-1 G→A IVS1-6 T→C IVS1-110 G→A cd 39 C→T IVS2-745 C→G	91–95	Mediterranean
cd 8 −AA cd 8/9+G IVS1-5 G→C cd 39 C→T cd 44-C IVS2-1 G→A		Middle Eastern
−619 bp deletion cd 8/9+G IVS1-1 G→T IVS1-5 G→C 41/42-TTCT		Indian
−28 A→G 17 A→T 19 A→G IVS1-5 G→C 41/42-TTCT IVS2-654 C→T		Thai
−28 A→G 17 A→T 41/42-TTCT IVS2-654 C→T		Chinese
−88 C→T −29 A→G IVS1-5 G→T cd 24 T→A IVS2-949 A→G, A→C	75–80	African/ African-American

Genotype–Phenotype Correlations

Any inherited or acquired condition that reduces the α/non-α-globin chain imbalance results in a lesser degree of globin α-chain precipitation and leads to a mild β-thalassemia phenotype. One of the most common and consistent mechanisms is homozygosity or compound heterozygosity for mild and silent β^+-thalassemia mutations. In contrast, compound heterozygosity for mild/silent β^+ and severe mutations produces a variable phenotype, ranging from thalassemia intermedia to thalassemia major. Compound heterozygosity for β-thalassemia and hemoglobin E (HbE) results in a wide range of clinical phenotypes, often severe, although sometimes mild and even clinically asymptomatic. HbE is a thalassemic structural variant, characterized by the presence of an abnormal structure as well as a biosynthetic defect. The nucleotide substitution at codon 26, producing the HbE variant ($\alpha_2\beta_2$26 Glu→Lys), activates a potential cryptic RNA splice site resulting in alternative splicing at this position. The homozygous state for HbE results in a mild hemolytic microcytic anemia. The clinical picture resulting from homozygosity for β°- or severe β^+-thalassemia may be ameliorated by coinheritance of mutations in the gene encoding the α-globin chain (deletion of two α-globin genes or point mutations in the major α_2 gene in β°-thalassemia and even a single α-globin gene deletion in β^+-thalassemia) associated with α-thalassemia, which reduces the output of the genes encoding the globin α-chains and therefore decreases the α/non-α-globin chain imbalance. However, the presence of coinherited α-thalassemia does not always produce a consistent effect, thus precluding its use to predict phenotype. The coinheritance of some genetic determinants capable of sustaining a continuous production of globin γ-chains (HbF) in adult life may also reduce the extent of α/non-α-globin chain imbalance.[2,3,9] This effect may be related to the β-thalassemia mutation per se which increases the globin

Table 2 Mild β-globin gene mutations causing β-thalassemia

Mutation type		Mild β^+
Transcriptional mutants in the proximal CACC box		−90 C→T −88 C→T −88 C→A −87 C→T −87 C→G −87 C→A −86 C→T −86 C→G
TATA box		−31 A→G −30 T→A −29 A→G
5′ UTR		+22 G→A +10-T +33 C→G
Alternative splicing		cd19 A→C (Hb Malay) cd24 T→A
Consensus splicing		IVS1-6 T→C
Poly A site		AACAAA AATGAA
Mild ß°	Frameshift	cd6-A cd8-AA

Table 3 Silent β-globin gene mutations causing ß-thalassemia

Mutation type or location	Silent
Transcriptional mutants in	−101 C→T
the proximal CACC box	−92 C→T
5′ UTR	+1′ A→C
Alternative splicing	cd27 G→T (Hb Knossos)
Intervening sequence	IVS2-844 C→G
In the 3′UTR	+6 C→G
Poly A site	AATAAG

γ-chain (HbF) output. This occurs in δβ°-thalassemia, which is caused by deletions of variable size in the β-globin gene cluster, and in deletions removing only the 5′ region of the β-globin gene promoter, which also result in high levels of HbA_2. High γ-chain output may also depend on cotransmission of hereditary persistence of fetal hemoglobin (HPFH), resulting from single-point mutations of the Aγ or Gγ promoter. The most common is a single-base C to T substitution at position −158 upstream from the Gγ gene, which is silent in normal subjects and β-thalassemia heterozygotes, but leads to increased HbF production in patients with erythropoietic stress, as occurs in homozygous β-thalassemia. The −158 Gγ mutation is in linkage disequilibrium with the IVSII-I (G→A), frameshift 8 (-AA), and frameshift 6 (-A) mutations. This explains the mild phenotype that may result from the inheritance of these mutations. Another point mutation of the Aγ promoter (−196 C→T) is in linkage with the β° 39 nonsense mutation in Sardinians. Homozygosity for this chromosome is associated with a clinically silent phenotype. Finally, also coinheritance of heterocellular hereditary persistence of fetal hemoglobin (HPFH), which may be linked or unlinked to the β-globin gene cluster, may lead to a mild phenotype. To date, three loci have been mapped: one at Xq22.2–q22.3, one on 6q22.3–q23.1, and one on 8q (which interacts with the Gγ −158 C→T mutation), but many others are very likely to exist. In some cases, heterozygous β-thalassemia may lead to the phenotype of thalassemia intermedia instead of the asymptomatic carrier state. Known molecular mechanisms include the following:

1. Heterozygosity for mutations in the β-globin gene that result in hyperunstable hemoglobins (dominant β-thalassemia), which precipitates in the red cell membrane together with unassembled α-globin chains, resulting in markedly ineffective erythropoiesis. Most of these β-globin gene mutations lie in the third exon and lead to the production of a markedly unstable Hb variant often not detectable in peripheral blood. Up to now, 32 dominant β-thalassemias have been described; all of them, but one, i.e., Cd121 G→T described in several families, are private mutations reported in single families.
2. Compound heterozygosity for typical β-thalassemia mutations and the triple or (less frequently) quadruple α-globin gene arrangement (ααα/αα, ααα/ααα, or αααα/αα), which may increase the imbalance in the ratio of globin α/non-α-globin chains.

Other Modifying Factors

The clinical phenotype of homozygous β-thalassemia may also be modified by the coinheritance of other genetic factors mapping outside the β-globin gene cluster. The best known of these modifying genes is the Gilbert mutation [i.e., the presence of the $(TA)_7$ configuration instead of the $(TA)_6$ in the TATA box of the gene encoding uridin-diphosphoglucuronyltransferase], which, when combined with thalassemia major or thalassemia intermedia or the β-thalassemia carrier state, leads to increased jaundice and increased risk of gallstones. Less defined modifying factors are genes coding for hereditary hemochromatosis and genes involved in bone metabolism.[10]

β-Thalassemia Phenotype With Normal β-Globin Gene Sequences

A limited proportion of individuals with the β-thalassemia carrier phenotype show completely normal β-globin gene sequences as well as normal LCR sequences. Recently, mutations in the general transcription factor TFIIH coding for a specific helicase have also been shown to be associated with β-thalassemia carrier phenotype besides tricothiodystrophy. Furthermore, depending on the site of mutation, mutations in GATA-1 resulted either in dyserythropoietic anemia and thrombocytopenia or thrombocytopenia and β-thalassemia carrier phenotype. The results indicate that the β-thalassemia carrier phenotype may result from mutation in a transcription factor regulating the function of the β-globin gene.

CARRIER IDENTIFICATION

β-Thalassemia carriers are identified by analysis of red blood cell indices, which show microcytosis and reduced MCH and Hb analysis, which displays high HbA_2 (>3.5%). Pitfalls in carrier identification may result from several molecular mechanisms. Coinheritance of α-thalassemia (two α-globin gene deletions or point mutations of the major α_2 globin gene in β°-thalassemia, or even a single α-globin gene deletion in β^+-thalassemia) may

normalize the red blood cell indices of the β-thalassemia carrier. Nevertheless, the HbA_2 concentration remains in the β-thalassemia carrier range and therefore has diagnostic value. Coinheritance of δ-thalassemia, which reduces the increased HbA_2 levels typical of the β-thalassemia carrier state to normal, may cause confusion with the α-thalassemia carrier state. Differentiation of the double heterozygote state for δ- and β-thalassemia from the α-thalassemia heterozygote may be accomplished by globin chain synthesis analysis or globin gene analysis. Heterozygotes for mild β-thalassemia mutations (β^+ IVS-I nt 6 mutation, γδβ-, and δβ-thalassemia) may have normal HbA_2 levels. However, δβ-thalassemias are characterized by high HbF levels.[1,11]

Based on these features, screening for β-thalassemia should be carried out by red cell indices analysis, Hb electrophoresis, and HbA_2 quantitation. Molecular genetic testing to identify the β gene mutation is usually performed in the couples at risk for β-thalassemia and when the hematologic tests are not conclusive (i.e., borderline HbA_2, microcytosis with normal A_2 and F and normal iron, increased HbF). In some cases, molecular diagnosis may be helpful in patients to predict the clinical severity. Mutations are detected by several PCR-based procedures, such as reverse dot blot analysis or primer specific amplification using probes or primers complementary to the most common mutations present in the population or denaturing high-performance liquid chromatography. Sequence analysis is used if the previous methods fail to detect the mutation. Because of the high carrier rate, population screening is ongoing in several populations at risk (Sardinians, Continental Italians, Greeks, Cypriots) in Mediterranean area. In these populations, screening in combination with prenatal diagnosis has led to a marked reduction of the birth rate of homozygous β-thalassemia.

PRENATAL DIAGNOSIS

Prenatal diagnosis may be carried out for couples of whom both members are β-thalassemia carriers and for whom β-globin gene mutations have been identified by DNA analysis. Molecular genetic testing can be performed either on fetal DNA extracted from cells obtained by chorionic villi sampling (CVS) at about 10–12 weeks gestation or by amniocentesis at 14–18 weeks gestation. Diagnosis by DNA analysis of polar bodies and preimplantation diagnosis by analysis of cleavage embryo biopsy are also possible. Prenatal diagnosis by analysis of fetal cells in maternal blood is not yet available. However, analysis of fetal DNA in maternal plasma for the presence of the father's mutation may lead to prenatal exclusion of homozygous β-thalassemia.

CONCLUSION

β-Thalassemias are very heterogeneous at the clinical and molecular level. Clinically, we distinguish four clinical-hematological conditions of increasing severity, i.e., silent β-thalassemia, the β-thalassemia carrier state, thalassemia intermedia, and thalassemia major. At molecular level, β-thalassemias result from more than 200 different molecular defects. Molecular detection is possible with several PCR-based procedures. Preconception and prenatal diagnosis is available. Carrier screening and genetic counseling in the Mediterranean population at risk have led to a dramatic decrease in the birth rate of homozygous β-thalassemia. Treatment, based on periodic transfusion and iron chelation, has resulted in extended survival. Bone marrow transplantation from HLA identical siblings results in a >90% disease-free survival in patients in good clinical conditions. The lentivirus-mediated transfer of the β-globin or γ-globin gene in a thalassemic mouse has recently produced encouraging results.

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Biosensors—DNA-Based Sensor Technology

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Wenonah A. Vercoutere
NASA Ames Research Center, Moffett Field, California, U.S.A.

INTRODUCTION

Biosensors link biological probes to a signal transducer to convert chemical recognition into recordable data, ideally in real time. For medical genomics, biosensor technology focuses on recognizing nucleic acid sequences. The biosensor probes are typically single strands of DNA complementary to a target. The probes may be in solution or associated with a surface. Signals from probe–target recognition are converted by a variety of techniques. Recognition may be measured, for instance, as a change in fluorescence, color, acoustic wave resonance, optical resonance, electronic signal, or magnetic field. Medically useful genomic variations extend from broad-range gene expression patterns to single point mutations. This review summarizes DNA-based biosensor technology that is being developed to provide fast, simple, and inexpensive methods for measuring medically important genomic information.

CURRENT TECHNIQUES USED IN MEDICAL GENOMICS

Genomic variations that influence disease predisposition or response to pharmaceutical therapies (see pharmacogenetics) are not simple to characterize. Clinically useful techniques must be able to detect a few mutations, such as associated with hereditary hemochromatosis (two single nucleotide polymorphism sites), or many mutations, such as associated with cystic fibrosis (>1000 mutations). Differences in drug response can be monogenic, but more typically they are caused by a combination of several genes.[1] Recognition of onset of disease such as cancer, and monitoring treatments, depends on identification of neoplastic markers. Research continues with the goal to identify genetic mutations and gene expression changes associated with disease, disease progression, and response to treatment.

An assortment of methods is currently used in genomic research and for molecular diagnostics. DNA microarrays are the first widely used research tool to couple signal generation directly to DNA hybridization to measure genomic variations. For example, recent research using microarrays has identified gene expression changes associated with breast cancer stages.[2] However, microarray use as clinical tool is limited by the number of manual steps involved, the time required, cost, and often by the variability between samples and between laboratories.[3] Current clinical techniques frequently use ''home-brew'' assays and commercial instruments that require analyte-specific reagents (ASRs). In-house validation is essential and all laboratories are required to be CLIA (Clinical Laboratory Improvement Amendments) certified (http://www.cms.hhs.gov/clia).[4] Clinical laboratories currently identify single nucleotide polymorphisms (SNPs) and other diagnostic mutations using platforms that include allele-specific amplification, chemiluminescent forward dot blot assay, dot blot hybridization, gel-based restriction fragment length polymorphism (RFLP) analysis, oligonucleotide ligation, primer extension, multiplex PCR. Also in use are commercial kits such as mDx platform for hereditary hemochromatosis and Factor V_{Leiden} from Bio-Rad Laboratories (Hercules, CA) and the Amplichip CYP450 Array that identifies common mutations associated with poor or rapid drug metabolisms from Roche Diagnostics (Indianapolis, IN). Solution-based biosensors have debuted in the clinical laboratory in the form of fluorescence resonance energy transfer (FRET) probes used in real-time PCR. Systems using TaqMan™, molecular beacon, or Scorpion™ primers fluoresce as DNA target replication proceeds, and results can be generated within 20 min to 2 hr.[5] However, typical laboratory turnaround times using these tools are on the order of weeks; as with DNA microarray analysis, multiple manual steps are involved that take time and can introduce human error. Depending on the particular test, concerns also include sample size, cost, scalability, and limited adaptability.

DNA BIOSENSORS

The ideal biosensor for clinical use would be robust, simple and rapid to use, cost-effective, require minimal sample preparation, use small volumes, and have low nonspecific interference. It would also produce results that are nonbiased, and with low false positives or negatives.

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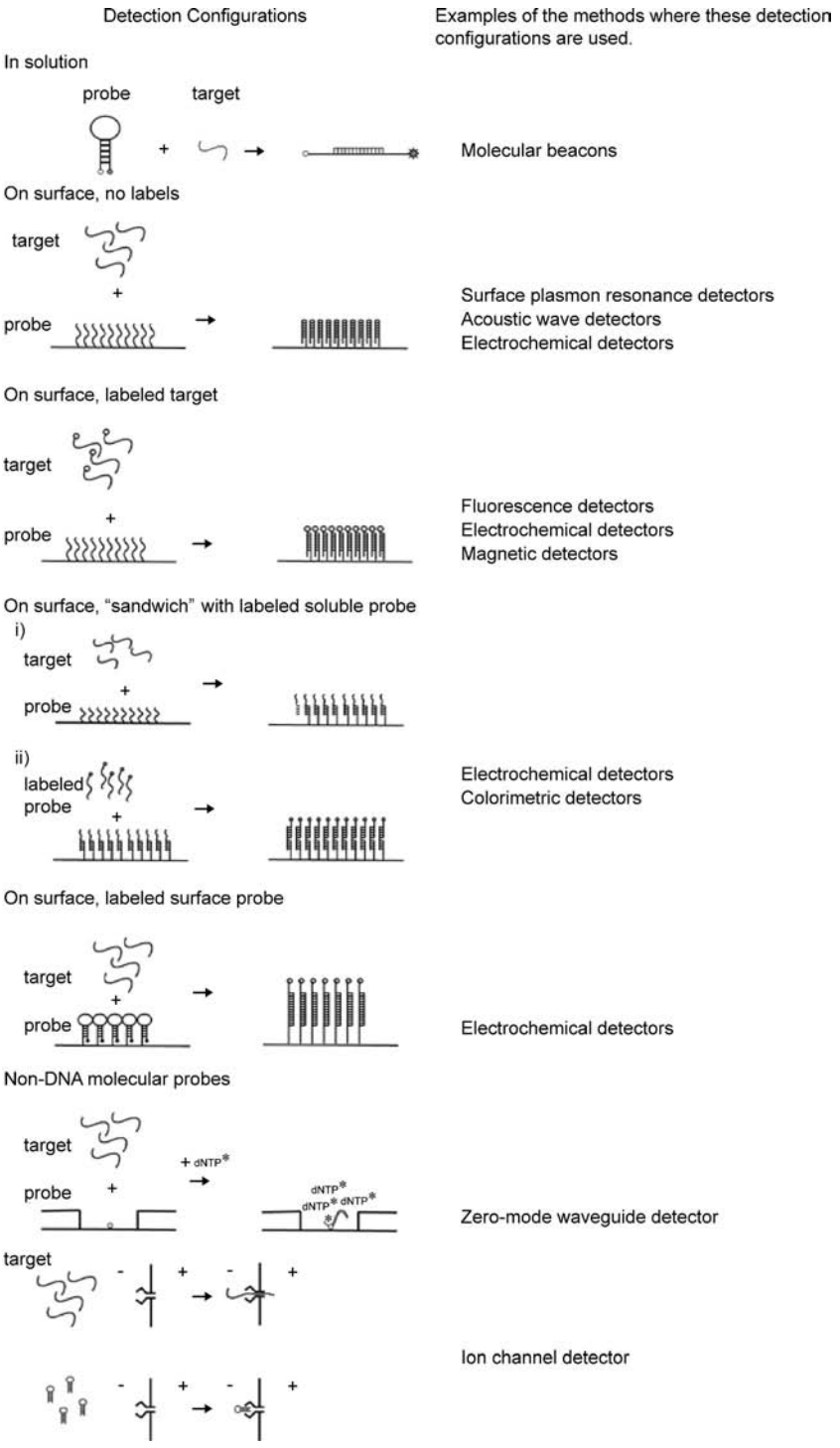

Fig. 1 Schematics showing different detection configurations with examples of the methods where they are used.

This device would potentially integrate multiple steps and be flexible enough to handle a broad spectrum of applications. Some biosensors are beginning to meet these criteria; most are still in development.[6,7] Currently, almost all DNA biosensors use DNA/DNA hybridization to obtain specificity (Fig. 1). Several novel methods use nonspecific proteins including enzymes and ion channels to detect sequence information from single molecules of DNA. The following are examples of DNA biosensors with a comparison of sensitivity and stage of development.

Evanescent and Acoustic Wave Sensors

A common form of evanescence wave detection used for biosensor applications is surface plasmon resonance (SPR). Ligand association (and dissociation) to surface-bound probe molecules is detected by changes in the refractive index of evanescence waves within a thin metal-coated quartz sensor adjacent to the sample flow chamber. This method is primarily used to study kinetics of protein–protein and protein–DNA interactions. Several reports demonstrate that SPR can also be used to monitor DNA and RNA hybridization. For example, Feriotto et al.[8] showed that SNPs associated with thalassemia could be distinguished by using PCR-amplified samples from normal, heterozygous, and homozygous thalassemia patients as the surface-bound molecule. Probe–target hybridization took 5 min per sample to read, and 1 to 3 μg of each PCR product was used. Surface plasmon resonance has also been applied in a microfluidics array format to detect 20 fmol of 1731-nucleotide RNA transcribed in vitro from the *uidA* gene of a transgenic *Arabidopsis thaliana* plant (20 nM in 1 μL).[9] The shift to array format and the significant reduction in sample size are improvements for SPR use as a tool to examine genetic mutations or gene expression. The requirement for specialized equipment, however, may limit the applicability for clinical use.

Acoustic wave biosensors measure mechanical wave propagation at the surface of a piezoelectric sensor that is sensitive to changes in mass, surface charge, and viscoelasticity. As a demonstration of concept, Furtado and Thompson[10] showed that eight synthesized 25-mer target DNAs each with a different single nucleotide mismatch could be discriminated in less than an hour. Increasing sensitivity to DNA hybridization or observing transcription at single nucleotide resolution may be achievable with these real-time resonance detection methods if they were coupled to a second detection method such as fluorescence microscopy.[11]

Fiber-Optic Sensors

Fiber-optic biosensors use total internal reflectance to measure fluorescent-labeled targets captured at the glass fiber surface. Fibers have been configured for use in large or small clusters. Large clusters of ~6000 fibers are used in an array format with probe-coated beads seated at the tip of each fiber (Illumina, San Diego, CA.). With this system, Epstein and colleagues[12] showed that zeptomole levels of fluoroscene-labeled 21-mer containing the cystic fibrosis F508C mutation could be identified within an hour (0.1 fM in 10 μL). Small (~4-fiber) clusters are individually coated with probes. Using 19-mer probes, this configuration was used to distinguish between two gene mutations associated with spinal muscular atrophy using Cy5-labeled 202-bp PCR products amplified from patient samples. When a time-derivative of the signal was applied, the difference was identified in less than 2 min.[13]

Colorimetric Sensors

There is emerging interest in sensitive colorimetric biosensor methods that do not require specialized instruments for signal detection. Nanoparticles, nanovesicles, and optical thin film have been used to transduce complementary hybridization into a color change. Aggregation of probe-labeled nanoparticles in the presence of complementary target is associated with a color shift that can be monitored using a UV–VIS spectrophotometer. This system was coupled in an array format with a chemoresponsive diffraction grating detector to show real-time detection of a synthesized 27-mer DNA target. The experimental detection limit was 0.8 fmol (1 pM in 800 μL), and an extrapolated detection limit of 33 attomol was calculated.[14] In a system using a dye-encapsulated nanovesicle label, Baeumner and colleagues[15] showed that sandwich hybridization in solution could be detected simply and with high sensitivity. Target DNA, capture probe, and labeled probe were mixed for 20 min, sampled with a membrane strip for 10 min, and then read with a reflectometer. This method showed sensitivity to 1 fmol (1 nM in 10 μL). Using an optical thin-film biosensor, Zhong and coworkers[16] printed DNA arrays that detected PCR products with a lower limit less than 5 fmol. The signal is visible to the naked eye, and data can be recorded using a digital camera, a dissecting microscope, and a standard light source. In this system, a 30–40-min oligo sandwich detection method was used, facilitated by a *Thermusthermophilus* ligase modified to have a 10-fold increase in efficiency. To compare accuracy with standard methods, DNA samples from 100 human subjects were tested for SNPs associated with increased risk of venous thromboembolism, and DNA samples from 50 individuals were tested for SNP genotyping. Results were as good as or better than TaqMan™ or RFLP results.

Electrochemical Sensors

Electrochemical detectors measure potential, resistance, or capacitance using electrodes functionalized with DNA probes. Techniques include using labeled second probes for sandwich hybridization or labeled surface probes for simultaneous target hybridization and signal generation. In an application of nanoparticles for electrochemical detection, Park and colleagues[17] used specific target–probe association to align nanoparticle-labeled probes across a gap. A final step of silver treatment to coat the

adhered gold particles completed a conducting bridge, and SNPs were distinguished at femtomolar levels. Park's research group is continuing to investigate applications of DNA-coated nanoparticles for low-level gene (and protein) detection.

Bernaki and coworkers[18] tested the accuracy of a low-density biosensor array called eSensor (Motorola Life Sciences, Pasadena, CA). This device uses a sandwich detection method on a pair of gold electrodes, with probes labeled with two different conducting ferrocenes. DNA samples from six B-lymphocyte cell lines were tested as part of an ongoing clinical validation process, four with hereditary hemochromatosis (*HFE* gene) mutations and two with cystic fibrosis (*CFTR* gene) mutations. After a 2-hr hybridization, the array was tested for conductance of the ferrocene conductance pairs representing wild-type and single nucleotide mutant. A signal of 6 to 40 nA indicated hybridization, showing reasonable selectivity from noncomplementary DNA, below 1 nA. Five molecular genetic laboratories tested the samples other than Motorola Life Sciences and used standard techniques including direct DNA sequence analysis, RFLP, and conformational gel electrophoresis. The electrochemical biosensor results concurred in all cases.

By labeling a surface-bound probe with an electrically active moiety, electronic detection can take place with a unimolecular reaction. Mao et al. electrochemically detected target 15-mers using a self-assembled monolayer of DNA hairpin probes end-labeled with redox-active thionine.[19] Binding of 2.5 pmol (50 nM in 50/μL) target DNA to the complementary loop sequence dissociated the hairpin stem and moved the active label away from the detection surface, reducing the potential measurably within about 25 min. Readings at two temperatures allowed detection of single nucleotide differences representing a p53 mutation with at least 10% change in signal. A similar system used hairpins labeled with ferrocene. In this system, 5-fmol (10 pM in 500/μL) target 17-mers were detected within 30 min.[20]

Magnetic Sensors

Magnetic biosensors detect hybridization using a signal probe labeled with a metal microbead. Miller and colleagues showed that the material used in hard-drive read heads, giant magnetoresistance (GMR) sensors, could be modified with DNA probes to detect target DNA.[21] Using commercially available chips, they showed eight DNA samples from pathogenic bacteria could be read within 30 min. Similar work compared probe concentrations and magnetic microbeads, and showed detection of 100 nmol (10 ng/μL) target DNA.[22] This technique has been studied by only a few groups, but shows promise because the GMR microchips and readers are already developed, and because each GMR sensor is capable of detecting a single magnetic bead, so that a single target molecule may be detectable.

Single Molecule Sensors

All DNA biosensors discussed so far use DNA as the biological recognition probe to obtain specificity. Several novel methods use nonspecific proteins such as ion channels or enzymes to detect sequence information from single molecules of DNA. The most commonly employed ion channel is the α-hemolysin ion channel reconstituted in an artificial bilayer. Using a 120-mV applied potential, the channel samples unlabeled DNA from solution. Capture of a DNA molecule is observed as an ionic current blockade. Blockade durations can last from 20 μsec to minutes depending on the length and stability of the molecule. Real-time identification using machine-learning analysis requires up to a 50-msec read of each molecule.[23] Watson–Crick base-pair differences at the termini of 9-bp DNA hairpins were distinguishable using this analysis method.[24,25] Standard experimental conditions were 10-μM target DNA in 70 μL. A reduction in volume would lower the concentration needed to maintain an adequate acquisition rate. However, identification of comparable sequence information from single-stranded DNA is not possible with the current system. Up to 12 nucleotides contribute to blocking current through the transmembrane compartment at a time, and the translocation rate of 2 μsec per nucleotide is at the limit of electronic instrument noise. A nanopore device capable of reading sequence from target DNA will likely incorporate an enzyme that reduces translocation speed from microseconds to milliseconds.

Another method to observe DNA, a single nucleotide at a time, used a surface-bound DNA polymerase. Levene and colleagues[26] used a 50-nm-wide well in a metal film as a zero-mode waveguide to restrict excitation light to the immediate vicinity of the enzyme. They observed real-time synthesis of a single DNA polymerase by monitoring the incorporation of fluorophore-labeled dCTP into a growing cDNA strand. If dNTPs with different fluorophores on each were used, it would be possible, in principle, to monitor the order of nucleotide incorporation and reconstruct the sequence.

CONCLUSION

The biosensors reviewed here each show improvement to some aspect of current mutation detection. A number have utility as research tools, including the nanovesicle-based colorimetric sensor and the α-hemolysin nanopore. A few DNA biosensors are beginning to meet the requirements

for clinical use, such as the eSensor. A key component to facilitating transition of DNA biosensors into clinical use will be the establishment of a set of reference samples such as used by Bernaki and colleagues to use for validation and calibration. In addition to being reliable, the most successful DNA biosensors will be flexible and scalable. This may be accomplished with biosensors that can be assembled into array format or that have interchangeable probe-coated surfaces. This may also be achieved using probes other than DNA that can give sequence information without specific base-pair hybridization, such as the DNA polymerase captured in a zero-mode waveguide, or a nanopore detector.

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Bladder Cancer—Identification of Bladder Cancer Biomarkers by Microarrays

Marta Sänchez-Carbayo
Memorial Sloan Kettering Cancer Center, New York, New York, U.S.A.

BLADDER CANCER

Bladder cancer is one of the most common malignancies in developed countries, ranking as the fourth most common cancer in men and the seventh most common in women.[1] Bladder cancer constitutes a spectrum of diseases that have been classified into three main groups with distinct clinical behavior, prognosis, primary managements, and molecular profiles: superficial (stages Ta-Tis-T1), deeply invasive (stages T2–4), and metastatic disease (N+/M+).[2] Most superficial cases are treated conservatively by transurethral removal of the tumor, followed by adjuvant intravesical therapy, without removal of the bladder. Muscle-infiltrating tumors are generally treated by cystectomy, plus systemic adjuvant chemotherapy or radiotherapy. Approximately 20% of the superficial tumors are cured by surgical removal of the presenting lesion, 50–70% recur one or more times, but never progress into invasive disease, and 10–30% progress to invasive and potentially lethal disease. In contrast, approximately 50% of patients with muscle-infiltrating tumors already harbor or will develop metastatic disease, and ultimately, the vast majority of patients with metastatic tumors die from their disease.

Bladder cancer, including some superficial lesions, has been reported to carry a significant number of genetic alterations at the time of diagnosis. A substantial body of work has suggested that superficial papillary tumors (pTa) differ from flat carcinoma in situ lesions (TIS) and muscle-invasive tumors in their molecular pathogenesis and pathways of progression. At least, these two major molecular pathways of bladder tumor development and evolution that can be followed are recognized (Fig. 1). The first, represented by papillary superficial tumors, is associated with chromosome 9 losses, including inactivation of CDKN2A (p16) on 9p and still unknown genes associated with telomeric 9q loci.[3,4] The second pathway includes inactivation of p53 on chromosome 17 (17p11.3) and RB on 13q14, adscribed to flat carcinoma in situ and pT1 tumors.[2–5] The number of genetic alterations is substantially higher in the invasive lesions, but there also appears to be a difference in the specific alterations present.

THE NEED FOR BIOMARKERS IN BLADDER CANCER

Bladder tumors are pathologically stratified based on stage, grade, tumor size, presence of concomitant carcinoma in situ, and multicentricity.[2] The chances of tumor progression are augmented with the increase of these pathological variables. Pathologically, most bladder tumors are transitional cell carcinomas. There is, however, increasing recognition of the prognostic importance associated with the metaplastic variants displaying squamous and glandular differentiation as part of their clonal evolution. The power of these histopathological variables and the tumor node metastases (TNM) categorization, in defining the clinical subtypes of bladder cancer and predicting the clinical outcome of individual patients, has certain limitations. Within each stage, it has been very difficult to identify clinically useful parameters that can predict risk of disease recurrence or progression. Numerous biological markers have been described for bladder cancer, some correlating with tumor stage and prognosis in patients affected with these neoplasms. Phenotypic features associated with tumor aggressiveness include cell cycle and apoptosis regulators, as studied by appropriate sensitive molecular techniques.[2–5] Remaining challenges are not only the identification of biomarkers of early diagnostic utility combining high sensitivity and specificity, but also progression and outcome-predictive markers in bladder cancer.

MICROARRAYS AS TARGET IDENTIFICATION TOOLS IN CANCER

Microarrays constitute a group of technologies characterized by the common availability of measuring hundreds or thousands of items, including DNA sequences, RNA transcripts, or proteins, within a single experiment using miniaturized devices. Hybridization-based methods and the microarray format constitute together an extremely versatile platform provided for both static and dynamic views of DNA structure, as well as RNA and protein expression patterns in cultured cancer cells and tumor

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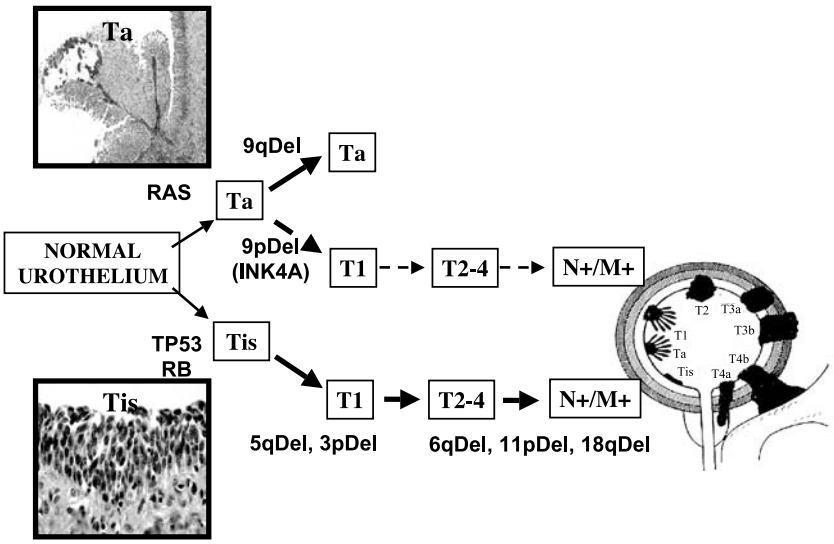

Fig. 1 Overview of the two major progression pathways described for bladder cancer outlining some of the main genetic alterations associated with these pathways. (*View this art in color at www.dekker.com.*)

tissues. The most widespread use of this technology to date has been the analysis of gene expression. There is an increasingly broad range of additional applications for microarrays, including genotyping polymorphisms and mutations, determining the sites of DNA-binding proteins, and identifying structural alterations using arrayed comparative genome hybridization (CGH). Powerful methods for comprehensive analysis of gene and protein expression patterns utilize not only microarray formats, but also differential display,[6] serial analysis of gene expression (SAGE),[7] protein composition-based approaches such as antibody microarrays,[8] high-throughput mass spectral analysis, or 2-D gel analysis.[9] Among these high-throughput techniques, DNA microarrays are the most utilized tools for target identification.

The development and implementation of high-throughput array technologies to primary human tumors is changing clinical management of patients with cancer. The histopathological diagnosis and classification of tumors are now being complemented by these comprehensive procedures, providing novel diagnostic, predictive, and therapeutic targets for the cancer patient. To the simplicity of the study of a single molecular marker, we may be able to integrate the complexity of multiple biological determinants, participating in signaling pathways and biological networks. Comprehensive information may, in turn, modify empirical clinical treatments for individualized mechanism-based therapies, as well as diagnostic and predictive molecular signatures for the management of patients with bladder cancer. The concept of individual biomarkers of diagnostic and/or prognostic utility is being progressively changed by the concept of cluster of genes or gene profiles characteristic of recurrence, progression, or survival.

IDENTIFICATION OF MOLECULAR TARGETS AND BIOMARKERS FOR BLADDER CANCER USING DNA MICROARRAYS

Microarray-based gene expression profiling to the study of bladder cancer can be divided into those analyzing in vitro systems and those centering on clinical material, with the objectives of achieving gene and pathway discovery, functional classification of genes, and a new classification based on tumor subtypes. Molecular profiling using DNA microarrays of in vivo models on bladder cancer has not been reported to date.

Bladder Cancer Studies Using In Vitro Models

Expression profiling using bladder cancer cell lines has been used to gain insight into the molecular events associated with clinical disease states, assigning potential functional roles to novel genes in both tumorigenic and tumor progression processes. Certain studies have focused on *gene and pathway discovery* associated to genistein[10] or 5-aza-2′-deoxycytidine[11] exposure. Other reports describe the *functional classification of genes* comparing the expression patterns of p53-mediated apoptosis in resistant tumor cell lines versus sensitive tumor cell lines using cDNA arrays,[12] or the expression patterns of a metastatic variant cell line to the respective parental

invasive cells.[13] Many molecular targets involved in bladder cancer progression have been identified in these studies. Comprehensive analyses using clinical specimens will also elucidate the clinical utility of these targets as biomarkers for patients with bladder cancer. An attempt to *tumor subtypes classification* of bladder cancer cell lines by means of gene expression analyses has rendered identification of biomarkers of clinical utility. Gene profiling identified novel biomarkers from in vitro analyses that were proven to be associated with clinical and histopathological variables when validated on tumor specimens using tissue microarrays at the protein level, using well-characterized antibodies and immunohistochemistry. Caveolin-1 and keratin 10 were differentially expressed in a squamous carcinoma cell line and certain invasive tumor cell lines. Cells clustered also based on their p53, RB, and INK4A status revealing E-cadherin, zyxin, and moesin as targets differentially expressed in these clusters. The expression of all these proteins in primary bladder tumors spotted on tissue microarrays was significantly associated with histopathological stage and tumor grade.[14]

Bladder Cancer Studies Using Clinical Specimens

Microarray analyses have been used to correlate changes in the expression of specific genes and groups of genes within distinct bladder subclasses. Such signature genes would ideally provide a molecular basis for classification, yielding insight into the molecular events underlying different clinical bladder cancer phenotypes.

The first report monitored the expression patterns of superficial and invasive tumor cell suspensions prepared from individuals and pools of normal and bladder tumors of tumors of different stages such as from pTA grade I and II and pT2 grade III and IV bladder cancer specimens.[15] Hierarchical clustering of gene expression levels grouped bladder cancer specimens based on tumor stage and grade. The most significant functional genes included those involved in cell cycle, cell growth, immunology, adhesion, transcription, and protein metabolism. Superficial papillary tumors showed increased transcription factor and ribosomal levels, as well as proteinase encoding genes up-regulation. In the invasive tumors, increased levels of cell cycle, growth factor networks, immunology-related and oncogene transcripts, and loss of cellular adhesion genes were observed.[15]

The combination of separate expression profiling studies of bladder tumors and bladder cancer cell lines has allowed the identification of the tumor suppressor role of KiSS-1 in bladder cancer progression.[16] Lower transcript levels of KiSS-1 were observed in cells derived from advanced bladder tumors and bladder carcinomas as compared with superficial tumors, and these ratios provided prognostic information. The expression patterns of KiSS-1 analyzed by in situ hybridization on tissue microarrays were associated with tumor stage, grade, and overall survival. Thus gene expression profiling identified a novel target involved in bladder cancer progression with clinical relevance.[16]

The most extensive expression profiling study of bladder tumors reported to date has dealt with the development of a predictive classifier of Ta, T1, and T2+ bladder carcinoma subclasses. The use of a support vector machine algorithm allowed prediction with 75% accuracy of these tumor subclasses in an independent set of patients, using cross-validation strategies to evaluate the clinical impact of the classifier defined using independent series of tumors. Smad6 and cyclin G2 were also identified as Ta/T1 classifier genes and their immunostaining patterns were validated on tissue microarrays by immunohistochemistry.[17] This study represents the first attempt to predict recurrence within 2 years for patients with bladder cancer.

Gene profiling has also successfully classified bladder tumors based on their progression and clinical outcome. Early-stage tumors showing gene profiles similar to invasive disease were identified. More importantly, carcinoma in situ from papillary superficial lesions and subgroups within early stage and invasive tumors displaying different overall survival were separated. Different techniques were used to identify molecular biomarkers of clinical significance. For example, p33ING1 was found to be significantly associated with pathological stage, tumor grade, and overall survival using tissue microarrays. Analysis of the annotation of the most significant genes revealed the relevance of critical genes and pathways during bladder cancer progression.[18]

BIOMARKER DISCOVERY USING OTHER MICROARRAY PLATFORMS

In addition to transcriptome expression microarrays, specific oligonucleotide microarrays have been applied to the study of DNA variation in clinical material. Multiple probes of short length that differ in sequence at a single base have been designed to identify simple polymorphisms and allelic variations in DNA. The primary applications of these types of microarrays have dealt with automated high-throughput identification of mutations in critical genes such as *TP53*, a valuable predictor for bladder cancer outcome,[19] and in the genotyping of single nucleotide polymorphisms (SNP).[20] High-throughput CGH arrays can also confirm chromosomal gains and losses alterations found at the genomic level in a comprehensive detailed manner. This provides a significant advantage over laborious pregenome mapping

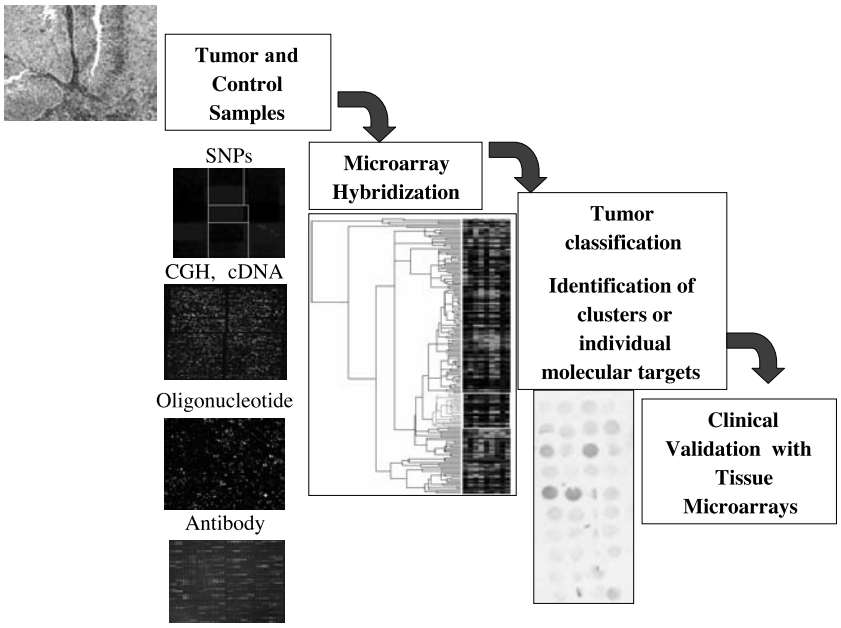

Fig. 2 General strategy used in tumor expression profiling for target identification. RNA isolation from cell lines, tumor biopsy, and control samples is followed by preparation of the hybridization probe, hybridization with the DNA microarray, data acquisition, and analysis. Verification of the results can be performed using different approaches, such as tissue microarray analysis. (*View this art in color at www.dekker.com.*)

and transcript identification strategies and allows study of genetic changes in tumor progression.[21]

It should be noted that microarrays technology is a convenient platform for assays involving biomolecules other than nucleic acids not widely utilized for patients with bladder cancer yet. Arrays of tissues, peptides, antibodies, proteins, and even cells have been developed.[8,9] This is further evidence of the strength and

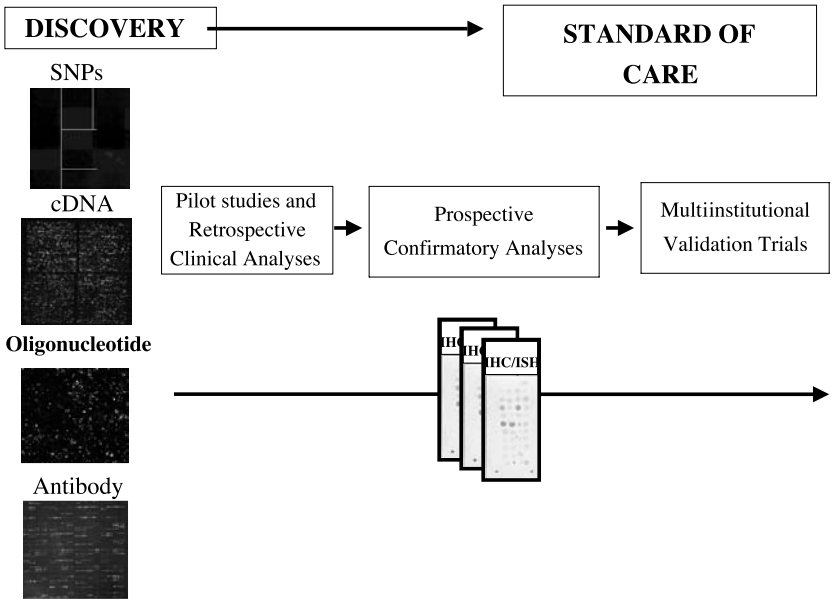

Fig. 3 The use of high-throughput technologies provides novel targets that might be developed into biomarkers of diagnostic and prognostic utility. The tumor marker development requires several steps before acceptance into clinical routine. (*View this art in color at www.dekker.com.*)

versatility for high-throughput screening for biomarker discovery using, for example, mass spectrometry. Protein microarrays also provide means of rapidly validating the genes identified by expression profiling using DNA microarrays at the protein level.

TISSUE ARRAYS FOR VALIDATION OF BIOMARKERS IDENTIFIED USING MICROARRAYS

Genomic analysis tools for biomarker identification can be complimented by the use of array-based high-throughput approaches to survey tumor biopsies for a large number of individual patients. In combination with the associated clinical data for these patients, this technology is ideally suited to a genomics- or proteomics-based discovery project and has the ability to significantly accelerate the transition of basic science findings into clinical applications (Fig. 2). The application of tissue microarrays represents a high-throughput approach for validation of potential novel markers for bladder cancer by immunohistochemistry or fluorescence in situ hybridization in paraffin blocks. Focus is intensified within this field to automate construction of tissue microarrays and generate arrays from frozen blocks.

CONCLUSION

The studies summarized here and others indicate that expression profiling provides a molecular means of identifying clinically important tumor subtypes and molecular targets not identified using standard methods. Gene expression profiling could define, at the molecular level, the individual clinical and histopathological phenotypes of bladder tumors. Reports using different microarray platforms and analyzing specific cancer subgroups are warranted to find consensus on subclasses and signatures of the disease with diagnostic and predictive utility (Fig. 3). Creation of international tumor banks represents an option that might facilitate interactive research among different laboratories. Further efforts using in vitro and in vivo models are warranted to characterize functionally the pathways by which many of the targets already identified to be involved in tumorigenesis or bladder cancer progression. The utility of the application of microarrays has not estimated yet many clinical issues in bladder cancer. Identification of Ta-T1-IS subtypes within superficial disease and patients more likely to develop positive lymph nodes or distant metastases are critical subclassification questions to be answered. Another area that will provide critical targets for clinical intervention is that of pharmacogenomics. In the near future, gene profiling will provide effective means to predict the response against specific therapeutic regimes based on the molecular signatures of the tumors associated with their chemosensitivity or resistance to anticancer drugs. Moreover, the discovery of molecular pathways altered in cancer progression as well as the identification of molecule-susceptible targets would lead to the development of novel alternative therapies. The combined information revealed by these studies also allows the identification of new molecular determinants involved in the progression of the disease with clinical diagnostic or predictive utility. The classical tumor marker concept of an individual biological determinant will be substituted by the use of cluster of genes as predictive classifiers. These genetic signatures will allow a better chance of cure by opting for individualized therapies and development of biomarkers of diagnostic and predictive value for patients with bladder cancer.

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Blood Group Genotyping

Lilian Castilho
Hemocentro Unicamp, Campinas, Brazil

INTRODUCTION

Accurate phenotyping of red blood cells (RBCs) can be difficult in transfusion-dependent patients such as those with thalassemia and sickle cell anemia because of the presence of previously transfused RBCs in the patients' circulation. Recently, the molecular basis associated with the expression of many blood group antigens was established. This allowed the development of a plethora of polymerase chain reaction (PCR)-based tests for the identification of blood group antigens by testing the DNA.

The determination of blood group polymorphism at the genomic level facilitates the resolution of clinical problems that cannot be addressed by hemagglutination. They are useful to 1) determine antigen types for which currently available antibodies are weakly reactive; 2) type patients who have been recently transfused; 3) identify fetuses at risk for hemolytic disease of the newborn; and 4) to increase the reliability of repositories of antigen-negative RBCs for transfusion.

BLOOD GROUP GENOTYPING

Blood group antigens are polymorphisms of proteins and carbohydrates on the outside surface of the red blood cell (RBC) (Fig. 1) and are defined by serum alloantibodies produced in response to an immunizing event such as transfusion or pregnancy. It is the antibody that causes clinical problems in transfusion incompatibility, maternal–fetal incompatibility, and autoimmune hemolytic anemia.

The major risks of transfusions are unexpected incompatibility reactions[1] and the transmission of infectious agents. Iron overload and alloimmunization are also frequently observed among some categories of chronically transfused patients. Alloimmunization leads to an increased risk of transfusion reactions, reducing the available pool of compatible blood for transfusion in subsequent crises. Alloimmunization is the source of a variety of problems during long-term medical and transfusion management, with the main problem being the identification of appropriate antigen-negative RBCs for transfusion.[2]

Thus in transfusion medicine much time and effort are expended in detecting and identifying blood group antibodies. Next to ABO, the most clinically significant antibodies are those in Rh, Kell, Duffy, and Kidd blood group systems.[3]

Red blood cell phenotyping is essential to confirm the identity of suspected alloantibodies and to facilitate the identification of antibodies that may be formed in the future. Accurate antigen typing of transfused patients is often a difficult task because of the presence of donor RBCs in the patients' circulation. Thus in these patients phenotyping can be time consuming and difficult to interpret. It is also complicated to type cells when a patient's RBCs have a positive direct antiglobulin test and no direct agglutinating antibody is available.

DNA technology led to the understanding of the molecular basis of many blood group antigens. The genes encoding 28 of the 29 blood group systems (only P remains to be resolved) have been cloned and sequenced[4–7] (Table 1), which has permitted the elucidation of the molecular basis of many common blood group antigens. There are many molecular events that give rise to blood group antigens and phenotypes (Table 2); however, the majority of genetically defined blood group antigens are the consequence of a single-nucleotide polymorphism (SNP). (For current information regarding blood group antigen SNPs, the following website is recommended: www.bioc.aecom.yu.edu/bgmut/index.htm). This knowledge allows the use of DNA-based assays to detect specific blood group SNPs and that can be used to overcome the limitations of hemagglutination assays.[8,9]

Several assays for blood group genotyping of patients have recently been developed to predict the blood group antigen profile of an individual, with the goal of reducing risk or helping in the assessment of the risk of hemolytic disease of the newborn (HDN);[10–16] they include PCR-RFLP, allele-specific PCR, sequence-specific PCR as single or multiplex assays, real-time quantitative PCR.

These assays can be applied for blood group antigens to type patients who have recently received transfusion; to type patients whose RBCs are coated with immunoglobulin; to identify a fetus at risk for HDN; to determine which phenotypically antigen-negative patients can receive antigen-positive RBCs; to type donors for antibody identification panels; to type patients who have an antigen that is expressed weakly on RBCs; to determine *RHD*

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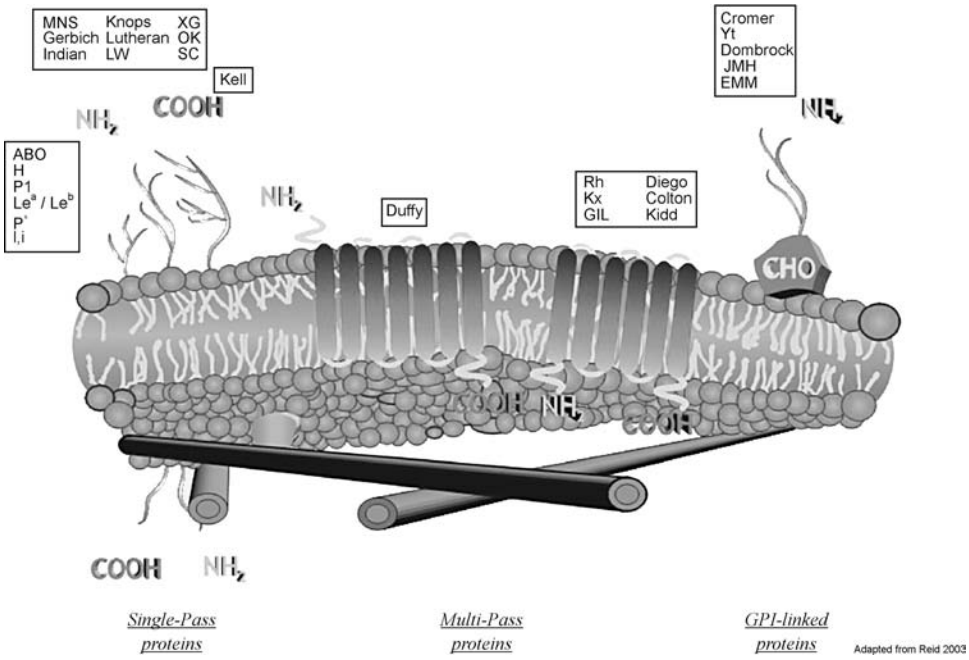

Fig. 1 Model of red blood cell membrane components that carry blood group antigens. (*View this art in color at www.dekker.com.*)

zygosity; to mass screen for antigen-negative donors; to resolve A, B, and D discrepancies.

APPLICATIONS TO TRANSFUSION MEDICINE

For Multiply Transfused Patients

The ability to determine a patient's antigen profile by DNA analysis when hemagglutination tests cannot be used is a useful adjunct to a serological investigation. Blood group genotyping in the transfusion setting is recommended for multiply transfused patients, as part of the antibody identification process.

Determination of a patient's blood type by DNA analysis is particularly useful when a patient who is transfusion-dependent has produced alloantibodies.[3] This is because identification of the patient's probable phenotype allows the laboratory to determine to which antigens the patient can and cannot respond to make alloantibodies.

We have demonstrated the relevance of genotype determination of blood groups for the management of multiply transfused patients with diseases such as sickle cell disease (SCD) and β-thalassemia[15,16] by allowing the determination of the true blood group genotype, and by assisting in the identification of suspected alloantibodies and the selection of antigen-negative RBCs for transfusion. Furthermore, we have observed that taking genotype into account allowed better selection of compatible units for patients with discrepancies between genotype and phenotype, leading to increased cell survival and a reduction of the transfusion frequency.

For Patients Whose RBCs are Coated with IgG

Patients with warm autoimmune hemolytic anemia (WAIHA), whose RBCs are coated with IgG, cannot be accurately typed for RBC antigens, particularly when directly agglutinating antibodies are not available, or IgG removal by chemical treatment of RBCs is insufficient. We have shown that blood group genotyping is very important for the determination of the true blood group antigens of these patients.[17] After genotyping, the patients received antigen-matched RBCs that had better in vivo survival, as assessed by increases in hemoglobin levels and diminished frequency of transfusions.

The possibility to have an alternative to hemagglutination tests to determine the patient's antigen profile should be considered for multiply transfused patients and for patients with WAIHA by allowing the determination of the true blood group genotype and by assisting in the identification of suspected alloantibodies and in the selection of antigen-negative RBCs for transfusion. This ensures more accurate selection of compatible donor units and is likely to prevent alloimmunization and reduce the potential for hemolytic reactions.

Table 1 Blood group systems with gene names and chromosome location

ISBT number	System name	Gene name	Number of antigens	Chromosome location
001	ABO	*ABO*	4	9p34.1–q34.2
002	MNS	*GYPA*, *GYPB*, *GYPE*	43	4q28.2–q31.1
003	P	*P1*	1	22q11.2–qter
004	Rh	*RHD, RHCE*	48	1p36.13–p34.3
005	Lutheran	*LU* (*B-CAM*)	18	19q13.2
006	Kell	*KEL*	24	7q33
007	Lewis	*LE* (*FUT3*)	3	19p13.3
008	Duffy	*FY*	6	1q22–q23
009	Kidd	*JK* (*SLC4A14*)	3	18q11–q12
010	Diego	*DI* (*AE1*, *SLC4A1*)	21	17q12–q21
011	Yt	*ACHE*	2	7q22
012	Xg	*XG*	2	Xp22.32
013	Scianna	*SC*	4	1p34
014	Dombrock	*DO*	5	12p13.2–p12.1
015	Colton	*CO* (*AQPI*)	3	7p14
016	Landsteiner–Wiener	*LW* (*ICAM-4*)	3	19p13.3
017	Chido/Rodgers	*C4A*, *C4B*	9	6p21.3
018	Hh	*H* (*FUT1*)	1	19q13.3
019	Kx	*XK*	1	Xp21.1
020	Gerbich	*GE* (*GYPC*)	7	2q14–q21
021	Cromer	*CROM* (*DAF*)	11	1q32
022	Knops	*KN* (*CR1*)	5	1q32
023	Indian	*IN* (*CD44*)	2	11p13
024	OK	*OK* (*CD147*)	1	19p13.3
025	RAPH	*RAPH* (*CD151*)	1	11p15.5
026	JMH	*JMH* (*CD18*)	1	15q22.3–q23
027	I	*I* (*IGNT*)	1	6p24
028	Globoside	*P* (*βGalT3*)	1	3q25
029	GIL	*GIL* (*AQP3*)	1	9p13

For Blood Donors

DNA-based typing can also be used to antigen type blood donor both for transfusion and for antibody identification reagent panels. This is particularly useful when antibodies are not available or are weakly reactive. A good example is the Dombrock blood group polymorphism where DNA-based assays are used to type patients and donors for Do^a and Do^b to overcome the dearth of reliable typing reagents.[18,19] Furthermore, the newer technologies have the potential to screen pools of DNA for rare blood types. The molecular analysis of a variant gene can also assist in resolving a serological investigation.[20]

ABO Genotyping and Resolution of A and B Discrepancies

ABO blood grouping is crucial for safe blood transfusion. Since the cloning of the *ABO* gene in 1990,[21] progress has been made in the structural and functional analyses of the *ABO* genes and A and B transferases at the molecular level. A proportion of blood donors and patients who historically have been typed as group O are now being recognized as group A or group B with the use of monoclonal antibodies capable of detecting small amounts of the immunodominant carbohydrate responsible for A or B specificity. A typing result that differs from the historical record often results in time-consuming analyses. Because the bases of many weak subgroups of A and B are associated with altered transferase genes, PCR-based assays can be used to define the transferase gene and thus the ABO group.[22] A majority of the mutations are nucleotide substitutions, resulting in amino acid substitutions or a single nucleotide deletion/insertion, and correlate well with the presence of specific subgroup alleles.

Table 2 Molecular events that give rise to blood group antigens and phenotypes

Gene conversion or recombination events (MNs, Rh, Ch/Rg blood group systems)
Duplication of an exon (Gerbich)
Deletion of a gene, exon, or nucleotide(s) (ABO, Rh, MNS, Kel, Duffy, Dombrock)
Insertion of a nucleotide(s) (Rh, Colton)
Single nucleotide substitutions (most blood group systems)

Resolution of RhD Typing Discrepancies

A proportion of blood donors that historically have been typed as RhD-negative are now reclassified as D-positive, because of monoclonal reagents that detect small and specific parts of the D antigen. The molecular basis of numerous D variants can be used to identify the genes encoding altered RhD protein in these individuals.[23,24,33]

APPLICATIONS TO MATERNAL–FETAL MEDICINE

Alloimmunization against the RhD antigen during pregnancy is the most frequent cause of hemolytic disease of the newborn (HDN). Immunization occurs when fetal cells, carrying antigens inherited from the father, enter the mother's circulation following fetal–maternal bleeding. The mother, when not expressing the same antigen(s), may produce IgG antibodies toward the fetal antigen, and these antibodies can pass through the placenta causing a diversity of symptoms, ranging from mild anemia to death of the fetus. Apart from antibodies in the RhD blood group antigen, other specificities within the Rh system and several other blood group antigens can give rise to HDN, but RhD is by far the most immunogenic.[25]

Prenatal determination of fetal RhD status is desirable in pregnancies to prevent sensitization and possible hydrops fetalis in fetuses of RhD-negative mothers with RhD-positive fathers. Fetal DNA has been detected in amniotic cells, chorionic villus samples, and, as recently reported, in maternal plasma.[26–32] Polymerase chain reaction amplification of *RHD* in maternal plasma may be useful for the management of RhD-negative mothers of RhD-positive fetuses and for the study of fetal–maternal cell trafficking.

Before interpreting the results of DNA analysis, it is important to obtain an accurate medical history and to establish whether the study subject is a surrogate mother and whether she has been impregnated with nonspousal sperm.

The discovery of fetal DNA in maternal plasma has opened up new and exciting possibilities for the noninvasive prenatal determination of fetal blood group status. However, a number of technical issues still need to be addressed and large-scale multicenter clinical trials need to be carried out. When these issues are resolved, it is likely that the prenatal testing of fetal blood group type will be carried out routinely and safely.

LIMITATIONS AND FUTURE

It is important to note that PCR-based assays are more prone to different types of errors than those observed with hemagglutination assays. For instance, contamination with amplified products may lead to false-positive test results. In addition, the identification of a particular genotype does not necessarily mean that the antigen will be expressed on the RBC membrane.

A large number of people from a variety of ethnic backgrounds need to be studied to determine the occurrence of particular genotypes and to establish more firmly the correlation between blood group genotype and phenotype.

Advances are ongoing in the automation of SNP and DNA sequence analysis, and the success of sequencing the human genome has shown that the potential for phenotyping large numbers of samples has already been realized.[34] It should soon be possible to analyze major and many minor blood group alleles on a single synthetic chip. Indeed, it will be possible to test for many genetically defined conditions.

As automated procedures attain higher and faster throughput at lower cost, blood group genotyping is likely to become more widespread. We believe that the PCR technology may be used in a transfusion service in the next few years to overcome the limitations of hemagglutination.

CONCLUSION

The identification of the molecular basis of blood group antigens provides an insight into the generation of gene diversity in humans. A molecular understanding of blood groups has enabled the design of simple assays that may be used to facilitate the provision of blood to patients who require antigen-matched red cells, both by phenotyping the patients to determine their requirements and by phenotyping red cell units.

Transfusion-dependent patients have sickle cell disease, thalassemias, and aplastic anemias, and frequently become alloimmunized. Blood group genotyping contributes substantially to the safety of blood transfusion in these recipients. Although it is unlikely that molecular genotyping will replace hemagglutination any time in the near future, together these techniques have substantial value in the resolution of clinical laboratory problems and, consequently, in the quality of patient care.

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Blotting Techniques

Philip Lewis White
Sam Hibbitts
University of Wales College of Medicine, Cardiff, U.K.

INTRODUCTION

The capacity to separate nucleic acids and proteins by their molecular weight and identify fractions of interest by the hybridization of specific labeled probes is known as blotting. It was originally developed for the analysis of gel-fractionated DNA and was termed the Southern blot. Northern blotting was soon to follow and permitted the investigation of RNA templates by using an identical procedure. Since then, Western blotting was designed to analyze proteins and a procedure known as Dot/Slot blotting was developed to detect and quantify any nucleic acid sequence without the necessity of processing [restriction fragment length polymorphism (RFLP) and/or separation by electrophoresis] the nucleic acids.

The development of blotting techniques has helped revolutionize molecular biology. One of the benefits of detecting nucleic acids using a blot/probe system, rather than ethidium bromide staining, is that it provides an enhanced sensitivity and confirms the identity of the polymerase chain reaction (PCR) amplicon or target sequence. Initially, targets were visualized by hybridization with a radiolabeled (^{32}P) probe, but because of apprehensions over user safety/exposure and the difficulty with disposal of radioisotopes, a number of nonisotopic alternatives are now available allowing colorimetric, chemiluminescent, or fluorescent detection.

TECHNICAL OVERVIEW

The basis of all blotting techniques (Southern,[1] Northern[2] and Western blotting[3]) is the transfer of nucleic acids/proteins to a solid inert support/filter followed by hybridization with a specific labeled probe. The process involves depurination (for nucleic acids >5 kb) to allow a more efficient transfer and denaturation of double-stranded nucleic acids to provide a suitable target for the probe. Neutralization is required for nitrocellulose filters because nucleic acids are not retained on these supports at pH values above 9.0. Usually, transfer to the support is by capillary diffusion, although apparatus are available that enhance the transfer by using suction and fixation onto a nitrocellulose/noncharged nylon solid support by baking or UV cross-bonding, respectively. Hybridization of the specific probe is performed under optimal conditions, and washing to remove excess (non-specific) hybridization is necessary before the degree of hybridization is determined.

BASIC PROTOCOL FOR SOUTHERN BLOTTING

Using a Nitrocellulose Filter

Southern blotting is the term given to the procedure of transferring DNA from a gel matrix to a solid inert filter. DNA samples, fragmented by restriction digests or PCR amplified, are separated by agarose gel electrophoresis (0.5–2.5% agarose), stained with ethidium bromide (0.5 μg/mL) and photographed to record the position of molecular weight markers and the fragments of interest. The gel is rinsed, the DNA depurinated by a HCl (0.25 M) wash, and denatured by washing twice in a denaturing buffer (1.5 M NaCl, 0.5 M NaOH). For the DNA to be transferred to the nitrocellulose filter, it is necessary to neutralize it by washing twice in a neutral, high-salt buffer (1.5 M NaCl; 0.5 M Tris–HCl, pH 7.0). For all the washes, use 5- to 10-fold gel volumes of each buffer and incubate at room temperature for 20–30 min with gentle shaking.

To transfer the DNA to the filter, assemble the blot apparatus as shown in Fig. 1. Fill a tray/sandwich box with transfer buffer (20× SSC; 3 M NaCl, 0.3 M trisodium citrate) and place a 20× SSC-soaked wick (three sheets of Whatman 3MM paper) onto the glass support so that each end dips into the 20× SSC reservoir. The nitrocellulose filter should be cut to the exact size of the gel and wetted by floating on distilled water prior to soaking in 20× SSC. Expel any air bubbles trapped beneath the wick with a gloved hand before placing the gel on to the saturated wick. Apply the 20× SSC-soaked filter to the top of the gel and remove any bubbles as instructed earlier. To prevent the filter from touching the wick, use saran wrap to form a barrier around the edges of the gel. Place five sheets of dry 3MM paper onto the filter and stack paper towels (4–5 cm) on top, cover with a glass plate, and place

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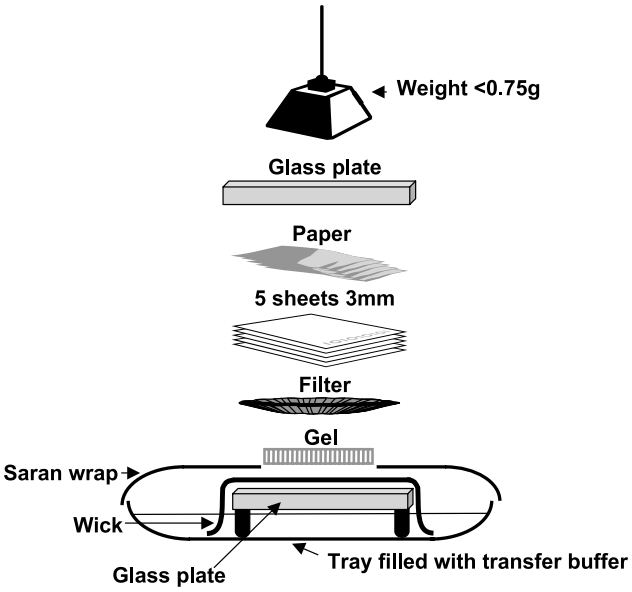

Fig. 1 Overview of nucleic acid blotting procedure.

a small weight (up to 500 g) at the top. The apparatus should be left overnight for optimal DNA transfer.

Disassemble the blotting apparatus, leaving a compacted gel/filter amalgamation. It is paramount to mark the position of the wells on the filter and to nick one of the corners to allow correct orientation. To remove excess salt and prevent the filter from becoming brittle, peel the filter from the gel and soak in a 6× SSC solution for 3 min before drying it on 3MM paper. It is recommended to confirm the DNA transfer by restaining the gel with ethidium bromide. After the filter has dried, sandwich it between two sheets of 3MM paper and bake it for 2–3 hr at 80°C under a vacuum to immobilize the DNA by expelling any water. The filter is now ready for probe hybridization, although it can still be stored for several months in a dry, dark location.

Using a Nylon Filter

Many groups now prefer the use of nylon filters, and varieties of charged (Hybond-N+) and noncharged nylon filters (Hybond-N) are commercially available. They have a high binding capacity for single-stranded nucleic acids; however, unlike nitrocellulose, nylon filters are capable of efficient double-stranded DNA retention. It is believed that nucleic acids bind to nitrocellulose in a noncovalent manner and baking will noncovalently bind nucleic acids to a nylon filter. Alternatively, a covalent association can be achieved by the UV irradiation of noncharged nylon filters and by simple alkaline treatment of positively charged filters. Further advantages of nylon-based filters include their ability to bind smaller oligonucleotides and their enhanced structural strength permits secure long-term storage and further analysis such as multiple reprobing.

The techniques for blotting on to a nylon filter are similar to those for nitrocellulose supports; however, when using commercially acquired filters, it is recommended that manufacturers' instructions are followed. In general, when performing Southern transfers to a nylon membrane, alkali blotting is the method of preference. Compared to high-salt blotting, alkali blotting allows quicker transfer of DNA molecules (within in 2 hr) while minimizing the possibilities for DNA reassociation. Furthermore, on transfer to positively charged nylon filters, the DNA becomes covalently linked—thus removing the need for baking or UV treatment.

The method is fundamentally the same; however, no neutralization stage is necessary and the blotting apparatus only requires a few minor alterations from what was previously described (Fig. 1). The 20× SSC transfer buffer is replaced with NaOH (0.4 mM) or NaOH (0.25 M) and NaCl (1.5 M) for positively charged and uncharged filters, respectively.[4] Because of its hydrophilic nature, it is not necessary to prewet the nylon filter; it can be placed on the gel without any pretreatment and left for a minimum of 2 hr for DNA transfer—after which the apparatus are disassembled, the filter marked as described, probed, and stored for reference or further use.

Alkali-induced covalent linking of DNA to nylon filters works most efficiently for positively charged supports, and although it does work for uncharged nylon filters, UV treatment is the preferred method when using the latter.[4] However, overexposure to UV irradiation may reduce the ability of the nylon membrane to hybridize a probe[5] and this limitation is enhanced when using positively charged filters.[4]

Alkali blotting is not suitable for DNA transfer to nitrocellulose filters as these supports require a neutral pH to retain nucleic acids, and furthermore, their lack of structural stability is accentuated by alkali treatment. Performing Northern blots by alkali transfer is not recommended, because RNA is hydrolyzed by prolonged (overnight) alkaline exposure;[4] however, the use of lower (0.2 M) NaOH concentrations for shorter (1 hr) transfer times has generated successful Northern blotting techniques.[6]

BASIC PROTOCOL FOR HYBRIDIZATION

The expression hybridization refers to the (re)association of complementary nucleic acid strands (DNA, RNA, or synthetically produced primers or probes) to form double-stranded complexes. The process starts with the

association of two short regions of complementary nucleic acid sequences, followed by a rapid and progressive linking of the base pairs on either end of the duplex region. The rate of hybridization and the stability of the complexes, once formed, are dependent on a number of factors. Temperature has an important role in both the rate and stability of the hybridization complex. Optimal hybridization occurs at 20–25°C and 10–15°C below the melt temperature of DNA and RNA complexes, respectively.[7,8] At suboptimal temperatures, reaction rates will be slowed and nonspecific hybridization may occur; however, if temperatures exceed the optimal range, then although stringency of the reaction may improve there may be a degradation of accurately associated dimers.

Hybridization time is also important and long incubation times may not provide the optimal results. There is a balance between achieving an optimal bind between the target and probe and the development of a nonspecific background signal or the hybridization of the probe to sequences similar to the target. Generally, incubations for less than 24 hr should not result in a high background signal, providing the probe concentration is not in excess. If the probe is in excess of the bound target, then binding to similar nontarget sequences may become problematic in the latter stages. For an overnight reaction, a usual probe concentration is 10 ng mL^{-1}.

Mismatches between the probe and target sequence will compromise the stability of the association and this may lead to a reduction in the optimal temperature to overcome the discrepancy. Even so, a 10% mismatch is usually tolerated. However, mismatches will also have a detrimental effect on the rates of hybridization and a 10% mismatch will reduce the hybridization rate.[7]

Other factors that need to be considered include the following:

1. Salt concentrations—generally concentrations of 1.0–1.5 M Na^+ (or equivalent, 5× SSC) are considered optimal.[9]
2. pH—reactions are usually performed at a neutral or weakly acidic pH.[4]
3. Dextran sulphate—the addition of dextran sulphate (10%) concentrates the nucleic acids by expelling them from the volume that it occupies, thus increasing the chances for the interaction between the probe and target sequence.[10]
4. Viscosity—the use of solution with a low viscosity will reduce the opportunity for the interaction between the probe and target sequence. The introduction of polyethylene glycol (PEG 8%) will increase the viscosity of less viscous solutions.[11]
5. Probe length—when probing a filter, the hybridization rate is directly proportional to the probe length when there is excess target DNA bound. Probes are usually >15 bp and <1.5 kbp[4] in length.

When performing hybridization, three steps are followed. The first stage is prehybridization, where the filter is washed with a protein, polymer, and sheared carrier DNA mix to block nonspecific binding. The next stage is hybridization and involves denaturing the probe before adding it to fresh, warm prehybridization solution, which is then applied to the filter and agitated overnight at the desired temperature. The filter is then washed for 30 min to remove any unbound or weakly bound probe using 0.2× SSC, 0.1% SDS solution. The stringency of the wash is temperature-dependent, with washing at room temperature and close to the probe Tm considered to be moderate and high, respectively.

The most common detection methods originally utilized ^{32}P radiolabeling of the oligonucleotide with autoradiography. The use of fluorescent dyes or lanthanide chelates, biotin-labeled nucleotide analogs, cross-linked enzymes such as horseradish peroxidase, or acridinium esters that can be detected by enhanced chemiluminescence are just some of the currently available alternatives.[12]

BASIC PROTOCOL FOR NORTHERN BLOTTING

Northern blotting is the transfer of RNA from a gel to a filter, and the method is comparable to that for Southern blotting with only a few modifications. By adding formaldehyde to the agarose gel, the RNA is denatured and secondary structures are prevented. Soak the gel in transfer buffer to dilute out the formaldehyde, cut off the marker lane for ethidium bromide staining, but do not stain the main body of the gel because this reduces RNA transfer.[13] Proceed as described earlier, although denaturing and neutralizing treatments are not essential if the RNA fragment is less than 5 kb. After the overnight transfer, the RNA can be cross-linked to the filter by baking or UV treatment prior to hybridization.

When compared to Southern blots, Northern blots, because of the ubiquitous nature of RNases, are highly variable in terms of sensitivity. As the presence of RNases is common, it is important to ensure all solutions are diethyl pyrocarbonate (DEPC)-treated, all plastics are baked prior to use, and, if possible, individual apparatus should be provided for RNA use. These preparations may help to provide more consistent results.

BASIC PROTOCOL FOR DOT/SLOT BLOTS

Dot blotting, also known as Spot or Slot blotting depending on the shape of the applied blot, is a rapid and simple alternative to Southern or Northern blotting procedures. It involves the direct application, either

manually (by pipette) or automatically (by suction apparatus), of the denatured nucleic acid to the filter. If DNA is being analyzed, usually 10–100 pg will be applied and can be resuspended in 0.4 M NaOH, high-salt solutions, or 6× SSC depending on which filter is being used. As soon as the sample has dried on the filter, it is exposed to the usual denaturing and neutralizing conditions before it is immobilized by baking or UV cross-linking. Positive and negative DNA controls must be included in these experiments, and it is further recommended to include standards that can be used for quantification. After application, the sample is air-dried and immobilized without any further treatments.

The major applications of Dot blotting are the rapid detection of specific nucleic acid sequences and the ability to quantify sequences in a complex sample, e.g., viral load in human blood. However, as Dot blotting does not utilize any fractionation methods, it is unable to differentiate true hybridization from nonspecific hybridization, and therefore it is essential to include the correct controls and standards, especially if accurate quantification is required.

BASIC PROTOCOL FOR WESTERN BLOTS

Western blots, otherwise known as Immune blots, have a method analogous to the one outlined above. In brief, proteins are fractionated and denatured by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The size-fractionated proteins are then blotted onto a nylon or nitrocellulose filter, where—once fixed and blocked—they are probed by antibodies specific for the target protein. This primary antibody can be labeled, but most often the filter is probed with a labeled secondary antibody raised against the first (primary antibody rabbit, secondary antibody antirabbit goat antibody), and it is this secondary antibody that is detected. The primary antibodies should be specific for a single protein, and bind to an amino acid sequence rather than any posttranslational modifications, e.g., phosphorylation, that may have been applied.[14] It is also beneficial for the antibody to be able to detect any epitopes of the protein, and so polyclonal antibodies are widely used, although they may create unsatisfactory high background levels.[14] Monoclonal antibodies do not have this problem, but being specific for a single epitope could be problematic if the targeted epitope has not renatured after electrophoresis.

CLINICAL AND GENETIC APPLICATIONS

Blotting techniques have widespread uses as both research and diagnostic tools:

1. They are essential in sequencing strategies for screening cosmid/phage clones and libraries.
2. They can be used to screen populations for polymorphisms.[15]
3. They can also be utilized for the mapping of unknown genes or sequences, e.g., to locate restriction sites for cloning[16] or inverse PCR.
4. Blotting techniques can be used to confirm the identity of PCR amplicons, which is especially useful if coamplification has occurred or is required.
5. They have various applications in medical molecular biology, e.g., screening for mutations,[17] confirming culture results,[18] or providing early diagnosis of infections where the alternatives are slower, less sensitive, and reproducible.[19]
6. Further medical applications involve the ability to quantify levels of nucleic acids[20] (e.g., the RNA copy number of infectious agents such as HIV) and proteins (e.g., hormones) or proteoglycans.[21]

CONCLUSION

The widespread and extensive applications of blotting techniques are undoubted. However, the development of real-time amplification systems has replaced their usage for diagnosing many infections and conditions. They will still be used for many applications in protein analysis, or where nucleic acids are not preamplified and blotting techniques will still provide a low-cost alternative.

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Molecular Detection and Epidemiological Typing of *Bordetella pertussis* and *Bordetella parapertussis*

Johanna Mäkinen
Matti K. Viljanen
Jussi Mertsola
Qiushui He
National Public Health Institute, Turku, Finland

INTRODUCTION

Pertussis has been classified by the American Centers for Disease Control and Prevention as one of the reemerging infectious diseases. The increase in the incidence of pertussis, the fact that other agents can cause a pertussis-like syndrome, and the possible adaptation of *Bordetella pertussis* populations to vaccine-induced immunity underscore the need for simultaneous detection, identification, and genetic typing of *B. pertussis* to enable rapid, sensitive, and specific diagnosis for treatment and prevention. This article discusses recent developments in the laboratory diagnosis of *Bordetella* infections and the epidemiological typing of *B. pertussis* as well as future prospects in the field.

BORDETELLAE

Bordetellae are small, gram-negative coccobacilli causing respiratory infections in humans and animals. The genus *Bordetella* comprises eight species; six of which have been isolated from humans. *B. pertussis* is pathogenic only for humans and causes pertussis (whooping cough), a contagious respiratory disease with severe clinical manifestations in infants. *Bordetella parapertussis* and *Bordetella holmesii* cause milder pertussis-like disease. In addition to these species, *Bordetella bronchiseptica* has been occasionally isolated from the human respiratory tract, *B. holmesii* and *Bordetella hinzii* from blood cultures of immunocompromised patients and *Bordetella trematum* from wound and ear infections. The remaining *Bordetella* species, *Bordetella avium* and *Bordetella petrii*, are not pathogenic in humans.[1]

DESCRIPTION OF THE INFECTION SPECTRUM

Whooping cough is acquired by inhalation of aerosols containing *B. pertussis*. The infection is characterized by bacterial colonization of ciliated respiratory epithelia in the trachea and bronchi. The incubation period is 6–20 days. Classical pertussis is an illness of three stages. It starts with a catarrhal stage, with nonspecific symptoms similar to those of the common cold (duration 1 to 2 weeks). At the paroxysmal stage (duration 2 to 6 weeks), the cough becomes more prominent with staccato attacks, postussive whooping, and vomiting. At the convalescent stage (duration several weeks), the frequency and severity of coughing attacks gradually decrease. The most severe cases of whooping cough occur in unvaccinated children under 1 year of age. This group accounts for most deaths.[1,2]

B. parapertussis and *B. pertussis* are almost identical at the DNA level and produce many similar virulence factors. However, *B. parapertussis* does not secrete pertussis toxin (PT).[3] In patients infected with *B. parapertussis*, the symptoms of whooping cough occur at lower frequencies and are of shorter duration than those of typical pertussis. A striking difference is the lack of lymphocytosis in children with *B. parapertussis* infections. Lymphocytosis during pertussis is thought to be caused by PT.[4]

PREVALENCE

Whooping cough is presently one of the 10 most common causes of death from infectious disease. The WHO estimates it to cause 50 million illnesses and over 350,000 deaths worldwide each year. After the introduction of whole-cell vaccines in the 1950s, its morbidity and mortality were significantly reduced in the developed world, whereas in the developing countries whooping cough has remained a major cause of infant mortality. In recent years, however, the incidence of pertussis has increased in a number of countries with high vaccination coverage.[5–7] Thus, the Centers for Disease Control and Prevention have classified pertussis as one of the reemerging infectious diseases.[8] This reemergence may be due to several factors such as waning vaccine-induced

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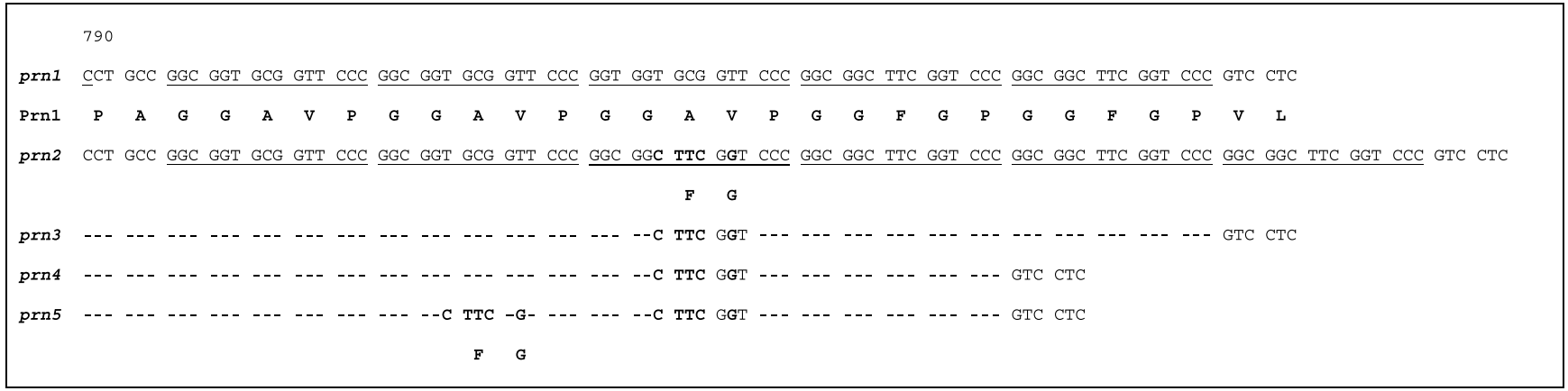

Fig. 1 Variable region 1 of the most common *B. pertussis* pertactin variants, Prn 1–5. Depicted are the nucleotide sequences of the pertactin genes and the corresponding amino acid sequences of the pertactins. The number in the upper left corner refers to the underlined residues and marks the start of an open reading frame. The dashes indicate identical bases relative to the vaccine-type pertactin, Prn1. Most clinical isolates collected in the 1990s expressed Prn2. (From Refs. [9,16,17].)

immunity, changes in vaccine production processes, improved surveillance, more sensitive laboratory diagnostic methods, or, possibly, expansion of strains antigenically distinct from the vaccine strains.[9]

In highly vaccinated communities, *B. pertussis* and *B. parapertussis* infections often go undiagnosed because pertussis is thought to be rare and adequate laboratory diagnostic tests are not used or available.[10] Infections caused by *B. parapertussis* are also more common than usually thought. In acellular vaccine efficacy trials, 2% to 25% of all isolates were *B. parapertussis*.[4,11] In addition to *B. pertussis* and *B. parapertussis*, *B. holmesii* has also been isolated from the respiratory tract of patients with pertussis-like symptoms.[12] However, its significance as a respiratory pathogen remains to be determined.

MANAGEMENT

The treatment of pertussis is primarily supportive, and adequate control of the disease depends on effective immunization. Currently, acellular pertussis vaccines, consisting of only a few antigens, are replacing whole-cell vaccines in many countries. These new vaccines are less reactogenic than the whole-cell vaccines. Several antibiotics eliminate *B. pertussis* from the respiratory tract, but erythromycin is recommended as first-line treatment because of the high concentration attainable in the serum and respiratory tract. Treatment during the catarrhal stage shortens and ameliorates the disease, and the onset of therapy even during the early paroxysmal stage has benefit. Erythromycin therapy terminates infectivity, and prophylaxis is recommended for prevention of household and school transmissions.[13] Patients should remain isolated as long as they are infectious, i.e., 5–7 days after the onset of erythromycin therapy. Infants under 1 year of age are at the greatest risk of complications and permanent sequelae.[2]

MOLECULAR CHARACTERIZATION OF *B. PERTUSSIS* AND *B. PARAPERTUSSIS*

Molecular characterization and typing of *B. pertussis* and *B. parapertussis* are used to obtain information on the changes in the phenotype and genotype of circulating isolates and in epidemiological studies. When polymorphisms in a number of *B. pertussis* genes encoding for virulence factors and surface-associated proteins as well as in housekeeping genes were studied, *B. pertussis* proved to be a rather conserved bacterium. Polymorphisms in recent isolates are restricted to genes encoding tracheal colonization factor, pertactin (Prn), and the S1 subunit (*ptxS1*) of PT.[9,14] Pertussis toxin and pertactin are important virulence factors and protective antigens. Pertussis toxin is a component of all and Prn of many new acellular vaccines and, thus, the degree of allelic variation in genes encoding these proteins has been studied intensively in many countries. The majority of the currently circulating isolates express PT and Prn types that are different from types included in the vaccines (Figs. 1 and 2).[9,15,16] It has been suggested that the reemergence of pertussis in highly vaccinated populations could be due to inadequate protection by currently used vaccines against these antigenic variant strains. For a better understanding of the causes for the reemergence of pertussis, population-based studies are necessary. Several molecular methods, such as serotyping, Western blotting, polymerase chain reaction, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism, ribotyping, and multilocus sequence typing have been applied to the characterization of *B. pertussis* isolates. The use of the various techniques hampers the comparison of typing data between countries. To make the data comparable, recommendations for standardized epidemiological typing of *B. pertussis* have been given. Currently, PFGE using the restriction enzyme *Xba*I (and *Spe*I when needed), serotyping using monoclonal

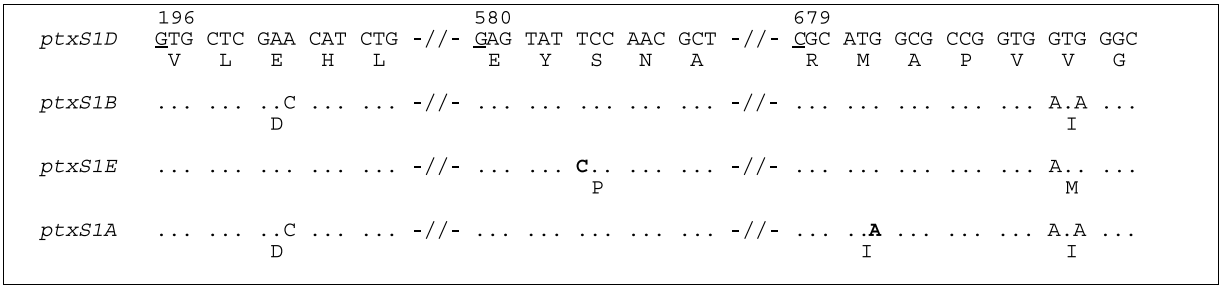

Fig. 2 Polymorphic region of *B. pertussis* pertussis toxin. Depicted are the nucleotide sequences of the toxin genes (*ptxS1*) and the corresponding amino acid sequences of the toxins (*ptxS1*). The numbers refer to the underlined residues and mark the start of open reading frames. The dots indicate identical bases relative to *ptxS1D*. The strains used for vaccine production express ptxS1B or ptxS1D, whereas the majority of currently circulating isolates express ptxS1A. (From Refs. [9,16,17].)

antibodies to fimbriae 2 and 3, and determination of PT and Prn encoding alleles are recommended for the standardized epidemiological typing of *B. pertussis*[17] *Xba*I PFGE is also recommended for the epidemiological typing of *B. parapertussis*.

MOLECULAR TESTS IN THE CLINICAL DIAGNOSTICS OF *BORDETELLA* INFECTIONS

Older children and adults often present with mild or atypical pertussis that easily goes unrecognized. In addition, other pathogens[18] can cause pertussis-like illnesses. These factors complicate the clinical diagnosis of pertussis and make the laboratory diagnosis a vital component of effective strategies against the disease in the community. Laboratory diagnosis requires isolation of *B. pertussis* or *B. parapertussis* from respiratory secretions, demonstration of specific antigens or nucleic acids in the above secretions, or detection of a serological response to infection. Culture and detection of antigens or nucleic acids yield positive results at the early stages of pertussis, whereas serology is usually the only diagnostic method that can be used at later stages, when microbial antigens and nucleic acids have disappeared from the infected host.

Bordetellae are fastidious organisms. Sampling time, sample collection and transportation methods, and culture conditions affect the sensitivity of culture. Furthermore, culture is a slow procedure; it may take up to 3 weeks before culture results are available for the physician. The direct fluorescence antibody test (DFA) uses polyclonal fluorescein-labeled antibodies to *B. pertussis* and *B. parapertussis* for the detection of organisms in nasopharyngeal smears.[19] The method is compromised by inadequate sensitivity, specificity, and subjectivity and should be used only in parallel with culture. The diagnostic sensitivity of serology is very much dependent on the timing of sample collection, choice of antigen, and the immunoglobulin class.[1,20] Assessments of IgG and IgA antibodies to PT and filamentous haemagglutinin (FHA) are the most useful assays. Only antibodies to PT are specific for *B. pertussis*, antibodies to FHA and Prn may be elicited by *B. parapertussis* and other bacteria.[21] The diagnostic efficacy of serology is rather low in the acute phase of the disease. At the later stages of pertussis, diagnosis is primarily based on serology. Without serology, adolescent and adult pertussis cases are largely overlooked, as the patients do not usually contact a doctor until after a long duration of symptoms.

Molecular diagnostic methods such as PCR have been widely applied to the direct detection of the DNA of fastidious pathogenic bacteria from clinical specimens. PCR has proved a rapid, sensitive, and reliable tool for the diagnostics of pertussis.[10] A variety of regions in the *B. pertussis* genome have been successfully used as targets, including the PT promoter region, repeated insertion sequence IS481, a DNA region upstream from the porin gene and the adenylate cyclase toxin gene. IS1001-based PCR has been applied to the diagnosis of *B. parapertussis* infections.[10]

The recent application of fluorescence techniques to in vitro nucleic acid amplification allows simultaneous amplification and sequence-specific detection of PCR products using hybridization probes.[22] The advantages of these real-time PCR methods are that PCR amplification can be carried out in less than an hour and no further analysis of the PCR amplicons (such as gel-electrophoresis and Southern blotting) is required. Because no PCR products need to be handled, contamination risk is minimized. Like with conventional PCR, the crucial issue of the use of these real-time PCR methods is finding good amplification targets allowing sensitive and specific detection of a given pathogen. The target gene region should have sufficient sequence variation to allow differentiation between *B. pertussis* and *B. parapertussis* and *B. holmesii*. However, the gene region should be conserved without any variation on the subspecies level in the regions where the probes bind. Further, multiple copies of the target gene region in the genome increase the sensitivity of the assay. Multiple copies of repeated insertion sequences IS481 and IS1001 are present in the genomes of *B. pertussis* and *B. parapertussis* and, therefore, IS-based PCR assays are widely used in diagnostic laboratories. The first diagnostic real-time PCR assay developed for simultaneous detection and identification of *B. pertussis* and *B. parapertussis* also targeted at IS481 and IS1001.[23] However, IS481 is also present in the genome of *B. holmesii*, and the major drawback of the IS-based assays is that *B. holmesii* and *B. pertussis* infections cannot be differentiated. The major limitation of the other target genes tested so far is that they are present singly or in low copy numbers and their amplification may not allow as good analytical sensitivity as that of IS-based assays.

Currently, rapid real-time PCR assays are gradually replacing conventional PCR assays in clinical diagnostic laboratories. One of the major challenges in pertussis diagnostics is finding new target gene regions for sensitive and specific detection and identification of Bordetellae directly from clinical specimens. However, PCR can probably never fully replace culture, as the collection of clinical isolates for research purposes is an important task for diagnostic laboratories.

MOLECULAR TESTING IN THE EPIDEMIOLOGICAL TYPING OF *B. PERTUSSIS* ISOLATES

The finding that the currently circulating strains harbor *ptxS1* and *prn* alleles different from those in strains used for vaccine production emphasizes the importance of monitoring the molecular evolution and antigenic variation of clinical isolates. In epidemiological studies, PFGE is used for the determination of the overall genotype of the isolates. Serotyping and the determination of *ptxS1* and *prn* alleles provide information about the expression and polymorphism of these virulence factors at a single-gene level.

To date, the only way to determine alleles encoding for important antigens has been PCR-based sequencing, a relatively time-consuming and expensive method. Thus, new simple methods suitable for large-scale screening of isolates are needed. Recently, real-time PCR using fluorescent-labeled hybridization probes has been applied to the determination of *prn* and *ptxS1* alleles of *B. pertussis*.[24,25] These assays proved a good alternative to sequencing as they correctly identified all *prn* and *ptxS1* gene variants, data analysis was uncomplicated and both *prn* and *ptxS1* typing results could be obtained within one working day. The disadvantage of the assays was that the PT encoding gene alleles of the two vaccine subtypes could not be differentiated. The main shortcoming of all hybridization probe-based methods, however, is that novel genotypes are missed and, therefore, these methods can never fully replace sequencing.

Studies have suggested that antigenic divergence between vaccine strains and circulating strains of *B. pertussis* affects the efficacy of pertussis vaccines and may have contributed to the disease burden as well as to recent pertussis epidemics.[9] Population-based studies should therefore be continued to ensure that antigenic shift among *B. pertussis* populations does not result in decreased efficacy of pertussis vaccines. Well-standardized, rapid, and reliable techniques are needed that further simplify typing procedures in pertussis research laboratories and facilitate the comparison of typing results from different countries.

CONCLUSION

The Sanger Centre had sequenced the genomes of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, and thorough analysis of the genomes will undoubtedly improve the understanding of the molecular pathogenesis of *Bordetella* infections. In the near future, novel analytical tools such as DNA microarrays, amplification on a chip, and direct sequencing of the amplicon will probably enable simultaneous detection, identification, and genetic typing of Bordetellae. The genomic information obtained may also promote the identification of new specific antigens to be used in pertussis vaccines as well as in diagnostic approaches and therapeutic interventions.

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Borrelia spp.

Maurice M. Exner
Quest Diagnostics Nichols Institute, San Juan Capistrano, California, U.S.A.

INTRODUCTION

The genus *Borrelia* comprises approximately 40 species that belong to the family Spirochaetaceae. Borreliae are vector-borne pathogens that cause Lyme disease and relapsing fever in humans, and spirochetosis/borreliosis in birds, cattle, sheep, and horses. Louse-borne relapsing fever epidemics appear to be decreasing over the years, with occasional outbreaks still occurring in parts of Africa. Endemic tick-borne relapsing fever still surfaces regularly in the Americas and Europe, although the number of reported cases remains low. Lyme disease, on the other hand, appears to be rapidly spreading on a global scale. This may be due to the ''promiscuous'' nature of Lyme borreliae, which have a wide variety of reservoirs and vectors compared to relapsing fever *Borrelia* species. While there are relatively few fatalities associated with Lyme disease or relapsing fever, significant morbidities can result from both, especially in the case of Lyme disease, in which chronic infection can last for years. An early and accurate diagnosis of either disease is extremely important, considering that both diseases respond well to early administration of antimicrobial agents. While a clinical diagnosis of relapsing fever can be relatively simple based on recurring febrile events, it is still confounded by the rarity of the disease, which often leaves it out of differential diagnoses (particularly in nonendemic areas). Diagnosing Lyme disease poses a particularly daunting challenge because of the protean manifestations of the disease, the variable stages of the disease, and the lack of sensitive, specific, and accurate diagnostic tools. Although the disease syndrome still relies on clinical findings for diagnosis, with confirmation by serological testing, molecular testing is proving to be sensitive and specific. Such tests are gradually gaining acceptance, and new molecular techniques may, in combination with serology, provide better insights into both diagnosis and treatment of the disease.

THE GENUS *BORRELIA*

The borreliae are motile, helical rods that range from approximately 5 to 30 μm in length and 0.2 to 0.5 μm in width.[1] They are similar in structure to other spirochetes, although *Borrelia* are generally longer and less tightly coiled. The organisms are highly motile, and they have 7 to 30 periplasmic flagella, which are partly responsible for maintaining the shape of the organism. The flagella are attached to the protoplasmic cylinder, which is enclosed by a cytoplasmic membrane and bounded by the periplasmic space and an outer membrane.

Borreliae are unique with respect to their genetic composition in that they possess a linear chromosome, along with a complement of linear and circular plasmids. The chromosome comprises approximately 950 kbp, whereas the plasmids may consist of another 40–50% more genetic material. The linear plasmids tend to encode surface proteins, whereas the circular plasmids encode proteins related to transmission and pathogenesis. Sequencing of the *Borrelia burgdorferi* genome has indicated that the organism possesses genes involved in replication, transcription, translation, and metabolism, although genes for biosynthetic pathways are lacking.[2]

Borrelial diseases are caused following the transmission of organisms to the host by means of an arthropod vector. All *Borrelia* species are vector borne; *Borrelia recurrentis* is transmitted by the human body louse, *Pediculus humanus humanus*, whereas all other species are transmitted by ticks. Hard-bodied ticks, predominantly of the genus *Ixodes*, are the vectors that are principally responsible for the transmission of Lyme disease-causing *Borrelia* species, whereas soft-bodied ticks of the genera *Ornithodoros* and *Argas* are typically responsible for the transmission of relapsing fever species.

RELAPSING FEVER

Relapsing fever is an acute, febrile illness that can be transmitted through lice or ticks. Disease is characterized by the sudden onset of high fever, chills, myalgia, delirium, joint pain, and nausea. The initial fever may occur 2 days to 2 weeks following infection, with symptoms that last 3–9 days. Successive relapses can occur after as little as 2–3 days, or as long as 2 months after the primary infection, and relapses can occur from 1 to 13 times. Secondary spirochaetemia and fever relapses occur because of antigenic variation in the variable major surface protein (vmp) of the organism. Rearrangement of

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silent and expressed genetic loci alters the *vmp* gene, and the result is an altered antigenic surface structure that allows the organism to temporarily evade the immune response.[3]

Louse-borne relapsing fever is caused by *B. recurrentis*, which enters the host through skin abrasions or through mucous membranes that have made contact with hemolymph from crushed lice. *Borrelia* are not transmitted through a louse bite, as they are not present in the louse saliva. *B. recurrentis* is the only *Borrelia* species for which humans are the sole host; all other species are maintained in various wild-animal reservoirs. Because of the nature of the vector, including the increased prevalence in regions with poor hygienic practices, louse-borne relapsing fever can result in epidemics, and it is often referred to as epidemic relapsing fever. Mortality rates can be greater than 50% for untreated cases, although this drops to less than 5% following antibiotic treatment. Currently, disease outbreaks are relatively rare, and they are primarily found in Africa, although historically, cases have been reported throughout Europe and Asia as well.

Tick-borne relapsing fever, or endemic relapsing fever, is isolated to regions containing the vectors and their reservoir hosts. Infection is a result of the direct transfer of *Borrelia* from the tick saliva to the host. The soft-bodied ticks, which are implicated as vectors, feed for only a short duration, and infection of the host occurs very rapidly, as opposed to the extended feedings that are required for transmission of Lyme-disease-causing spirochetes. Relapsing fever is caused by a number of different characterized *Borrelia* species, and most are associated with a species-specific vector. As with louse-borne relapsing fever, cases of the tick-borne variety are uncommon. For example, less than 1000 cases have been reported in the United States in the past 40 years. Nevertheless, it is not likely that tick-borne relapsing fever will ever be eradicated, considering that there are a number of natural reservoirs, and that vertical transmission can occur in ticks.

While the documentation of recurring febrile episodes in a patient is diagnostic for relapsing fever, the rarity of the disease, the variable pattern of recurrences (or the lack of recurrences), and the fact that patients may not recall being bitten by a tick can make diagnosis difficult. Laboratory diagnosis can be helpful in some instances. Organisms can be visualized by microscopy (using Giemsa or Wright stains) in blood smears obtained during a spirochaetemic/febrile phase, or they may be directly viewed using darkfield microscopy. It has been estimated that the detection sensitivity of microscopy is approximately 70%.[4] In addition to direct observation, organisms can be cultured from specimens, and the cultures can then be monitored by microscopy for the growth of *Borreliae*. Aside from microscopic techniques, there have been few advances in relapsing fever diagnosis. This is, for the most part, due to the relatively few outbreaks that occur, and the consequent lack of utility for such tests. Serology is generally not used in diagnosis, and although molecular detection methods involving amplification of specific *Borrelia* genes using the polymerase chain reaction (PCR) have been devised,[5] they are rarely utilized. The low disease prevalence coincides with a low demand for these tests, and it also means that such tests are not considered to be useful for routine differential diagnoses of febrile illnesses.

LYME DISEASE

The causative agent of Lyme disease, *B. burgdorferi*, was first identified in 1982,[6] five years after the description of an epidemic of arthritis cases among children in Lyme, Connecticut. Lyme disease occurs following the transmission of *B. burgdorferi* to a patient via the bite of an infected tick. Infection is often accompanied by a distinctive bulls eye rash, erythema migrans (EM), which occurs in 60–80% of cases, or it may occasionally be associated with acrodermatitis chronicum atrophicans (ACA), which is a rash found on the legs and hands. The disease originally manifests itself with flu-like symptoms, including low-grade fever, myalgia, fatigue, headaches, and arthralgia. In the early stages of the disease, antibiotic therapy is usually successful, but without treatment, the organisms disseminate to a number of different tissues, including joints, neural tissue, and the heart. Chronic disease can then develop, causing a variety of neurological/cognitive malfunctions, along with severe arthritis, which may be exacerbated by autoimmune reactions in the host.[7]

The identification of Lyme borreliae throughout the world has led to the creation of three different genospecies of *B. burgdorferi* sensu lato. All have been implicated with Lyme disease, although each appears to have distinct pathologies. *B. burgdorferi* sensu stricto is found in North America and parts of Europe, and is most often associated with arthritis and disseminated disease. *Borreliae garinii* is most commonly found in western Europe and is more associated with neurological symptoms, whereas *Borreliae afzelii*, which is associated with ACA, is most commonly found in central Europe, Scandinavia, and Asia.[8] In addition to the three major genospecies, there are a number of other emerging species that are phylogenetically related and that have been isolated from ticks, but none has been proven to cause disease in humans.

The antigenic structure of *B. burgdorferi* has similarities to that of relapsing fever borreliae in that they all

undergo antigenic variation. *B. burgdorferi* normally express two major surface lipoproteins, OspA and OspB, while they are within a tick, but upon transfer to a mammalian host, these proteins are down regulated, and a new lipoprotein, OspC, begins to be expressed.[9] *B. burgdorferi* also possesses a number of paralogous gene families, including the *vlsE* genes, which consist of a number of silent cassettes that can be inserted into an expression site. This results in antigenic variations that increase in frequency throughout the duration of infection.[10] The combination of antigenic variation with the paucity of organisms present in late stages of infection may contribute to the establishment of chronic infection.

Lyme disease, which was originally thought to be confined to the northeastern United States and parts of Europe, is now being reported in North and South America, Europe, Asia, and even Australia. This geographical spread brings a concomitant increase in disease prevalence. According to the Centers for Disease Control, in the United States alone, reported incidences of Lyme disease have increased from 2386 cases in 1987, to 12,901 cases in 1997, and to 23,763 in 2002.[11] With such a rise in prevalence, and with the serious sequelae of untreated disease, proper diagnosis becomes increasingly important. Lyme disease diagnosis is still, for the most part, based on clinical findings, including the presence of EM, ACA, and history of a tick bite. Laboratory diagnosis during the early stages of the disease can confirm clinical observations, and considering that organisms are most abundant in host tissues during the production of EM, it is at this time that laboratory confirmation by culture, biopsy examination, or molecular detection is most successful. Although culture is the ''gold standard'' for detection, it is rather insensitive and impractical, particularly during disseminated disease. At this stage, serological and molecular tests gain increased utility. Serological analysis is currently the most commonly used method for laboratory diagnosis, since established recommendations have been set forth for such tests. Despite the utility of serology, false positive results may occur, and false negative results can be common, because a number of patients do not develop immune responses following infection.[12] In addition, serological testing cannot distinguish past from current infections, nor can it indicate clearing of organisms following therapy.

In many instances, PCR is being used as an alternative to serological testing, as it has been shown to be more sensitive and specific. Polymerase chain reaction has been used to detect organisms in skin biopsies, spinal fluid, urine, synovial fluid, and joint tissue. Studies indicate that PCR analysis of biopsies from EM or ACA lesions provides up to 80% and 92% detection sensitivity, respectively.[13] Furthermore, urine specimens from patients with EM show 13% to 100% sensitivity for the detection of *B. burgdorferi* DNA.[14–16] For patients with symptoms of neuroborreliosis, detection of *B. burgdorferi* DNA in CSF has a sensitivity of 17% to 79%, along with 7% to 45% sensitivity from urine specimens from the same patients.[15,17] With respect to patients diagnosed with Lyme arthritis, detection sensitivities from synovial fluid and urine are 85% and 79%, respectively.[15,18] For all disease manifestations, increased detection sensitivity is achieved when analyzing multiple specimen types from a single patient.

The variability in observed assay sensitivities between studies is in part due to the lack of PCR assay standardization. There is currently no consensus regarding which PCR target provides the best sensitivity, but in general, detection methods using chromosomal targets, such as the flagellin gene, appear to be more effective than those targeting plasmid encoded genes, such as the *ospA* gene. Further variation in sensitivity can be caused by differences in the disease stage at which specimens are drawn, as the number of organisms present in the host tends to decrease with extended lengths of infection. Assay sensitivity can also be affected by the disposition of the patients, particularly with respect to administered treatment. Studies have consistently demonstrated decreased detection of organisms following antibiotic therapy. Despite variations in assay sensitivity, most described PCR assays show specificities approaching 100%. False positive results are always a concern with PCR assays, because of the target amplification and the ensuing possibility for contamination, but, nevertheless, PCR has become a routine technique for many laboratories, such that specificity problems resulting from cross contamination can be minimized.

CONCLUSION

While relapsing fever is generally in decline, or is at least limited to a few endemic areas, Lyme disease appears to be rapidly spreading across the globe. The geographical spread coincides with the inception of a variety of new reservoirs and vectors, which is in turn initiating the evolution of new Lyme disease-causing *Borrelia* species. Corresponding with the incidences of each disease and the impact on public health is the fact that only a modest amount of research is being done with respect to treatment and diagnosis of relapsing fever, whereas Lyme disease research is burgeoning. Despite the volume of study being conducted on Lyme disease, both clinical and laboratory diagnosis are far from perfect. The multitude of different symptoms presented, combined with the variety of emerging strains, complicates diagnoses. Neither clinical findings nor serological or molecular laboratory diagnosis is able to detect all forms of disease. Established

serological tests are often challenged by conflicting clinical findings, and molecular testing, while often specific, has yet to be standardized, despite being in use for the past 15 years. Because of the changing face of this disease and its causative agent, it is likely that an accurate and sensitive laboratory diagnosis will always be elusive, and it will take a combination of both serology and molecular detection techniques to provide the greatest chance for success.

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Branched DNA Signal Amplification

R. Stefan Ross
University of Essen, Essen, Germany

INTRODUCTION

Techniques for detection and quantitation of a given nucleic acid sequence can be divided into three categories: 1) approaches for target amplification including polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), or strand displacement amplification (SDA), 2) probe amplification systems such as ligase chain reaction (LCR), and 3) methods that do not alter the number of target molecules but dramatically increase the signal generated by nucleic acid sequences present in the original specimen. One of the signal amplification strategies involves the so-called branched DNA (bDNA) and the following article will attempt to provide a short overview of the basic principles as well as specific clinical and research applications of this technology.

PRINCIPLES OF bDNA TECHNOLOGY

The basic principles of bDNA technology will be described here taking the commercially available VERSANT™ assays (Bayer Diagnostics, Berkeley, CA) for determination of viral loads as an example. After release of genomic DNA or RNA by a lysis reagent, capture of the target nucleic acids to the bottom of a microtiter plate well is mediated by probes termed capture extenders (Fig. 1). Then, a second series of target-specific probes hybridize to various sites of the captured DNA or RNA. These label extenders are designed in such a way that two of them must be bound to adjacent regions of the target to enable efficient hybridization of the preamplifier molecule. The resultant cruciform label extender–preamplifier complex and the incorporation of nonnatural, synthetic nucleotides (i.e., iso-C and iso-G) into selected target and amplification probes significantly reduce the assay background by minimizing nonspecific probe interactions. The bDNA amplifiers as the key element of the whole technology finally hybridize to the preamplifiers. Every bDNA molecule has 15 arms, and three sites for hybridization of alkaline phosphatase-labeled probes are located on each of the branches resulting in a tremendous enhancement of the generated signal. The enzyme catalyzes the dephosphorylation of the chemiluminescent substrate Lumi-Phos Plus™, and the intensity of the light emission is directly proportional to the original amount of target sequence present in the sample. Quantitation is possible by a standard curve defined from preparations containing known concentrations of recombinant DNA.[1–4]

bDNA technology, in principle, can be used for detection and quantitation of every known nucleic acid sequence. The specificity and sensitivity of the methodology, however, largely depend on the judicious design of the probe sets. This task is partly facilitated by a commercial software application (ProbeDesigner™), which allows for the recognition of unwanted nonspecific hybridization events and provides an algorithm for the simultaneous design of probe sets for multiple targets.[5] Because bDNA amplification is a nonenzymatic process that does not change the original amount of target nucleic acid sequences, this technique, in comparison to enzymatically mediated amplification systems, is less prone to contamination, sample-to-sample variation, and the influence of inhibitory substances contained in clinical specimens. On the other hand, target amplification in general still has greater analytical sensitivity than bDNA and provides greater feasibility in design when novel targets are approached because only one appropriate pair of primers and not a whole set of hybridization probes is needed.

INFECTIOUS DISEASE APPLICATIONS

The development and ongoing improvement of bDNA technology during the past decade was mainly driven by the increasing demand for quantitation of nucleic acids in clinical virology. Understanding the natural history and pathogenesis of chronic viral infections caused, for instance, by hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) has been greatly supported by accurate determinations of viral load. Consequently, measurements of HBV DNA, HCV RNA, and HIV RNA are extensively used in today's clinical practice to monitor the efficacy of antiviral treatment, predict the outcome of therapeutic strategies,

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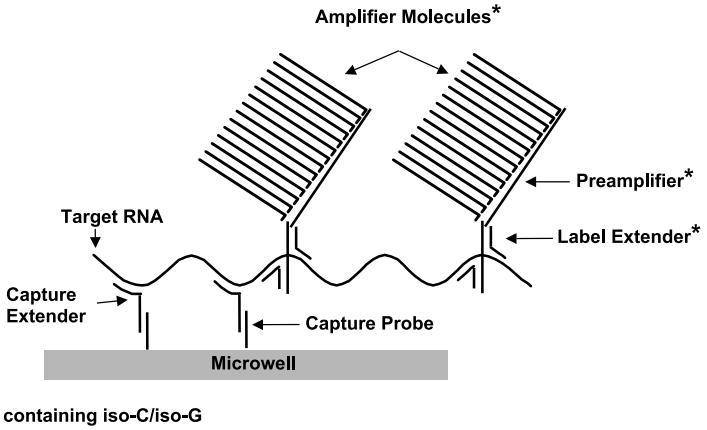


Fig. 1 Overview on the different hybridization probes and amplifier molecules used in the third-generation VERSANT bDNA assays. Iso-C/iso-G: nonnatural, synthetic nucleotides to minimize nonspecific hybridization events (from Bayer Diagnostics).

assess disease progression, and detect the potential emergence of drug-resistant viral strains.[6]

HBV DNA Quantitation

For quantitation of HBV DNA, a commercial third-generation assay is available from Bayer Diagnostics (VERSANT HBV DNA 3.0). Results of a multicenter evaluation showed that the specificity of this test was 99.3% using 600 HBV seronegative samples at four different sites. Quantitation was possible between 2×10^3 and 1×10^8 copies HBV DNA/mL and the assay proved to be linear throughout its entire dynamic range. Mean coefficients of variations of 7.5% and 13.6% were established for intra- and interassay imprecisions, respectively. Furthermore, studies revealed that HBV DNA concentrations obtained by bDNA 3.0 differed by less than threefold from the expected values for HBV isolates of genotypes A to F, suggesting that quantitation is not significantly affected by HBV genotypic variability.[7]

Because HBV DNA quantitative units reported by the various commercially available assays currently do not represent the same actual amount of HBV DNA in a given sample, these tests cannot be used interchangeably in the clinical laboratory. Therefore, like bDNA 3.0, all quantitative assays should in future be calibrated against the WHO HBV DNA standard[8] to achieve better comparability of the results and aid in the establishment of reliable, clinically relevant thresholds for HBV DNA loads that are yet poorly defined.[9] bDNA technology in the format of the VERSANT HBV 3.0 assay meets most of the clinical demands for HBV DNA quantitation, but is still not sensitive enough to answer all analytical questions in this field; for example, it is not suited to decide whether healthcare workers who are HBV carriers can be allowed to perform exposure-prone procedures under the strict guidelines recently issued by the UK Department of Health (decision threshold: 1000 copies HBV DNA/mL[10]).

HCV RNA Quantitation

Quantitation of HCV RNA by bDNA technology is possible with the commercial VERSANT HCV RNA 3.0 assay. The performance characteristics of this test were established in our laboratory during the so-called ''beta trials'' before licensing and are given in Table 1.[4] Our findings were essentially confirmed by other studies,[11,12] and showed that the assay, from an analytical point of view, can be used as a routine tool for HCV RNA quantitation in clinical laboratory settings.

Table 1 Analytical performance characteristics of VERSANT HCV RNA 3.0 assay

Specificity (%)	98
Sensitivity (IU/mL)[a]	1000[b]
Reproducibility	
Intraassay [CV (%)][c]	6.8
Interassay [CV (%)]	11.2
Dynamic range (IU/mL)	1×10^3 to 7.7×10^6 [b]
Influence of HCV genotype[d]	None for types 1 to 4

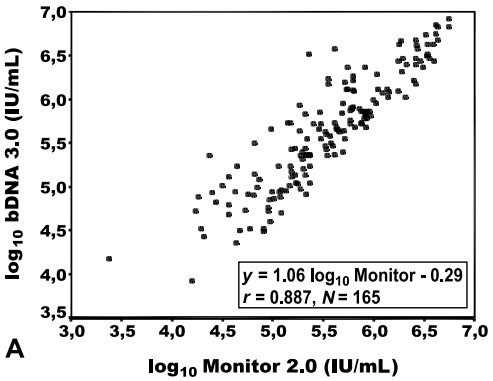
[a]International units/mL (IU/mL) of the first international WHO standard for HCV RNA.[13]

[b]Detection limit stated by the manufacturer: 615 IU/mL.

[c]Mean coefficients of variation (CVs) calculated from non-$\log_{10}$-transformed results.

[d]Two hundred samples were included in statistical analysis: 129 with HCV genotype 1, 21 with genotype 2, 42 with genotype 3, and 8 with genotype 4 HCV isolates, respectively.

Source: Ref. [4].



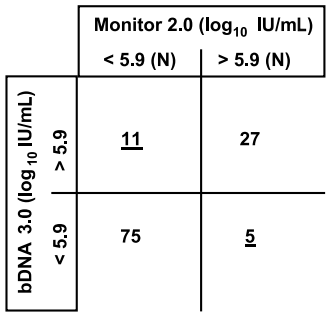


Fig. 2 (A) Scatterplot of $\log_{10}$ HCV RNA IU/mL values as determined by VERSANT HCV RNA 3.0 and Cobas Amplicor HCV Monitor 2.0 assays in 165 clinical samples. In the insert, the conversion equation and the correlation coefficient calculated by nonparametric regression analysis are given.[4] (B) Classification of test results obtained by both methods in sera of 118 patients chronically infected with HCV genotypes 1 and 4 according to the decision threshold of $\log_{10}$ 5.9 IU/mL (i.e., 800,000 IU/mL[14]). Numbers of discrepant results are indicated by underscore.

In contrast to the situation in HBV DNA viral load determinations, commercially available packages for HCV RNA quantitation have already been universally calibrated against an international WHO standard.[13] These attempts, however, did not completely lead to the expected widespread comparability of the results obtained with different methodologies. When testing 165 sera from patients infected with HCV in parallel with both HCV RNA bDNA 3.0 and the PCR-based Cobas Amplicor HCV Monitor™ 2.0 assay (Roche Diagnostics, Branchburg, NJ), we recorded a mean absolute difference between the results of $\log_{10}$ 0.23 (i.e., 1.7-fold) (Fig. 2A). Eleven percent of the paired results differed by even more than threefold,[4] i.e., a deviation that is generally regarded as clinically important in consecutive viral load determinations.[9] Such discrepancies may be particularly problematic in the clinical context if absolute HCV RNA concentrations are used for decision making. For instance, until recently a threshold value of 800,000 IU HCV RNA/mL was applied to tailor the initial duration of antiviral combination therapy in naïve patients chronically infected with HCV genotypes 1, 4, and 5.[14] Agreement between the HCV bDNA 3.0 and HCV Monitor assays in predicting viral loads either above or below 800,000 IU HCV RNA/mL (i.e., $\log_{10}$ 5.9) according to our results was only moderate, because about 14% of the samples were classified differently by both tests (Fig. 2B) and these misclassifications would have led to a wrong assignment of the initial duration of antiviral treatment in a considerable number of patients.[4] Germer and coworkers reported very similar observations,[15] indicating that HCV RNA concentrations close to any clinically defined threshold values should generally be interpreted with caution.

HIV-1 RNA Quantitation

HIV-1 RNA quantitation by bDNA signal amplification with the VERSANT HIV-1 RNA 3.0 assay was assessed in a large multicenter trial preceding U.S. product registration. In this evaluation, the test showed a specificity of 97.6% and a clinical sensitivity between 98.7% and 65.3%, depending on the HIV-1-infected patient's $CD4^+$ T-cell counts. As detection limit, 75 HIV-1 RNA copies/mL were established and the assay was linear up to 500,000 HIV-1 RNA copies/mL. Mean intra- and overall interassays imprecisions were 18.1% and 21.3%, respectively.[16] The test could quantify HIV-1 group M subtypes, but must not be used for detecting other subtypes of HIV-1 or HIV-2 strains.[17]

Commercial assays for quantitation of HIV-1 RNA in clinical samples lack standardization and so far have not been universally calibrated against the appropriate WHO reference preparation, which was available since 2001.[18] Consequently, quantitative values obtained with the third-generation bDNA assay were, on an average, 39% lower than the measurements recorded with the PCR-based Roche Cobas Amplicor HIV-1 Monitor (version 1.5) and the differences were consistent across the whole common reportable range of the two tests.[16] Despite these striking discrepancies, the treatment guidelines of the Department of Health and Human Services for HIV-infected adults and adolescents state that an HIV-1 RNA concentration of 55,000 copies/mL, as determined by either RT-PCR or bDNA, might represent a useful laboratory marker for the decision whether an asymptomatic patient with a $CD4^+$ T-cell count of greater than 350/mm^3 should be treated with antiretroviral agents or instead deferred from therapy.[19]

Other Pathogens

Besides its extensive use for viral load determinations, bDNA signal amplification has not yet gained widespread application in other fields of diagnostic clinical microbiology. Only a few prototype assays were reported for detection and quantitation of pathogens other than HBV, HCV, and HIV, including tests for GB virus C, cytomegalovirus (CMV), *Trypanosoma brucei* species, which cause the human sleeping sickness, and *Yersinia pestis*, the etiological agent of plague.[3,20] bDNA technology was also occasionally used to identify the mecA gene of methicillin-resistant *Staphylococcus aureus* isolates from clinical specimens. The obtained results suggested that the bDNA assay enabled mecA detection directly from blood culture bottles without the need for any subcultivation and was as sensitive and specific as the PCR-based comparative method.[21]

QUANTITATION OF mRNA BY bDNA SIGNAL AMPLIFICATION

bDNA technology during recent years has been increasingly adapted to the requirements of research laboratories and is now, for instance, a powerful tool for high-throughput mRNA quantitation from cell lysates or tissue homogenates. In this format, the assays are appropriate for a variety of research applications covering such different fields as regulation of gene expression, identification and validation of targets, secondary screening of biological agents, in vitro toxicology, metabolic and cell-signaling pathway studies, and cancer biology.[3,22] Although commercial kits for mRNA quantitation by bDNA signal amplification (QuantiGene™) are available from Genospectra (Fremont, CA), users, of course, still have to design a target probe set for the gene of interest. In this respect, they may rely on the help of the already mentioned ProbeDesigner software package, which allows for the prediction of nonspecific hybridization events, thereby reducing the overall assay background.[5]

bDNA SIGNAL AMPLIFICATION FOR IN SITU HYBRIDIZATION

bDNA signal amplification from a principal point of view offers a number of advantages for in situ hybridization (ISH) when compared to alternative PCR-based methods or catalyzed reporter deposition signal amplification: The methodology is not affected by polymerase inhibitors present in tissue specimens or endogenous biotin, intricate cell morphology is better preserved because no high-temperature incubations are necessary, and potential diffusion of amplification products away from the target site is not a significant concern with bDNA ISH, which therefore might provide a more accurate subcellular localization of nucleic acid sequences.[23]

bDNA ISH, e.g., could detect about one or two copies of human papillomavirus (HPV) DNA in cervical cancer cell lines and proved to be highly specific in discriminating cells infected with HPV type 16 from those with HPV type 18 DNA.[23] The same technique was subsequently applied to normal and HPV-infected cervical biopsy specimens and the obtained results led to the preliminary interpretation that bDNA ISH, as an adjunct to conventional histopathology, can perhaps improve future diagnostic accuracy at least in cases of HPV-mediated neoplastic disease.[24]

CONCLUSION

This article attempted to give a concise overview on the basic principles as well as the current clinical and research applications of bDNA technology. The widespread use of analytically reliable bDNA assays for viral load determinations during the past decade considerably increased our knowledge on the natural history and pathogenesis of HBV, HCV, and HIV infections; at present, quantitation of viral nucleic acids is an integral part of the clinical management of patients chronically infected with these pathogens. Notwithstanding its indisputable merits, bDNA technology still awaits the major breakthrough in other fields of diagnostic clinical microbiology and has just started to enter the wide area of possible research applications. At present, however, the future development and fate of bDNA methodology is difficult to predict; thus we will most probably only know in a few years which group of the various prophets is right: the ''bDNA supporters,'' who are envisioning further spread of the technology and are waiting for the creation of new applications by ongoing analytical refinement, or the ''bDNA skeptics,'' who believe that the technique has already reached its inherent limits and, consequently, will be replaced soon by newer and even more powerful approaches for nucleic acid quantitation.

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Brucella spp.

Vito G. DelVecchio
Cesar V. Mujer
Vital Probes, Inc., Mayfield, Pennsylvania, U.S.A.

INTRODUCTION

Brucellae are aerobic, gram-negative, nonmotile, facultative intracellular, non-spore-forming coccobacilli. The genus *Brucella* belongs to the α-2 subgroup of the class Proteobacteria and is composed of six currently recognized nomen species: *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella neotomae*, *Brucella ovis*, and *Brucella suis*. Several other strains have been isolated from marine mammals and have been grouped generically as *Brucella maris*.

Among the various nomen species, *B. abortus*, *B. melitensis*, and *B. suis* have been reported to cause human brucellosis, also called Malta fever, a chronic febrile illness characterized by undulating fever, arthritic pains, and some neurological disorders. Currently, there is no acceptable human vaccine, and treatment is via prolonged antibiotic therapy that usually lasts for a year. Human infection occurs by direct contact with tissues and fluids from infected animals, consumption of contaminated dairy products, and inhalation. Brucellosis is also a major zoonotic disease that causes abortion and sterility in wild and domesticated animals. The World Health Organization considers *B. melitensis* as the most important zoonotic agent because it is extremely infectious, partly because of its aerosolic nature. Because of its ability to cause a debilitating disease in humans, it is considered a potent biological warfare agent. It can also bring havoc to the economy when used as a weapon for agroterrorism.

BRUCELLA GENOMES

The *B. melitensis* strain 16M (Biovar 1, ATCC 23456) genome was the first to be sequenced, closed, and annotated from among the six *Brucella* nomen species.[1–4] This was followed by the genome of *B. suis* strain 1330 (Biovar 1).[5] The total genome size of *B. melitensis* is 3.30 Mb, similar to that of *B. suis* (3.31 Mb). The genome of *B. melitensis* is distributed over two circular chromosomes of 2.12 and 1.18 Mb, whereas that of *B. suis* is distributed at 2.11 and 1.20 Mb.[4,5] Their chromosome maps can be accessed at NCBI (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html).

Some general features of the *B. melitensis* and *B. suis* genomes are presented in Table 1. The *B. melitensis* genome encodes approximately 3197 ORFs whereas that of *B. suis* encodes 3388 ORFs. A GC content of 57% was noted for both species. In *B. melitensis*, the origins of replication are similar to those of other α-Proteobacteria. Housekeeping genes, including those involved in DNA replication, transcription, translation, core metabolism, and cell wall biosynthesis, are distributed on both chromosomes. Type I, II, and III secretion systems are absent, but genes encoding sec-dependent, sec-independent, and flagella-specific Type III, Type IV, and Type V secretion systems as well as adhesins, invasins, and hemolysins were identified.[4,5]

B. melitensis and *B. suis* genomes shared extensive similarity and gene synteny. Greater than 90% of their genes showed 98% to 100% identity at the nucleotide level. Those genes with less than 95% identity included mostly the hypothetical genes, the *UreE* urease component, the outer membrane proteins, membrane transporters, a putative invasin, and Shd-like adhesins. These genetic differences may significantly contribute to the differences in pathogenicity or host preference between the two organisms. A total of 7307 single nucleotide polymorphisms were noted between *B. melitensis* and *B. suis*. This interspecies frequency is lower than some of the intraspecies frequency reported in some other sequenced organisms.[5] Based on whole genome nucleotide alignments, 42 *B. suis* and 32 *B. melitensis* genes are completely absent from each other's genome. This difference may be due to insertion or deletion events that possibly took place during the course of the species evolution.[6,7] Overall, analysis of the *Brucella* genomes has not revealed any traditional virulence factors, toxin genes, or pathogenicity islands.[8] A study of the virulence mechanism of Brucellae is currently an active area of

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Table 1 Comparative general features of the *B. melitensis* and *B. suis* genomes

Genomic features	*B. melitensis*	*B. suis*
No. of chromosomes	2	2
Size of chromosome I	2,117,144 bp	2,107,792 bp
Size of chromosome II	1,177,787 bp	1,207,381 bp
DNA total sequence	3,294,931 bp	3,315,173 bp
DNA-coding sequences	2,874,027 bp (87%)	ND[a]
GC content	57.0%	57.2%
Plasmids	None	None
Total no. of ORFs	3,197	3,388
Chromosome I	2,059	2,185
Chromosome II	1,138	1,203

[a]Not determined.

research that may be deciphered by comparative proteomics analysis.

BRUCELLA PROTEOMES

Proteome refers to the entire set of proteins expressed by an organism at a specific time and under defined environmental conditions. Early studies of the proteomes of laboratory-grown *B. melitensis* strains B1115 and *B. ovis* were conducted by Teixeira-Gomes et al.[9,10] on silver-stained 2-DE gels. In these and other previous 2-DE studies, protein spots were identified by Edman sequencing and Western blotting (for a review, see Ref. [11]). An examination was also made of the proteomes of *B. melitensis* 16M and *B. abortus* grown under conditions of heat, acid, and oxidative stress. These early studies demonstrated that differentially expressed proteins were either induced or repressed under these conditions. Using traditional biochemical approaches, about 54 proteins were identified.[11]

The availability of a completely sequenced and annotated *B. melitensis* genome paved the way for a rapid global analysis of its proteome (Fig. 1).[12] Using whole-cell protein extracts of laboratory-grown *B. melitensis* 16M, a comprehensive proteome initiative was undertaken using a series of 2-D gel electrophoresis of narrow overlapping IPG strips from pH 3.5 to 11.0 and MALDI-TOF MS (for a review, see Ref. [13]). To date, more than 1000 nonredundant protein spots were detected and 557 proteins were identified representing 232 discrete ORFs. As shown in Fig. 2, the corresponding ORFs of the identified proteins are distributed evenly between each of the two circular *B. melitensis* chromosomes, indicating that both replicons are functionally active.

A global comparative proteome analysis of the virulent 16M and vaccine Rev1 strains of *B. melitensis* has been reported.[14] Significant differences in the expression levels of several proteins involved in various metabolic pathways especially those regulating iron acquisition, sugar binding, protein biosynthesis, and lipid degradation

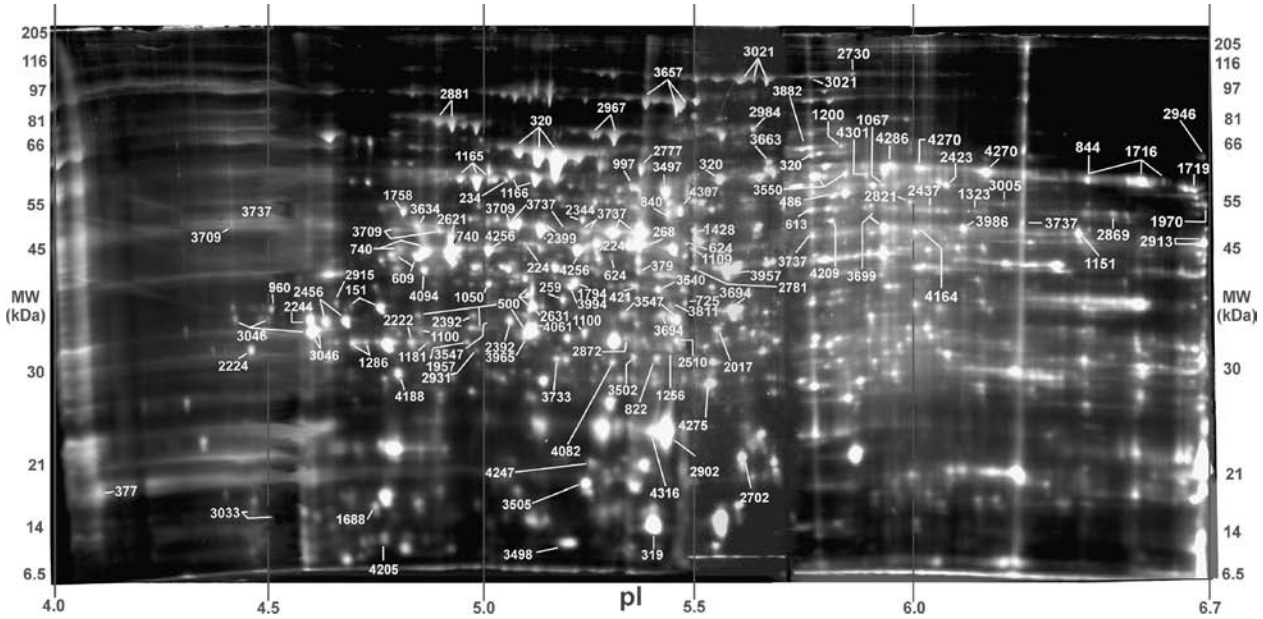

Fig. 1 Partial global proteome map of *B. melitensis* 16M. The map is a manually seamed, composite image of four narrow pH range 2-D gels, each containing 40–120 μg total protein extracted from chloroform-killed cultures of *B. melitensis* 16M. Gels were stained with SYPRO® Ruby for 12 hr before imaging under 470-nm light. (From Ref. [11].) (*View this art in color at www.dekker.com.*)

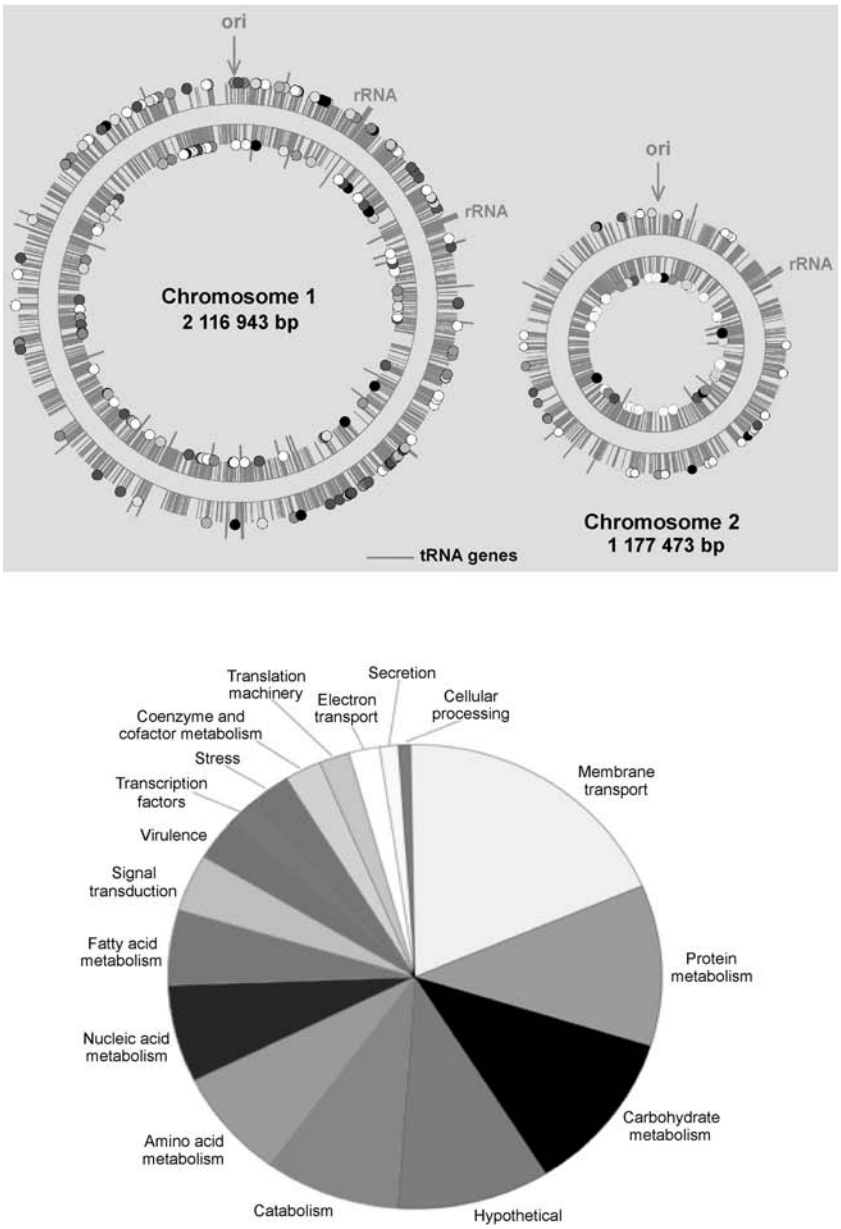

Fig. 2 Graphical representation of sequenced chromosomes 1 and 2 of *B. melitensis* 16M (top) and expressed ORFs categorized into major cellular functions (bottom). The colored spheres on chromosomes 1 and 2 represent the proteome under laboratory conditions. (*View this art in color at www.dekker.com.*)

were noted. One major pathway associated with the survival of pathogens inside the host cell is that regulating iron metabolism. During infection, a macrophage reduces iron by producing chelating agents and actively exporting iron from the phagosome where the pathogen multiplies. In response, the pathogen synthesizes proteins designed to compete with the host for iron. One such protein is bacterioferritin whose accumulation is significantly greater in the vaccine strain Rev1 than in the virulent strain 16M. Likewise, Rev1 overexpressed an iron-regulated outer membrane protein and an iron (III)-binding periplasmic proteins which are normally derepressed during low iron availability. Such a misregulated system leads to unnecessary expenditure of energy and as a consequence Rev1 upregulated other energy-generating pathways, i.e., β-oxidation of fatty acids and protein synthesis to generate more reducing equivalents for the production of ATP. The significant accumulation of enoyl coenzyme A (CoA) hydratase and acyl-CoA dehydrogenase in Rev1 indicated the upregulation of fatty acid

metabolism. Both enzymes are involved in the synthesis of acetyl-CoA that consequently enters the TCA cycle. Further metabolic pathway analysis will unravel more phenotypic differences including virulence and host preference among the various nomen species of *Brucella*.

DIAGNOSIS

The diagnosis of human brucellosis is conventionally performed using serological and bacteriological tests. The Rose Bengal test,[15] the serum agglutination test, and the complement fixation test are the most accepted serological tests used worldwide. These tests are based on a reaction between *Brucella* cell antigen and antibodies produced in response to the infection. The accuracy of the Rose Bengal test is over 99%, but it can give false positive results with sera from patients infected with *Yersinia enterocolitica* or other cross-reactive organisms.[16] In contrast, the bacteriological diagnosis is based on the recovery of bacteria from the patient's blood. The biphasic method which uses both solid and liquid media in the same container has been recommended and is widely used.[17] Using this method, it may take from 3 to 35 days to determine whether the hemoculture being tested is positive.[18] The variation of bacterial growth in blood culture is due to a number of factors including the culture medium, the number of circulating bacteria, the time of blood collection, the volume of blood used for culture, the host's immune response system, and the intracellular characteristics of the bacteria.[19]

Molecular-based assays using PCR have been developed that can be used in detecting various nomen species of *Brucella*.[20–23] Compared to standard identification methods, PCR-based assays are fast, less expensive, highly accurate, and safe to perform. For instance, with real-time PCR specific for *B. abortus*, *B. melitensis*, and *B. suis*, the assay can be accomplished in less than 30 min.[24] This assay utilizes an upstream primer that is derived from the 3′ end of the genetic element *IS711*, whereas the downstream primers and probes are designed from signature sequences specific to a species or biovar. In addition to PCR-based assays, Western blot analysis of cytoplasmic proteins has the potential to differentiate past from subclinical infection.[25] Despite their advantages, molecular diagnostic tests have not been approved for clinical use by FDA.

CONCLUSION

The sequencing and complete annotation of the *Brucella* genomes have paved the way for a rapid and comprehensive characterization of the *Brucella* proteomes. By globally defining differentially regulated biochemical pathways, it is possible to identify factors that confer host preference, survival mechanisms inside host macrophages, and attenuation of virulence among *Brucella* species. Further, proteomics data may be used in the development of diagnostic assays, antimicrobials, and next generation of vaccines. This is important as there is no effective human vaccine and all available vaccines are pathogenic to humans. Future studies on the secretome and membrane proteins will also be most valuable in determining pathogenicity and host preference. The advances in genomics and proteomics have given rise to the development of powerful and highly specific diagnostic tools that may soon be used in conjunction with conventional methods for the rapid and accurate detection of *Brucella* infection in humans.

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Brugada Syndrome

Robert Roberts
Ramon Brugada
Baylor College of Medicine, Houston, Texas, U.S.A.

INTRODUCTION

Since its initial description in the early 1990s, Brugada syndrome has attracted progressively more attention in the medical community. First, the disease takes the life of individuals in their 40s, during the most productive years. Second, the delineation of the disease coincided with a burst of activity in molecular biology and at the beginning of a golden age with the Human Genome. Third, once thought to be rare, the Brugada syndrome is now recognized to have a relatively high incidence, particularly in Southeast Asia. Thus, the past decade has witnessed a rapid growth in our understanding of the cellular and genetic basis of Brugada syndrome.

Basic research into the mechanisms underlying the Brugada syndrome is in its infancy and the next 10 years will provide a greater understanding of this lethal disease. What started as an electrocardiographic curiosity has become a great challenge for electrophysiologists, cardiologists, biophysicists, and molecular biologists.

CLINICAL FEATURES AND DIAGNOSIS

Brugada syndrome is characterized by a typical electrocardiographic pattern of ST segment elevation in the precordial leads, with or without right bundle branch block, malignant arrhythmias, and a structurally normal heart (Fig. 1).

It is certainly a challenge to risk stratify patients with Brugada syndrome. There have been many uncertainties which can only be clarified with further experience as more patients and families are being identified and followed for clinical outcome. Electrocardiographic criteria are used to try to risk stratify patients, but there is some disagreement on the criteria to be applied. Some of the conclusions are as follows. Symptomatic patients are at high risk. There is a consensus that symptomatic patients with documented ventricular fibrillation have close to a 50% chance of having another event within 5 years. If the patient experienced syncope, the recurrence rate is 20%.[1] Individuals with normal ECG at baseline and abnormal after antiarrhythmics have a good prognosis. This is the other side of the spectrum. Individuals that have the mutation but require an antiarrhythmic challenge to develop the abnormal electrocardiographic pattern probably also have a genetic background that is protecting them. A note of caution is required in this analysis; many of these are relatively young individuals, much younger than the symptomatic group. What will happen in the future remains unknown.[1] Individuals with an electrocardiographic pattern that normalizes are at the same risk as patients with a consistent ECG abnormality. If the substrate for arrhythmias depends on the transmural voltage gradient, one might postulate that patients would develop reentrant arrhythmias more often with the ST segment elevation. It is of course possible the ST segment elevation appears just moments before the episode of ventricular fibrillation. More data and patients will be required to assess the risk of transient vs. continuous ST segment elevation.

Those with a coved-type ECG are at higher risk than saddleback type. Actually, our group does not accept a diagnosis of Brugada syndrome without a coved-type ECG either before or after sodium blocker drug challenge. Again, this may correspond to the severity of the defect that throws off the balance of currents during the early phases of the action potential, as previously discussed. Our current understanding of basic mechanisms is that the substrate for VT/VF is unlikely to be present when only a saddleback type of ST segment elevation is manifest, but more likely in the presence of a coved type. Consistent with this hypothesis is the clinical observation that saddleback ST segment elevation usually progresses to a coved type before VT/VF is observed.

The use of potent sodium channel blockers to diagnose patients at risk for Brugada syndrome has raised some controversy. There is a wide variety of medications used to unmask the disease. False negative results have been reported with procainamide and flecainide.[2] False positive results have been reported with flecainide in patients with LQT3.[3] The value of the antiarrhythmic challenge will be able to be ascertained once more people are tested and genotyped for mutations.

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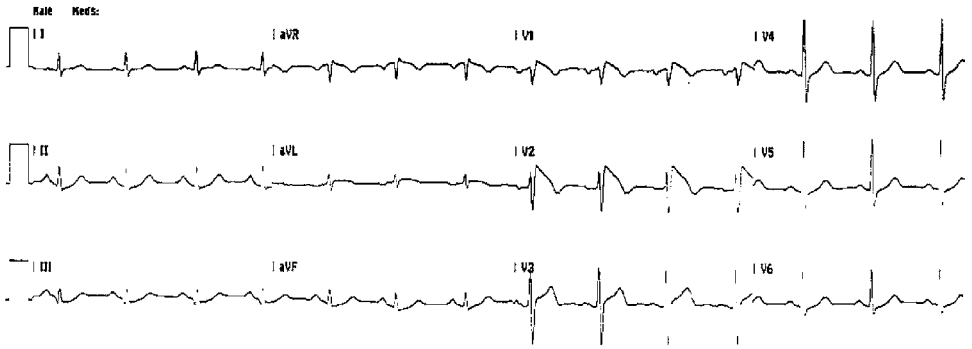

Fig. 1 Typical electrocardiogram of a patient with Brugada syndrome. Please notice the ST segment elevation in leads V1 to V3.

GENOTYPE–PHENOTYPE CORRELATION

The myocardium is a heterogeneous milieu with multiple cellular subtypes having different functional and electrophysiological properties.[4] These electrophysiological distinctions are due to variations in the expression of ionic currents, especially between epicardium and endocardium. These differences contribute to the development of ST segment elevation and the substrate for reentrant arrhythmias. Experiments involving the arterially perfused right ventricular wedge preparation have shown the epicardial action potential notch is responsible for inscription of the electrocardiographic J wave and accentuation of the notch leads to amplification of the J wave, resulting in an apparent elevation of the ST segment.[5] This has been shown in pathophysiological states such as hypothermia, where an increase in the notch in the epicardium, but not the endocardium, causes an elevation of the J point and ST segment in the electrocardiogram. If the epicardial action potential repolarizes before that of the endocardium, the T wave will remain positive and the electrocardiogram will show a saddleback type of ST segment elevation. Further accentuation of the pathophysiological state will accentuate the notch and prolong the epicardial action potential so that it repolarizes after the endocardial response, thus leading to inversion of the T wave. The result is an electrocardiogram with a coved ST segment elevation and a negative T wave as observed in Brugada syndrome shown in Fig. 1. Progressive accentuation of the notch will eventually lead to loss of the action potential dome and marked abbreviation of the epicardial response.[6] The end result will be the creation of a transmural gradient between epicardium and endocardium, and thus the substrate for reentrant arrhythmias.[7] This is the mechanism believed to induce ventricular arrhythmias and sudden death.

Experimental models of the Brugada syndrome created by exposing right ventricular wedge preparations to a variety of pharmacological agents have highlighted the importance of I_{to}. The high expression of I_{to} during phase 1 of the action potential plays a pivotal role in the electrocardiographic pattern of the Brugada syndrome. It is the balance of currents active during phase 1 that determines the degree of ST segment elevation. The use of quinidine, a class IA antiarrhythmic with action to block I_{to}, among other currents, has been shown to be capable of restoring the action potential dome in epicardium, thus normalizing the ST segment elevation.[6] It is certainly a finding which could have clinical and therapeutic relevance. There is a group who advocate the use of this antiarrhythmic to treat Brugada syndrome[8] and there has been a case report in the literature of decreased ST elevation with the use of quinidine.[9]

Sodium channel blockers also increase the notch of the epicardial action potential and thus give rise to an ST segment elevation in the wedge preparation.[6] This has also proved useful in clinical practice as a form of diagnostic tool to unmask the electrocardiographic pattern in individuals suspected of having the Brugada syndrome. Sodium blockers such as ajmaline, procainamide, flecainide, and pilsicainide are now being used as diagnostic test to identify individuals with concealed Brugada syndrome.[10]

Two intriguing questions have arisen in recent years. One is why is this electrocardiographic pattern only present in the right precordial leads, and the second is why the Brugada phenotype is so much more prevalent in males vs. females of Southeast Asian origin. In the experimental myocardial, I_{to} is much more prominent in males than in females[11] and is much greater in the right ventricle than in the left ventricle of the heart.[12]

GENETICS

The first gene responsible for the disease was identified in 1998 by Chen and coworkers.[13] This gene, the α-subunit of the cardiac sodium channel gene, *SCN5A*, is

responsible for the phase 0 of the cardiac action potential. The identification of mutations in *SCN5A* causing the disease and the decrease in availability of sodium ions indicates that a shift in the ionic balance in favor of I_{to} during phase 1 of the action potential is the determinant of the disease.[7] To date, this is the only gene linked to Brugada syndrome. *SCN5A* has been identified in approximately 25% of the patients with Brugada syndrome, indicating there is at least another gene responsible for the disease. In 2002, a second locus on chromosome 3 was identified, although the gene responsible has not been identified.[14] Close to 60 different mutations in *SCN5A* have been reported to date and approximately half of them have been biophysically characterized. The common denominator in the analysis of the mutations is the decrease in Na current availability by two main mechanisms: lack of expression of the mutant channel, or acceleration of inactivation of the channel.[15] In the case of *T1620M* mutation, the alteration in the ionic currents worsened at higher temperatures.[16] This has clinical significance as several cases of ventricular fibrillation in patients with Brugada syndrome have been precipitated during febrile states.[17–19]

Brugada syndrome is inherited as an autosomal dominant disorder and usually the disease manifests itself in individuals at risk in their 40s. However, it has also been described as causing sudden infant death syndrome (SIDS).[20] In addition to Brugada syndrome, mutations of *SCN5A* can lead to a large spectrum of phenotypes, including long-QT syndrome (LQT3),[21] isolated progressive cardiac conduction defect,[22] idiopathic ventricular fibrillation,[13] and sudden unexplained nocturnal death syndrome (SUDS or SUNDS).[23] These are all considered allelic diseases, caused by mutations in the same gene. Electrocardiographic, clinical, and biophysical data have clarified the relationship between these diseases in part.

When comparing to LQT3, Brugada syndrome could be considered a mirror image. Biophysical data indicate that LQT3 mutations cause a delayed inactivation of the channel,[21] which is exactly the opposite to Brugada syndrome, where there is an accelerated inactivation.[16] The difference between the two diseases is, however, difficult to identify in some cases, and one family was described manifesting the phenotype of both Brugada and long-QT syndromes.[24] Likewise, the line between progressive conduction disease and Brugada syndrome is closer than ever after the publication of a paper with a family displaying both diseases.[25] Whether they represent variable phenotypic expression of the same disease is difficult to ascertain. All affected family members in this family, with Brugada syndrome or conduction disease, had a mutation that proved lethal to some of its members. This certainly raises important issues regarding therapy, prevention, and risk stratification.

Recent studies have shed some light on the distinctions or lack thereof between Brugada syndrome and sudden unexpected death syndrome (SUDS) in Southeast Asia. Sudden unexpected death syndrome is very prevalent in Southeast Asia. In countries such as Thailand, it is believed to affect up to 1% of the population, and it is the most common cause of death in young males, second only to car accidents.[26] The patients commonly die at night and the male-to-female ratio is on the order of 10:1. Electrocardiographically, the disease is identical to Brugada syndrome. It is also caused by mutations in *SCN5A* and biophysical data indicate a nonworking *SCN5A* or accelerated inactivation.[23] These characteristics are similar to those of the Brugada syndrome, indicating they are the same disease.

TREATMENT

The treatment of choice is an indwelling defibrillation. The question is who should receive it. The most controversial issue is the value of electrophysiological study as an indicator of prognosis. Studies from Priori et al.,[2] Kanda et al.,[27] and Eckardt et al.[28] have not found an association between inducibility and recurrence of events. Our data of close to 700 patients indicate that EP study inducibility is prognostic of risk. The use of electrophysiological data is probably not clinically necessary in the patient who has recovered from sudden cardiac death. These patients require a defibrillator. The controversy is how aggressive to be with asymptomatic patients. There is no doubt that asymptomatic patients are also at risk. Brugada syndrome generally affects individuals in their 40s, despite the fact that the genetic predisposition is present since birth. What determines the likelihood of a patient becoming symptomatic is at present unknown. Unfortunately, all symptomatic patients were previously asymptomatic for many years. Identifying which group will become symptomatic is a critically important preventative measure. Our group has shown that asymptomatic patients who are inducible have a 14 % chance of having an event. If they are noninducible they have less than a 2% of chance of having an event. We therefore advocate the implantation of a defibrillator in asymptomatic inducible patients. The remainder require close follow-up until evidence-based data provide further guidance for risk stratification.

GENETIC COUNSELING

When studying a monogenic disease such as Brugada syndrome, it must be understood that the presence of the mutation does not always mean the person will develop

the disease (varying penetrance). Brugada syndrome is inherited as an autosomal disorder which means the disease will be present in each generation and 50% of the offspring are expected to inherit the mutant gene and thus be at risk of developing the disease. Like most autosomal dominant disorders, penetrance is age dependent tending to increase with age. Several factors remain unknown as to why onset of the disease is so age dependent. Genes continuously interact with the environment and determine the phenotype, its variability, and its age of onset. The family should undergo ECG screening and genetic testing if possible. However, it is embedded in the general population that genetics determines everything, and they expect the worse from a positive diagnosis. Genetic counseling becomes, in this issue, the most important tool to put this family back to a normal lifestyle.

SCREENING FOR THE GENETIC DEFECT

Genetic screening in countries such as the United States is performed only as a research procedure. In Europe, genetic screening has been accepted in certain countries as a routine medical procedure. It is of note that only one gene, the cardiac sodium channel, has been identified and thus may only account for a small percentage of inherited Brugada syndrome. A second locus has been identified on chromosome 3 but the gene has not been isolated. We recommend sequencing the whole of the cDNA for cardiac sodium channel. This is necessary as over 60 mutations have already been identified in the sodium channel to be responsible for Brugada syndrome. It is recommended that all of the exons be amplified from genomic DNA. The sequencing primers are available online in GenBank.

CONCLUSION

The past decade has witnessed the identification of a new clinical entity responsible for sudden death in the young and the evolution of a strategy to diagnose, risk stratify, and treat patients with this syndrome. This has been possible thanks to the efforts of many centers around the world and to the collaboration of hundreds of physicians. This is still a very new disease, with a high social impact because it involves death of relatively young individuals. Through research and continued collaboration among the basic and clinical groups involved, we look forward to advances that will enable us to better identify those at risk and provide the means to treat them more effectively, so as to reduce the burden of this disease on the affected families.

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Burkholderia pseudomallei

Sumalee Tungpradabkul
Mahidol University, Bangkok, Thailand

INTRODUCTION

Burkholderia pseudomallei is a causative agent of melioidosis, an infectious disease being recognized increasingly as an important cause of morbidity and mortality in many regions of the world. The organism is a free-living environmental saprophyte bacterium that is limited in its distribution to tropical, subtropical, and in endemic areas especially in the tropical regions of Southeast Asia and in the northern parts of Australia. The increase of worldwide travel and spreading of *B. pseudomallei* to humans and animals results in the potential for melioidosis to be found around the world. The organism is commonly found in water and soil and is usually transmitted to humans through skin abrasion or by respiratory route.[2] Infection by this organism exhibits interesting clinical features including its ability to cause severe disease, resulting in highly fatal septicemia. The incubation period can be days to many years after infection. This disease affects all age groups in both sexes[3] and could be also found in healthy person or in immunodeficient conditions such as diabetes mellitus.[4] Because this disease may be acute or chronic and can be symptomatically confused with other disease and septicemia caused by other gram-negative bacteria,[2] the most reliable and simple diagnosis is bacterial culture using selective medium. The disadvantage of using bacterial culture is that it is time consuming and the result obtained is often too late to be useful. Moreover, the method is limited to identify noncultivated bacteria and to distinguish the closely related species such as *Burkholderia cepacia*, *Burkholderia mallei*, and *Burkholderia thailandensis*, a nonvirulent species, as well. In this article, the development of diagnosis methods including the advanced knowledge of genomic and proteomic study will be therefore discussed.

BACTERIOLOGICAL CLASSIFICATION

Burkholderia, formerly called *Pseudomonas* in homology group II, was transferred to a new genus (*Burkholderia*) including *B. cepacia*, *B. pseudomallei*, *B. mallei*, and *Burkholderia pickettei* in 1992 by Yabuuchi et al.[5] using 16S rRNA sequence, DNA–DNA homology values, cellular lipid and fatty acid composition, and phenotypic character. Recently, a new species, *B. thailandensis*, is also classified in the *Burkholderia* genus.[6]

B. pseudomallei is gram-negative rod, nonacid-fast, non-spore-forming, and has bipolar flagella when stained with Sudan black. The organism is a causative agent of melioidosis. For a recent review, see Ref. [1].

B. cepacia, which is placed within the genus *Burkholderia*, has emerged as an increasingly important opportunistic pathogen, particularly in relation to patients suffering from cystic fibrosis.[7] There are several distinct biochemical characters to separate *B. cepacia* from *B. pseudomallei*. However, there are some evidences indicating the similarities of these two species such as the same fatty acid pattern and the same pH optimum for their phosphatase activity.[5] Diagnostic bacteriology concerning *B. pseudomallei* and *B. cepacia* is attracting much attention in melioidosis areas.

B. mallei is a nonmotile, gram-negative rod-shaped bacterium that stains irregularly with methylene blue.[8] In contrast to *B. pseudomallei*, growth of *B. mallei* is more slowly on nutrient agar. Like other *Burkholderia* genus, *B. mallei* is an obligate aerobe. The colonial appearance of *B. mallei* overlaps in appearance with 24-hr *B. pseudomallei* colonies and it is difficult to distinguish them. *B. mallei*, a causative agent of glanders disease, is a seriously infectious disease in equine.[8] Human glanders is rare but can be found primarily in veterinarians, horse and donkey caretakers, abattoir workers, and laboratory workers.[9] *B. mallei* and *B. pseudomallei* are very similar with respect to their nutritional, biochemical, and genetic properties, and in addition, glanders bears a striking resemblance to melioidosis both clinically and pathologically. However, they are epidemiologically dissimilar.

B. thailandensis is a new species which is found in soil isolates (environmental isolates) from the northeastern Thailand. This species was classified in the *Burkholderia* genus in 1998 by Brett et al.[6] There are some evidences which support the significant genotypic dissimilarities exhibited between this organism and the true *B. pseudomallei* strains. Although this organism is almost identical to *B. pseudomallei* in terms of morphology and antigenicity, some differences could be demonstrated. The significant differences in the sequences of 16S rRNA

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gene,[10] the exoenzyme production,[10] the flagellin gene,[11] and the hamsters virulence are seen when compared between these two species.

GENOME STRUCTURE

Recently, the complete three genomic sequences of the genus *Burkholderia* have been determined. All are virulent species—*B. cepacia*, *B. pseudomallei*, and *B. mallei*. The *B. cepacia* (the former name) or *Burkholderia cenocepacia* genome consisted of three chromosomes of 3.87, 3.22, and 0.88 Mb with a total size of 8.1 Mb. The G+C content of this genome is 66.9%. The genome of *B. pseudomallei* strain K96243 is 7.24 Mb, with a G+C content of 68.06% consisting of two chromosomes of 4.07 and 3.17 Mb.[12] These genome data have been produced by the *B. cenocepacia* and *B. pseudomallei* Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/Projects (accessed November 2003). In *B. pseudomallei* genome, there are approximately 7000 open reading frames, with 60% code for proteins of unknown function. There are no plasmids. The *B. mallei* strain ATCC23344 has a smaller genome of about 5.9 Mb and 5000 open reading frames.[13] The *B. mallei* genome data can be obtained from the Institute for Genomic Research (TIGR) Web site at http://www.tigr.org (accessed November 2003). The main differences between *B. pseudomallei* and *B. mallei* are in the cell envelope genes, which are more in *B. pseudomallei*, and in the energy metabolism genes as well. *B. pseudomallei* also has very few insertion sequences (IS) compared with *B. mallei*. This is consistent with *B. pseudomallei*, the ancestral organism of *B. mallei*, which has become an obligate pathogen.

To date, the proteomic profiles of *B. pseudomallei*, both extracellular and intracellular proteomes, are under investigation. However, many genes with coding regions have been studied and the sequences are available in the public databases.

IDENTIFICATION AND DIAGNOSIS

Bacteriological Examination

Isolation and identification of *B. pseudomallei* by cultivation remains the method of choice for definitive diagnosis of melioidosis. It is relatively simple and economical to perform. It needs neither elaborate nor expensive equipment but requires experienced personnel, particularly in the interpretation of the results. Its main drawback is that it takes at least 3–4 days to obtain the results, and by that time, it may be too late for successful management, as a high percentage of patients admitted for acute septicemia die within 24–48 hr of admission. Recently, an automatic BacT/Alert® nonradiolabeled blood culture system can be used to overcome this problem. Culture of *B. pseudomallei* was detected to be positive approximately 62% within 24 hr and 90% within 48 hr.[14] Although the use of a sensitive automated system has considerably cut down the incubation time normally required for the less sensitive semiautomatic or manual systems, its remaining drawbacks are the running cost and its availability only in large hospitals.

Some clinical specimens, such as throat swab and sputum, are generally heavily contaminated by other organisms. The use of selective media, a modified Ashdown medium containing additional chemicals such as colistin on MacConkey's agar or crystal violet, glycerol, neutral red, and gentamicin on trypticase soy agar, has been devised for isolation of *B. pseudomallei* from clinical specimens.[15] Availability of a costly API 20NE test panel has considerably simplified the identification of *B. pseudomallei*. However, the API test has been reported to misidentify for *Chromobacterium violacium* and other bacteria.[16]

However, four species—*B. pseudomallei*, *B. mallei*, *B. cepacia*, and *B. thailandensis*—are similar in many biochemical properties. To differentiate *B. pseudomallei* from *B. mallei*, motility assay is the method used to distinguish these organisms because *B. mallei* is nonmotile as a result of a lack of flagella. To distinguish *B. pseudomallei* from *B. cepacia*, the Minitek disc system was developed in 1992 as a tool for the differentiation.[17] In case of *B. thailandensis*, the ability to assimilate L-arabinose by *B. thailandensis* is used to differentiate this organism from *B. pseudomallei*.[18] At present, other specific methods based on immunology and DNA technology have been developed to use in identification and diagnostic detection of *B. pseudomallei*.

Immunological Diagnosis

Rapid and specific immunological diagnosis is used to overcome the problem of time-consuming bacteriological examinations. However, regardless of the detection of antibodies or antigens, with the immunological assays, one needs highly specific reagents. Several immunological assays have been developed and used for the detection of either *B. pseudomallei* antibodies or antigens (for review, see Ref. [19]).

Antibody detection

The first method is agglutination, which is simple but gives low sensitivity in detecting antibody to *B. pseudomallei*. In 1970, two methods, the complement fixation test (CF)

and the indirect hemagglutination test (IHA), were developed. An IHA test is still being widely used in many endemic areas of infection. One of the main drawbacks of these two assays that limit their value in clinical situations is the presence of background antibody in some healthy individuals in the endemic area. This is largely attributable to unrecognized subclinical infection in these individuals. In 1981, Ashdown had developed indirect fluorescent antibody staining (IFA) by applying immunofluorescence to detect the antibody in human serum after infection. The IgM antibody could be detected rapidly by fluorescence electroscopy. This method has been very useful for screening of melioidosis. The disadvantages of the method are the expensive microscopy and the need for skilled persons to interpret the results. Enzyme-linked immunosorbent assay (ELISA) has sensitivity, specificity, stable inexpensive reagents, and simple equipment, which is appropriate for use in determination of antibody present in melioidosis patients in developing country. This method has been performed to detect antibody to crude antigen, exotoxin, and endotoxin. Other detection methods such as gold blot have also been developed to detect IgM and IgG antibodies. However, the method has not been evaluated for their clinical usefulness in a large-scale field trial. More recently, recombinant antigens specific for *B. pseudomallei* have been produced and appeared to be highly specific and useful for the diagnosis of melioidosis.

Antigen detection

This approach is more logical and is superior to antibody detection because it indicates active disease. Many immunological methods have been developed for the detection of *B. pseudomallei* antigen, including the detection of soluble secreted product in blood and urine, and the detection of whole organisms in pus, wound, sputum, and throat swabs. The first method was undertaken by Ismail et al. in 1987, when they developed a monoclonal antibody-based assay for the quantitation of exotoxin. This assay could detect toxin in the range of 16 ng/mL of culture supernatant fluid. Similarly, in 1990, Wongratanacheewin et al. have developed a polyclonal antibody-based avidin–biotin ELISA which could detect *B. pseudomallei* antigens in the culture filtrate at a concentration as low as 4 ng/mL. However, both methods have never been evaluated in real clinical situations. More recently, a highly sensitive assay was developed for the detection of antigen, most likely the LPS at a concentration of 12.2 ng/mL, in the urine of patients with systemic and localized infection. When applied to clinical specimens, a sensitivity of 81% and a specificity of 96% have been achieved. The assay is, however, more complicated and more costly to perform and thus may not be very practical for routine use in smaller health centers with a limited health budget. Recently, another antigen-detecting assay based on the use of monoclonal antibody reactive against 200-kDa antigen secreted by *B. pseudomallei* was developed. This sandwich ELISA detection was shown to be highly reliable giving 75% sensitivity and 95% specificity. Other assay systems have been developed and evaluated including latex agglutination and immunofluorescence. The former is not sensitive enough for routine use and needs prior concentration, whereas the latter requires a fluorescent microscope that is not readily available in most laboratories in the endemic area.

Molecular Diagnosis

Because in a medical microbiological laboratory, pathogenic microorganisms particularly need to be identified under killed conditions, molecular diagnosis in different clinical specimens has been successfully used for the diagnosis of a large number of infectious diseases of man and animals and, more recently, evaluated for its reliability in melioidosis. In detection of melioidosis, the hybridization technique does not have sufficient sensitivity[20] and therefore in more recent years, the PCR approach has been more commonly performed with satisfactory results (for review, see Ref. [21]). Different sets of specific primers have been evaluated including the regions in the 23S rRNA, 16S rRNA, a junction between 16S and 23S rRNA, ribosomal protein subunit 21 (rpsU), and specific sequences designed from a specific DNA region. Using these specific primers, as few as a single bacterium present in the clinical specimens such as blood samples[21] or spleen tissue samples[22] can be successfully amplified in laboratory scale and detected with sensitivity approach to 100%. However, none of these has been subjected to critical evaluation in the real clinical situation. Because low numbers of bacteria in samples are suspected to be one of the causes of the low sensitivity of PCR, the amount of bacteria in blood of around half of septicemic melioidosis patients is beyond detection. Adding steps for concentrating the bacteria from blood could definitely improve the sensitivity.[21]

Molecular diagnosis method for discriminating among the three virulent species, *B. pseudomallei*, *B. cepacia*, and *B. mallei*, is more interesting. PCR-RFLP of the flagellin gene sequence has been applied for identification of *B. pseudomallei* and *B. cepacia* from the clinical isolates.[23] Surprisingly, the molecular system based on the flagellin gene cannot be applied for differentiation of *B. pseudomallei* and *B. mallei*,[24] although *B. mallei* is a nonmotile species. The PCR-based differentiation of the 23S rDNA sequence between *B. pseudomallei* and *B. mallei* has been reported using a species-specific primer

in which one nucleotide is substituted for a *B. mallei* template. Recently, PCR-RFLP based on a specific DNA fragment has been developed to differentiate *B. pseudomallei* and *B. mallei*.[25] Although these methods have never been evaluated in real clinical situations, these could be applied for epidemiological study and medical microbiological laboratory in identifying under kill conditions or noncultivation pathogenic bacteria as well. More recently, the PCR based on specific flagellin gene primers can be applied to epidemiological identification of *B. pseudomallei* and *B. thailandensis* and the mixed population of these two species as well.[26] The difference between the 16S rRNA gene sequences of *B. pseudomallei* and that of *B. thailandensis* is only 1%, and it is therefore not discriminative enough to distinguish the two species. The groEL gene sequence is better for distinguishing between *B. thailandensis* and *B. pseudomallei*, and the GroEL amino acid and groEL nucleotide sequences of this single gene locus may potentially be useful for a two-tier hierarchical identification of medically important *Burkholderia* at the genus and species levels.[27]

CONCLUSION

Melioidosis undoubtedly qualifies as an emerging infection because there is no doubt that it has been recognized more frequently during the past two decades, both within established endemic areas and elsewhere. This is probably largely because of greater familiarity with the organism and the disease it causes and of improvements in laboratory knowledge and facilities. Our current understanding of the molecular structure in the genome level of *B. pseudomallei* and other related species such as *B. cepacia* and *B. mallei* has also increased a possible way for rapid diagnosis. However, the culture method remains the method of choice, not only because of its reliability, but also because of its simplicity and cost. Its only drawback is that it takes up a great deal of time before a definitive diagnosis can be made. To help speed up the time needed for bacterial identification, a combination of the culture method with anyone of the immunological and molecular diagnostic methods discussed in this article could be used and results evaluated.

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Butyrylcholinesterase Gene Polymorphism

Weidong Liu
Toshikazu Hada
Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

INTRODUCTION

Human butyrylcholinesterase (BChE: EC 3.1.1.8; OMIM 177400), also called serum cholinesterase and pseudocholinesterase, is one of the enzymes routinely measured for liver function in serum. Butyrylcholinesterase is a plasma esterase that hydrolyzes acetylcholine, other choline esters, and choline ester-containing drugs such as succinylcholine. Except for agricultural chemical poisoning and hepatic dysfunction, low serum cholinesterase is usually caused by mutations in the BChE gene. To identify inherited abnormalities, both biochemical and genetic methods are used. Phenotypic and genetic polymorphism exist in the BChE gene in different ethnic groups; some of the polymorphism has been related to drug metabolism and disease occurrence. Here the detection and clinical implications of phenotypic and genetic polymorphism are described.

PHENOTYPIC POLYMORPHISM

Serum BChE activity is determined by using benzoylcholine chloride as a substrate. Phenotypes were determined by the inhibition of cholinesterase activity by 10 μmol/L dibucaine or by 50 μmol/L sodium fluoride. Established normal inhibitory values were $\geqq 77\%$ for dibucaine number (DN) and $\geqq 55\%$ for fluoride number (FN).[1] Three main BChE phenotypes have been defined: 1) the U form with normal DN and FN; 2) the A (atypical) form, less inhibited by dibucaine than the U phenotype; and 3) the F form with lower dibucaine and fluoride inhibition. They are also called ''qualitative variants.'' The quantitative variants such as the S- (silent form), H-, J-, and K-variants are the diminished numbers of effective circulating molecules and are present in approximate concentrations of 0%, 10%, 30%, and 70% of normal, respectively.[1,2]

Human BChE protein also shows a complex molecular polymorphism. Numerous forms of BChE protein can be detected by gel electrophoresis. Four main forms, named C1, C2, C3, and C4 according to their electrophoretic mobility on starch gel, have been identified. C1, C3, and C4 are monomer, dimer, and tetramer forms, respectively. The C2 component is a BChE monomer–albumin conjugate.[3] The C5 variant is considered a polymorphism in different ethnic groups and may be related to hypercholinesterasemia. Other components often observed depend on storage, partial proteolysis, and/or electrophoresis conditions.[3] Familial hypercholinesterasemia was reported with C_5 (+) or (−) band.[4,5]

GENETIC POLYMORPHISM

The human BChE gene is located on chromosome 3q26. The genomic region for BChE spans about 70 kb and has four exons and three large introns.[6] Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), PCR-restriction fragment length polymorphism (PCR-RFLP), and competitive oligo-priming-PCR (COP-PCR) were used to detect the common variants of the BChE gene.[7,8] For rare mutations, sequencing the PCR products is necessary. Usually, a DNA sample is extracted from peripheral blood, and exons 2, 3, and 4, which encode the mature protein of the BChE gene (Genbank Accession Number: J02879), are individually amplified by the PCR method. A total of 35 cycles of amplification are performed, each round consisting of a denaturation step at 94C for 1 min, an annealing step at 55C for 1 min, and an extension step at 72C for 1.5 min. After the sizes and homogeneity of the PCR products are confirmed by agarose gels stained with ethidium bromide, direct sequencing is performed using a dye terminator cycle sequencing core kit and a DNA sequencer.[9,10] The primers used for PCR and sequence are listed in Table 1.

Two genetic polymorphisms in the noncoding region of BChE were found early in 1990. At nucleotide −116, the frequency of G is 0.92 and that of A is 0.08. At nucleotide 1914, the frequency of A is 0.74 and that of G is 0.26.[11] These frequencies are considered to have no effect on BChE activity. At least 49 mutations have been reported that are associated with low serum BChE activity, including 15 truncating and 34 nontruncating mutations (Fig. 1). The genetic mutation related to the atypical phenotype is D70G. Two fluoride-resistant phenotypes

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Table 1 Primer sequences used to amplify the butyrylcholinesterase gene

Primer	Nucleotide location	Sequence (5′ to 3′)	Amplified region
AP3′	Intron 1	TAATATGCTATATGCAGAAGGC	Exon 2, locus 1
C2-1	106 to 127	ATCGAAGTCTACCAAGAGGTGG	
M-6	−7 to 15	ACATACTGAAGATGACATCATA	Exon 2, locus 2
M-115	366 to 345	TGTTCCAGTTTGAAAACCACCA	
C2-2	284 to 305	TAAATGTATGGATTCCAGCACC	Exon 2, locus 3
C2-4	607 to 586	AAGCTGCTCCTGCACTTTCTCC	
C2-3	514 to 535	CAGTTGGCTCTTCAGTGGGTTC	Exon 2, locus 4
C2-5	823 to 802	TCAGAAGAATTTCTTGGGGATC	
M-243	706 to 727	CTTTATGAAGCTAGGAACAGAA	Exon 2, locus 5
C2-7	991 to 970	CTAAAAAAGCTGTCCCTTCATC	
C2-6	910 to 931	GACATATTACTTGAACTTGGAC	Exon 2, locus 6
C2-9	1248 to1227	GGCATTATTTCCCCATTCTGAG	
C2-8	1188 to 1209	TAATTTCATATGCCCTGCCTTG	Exon 2, locus 7
AP4′	Intron 2	ACGGATCAAACCAAGCCAGAGAAC	
AP11′	Intron 2	AGCTCTGTGAACAGTGTTAGAA	Exon 3
AP12′	Intron 3	CACCGTGCCTTGGAGAGTATAC	
AP5′	Intron 3	CTGTGTAGTTAGAGAAAATGGC	Exon 4
AP6′	1844 to 1822	CCTTCTGGCATTTTTGTTTCAGC	

Primers come from Refs. [9] and [10]. Nt: nucleotide.

are F-1 for T243M and F-2 for G390V. K-, J-, and H-variants are A539T, E497V, and V142M, respectively. Other than the truncating mutations, some of the non-truncating mutations such as P100S and D170E, some compound heterozygous mutations (e.g., D70G linked to A539T), and multiple mutations (Q119X, L330I, and A539T) are attributed to silent variants (S form). Heterotypic mutations (D70G and D70H, 943–944insA, and T315S) have also been reported in the BChE gene.[1,2,7,9,12] When multiple point mutations occur simultaneously in a BChE molecule, one mutation may magnify or counteract the effect of another mutation. Therefore the phenotype of BChE cannot exactly reflect the genotype.[2,9]

The frequency of the silent BChE phenotype (S form) is estimated to be ~1/100,000 in the majority of the populations investigated, but it is present in ~1–2% in Eskimos and Vysyas from Andhra Pradesh.[12] In unselected populations in Caucasian-origin Italians, the atypical variant allele frequency is 0.009 and the K variant is 0.128, similar to some previously published data for different ethnic groups. The paper also reported that low

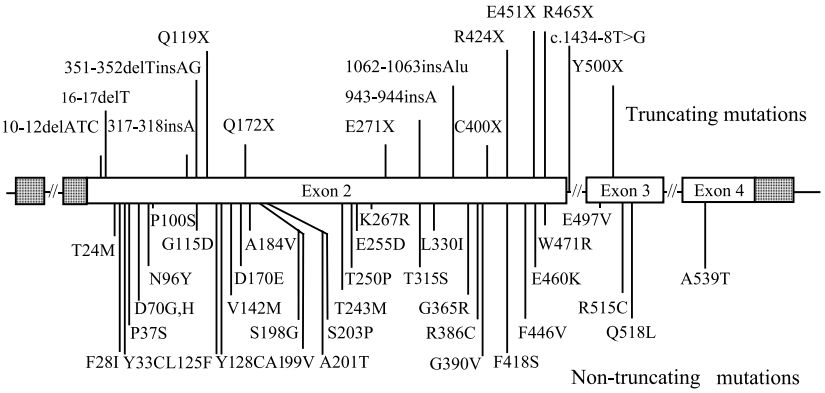

Fig. 1 Mutations found in the serum cholinesterase gene. (Mutations F28I, N96Y, 317–318insA, R386C, R424X, and E460K come from Ref. [7]. All of the others come from Ref. [2].)

serum cholinesterase is 2.2% and low FN or DN/FN is 1.1% in these peoples.[1] These values show that polymorphism is very common in normal populations.

Distinct ethnic differences in genetic mutations of the human BChE gene have been reported. The P100S, L330I, G365R, and R515C were only found in Japanese, whereas A (G70D), F-1 (T243M), F-2 (G390V), J (E490V), and H (V142M) variants existed mostly in European, American, and Australian populations.[1,2,7–13]

K-variant polymorphism has a similar high frequency (>0.1) in Americans, Chinese, Italian, Australian, and Japanese.[1,2,8] However, the K-variant was not found in Georgian and Ashkenazi Jews.[14] The K-variant is associated with Alzheimer's disease in Europeans,[6] but not in Asians such as Chinese and Japanese.[13,15] The K-variant might be a protective factor, presumably because of its reduced enzymatic activity, and the wild-type allele of BChE could be a risk factor for Alzheimer's disease.[6] Indeed, some effective therapeutic agents such as donepezil and tacrine currently used to treat Alzheimer's disease are cholinesterase inhibitors.[6]

No mutation has been found in the BChE gene in patients with hypercholinesterasemia. Some patients with hypocholinesterasemia have had no mutations found in the BChE gene. It is suggested that some other protein has affected BChE activity in the serum.

Patients with inherited a-, hypo-, or hypercholinesterasemia generally have no signs or symptoms and can lead a healthy life. Gene diagnosis is used to distinguish between hypocholinesterasemia caused by the genetic factor or liver disease. If a muscle relaxant such as succinylcholine or mivacurium is injected in patients with low serum cholinesterase, BChE activity greater than 30% of normal is necessary to metabolize the drug safely.[1] If prolonged apnea has been caused by administration of succinylcholine or mivacurium, an injection of fresh plasma or refined BChE should be used immediately. Butyrylcholinesterase may also be used as a medicine to treat poisoning caused by cocaine or organophosphorus pesticides.[16,17]

CONCLUSION

The genetic polymorphism of the BChE gene is very common in the normal population. It can be detected by PCR-SSCP, PCR-RFLP, COP-PCR, or by directly sequencing the PCR product. The phenotype of BChE cannot exactly reflect the genotype. Genetic methods can be used to distinguish inherited hypocholinesterasemia from acquired. Distinct ethnic differences exist in the BChE gene. Muscle-relaxant drugs should be used carefully with patients having low serum cholinesterase. Research on the polymorphism of the BChE gene may be useful in clarifying the mechanism and treatment of some nerve diseases.

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CADASIL

Marie Magdeleine Ruchoux
Parc EuraSanté, Lille, France

Michel Franck
Ecole Nationale Vétérinaire de Lyon, Marcy l'Etoile, France

INTRODUCTION

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited syndrome that leads to dementia. The key features of the disorder are migraine, recurrent subcortical events, mood disorders, and dementia in association with diffuse leukoaraiosis on neuroimaging. The CADASIL or *Notch3* gene encodes a transmembrane (TM) receptor exclusively expressed in vascular smooth muscle cells (VSMCs) in adult human tissues. The brain pathology is typical of subcortical vascular dementia with a diffuse leukoencephalopathy and numerous small deep infarcts. This is underlaid by a systemic angiopathy characterized by prominent VSMC alterations in humans as well as in Notch3 transgenic mice. So far, mechanisms of such VSMC destruction are not elucidated and further analysis of the *Notch3* pathway and of a more adapted animal model will provide insights into the CADASIL VSMC physiology. The next and most important step is to find preventive treatments for this dramatic condition.

CLINICAL DESCRIPTION

Four main symptoms are observed in CADASIL: migraines with aura, ischemic attacks, mood disturbances, and cognitive decline or dementia. The clinical presentation is highly variable between and within families. The age of onset varies greatly, depending on the criterion used for the onset of the disease.[1–3] Migraine attacks may begin even before age 10 years, but more commonly during the second and third decades. The most constant finding is a history of ischemic events, transient or completed, which are present in 87% of symptomatic individuals. They are often recurrent over years, leading to gait difficulties, pseudobulbar palsy, and incontinence. All subjects older than age 66 years have developed ischemic signs and all deceased subjects had suffered from strokes.[3] Mood disturbances such as severe depression of the melancholic type are present in more than 20% of cases. Rare manic episodes and psychotic disorders were found in families with a common phenotype.[3] The second most frequent feature is the development of cognitive deficits (59% of symptomatic cases), which displays a slowly progressive course with additional stepwise deterioration similar to sporadic Binswanger's disease. It happens between the ages of 40 and 70 years, with about 80% of the CADASIL patients aged over 65 years being demented. Dementia is invariably of the subcortical vascular type (SVD), with apragmatism, apathy, and memory impairments, whereas the patients perform relatively well in the routine Mini Mental Status Examination (MMSE). Epileptic seizures occur in about 6–10% of the patients. Routine laboratory examinations are usually noncontributory.

The differential diagnosis of CADASIL is broad. Before their final diagnosis, many of the patients are diagnosed as having multiple sclerosis, cerebral vasculitis, Binswanger's disease, leukoencephalopathy of indeterminate cause, or Alzheimer's disease.[1–5]

NEUROIMAGING

The two key neuroimaging features in CADASIL are multiple focal lacunar infarcts and diffuse white matter (WM) T2-weighted (T2w) hyperintensities observed with magnetic resonance imaging (MRI) (Fig. 1A and B) corresponding to the diffuse rarefaction of WM or leukoaraiosis on computed tomography (CT) scanning.[3] MRI is an essential tool for the CADASIL genetic study because neuroimaging penetrance of CADASIL is complete or almost complete between the ages of 30 and 40 years.[4] Changes highly suggestive of CADASIL diagnosis were already detected in asymptomatic mutation carriers, even in an 18-year-old CADASIL subject.[3] Areas of high T2w and fluid attenuated inversion recovery (FLAIR) signal intensity within the anterior temporal and paramedian superior frontal WM are useful radiological signs that help to differentiate CADASIL from subcortical arteriosclerotic encephalopathy.[4] Conversely, the absence of periventricular hyperintensities in practice excludes the diagnosis of CADASIL.[4]

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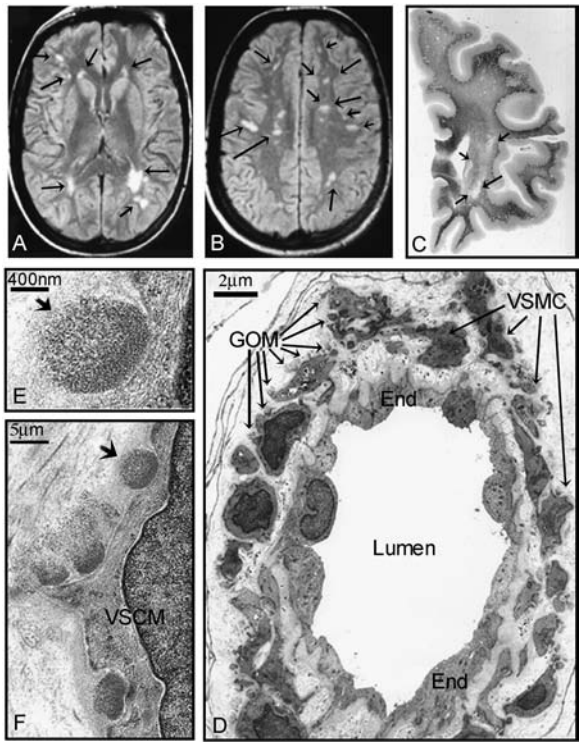

Fig. 1 (A and B) A 47-year-old patient's MRI showing diffuse hyperintense foci of white matter in T2w images. (Courtesy of Dr. C. Lucas.) (C) Brain coronal section presenting diffuse myelin pallor and some lacunes (arrow) (Luxol; Kluver–Barrera method). (D) Skin arteriole. Enlarged lumen surrounded with endothelium (end) widely separated from VSMCs. Irregular-shaped VSMCs, in part destroyed and surrounded with numerous GOMs (arrow; ×1670). (E and F) GOMs within VSMC infoldings (E, ×27,800; F, ×12,930).

PATHOLOGY

Pathological findings on autopsy of CADASIL patients include multiple small, deep infarcts in WM, deep gray matter in the brainstem, and a diffuse myelin loss in the hemispheric WM (Fig. 1C). The cortex, cerebellum, and other tissues seem well preserved. Histological sections show a widespread angiopathy first described in small- and medium-sized brain arteries.[3] Finally, lesions occur throughout the systemic blood vasculature including capillaries and veins.[5]

This angiopathy is characterized by a slight to marked thickening of the wall depending on the localization, destruction of VSMCs, and wall accumulation of eosinophilic smudgy material, which is periodic acid Schiff-positive (PAS^+) but negative in Congo red staining, ruling out the diagnosis of amyloid angiopathy.[3,5,6] The ultrastructural CADASIL hallmark is a granular osmiophilic material (GOM) without filament-like profiles[5,6] surrounding VSMCs and often lodging within VSMC infoldings (Fig. 1D–F). So far, the GOM nature is not known. An important breakthrough in understanding CADASIL vascular wall changes is the finding that the Notch3 protein expression is restricted to VSMCs[7] and that its extracellular domain (NECD) accumulates within CADASIL patients' vessel walls, in close vicinity to GOMs and can be visualized immunohistochemically.[7] Given the uncertain specificity and sensitivity of this technique,[8] GOMs observed in skin biopsies continue to remain a diagnostic tool.[3,5,6] Moreover, it appears to be detectable early, before age 20 years in skin vessels.[3] Nevertheless, we did not find

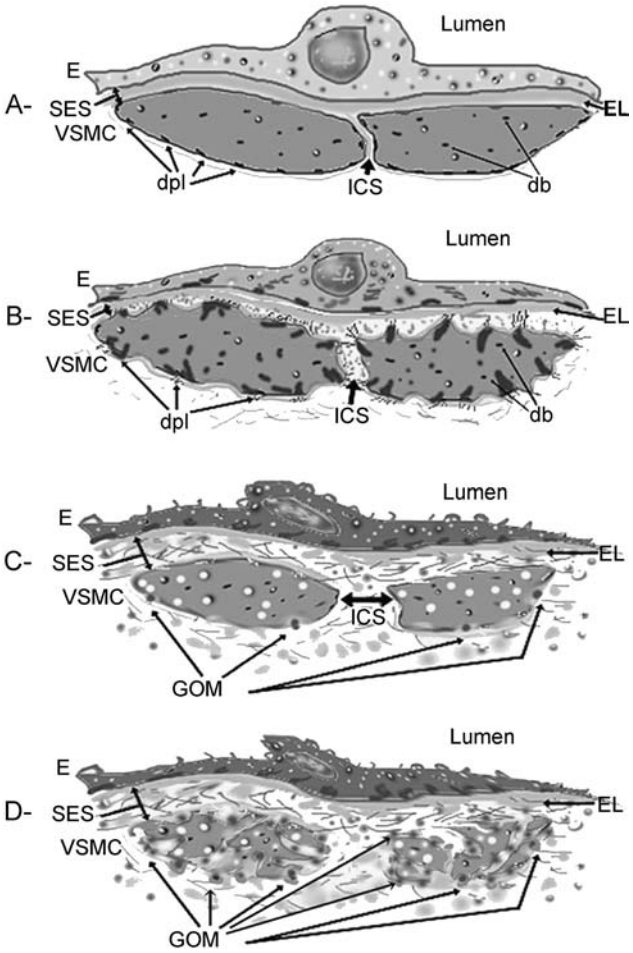

Fig. 2 Schematic representation of vascular changes in CADASIL patients and transgenic mice. (A) Normal vessel wall. (B) First modifications observed within the vessel wall of young CADASIL patients (8 years old) and of young mice before 10 months. (C and D) Evolution of VSMC alterations with a dramatic separation of the different vessel wall cells and the presence of GOM (arrow) observed on and after 20 years in humans and 14 months in transgenic mice. E, endothelium; SES, subendothelial space; EL, elastic lamina; dpl, dense plaque; db, dense core; ICS, intercellular space.

GOM in very young patients (8 years old), whereas VSMC alterations were already present, suggesting that VSMCs are the target of Notch3 mutations. In a study of 50 CADASIL cases, Brulin et al.[9] confirmed that skin VSMC alterations begin early; since age of 20 years, VSMCs are already isolated, irregular-shaped, and surrounded with an increased extracellular matrix, which seems to just replace the VSMC destruction (Figs. 1D and 2B and C). VSMC destructions increase with the patient's age. Finally, vessel wall VSMCs are barely recognizable at 60 years (Fig. 2D) Interestingly, no real stenosis (lumen diameter decreasing) is found compared with 20 younger controls even though vessel walls are thickened. These findings are also observed in brain tissues from five CADASIL cases.[9]

GENETICS

Genetic linkage analysis and DNA[10] sequencing have shown that CADASIL is caused by mutations in the human *Notch3* gene at locus chromosome 19p13.[11]

Notch3 belongs to the family of highly conserved Notch/LIN-12 receptors, which includes four members in vertebrates.[12] This gene has 33 exons and encodes for a TM receptor that is a component of an intercellular signaling pathway essential for controlling cell fate during development in a wide range of organisms, including insects, nematodes, and mammals.[13] The Notch3 molecule is synthesized as a 2321-amino acid precursor containing all typical Notch motifs, namely, an extracellular region exhibiting a signal sequence and an extracellular domain (NECD) with 34-tandem epidermal growth factor (EGF)-like repeats followed by three Notch/LIN-12 repeats, a TM segment, and an intracellular domain (NICD) containing six cdc10/ankyrin repeats flanked by two nuclear localization signals (Fig. 3).[12,13] During maturation and activation, Notch3 undergoes three proteolytic cleavages (Fig. 3). The first cleavage (S_1) occurs in the Golgi apparatus where a furine-like convertase cleaves Notch between its NCID and its NECD. After the reappearance of these two domains, they bind to each other to generate a heterodimeric receptor. At

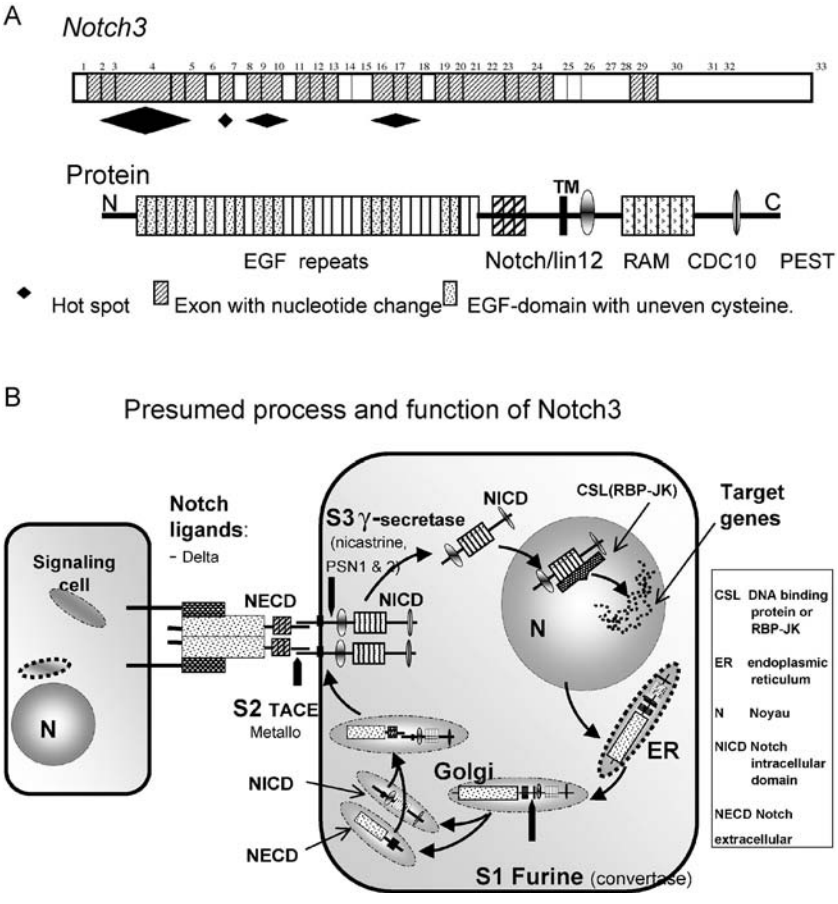

Fig. 3 (A) Preferential location of mutations on *Notch3* EGF repeats involved in CADASIL Notch3 protein. (B) Schematic representation of the three cleavages (S_1, S_2, and S_3) that occur during Notch3 maturation.

the cell surface, the receptor interacts with ligands, Jagged-1 or Delta-1, which are TM proteins expressed to juxtaposed cells.[12] Ligand interaction leads to a second cleavage (S_2) of the receptor. A metalloprotease ADAM-17, also called TNFα-converting enzyme (TACE), cleaves the NECD, precisely 12 amino acids outside of the plasma membrane. This cleavage releases the NECD to the extracellular space. The third cleavage is the result of the γ-secretase complex formed by nicastrine, preseniline1, and preseniline2. This last cleavage releases the NCID, which translocates to the nucleus, where it regulates transcription associated with a DNA-binding protein CSL (RBP-Jk) (Fig. 3).[12]

Molecular screening of all 33 coding exons within a large panel of CADASIL patients revealed that most of the mutations are missense mutations;[11] other mutations including splice site mutations and deletions, which have been identified in a few patients, lead to small in-frame deletions.[14] All the mutations are located within the EGF-like repeats of the NECD, with a strong clustering within exons 3 and 4, which encode the first five EGF repeats (Fig. 3).[15] CADASIL mutations are highly stereotyped, leading to an odd number of cysteine residues within an EGF-like repeat.[15]

GENOTYPE–PHENOTYPE CORRELATION

No correlations are found between a mutation and a clinical phenotype because a family unique presentation such as migraine or stroke is also found with several other mutations.[2,3] Additionally, we did not notice neither different clinical presentations nor different skin vessel wall lesions in CADASIL patients with mutations located either in the first or very last exons. Interestingly, a homozygous patient followed in Finland was indistinguishable when compared with an age-matched heterozygous patient with the same R133C Notch3 mutation. The double dose of gene defect does not appear to aggravate the symptoms, which indicates that either mutated Notch3 receptors retain some of their function, or other molecules can compensate for the loss.[16]

MANAGEMENT AND GENETIC COUNSELING

The broad variety of CADASIL symptoms in affected families often leads to its underdiagnosis and misdiagnosis. A truly negative family history by no means excludes this diagnosis because a patient with a de novo Notch3 mutation has been reported.[17] Consequently, patients presenting with neurological deficits, leukoaraiosis, and a positive family history will benefit from a mutation analysis of hotspot 3–4–5–11EGF-encoding exons. In case of negative result, a skin biopsy is performed for ultrastructural and immunohistochemical studies. A positive result leads to a mutation analysis of all EGF-encoding exons and then to a mutation screening of remaining *Notch3*.

ANIMAL MODEL

Similarities were found between *Drosophila*-lethal Abruptex and human CADASIL because they are precisely analogous at the molecular level and both are genetically dominant. This model was proposed to provide more general insights into the VSMC biology because its mutations also lead to an odd number of NECD cysteine.[18] Recently, transgenic mice in which the SM22α promoter drove the expression of the full-length human Notch3 with the Arg90Cys mutation (one CADASIL archetypal mutation) were generated.[19] These A90C-Tg mice showed GOM deposits, NECD accumulation, and evidence of VSMC degeneration strikingly similar to those observed in CADASIL patients. Vessel changes were systemic but more prominent in the tail arteries. Time course analysis of vessel changes revealed that VSMC damages were present before NECD and GOM accumulation occurred (Fig. 2B and C), and that the first ultrastructural findings were the disruption of normal VSMC anchorages to adjacent extracellular matrix and cells (Fig. 2B). Unfortunately, these A90C-Tg mice showed no evidence of brain damage; nevertheless, they modeled the early stage of the human disease before onset of clinical symptoms. One knows the difficulty in predicting the accuracy with which animals will model a corresponding human disease. The mouse with its short life expectancy and its brain anatomy is actually far from humans. SVD model requires animals with brain macroscopically similar to that of humans. In the future, these facts must be taken into account in studying diseases similar to those of humans.

PATHOGENESIS

In the absence of stenosis and occlusions in CADASIL vasculature, hemodynamic troubles need to be explained in the light of typical vessel changes. The extensive destruction of VSMCs and pericytes suggests that hypotonicity may play a more important role in the pathogenesis of cerebral ischemia than vascular occlusion. Several studies, employing different specialized imaging techniques, have indicated a reduced baseline cerebral blood flow and a reduced cerebrovascular reactivity in CADASIL patients and in asymptomatic mutation carriers.[20–22] Moreover, diffusion tensor MRI and proton

MR spectroscopy studies indicate that cerebral tissue damage is much more widespread than is evident on conventional MRI.[21,22] It correlates with previous pathological observations, showing a diffuse angiopathy as severe in WM as in the cortex[5,6] but with different consequences depending on the vascular density.[5,6] Interestingly, the dependence of endothelial permeability on the presence of pericytes was recently demonstrated.[23] Pericyte alteration and destruction observed in CADASIL probably result in a decrease in permeability because VSMCs and pericytes secrete vascular endothelial growth factor (VEGF), a growth factor that is also the most potent permeability factor.[24] Therefore, leukoaraiosis could be the result of both oligemia and decreased permeability. The latter could explain why the only tissues affected are those depending on the blood–brain barrier because all the exchanges rely on a perfectly working capillary bed.

Now, the utmost question is: On the molecular and cellular levels, what sequence of events leads from Notch3 mutation to the characteristic VSMC alterations? Recent data suggest that trafficking and localizing, rather than signaling, of Notch3 are affected: cultured human embryonic kidney cell lines expressing mutant *Notch3*^{R142C} showed a higher propensity to form intracellular aggregates.[13] On the opposite, the link between this cleavage product and the VSMC destruction is not explained.

CONCLUSION

So far, over 500 families have been identified worldwide in many different ethnic groups.[3] With the awareness of the hereditary aspects of CADASIL and Notch3 mutation analysis, special care and management of CADASIL patients are improving and genetic counseling is being provided to patients and their families. In the absence of preventive therapeutics, the combined efforts of research groups are focused on finding an animal model presenting brain parenchyma damage comparable to that of humans. This could be the key to unveiling the sequence of events leading from mutant gene to VSMC destruction and, in the future, could function as a model for therapeutic testing.

In the SVD field, the identification of CADASIL as a hereditary systemic vascular disease represents a paradigm for research, and the significance of the findings goes well beyond the condition itself because new disease identifications are likely to be in progress.

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Calicivirus

C. Anthony Hart
A. D. Radford
University of Liverpool, Liverpool, U.K.

INTRODUCTION

The human caliciviruses have recently been subdivided into the *Norovirus* and the *Sapovirus* genera. Norwalk agent was the first human viral enteropathogen to be described. It was designated the cause of ''Winter vomiting disease'' and named after Norwalk, Ohio, USA, in 1972. They do not have a classic calicivirus morphology on electron microscopy and were also referred to as small round structured viruses (SRSV). The sapoviruses are named after a virus found in Sapporo, Japan, in 1982. They have the classical cup-shaped depressions (calyx is Greek for a cup) in their surface when viewed by negative stain electron microscopy. The noroviruses are an important cause of vomiting with or without diarrhea and are responsible for large outbreaks of disease in the community, in hospitals, or on cruise liners. After rotavirus they are the most important etiological agents of gastroenteritis. The sapoviruses cause mild diarrheal disease predominantly in children.

THE VIRUSES

The family Caliciviridae is a group of small (27–40 nm) unenveloped round viruses with a linear, positive strand, unsegmented RNA genome.[1] The family includes vesiviruses that infect cats (feline calicivirus) and pigs (vesicular exanthema of swine), the lagoviruses of hares and rabbits (rabbit hemorrhagic disease), and the human caliciviruses (genera: *Norovirus* and *Sapovirus*). Hepatitis E virus has been removed from the family Caliciviridae and is currently of ''unclassified status.''[2]

Noroviruses (prototype strains Hu/NLV/Norwalk virus/8FIIa/1968/95) are 38 nm in diameter by electron cryomicroscopy (Table 1). By negative stain electron microscopy they are 27–32 nm in diameter with a feathered edge (Fig. 1). The capsid is made from a single major capsid protein that can self-assemble in the absence of viral RNA to form viruslike particles (VLP). Most of our information on virus morphology comes from VLPs. The capsid comprises 90 dimers of the capsid protein which form particles with T-3 icosahedral symmetry. There are two major capsid domains: the shell (S) and protruding (P) arm. The P-domains form archlike structures that give the virus its feathery outline. The S-domain forms a β-barrel and is at the N-terminus. The P-domain is the carboxy half of the protein and shows sequence variability. It is divided into the P1 [amino acids (αα) 226–278 and αα 406–520] and the P2 (αα 279–405) subdomains. The P2 subdomain shows the highest sequence variability. The P1 subdomain forms the sides of the archlike structure and the P2 subdomain is at the top of the arch. P2 is a major antigenic site and is involved in receptor binding.

It has thus far proved impossible to maintain noroviruses (or sapoviruses) in artificial culture so knowledge of the genome is derived from analysis of cDNA copies of virus genome extracted from human stool samples and by comparison with the caliciviruses that are easier to grow [feline calicivirus (FCV) and vesicular exanthema of swine virus (VESV)] in culture. The norovirus genome is a positive sense polyadenylated single-stranded RNA of around 7.6 kb. It is organized as three open reading frames (ORF). The first ORF at the 5′ end encodes a large nonstructural polyprotein of predicted mass 193.5 kDa or 1738 (αα). This contains motifs with similarity to the helicase, serine protease, and RNA-dependent RNA polymerase of picornaviruses, and is thought to be post-translationally cleaved by proteolysis to the individual enzymes. ORF2 encodes a protein of 533 αα, which is the capsid protein, and can self-assemble to form VLPs when expressed using a baculovirus vector in insect cells.[3] At the 3′ end ORF3 encodes a small (212 αα) basic protein, which is a minor structural protein.[4]

The sapoviruses (prototype strain Hu/SLV/Sapporovirus/1982/JR) are of similar size to noroviruses but have a much more distinct morphology. They have 32 cup-shaped depressions at each of the fivefold and threefold axes. These lead to their characteristic ''Star of David'' appearance (Fig. 2) on negative stain electron microscopy. The genome of sapovirus Manchester is slightly shorter than that of noroviruses at 7.4 kb, and it is arranged differently. ORF1 encodes both the nonstructural proteins and the capsid protein, which is found in-frame at the end of the nonstructural ORF.[5] ORF2 encodes a basic protein

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Table 1 Norovirus genogroups

Genogroup	Viruses and hosts
I	*Humans*: GI-1 (Norwalk), GI-2 (Southampton), GI-3 (Desert Storm), GI-4 (New Orleans, Chiba), CI-5 (Apalachicola Bay, Musgrove), GI-6 (Hesse)
II	*Humans*: GII-1 (Hawaii, Wortley), GII-2 (Melksham), GII-3 (Toronto), GII-4 (Bristol), GII-5 (Hillingdon, White River), GII-6 (Seacroft, Florida), GII-7 (Leeds, Gwynedd), GII-3 (Idaho Falls), GII-n (Erfurt). *Pigs*: SW/NLV/SW43/1992/JP
III	*Cattle*: GIII-1 (Jena)
IV	*Humans*: GIV-1 (Fort Lauderdale, Alphatron)
V	*Mice*: GV-1 (murine norovirus)

similar to that of ORF3 of noroviruses. There is another potential ORF within the capsid gene that might encode a further basic protein of unknown function.

VIRAL VARIABILITY

Both genomic and antigenic analyses have shown that the noroviruses are highly variable. They are divided into five major genogroups within which are clustered a number of genotypes on the basis of sequence variations in polymerase and capsid genes. Most human infections are due to genotypes I and II with genogroup II being responsible for the majority (73%) of outbreaks of disease.[6] Genotype IV viruses have also been detected in humans, but their role in disease is unclear. Genogroup II noroviruses have recently been detected in pigs,[7] but so far genogroup III viruses are restricted to cattle, and the recently described genogroup V norovirus was found in a mouse.[8] However, the genome is constantly mutating and within each genogroup and genotype variants can be found. There is even evidence for mutation of a genogroup II norovirus from a heart transplant patient with chronic diarrhea lasting over 2 years.[9] The mutations tend to accumulate in the P2 region of the capsid protein, which is a major target for the immune response. There are fewer studies of sapovirus variability. Studies based on part of the RNA polymerase (278–286 nucleotides) and capsid genes have divided sapoviruses into five genogroups. As with the noroviruses there are a number of genotypes within the genogroups and increasing evidence of great variability.[10]

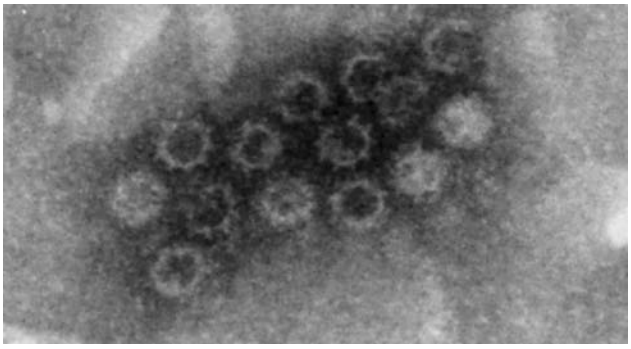

Fig. 1 Negative stain electron micrograph of noroviruses showing their ''feathery edge.''

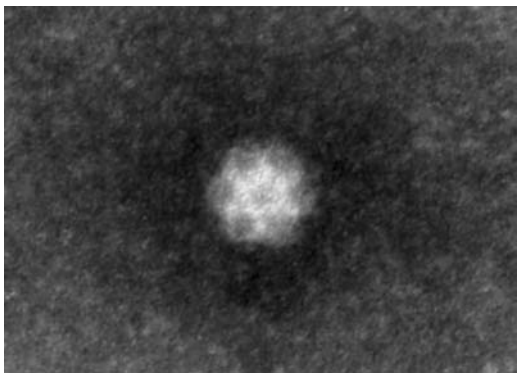

Fig. 2 Negative stain electronmicrograph of a sapovirus demonstrating cup-shaped depressions and the classic ''Star of David'' appearance.

REPLICATION, PATHOGENESIS, AND IMMUNITY

Neither human noroviruses nor sapoviruses have been cultured in cell lines or intestinal organ culture so little is known of their replication in vivo or in vitro.

Both viruses are excreted in feces and vomitus, and infection is acquired by ingestion or even inhalation of aerosolized vomit. The incubation period ranges from 10 to 51 hr with a mean of 24 hr in volunteers.[11] The site of infection in the gastrointestinal tract is unknown but from the short incubation period it is likely to include the jejunum.[12] The receptor(s) for noroviruses appear to be the ABH histo-blood group antigens and heparan sulfate

proteoglycans.[13] How human caliciviruses cause disease expression is not known. Although it was previously thought that virus shedding was short-lived, there is increasing evidence for virus excretion before onset of disease and for several weeks after clinical recovery.[11,14,15] Immunity to human caliciviruses is poorly understood. In challenge experiments, six volunteers who developed gastroenteritis after ingesting Norwalk virus developed disease when challenged again with the same virus 27–42 months later, despite a brisk antibody response.[11] There does, however, appear to be a correlation between antisapovirus antibodies and immunity to infection.

EPIDEMIOLOGY

The human caliciviruses have a worldwide distribution being detected in every country they have been sought.[11] In a community-based study in Holland 20% of cases of gastroenteritis were due to noroviruses and 6% to sapoviruses.[15] Noroviruses were detected in all age groups whereas sapoviruses were found only in children with 19% of cases being found in those under 6 months.[15] Seroprevalence studies in developed countries indicate that antibodies to noroviruses are acquired gradually in childhood, and by adulthood 50% are seropositive.[11] In contrast, in some developing countries norovirus seropositivity reaches 100% by 4 years of age.[11] Virtually all adults have antibody to sapoviruses and in Kenya most children are seropositive by 2 years of age.[11] Sapovirus infections appear to be endemic particularly in infants and, in temperate countries, predominate in the cooler winter months often before the rotavirus season. In contrast, noroviruses, while also able to cause sporadic disease, are frequently associated with explosive outbreaks of disease being responsible for up to 80% of outbreaks. These can be community-wide but cause particular problems in health-care institutions such as hospitals and nursing homes. Outbreaks on holiday cruise ships attract great publicity.[16] Finally, food- and waterborne outbreaks occur frequently. Shellfish such as oysters and mussels, which are filter feeders and concentrate noroviruses discharged into the sea in human excreta, are particularly associated with outbreaks. However, other foodstuffs including raspberries and delicatessen food contaminated at source or during preparation also precipitate outbreaks.[14,17] There is recent evidence for a global epidemic of infection with a single GII-4 norovirus which was detected in 55% of unrelated outbreaks in the United States and in seven countries on five continents.[18] Its emergence was related to an increase in the prevalence of norovirus gastroenteritis.

THE DISEASE

In general, norovirus infection is associated with sudden onset of nausea and vomiting that is severe, projectile, and frequent. However, a significant proportion of patients report diarrhea, abdominal cramps, headache, myalgia, and pyrexia.[11,15,16] In the elderly, however, disease can be severe and cause death. In a study of diarrheal disease in the United Kingdom there were 43 deaths in 38 outbreaks in hospitals or elderly care facilities,[19] and in the United States norovirus is estimated to account for 7% of fatalities due to foodborne disease.[20] Disease manifestation persists for a median of 5 days with diarrhea persisting for a median of 4 days. The median duration of illness decreased with age.[15] Chronic diarrhea can occur in the immunocompromised.[9] Sapovirus gastroenteritis is more often associated with diarrhea than vomiting; however, nausea and vomiting precede diarrhea in a proportion of cases. Disease persists for a median of 6 days.

DIAGNOSIS

This can be by visualization of the virus by electron microscopy, detection of antigens, detection of genome by RT-PCR, or detection of specific antibody. Electron microscopy is how the viruses were first detected; however, the sensitivity of the test is low. Feces must contain at least 10^6 virus particles per milliliter. The virus is small and detection requires an experienced electron microscopist. Finally, the quantity of virus excreted drops rapidly below detectable levels after onset of disease. Antigen detection has used immune adherence hemagglutination, radioimmunoassay, and enzyme immunoassay (EIA) formats.[1] Enzyme immunoassays for antigen detection are now available commercially. However, they are neither highly specific nor sensitive at detecting all genogroups of noroviruses.[21] There are no commercially available antigen capture EIAs for sapoviruses. Genome detection by reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive and specific method available for detection of human caliciviruses.[1,22,23] However, selection of the correct primers is of major importance. Even relatively well-conserved regions of the genome such as the helicase or polymerase regions have nucleotide identities as low as 36% between genera and genotypes.[1] However, broadly reactive primer sets targeting the RNA polymerase of circulating noro- and sapoviruses have been designed which perform well[22] but may cross-react with the polymerase of other viruses.[24] It may therefore be necessary to screen for the two genera separately. A highly sensitive broadly based quantitative RT-PCR has recently been

Table 2 Examples of primers used for the detection of human calicivirus from feces

Primer	Sense	Sequence	Amplicon size
Norwalk-like viruses[25]			
JV_{12}	Outer+	5′ATA CCA CTA TGA TGC AGA TTA 3′	333 bp
SM_{31}	Outer−	5′CGA TTT CAT CAT CAC CAT A 3′	
N_1	Inner+	5′ GAA TTC CAT CGC CCA CTG GCT 3′	114 bp
E_3	Inner−	5′ ATC TCA TCA TCA CCA TA-3′	
(A nested RT-PCR)			
Sapporo-like viruses[26]			
Sapp 36	+	5′-GTT CGT GTT GGC ATT AAC A-3′	470 bp
Sapp 35	−	5′-GCA GTG GGT TTG AGA CCA AAG-3′	
Both NLV and SLV[22]			
P289	−	5′-TGA CAA TGT AAT CAT CAC CAT A-3′	319 bp (NLV), 339 bp (SLV)
P290	+	5′-GAT TAC TCC AAG TGG GAC TCC AC-3′	

devised for noroviruses.[23] Some primer sets used to detect the human caliciviruses are shown in Table 2. It is likely that new primer sets will be required as our understanding of the range and variability of human caliciviruses expands. Antibody detection is used only for epidemiological purposes and numerous different antigens are needed.

TREATMENT AND PREVENTION

There is no specific treatment nor method of prevention of infection. Viruslike particles stimulate specific immune responses and are currently being tested for efficacy in vaccine.[11,15,27] In the face of outbreaks the only methods of prevention are isolation of cases and use of disinfectants.

CONCLUSION

Noroviruses are the most frequent causes of outbreaks of gastroenteritis. They are genetically and antigenically highly diverse and are able to mutate in a human host. Their major disease manifestation is vomiting. Sapoviruses tend to cause mild diarrhea predominantly in children.

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Campylobacter spp.

Kieran Jordan
Caroline Murphy
Teagasc, Moorepark, Fermoy, Co. Cork, Ireland

Cyril Carroll
National University of Ireland, Galway, Ireland

INTRODUCTION

Campylobacter spp. are the most common cause of bacterial foodborne illness worldwide. Despite this, they are poorly understood in terms of growth, survival, and pathogenesis. The recent publication of the genome sequence for *Campylobacter jejuni* NCTC11168 presents an opportunity to advance our knowledge of this organism, its detection, its mechanisms of survival, and its pathogenesis. Therefore, the application of genomics and proteomics may answer important questions about *Campylobacter* spp. and contribute to the development of effective control procedures that will reduce the incidence of *Campylobacter*-related illness.

THE GENUS *CAMPYLOBACTER*

Members of the genus *Campylobacter* are gram-negative, nonspore-forming, motile-curved, or S-shaped spiral cells (0.5–0.8 μm long and 0.2–0.5 μm wide) during logarithmic growth. In stationary phase cultures or on exposure to air or adverse conditions, the cell morphology changes to coccoid, even in the absence of de novo protein synthesis. Although there are 16 members of the genus identified to date, those associated with human disease are *C. jejuni*, *Campylobacter coli*, *Campylobacter lari*, and *Campylobacter upsaliensis*, and it is these species that will be primarily considered. Optimum growth occurs in a microaerobic environment of 5% oxygen, 10% carbon dioxide, and 85% nitrogen. Iron is an essential nutrient, but intracellular iron homeostasis must be maintained, because iron in combination with oxygen leads to the generation of toxic reactive oxygen species.[1] *Campylobacter* species may grow over a wide spectrum of temperatures ranging from 25°C to 42°C.[2] With regard to survival in a hostile environment, *Campylobacter* spp. can enter a viable but nonculturable (VNC) state; recovery from that state is variable and may be due to strain differences.[3]

PATHOGENESIS

Campylobacteriosis is the leading cause of foodborne illness worldwide. The clinical manifestations of the disease are very diverse, ranging from mild, noninflammatory watery diarrhea to more severe inflammatory diarrhea with abdominal cramps. The incubation time can be 1–7 days, and although severe illness can last more than a week, the disease is generally self-limiting and complications are rare. Antibiotics may be used in such clinical circumstances as high fever, bloody stools, prolonged illness with symptoms lasting more than a week, pregnancy, infection with human immunodeficiency virus (HIV), and other immunocompromised states, although they are not generally required. Erythromycin is the drug of choice, and resistance to it remains relatively low following decades of use.[4] It is estimated that about 1 in 1000 cases of campylobacteriosis results in the neurological disorder Guillain–Barré syndrome.[5] Another related neurological disorder, Miller–Fisher syndrome, is also associated with *C. jejuni*.

Campylobacter spp. colonize the gastrointestinal tract of animals that act as a vehicle of transmission. In the human host, they colonize the ileum and colon, with colonization being facilitated by both the spiral shape (which helps cells to corkscrew their way through the mucous) of the cell and bacterial motility. The mechanisms by which *Campylobacter* spp. cause disease are poorly understood, but involve toxin production and/or damaging of the host epithelial cells by invasion, or inflammatory response. *Campylobacter* toxins play a significant role in pathogenesis and, recently, cytolethal distending toxins (CDTs) of *C. jejuni* have been shown to be of particular significance.[6] CDTs have a mode of action different from other toxins (e.g., enterotoxin), which is not fully understood. The most important virulence factor appears to be the flagellum. Chemotaxis toward mucin, adherence, iron metabolism, invasion, and intracellular survival are other important virulence factors. Given the large number of genes probably involved in virulence, effective regulation of virulence is essential.

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The most important virulence regulators appear to be the ferric uptake regulator, two-component regulatory systems, and *fla* gene regulation.[7]

EPIDEMIOLOGY AND TYPING

Although the origin of a particular *Campylobacter* infection is rarely traced, transmission probably occurs via fecal contamination. Other sources of *Campylobacter* infection include unpasteurized milk, water, and pet contact. The infective dose is low, being about 800 cells ingested.

Campylobacter species represent a taxonomically heterogeneous group and many typing methods have been used to study the epidemiology of *Campylobacter* infections. These include biotyping, serotyping, phage typing, auxotyping, ribotyping, multilocus enzyme electrophoresis, amplified fragment length polymorphism (AFLP), and pulse field gel electrophoresis (PFGE). Biotyping campylobacters is of limited value as strains have relatively fastidious growth requirements and only a few biochemical tests give adequate discrimination. Two serotyping methods are most often used: the Penner scheme and the Lior scheme. These serotyping methods are primarily used by a few reference laboratories because of the time and expenses involved in maintaining good-quality antisera.

DNA-based typing methods have advantages over phenotypic-based methods in that they are faster, more discriminatory, and widely available.[8] Each method has advantages and disadvantages, but for its speed and simplicity, typing of the flagellin gene (*fla* typing) has been widely used. The primers A1 (5′-GGA TTT CGT ATT AAC ACA AAT GGT GC-3′; forward) and A2 (5′-CTG TAG TAA TCT TAA AAC ATT TTG; reverse), followed by digestion with *Dde*I,[9] are the most widely used methods. This has been used in efforts toward interlaboratory standardization.[10] PFGE, based on standardized protocols, has also been used for subtyping *C. jejuni* strains in PulseNet laboratories and is, at present, considered the gel standard genotyping method.[11]

The *Campylobacter* genome contains highly variable ''plastic'' regions, which gives some strains the ability to disguise themselves and avoid the host immune system. This plasticity can distort the results of genotyping methods for *Campylobacter* spp.

ISOLATION AND DETECTION

There are many culture-based methods for the isolation and detection of *Campylobacter* spp. These include basal media such as Colombia, Brucella, and *Campylobacter* Blood-Free Selective Medium. The addition of antibiotic supplements (such as Bolton, Skirrow, or Preston) as selective agents and blood or charcoal (to quench toxic compounds or to prevent accumulation of photochemically generated oxygen derivatives) is necessary to improve isolation. Other media such as *Campylobacter* cefoperazone deoxycholate selective agar (CCDA) contain the antifungal agent amphotericin B and the broad-spectrum antibiotic cefoperazone, which inhibits both yeast and fungi. The medium and incubation temperature used can influence the genotype of *Campylobacter* spp. detected,[12] rate of survival (Murphy et al., unpublished data), oxygen tolerance,[13] and percentage isolation.[14] Using culture-based methods, presumptive results can be obtained in 2–3 days, whereas definitive results can take 3–5 days.

The enrichment of samples, combined with a direct method of detection, is frequently used to enumerate *Campylobacter* spp., especially where cell numbers are low.[15] The use of a resuscitation incubation period at 37°C facilitates the recovery of injured cells. The incubation temperature of 42°C, which is used routinely for the isolation of thermotolerant *Campylobacter* spp., can inhibit many nonthermotolerant species associated with gastroenteritis.

Genetic-based methods of detection offer an alternative to culture-based methods. These methods are sensitive, specific, rapid, and less cumbersome than traditional culture-based methods. An extensive range of polymerase chain reaction (PCR) assays, based on different target genes and primers, has been described for the detection and identification of *Campylobacter* spp. Of 26 primer pairs tested, the combination of OT1559 (5′-CTGCTTAACA-CAAGTTGAGTAGG) and 18-1 (5′-TTCTGACGGTAC-CTAAGGAA), targeting the 16S rRNA gene, was found to be most selective. This primer pair was then used in a multicenter international collaborative trial, testing 10 target and 8 nontarget strains. The results showed 93.7% sensitivity and 100% specificity.[16] With multinational disease outbreaks occurring, the move toward standardization of methods used for isolation and epidemiology is essential.

There is no simple method for the identification of *Campylobacter* to subspecies level. Discrimination between *C. jejuni* and *C. coli* can be achieved by using a combination of PCR amplification with a DNA probe hybridization step to detect and discriminate subspecies[17] by PFGE or multiplex PCR.

SURVIVAL

Most foodborne pathogens are robust organisms capable of surviving the stressful environment outside the host. Various mechanisms have evolved to enable survival of oxidative stress, starvation, heat, cold, and so on.

Campylobacter spp. lack many of these important survival mechanisms.[3] In particular, they lack the typical stationary-phase response mediated by rpoS.[18] In a farm environment, *C. jejuni* can be isolated from animals, milk, slurry, farm vehicles, water, and so on. They can also be isolated during food processing and from various food samples. This implies that, although they are apparently sensitive, they must be capable of surviving environmental stress by as yet unidentified mechanisms. The VNC state is one such possible mechanism, but two others have been recently reported. These are the production of an extracellular protein that confers stress resistance[19] and the observation of an adaptive tolerance response.[20] The indications are that *Campylobacter* spp. are quite robust in their ability to survive outside the host; otherwise, they would not be the principal cause of foodborne illness worldwide.

GENOMICS AND PROTEOMICS

The amount of DNA sequence data available and the methods used to exploit it have grown exponentially in recent years. The aim of this work is to understand how the various components of an organism interact to function properly, not just to catalogue a list of genes and how they function. Genomics describes the study of the genetic makeup of an organism, whereas proteomics is the study of the protein composition of the cell. There are more proteins in an organism than there are genes because transcriptional control, posttranslational modification, and so on can lead to multiple products form the one gene—hence the need to study the interrelationship between genomics and proteomics, which is termed functional genomics.

The recent publication of the genome sequence of *C. jejuni* NCTC11168 has shown that it has a circular chromosome of 1.6 Mbp, a G+C content of 36%, and a predicted 1654 proteins.[21,22] One of the most notable features of the genome sequence was the presence of hypervariable sequences associated with genes encoding biosynthesis/modification of surface structures, or linked to hypothetical genes. Whole genome comparison of different *C. jejuni* isolates has quantified this degree of genetic heterogeneity among different strains. Pearson et al.[23] have shown that 16.3% of the genes are absent or divergent in one or more of the 18 strains tested. Despite this, the genome sequence contains virtually no transposons, phage remnants, or insertion elements, and few repeat sequences, raising the question as to the source of the heterogeneity. The sequencing of a second strain of *C. jejuni*, which should be available in 2004, and the availability of the genome sequence of *C. coli*, *C. lari*, and *C. upsaliensis*, which are in progress,[24] should give a better understanding of genetic heterogeneity in *Campylobacter* spp.

There has been an upsurge in research capitalizing on the availability of the genome sequence. DNA microarrays are being used for genome analysis, gene expression, identification, and typing (called genomotyping). Large biotechnology companies are using DNA-based microarrays to develop new drugs. This can avoid large-scale, expensive mode-of-action studies. Gene expression studies (transcriptomics) have tended to predominate functional genomics. Although this does have the advantage of showing what genes are turned on and when, it has the disadvantage that it gives no information on the expression of these genes as functional proteins. Two-dimensional (2-D) gel electrophoresis is the best method to study protein expression. However, protein concentrations less than 1 ng cannot be routinely measured; certain proteins (e.g., hydrophobic membrane-bound proteins) are not suitable for separation by 2-D gels, and differences in abundance of proteins cannot be accounted for by this method. Until further development of new technologies, gene expression studies, in association with 2-D gel electrophoresis, will be necessary to yield maximum information. New technologies, such as polysome-bound mRNA profiling, will bridge the gap between genomics and proteomics, as it will give information on every level of regulation between transcription and translation. However, it cannot be used to study posttranslational modification.[25]

Although the ''genomic'' understanding of *Campylobacter* spp. has been advanced recently, research on proteomics has lagged behind. Very little information relating to protein expression by *Campylobacter* has been published. We have studied the proteomics of *C. jejuni* during induction of an adaptive tolerance response to acid and aerobic conditions. Under conditions of induction, 11 proteins were upregulated, 21 were downregulated, and 60 ''new'' proteins were identified. These ''new'' proteins could be due to upregulation of low-abundance proteins not detected in uninduced samples. Identification of these proteins by matrix-assisted laser desorption initiation time of flight (MALDI-TOF) analysis showed the induction of global regulatory systems, such as two-component regulators, methylation proteins, and heat shock proteins. The generation of mutants and the complementation of these mutants with the expressed gene on a plasmid are necessary to show functional relationships in survival and pathogenesis.

CONCLUSION

Advances in the genomics of *Campylobacter* spp. have resulted from the publication of the genome sequence.

Whole genome-based DNA microarrays are being used for the detection, isolation, and typing of *Campylobacter* spp. When developed further, these methods will revolutionize the epidemiology of *Campylobacter* spp. Gene expression studies, in association with protein expression studies, should begin to provide the type of information that will result in a better understanding of *Campylobacter* pathogenesis and virulence, and the mechanisms by which they cause disease, although development of new technologies will be necessary to bridge the gap between genomics and proteomics. Although advancing the scientific understanding of *Campylobacter* spp. will undoubtedly result from this research, the application of this information to achieving a reduction in the occurrence of *Campylobacter*-related illness is essential.

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Cancer Chemotherapy—Overview on the Clinical Impact of Polymorphisms in Metabolizing Enzymes

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Ikuo Sekine
Nagahiro Saijo
National Cancer Center Hospital, Chuo-ku, Tokyo, Japan

INTRODUCTION

Cancer chemotherapy has progressed greatly over the past five decades, providing not only temporary control of the metastatic disease that develops in 60% to 70% of cancer patients during their lifetime, but also curative therapeutic interventions for patients with some types of locally advanced solid tumors and hematologic malignancies. Cancer chemotherapy is distinct from other general drug therapies because of the following characteristics: 1) advanced cancer remains a life-threatening disease in many cases; 2) the therapeutic index, the ratio of the dosage required to produce toxicity and an antitumor effect, is narrow; 3) patients are treated with or close to the maximum tolerated dosage, with toxicity levels so severe that treatment-related death occurs in about 1% of patients who receive chemotherapy; 4) chemotherapy drugs are administered intermittently, usually on a weekly or monthly basis; 5) the delay between the administration of anticancer agents and their clinical effects is a matter of days to weeks; and 6) two to four anticancer agents are often administered as combination chemotherapy for many tumors, with drug–drug interaction, significantly affecting the pharmacokinetics of each agent in some cases. Until recently, the therapeutic dosage of anticancer agents was determined empirically in Phase I trials, without any consideration of interindividual variability in drug pharmacology. The plasma drug concentration, however, can vary greatly between individuals of the same weight and with the same drug dosage, leading to lethal toxicity or no antitumor effects. Genetic polymorphisms in drug metabolizing enzymes are therefore of utmost importance for achieving a favorable outcome from drug therapy.

TYPES OF ENZYMES

Dihydropyrimidine Dehydrogenase

Dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2), the first and rate-limiting enzyme in the three-step catabolic pathway of the endogenous pyrimidine bases uracil and thymine, also catalyzes the reduction of 5-fluorouracil (5-FU) to dihydrofluorouracil. The human DPD enzyme is a homodimer of a 111-kDa subunit with 1025 amino acids residues containing FMN, NADPH, and flavin adenine nucleotide (FAD) binding sites; the enzyme is encoded by a single gene (DPYD gene) with 23 exons mapped to chromosome 1p22.[1,2]

Familial DPD deficiency was first described in 1984 in pediatric patients exhibiting thymine–uraciluria with a variety of neurologic symptoms. Of the more than 20 DPYD allelic alterations, the most common variant in patients with DPD deficiency is the homozygous deletion of exon 14 that is caused by a G to A point mutation at the splice donor site (DPYD*2A variant). This variant is detected in 24% to 52% of patients with DPD deficiency.[2,3] Screening for the presence of this variant in the general population revealed that it was found in 1.2% or less of individuals from various ethnic backgrounds.[2,3] Dihydropyrimidine dehydrogenase activity is also regulated at the levels of transcription and translation. Expression of the DPYD gene and DPD activity in the liver and colonic tissues varies markedly. Dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells from the general population and from unselected cancer patients varies widely among individuals, with a significantly low enzyme activity found in 3–6% of these populations.[1,2]

As severe toxicity with neurologic symptoms associated with the administration of 5-FU in a patient with familial pyrimidiuria was first described in 1985, several patients with DPD deficiency have developed severe 5-FU-induced toxicity. However, pretreatment analyses of DPD activity in human peripheral blood mononuclear cells showed a weak or no correlation with 5-FU clearance and failed to predict a risk of toxicity in patients receiving 5-FU.[2] A genotyping test for DPYD mutations explained the reduction in DPD activity in only a limited number of patients. The sensitivity of the test for identifying patients at high risk for severe 5-FU toxicity was also low. Thus, no single test has been validated as a tool for individualizing the dosage of 5-FU.[2]

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Methylenetetrahydrofolate Reductase

Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which donates a methyl group to homocysteine to produce methionine. This enzyme has a central regulatory role in the metabolism of folate and methionine, both important factors in DNA synthesis, repair, and methylation.[4] The molecular weight of this enzyme is 77 kDa, and the enzyme is encoded by a gene with 11 exons located on chromosome 1p36.3.

Severe MTHFR deficiency was first described in 1972 in a 15-year-old boy who exhibited mental retardation, progressive neurologic deterioration, and homocystinuria. Of the 33 polymorphisms identified in the MTHFR gene, the most common was a C to T transition at nucleotide 677 (C677T), corresponding to an alanine to valine change at codon 222. Homozygosity for this mutation results in an enzyme activity level that is equal to 30% of the wild-type enzyme activity level found in vitro and is found in 10–16% of both Caucasian and Asian populations. Heterozygosity, resulting in 60% of the enzyme activity level found in vitro, is found in up to 40% of the population.[4,5] This variant is associated with an increased risk for various common cancers, probably as a result of the insufficient methylation of genomic DNA.[6]

An association between the MTHFR C677T polymorphism and severe methotrexate (MTX) toxicity has been reported in patients with breast cancer receiving cyclophosphamide, MTX, and 5-FU; patients with chronic myelogenous leukemia following hematopoietic cell transplantation; patients with acute leukemia undergoing maintenance chemotherapy; patients with ovarian cancer treated with carboplatin and MTX; and patients with rheumatoid arthritis receiving MTX.[7] As MTHFR does not metabolize MTX directly and is not the primary target of this agent, an imbalance in folate pools and a reduced ability to recycle folate have been proposed as important mechanisms in the development of MTX toxicity.[5]

Cytochrome P450

The cytochrome P450 enzymes are a superfamily of heme proteins that carry out the oxidative, peroxidative, and reductive metabolism of many structurally unrelated compounds, including endogenous steroids, fatty acids, and bile acids, as well as xenobiotics such as environmental pollutants. They are also responsible for the metabolic activation and/or inactivation of many drugs, including anticancer agents.[1,8]

CYP1A2 accounts for nearly 15% of the cytochrome P450 found in the human liver and metabolizes more than 20 drugs including caffeine, theophylline, imipramine, clozapine, and propranolol. Dacarbazine requires oxidative *N*-demethylation by CYP1A2 for its activation to exhibit a DNA-alkylating action.[1] Reported interindividual variations in CYP1A2 expression range from 15- to 60-fold in a number of ethnic and geographically distinct populations. The human CYP1A2 gene, consisting of seven exons, is located on chromosome 15. Among the 14 known variant alleles of CYP1A2, CYP1A2*1C and CYP1A2*1K lead to a reduction in enzyme activity, but their clinical significance in dacarbazine metabolism and toxicity is unknown.

CYP2C8 catalyzes the formation of 6-alpha-hydroxypaclitaxel, one of the major paclitaxel metabolites in human plasma and bile. The CYP2C8 gene is one of four CYP2C gene family members located on chromosome 10q24. Six variant alleles have been reported, and CYP2C8*3 is associated with a low enzymatic activity and a reduction in the metabolism of paclitaxel.[9]

CYP3A4 accounts for as much as 60% of the cytochrome P450 enzymes in the human liver and is responsible for the oxidative metabolism of an estimated 50% to 60% of all clinically used drugs, including anticancer agents such as teniposide, etoposide, cyclophosphamide, ifosfamide, vindesine, vinblastine, vincristine, vinorelbine, paclitaxel, docetaxel, and irinotecan.[1] The CYP3A4 gene has been mapped to 7q22.1, and 24 variant alleles have been reported. A more than 10-fold interindividual variation in the clearance of CYP3A4 substrate drugs is seen in vivo, but structural mutations in the CYP3A4 gene do not seem to be a major factor in this functional variation.[10] In contrast, the expression levels of CYP3A4 in the human liver vary up to 60-fold in the general population. CYP3A4 expression is known to be induced by various therapeutic agents, including glucocorticoids, rifampicin, barbiturates, and other xenobiotics. Recently, nuclear receptors, such as the constitutive androstane receptor (CAR) and pregnane X receptor (PXR), have been reported to mediate such inductions and may play an important role in the large variation in CYP3A4 expression.[11]

CYP3A4 is involved in the metabolic pathways of many anticancer agents, and it may be possible to predict the pharmacokinetics and toxicity of an anticancer agent by its effect on CYP3A4 activity, as shown in an excellent correlation between docetaxel clearance and pretreatment CYP3A4 activity measured by excreted 6-beta-hydroxycortisol.[12] In addition, the inhibition or induction of CYP3A4 activity by a drug may lead to an alteration in the pharmacokinetics and toxicity profile of an anticancer agent. The sequence of cisplatin followed

by paclitaxel administered in a 24-hr infusion induced a 33% reduction in the clearance rate of paclitaxel and a more profound neutropenia than the reverse drug sequence in a Phase I study.[13] This finding can be explained by the reduction in CYP3A4 activity produced by cisplatin, although the mechanisms responsible for this sequence-dependent drug–drug interaction are not entirely understood. Enhanced vincristine toxicity was observed in two patients with acute lymphoblastic leukaemia who concomitantly received itraconazole, which inhibits CYP3A4 activity.[14] The CYP3A4-inducing antiepileptic agents carbamazepine, phenytoin, and phenobarbital decreased the steady-state paclitaxel concentration and toxicity in patients with glioblastoma multiforme, leading to a 42% higher maximum tolerated paclitaxel dosage in patients who received the antiepileptic agent, compared to that in patients who did not receive any of these agents.[15] These antiepileptic agents also increased the systemic clearance of vincristine by 63% in patients with brain tumors, compared with a control group.[16]

Thiopurine *S*-Methyltransferase

Thiopurine *S*-methyltransferase (TPMT; E.C. 2.1.1.67) is a cytosolic enzyme that catalyzes the *S*-methylation of aromatic and heterocyclic sulfhydryl compounds.[1] The natural substrates for TPMT remain unknown, but this enzyme is involved in the intracellular inactivation of 6-mercaptopurine, 6-thioguanine, and azathioprine. The TPMT gene consists of 10 exons located on chromosome 6p22.3. Thiopurine *S*-methyltransferase activity in erythrocyte lysates is well correlated with the level of TPMT activity in the liver and kidney. An inverse correlation between TPMT activity and the accumulation of 6-mercaptopurine metabolites in erythrocytes has been established. Patients with TPMT deficiency are at a high risk of potentially fatal hematopoietic toxicity after receiving a standard dosage of 6-mercaptopurine, and the mercaptopurine dosage should therefore be reduced by 10- to 15-fold.[17]

Thiopurine *S*-methyltransferase activity is inherited as an autosomal codominant trait. Population studies in Caucasians have shown a trimodal distribution of TPMT activity, with about 90% of subjects having high activity, 10% of subjects having intermediate activity, and 0.3% having no activity. Of the 10 mutant alleles resulting in a low TPMT activity, TPMT*2, TPMT*3A, and TPMT*3C account for 80–95% of the intermediate or low enzyme activity cases. The frequencies and patterns of these alleles exhibit ethnic variations. TPMT*3A is the most prevalent mutant allele among Caucasians, whereas TPMT*3C is the most common mutant allele among Asians.[17] A variable number tandem repeat (VNTR) polymorphism within the TPMT promoter has also been reported. The VNTR length varied from three to nine repeats, and an inverse relationship existed between the sum of the repeat units on the two alleles and the level of erythrocyte TPMT activity, although the effects of this relationship were quantitatively small.[17]

Uridine Diphosphate Glucuronosyltransferase

Uridine diphosphate glucuronosyltransferases (UGTs; E.C.2.4.1.17) are a family of enzymes in the endoplasmic reticulum that catalyze the conjugation of hydrophobic endogenous and xenobiotic compounds into hydrophilic glucuronides with UDP-glucuronic acid.[18] The human UGT family is divided into two major classes, UGT1 and UGT2, based on an amino acid sequence comparison. At least 15 human UGT cDNAs have been identified to date. Of these, eight are classified as UGT1 isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, and 1A10) encoded by the UGT1A genes located on chromosome 2q37, and seven are classified as UGT2 isoforms (UGT2A1, 2B4, 2B7, 2B10, 2B11, 2B15, and 2B17) encoded by the UGT2 genes located at chromosomes 4q13 and 4q28.

The UGT 1A1 isoform glucuronidates bilirubin and SN-38, an active metabolite of irinotecan. An absence or reduction in UGT1A1 function leads to three inherited forms of disorders in humans: Crigler–Najjar syndrome type I, Crigler–Najjar syndrome type II, and Gilbert's syndrome. All of these disorders are the results of either mutant UGT1A1 alleles or mutations in the UGT1A1 promoter sequences.[18] The insertion of TA bases in the repetitive TATA-box upstream of the UGT1A1, the TA[6] allele (UGT1A1*28), causes a reduction in the expression of the gene when compared with the wild-type TA[5] allele. UGT1A1*28 is a relatively common polymorphism, and heterozygosity for the mutated allele and homozygosity for the mutated alleles are found in 55% and 11%, respectively, of the European population and in 28% and 2%, respectively, of the Asian population.[1] Heterozygosity and homozygosity for UGT1A1*28 are both significant risk factors for severe irinotecan toxicity. Other UGT1A1 variant alleles, UGT1A1*6 and UGT1A1*7, are not associated with irinotecan toxicity.[19]

Two other UGT isoforms, UGT 1A7 and UGT 1A9, also glucuronidate SN-38, in vivo, but their clinical significance with regard to irinotecan toxicity is unknown.

Glutathione *S*-Transferase

Glutathione *S*-transferase (GST; EC.2.5.1.18) catalyzes the glutathione conjugation reaction in detoxification pathways for a wide variety of electrophilic compounds including anticancer agents to make them more water-soluble, less toxic, conjugates.[1] The human cytosolic GST enzymes, classified into six families, α, μ, ω, π, θ, and ζ, comprise heterodimers or homodimers of subunits. Orally administered busulfan, a bifunctional alkylating agent, is eliminated by conjugation with glutathione in the intestinal mucosa, and the large interpatient variation in busulfan clearance is associated with the activity of GSTs, especially the α-isoform. In addition, alteration of GSTs in tumor cells is considered to be associated with their resistant phenotypes against anticancer agents through direct detoxification and inhibition of the MAP kinase pathway. The expression of GST-π in non-small cell lung cancer tissue was significantly related to clinical response to cisplatin-based chemotherapy in small case series studies.[20]

CONCLUSION

Individual variations in responses to anticancer agents can be partly explained by genetic polymorphisms in their metabolizing enzymes. Genotype–phenotype correlations, however, are still poorly understood because epigenetic influences on gene expression make it difficult to estimate the exact effects of genetic conditions on the activities of metabolizing enzymes. To enable customized and individualized cancer chemotherapies in which the most favorable dosage and drug combination are administered, further studies should focus on parameters that are closely correlated with the pharmacokinetics and pharmacodynamics of an agent and on well-designed clinical trials to evaluate their clinical usefulness.

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Cancer Chemotherapy—Polymorphisms in DPD, TPMT, UGT1A1

Thomas Szekeres
University of Vienna Medical School, Vienna, Austria

Alexander C. Haushofer
General Hospital of St. Pölten, St. Pölten, Austria

Monika Fritzer-Szekeres
University of Vienna Medical School, Vienna, Austria

INTRODUCTION

Chemotherapy is still one of the main treatment options for the therapy of various malignancies. Anticancer drugs have to be metabolized by several enzymes and can also be degraded by specific enzymes. As activities of these enzymes may vary from patient to patient and may significantly influence the treatment outcome, understanding of the effects of increased or decreased drug-metabolizing enzyme activities help in the design of the optimal chemotherapeutic regimen. More recently, these treatment attempts may even be designed for every patient based on their genetic pattern. Considering the narrow therapeutic range of many anticancer compounds, precise dosing of such drugs is mandatory. The determination of genetic conditions, i.e., polymorphisms of specific genes encoding enzymes, might help to predict the effects of selected anticancer drugs. Thus ineffective administration of chemotherapeutic drugs or even the occurrence of severe toxic side effects could be avoided.

In this article, we focus on three enzymes that play a major role for effective treatment with 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), or irinotecan. The enzymes involved in the development of severe toxicity associated with these drugs are dihydropyrimidine dehydrogenase (DPD), thiopurine *S*-methyltransferase (TPMT), and UDP-glucuronosyltranferase 1A1 (UGT1A1). The genes encoding for these enzymes can be examined prior to chemotherapy using molecular methods for genetic testing and thus possible side effects can be predicted.

DIHYDROPYRIMIDINE DEHYDROGENASE

The chemotherapeutic compound 5-Fluorouracil (5-FU) is a widely used drug for the treatment of various types of cancer, such as colon or breast tumors. Optimal combinations with other chemotherapeutic compounds have been developed because of its limited efficacy when used alone. More recently, prodrugs of 5-FU were invented that can be administered orally. In particular, for patients suffering from advanced colon or breast cancer, oral administration can significantly improve the quality of life.

The therapeutic effects of 5-FU are caused by the inhibition of RNA and DNA synthesis. 5-FU is metabolized to 5-fluorouridine and further phospholylated to 5-fluorouridinetriphosphate, which replaces uridine 5′-triphosphate (UTP) in RNA. On the other hand, 5-FU can be converted to 5-fluorodeoyxyuridine and further to the respective 5′-triphosphate, which causes the inhibition of DNA synthesis. The monophosphate of 5-fluorodeoxyurdine (5-FdUMP) itself is an inhibitor of the enzyme thymidylate synthase, which is responsible for the conversion of dUMP to dTMP.

The degradation of 5-FU is metabolized by the enzyme dihydropyrimidine dehydrogenase (DPD), which generates dihydro-5FU (5-FUH_2), an inactive metabolite of 5-FU. The metabolic pathway is depicted in Fig. 1.

Therefore, patients with increased activity of DPD might require higher 5-FU doses to achieve antitumor activity. Decreased activity of this enzyme, however, might cause higher 5-FU levels with the risk of life-threatening toxicities.[1] DPD deficiency might have been the cause of severe neutropenia in patients after 5-FU administration.[2–4] Even lethal toxic effects were reported in DPD-deficient patients. Because of the very narrow therapeutic range of 5-FU, knowledge of the activities of these metabolizing enzymes might yield essential information for individual dosing. Thus ineffective doses or life-threatening toxicities at doses in the regular range may be avoided. Significant reduction of DPD activity resulting in severe 5-FU toxicity can even be caused by the inactivation of one allele of the DPD gene. Various mutations of the DPD gene (DPYD),

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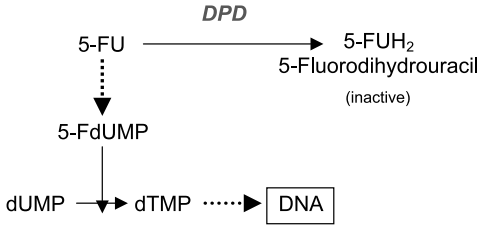

Fig. 1 The metabolic pathway of DPD action. (*View this art in color at www.dekker.com.*)

which are associated with reduced enzyme activity and increased risk of toxicity, were identified.[5,6] For instance, a mutation for DPYD*9A was identified in patients suffering from toxic 5-FU side effects. However, DPYD mutations do not always result in reduced enzyme activity (biochemical phenotype). There are patients who have inherited DPD deficiency and are at high risk for 5-FU toxicity, but many others inherit variant alleles without clear association between genotype and 5-FU toxicity. However, van Kuilenburg and coworkers[6] described the correlation of the rather frequent (1.8% heterozygotes in the normal population) mutation (IVS14+1G<A) of the DPYD, which might also be associated with 5-FU toxicity. They even described the lethal outcome of 5-FU treatment in a patient who was homozygous for this mutation. So far, various DPYD allelic variants are known. In some cases, these genetic variants correlate with altered DPD activity; in other cases, this connection remains to be further elucidated. However, some of the DPD gene mutations such as DPYD*2A and DPYD*9A are considered to be of clinical relevance. This renders DPYD as a potential candidate for pharmacogenetic profiling prior to chemotherapy with 5-FU, and thus adverse drug effects might be avoided. To be considered beneficial for all patients, a closer correlation between genotype and phenotype needs to be established, and then all relevant genetic alterations, i.e., single nucleotide polymorphisms (SNPs) have to be tested prior to therapy. This could lead to the development of personalized therapy for every patient with maximum effect and minimum toxicity.

C

THIOPURINE *S*-METHYLTRANSFERASE

Childhood acute lymphoblastic leukemia (ALL) is the most common childhood malignancy and can be cured in most cases via chemotherapy. ALL is treated by a combination of various drugs; however, 6-mercaptopurine (6-MP) is one of the drugs required to maintain remission. It is mandatory to adjust 6-MP dosage according to the toxicity of 6-MP, which is mainly caused by the accumulation of the active metabolite 6-thioguanine triphosphate (6-TGTP) via the metabolite 6-thioinosine-monophosphate (6-TIMP; metabolic pathway shown in Fig. 2). 6-MP is an antimetabolite, which has to be activated to its triphosphate in order to be incorporated into DNA. On the other hand, 6-MP, but also 6-TIMP, can be inactivated by the enzyme TPMT to their inactive methylated metabolites 6-methylmecaptopurine (6-MMP) or 6-methylthioinosinemonophosphate (6-MTIMP). Thus decreased TPMT activity may lead to accumulation of the active 6-MP metabolite, and increased activity may lead to ineffective drug concentrations. Another drug which is methylated by TPMT is 6-thioguanine (6-TG), and indirectly the compound azathioprine, which is a prodrug of 6-MP. 6-TG is also being used as cytotoxic agent, and azathioprine is used as an immunosuppressant to treat rheumatic diseases. In all cases, elevated or depressed TPMT activity can cause ineffective accumulation of drug metabolites or toxic side effects, respectively. This can even include fatal myelosuppression after a standard dose of a thiopurine drug. Various other cases of TPMT-deficient patients showing severe side effects after 6-MP treatment are known. Therefore, the determination of polymorphisms of the TPMT gene prior to therapy might help to identify patients at risk of developing toxic side effects from 6-MP therapy. Genetic diversity (e.g., 238G>C, 460G>A, 719A>G) causes enhanced proteolysis of TPMT proteins and correlates

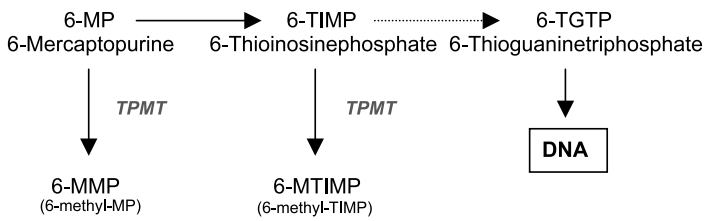

Fig. 2 The metabolic pathway of TPMT action. (*View this art in color at www.dekker.com.*)

with decreased TPMT enzyme activity and increased risk of treatment.[7] However, adverse drug reactions, such as hepatic toxicity, cannot be linked to TPMT polymorphisms and activity. Some TPMT variant alleles, which are associated with low enzyme activity, are known.[8–12] In Caucasians (55–70%), however, TPMT*3A is the most common variant allele attributed to low TPMT activity.[13] TPMT*3C is more common among Asians compared to Caucasians, where this variant is quite rare (<1%). The wild-type TPMT gene is associated with higher TPMT activity, and heterozygosity or homozygosity for one variant allele show significantly reduced enzyme activity.[14–16] It has to be considered that molecular testing, which has to be performed prior to therapy, might vary from one ethnic group to another, and still be unable to provide 100% safety. Some still unknown mutations might also be responsible for decreased TPMT activity.[17] Thus TPMT phenotyping is being performed in various establishments. The enzyme activity of erythrocytes correlates well with the activity of bone marrow cells and might therefore be measured prior to therapy. However, significant variability of enzyme activities in wild-type patients are known and cannot yet be clearly linked to genetic patterns. Even in TPMT heterozygotes, an increased risk of thiopurine hematopoietic toxicity was observed by Relling et al.[18] Individual dosing of 6-MP might help to successfully treat ALL, and therefore the aim is to find all genetic variants that are linked to altered TPMT activity. In such a way, genotyping might help to determine the individual dose needed for effective cancer chemotherapy.

UDP-GLUCURONOSYLTRANFERASE 1A1

UDP-glucoronosyltransferases are enzymes responsible for detoxification and elimination of various metabolites and drugs. These enzymes also catalyze the inactivation of irinotecan (CPT-11), an analog of the alkaloid camptothecin. CPT-11 is clinically used for the treatment of refractory and advanced colorectal cancers. Dose-limiting toxicities are diarrhea and pancytopenia. CPT-11 is metabolized to its active product, SN-38, by the enzyme carboxylesterase 2. As depicted in Fig. 3, UGT1A1 is responsible for the inactivation of SN-38, the most relevant metabolite of CPT-11, to the glucuronide of SN-38. CPT-11 can also be directly inactivated by formation of the inactive oxidation product, aminopenthan carboxylic acid (APC). Responsible for this step are cytochromes *P*450 3A4 and 3A5, as shown in Fig. 3. However, the formation of the SN-38 glucuronide is the most important inactivation pathway of CPT-11. This step is catalyzed by UGT1A1, an enzyme that is also responsible for bilirubin conjugation. The formation of glucuronide is the limiting step for renal clearance, which provides the solubility in water. Therefore, decreased UGT1A1 might not only cause hyperbilirubinaemia (unconjugated bilirubin), but also the accumulation of SN-38 and the toxic side effects of CPT-11 treatment. A condition based on a genetic defect of the UGT1A1 gene is known as Gilbert's syndrome, which is associated with increased bilirubin concentrations and occasionally severe CPT-11 toxicity, as previously described by Wasserman et al.[19] The genetic defect associated with Gilbert's syndrome is a TA insertion in the promotor region of the gene;[20] the variant allele is named UGT1A1*28.[21] Another mutation of the gene, which is mainly found among Asians is a G211A mutation (UGT1A1*6).[22] In Asian populations, Gilbert's syndrome is a result of missense mutations (either homozygous or heterozygous) in the coding sequence. Another important regulatory element of UGT1A1 is the phenobarbital-responsive enhancer module (PBREM), which might contain variants affecting inducible gene expression. Innocenti et al.[23] recently showed that the haplotype structure of promoter is probably different between Caucasians and African-Americans. The mutations result in reduced enzyme activity and the likelihood of toxic side effects of CPT-11 treatment.

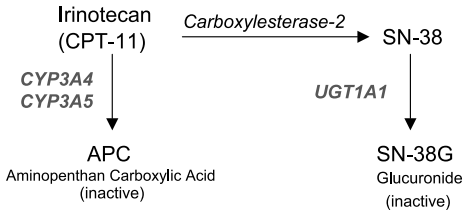

Fig. 3 The metabolic pathway of UGT1A1 action. (*View this art in color at www.dekker.com.*)

CONCLUSION

Chemotherapy remains an important option for the treatment of various malignancies. Drugs have to be activated and inactivated by various enzymes. We have described three enzymes (DPD, TPMT, and UGT1A1) responsible for the degradation of 5-FU, thiopurines, and CPT-11—all of which are widely used anticancer agents. As the activities of these enzymes might vary from patient to patient, standard doses could either cause

severe toxicities or be ineffective. Therefore, the future goal should be to individualize dosing according to the genetic pattern of the patient reflected in different enzymatic activities. Molecular testing prior to therapy for identifying factors which predict the treatment outcome and supports individualized therapy are needed. One approach is to determine the variants of the genes coding for the above mentioned enzymes. These genetic polymorphisms should be associated with altered enzyme activities. Thus doses could be predicted for every single patient. As described, a number of genetic variations and mutations are known for the enzymes. Some of them translate into altered enzyme activities. However, because of the huge number of mutations—not always linked to a different phenotype—and because of the genetic differences between ethnic groups, until now it has not been possible to draw final conclusions, which are advantageous for all patients. Nevertheless, for the described enzymes, genetic testing may provide additional information and help to predict the individual dosage for many patients. We hope that, as a result of intensive research in this field, ''personalized therapy'' with drugs having a narrow therapeutic window, such as anticancer compounds, might be possible in the near future.

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Candida spp.

Philip Lewis White
Sam Hibbitts
University of Wales College of Medicine, Cardiff, U.K.

INTRODUCTION

With the number of immunocompromised patients at risk for fungal infections on the rise and with yeasts representing the most common fungal isolates from human patients, concerns over infections caused by the genus *Candida* continue to expand. In 1999, *Candida* species were reported as the fourth most frequent cause of nosocomial blood-borne infection within the critically ill population, and with this population ever increasing, it seems reasonable to predict a mounting number of deep-seated candidal infections.[1] However, improved awareness/diagnosis of the disease, combined with the development of novel detection techniques, may have produced a deceptive increase in the incidence of candidosis.

Candidal infections can target almost every tissue in the human body, with superficial infections being the most common and with invasive infections providing the greatest concerns for clinicians. The genus *Candida* contains approximately 200 species, but few species are considered as opportunistic pathogens, although it is probably only a matter of time until further species would have been considered as causative agents for a case of superficial or deep-seated candidosis.

TYPE OF PATHOGEN

Candida species are primarily saprophytic eukaryotic organisms and only occasional opportunistic pathogens. Many species exhibit a bitrophic existence, spending much of their life as common commensals of the gastrointestinal (GI) tract and mucous membranes of humans only to become pathogenic because of debilitation of the host. Indeed, the most significant source of *Candida* causing candidosis is endogenous.[2]

Despite the main cause of invasive candidosis (IC; *Candida albicans*) being considered as an obligatory animal saprophyte,[2]*Candida* species, including *C. albicans*, are frequently isolated from nonanimal environments. Indeed, *Candida* is an important cause of nosocomial bloodstream infections, with 97% of candidemias attributed to *C. albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*.[3]

RANGE OF INFECTIONS

Superficial Candidosis

For an informative review of superficial candidosis including the symptoms, predisposing factors, and treatment for oral, genital, cutaneous, and chronic mucotaneous candidosis, and candidosis of the nail, then refer to the comprehensive book by Odds.[2] The following text represents a summary of some of the information available.

Oral Candidosis

Oral candidosis (OC) is one of the most frequent human fungal infections. Commonly affecting the young, the aged, and the immunosuppressed, it has various clinical forms [pseudomembranous candidosis (oral thrush), atrophic candidosis, hyperplastic candidosis, and angular cheilitis]. Its diagnosis can be complicated by a lack of culture or symptoms; however, because *Candida* species are regular human commensals (30–50% of the normal population),[4] then an oral culture of *Candida* does not confirm a case of OC and only histological evidence can validate an infection.

C. albicans is the most prevalent cause of OC (60–80% of yeasts isolated from the mouth), with at least eight other species documented as causative agents of OC and with non-*C. albicans* emerging as relatively common causes of OC.[4] Furthermore, novel species of *Candida* (*Candida dubliniensis*)[5] have been, and will continue to be, isolated over time. The treatment regime is usually topical; however, oral fluconazole has proven successful with acquired immunodeficiency syndrome (AIDS) patients, although monitoring for fluconazole-resistant strains is essential.

Genital Candidosis

Candida species are common organisms of the female genital tract, which are isolated from 20% of normal women.[4] *C. albicans* is the most common cause of vaginal candidosis (VC) (up to 89% of infections),[6] although *C. glabrata* is emerging as an important pathogen that could become resistant to fluconazole treatment.[7]

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Affecting 75% of women during their lifetime,[4] VC is a common problem. As the prevalence of *Candida* detected by culture is the same in women with or without symptoms, meaningful diagnosis is difficult and both culture and clinical symptoms should be evaluated to define the condition.[2]

VC can be a severe source of irritation and discomfort to the patient, but the condition can have more serious consequences. Infection with VC has been considered as a risk factor for human immunodeficiency virus (HIV) infection[8] and gynecological surgery performed on patients with VC has led to systemic candidal infections.[2] VC has also been transmitted congenitally from mother to child,[2] with some research indicating that treatment for VC during pregnancy actually reduced the opportunity for premature births.[9]

Penile candidosis is far less common than vaginal infection, although it can be a result of sexual transmission. It is usually an uncomplicated infection that responds to topical antifungal therapy.

Invasive Candidosis

IC (deep-seated candidosis) is a worldwide disease that targets the immunocompromised population, with *Candida* species able to infect virtually every deep organ. Localized systemic candidosis is the term given to an infection of an individual organ, whereas disseminated candidosis (*Candida* septicemia, candidemia) involves the infection of several organs where the disease has been propagated by the bloodstream. This classification is not rigid and the diagnosis of systemic candidosis in one organ may be the initial sign of disseminated candidosis observed earlier at that particular site.[2]

The population at risk for IC will generally be immunocompromised, usually as a result of treatment for cancer, organ transplantation, HIV, or intensive care unit (ICU) treatment. However, surgery, antibiotic use, a central venous line, prematurity, parenteral nutrition, damage to the GI mucosal membrane, and neutropenia have been indicated as predisposing factors to infection.[10] Furthermore, several of these risk factors may be combined in certain case scenarios.[10]

C. albicans is the most common cause of IC, although the contribution of non-*C. albicans* species causing IC is rising. There are distinct geographical and patient group distributions to the species causing non-*C. albicans* infections,[11] and recent publications have highlighted the diagnosis of polycandidal IC, which could be significant when initiating the correct antifungal therapy.[12]

With high mortality rates associated with IC (up to 40%), early diagnosis and treatment of IC are essential. However, the diagnosis of IC can be problematical, with up to 50% of patients with autopsy-proven IC having multiple negative blood cultures.[13] The British Society for Medical Mycology has produced a review article highlighting the problems and proposing standards that should be met to provide optimal surveillance and diagnostic programs for invasive fungal infections.[13]

Treatment for IC involves the use of a systemic antifungal agent, such as fluconazole or amphotericin B, with or without flucytosine, plus the removal of central venous catheters. Fluconazole should not be used with *C. krusei* or *C. glabrata* infections. Recent developments in antifungal therapy have seen the emergence of a new triazole ''voriconazole'' and a novel group of drugs, the echinocandins, of which ''caspofungin'' is the first licensed drug. Both of these have given promising results in treating IC, with the added benefit of being effective against *Aspergillus* infections.

MOLECULAR CHARACTERIZATION

The Stanford Genome Technology Center has recently sequenced the entire diploid genome of *C. albicans*. Covering 1.6×10^7 bp, with eight pairs of chromosomes and containing over 9000 genes encoding proteins of at least 100 amino acids, it is larger and shares only 60% gene homology with the genome of *Saccharomyces cerevisiae*—the organism often used to predict antifungal targets in *C. albicans*.[14,15] Compared to *S. cerevisiae*, *C. albicans* has more genes that are specific to it—a factor that could account for its pathogenicity and ability to colonize various environments.[16]

Within the genome, 567 genes have been designated as essential for survival, with an estimated 1400 genes required for growth.[15] The genome contains several large gene families encoding proteases, lipases, and cell wall proteins, and, together with genes distinct to *C. albicans*, are considered good targets for antifungal agents.[14] Furthermore, different strains of *C. albicans* have varying numbers of rDNA units, with some strains possessing over 200 copies,[17] making it an ideal target for molecular-based identification methods.

MOLECULAR TESTING

With an increasing number of cases of IC but lack of a sensitive detection method, it has become essential to develop techniques that overcome traditional limitations. Molecular methods detecting nonculturable/nonviable cells or circulating free fungal DNA have been developed, which may provide an alternative to culture-based

techniques. Owing to these culture limitations, most diagnostic molecular mycology techniques have focused on detecting IC, with little work performed on detecting superficial candidosis.[18,19] Although it is likely that the methods used to detect IC could detect a case of VC or OC, it is uncertain whether the result gained would provide any additional information, at extra expense, over culture. This is not the case for IC, where the samples tested should be from a sterile site and are unlikely to be culture-positive. With this in mind, the rest of the review will follow the current trend and concentrate on molecular testing for IC.

The field of diagnostic molecular mycology has, to a certain degree, been led by the Tubingen group, which has published methods using traditional molecular techniques,[20] polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) methods,[21] and real-time PCR methods[22] for detecting *Candida* but has also highlighted some of the limitations of molecular mycology in a diagnostic scenario.[23] However, many groups have now published alternative methods that provide equally sensitive and specific methods for different amplification platforms, or to meet different requirements (Table 1).

Before performing any amplification procedure, it is essential to obtain high-quality nucleic acid. With the current increased interest in diagnostic molecular mycology, numerous protocols for extracting fungal nucleic acids from various sample types have been published. The recommended specimen is ethylenediaminetetraacetic acid (EDTA) whole blood. Permitting the detection of free circulating candidal nucleic acids plus the extraction of nucleic acids from phagocytosed and circulating cells (lost in serum samples), it may generate higher fungal nucleic acid yields.

All whole blood fungal nucleic acid extractions have one thing in common—their laborious nature. All should have effective red cell and white cell lysis; mechanical, chemical, or enzymatic disruption of the fungal cell wall; lysis of the fungal cell membrane to release the nucleic acids; and then purification of the nucleic acids. In 1997, Loeffler et al.[24] published a combined heat–alkali–enzymatic extraction procedure that is widely used. Combining ''in-house'' steps with the commercial Qiagen Tissue kit, it achieved good sensitivity and could be performed within the working day. Originally, the method used the enzyme Zymolase to form spheroplasts; however, Zymolase can be contaminated with DNA from *S. cerevisiae*,[23] which would be problematical when performing PCR with pan-fungal/yeast primers/probes. To overcome this problem, recombinant lyticase has been extensively employed, although this increases the cost of the assay.

Recently, a semiautomated method using some ''in-house'' steps to lyse blood cells, mechanical disruption of the fungal cells by bead beating and automated nucleic acid release, and purification by the Roche MagnaPure has been presented.[25] It also achieves high sensitivity and reduces the hands-on time by approximately half, but does require the purchase of an expensive piece of equipment.

On obtaining high-quality nucleic acids, various molecular methods can be performed to test for the presence of candidal DNA (Table 1). More traditional molecular techniques for detecting candidal DNA have been highlighted previously.[26] Despite having good sensitivity and specificity, their major disadvantage is the need for postamplification handling, which increases the turnaround time and possibility of contamination.

With the development of many real-time nucleic acid amplification systems, many research works have focused on developing assays to detect invasive fungal infections. The detection of *C. albicans* DNA using the Roche Light-Cycler™ and hybridization (fluorescence resonance energy transfer, FRET) probes has given high sensitivities (5 cfu/mL whole blood) and specificities,[22] but the system was specific for *C. albicans* only and did not detect other pathogenic *Candida* species. More recent research overcame this problem by designing a single probe that could detect—but not differentiate—seven pathogenic *Candida* species to the same sensitivity.[26] Originally, the assay used the less familiar (first-generation) Biogene Light-Cycler™, but by slight modifications to the probe sequence and by changing the fluorescent labeling, the assay can now be used on the Roche Light-Cycler as a hydrolysis (TaqMan) probe system (Table 1).

Because of the limited number of detection channels on Light-Cyclers to date, multiplexing of PCRs has not been an option. However, by using the TaqMan PCR system, it is possible to detect and differentiate *C. albicans*, *Candida kefyr*, and *C. glabrata* in one reaction and *C. parapsilosis*, *C. tropicalis*, and *C. krusei* in a second multiplexed reaction.[27] The method provides a specific alternative to culture-based identification for clinical isolates and, although not stated in the publication, has good sensitivity (10^1 cfu) when extracting from whole blood (M. Guiver, personal communication).

A good alternative to PCR is isothermal amplification of RNA by Nucleic Acid Sequence-Based Amplification (NASBA).[28] Using avian myeloblastosis virus reverse transcriptase (AMV-RT), RNAse H, and T7 RNA, polymerase amplification occurs at 41°C, removing the need for a separate RT step or for PCR machines. Detection can be achieved by enhanced chemiluminescence (end point) or by molecular beacon (real time), and, like other molecular assays, NASBA has high sensitivity and specificity, but because of the ubiquitous nature of RNases, there may

Table 1 Recently published molecular methods used to detect *Candida* species

Method	Target	Primer sequences	Probe sequence/enzyme	PCR conditions
PCR/Southern blotting[20]	*C. albicans* 18S rRNA gene[a]	5′-ATT GGA GGG CAA GTC TGG TG-3′; 5′-CCG ATC CCT AGT CGG CAT AG-3′ (concentration 2 μM)	TCT GGG TAG CCA TTT ATG GCG AAC CAG GAC (concentration 30 pM)	50 μL reaction; 0.5 mM dNTPs, 1.0 mM $MgCl_2$, 1.5 U *Taq* polymerase; 35 cycles: 94°C/30 sec, 62°C/1 min, 72°C/2 min; 1 cycle: 72°C/5 min; blotting: alkali blotting to a nylon filter; antidigoxigenin–alkaline phosphatase labeling
PCR-RFLP[31]	P450 lanosterol 14 α-demethylase gene	5′-ATG ACT GAT CAA GAA ATY GCT AA-3′; 5′-TAA CCT GGA GAA ACY AAA AC-3′ (concentration 0.1 μM, approximation)	*Sau*3A, *Hin*cII, and *Nsi*I	100 μL reaction; 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 U *Taq* polymerase; 1 cycle: 94°C/3 min, 59°C/1 min, 72°C/1.5 min; 40 cycles: 94°C/45 sec, 59°C/1 min, 72°C/1.5 min; restriction fragment length polymorphism (RFLP) as instructed by manufacturer
FRET probes/ Light-Cycler PCR[22]	*C. albicans* 18S rRNA gene	5′-ATT GGA GGG CAA GTC TGG TG-3′; 5′-CCG ATC CCT AGT CGG CAT AG-3′ (concentration 0.625 μM)	5′-AGC CTT TCC TTC TGG GTA GCC ATT (fluorescein)-3′; 5′-($LC_{red\ 640}$) TGG CGA ACC AGG ACT TTT ACT TTG A-3′ (concentration 0.2–0.4 μM)	20 μL reaction; 1× Roche LC hybridization reaction mix (containing *Taq* polymerase, dNTPS), with 3 mM $MgCl_2$; 45 cycles: 95°C/1 sec, 62°C/15 sec, 72°C/25 sec[b]

TaqMan PCR[27]	*C. albicans* ITS2 region[c]	5′-GGG TTT GCT TGA AAG ACG GTA-3′; 5′-TTG AAG ATA TAC GTG GTG GAC GTT A-3′ (concentration 0.2 μM)	5′-(FAM) ACC TAA GCC ATT GTC AAA GCG ATC CCG (TAMRA)-3′ (concentration 0.1 μM)	25 μL reaction; 12.5 μL of TaqMan PCR Master Mix; 50°C/2 min, 95°C/10 min; 45 cycles: 95°C/15 sec, 60°C/1 min
Hydrolysis probe/ Light-Cycler PCR[26][d]	*Candida* species 18S rRNA gene	5′-CTC GTA GTT GAA CCT TGG-3′ 5′-GCC TGC TTT GAA CAC TCT-3′ (concentration 0.75 μM)	5′-(FAM) ATC TTT TTG ATG CGT ACT GGA CCC TG (TAMRA)-3′ (concentration 0.4 μM)	20 μL reaction; 1× Roche Faststart LC hybridization reaction mix (containing *Taq* polymerase, dNTPS), with 4 mM $MgCl_2$; 1 cycle: 95°C/15 min, up to 60 cycles: 95°C/15 sec, 62°C/30 sec
NASBA RNA detection[28]	*C. albicans*, *C. parapsilosis*, *C. tropicalis* 18S rRNA gene[e]	5′-ATG TCT AAG TAT AAG CAA TTT A-3′; 5′-*AAT TCT AAT ACG ACT CAC TAT AGG GAG AGA* CAT GCG ATT CGA AAA GTT A-3′[f] (concentration 0.27 μM)	5′-(Biotin) ATC CCG ACT GTT TGG AAG GGA TGT-3′	20 μL reaction (for buffer mix, see original article); incubate buffer mix at 65°C/5 min, 41°C/4 min, and then add enzyme mix (see original article); incubate at 41°C/90 min; NASBA products are detected by incubating with capture probe-coated magnetic beads

[a]By using different probes, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* can be detected.
[b]A melt program can be included. If the Faststart LC hybridization reaction mix is used, then an initial denaturation of 95°C/10 min should be included.
[c]By using different primers and probes, *C. glabrata*, *C. kefyr*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* can be detected. Multiplexing is also possible.
[d]Species detected by the probe: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. kefyr*, *C. krusei, C. parapsilosis*, and *C. tropicalis*. The system was originally developed for use on the Biogene Light-Cycler using a Cy5-labeled probe. By modifying the probe sequence and labeling, it can now be used on the Roche system. The conditions above are for the Roche system.
[e]By using different probes, *C. glabrata*, *C. krusei*, *Candida lusitaniae*, and *C. tropicalis* can be detected.
[f]Bases in italics represent the T7 promoter sequence.

be problems with target degradation, leading to false-negative results.

Interest in the epidemiology of fungal infections has grown concurrently with the interest in molecular diagnostics. However, the DNA fingerprinting of *Candida* is a field that should be addressed in detail, with the current review of Soll[29] being a good place to start.

CONCLUSION

With the limitations of culture techniques for detecting IC being well publicized, it is only a matter of time before nonculture techniques become routine. However, with molecular techniques still to be evaluated on a large scale and with the existence of other nonculture methods (e.g., ELISA), then the correct plan of action is still not clear and is far from being decided. Furthermore, with the existence of resistant *Candida* strains that would be identified if cultured, then traditional methods may still have benefits despite recent publications targeting resistant *Candida* species.[30]

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Capillary Electrochromatography

Vladislav Kahle
Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Brno, Czech Republic

INTRODUCTION

Capillary electrochromatography (CEC) is a quickly developing high-performance analytical separation method based on a combination of principles of liquid chromatography (LC) and capillary electrophoresis (CE). In CEC, the driving force dragging the mobile phase through the separation column is electroosmotic flow (EOF), which is induced by applied electric field. The method enables the separation of neutral analytes with selectivity typical of LC, whereas the charged analytes' migration velocity, high separation efficiency, and simplicity of instrumentation correspond to CE. Important progress was made in the column technology, allowing a burst of applications, particularly in pharmaceutical chemistry and biochemistry. In spite all of this, CEC at the present time is not considered a mature technique. Intensive development and associated lack of specialized instrumentation characterize the current situation. Nevertheless, it is obvious that CEC is a promising analytical tool in many important application areas.

BASIC CONCEPTS OF CEC

History

The roots of CEC can be traced back to several decades (e.g., Ref. [1]), but only the experiment of Jorgenson and Lukacs[2] demonstrated the real separation power of the new emerging analytical method. After that, Knox and Grant[3,4] used very small silica particles to gain extremely high separation efficiencies, and a steep growth of interest documented by an exponential increase in the number of articles began, giving a cumulative number of some 1300, to date. All this information is considered in several discussed general reviews[1,5–14] and monographs.[15–17]

EOF

In CEC, the EOF is harnessed to propel the mobile phase through the column unlike the pressure-driven flow in LC. EOF results from electrophoretic movement of the diffuse part of the electric double layer and its magnitude, measured as linear velocity u_{eo} by a neutral marker, are given by the Helmholtz–Smoluchowski equation:

$$u_{eo} = (\varepsilon_o \varepsilon \zeta E)/\eta$$

where ε_o is the permittivity of vacuum, ε is the dielectric constant of the mobile phase, ζ is zeta potential, E is electric field strength, and η is viscosity. In comparison with the pressure-driven parabolic flow in LC, the EOF is less dependent on the pore diameter and its flow profile is flatter, which both lead to a decrease of peak broadening and to a higher separation efficiency of CEC. Because the whole column operates as an electroosmotic pump, smaller sorbent particles and longer columns could be used in comparison with LC, which also facilitates a higher efficiency. The direction of EOF is from anode to cathode in the negatively charged silica-based packing. EOF of the opposite direction is also possible in packings with a positively charged surface (e.g., in polymeric monoliths containing quaternary ammonium groups).

Separation Mechanisms

Two basic mechanisms influence the migration velocity of analytes:[15,18] a partition between solid and liquid phases as in LC in the case of neutral analytes (characterized by capacity factor k) and electrophoretic migration as in CE for charged analytes (characterized by effective mobility $\mu_{eph,eff}$). Other separation mechanisms (e.g., ion exchange, size exclusion, chiral recognition, and molecular imprinting) can also participate.[14]

Figure 1 can be used to compare some separation characteristics of LC, CE, and the hybrid of both CEC: 1) the expected higher separation efficiency of CEC in comparison with LC is clearly visible in Fig. 1; 2) the higher selectivity of CEC compared with CE—originating from the broad range of partition coefficients, on one hand, and relatively small difference in solutes mobilities, on the other hand—is also obvious from Fig. 1; 3) in CEC, the separation window is wider ($-1<k<\infty$) than in LC ($0<k<\infty$) because of the increased velocity of charged solutes comigrating with EOF; and 4) the combination of two orthogonal separation mechanisms, viz. partition and

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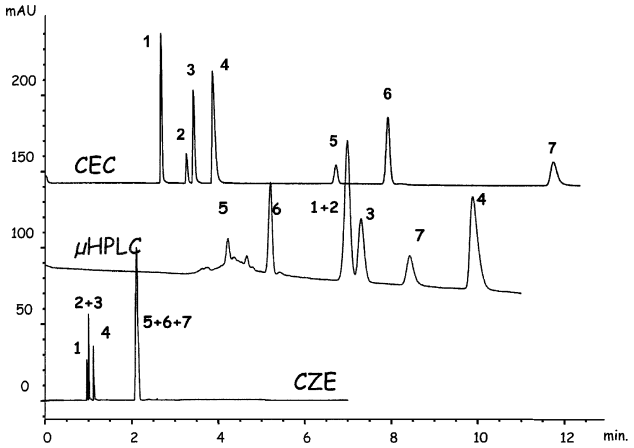

Fig. 1 Comparison of separation of drug mix by capillary electrochromatography (CEC), microliquid chromatography (μLC), and CE. Column (CEC and μLC): Spherisorb ODS-I, 3 μm, 250 (335) × 0.1 mm, (CE) uncoated fused silica capillary 250 (335) × 0.075 mm. Eluent: ACN 25 mM phosphate, 0.2% hexylamine, pH 2.5 (80:20), voltage (CEC and CE): 25 kV, pressure (μLC): 200 bar, temperature: 20°C. Peaks: 1=procaine, 2=timolol, 3=ambroxol, 4=metoclopramide, 5=thiourea, 6=naproxene, 7=antipyrine. (From Ref. [19].)

migration, gives rise to a much larger potential to manipulate selectivity.

Column Technology

A typical CEC column[1,5,6,10,20] is a fused silica capillary (50–100 μm ID, 200–500 mm long), filled either by particulate or monolithic[21] stationary phase. The small column internal diameter is dictated by the necessity of heat dissipation. Open-tube CEC columns (10–25 μm ID) were also used, but these suffer from smaller detection sensitivity. The basic problem of particle packed column is frit, which is unavoidable to keep the tiny sorbent particles (1.5–10 μm in diameter) in the original position. The ultimate solution here is the use of silica or organopolymeric monolithic columns.[17]

CEC INSTRUMENTATION

The setup of an electrochromatograph is, at least in principle, simple (Fig. 2). Identically as in LC and CE instruments, it comprises three basic parts: injector, separation column, and detector. The high-voltage power supply serves the mobile-phase propulsion through the column by EOF, instead of the pump used in LC. Mostly, either commercial electrophoreographs or laboratory-made devices are used,[22] still substituting specialized CEC instrumentation. Sometimes, a slightly more complicated setup incorporating hydraulic pressure capabilities is employed. The pressure can be applied the same either on both ends of the CEC column maintaining the bubble suppression, or on the injection side only. The latter method is called pressure-assisted CEC and forms a connecting link to pressure-driven LC.

For the injection, the dip of the end of the CEC column into the sample vial and subsequent electrokinetic sample sucking is frequently used (Fig. 2, position A), which is very simple but could bring about a decrease of repeatability. To overcome this problem, another sample injection method exploiting a sample and mobile-phase flow splitter was developed.[10,22]

Because of the predominantly chromatographic character of CEC separation, the mobile-phase gradient elution is important to attain higher peak capacity and shorter separation time, especially for complex samples

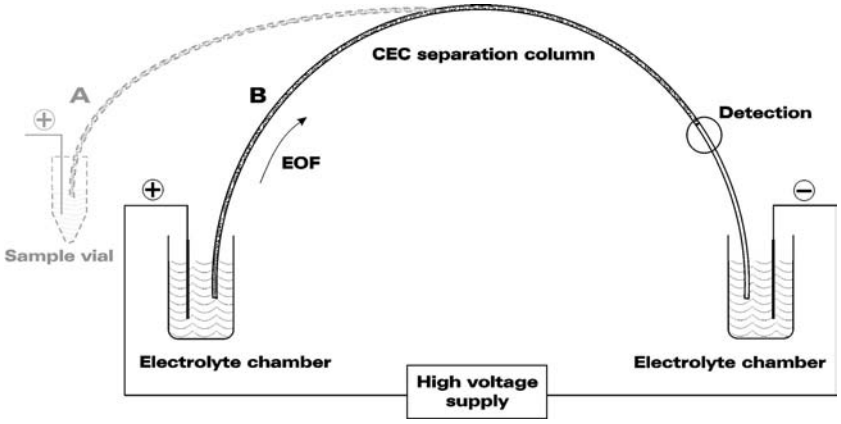

Fig. 2 Schema of electrochromatograph. (A) Electrokinetic sample injection. (B) CEC separation.

containing analytes of a wide polarity range. An array of gradient elution methods based on various principles was suggested.[7,10–12,22]

The methods, serving for detection of separated analytes, are in CEC essentially the same as in CE.[1,6–8,22] The on-column ultraviolet (UV) photometry is most often used but suffers from a decreased sensitivity because of the short optical path length. Methods increasing the path length (e.g., Z cell) are sometimes used, but increased peak dispersion and the resulting loss of efficiency are to be expected. The basic problem of the otherwise extremely sensitive (laser-induced) fluorometry is a lack of native fluorophores. Then the derivatization by reactive fluorescence tags comes into consideration,[22,23] bringing the benefit of a high-sensitivity detection of otherwise poorly detectable analytes. The instrumentally complex but highly specific and informationally rich method is the coupling of CEC to mass spectrometry,[17,22] which is especially important for proteomic studies.[24]

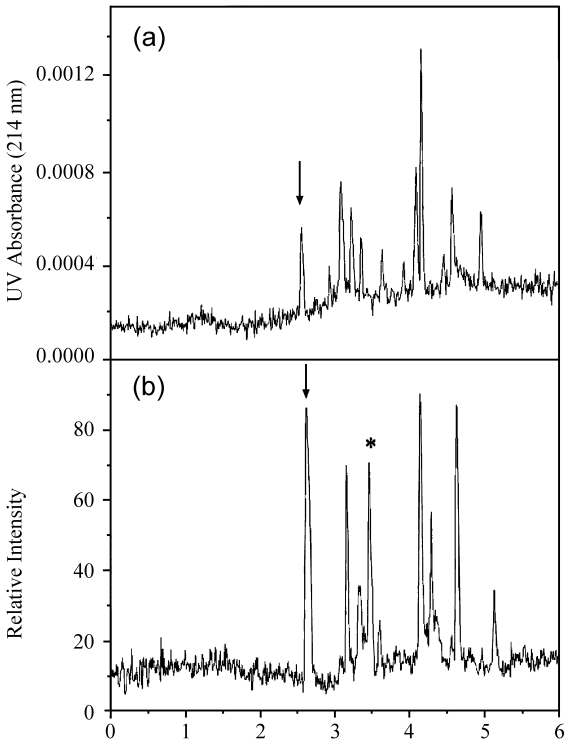

Fig. 3 UV trace (a) and TIC (b) of gradient CEC separation of a tryptic horse heart myoglobin digest. Conditions: 0–35% acetonitrile gradient in 6 min; column length, 40 cm (for UV detection, 35 cm to UV detector); separation voltage, −14 kV; injection, −2000 V × 5 sec; UV detection at 214 nm; MS detection speed, 8 Hz. (From Ref. [28], with permission from the American Chemical Society.)

APPLICATIONS

Originally, the CEC was developed for the separation of neutral analytes to extend the possibilities of CE, and a typical application in these early days was analysis of polynuclear aromatic hydrocarbons. Since then, the application range of CEC has increased dramatically and, nowadays, it covers analyses typical of LC but also of CE. Various previously mentioned separation modes were applied. Organic molecules of a wide range of functionalities; molecular weight; neutral, acidic, or basic origin; achiral or chiral origin; and synthesized or natural origin were successfully separated. Hundreds of application articles appeared in the literature and most of them were collected in several reviews.[1,7–9,11,25,26] The application of CEC in the pharmaceutical industry was critically reviewed.[27] From a proteomic point of view, the separation of peptides and proteins is of great importance (Fig. 3).

CONCLUSION

CEC is a modern and rapidly developing analytical separation method, often designated as a hybrid of electrophoresis and chromatography. The reasons for the growing popularity of CEC are high separation efficiency, simplicity of instrumentation, low operating costs resulting from miniaturization, low sample consumption, and expectations of selectivity tuning in the case of charged analytes. It is supposed that its importance will grow in connection with the solution of actual challenges, which are column technology, lack of specialized instrumentation, and better understanding of separation mechanisms, to name the most significant.

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Capillary Electrophoresis

Ronald J. A. Trent
Melody Caramins
Bing Yu
Royal Prince Alfred Hospital, Camperdown, Australia

INTRODUCTION

The range of DNA changes detected in diagnostic genomics include 1) single-base changes that include normal variations (DNA polymorphisms such as SNPs) and DNA mutations, 2) deletions that can be small (the ΔF508 three-base-pair deletion in cystic fibrosis) or very large (some examples of α° thalassemia), 3) rearrangements, e.g., the flip-tip on the X chromosome leading to hemophilia A, 4) sizing of simple tandem repeats such as microsatellites or expansions in triplet repeats found in a number of neurological disorders, and 5) epigenetic effects such as DNA methylation associated with gene silencing.

To detect these changes in DNA, four broad classes of DNA tests are used. These tests are based on 1) sizing, 2) hybridization, 3) sequencing, and 4) quantitation. For most DNA diagnostic assays, efficient and reproducible separation of DNA fragments and accurate sizing are key requirements. Traditionally, this has been undertaken by slab gel electrophoresis using polyacrylamide or agarose. These electrophoretic techniques are slow, and the accuracy is limited if visual inspection is used for sizing. As well as being time consuming, the traditional electrophoretic approaches are less amenable to automation.

Capillary electrophoresis is now replacing slab gel electrophoresis because it is faster, more sensitive and reproducible, and allows greater flexibility in the design of diagnostic DNA assays. The modern DNA laboratory will utilize capillary electrophoresis in conjunction with robotics to ensure that diagnostic DNA tests are efficiently and accurately undertaken.

PRINCIPLES OF CAPILLARY ELECTROPHORESIS

Capillary electrophoresis has proven to be a versatile technique for analyzing a wide range of analytes. Variations include 1) open tubular or capillary zone electrophoresis (CZE), 2) capillary gel electrophoresis (CGE), 3) micellar electrokinetic chromatography, 4) capillary isoelectric focusing, 5) capillary isotacophoresis, and 6) capillary electrochromatography (CEC).[1–3] For the purpose of this overview, CGE will be described to illustrate its utility in DNA analysis with a brief comment on other analytes. Capillary zone electrophoresis is used for the analysis of a wide range of charged simple organic molecules, inorganic ions, peptides, and proteins. Capillary gel electrophoresis is the method of choice for sizing and sequence analysis in the DNA diagnostic laboratory.

A simplified diagrammatic representation of CGE is given in Fig. 1. A constraint with the traditional slab gel electrophoresis is the heat generated by the applied voltage, which is limited to 5–40 V/cm. In contrast, a capillary is more effective in dissipating heat, thereby allowing higher voltages (up to 300 V/cm). This leads to shorter running times and sharper bands. The migration of an analyte is influenced by size, charge, and unique to CGE, is the electroosmotic flow (EOF). Only picogram amounts need to be loaded as the detection system can use a laser–CCD camera system. Software will allow the appropriate loading and running conditions to be selected and is also used for analysis and interpretation of the results.

THE CAPILLARY

Capillaries can be made of fused silica, glass, or Teflon. The most popular is fused silica, which is externally coated with polyimide to give it greater strength and flexibility. The polyimide layer is usually removed at the detector end to enable online detection. Capillary lengths range from 10 to 100 cm, and internal diameters vary from 20 to 100 μm. These narrow bores improve heat dissipation due to a higher surface area to volume ratio.

Manufacturers recommend that capillaries are changed after a defined number of injections in diagnostic work. In our laboratory, the capillaries are replaced after 200 injections. Multichannel CGE, such as that used in the ABI Prism 3700 DNA Analyzer®, allows simultaneous

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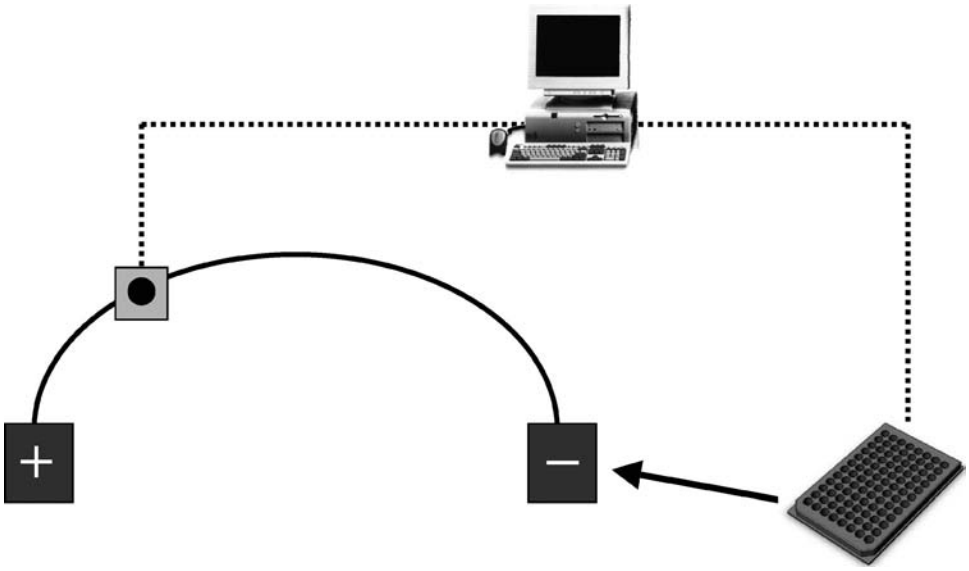

Fig. 1 Simple representation of a CGE apparatus. The + and − represent the anode and cathode, respectively, joined through a capillary (solid line). The sample is loaded automatically and the end products are detected through a laser–CCD system (▣). Software allows the injection and electrophoresis conditions to be selected, and fragments to be characterized based on their size and color. (*View this art in color at www.dekker.com.*)

analysis of information from 96 capillaries, thereby facilitating high-throughput DNA analysis.

SAMPLE LOADING

Capillary gel electrophoresis requires smaller amounts of sample compared to slab gels (nanoliters compared to microliters) to avoid excessive band broadening.[4] Loading is automated, and injection volume can be controlled and standardized by varying the force and time. Samples can be loaded in a number of ways including hydrodynamic or electrokinetic injections. For hydrodynamic loading, the capillary is subjected to a pressure difference between its extremities. The pressure difference can result from gravity, application of pressure inside the sample vial, or aspiration of the sample through the capillary tube. Hydrodynamic injection produces more reproducible sample loading. For electrokinetic injection, the capillary tip is dipped into the sample for a fixed time and a potential is applied. Electrokinetic injection results in the best flow profiles but can give a biased sample because more mobile ions can be preferentially introduced.[4]

SAMPLE MIGRATION

The mobility of sample ions under the influence of applied voltage is dependent on their charge/mass ratio. Therefore, when separating a sample, smaller, more highly charged ions will be detected first, followed by larger, lesser-charged ions. Application of voltage across a capillary filled with electrolyte causes a bulk EOF of solution along the capillary.[2] This results from a characteristic of fused silica capillaries in which the inner wall becomes covered with acidic silanol groups. The degree of EOF is dependent on the pH. At a pH below 4, the ionization is small, and so the EOF flow rate is not significant. Silanol groups become fully ionized at pH>9, which produces a strong EOF. To maintain electrical neutrality, cations build near the surface. Under the influence of an applied voltage, these cations, and the water molecules dissolving them, migrate toward the cathode, resulting in a net solution flow along the capillary. At pH of 7 or more, the EOF is strong enough to sweep anions to the cathode regardless of their charge, thereby allowing separation and analysis of analytes with mixtures of size and charge.[2]

If EOF is undesirable for analysis, it can be reduced or eliminated by coating the internal surface of the capillary or adding surfactants and/or buffer additives to the separation buffer.[4] Reducing or eliminating the EOF leads to a more efficient and reproducible separation of DNA fragments. Without the effect of EOF, the order in which DNA fragments migrate in gels with CGE is dependent on their molecular size, with smaller fragments having a shorter migration time (like slab gel electrophoresis).[3]

POLYMER GEL MATRIX

Capillary gel electrophoresis can be used for separation of small DNA fragments such as nucleotides or small oligonucleotides (10–150 bases in size).[3] For larger

DNA fragments, electrophoresis must be undertaken with a separation matrix such as agarose, polyacrylamide, or a liquid polymer to act as a molecular sieve. For CGE, there are two types of gel matrices in use: relatively high viscosity cross-linked (i.e., chemical) or low viscosity non-cross-linked (i.e., physical). The latter are more flexible, and hence widely used because of the following properties: 1) they can operate at more extreme temperatures, and 2) they are easily replaced by rinsing the capillary, thus minimizing contamination and enhancing convenience. With both types of gel matrices, capillaries that are modified by coating agents can be used to eliminate the EOF.[4]

Commercially produced polymers are available. Liquid polymers made in-house are cheaper but formulation is more likely to vary between batches thereby affecting reproducibility and sensitivity. Although costly, commercial polymers are produced to specific standards and consistent quality, which are important considerations in the DNA diagnostic laboratory.

Products such as glycerol, mannitol, urea, and formamide can be added to the sieving media for different purposes. For example, 5% glycerol can be used to preserve secondary structures when SSCP analysis is performed. Formamide is used to keep double-stranded DNA denatured when DNA fragment analysis is required.

DETECTION SYSTEMS

Ultraviolet (UV)–visible based detection is used on many CGE applications. This type of system is applicable to a wide range of analytes, and is sensitive enough for many applications. However, the detection limit with UV is limited because of the small internal diameter of the capillary. For example, with this mode of detection, a DNA fragment of approximately 200 bp would need to have a concentration of about 0.5 μg/mL.[3] Therefore, detection systems based on laser-induced fluorescence are preferred if lower detection limits are needed.

Laser-induced fluorescence is also attractive because it allows the detection of multiple parameters in the same tube; that is, multiplexing and DNA sequencing become possible. DNA primers can be labeled with different fluorophores. On passing through the detection window in the capillary, the fluorescein-labeled polymerase chain reaction (PCR) products are excited by the laser and emit fluorescence with various emission spectra depending on the dye. The emitted fluorescence is detected by a CCD camera and the information is relayed to the computer, where data collection software processes it and displays the information as a graph of fluorescence color and intensity against size. Multicolor fluorescence detection also allows the use of in-lane size standards, thereby increasing accuracy and reproducibility when unknown samples are being sized.

DNA MUTATION DETECTION

Capillary gel electrophoresis is ideal for detecting DNA fragment sizes that include small or large deletions, insertions, allele-specific amplification fragments, and oligonucleotide ligation or primer extension products. For example, a common mutation in the genetic disorder cystic fibrosis is ΔF508 mutation. This involves a 3-bp deletion in the *CFTR* gene. To detect this deletion by CGE, we label the forward primer with FAM, a fluorophore that emits a blue light spectrum on laser excitation. After PCR amplification, the product is separated on CGE. The mutant, shorter allele emits a blue fluorescence at 91 bp, compared with the normal allele that produces a peak of blue fluorescence at 94 bp. The two peaks are easily distinguishable. As well as separating wild type and mutant alleles on the basis of size, it is possible to distinguish point mutations on the basis of color. This is illustrated by SNP analysis (Fig. 2a).

For DNA sequencing, the four terminating dideoxynucleotides are each labeled with different flurophores. Each time a ddNTP is incorporated into the extending product, it results in termination of product extension, with the terminal nucleotide identified by its fluorescent label. The final product is composed of a heterogeneous population with different-sized products that can be distinguished by their various spectral emissions. With the fluorescent labeling, the entire reaction can be run in the same capillary; that is, multiplexing is possible, which was also illustrated in the earlier SNP example.

GENOTYPING

Applications for genotyping are extensive, ranging from linkage mapping for research or diagnostic purposes to forensic or paternity testing, microbiological typing, and veterinary or agricultural trait mapping.[5–7] Electrophoretic conditions with high precision are essential in many of these applications. For 2-bp microsatellites, standard deviations of ±0.3 nucleotide are required to ensure with 99.7% probability that the tested alleles are identical or different.[8] This level of precision is achievable with CGE.

Capillary gel electrophoresis can be problematic when it is used to determine the absolute size of a DNA fragment. This is illustrated by DNA triplet repeat expansions, such as those in Huntington disease.[9]

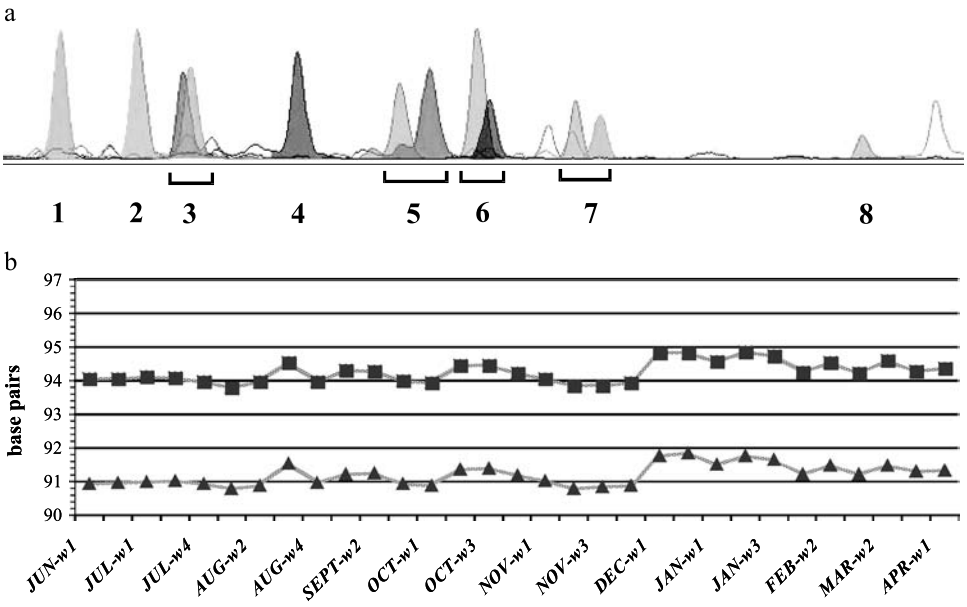

Fig. 2 (a) Detecting eight SNPs in the 5′UTR and intron 2 of the *MT3* gene. The various alleles are numbered 1–8 and demonstrate the utility of CGE in multiplexing and distinguishing various fragments on the basis of size and/or color. SNPs #3 and #6 illustrate how comigrating alleles for each SNP are distinguishable by primers labelled with two different fluorochromes. SNPs #4 and #6 are distinguishable by their migratory distance. (b) QA graph for normal and mutant (ΔF508) fragments for cystic fibrosis. It can be seen that over a period of 10 months, there has been little drift outside the acceptable limit of 1 bp for the mutant (91 bp) and normal (94 bp) fragments. (*View this art in color at www.dekker.com.*)

Although the resolving power of CGE can distinguish variations in DNA fragments as small as 1 bp, the sizing of a DNA fragment can be a potential source of error. With the Huntington disease example, we observed that the sizes of triplet repeats determined through CGE were always one to two repeats (3–6 bp) less than the gold standard obtained by the ^{32}P slab gel technique.[10] This discrepancy is unacceptable in the above triplet repeat scenario for which an accurate measure of the repeat number is essential because the cutoff for predicting the development of disease occurs exactly at 40 repeats.

Sizing in CGE depends on the algorithm that converts mobility data for an unknown peak into size information based on size vs. mobility results from the size standards.[11] Incorrect size calls can come from poor resolution of two or more peaks, and of particular relevance to CGE, the aberrant mobility of unknown peaks. Incomplete denaturation leading to sequence-specific secondary structures is a recognized cause of aberrant mobility and can be avoided by optimizing the denaturing conditions in CGE.[11] Another way around this potential problem is to normalize the automated size measurements to known fragment sizes. We take the latter approach in Huntington disease by incorporating a CAG allelic ladder with known CAG fragment sizes as an additional sizing standard, thereby allowing exact sizing of the triplet repeat.[12]

QUALITY ASSURANCE

Technology based on CGE facilitates QA activities. In the laboratory, regular monitoring of graphs representing the known PCR product sizes for the various assays are kept for these purposes (Fig. 2b). A drift of more than 1 bp in the case of cystic fibrosis testing for the ΔF508 mutation is unacceptable, requiring the controls to be rerun, and requiring a review of all reagents. A persisting drift could indicate the possibility of hardware or software problems.

OTHER APPLICATIONS

Although we have focused on the genomic uses of CGE, the technology is universally applicable to the separation of a wide range of analytes including peptides, proteins, carbohydrates, drugs for clinical or forensic applications, as well as applications in biotechnology and environmental analysis.[13–15]

The review by Thormann[13] compares capillary electrophoresis to current analytical approaches in licit and illicit drug assays including high-performance liquid chromatography (HPLC), gas chromatography, and high-throughput automated immunoassays and photometric assays. It concludes that capillary electrophoresis has yet

Table 1 Some examples of capillary electrophoresis platforms

Manufacturer	Instrument	Web site
Applied Biosystems	Options from a single capillary to combinations of 4, 16, 48, and 96 capillaries. The most recent platform (3730) incorporates a new detection technology as well as allowing active control of running temperature from 18°C to 70°C. The option to control the running temperature provides an additional variable for DNA mutation testing, e.g., a technique such as SSCP	http://www.appliedbiosystems.com/
Beckman Coulter	P/ACGE-MDQ is a single-capillary system designed for general uses for protein, organic compounds, and nucleic acid analyses. CGEQ 8000 is an eight-capillary system	http://www.beckmancoulter.com/
SpectruMedix	96-channel capillary system capable of handling six different sample trays and up to 30-color capacity	http://www.scientificassociates.8m.com/

to realize its full potential, and is more likely to complement the existing technologies than replace them. However, the reproducibility coupled with the potential to perform drug measurements in nanoliter volumes of body fluids is an attractive option in a clinical chemistry, pharmacology, or forensic laboratory setting.

CONCLUSION

Capillary gel electrophoresis has revolutionized the work flow in the DNA diagnostic laboratory. It provides opportunities for increasing the level of automation at all steps from sample receipt to the generation of a computer-based result. As well as its inherent high sensitivity, the robustness and excellent reproducibility of this technology are critical parameters in an environment requiring accuracy and ongoing QA. Improved efficiency and faster turnaround times come from the multiplexing potential available from the use of fluoroscein-labeled primers or capillary arrays. From the health and environmental perspective, the avoidance of potentially hazardous reagents such as acrylamide and ethidium bromide used in conventional DNA slab gel electrophoresis is an additional benefit of CGE. Hardware and CGE reagent costs are high, but this is offset by the saving in time and human resources. There are a number of platforms now available, and increasing competition will ensure that costs continue to fall (Table 1). A wider range of instrumentation options is described in Ref. [16].

This overview started with a description of six types of capillary electrophoresis. However, there is considerable potential for innovation with this technology. As the Human Genome Project expands the opportunities in proteomics, combinations of capillary electrophoresis with techniques such as mass spectrometry will identify novel ways in which to analyze proteins and peptides.[14] Developments in nanotechnology coupled with capillary electrophoresis (microchip capillary electrophoresis) will mean faster analysis times, decreased costs and waste, and portability that would open up options for point-of-care DNA diagnostic testing.[17]

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Cardiomyopathy, Dilated (Familial)

Dagmar I. Keller
Gisèle Bonne
INSERM U582, Paris, France

INTRODUCTION

Dilated cardiomyopathy (DCM) is a cardiac disorder characterized by dilatation of the left ventricle (LV) or both ventricles, with impaired systolic function. Familial DCM (FDCM) accounts for 25% of DCM. The clinical course of DCM/FDCM is determined by heart failure and arrhythmia with high morbidity and mortality despite progress in disease management. FDCM is inherited autosomal-dominant, autosomal-recessive, or X-linked. Mutations in at least 19 genes have been identified, indicating wide genetic heterogeneity and resulting in pure FDCM or distinct phenotypes of FDCM with other cardiac, muscle, or specific pathologies. Diseased proteins are localized in different cellular compartments. Because of wide phenotype and genotype heterogeneity, precise phenotype–genotype correlations are not available yet and predictors for clinical outcome are still largely undetermined.

CLINICAL DESCRIPTION

DCM is defined by the World Health Organization (WHO) as myocardial disorder ''characterized by dilatation and impaired contractility of the left ventricle or both left and right ventricles. It may be idiopathic, familial/genetic, viral and/or immune, alcoholic/toxic, or associated with recognized cardiovascular disease in which the degree of myocardial dysfunction is not explained by the abnormal loading conditions or the extent of ischemic damage. The histological findings are frequently nonspecific. The usual presentation of the disease is heart failure, which is often progressive. Arrhythmias, thromboembolism, and sudden death are common and may occur at any stage.''[1]

The prevalence of DCM in the general population is 40–50 cases per 100,000. Almost 50% of DCM cases are idiopathic, with an incidence of five to eight cases per 100,000 per year.[2] At least 25% of DCM cases are familial,[3] inherited most frequently autosomal-dominant, besides autosomal-recessive and X-linked forms. FDCM is clinically heterogeneous with distinct phenotype groups arising from various inheritance patterns.[4] FDCM can be pure or combined with other cardiac diseases, in particular conduction system defects, skeletal myopathies, and sensorineuronal deafness, or in the context of inherited multisystem diseases. In fatal infantile forms of FDCM, mitochondrial DNA mutations have been found.

Besides family history, no clinical parameter can distinguish familial from nonfamilial DCM.[5] Both forms are diagnosed by transthoracic echocardiography for assessment of LV structure, dimension, systolic/diastolic function, and heart valve morphology/function. Hallmark findings are marked dilatation of the LV cavity with normal wall thickness and globally reduced systolic function. Regional wall abnormalities do not exclude FDCM/DCM. Electrocardiogram (ECG) and ambulant ECG monitoring reveal conduction system defects such as atrioventricular conduction blocks, sinus node dysfunction, and atrial and ventricular tachyarrhythmias.

FDCM/DCM is a leading cause of heart failure and arrhythmia. Symptoms of congestive heart failure are dyspnea on exertion, decreased exercise tolerance, orthopnea, paroxysmal nocturnal dyspnea, fatigue, edema, and abdominal distension. Chest pain results from limited coronary vessel reserve. Ventricular arrhythmia leads to palpitations, syncope, and sudden cardiac death (SCD). Severe LV dilatation and dysfunction result in thromboembolic complications.

Despite progress in therapy, 5-year mortality is high (between 15% and 50%). SCD accounts for 30% of deaths and occurs irrespective of LV systolic function.

DIFFERENTIAL DIAGNOSIS

FDCM is distinguished from other specific cardiac diseases and systemic processes resulting in secondary ventricular dilatation and dysfunction:[1] eccentric hypertensive cardiomyopathy, ischemic cardiomyopathy, decompensated valvular heart disease, alcoholic cardiomyopathy, and myocarditis. Less common are cardiomyopathies resulting from amyloidosis, sarcoidosis, hemochromatosis and other metabolic disorders, and peripartum cardiomyopathy. Doxorubicin can cause toxic cardiomyopathy.

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MANAGEMENT

Pharmacological therapy is the standard approach in heart failure treatment.[6,7] Angiotensin-converting enzyme (ACE) inhibitors reduce preload and afterload, and wall stress. ACE inhibitors reduce mortality, hospitalization rate, and progression of heart failure, and are indicated for all patients with systolic LV dysfunction. Angiotensin II receptor antagonists have equal effects on symptoms but without the bradykinin-related side effects of ACE inhibitors. Reduction in mortality has been shown for these drugs. The major advancement in heart failure therapy is the use of low-dose β-adrenergic blockers. The effect of β-blockade on mortality is additional to the effect of ACE inhibitors, and benefit is independent of the severity of heart failure. Digitalis is a positive inotropic agent and provides symptomatic benefit without effect on mortality. Diuretics are indicated in symptoms of volume overload despite the use of ACE inhibitors and digitalis. Spironolactone is a potassium-sparing diuretic agent and has been shown to reduce mortality. First-generation calcium channel antagonists are contraindicated in standard treatment guidelines in heart failure because they reduce survival rate and exacerbate symptoms.

Anticoagulants or antiplatelet agents are recommended in LV ejection fraction less than 30%. Atrial fibrillation requires chronic anticoagulation.

Cardiac resynchronization with biventricular pacing improves symptoms in advanced heart failure. Patients with impaired systolic function and at high risk for SCD benefit from biventricular pacing properties of an implantable cardioverter defibrillator (ICD). Implantable cardioverter defibrillator implantation is recommended in *LMNA* mutation carriers. Higher-degree atrioventricular conduction defect requires pacemaker therapy. Benefits of LV assist devices are tempered by a high complication rate. Heart transplantation in end-stage disease improves survival and quality of life. Monitored physical conditioning with general education improves exercise capacity and quality of life.

MOLECULAR GENETICS

Familial DCM is genetically heterogeneous because various inheritance patterns are involved, and many chromosomal loci and disease genes have been identified by genetic studies in affected families (Table 1).

Autosomal-Dominant FDCM

Fifteen loci and 12 genes, encoding proteins of different cellular compartments, have been identified to be involved in autosomal-dominant FDCM with pure DCM. δ-Sarcoglycan, encoded by *SGCD*, is one of the four (α, β, δ, and γ) proteins forming the sarcoglycan complex—a component of the glycoprotein transmembrane complex (DCG) in the plasma membrane. *DES* and *VCL* encode cytoskeletal proteins, desmin, an intermediate filament, and metavinculin, respectively. *PLN* encodes phospholamban in the sarcoplasmic reticulum, a major substrate for cAMP-dependent protein kinase in cardiac muscles. It is an inhibitor of cardiac muscle sarcoplasmic reticulum Ca^{2+}-ATPase. Mutations have been identified in sarcomeric proteins: actin encoded by *ACTC*, cardiac troponin T encoded by *TNNT2*, cardiac myosin-binding protein C encoded by *MYBPC3*, β-myosin heavy chain encoded by *MYH7*, α-tropomyosin encoded by *TPM1*, and titin encoded by *TTN*. Telethonin/TCap is encoded by *TCAP* and is a substrate of titin. TCap interacts and colocalizes with the muscle limb protein (MLP), which is a Z-disc protein encoded by *CSPR3*. Recently, MLP mutations have been identified in DCM families.[8]

Four disease loci have been related to autosomal-dominant FDCM with a conduction defect. The only gene identified is *LMNA* encoding lamins A and C, components of the nuclear envelope, produced by alternative splicing.[9] *LMNA* mutations have been found in FDCM associated with atrial fibrillation[10] and in FDCM with LV apical aneurysm.[11] For autosomal-dominant FDCM with skeletal myopathy and conduction defect, the only identified disease gene is again *LMNA*. Mutations are associated with autosomal-dominant Emery–Dreifuss muscular dystrophy (AD-EDMD)[12] and limb girdle muscular dystrophy type 1B (LGMD1B), and were recently described in a myopathy specifically affecting the quadriceps muscle.[13]

Autosomal-Recessive FDCM

Autosomal-recessive FDCM is rare and cosegregates with skeletal myopathies. Mutations in four genes have been identified. A homozygous *LMNA* mutation was identified in autosomal-recessive Emery–Dreifuss muscular dystrophy (AR-EDMD).[14] *SGCB* encodes β-sarcoglycan, a component of the sarcoglycan complex linking the extracellular matrix to the dystrophin. Mutations in *FKRP* were identified in two allelic disorders: a form of limb girdle muscular dystrophy (LGMD2I) and a form of congenital muscular dystrophy (MDC1C). *FKRP* encodes the fukutin-related protein, a newly identified protein implicated in glycosylation of α-dystroglycan, another component of the DCG complex.[15,16] Mutations in *DSP*, encoding desmoplakin, a protein of the desmosome complex, cause DCM associated with wooly hair and keratoderma.

Table 1 DCM genes and phenotypes

Inheritance	Chromosome	Gene (OMIM number)	Protein	Phenotype
AD	1q32	*TNNT2* (191045)	Cardiac troponin T	Pure DCM
AD	2q31	*TTN* (188840)	Titin	Pure DCM
AD	2q35	*DES* (125660)	Desmin	Pure DCM
AD	5q33	*SGCD* (601411)	δ-Sarcoglycan	Pure DCM
AD	6q22.1	*PLN* (172405)	Phospholamban	Pure DCM
AD	10q22.1	*VCL* (193065)	Metavinculin	Pure DCM
AD	11p11.2	*MYBPC3* (600958)	Cardiac myosin-binding protein C	Pure DCM
AD	11p15.1	*CSRP3* (600824)	Muscle limb protein	Pure DCM
AD	14q12	*MYH7* (160760)	β-Myosin heavy chain	Pure DCM
AD	15q14	*ACTC* (102540)	α-Actin	Pure DCM
AD	15q22.1	*TPM1* (191010)	α-Tropomyosin	Pure DCM
AD	17q12	*TCAP* (604488)	Telethonin/TCap	Pure DCM
AD	6q12–q16			Pure DCM
AD	9q13			Pure DCM
AD	9q22–q31			Pure DCM
AD+C	1q21.2	*LMNA* (150330)	Lamins A and C	DCM+CD
AD+C	1q21.2	*LMNA* (150330)	Lamins A and C	DCM+atrial fibrillation
AD+C	1q21.2	*LMNA* (150330)	Lamins A and C	DCM+apical aneurysm
AD+C	2q14–q22			DCM+CD
AD+C	3p22–25			DCM+sinus node dysfunction
AD+C	10q21–q23			DCM+mitral valve prolapse
AD+C+M	1q21.2	*LMNA* (150330)	Lamins A and C	AD-EDMD
AD+C+M	1q21.2	*LMNA* (150330)	Lamins A and C	LGMD1B
AD+C+M	1q21.2	*LMNA* (150330)	Lamins A and C	DCM+quadriceps myopathy
AD+C+M	6q22–23			DCM+CD+LGMD
AD+C+O	6q23–q24			DCM+CD+hearing loss
AR+C+M	1q21.2	*LMNA* (150330)	Lamins A and C	AR-EDMD
AR+M	4q12	*SGCB* (600900)	β-Sarcoglycan	LGMD+severe DCM
AR+M	19q13.3	*FKRP* (606596)	Fukutin-related protein	DCM of LGMD2I
AR+M	19q13.3	*FKRP* (606596)	Fukutin-related protein	DCM of MDC1C
AR+O	6p24	*DSP* (125647)	Desmoplakin	DCM+wooly hair/keratoderma
XL	Xp21.2	*DYS* (300377)	Dystrophin	Pure DCM
XL	Xq28	*TAZ* (300394)	Tafazzin	DCM fatal in infancy
XL+M	Xq28	*TAZ* (300394)	Tafazzin	DCM of Barth syndrome
XL+M	Xq28	*EMD* (300384)	Emerin	XL-EDMD
XL+M	Xp21.2	*DYS* (300377)	Dystrophin	DCM of DMD
XL+M	Xp21.2	*DYS* (300377)	Dystrophin	DCM of BMD

AD, autosomal dominant; C, cardiac; M, muscular; O, other; AR, autosomal recessive; XL, X-linked; DCM, dilated cardiomyopathy; CD, conduction defect; EDMD, Emery–Dreifuss muscular dystrophy; LGMD, limb girdle muscular dystrophy; MDC, congenital muscular dystrophy; DMD, Duchenne-type muscular dystrophy; BMD, Becker-type muscular dystrophy.
Number in parentheses represents an accession number in Online Mendelian Inheritance in Men (OMIM) (http://www.ncbi.nlm.nih.gov).

X-linked FDCM

X-linked FDCM leading to pure DCM is associated with mutations in the cytoskeletal protein dystrophin, encoded by *DYS*. Mutations were identified in Duchenne and Becker-type muscular dystrophy, in which DCM is a constant feature. Mutations in tafazzin, a protein of unknown function encoded by *TAZ*, result in early-onset DCM with lethality in infants and Barth syndrome. Emerin, an integral protein of the inner nuclear membrane binding to lamin A/C, is encoded by *EMD* and is associated with X-linked Emery–Dreifuss muscular dystrophy (XL–EDMD).

GENETIC TESTING

Mutations in disease genes can be detected after gene amplification by polymerase chain reaction (PCR), followed by a mutation screening technique (single-strand

conformation polymorphism, denaturing high-performance liquid chromatography, or others) and finally by sequencing. These techniques are highly specific, if different conditions are analyzed and if the PCR primers largely surround the exons and important intronic sites. The sensitivity depends on several factors, particularly one cannot exclude the implication of another as-yet identified gene and/or that PCR primers may hide a point mutation.

MOLECULAR MECHANISMS

Mutations in *LMNA* produce up to eight different disorders, now called laminopathies, and it is not clear how they do so. To date, 35 *LMNA* mutations have been identified in autosomal-dominant FDCM with conduction disease without associated skeletal symptoms: 23 missense and 6 nonsense mutations, and 6 insertions/deletions with frameshift leading to truncated proteins. These mutations are located all along the gene, with 31 being common to both lamins A and C, 1 being specific for lamin C, and 3 being specific for lamin A. More than 101 *LMNA* mutations have been identified in families with autosomal-dominant skeletal myopathies (EDMD or LGMD1B), in which DCM-conduction defect is a constant feature at an adult age. These mutations are located all along the gene. Thus there is no specific ''DCM-restricted domain'' in lamin A/C.

Mutations in sarcomeric genes can cause both FDCM and familial hypertrophic cardiomyopathy (FHC). Sarcomeric gene mutations in the two distinct clinical entities can result in energetic compromise and alterations in myofibrillar calcium sensitivity, suggesting a unifying pathophysiological mechanism in both FDCM and FHC.

Mutations in cytoskeletal proteins leading to FDCM are thought to produce reduced force transmission from the sarcomere to the extracellular matrix (for review, see Ref. [17]).

Mechanisms by which FDCM is associated with skeletal symptoms still need to be elucidated. A possible key role might be attributed to modifier genes, which influence phenotype expressivity. Environmental factors may contribute to phenotypical variability.

GENOTYPE–PHENOTYPE CORRELATION

Genotype–phenotype correlations are not yet determined for the majority of disease genes because of the wide phenotype and genotype heterogeneity and the lack of informational data obtained from families carrying the same FDCM gene mutation. Data about natural disease history are available for *LMNA* mutations.[18] Compared to noncarriers, FDCM patients with *LMNA* mutations had significantly poorer cumulative survival with more severe and progressive DCM.

GENETIC COUNSELING

Genetic counseling is preliminary and under development. Limitations are wide genetic heterogeneity and lack of genotype–phenotype correlation. Screening of first-degree relatives focuses on history, physical examination, and clinical tests. If a disease mutation is known in a family, genetic testing can be used for evaluation of relatives at risk for developing FDCM. Noncarriers might be reassured that they will not develop FDCM in the context of a specific mutation. The complex genotypical and phenotypical variability underlines the necessity for further extensive genotype–phenotype correlation studies on large families.

CONCLUSION

FDCM shows a wide genotype heterogeneity with various inheritance patterns and a large number of involved genes. The major goals are to identify all genes implicated in FDCM, to study large informative families, to correlate genotypes and phenotypes, and to identify patients at high risk for SCD. Experimental approaches using animal models will help in elucidating pathophysiological mechanisms involved in disease development.

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Cardiomyopathy, Hypertrophic (Familial)

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Dagmar I. Keller
Lucie Carrier
INSERM U582, Paris, France

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a myocardial disease characterized by left and/or right ventricular hypertrophy and dysfunction. HCM shows wide phenotypical heterogeneity with regard to age of onset, degree and distribution of hypertrophy, and type and severity of clinical manifestations. The familial form of HCM (familial hypertrophic cardiomyopathy, or FHC) is genetically heterogeneous. The typical form is caused by mutations in 12 different genes encoding proteins of the contractile apparatus. The variability of the phenotype depends not only on the major gene mutation, but on the complexity of the genetic status and modifier genes. Molecular genetic studies have given insights into the pathogenesis of FHC and have elucidated ''poison peptide'' and ''haploinsufficieny'' as molecular mechanisms. A common underlying mechanism could be energy depletion with a key role in hypertrophy development.

CLINICAL DESCRIPTION

HCM is a primary myocardial disease defined by the World Health Organization (WHO) as ''left and/or right ventricular hypertrophy, which is usually asymmetric and involves the interventricular septum.''[1] Histological features include myocyte hypertrophy and disarray, and increased interstitial fibrosis.

The diagnosis of HCM is made by clinical examination, electrocardiogram, and transthoracic echocardiography for the detection of unexplained left ventricular hypertrophy (LVH) greater than 13 mm in the absence of another cardiac or systemic disease. LVH is typically asymmetric with a diffuse or segmental (subaortic, midventricular, or apical) pattern and is usually associated with a hyperdynamic left ventricle (LV).[2] The differentiation of HCM from physiologically mild LVH, such as in athletic hearts, is crucial. Distinction in a nonobstructive or obstructive form of HCM is based on the presence or absence of a systolic outflow tract gradient under resting and/or provoking conditions. Symptoms in HCM are complex.[3] Exertional dyspnea, typical or atypical angina, and loss or impairment of consciousness are produced by multiple mechanisms. LV outflow tract obstruction depends on both the LV contractility state and loading condition. Mitral insufficiency from distortion of the mitral valve apparatus can be severe and can contribute to symptoms of heart failure. Diastolic dysfunction is a hallmark finding in HCM and can lead to diastolic heart failure. Myocardial ischemia results from a misbalance of oxygen demand/supply in the hypertrophied myocardium even in the absence of coronary artery disease. Sustained ventricular tachycardias and ventricular fibrillation can lead to syncope and sudden cardiac death (SCD); embolic complications of atrial fibrillation may increase morbidity.

The clinical course of FHC does not follow a unique pattern. The age of onset varies widely between 15 and 60 years, depending on the genetic background. SCD is often the first manifestation in young individuals and competitive athletes. Patients can exhibit no or only mild symptoms over the long term, or the disease can progress to systolic dysfunction and heart failure.

DIFFERENTIAL DIAGNOSIS

All other causes of LVH have to be excluded as longstanding, insufficiently treated or untreated hypertension and aortic stenosis. The distinction of LVH as physiological adaptation in athletes is mandatory. Systemic disease with infiltrative heart processes can cause an HCM-like phenotype: amyloidosis, Noonan's syndrome, multiple lentigines syndrome, Friedreich's ataxia, mitochondrial cytopathies, and hemochromatosis.[4]

MANAGEMENT

Therapeutical Approaches for HCM Are Symptom-Oriented

β-Blockers act as negative inotropic drugs and are used as first-line drugs in patients with or without LV obstruction, and are the preferred treatment in symptomatic HCM patients with an LV outflow tract gradient only by exertion.[5] Verapamil, a calcium antagonist with negative

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inotropic properties, is used in both obstructive and nonobstructive forms in patients without severely disabling symptoms. Disopyramide, a class IA antiarrhythmic drug with negative inotropic effects, is reserved for patients with outflow tract obstruction not responding to β-blocker and verapamil. End-stage HCM requires adapted therapy including angiotensin-converting enzyme (ACE) inhibitors or angiotensinogen II receptor blockers, diuretics, spironolactone, and digitalis.

Septal myectomy can abolish the gradient caused by severe outflow tract obstruction. Alcohol septal ablation initiates controlled septal myocardial infarction to reduce the gradient. Dual-chamber pacing allows substantial reduction of the gradient and more aggressive medical treatment. Patients at high risk for SCD with important outflow tract gradient benefit from biventricular pacing properties of an implantable cardioverter defibrillator.

Risk stratification for SCD in HCM patients is currently based on available clinical data, but does not yet allow to precisely identify all patients at high risk.[6]

MOLECULAR GENETICS

In about 60% of cases, HCM is inherited as autosomal-dominant FHC. The prevalence is 1/500 in the common adult population. In the typical form of FHC, mutations in 12 different disease genes encoding proteins of the sarcomere have been identified (Table 1 and Fig. 1). Four genes encode the thick filaments: *MYH7* for β-myosin heavy chain (β-MyHC) and *MYH6* for α-myosin heavy chain (α-MyHC), *MYL3* for essential myosin light chain (MLC-1), and *MYL2* for regulatory myosin light chain (MLC-2); five genes encode the thin filaments: *ACTC* for α-cardiac actin (α-cAct), *TNNT2*, *TNNI3*, and *TNNC1* for the cardiac troponins T, I, and C (cTnT, cTnI, and cTnC), and *TPM1* for α-tropomyosin (α-TM). *MYBPC3* encodes the cardiac myosin-binding protein C (cMyBP-C), *TTN* encodes titin, and *CRP3* encodes the muscle LIM protein (MLP), which is a Z-disc protein stabilizing the contractile apparatus. Recently, a mutation in the promoter region of the phospholamban gene (*PLN*) has been reported.[8] Patients presenting FHC associated with the Wolff–Parkinson–White syndrome plus ventricular preexcitation exhibited mutations in *PRKAG2*, encoding the γ_2-regulatory subunit of AMP-activated protein kinase (AMPK), a metabolic protein.

Today, about 200 mutations in all these disease genes are known (http://www.angis.org.au/Databases/Heart/heartbreak.html). Eighty percent of the genotyped families present a mutation in *MYH7* and *MYBPC3*;[9] at least these two genes should be systematically screened for mutations in routine diagnostics.

GENETIC TESTING

Mutations in disease genes can be detected after gene amplification by polymerase chain reaction (PCR), followed by a mutation screening technique (single-strand conformation polymorphism, denaturing high-performance liquid chromatography, or others) and, finally, by sequencing. These techniques are highly specific if different conditions are analyzed and if the PCR primers largely surround the exons and important intronic sites. Sensitivity depends on several factors; particularly, one cannot exclude the implication of another as-yet identified gene and/or that PCR primers may hide a point mutation.

Table 1 FHC genes, loci, and proteins

Gene	Locus	Protein
MYH7	14q11.2–q12	β-myosin heavy chain (β-MyHC)
MYH6	14q11.2–q12	α-myosin heavy chain (α-MyHC)
MYL3	3p21.2–p21.2	Ventricular essential myosin light chain (MLC-1s/v)
MYL2	12q23–q24.3	Ventricular regulatory myosin light chain (MLC-2s/v)
ACTC	15q14	α-cardiac actin (α-cAct)
TNNT2	1q32	Cardiac troponin T (cTnT)
TNNI3	19p13.4	Cardiac troponin I (cTnI)
TNNC1	3p21.3	Cardiac troponin C (cTnC)
TPM1	15q22.1	α-tropomyosin (α-TM)
MYBPC3	11p11.2	Cardiac myosin-binding protein C (cMyBP-C)
TTN	2q24.3	Titin
CRP3	11p15.1	Muscle LIM protein (MLP)
PRKAG2	7q36	AMP-activated protein kinase (AMPK)
PLN	6q22.1	Phospholamban

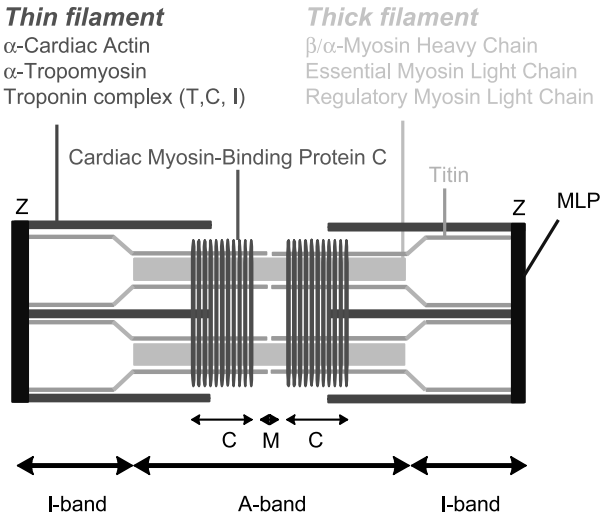

Fig. 1 Localization of FHC disease proteins in the sarcomere. (Adapted from Ref. [7].) (*View this art in color at www.dekker.com.*)

MOLECULAR MECHANISMS

Genetically modified models of FHC elucidate the involved molecular mechanisms. Ex vivo analysis in cellular models was used to analyze the impact of exogenous mutants on sarcomeric structure and contractility. Histopathology or molecular consequences of mutations at mRNA or protein levels were assessed on human cardiac or skeletal muscle biopsies. In vitro analysis of purified mutant proteins has given insights into mechanical and enzymatic alterations of specific molecules. In vivo studies in animal models allow several measurements.

Most of the FHC gene mutations are missense mutations. The mutated proteins are stable and incorporated into the sarcomere after gene transfer in various cellular models (for review, see Ref. [10]) and present in human skeletal biopsies carrying a heterozygous mutation in the *β-MyHC* or *α-TM* genes.[11,12] Mutated proteins act as poison polypeptide and lead to FHC by a dominant negative effect on sarcomere structure and/or function.

Most of the *MYBPC3* mutations result in a frameshift and may produce a C-terminal truncated protein, which is faintly accumulated in fetal rat cardiomyocytes,[13] even after infection of neonatal rat cardiomyocytes with adenovirus, in which they are degraded by the proteasome.[14] Truncated protein was not detected in myocardial tissue of patients carrying a frameshift *MYBPC3* mutation.[15–17] Altogether, these data strongly suggested that frameshift mutations act as ''null allele'' leading to haploinsufficiency. To further elucidate this hypothesis, we developed cMyBP-C null mice by gene targeting.[18] The heterozygous cMyBP-C-deficient mice, carrying only one functional allele, displayed asymmetric septal hypertrophy with myocardial disarray at 10–11 months of age. Therefore, these mice represent the first model with the major feature of human FHC, which is usually asymmetric hypertrophy predominantly affecting the interventricular septum.

The underlying pathophysiological mechanism by which missense and frameshift mutations cause LVH remains to be determined. The conventional concept of decreased contractility caused by mutant sarcomeric proteins, resulting in neuroendocrinal and mechanoreceptor responses and compensatory hypertrophy, has been inconsistent with experimental and clinical data (for review, see Ref. [19]). Energy metabolism may play a key role as trigger for the development of HCM,[20] which is supported by HCM-like phenotypes caused by mutations in metabolic proteins. Patients with a *MYH7*, *MYBPC3*, or *TNNT2* mutation exhibit impaired energy metabolism irrespective of the LVH degree.[21] We proposed that the link between mutation and energy depletion might be a disproportional alteration of mechanical and enzymatic properties of mutant sarcomeric proteins, which can result in inefficient ATP utilization.[22]

GENOTYPE–PHENOTYPE CORRELATION

The FHC phenotype shows wide interfamilial and intrafamilial heterogeneity depending on the major gene mutation, the complexity of the genotype, and the influence of modifier genes and environmental factors. Phenotypes resulting from *MYH7* mutations are usually associated with early LVH onset. Particular *MYH7* mutations are associated with high SCD risk: Arg403Gln, Arg719Trp, Arg453Cys, and Arg723Gly. The Gly256Glu, Val606Met, or Leu908Val *MYH7* mutations have a good prognosis in regard to arrhythmic events.[23] Mutations in *MYBPC3* correlate with mild hypertrophy in young patients, late onset of symptoms, and good prognosis before the age of 40 years.[24] Minimal LVH is found in *TNNT2* mutations, but can be associated with high SCD risk.[25] Studies of *MYBPC3* and *TNNT2* mutations have shown incomplete penetrance and disease expression in adult patients, often associated with minimal or absent LVH.

Five percent of families present individuals with a complex genetic status as homozygotes, double heterozygotes, or compound heterozygotes.[9] Compound and double heterozygotes for mutations in *MYBPC3* and *MYH7* develop a higher degree of LVH compared with single heterozygotes. Homozygotes for a mutation in

MYBPC3 or *MYH7* exhibit SCD at young age, atrial arrhythmias, and congestive heart failure.[9]

Genetic studies have revealed the presence of clinically healthy individuals carrying mutant alleles, which is associated in first-degree relatives with a typical phenotype of the disease.[26,27] This variable expressivity could reflect the existence of modifier genes or polymorphisms, which could modulate the phenotypical disease expression. Components of the renin–angiotensin–aldosterone system have been analyzed in patients with FHC (for review, see Ref. [28]).

GENETIC COUNSELING

The complexity of the wide phenotypical heterogeneity in the context of a complex genotype necessitates a multidisciplinary and multistep approach. Good clinical practice presumes interactive work between cardiologists, geneticists, and psychological support.[29] Genetic counseling is used for a general disease information and specifically for prenatal counseling and/or diagnostics if one parent is affected and predictive diagnosis in phenotypical healthy family members. Genetic testing of first-degree family members and relatives should be encouraged. If mutation analysis is not possible, repetitive clinical examination for HCM diagnosis is recommended.

CONCLUSION

FHC is an inherited cardiac disorder associated with a wide phenotype and genotype heterogeneity. Careful phenotype characterization and genetic studies are important in critical decision making in regard to optimal patient management. Further genotype–phenotype correlation studies and analysis of clinical data may, in the future, allow to identify FHC individuals at high risk for SCD.

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Certified Reference Materials for Genetic Testing

David E. Barton
Our Lady's Hospital for the Sick, Dublin, Ireland

Christoph Klein
Institute for Reference Materials and Measurements (IRMM), Geel, Belgium

Glyn Stacey
National Institute for Biological Standards and Control, Herts, U.K.

INTRODUCTION

Until recently, molecular genetic testing was confined to specialist laboratories with substantial expertise in this area, but such tests are now increasingly being carried out in routine clinical laboratories. The development of systems to ensure the quality of testing procedures and methods has lagged behind the extremely rapid growth in the application of DNA-based testing in medicine. Concerns over the accuracy of genetic test results are given added importance because individuals are usually only tested once in their lifetime, and the consequences of an incorrect result may be far-reaching. Public perception currently holds that genetic tests are highly accurate and represent ''state of the art'' technology. These factors combine to make total quality in molecular genetic testing a matter of primary importance, if a loss of public confidence is not to result.

A broad range of products and services benefit from the development of technical standards aimed at raising levels, of safety, reliability, interchangeability efficiency and other aspects of quality. National standards institutes work together under the International Standards Organisation (*www.iso.ch*) to develop International Standards and within Europe these are developed as European Norms (ENs). These standards refer to the use of appropriate Reference Materials (RMs) to validate test equipment or testing methods as an important part of any analytical testing system. Currently, no CRMs are available for molecular genetic testing. The IVD Directive of the EC (98/79/EC) requires, amongst other things, in vitro diagnostic medical device (IVD) manufacturers to assure the traceability of values assigned to their calibrators and control materials through available reference measurement procedures and/or available reference materials of a higher order (e.g., established International Standards.)

Thus, the demand for reference materials for genetic testing is driven by a combination of regulatory requirements and the desire of testing laboratories to be assured that their results are correct. This entry explores some of the issues which must be considered when designing RMs for molecular genetic testing and describes current research and development efforts in this area.

OVERVIEW

Reference Measurement Systems and Traceability Chain

In order to harmonize, standardize, and improve the quality of genetic diagnostic services the establishment of reference measurement systems is of pivotal importance. Diagnostic measurements must be accurate, precise, specific, comparable among laboratories, and traceable to the available reference measurement procedures and available reference materials of a higher order. Manufacturers are obliged to demonstrate traceablitity to a CRM according to the IVD directive 98/79/EC. Certified reference materials are reference materials qualified with a certificate stating assigned values and uncertainties (i.e., expected variability in the values), intended use, information about the matrix (the form of the material), and commutability. The three elements of reference measurement systems (RMS) are internationally accepted agreed reference measurement procedures (RMP), CRM, and reference laboratories.

The standard EN ISO 17511:2003 refers to the RMS, and it requires for measurement of quantities in biological samples: definition of the analyte with regard to the intended use of the measurement results, RMP for the selected quantity in samples, and suitable reference materials for the selected quantity, e.g., primary calibrators and secondary matrix-based calibrators that are commutable (i.e., the range of assays in which the CRM will perform correctly).

Traceability is defined[1] as a property of the result of a measurement, or the value of a standard, whereby it can be

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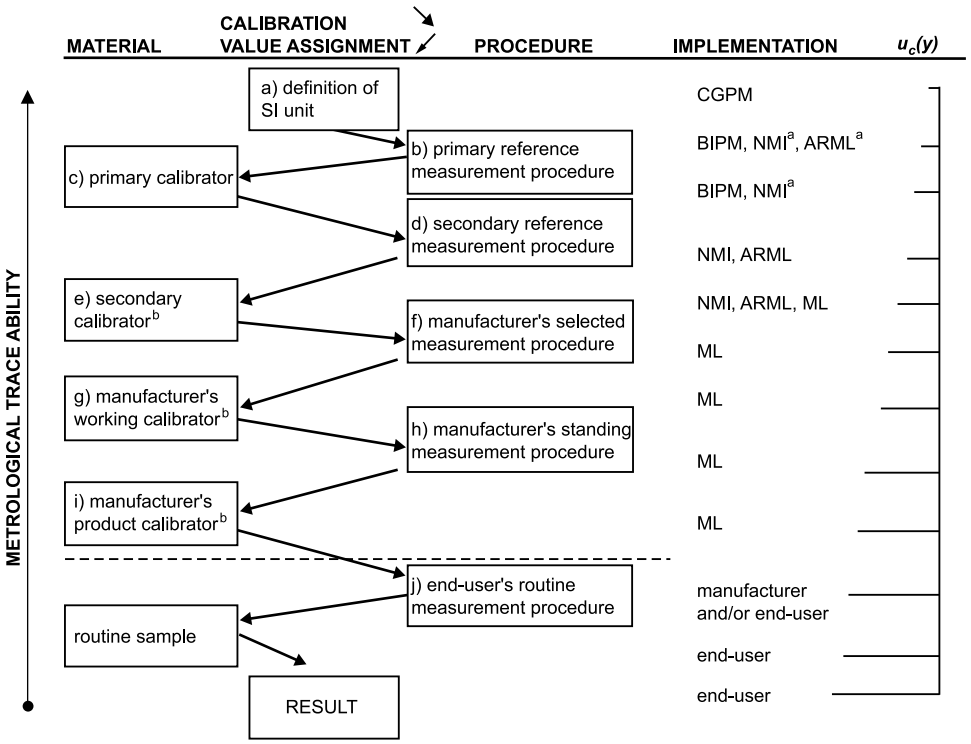

Fig. 1 Calibration hierarchy according to one scheme given in EN ISO 17511.

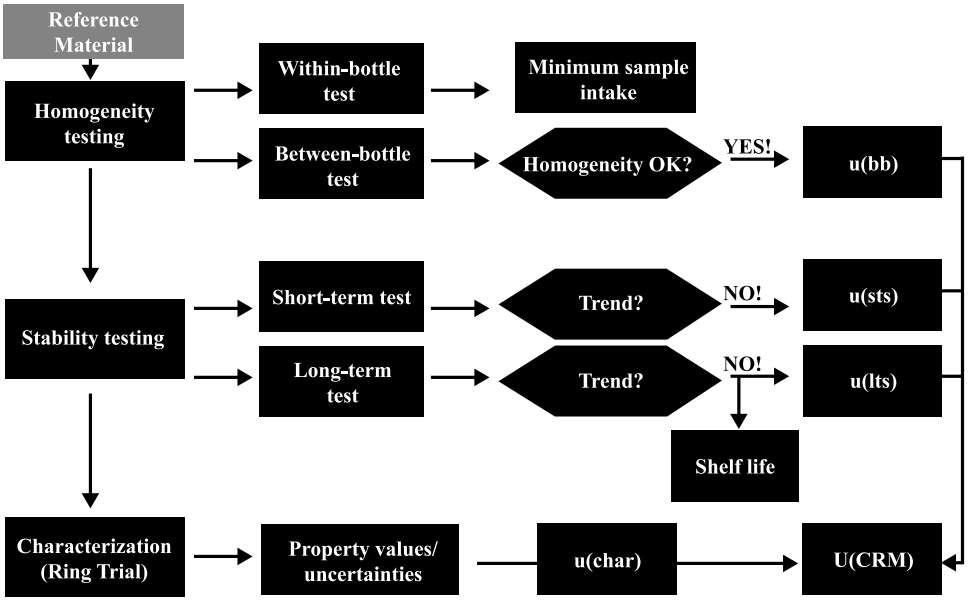

Fig. 2 Typical certification campaign scheme.

related to stated references, usually national or international standards, through an unbroken chain of comparisons (Fig. 1) all having stated uncertainties. The certification of a CRM is an integrated process of a correct preparation, homogeneity, and stability demonstration, and accurate and traceable characterization that leads to a certified value together with its total uncertainty as shown in Fig. 2.

In fact the Guide to Expressing Uncertainty in Measurement (GUM), revision of ISO Guide-35,[2] defines that all components of uncertainty of ''the sample on the desk of the user'' should be properly accounted for. The uncertainty of a CRM is calculated as the sum of the different uncertainties coming from different measurements. The role of CRMS is to facilitate measurements that are traceable, reliable, and comparable, and in which users can have confidence. Commutable CRMs (i.e., CRMs that will perform correctly in a wide variety of assays) are necessary to cover the broad scope of methodological approaches used.

DEVELOPMENT OF REFERENCE MATERIALS

Overview

Genetic testing involves the determination of whether a particular DNA sequence is present or absent in a patient's sample. In some cases, tests are designed to determine the presence or absence of known mutations (mutation testing), whereas in other cases the sample is screened for any deviation from the normal DNA sequence (mutation scanning).

There exist a large number of approaches for both mutation testing and mutation scanning. The situation is further complicated by the existence of many different types of genetic mutation (point mutations, deletions large and small, gene rearrangements, duplications, triplet repeat expansions), each of which may require different testing or scanning technologies. There is no ''gold standard'' testing or scanning method which will detect all possible mutation types. Although many genetics tests are qualitative in nature (presence or absence of a DNA sequence), some are quantitative. These involve either the measurement of the number of copies of a gene or part of a gene (to test for duplications or deletions) or the measurement of the number of copies of the repeated sequence in short repeated stretches of DNA which may be located adjacent to or within genes. Preparation of certified reference materials for molecular genetic testing had until recently not been attempted, and there are as yet no CRMs available in this field. Currently, molecular genetics tests are validated using reference materials which are often derived from research laboratories. These materials are deemed to be valid on the basis that an expert laboratory has determined that the material contains (or does not contain) a particular mutation. The materials are usually distributed on an informed peer-to-peer basis in response to personal requests made to the expert laboratory. There are few instances of organized distribution of genetic reference materials which have been validated in a methodical way by more than one laboratory. One exception involves the materials distributed for the purposes of external quality assurance (EQA) schemes. These materials are routinely validated by two laboratories before distribution, and the consensus result obtained by scheme participants provides further validation. However, the amount of such materials remaining in participating laboratories after completion of EQA schemes is usually small, perhaps only sufficient for a few assays. For quantitative tests, this may be sufficient to calibrate in-house materials which can then be used as reference samples and run with each batch of tests. Qualitative tests, however, require positive and negative control material which can be run with each batch of test samples. It is not unusual for a laboratory to test many hundreds of samples without ever encountering a positive result.

Thus a continuing supply of reference material which is certified to give a positive result is required for each test. The material left over from EQA schemes cannot fill this requirement. Furthermore, many laboratories are beyond the reach of the EQA schemes which are currently in place and have no access to these materials. These laboratories are likely to be the ones most in need of external validation of their tests through the use of appropriate reference materials. The fact that many laboratories fail to provide correct mutation detection in the ongoing European EQAs[3] further stresses the need for the availability of *certified* RMs.

The IVD Directive

The EU Directive on in vitro Diagnostic Medical Devices (98/79/EC), which came fully into force in Member States in December 2003, requires that all in vitro diagnostic devices carry the CE mark. The Directive explicitly covers materials transferred between laboratories as described above. Paragraph (9): ''Whereas, although internationally certified reference materials and materials used for external quality assessment schemes are not covered by this Directive, calibrators and control materials needed by the user to establish or verify performances of devices are in vitro diagnostic medical devices.'' Manufacturers of kits for genetic tests also have to obtain CE marking for their products under this Directive. For this, they are obliged to assure the traceability of values ''through available reference material

procedures and/or available reference materials of a higher order'' (Annex 1, Section 3). The expected rapid rise in the number of such kits on the market, and the increasing proportion likely to be designed for direct use by the public, adds urgency to the requirement for the availability of CRMs for genetic testing.

Validation Methodologies

In the absence of any ''gold standard'' method for genetic testing, reference materials must be certified by a consensus approach. The range of approaches used for each RM will be determined by the nature of the genetic change contained in the RM. For example, large triplet repeat expansions (fragile X syndrome, myotonic dystrophy) are not amenable to standard polymerase chain reaction (PCR) amplification and must be analyzed by Southern blotting or by a modified PCR method, whereas single-base substitutions are not readily detected by Southern blot, but can be ascertained by a wide range of other methods. In each case, the widest range of methods compatible with the particular RM should be used, with particular emphasis placed on using methods which are as independent of each other as possible, such as denaturing gradient gel electrophoresis, or sequencing using different protocols and hardware. This will be important, not only to assure that the characteristic assigned to the RM (presence/absence of a mutation) is correct, but also to ensure that the RM will be useful in calibrating or validating as wide a range of test methodologies as possible. Wherever possible, the methodology employed to verify the RM should include direct determination of the DNA sequence, using multiple replicates in different centers.

Types of Reference Material

Besides the testing methodology employed, the other major variable to be considered in designing a RM is the type of material which should be used. One can envisage many types of material as potentially suitable (Table 1), such as cultured cell lines, genetically engineered cell lines, genomic DNA (gDNA), recombinant (cloned) DNA fragments (rDNA), PCR amplified DNA, synthetic DNA (sDNA), and dried blood spots.

The parameters to be considered in selecting a reference material are as follows.

1. *How similar is the reference material to the material usually received for testing?*

   Most laboratories receive samples of biological material taken directly from patients for testing. This may be blood, mouthwash, chorionic villi, or indeed any tissue containing nucleated cells. DNA is extracted from these samples in the testing laboratory, by one of a large variety of methods. Laboratories have in general optimized their testing procedures to work reliably on DNA prepared by their selected protocol. Some testing protocols require DNA to be prepared by embedding whole cells in agarose blocks. It is impractical to prepare a RM using a patient's sample directly, as sufficient material would not be available for a useful batch. As CRM producers guarantee, certify, and monitor stability of the CRMs, these materials must be produced, stored, and delivered in a stable defined format, which is different to a native patient sample. Thus the practical material most similar to the usual samples will be cultured cells or immortalized cell lines. As most testing requires purification of genomic DNA as a first step, purified genomic DNA must be the next most suitable material in terms of similarity to the usual test material. However, both of these types of material have certain drawbacks (Table 1). No matter how the DNA for an RM is prepared, it will be different from the DNA routinely tested in most testing laboratories in terms of purity from contaminants, salt concentration, average fragment size, and many other parameters, and this may interfere with the proper functioning of tests in some laboratories. The most one can expect from a RM based on purified DNA is that the DNA is of the highest purity required for the range of assays in

Table 1 Choosing a reference material formulation/presentation

Type	Similar to usual samples	Versatile	Stable	Economical to produce	Storage cost	Ethical issues
Cell line	+++	+++	−	+	−	+
Genomic DNA	++	++	+*	++	++	+
Recombinant DNA	+	++	++	+++	++	++
PCR product	+	++	++	+++	++	++
Synthetic DNA	−	−	++	++	++	+++

+++, excellent; ++, very favorable; +, moderate; −, less favorable.
*If freeze-dried, rating becomes ++.

which it is to be used and that all of its physical and chemical properties are fully documented.

2. *How versatile is the material? i.e., is it technically suitable for use in all the possible testing methodologies?*

   As indicated above, a wide range of molecular genetic techniques can be used to test for the same mutation. Some forms of RM will be suitable for a wider range of tests than others. In essence, the more like total genomic DNA a RM is the more versatile it will be, so this parameter is linked to the previous one. Reference materials based on short DNA fragments, whether cloned, produced by PCR, or chemically synthesized will not be useful RMs for tests which involve Southern blotting or other long-range techniques, but they may be excellent RMs for short-range techniques. The testing methodologies employed are tailored to the type of mutation under study: short-range techniques for point mutations and long-range techniques for large triplet repeat expansions and major gene rearrangements. Thus the optimal type of RM will depend on what type of mutation the RM is designed for. When RMs for highly multiplexed tests are required, only artificial RMs will normally be possible, as natural sources will only contain at most two mutations.

3. *How stable is the material likely to be?*

   As a batch of RMs must have a reasonable shelf life to be useful (5 years is a sensible target), the stability of the proposed RM is of central importance. Experience of maintaining DNA banks in diagnostic and research centers tells us that purified DNA is one of the more stable biomolecules, and may survive intact in solution at 4°C or even room temperature for many years, as long as it is stored in a suitable solution and at high concentration. Freeze-dried DNA may be stable at room temperature.

   Intact cells are likely to be the least stable of all the forms of RM, as they need to be preserved at at least −70°C and preferably in liquid nitrogen to ensure their integrity. The other RM types, all consisting of purified DNA, may have similar stability characteristics, but this will need to be determined experimentally. The very large fragments in purified genomic DNA are more likely to be affected by physical shearing on storage in liquid, or on freezing and thawing, than the shorter fragments in PCR products or recombinant DNA. Dried blood in spots appears to be reasonably stable over several years at room temperature.

4. *How expensive or economical is it to produce large batches of the material?*

   In order to meet the requirements of a certified reference material, the material must be capable of being prepared in large batches suitable for many hundreds or even thousands of tests. This is possible for all of the RM types mentioned above, but is likely to be much more economical in the cases of PCR products and cloned DNA fragments. A 20 μL PCR reaction, diluted one million-fold for use as an RM, yields 20 liters of the product.

5. *How expensive or economical is it to store and to transport the material?*

   With RMs for molecular genetic testing, we are dealing with very small quantities of material in each aliquot, so bulk is not a problem to be considered. The parameters contributing to the cost of storage and transport are stability (discussed above) and hazard. Storage and transport costs will of course rise sharply with decreasing stability because of the requirement for refrigeration/freezing. Hazards associated with biological materials include pathogens which may be harbored by cell lines but most potential contaminants are unlikely to be present in purified DNA.

6. *Are there ethical or ownership issues associated with the material?*

   It is clear that apart from synthetic DNA, which is the least suitable material from other points of view, all types of RM are derived directly or indirectly from a human source. Full ethical approval must be obtained from donors of material for the establishment of cell lines, or the use of blood or other samples in RM development. Concerns may remain, however, that consent may be later withdrawn or challenged, or that testing of the RM for mutations other than the one specified in the certification process might constitute an invasion of the donor's genetic privacy. Furthermore, materials derived from human sources may be the subject of ownership claims when used for commercial purposes. Thus the further removed from a human source an RM is the less likelihood there is that such problems may arise. Cloned DNA fragments are removed from their genomic context, but their derivation must be documented under Genetically Modified Organism (GMO) regulations. Polymerase chain reaction products must be derived from a genomic DNA sample, which is traceable to an individual.

   The approach must then be to minimize the potential consent and ownership problems by obtaining appropriate informed consent and releases for each human source. Other legal issues concern transportation of RMs over boundaries. Often, a human- or biological-source-derived material is deemed to be potentially hazardous, which may hinder distribution of RMs in international initiatives.

Some types of reference material and their suitability under the headings discussed are summarized in Table 1.

Ongoing Initiatives to Develop Reference Materials

Several initiatives are ongoing to produce RMs for genetic testing in Europe and North America. In Europe, the CRMGEN project, funded by the European Union's Fifth Framework for Research, is developing reference materials in the form of cell lines, genomic DNA, PCR products, and plasmids for a range of common genetic tests (www.crmgen.org). Based on the expertise gained in the project, guidelines for the future production and validation of RMs will also be developed and published. The European Commission's Institute for Reference Materials and Measurements (IRMM) is collaborating with the International Federation of Clinical Chemistry (IFCC) to produce plasmid-based RMs for prothrombin variants. In the United Kingdom, the National Institute for Biological Standards and Control (NIBSC) is working on a number of gDNA RMs for Factor V Leiden testing based on cell lines.

In the United States, the Centers for Disease Control and Prevention are coordinating efforts to source, produce, and validate new reference materials for genetic testing[4] which are ongoing both in academic centers and in the private sector.[5]

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Chemical Cleavage of Mismatch (CCM)

Neil V. Whittock
Peninsula Medical School, Exeter, U.K.

Louise Izatt
Guy's Hospital, London, U.K.

INTRODUCTION

The reference standard essential for all molecular biology is direct nucleotide sequencing. This ''Gold'' standard is used for studies ranging from gene mutation screening in human disease to the sequencing of an entire genome. Sequencing protocols had been developed that used either chemicals or dideoxy nucleotides, although the former became less popular because of the use of toxic chemicals. If direct nucleotide sequencing was less expensive then there would not be the need for mutation screening techniques.

MUTATION DETECTION

Because of the cost of the direct sequencing techniques described above,[1,2] many mutation detection procedures have been developed. The most important criteria for an efficient mutation screening technique is the detection of new mutations within a gene. Several methods have been developed to screen for sequence changes in either cellular RNA or genomic DNA, including denaturing gradient gel electrophoresis (DGGE),[3] chemical cleavage of mismatch (CCM),[4] single-stranded conformation polymorphism (SSCP),[5] heteroduplex analysis (HA),[6] conformation sensitive gel electrophoresis (CSGE),[7] and denaturing high-performance liquid chromatography (DHPLC).[8] These methods detect all sequence changes such as nonsense, frameshift, splice site, and missense mutations as well as polymorphisms, whereas the protein truncation test (PTT)[9] screens specifically for truncating mutations. The detection efficiency of these different methods varies significantly depending on the size of the template screened. Single-stranded conformation polymorphism has been one of the most popular screening procedures used over the last decade because of its simplicity along with a sensitivity of >95% for fragments of approximately 150 bp. However, this sensitivity decreases to <5% for fragments of around 600 bp.[10] Conformation sensitive gel electrophoresis is also technically simple and has a sensitivity of 90–100 % for fragments of up to 450 bp.[11] Denaturing gradient gel electrophoresis requires much optimization for each fragment screened, but will yield a sensitivity of ~99% for fragments of up to 500 bp.[12] Chemical cleavage of mismatch has a sensitivity of between 95% and 100% for fragments >1.5 kb in size[13] and is perfect for screening larger fragments while still achieving a high sensitivity of detection.

CHEMICAL CLEAVAGE OF MISMATCH

Early studies demonstrated that mismatched bases became chemically reactive. Mismatched guanine and thymine react with the compound carbodiimide,[14] and mismatched cytosine reacts with hydroxylamine (NH_2OH). Mismatched thymine also reacts with osmium tetroxide (OsO_4),[4] potassium permanganate/tetramethylammonium chloride ($KMNO_4$/TMAC),[15] and potassium permanganate/tetraethylammonium chloride ($KMNO_4$/TEAC).[16] These chemical reactions were exploited in the development of the mismatch detection techniques, chemical cleavage of mismatch,[4] and the carbodiimide method.[14] Chemical cleavage of mismatch or chemical mismatch cleavage (CMC) was developed for use in the detection of single-base mismatches in cloned DNA, and with the advent of PCR, was employed to the screening of genomic DNA and mRNA.[4] Chemical cleavage of mismatch is based upon the detection of base mismatches in double-stranded DNA hybrids called heteroduplexes. These heteroduplexes are generated by mixing probe (wild type) and target (patient) DNA. When the sequence is different in the target compared to the probe, which can be caused by mutation or polymorphism, the usual Watson–Crick base pairing is disrupted. These free bases can then be chemically modified whereby they become substrates for piperidine cleavage of the DNA strand at or adjacent to the mismatch. Probe and/or target DNA can be fluorescently or radioactively labeled with the subsequent detection of cleavage products using either an automated fluorescent analyzer or autoradiography in conjunction

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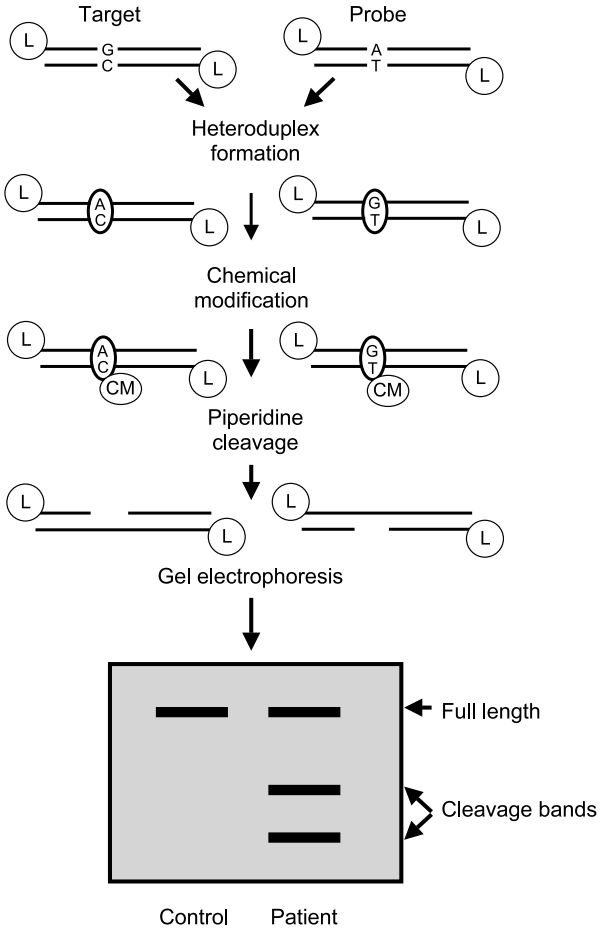

Fig. 1 Principle of the CCM mutation screening technique using labeled target and probe. Target and probe are hybridized to form the heteroduplexes, A–C and G–T (circled). The mismatches are then chemically modified and cleaved. Cleavage products are electrophoresed through a polyacrylamide gel and detected using either fluorescence or autoradiography. L—label, CM—chemical modification.

with denaturing polyacrylamide gel electrophoresis (Fig. 1).

Practical Considerations

PCR template

Chemical cleavage of mismatch gene-screening protocols can be designed using either patient genomic DNA as the template for PCR or mRNA isolated from specific tissues such as muscle or skin, or ectopic mRNA from peripheral blood lymphocytes. However, when total RNA is used as the template, a nested PCR protocol will probably be required to obtain enough DNA for the CCM procedure.

Labeling of fragment

The original CCM studies used DNA probes that had been radioactively end-labeled.[4] This technique was thought to detect nearly 100% of single-base substitutions,[17] but was considered dangerous because of the use of radioactivity along with toxic chemicals such as osmium tetroxide. However, the introduction of fluorescent-based technology has greatly improved the safety aspect of the procedure. Fluorescent CCM (FCCM), or fluorescence-assisted mismatch analysis (FAMA), has been developed whereby DNA can now be either internally labeled using fluorescently labeled dUTPs,[13] or end-labeled using fluorescently labeled primers,[18] although both procedures will achieve the same sensitivity of detection. Subsequent cleavage product analysis yields precise information on the position of the sequence change from either end of where the mismatch is positioned. A product of 850 bp may yield products of 700 and 150 bp, although the scientist will not know whether the mutation is 150 bp from the 5′ end of the fragment or 150 bp from the 3′ end. This can be overcome by using a protocol that utilizes different fluorescent labels on the 5′ and 3′ primers. To obtain 100% sensitivity of mismatch detection it will be necessary for both probe and target to be labeled. Some reports have demonstrated that a number of thymine–guanine mismatches are resistant to modification by osmium tetroxide.[17] However, the complementary DNA strand heteroduplex will consist of an adenine–cytosine mismatch that can be modified by hydroxylamine.

Heteroduplex formation

The correct formation of the probe/target heteroduplex is essential for chemical modification, although this can be inhibited by the sequence context and/or size of the fragment being studied. Autosomal dominantly inherited disorders will demonstrate a mutation on one chromosome and thus the patients DNA will be heterozygous and can naturally form a heteroduplex. However, autosomal recessively inherited disorders may demonstrate homozygosity and will not form a heteroduplex. Here, or where the mode of inheritance is not known, the patient DNA should be mixed with control DNA. Heteroduplex formation is performed by the denaturation of the double-stranded DNA followed by controlled cooling to permit annealing of the target and probe DNA strands.

Chemical modification

The majority of studies to date have used a combined hydroxylamine/osmium tetroxide protocol. However,

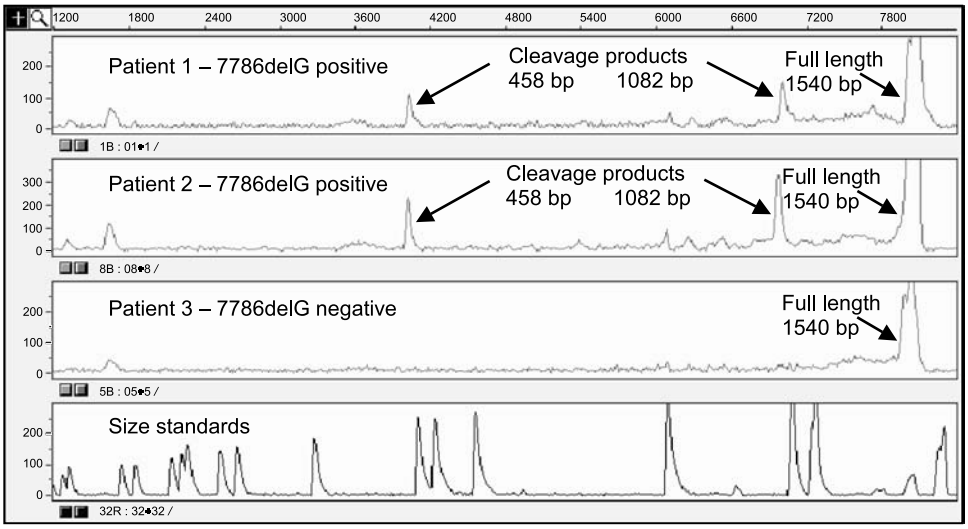

Fig. 2 FCCM analysis of the common British *COL7A1* mutation 7786delG. FCCM of fragment 19 (exons 103–108) for two patients heterozygous for the mutation 7786delG (lanes 1–2) and a negative control (lane 3). Lane 4 contains the size standards. Cleavage peaks (458 + 1082 bp) in lanes 1 and 2 are absent for the negative control. The uncleaved peak is 1540 bp.

because of the toxic nature of osmium tetroxide, work has been undertaken to validate a safer replacement. Potassium permanganate/tetraethylamine chloride[16,19,20] is now preferred because in addition to resolving the safety concerns of osmium tetroxide it can also overcome the lack of reactivity of some thymine–guanine mismatches.[17] Recently, a combination single-tube protocol has been developed, termed Single-Tube Chemical Cleavage of Mismatch Method, that permits the chemical modification of heteroduplexes by potassium permanganate/tetraethylamine chloride and hydroxylamine without the need for intermediate purification steps.[19]

Solid-phase technology

The original CCM protocol[4] required many lengthy ethanol precipitation stages that limited the quantity of work performed each day. However, the development of an ultrarapid protocol that made use of biotinylated PCR primers, in conjunction with streptavidin-coated paramagnetic beads, yielded a solid-phase CCM protocol. Exploitation of the affinity of biotin for streptavidin along with microtitre plate technology permits easy and effective purification of the cleavage products, thereby allowing high throughput screening to be performed.[13] Very recently, the solid-phase CCM protocol has seen further development whereby the DNA is immobilized on solid silica supports. This version of solid-phase CCM is fast and sensitive for detection of sequence changes.[21]

Detection protocols

Depending on the labeling protocol used, cleavage products can be detected using standard denaturing polyacrylamide gel electrophoresis (PAGE) apparatus and detected using standard X-ray film, or on a fluorescent DNA fragment analyzer utilizing PAGE or, more recently, capillary electrophoresis.

CLINICAL APPLICATIONS

Chemical cleavage of mismatch has been applied to the screening of many diseases including hemophilia A (*factor VIII*),[22] Fabry disease (α-*galactosidase A*),[23] hereditary breast and ovarian cancer (*BRCA1*),[24] Fanconi anaemia (*FANCC*),[25] dystrophic epidermolysis bullosa (*COL7A1*)[26] (Fig. 2), and ataxia telangiectasia (*ATM*).[27]

CONCLUSION

Since its development over 15 years ago, CCM has been shown to be a very efficient method for the detection of sequence changes in DNA and RNA. Although it is technically more involved than other gene-screening techniques such as SSCP, HA, or CSGE, and can include the use of toxic chemicals, it is ideally suited to screening relatively large fragments in excess of 1.5 kb

and will accurately pinpoint the position of the sequence change. To date, apart from direct nucleotide sequencing, FCCM remains the fastest, most accurate, and sensitive fluorescent mutation screening technique available, and these high standards will only improve in the future with the optimization of fluorescent and solid-phase technologies.

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Chlamydia pneumoniae, Chlamydophila pneumoniae

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Sven Poppert
University Hospital of Ulm, Ulm, Germany

Udo Reischl
University of Regensburg, Regensburg, Germany

Andreas Essig
University Hospital of Ulm, Ulm, Germany

INTRODUCTION

Chlamydia pneumoniae causes usually mild infections of the respiratory tract in humans. It has further been associated with diverse chronic inflammatory diseases such as atherosclerosis and asthma. *Chlamydia pneumoniae* is a member of the order Chlamydiales which contains a couple of other human pathogens and animal pathogens. A common feature of all chlamydiae is a unique biphasic, strictly intracellular life cycle. Metabolically inactive elementary bodies (EBs) infect the cells, then they transform into reticulate bodies which multiply inside special inclusions. Because of this developmental cycle, chlamydiae are difficult to culture, posing special challenges to diagnosis and research. Research on these bacteria is further complicated by the lack of gene transformation systems.

DESCRIPTION OF THE GENUS

Taxonomy

C. pneumoniae belongs to the genus *Chlamydia*, which also contains the species *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydia pecorum*. Due to the detection of new *Chlamydia*-like organisms and genetic analysis, in 1999 a new taxonomy was proposed which divides the common chlamydiae into the two families, *Chlamydophila* (including *C. pneumoniae*) and *Chlamydia*, and adds two more families containing the newly detected chlamydia-like organisms (Table 1).[1] This proposal, however, is still controversial, leading to the concomitant usage of old and new taxonomies in current publications.[1,2]

Life Cycle

A common feature of all chlamydiae is the unique biphasic, strictly intracellular life cycle. The cycle starts with the attachment of small ($\sim$0.3 μm), metabolically inactive EBs to the host cell. After internalization, these transform into reticulate bodies (RBs; $\sim$1 μm) which multiply by binary fission inside an inclusion. In the case of *C. pneumoniae*, these RBs begin to reorganize to EBs after 2 days, until finally the inclusion and cell rupture thereby releasing new EBs (Fig. 1).

In vitro a third state, the so-called persistent or aberrant state, can be induced by IFN-γ, antibiotics, or depletion of nutrients.[3] When the inducing stimulus is removed these forms can reenter the normal developmental cycle.

Macromolecular Composition

Chlamydiae have a bilayered cell membrane similar to other Gram-negative bacteria. The cell membrane contains species-specific antigens such as the major outer membrane protein (MOMP), as well as common antigens such as lipopolysaccharide (LPS).[4] Major outer membrane protein is the most prominent membrane protein making up about 60% of the surface proteins. Variable domains of this protein allow the division of *C. trachomatis* but not *C. pneumoniae*, into different serovars. In the genome of *C. pneumoniae* open reading frames of 21 putative polymorphic membrane proteins (Pmp) have been found.[5] For some, presence on the surface of chlamydiae and immunogenicity could be demonstrated.[4,6]

All chlamydiae share the same LPS structure[7] with a comparably low number of acylic groups, which is probably responsible for the low endotoxic activity. Lipopolysaccharide is used as a group complementation fixation antigen and recombinant ELISA–antigen for chlamydial serodiagnosis. Heat shock proteins, such as the genus-specific heat shock protein 60 (HSP60), are additional important immunogenic antigens although they are scarcely expressed on the surface. Also of interest is a Type III secretion apparatus that is expressed on the surface of reticulate bodies as well as EBs.[5]

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Table 1 Proposed taxonomy of Chlamydiales

Order	Chlamydiales
Family I	Chlamydiaceae
Genus	*Chlamydia*
Species	*Chlamydia muridarum* sp. nov.
	Chlamydia suis sp. nov.
	Chlamydia trachomatis
	Biovar trachoma
	Biovar LGV
Genus	*Chlamydophila*
Species	*Chlamydiophila abortus* sp. nov.
	Chlamydiophila caviae sp. nov.
	Chlamydiophila felis sp. nov.
	Chlamydiophila pecorum comb. nov.
	Chlamydiophila pneumoniae comb. nov.
	Biovar TWAR
	Biovar Koala
	Biovar Equine
	Chlamydiophila psittaci comb. nov.
Family II	Simkaniaceae fam. nov.
Species	*Simkania negevensis* sp. nov.
Family III	Parachlamydiaceae fam. nov.
Species	*Parachlamydia acanthamoebae* sp. nov. and others
Family IV unnamed	
Species	*Waddlia*

Source: From Ref. [1].

Genome

The genomes of four *C. pneumoniae* strains (CWL029, AR39, J138, TW183) have been sequenced and published.[5,8–10] Additionally, the genomes of three *C. trachomatis* strains (trachoma serovar D: D/UW-3Cx; serovar L2 L2/434/Bu; Nigg-MoPn, proposed *Chlamydia muridarum*), one *C. psittaci* (GPIC, proposed *Chlamydia caviae*)[2–4] and one parachlamydial strain[1] are available and more genomes are soon to be published.

With slightly more than 1 million base pairs (1.23 million for *C. pneumoniae*) chlamydiae possess one of the smallest genomes in the bacterial world.[8,10] The different chlamydial genomes have a homology of about 80% and there are very few genetic differences within the species of *C. pneumoniae*.

Chlamydiae were previously considered to be ATP-parasites but interestingly possess putative proteins for the synthesis of ATP.[5] The presence of two genes encoding (ADP)ATP-translocases, however, make it likely that chlamydiae additionally import host cell ATP.[11]

In some strains of *C. pneumoniae*, and also *C. psittaci*, different bacteriophages have been demonstrated.[8]

Additional contemporary information about the chlamydial genomes can be accessed on the Internet: http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html, http://www.tigr.org/tdb/, http://chlamydia-www.berkeley.edu:4231/.

CLINICAL SIGNIFICANCE

Respiratory Tract Infections

C. pneumoniae is endemic worldwide and is responsible for an estimated 10–20% of community acquired pneumoniae. The first contact with *C. pneumoniae* usually occurs at a young age leading to a seroprevalence of 50% to 60% by the age of 30, which slowly rises with age.

Infection is transmitted via respiratory secretions. After a variable incubation period of up to 4 weeks the disease begins with growing hoarseness and aching throat, mild cough, and fever and does later develop into an atypical pneumonia.[12,13] In rare cases infection may also present as sinusitis or otitis media or lead to the aggravation of asthma.[14] In immunocompetent individuals the infection is usually self-limiting, but may be complicated by superinfections. In immunocompromised persons infection may take a more severe course. Reinfections do occur but are generally milder than primary infections.[13]

C. pneumoniae and Atherosclerosis

Since the first report of a link between *C. pneumoniae* and coronary heart disease (CHD) in 1988,[15] the chlamydia-arteriosclerosis theory has been challenged by a vast number of epidemiological and experimental studies, resulting, however, in contradictory outcomes. Recent meta-analysis of serological studies did not confirm the early reports of a serologic correlation.[16,17] But direct investigation of arteries by PCR and immunohistochemistry revealed that 25% to 50% of atherosclerotic vessels contain chlamydiae compared to only 2% of healthy vessels.[17,18] There is, however, considerable variation among different studies and only poor correlation between the different detection methods.[17]

In vitro, chlamydiae were able to infect all cells relevant in the development of arteriosclerosis, including macrophages, endothelial cells, and smooth muscle cells.[19] *Chlamydiae* further promoted the expression of proatherogenic receptors, second messengers, and enzymes in cell culture experiments.[19]

While early studies addressing antibiotic intervention of arteriosclerosis seemed to show a positive effect, this result has not been confirmed in recent studies.[20]

Animal experiments with mice and rabbits have also produced conflicting results concerning the development

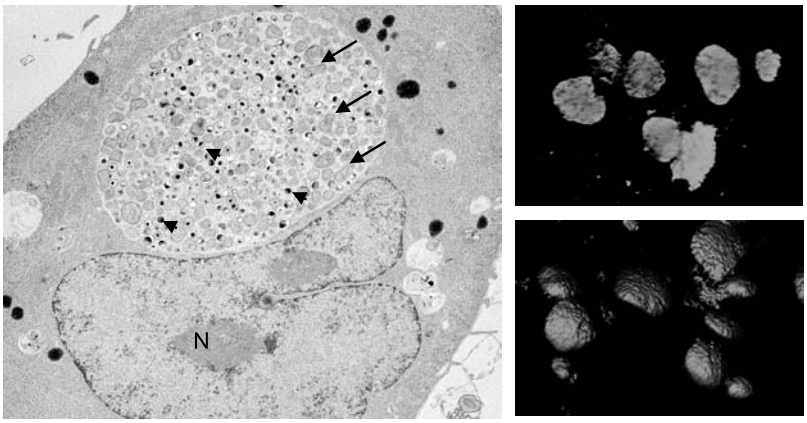

Fig. 1 *C. pneumoniae* in HeLa cells 72 hr post infection. The transmission electron microscopic image shows an inclusion containing reticulate bodies which are still dividing (long arrows) and already developed elementary bodies (short arrows) as well as intermediate forms (N=nucleus of the cell). The right pictures represent 3-D images of an immunofluorescent staining with a FITC-labeled antibody targeting chlamydial LPS (green) and a fluorescence in situ hybridization with a Cy-3-labeled DNA-probe targeting specific sequences on the chlamydial ribosomal RNA (red), respectively. Some inclusions are already rupturing and their infectious content is spreading ready to infect new host cells. (Photos courtesy of Poppert and Horn.) (*View this art in color at www.dekker.com.*)

of arteriosclerosis after infection with *C. pneumoniae*.[21,22] Although the enthusiasm regarding the detection of a bacterial cause of atherosclerosis has been dampened by the contradictory results, the debate is still ongoing.

C. pneumoniae and Other Chronic Diseases

A considerable number of papers report on a link between *C. pneumoniae* and chronic diseases such as multiple sclerosis,[23] lung cancer,[24] chronic fatigue syndrome, morbus Alzheimer, and various others. However, these reports mainly rely on rather small studies and are, as those for arteriosclerosis, hampered by the lack of standardized and validated detection methods.

PATHOGENETIC MECHANISMS

The various chlamydial infections show an analogous pattern of pathology.[25] Initially, polymorphonuclear leukocytes infiltrate the locus of infection followed by lymphocytes, macrophages, and plasma cells. A strong Th1 response seems to be of major importance for the clearance of infection.

Recurrence of infection frequently occurs. This may be explained by the survival of chlamydiae inside the protected cell, possibly in the aforementioned so-called persistent state. Another possibly important factor is the ability of chlamydiae to suppress apoptosis.[26]

DIAGNOSIS

Laboratory diagnosis of *C. pneumoniae* infections can be performed by culture, serology, and nucleic acid amplification tests.[27] In tissues, chlamydiae can additionally be targeted by immunohistochemistry and in situ hybridization techniques.

The most frequently used approach for the diagnosis of *C. pneumoniae* infections is serology. Although, often, it can only retrospectively demonstrate a significant rise in antibody titers. In primary infection, specific IgM is detectable from 2 to 3 weeks until 2 to 6 months after infection. IgG antibodies may not appear until 2 months after infection. In reinfection, complement fixation as well as IgM antibodies often remain negative while the IgG titer may rise to 1:512 or more as soon as 1 week after infection.[28,29] There are yet no validated serologic markers for persistent or chronic infection, but IgA antibodies in combination with high titers of IgG antibodies may be indicative.[29]

The most used and recommended serologic test is the microimmunofluorescence test (MIF), which uses purified formalinized EBs from *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* that are fixed on glass slides in distinct, small spots. It can therefore simultaneously determine species-specific antibodies to the three chlamydiae.[29] The chlamydial complement fixation test, based on the common lipopolysacharide antigen (LPS), may react more promptly, but has a low sensitivity for reinfection. It does not distinguish between various species of chlamydiae and is therefore generally not recommended.[27] The same

is valid for whole inclusion fluorescence tests that are not species-specific either and not yet sufficiently validated to be recommended.[27] Enzyme immunoassays (EIA) offer a more automated and standardized workflow. Evaluations of these tests are currently underway.[27]

Culture of *C. pneumoniae* is hampered by great technical effort and limited sensitivity. Therefore it is not used routinely, but remains essential for the determination of resistance and the characterization of strains for research purposes. Cultured chlamydiae can be identified by PCR[30] or by species-specific antibodies (Fig. 1). We have recently developed a probe set for differentiation of chlamydiae by fluorescent in situ hybridization (FISH) that can be implemented for this purpose and may also be used for the detection of chlamydiae in clinical samples (Fig. 1).[31]

A variety of DNA amplification methods has been described using different formats (nested,[32] nonnested, real-time LightCycler PCR,[33] and enzyme time-released touch down PCR.[34]) and targeting different gene sequences including the ompA gene, 16SrRNA gene, omcB, and the Pst I fragment.[17] These methods are increasingly used to detect *C. pneumoniae* in respiratory specimens as well as in tissues; however, standardization of these methods is urgently required to avoid discrepant results.[27]

Immunohistochemistry (IHC) is used for the detection of *C. pneumoniae* in tissues for research issues such as the association of chlamydiae with arteriosclerosis.[27] Contrary to PCR and culture, this technique allows for the preservation of the tissue structure and localization of the antigen. Results, however, have to be interpreted carefully as artifacts and positive stainings are difficult to distinguish.[27]

An additional technique preserving the histology of the tissue is in situ hybridization with labeled DNA probes.[27,31] This technique is hampered by similar problems as seen in IHC.

THERAPY

The recommendations for antibiotic treatment of *C. pneumoniae* infections are based on in vitro studies, animal experiments, case reports, and experiences with the treatment of other chlamydial infections. Therapeutic regimens for *C. pneumoniae* include doxycycline, erythromycin, or clarithromycin for 2–3 weeks or a 5-day course of azithromycin. For recurrent infection a second course with a different antibiotic is recommended. There are singular publications of development of resistance in *C. trachomatis*, but resistance in *C. pneumoniae* has never been reported. However, antibiotic testing is not standardized and valid studies comparing various therapeutic regimens do not exist.

CONCLUSION

Diagnosis of *C. pneumoniae* is still unsatisfactory. Internationally standardized and validated assays especially for nucleic amplification and antibody detection are warranted. The first steps in this direction have been taken for example with the formulation of recommendations from the Centers of Disease Control (United States).[27] The standardization of diagnosis may eventually allow determining the contribution of *C. pneumoniae* in chronic inflammatory disease more precisely.

The known sequences of the chlamydial genomes were already the base for many elucidating studies and will be the base for further research.[8–11] Methods for the genetic transformation of chlamydiae are not in sight, but transcriptional analysis will further contribute to elucidate mechanisms of chlamydial infection by identifying the involved genes and their interplay. The microarray techniques are increasingly implemented for transcriptional profiling of global gene regulation and more results are soon going to be published.[35] Functional research on chlamydial proteins will also base on the expression of chlamydial proteins in transformable organisms such as *Escherichia coli*. Two-dimensional (2-D) gel electrophoresis is a promising technique for the analysis of protein expression in chlamydiae and some of the abovementioned data have already been derived from 2-D gel electrophoresis partly in genomic–proteomic approaches[3,4,6] (more contemporary information about chlamydiae and proteomics is available at http://www.gram.au.dk). In addition to enhancing the functional understanding of chlamydiae, proteomics might help to identify proteins for diagnostic purposes and even targets for the development of a vaccine.

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Chlamydia trachomatis

Hsi Liu
Centers for Disease Control and Prevention, Atlanta, Georgia, U.S.A.

INTRODUCTION

Chlamydiae are gram-negative intracellular bacteria which contribute to a wide range of acute and chronic diseases. Four species are included in the genus *Chlamydia*: *C. trachomatis*, *C. pneumoniae*, *C. psittaci*, *and C. pecorum*. *Chlamydia psittaci* and *C. pecorum* are animal pathogens with humans becoming infected only as a result of exposure to infected animals. In contrast, *C. trachomatis* and *C. pneumoniae* infections appear to be restricted to humans, with *C. pneumoniae* having recently emerged as a significant cause of respiratory tract disease, with no obvious animal host.

BIOLOGY OF THE ORGANISMS

Chlamydiae are obligate intracellular bacteria of limited metabolic capability, characterized by a dimorphic growth cycle. The infectious form is called the elementary body (EB) and is a condensed sporelike spheroid. The EB is metabolically inactive, consisting of a tightly compacted chromosome or nucleoid, and an outer membrane of covalently linked lipopolysaccharides. When it encounters host cells, the EB is taken in by receptor-mediated endocytosis. It has been suggested that a trimolecular mechanism is used to bridge a host receptor with a chlamydial receptor by glycosaminoglycan.[1] The mannose receptor has also been suggested to have a role in the EB's entry into the cell.[2] The engulfed EB is enclosed in an endosome which does not fuse with a lysosome.

Several hours after entering the cell, the EB converts to a metabolically active form called the reticulate body (RB) that undergoes binary fission, forming microscopically visible inclusions containing hundreds of organisms within 24 hr. In a further 24–48 hr, the reticulate bodies condense to transform into infectious EBs that are liberated as a result of cell lysis. Polysaccharide components may form within the inclusions after 48 to 60 hr of development, depending on the chlamydial species. *Chlamydia trachomatis* can be distinguished from *C. psittaci* and *C. pneumoniae* as a result of this polysaccharide material, which stains brown with iodine. *Chlamydia psittaci* and *C. pecorum* essentially cause infections of animals, with humans becoming infected as a result of exposure to infected secretions of those animals. In contrast, *C. pneumoniae* has recently emerged as a significant cause of respiratory tract disease in humans, with no obvious animal host.

The human pathogen *C. trachomatis* has been further subdivided into 15 serovars (A–K and L1–L3), based on the monoclonal antibodies that identify epitopes located in the major outer membrane protein (MOMP). *Chlamydia trachomatis* can also be classified into two biovars, based on the diseases it causes. Serovars A, B, Ba, and C have been associated with the eye disease trachoma, and serovars D, E, F, G, H, I, J, and K with genitourinary tract infections. Both diseases have worldwide distribution. Together, they are termed the trachoma biovar. The L1, L2, and L3 serovars are associated with the more invasive sexually transmitted disease (STD) lymphogranuloma venereum (LGV), which is prevalent in sub-Saharan Africa, South America, and Southeast Asia (the LGV biovar).

TRACHOMA

Trachoma remains the most common cause of preventable blindness in the world. It is found in communities with poor hygiene or sanitation facilities and inadequate access to potable water. The infection is endemic in many tropical and subtropical areas, especially countries in northern and southern Africa, the Middle East, and on the Indian subcontinent. It is transmitted by direct contact or by flies, which act as mechanical vectors. Trachoma presents as conjunctivitis of both the palpebral and bulbar conjunctivae, followed by the formation of lymphoid follicles; the sequelae, entropion, and trichiasis arise as a result of conjunctival scarring, causing corneal damage. Repeated infection and associated bacterial superinfection can result in visual impairment and blindness. The conjunctival epithelium of infected children is the most important reservoir of infection in the affected communities. High chlamydial loads occur in very young children and have been directly correlated with severity of inflammatory changes.[3]

Diagnosis

Trachoma is frequently diagnosed based on its clinical presentations. In the laboratory, Giemsa staining of

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conjunctival scrapings can identify cytoplasmic inclusions within conjunctival epithelial cells, and organisms can be detected by tissue culture method or by nucleic acid amplification tests (NAAT) such as PCR.[3,4]

Treatment and Control

Recent studies have shown that a single dose of azithromycin is as effective as a long course of topical tetracycline (42 doses over a 6-week period) in treating trachoma, and the effect of treatment lasts for 1 year.[3,4] However, only small differences in effectiveness have been found between mass treatment programs and programs treating active cases only.[5] WHO has set a goal of eliminating trachoma by 2020, using the SAFE program.[6,7] ''SAFE'' stands for *surgery* to correct lid deformities, *antibiotics* to treat active disease, *face-washing*, and *environmental controls* to reduce transmission.

GENITOURINARY CHLAMYDIAL INFECTION

Chlamydia trachomatis infection of the genitourinary tract is the most common bacterial STD in both the United States (>4 million new cases per year) and worldwide (>50 million new cases per year). Many infected individuals of either sex are asymptomatic; but symptomatic urethritis is common in men, whereas women may present with a mucopurulent cervicitis and acute salpingitis. The most serious complication of infection in men is epididymitis. Without treatment, more than 40% of infected women may develop pelvic inflammatory disease, which may lead to tubal infertility or increased risk of ectopic pregnancy. Pregnant women may transmit their infection to their babies as they pass through the infected endocervix at the time of delivery. The baby may develop a mild, neonatal conjunctivitis and/or afebrile pneumonia. Infection stimulates host inflammatory and immune responses, but the immunity is of short duration and reinfection occurs frequently.

Diagnostic Tests

A definitive diagnosis of chlamydial genital infection can be established solely on the basis of laboratory testing. In the past, the standard laboratory test for *C. trachomatis* genital infections was isolation of the causative organisms in cell culture. By definition, this test is 100% specific but it lacks sensitivity when compared to the newer NAATs. Several commercial tests are currently available (Table 1), and new tests are being developed every year.[8–11] The Roche Amplicor and Cobas Amplicor CT/NG tests use polymerase chain reaction;[12] the Becton-Dickinson B-D-ProbeTec test uses strand displacement amplification;[13] and the GenProbe Aptima test uses transcription-mediated amplification. All three tests amplify the cryptic plasmid which is found in >99% of the clinical isolates.[14] Unfortunately, the Abbott LCx test, which employs the ligase chain reaction, is no longer commercially available. The NAATs are extremely sensitive and can be applied to noninvasive specimens such as first-catch urine and self-administered vaginal swabs. However, NAATS detect dead as well as living organisms and may give false-positive results, particularly in low-prevalence settings, and they are relatively expensive. Liquid Pap smear medium for CT/NG collection has also been used with acceptable results.[15] As with all testing, each laboratory must establish an ongoing quality-assurance program to validate the test results.[16] Two nonamplified nucleic acid probe tests are commercially available which can diagnose both *C. trachomatis* and *Neisseria gonorrhoeae* simultaneously, but cannot differentiate between the two, requiring additional tests for verification. The GenProbe PACE 2 test uses a DNA probe that is specific for the rRNA. The Digene Hybrid Capture II test uses an RNA probe that is specific for the DNA of *C. trachomatis* and targets both the genomic DNA and cryptic plasmid.[17] The commercial DNA probe tests, direct immunofluorescence tests, and antigen-capture ELISA tests are all used in peripheral laboratories, but lack sensitivity when compared to NAATs. Serological tests are of little value in the diagnosis of *C. trachomatis* infections caused by serovars A–K except in the case of detection of specific IgM responses in babies presenting with neonatal pneumonia where high titers of antibody may be detected when using an indirect immunofluorescence test.

LYMPHOGRANULOMA VENEREUM

Lymphogranuloma venereum (LGV) is caused by *C. trachomatis* serovars L1, L2, or L3. The LGV organisms are more invasive than those that cause other sexually transmitted infections and may spread systematically via the lymphatics.

Diagnosis

Lymphogranuloma venereum is usually diagnosed on the basis of clinical symptoms, signs, and exclusion of other relevant diseases, especially chancroid and syphilis. Serology (with detection of high titers of broadly cross-reactive antichlamydial antibody), isolation of *C. trachomatis* from the site of infection, and application of molecular tests can all assist in the diagnosis; but all tests for LGV are subject to the same constraints as tests for

Table 1 Comparison of Chlamydia tests

Commercial name	Company	Advantages	Disadvantages
Amplification tests			
Amplicor CT test	Roche	• Cryptic plasmid as target and MOMP was used as alternate target • Endocervical swab • Male urethra • Male and female urine	
Cobas Amplicor GC/CT	Roche	• Automated test	• Requires special instrument
Ligase Chain Reaction (Lcx; LCR)	Abbot	• FDA approved since 1995 • PCR+ligase • Automatic test • Less contamination problem • Cryptic plasmid as target and MOMP is used as alternate target • Used by 13.5% of laboratories	• Discontinued
Hybridization tests			
ProbeTecET	Becton-Dickson	• FDA approved since 1999 • Detects both GC and CT • Strand displacement amplification • Fast throughput • Endocervical swap • Male urethra • Male and female urine	• Report variations from site to site
Gen-Probe Amplified CT	Gen-Probe	• Transcription Mediated Amplification (TMA) of rRNA • Use 16s RNA as alternate target • Male and female urine • Male and female swab	• Labor-intensive
Gen-Probe PACE 2C	Gen-Probe	• A screening test • Has several versions • Competitive probe version uses labeled probe • Low cost • Used by 41% of laboratories (Pace 2C); used by 21.5% of laboratories (Pace 2)	• May be less sensitive than NAATS • Cannot be used in urine
Digene Hybrid Capture II (HCII)	Digene	• Antibody capture of DNA • Signal amplification test • Detect both GC and CT	• Does not differentiate CG/CT • Less sensitive
Gen-Probe Hybridization Assay	Gen-Probe	• rRNA hybridize to CT and CG • Specimens do not need to be refrigerated and last for 7 days	

genitourinary infection. Lymphogranuloma venereum infection is usually confirmed after isolating the organism and sequencing the MOMP gene, a time-consuming process. A combined PCR and restriction fragment length polymorphism (RFLP) test has been used to diagnose and differentiate LGV from trachoma biovars.[18] Currently, the only specific test which can rapidly differentiate LGV from the trachoma biovar is a multiplexed real-time PCR developed at the Centers for Disease Control and Prevention. This test is based on a gene deletion of the polymorphic membrane protein H (*pmpH*) gene in the trachoma biovar, which differentiates it from the LGV biovar.

GENOMICS AND MOLECULAR TYPING

The Chlamydia genome is composed of a chromosome of approximately 1.04 million base pairs and a plasmid of 7493 base pairs.[19] The trachoma and LGV biovars have high gene sequence homology and usually only single

base-pair mutations are found between the two. Several genes show heterogeneity that is important.

The major outer membrane protein (MOMP) gene exhibits extensive sequence variation within the serovars. The differences are mainly clustered in four regions (VD1 through 4), which form the basis of serovar differentiation, using sequencing methods and antibody typing.[20,21] The finding that the tryptophan synthase gene becomes inactivated in the ocular infection could prove to be useful in differentiating ocular chlamydial isolates from those normally causing genital tract disease.[22] The finding of polymorphism in the *pmpH* gene has enabled us to differentiate the trachoma from the LGV biovars.[23]

CONCLUSION

Our understanding of the biology of *C. trachomatis* has progressed tremendously, leading to development of better diagnostic methods. While clinicians should understand each type of test and its limitations, in general molecular tests such as NAATs are likely to continue to replace traditional tests in the future.[24]

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Chromogenic In Situ Hybridization (CISH)

Bae-Li Hsi
National Health Research Institutes, Taiwan, Republic of China

INTRODUCTION

In situ hybridization (ISH) on routine formalin-fixed paraffin-embedded (FFPE) tissues faces several technical challenges. It has not always been easy to consistently obtain satisfactory ISH results on FFPE tissue sections. The major obstacle is the permeability of the section to the probes and detection reagents. The method that gave the most consistent results in our hand uses microwave to heat slides in an EDTA buffer to a temperature close to boiling for 15 min. The step is similar to the heat-induced antigen-retrieval step that has been popular in the immunohistochemistry laboratories. The sections were then digested by pepsin (Zymed Laboratories, South San Francisco, CA) or proteinase K (DAKO Co., Carpinteria, CA). For the ease of use and consistency of results, we recommend the use of commercially available, ready-to-use digestion agents. The amount of time required for the digestion varies according to the type of tissues and method of fixation; consistency is essential so that the result can be easily reproduced.

There are several difficulties of using traditional fluorescent in situ hybridization (FISH) on FFPE sections. The autofluorescence of formalin-fixed tissues often obscures the real fluorescence signals. Tissue morphology is difficult to appreciate under dark-field microscopy at high magnification. Moreover, FISH slides cannot be stored or archived for longer periods of time. We demonstrated here the use of chromogenic in situ hybridization (CISH) for the study of genomic alteration in cancer on FFPE sections. The technique allows ISH data to be interpreted using conventional bright-field microscope.

The detail protocols for two experiments were shown. Although commercial kits are available for both experiments, more detail information was given; thus readers can use it as model to develop protocols for their own experiments. The first experiment uses two-color CISH to examine the translocation of *MYC* gene on chromosome 8 in Burkitt lymphoma. The second example demonstrates combined IHC and CISH for HER2 protein expression and gene amplification in breast cancer.

PROBE DEVELOPMENT

With the completion of human genome program and the easy accessibility of human genome information, ISH probes can now be easily selected and prepared. The essential information is provided in the following protocol. More detailed protocols can be obtained from standard molecular biology texts or technical manuals of commercial suppliers.

Bacterial artificial chromosomes (BACs) were selected from the genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) and ordered from CHORI (http://www.chori.org) or Research Genetics (http://www.resgen.com). Usually, two closely positioned BACs of 200 to 300 kb were used together as one probe. Thus, the final labeled probe covers about 400- to 600-kb genomic DNA. DNA of human insert from the BACs was prepared using standard protocol.[1] The probes were labeled by random priming with biotin or digoxigenin using a commercial labeling kit (Invitrogen, Co., Carlsbad, CA; Roche Applied Science, Indianapolis, IN). The labeled probes were purified using MicroSpin S-200 HR column (Amersham Biosciences, Piscataway, NJ), and the quantity and quality of labeled DNA were evaluated by gel electrophoresis or spectrophotometry (usually 5 μg from one labeling reaction). Cot-1 DNA (Invitrogen) was added to the labeled probe (10 times the quantity of labeled probe, e.g., 50 μg of Cot-1 for 5 μg of labeled probe). This anneals with, and blocks, the nonspecific repeating sequences, such as *alu* or LINE sequences.

The probes used for the two examples were prepared before the completion of the human genome program. The selection of probe was slightly different from the method described above. The probes for MYC translocation were YAC probes. The human DNA inserts in the YAC were isolated by pulsed-field gel electrophoresis. For the HER2 probe, the two BACs were selected by ''BLAST'' HER2 gene sequence in the National Center for Biotechnology Information (NCBI) database. The nonspecific repeating sequences were removed in the commercial version of the probe.[3] Thus, no blocking DNA, such as Cot-1 DNA, was added to the probe. All probes are available from Zymed Laboratories.

MYC TRANSLOCATION IN BURKITT LYMPHOMA

The translocation of *c-MYC* gene on 8q24 is a hallmark of Burkitt lymphoma. Two yeast artificial chromosomes

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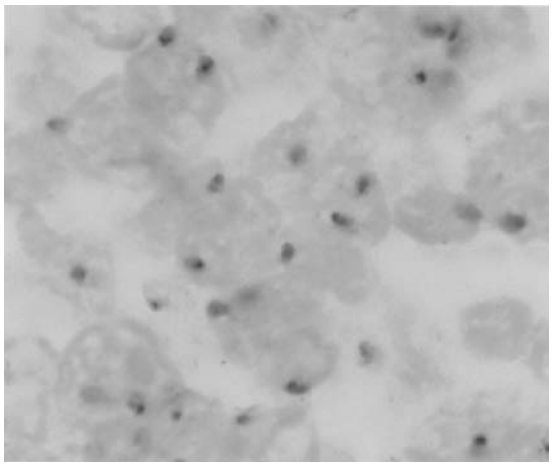

Fig. 1 CISH of MYC on a sample with normal chromosome 8. Majority of red and brown signals are adjacent to each other. The red and brown signals could be clearly differentiated. (*View this art in color at www.dekker.com.*)

(YACs) on the centromeric side of *c-MYC* gene and two YACs on the telomeric side of *c-MYC* gene were selected. The centromeric probe was labeled with digoxigenin and the telomeric probe was labeled with biotin. On the normal chromosome, the two probes were close together. On the abnormal chromosome, the signals of the two probes were apart.

Four-micrometer sections were cut from FFPE tissues and placed on Superfrost Plus slides (Fisher Scientific). The slides were dried in an oven at 37°C overnight. After dewaxing and dehydration, the slides were placed in a preheated pretreatment solution (100 mM Tris–base, 50 mM EDTA, pH 7) in a microwave oven (GE Appliances, model JE1390GA 001) at 199°F for 15 min. After pretreatment, the slides were rinsed with PBS and digested with Digest-All 3 (Zymed) at 37°C for 2 min. The slides were rinsed in PBS and postfixed in 10% buffered formalin for 1 min, dehydrated in graded ethanol, and air dried.

Ten microliters (50 ng) of MYC probe (Zymed) was added over the section and covered with a coverslip. The edges of the coverslip were sealed with rubber cement. The slides were placed in HYBrite slide warmer (Vysis) at 94°C for 3 min and incubated in a humidified chamber at 37°C overnight. The stringency wash was carried out in 0.5× SSC at 72°C for 5 min. For detection, the sections were preblocked with CAS-Block (Zymed) for 10 min and incubated with enzyme conjugate containing alkaline phosphatase (AP)-labeled antidigoxigenin (Roche Applied Science) and horseradish peroxidase (HRP)-labeled streptavidin (Zymed) at 1:500 dilution in antibody diluent (10% normal goat serum in CAS-Block) for 60 min. After washing in PBS/T (0.025% Tween-20 in PBS), the color for HRP was developed using DAB (Zymed) and the color for AP was developed with Fast Red (Zymed) according to the manufacturer's instruction. The slides were counterstained with hematoxylin and mounted in Glycergel (DAKO).

CISH allowed the morphology of the tissue sections to be easily examined. The area of tumor cells could be identified at low magnification; chromosome ISH results on these tumor cells were then examined using 100× objective. The microscope must be of sufficiently high quality to allow the brown color of DAB and red color of Fast Red be clearly differentiated (Figs. 1 and 2) at high magnification.

In the ideal condition, signals on a tissue section that do not have chromosome translocation at 8q24 should all be shown as adjacent red/brown signals. However, often, only part of each nucleus was present on the section. If the sectioning cut through chromosome 8 at area close to 8q24, then individual red or brown signal may be observed on normal tissue samples. In a 4-μm-thick section of normal lymph node, up to 5% of the signals may be shown as single red or single brown signal.

Because of sectioning effect, many nuclei will have incomplete signal profiles. The traditional way of reading FISH results is to look for a nucleus containing all four of the expected red and brown signals. However, a simpler approach is to identify regions predominantly composed of neoplastic cells, then simply record the total number of red, brown, and red/brown signals in that region. The tumor with the translocation should have about 1/3 of all

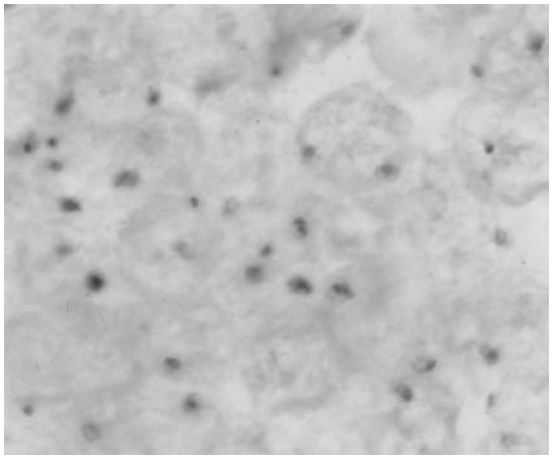

Fig. 2 CISH of MYC on a sample with 8q24 translocation. The image shows individual red, individual brown, and red/brown adjacent signals. (*View this art in color at www.dekker.com.*)

the signals shown as red/brown, 1/3 as red only, and 1/3 brown only.

Using this method of recording data for translocation study, the counting from five samples that did not have 8q24 translocation showed that around 90% of signals were red/brown and about 5% were either individual red or individual brown. The counting from five samples that had 8q24 translocation showed that around 40% of signals were red/brown and around 30% were either individual red or individual brown.

COMBINED IHC AND CISH OF HER2 IN BREAST CANCER

The protocol described here allows immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) to be combined, such that genetic alternation and protein expression were evaluated using an ordinary light microscope. This technique can have many uses. It can be used, as in this experiment, to evaluate the genetic modification of the gene and the resulting alternation of gene expression;[4] or IHC can be used simply as marker to differentiate different cell type.

The FFEP sections were prepared and pretreated with pretreatment solution as previously described. For immunohistochemistry, the sections were preblocked with CAS-Block for 10 min, then incubated with rabbit antibody to HER2 (Zymed) for 30 min. After washing in PBS/T, the slides were incubated with AP-labeled goat antirabbit IgG (Zymed) at 1:100 dilution in antibody diluent for 30 min and washed with PBS/T. Then, the color of AP was developed with Vector-blue (Vector). The sections were then digested with Digest-All 3 and in situ hybridization was carried out according to the protocol described in the previous section using digoxigenin-labeled HER2 probe (Zymed). For detection, the slides were first incubated with CAS-Block for 10 min, and then incubated with FITC–antidigoxigenin (Roche Applied Science) at 1:500 dilution in antibody diluent for 60 min. After washing in PBS/T, the slides were further incubated with HRP–anti-FITC (Zymed) for 60 min and washed in PBS/T. Then, the color of HRP was developed with DAB (Zymed). The slides were counterstained with hematoxylin and mounted in Glycerogel (DAKO).

The IHC results and CISH of highly amplified *HER2* gene can be viewed at 10× objective. The tumor cells with cluster of amplified *HER2* gene usually are also strongly positive for the HER2 IHC (Fig. 3). The normal cells are negative of HER2 IHC and the nuclei contain only one or two CISH signals for the *HER2* gene. Using conventional bright-field microscopy, the nonamplified HER2 signal could be observed with 40× objective. However, because of the reflective nature of DAB, the signal can be observed at 10× using phase-contrast microscopy.

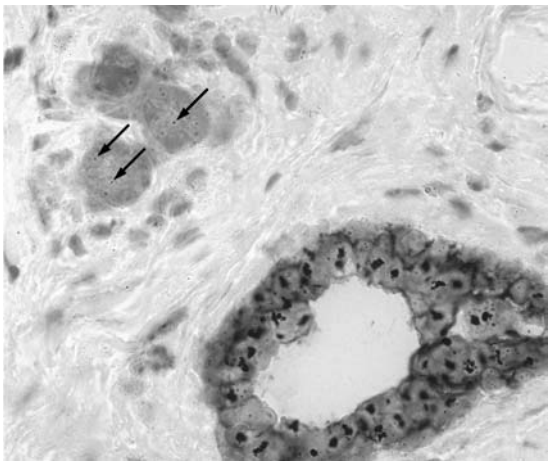

Fig. 3 HER2 IHC/CISH in 4-μm-thick paraffin section of breast carcinoma open biopsy. Tumor cells with clusters of highly amplified *HER2* gene and strong IHC staining can be seen. The arrows point to normal HER2 signals in cells of a normal breast epithelium, which is negative for IHC staining. (*View this art in color at www.dekker.com.*)

CONCLUSION

We demonstrate here the technique of CISH on FFPE tissue sections. It has many advantages over traditional FISH. However, CISH also offer much more limited option than FISH in image analysis. The FISH signals can be computer enhanced, quantified, and analyzed. Computer-assisted analysis also allows multiple probes labeled with several different colors of fluorochrome to be used. Furthermore, the experimental procedure for FISH using directly labeled probe is less time consuming than CISH. However, in routine pathology, the convenience of CISH often outweighs its shortcoming. The most important advantage is that the morphology of tissue can be clearly visualized. They can then be compared with sections stained with other traditional methods, e.g., H+E and IHC, using conventional bright-field microscopy. The slides can also be archived as easily as other routinely prepared slides.

The combination of IHC with CISH, such as in the HER2 example, allows the confirmation of IHC results by ISH. Often, the CISH result was much more quantitative and less variable than the IHC data. More

importantly, it allows the potential of examining gene modification and the consequence of protein expression on tissue section.

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Chronic Myelogenous Leukemia—Molecular Diagnosis and Monitoring

Jo-Anne Vergilio
Brigham and Women's Hospital, Boston, Massachusetts, U.S.A.

Adam Bagg
Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder with a well-known molecular pathogenesis, the understanding of which has resulted in the recent exciting development and application of targeted molecular therapy. Molecular techniques play an integral and well-established role in the diagnosis and monitoring of CML, with the latter detection of minimal residual disease being pertinent to both a determination of therapeutic response and efficacy, as well as in the detection of early relapse.

CLINICAL DESCRIPTION

Chronic myelogenous leukemia originates from the transformation of a hematopoietic stem cell with resultant expanding myelopoiesis that, when untreated, characteristically evolves through three phases: a chronic phase manifested by myeloid hyperplasia with circulating granulocytes in all stages of maturation; an accelerated phase of shorter duration during which myeloid elements begin to lose the ability to differentiate; and inevitably, a blast phase of acute leukemia that is usually of myeloid (70%), but can be of lymphoid, phenotype.

Although some patients are asymptomatic with coincidental detection of a leukocytosis on routine medical evaluation, the chronic phase of disease typically has an insidious onset with symptoms related to hypermetabolism, including fatigue, anorexia, weight loss, and night sweats. Massive splenomegaly is common. With disease progression, patients typically develop worsening anemia and thrombocytopenia. Without treatment, the median survival of CML is 4–5 years from diagnosis.

GENOTYPE–PHENOTYPE CORRELATION

The reciprocal t(9;22)(q34;q11) translocation is probably the initial transforming event in the development of CML. This translocation yields a shortened chromosome 22 that is the Philadelphia (Ph) chromosome. With the translocation, two distinct genes are fused: 1) *BCR* that encodes a cytoplasmic protein of uncertain function with oligomerization, serine threonine kinase, and GTPase-activating domains; and 2) *ABL* that encodes a nonreceptor tyrosine kinase normally localized to the nucleus.[1] The resultant chimeric gene and fusion transcript yield a protein with constitutively heightened tyrosine kinase activity that is relocated from the nucleus to the cytoplasm and phosphorylates a variety of cellular substrates with consequent proliferative, growth factor independent, antiapoptotic, and defective adhesive properties in the transformed cells.

The subsequent occurrence of specific cytogenetic and molecular genetic events, in addition to t(9;22), heralds disease progression prior to hematological and/or clinical manifestations. The acquisition of +Ph, isochromosome 17q, and +8 commonly indicates an impending blast crisis while molecular abnormalities associated with disease progression include overexpression of *BCR–ABL*, upregulation of the *EVI1* gene, mutations in tumor suppressor genes *P16*, *P53*, *CDKN2A*, and *RB1*, and aberrant DNA methylation of the translocated *ABL* allele.[2,3] Deletion of the derivative chromosome 9 is an independent poor prognosticator that predicts rapid progression to blast crisis with shortened survival.[4]

PREVALENCE

Chronic myelogenous leukemia comprises ~20% of all leukemias, with a worldwide incidence of 1–1.5 cases per 100,000 population per year. The median age at diagnosis is 50 years, although pediatric cases have also been reported.

DIFFERENTIAL DIAGNOSIS

An important entity in the differential diagnosis of CML is a leukemoid reaction that is nonneoplastic in origin,

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arising secondary to other causes, including infection. Other myeloproliferative/myelodysplastic disorders, such as atypical chronic myelogenous leukemia and chronic myelomonocytic leukemia, must also be distinguished from CML given their similar clinical presentations; however, both lack the *BCR–ABL* translocation.

MANAGEMENT

Allogeneic stem cell transplantation (ASCT) is the only proven curative therapy for CML with cure rates of ~70–80% in young (age <40 years) chronic-phase patients who have HLA-matched donors and undergo transplantation within 1 year of diagnosis.[5] However, the associated morbidity and mortality of ASCT is significant; furthermore, most (~65%) patients do not have a suitably matched donor while older patients are often suboptimal candidates for transplantation.

An appreciation and comparison of other treatment options requires an understanding of what constitutes an efficacious therapeutic response; this is gauged using three basic parameters: hematological, cytogenetic, and molecular. Hematological remission is achieved when the blood counts and spleen size have normalized. Cytogenetic response is quantified and graded based upon the percentage of residual Ph+ cells. Traditionally, cytogenetic response serves as the ''gold standard'' for analysis, providing an important predictor of patient survival. Once conventional hematological and cytogenetic remission has been achieved, monitoring then relies upon minimal residual disease assessment using more sensitive molecular techniques to detect the *BCR–ABL* fusion transcript. With successful ASCT, both hematological and cytogenetic remission are achieved and the *BCR–ABL* transcript is no longer detectable.

In patients who are not suitable candidates for ASCT, alternative therapies include interferon-alpha[6] and, more recently, imatinib mesylate (or Gleevec, STI571). Molecular targeted drug therapy with imatinib, a synthetic tyrosine kinase inhibitor, has shown activity in all phases of CML with substantial responses in newly diagnosed patients in chronic phase.[7–10] In an on-going randomized Phase III trial (IRIS), the observed rate of complete cytogenetic remission in newly diagnosed CML patients was 76% with imatinib vs. 15% with interferon plus Ara-C (median follow-up 18 months).[8] Although long-term outcome data are not yet available, imatinib has emerged as the therapy of choice for initial, although still noncurative, treatment of CML as the *BCR–ABL* fusion transcript persists at low levels. One major obstacle to treatment has been the development of therapeutic resistance, particularly in individuals with advanced disease. Mechanisms of resistance include 1) amplification of the *BCR–ABL* chimeric gene at the genomic or transcript level, and 2) point mutations in the kinase domain of *BCR–ABL* (altering its responsiveness to imatinib).[11]

MOLECULAR GENETICS

The t(9;22) translocation can result from several different breakpoints in the *BCR* and *ABL* genes, each with different chimeric fusion proteins that confer somewhat specific clinicopathological features and indicate that this translocation is not pathognomonic of CML (Fig. 1A and B). These breakpoints, indistinguishable by traditional karyotyping, can only be differentiated using molecular techniques. In each, most of *ABL* is juxtaposed to variable 5′ portions of *BCR*. Whereas the breakpoint involving *ABL* is relatively conserved, usually arising in the intron before exon 2 (a2), the breakpoints involving *BCR* are more variable. Breakpoints arising in the major breakpoint cluster region (*M-bcr*) are by far the most common in CML. They occur after either exon 13 (e13 or b2) or exon 14 (e14 or b3), encode a p210 fusion protein, and are present in the vast majority (~99%) of patients with CML.

MOLECULAR GENETIC TESTING

Two broad scenarios necessitate molecular testing in CML: diagnosis and subsequent therapeutic monitoring.

Diagnosis

Whereas cytogenetics identifies the presence of the t(9;22) translocation as well as other chromosomal abnormalities, documentation of the t(9;22) is successful in only ~95% of cases of CML. In one-half of the remaining patients with a normal karyotype, the *BCR–ABL* fusion is only detectable at the molecular level, due to a submicroscopic genetic fusion. In those patients with a normal karyotype, who have the clinical and hematological profile of CML, molecular testing serves a primary role in identifying such patients. In the remaining patients negative for both the Philadelphia chromosome and the *BCR–ABL* fusion, alternative diagnoses must be considered.

Qualitative RT-PCR

Methodologically, qualitative RT-PCR can be performed with a simple, nested, or multiplex approach. Nested

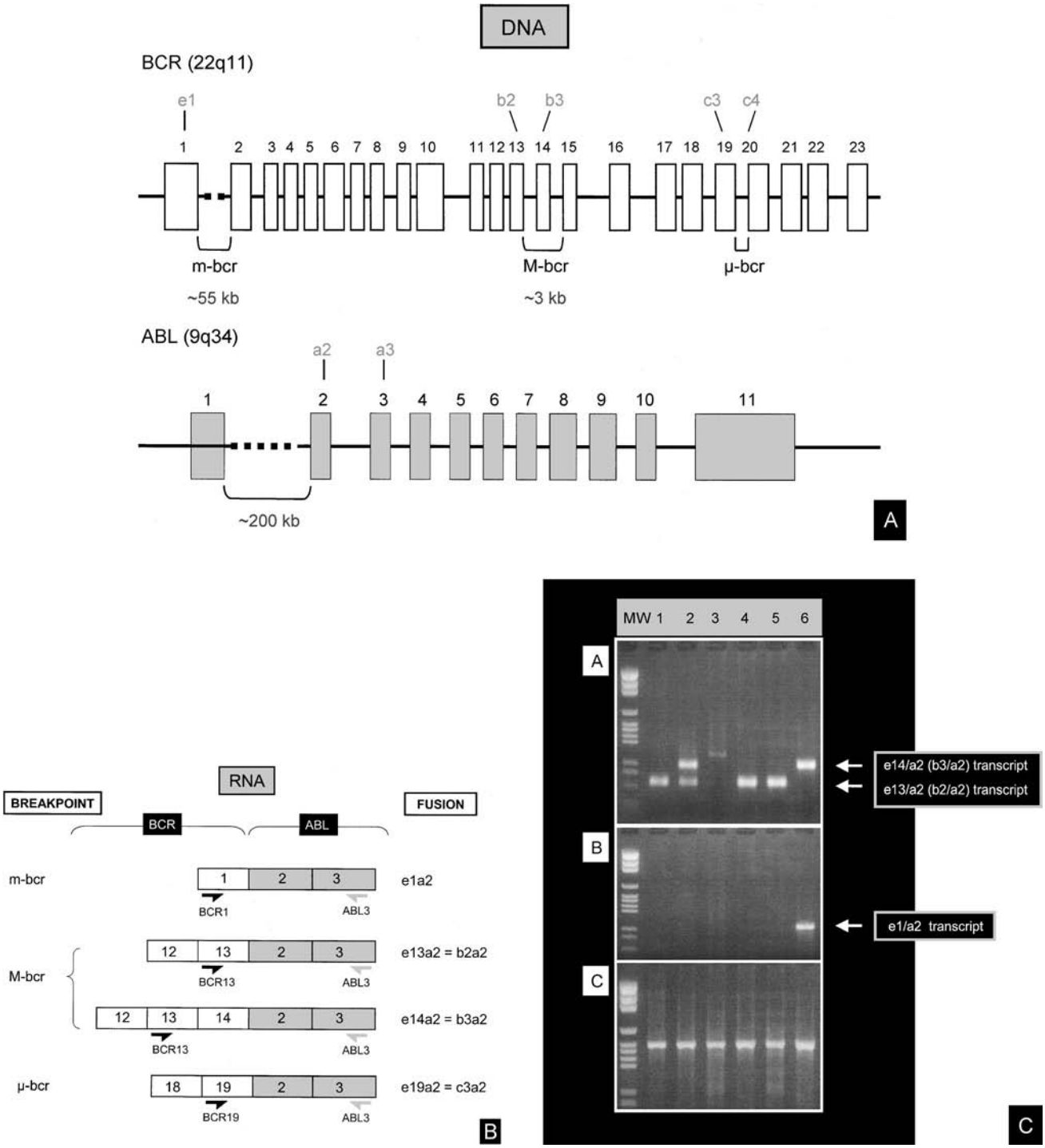


Fig. 1 Schematic representation of (A) the *BCR* and *ABL* genes demonstrating the primary breakpoint cluster regions (bcr) and (B) the RNA/cDNA structure of the most common chimeric transcripts and the primers used for their RT-PCR amplification. The μ-bcr occurs in the uncommon chronic neutrophilic leukemia and is not routinely evaluated. (C) Conventional qualitative RT-PCR for detection of the most common *BCR–ABL* fusion transcripts using gel electrophoresis. Upper panel (A): *BCR* exon 13 (b2) and *ABL* exon 2 (a2) primers. Middle panel (B): *BCR* exon 1 (e1) and *ABL* exon 2 (a2) primers. Lower panel (C): β2-microglobulin primers. Lanes: (1) intron 13 breakpoint, (2) intron 14 breakpoint with alternative splicing to *BCR* exon 13, (3) ? novel breakpoint, ? nonspecific band, (4) intron 13 breakpoint, (5) intron 13 breakpoint, (6) intron 14 breakpoint with alternative splicing to *BCR* exon 1. See text for details.

approaches are to be avoided for routine laboratory practice given the significant risk of contamination and false positivity. With nonnested, simplex RT-PCR, one assay is performed using a single pair of primers. Primers for *BCR* exon 13 (b2) and *ABL* exon 2 (a2) identify both the e13a2 (b2a2) and e14a2 (b3a2) fusion transcripts that differ in size by 75 bp (Fig. 1C, lanes 1 and 6, upper panel). Use of this primer set allows for the molecular detection of CML in nearly all cases (~99%); however, in rare instances, variant breakpoints may occur, yielding a false-negative result or alternatively sized RT-PCR product. The incorporation of an *ABL* exon 3 (a3) primer, in place of an a2 primer, permits the routine detection of rare *ABL* intron 3 breakpoints and has been recommended by some for routine evaluation.[12]

Multiple fusion transcripts may be detectable with a single *BCR* intron 14 breakpoint due to alternative splicing of the primary transcript that yields either an accompanying e13a2 (Fig. 1C, lane 2, upper panel) or an e1a2 (Fig. 1C, lane 6, upper and mid panels) transcript. Identification of the e1a2 transcript requires a separate BCR primer targeting BCR exon 1 (e1) (Fig. 1C, mid-panel, lane 6), and although not essential to the diagnosis of CML, some studies suggest its presence to be a poor prognosticator associated with metamorphosis.[3]

Interpretation

A negative RT-PCR result renders CML extremely unlikely, but does not definitively exclude the diagnosis given the rare variant breakpoints alluded to above. Similarly, a positive RT-PCR result does not definitively invoke a diagnosis of CML, given that *BCR–ABL* fusion transcripts have been identified in the blood of as many as two-thirds of healthy individuals using an extremely sensitive nested RT-PCR technique.[13] However, whereas the majority of CML patients express either the e13a2 or the e14a2 chimeric transcripts, healthy individuals express primarily the e1a2 type. Furthermore, the extreme level of sensitivity achieved ($\sim 10^{-8}$) with this nested technique is neither required nor recommended for diagnostic clinical testing, and thus misdiagnosis should not be an issue.

Monitoring

An understanding of laboratory monitoring is predicated on an appreciation of therapeutic goals and options. The goal of therapy is to eliminate disease; however, if eradication of the neoplastic clone cannot be achieved then therapeutic intent relies on tumor control with an attempt to forestall disease progression.

Molecular monitoring with qualitative RT-PCR originated in the posttransplantation setting. Here it serves two distinct functions: first to document remission and then to monitor for disease relapse. In the latter, molecular monitoring permits early disease detection (prior to hematological or cytogenetic manifestations), when the tumor burden is low and presumably more amenable to treatment. At molecular relapse, therapeutic options include the withdrawal of immunosuppressive agents and/or the administration of donor lymphocyte infusions (DLI) with an improved response when implemented prior to overt hematological relapse.

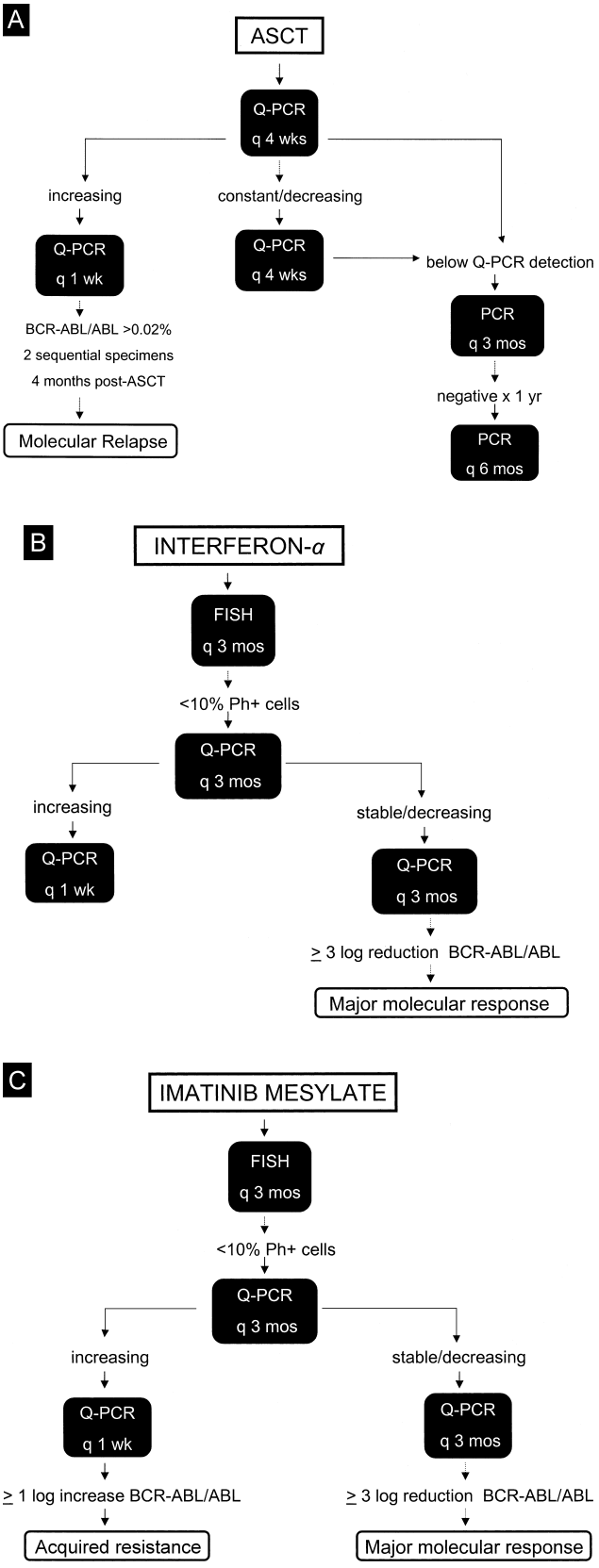
Therapeutic context dictates the ramifications of any test result. In the setting of ASCT, the interval post-transplant, the type of transplant (i.e., unrelated vs. allogeneic-related matched), and the presence or absence of T-cell depletion may all impact the prognostic relevance of qualitative test results. Most patients are qualitatively RT-PCR positive in the first 6 months following ASCT and such results are thus of little consequence. Six to 12 months after ASCT, however, positive qualitative results are highly predictive of subsequent relapse.[14,15] Here RT-PCR positivity precedes cytogenetic and hematological relapse by several months.

Although qualitative RT-PCR has been the mainstay of minimal residual disease assessment, a single positive qualitative result is, in isolation, of little clinical predictive value.[16] In contrast, quantitative RT-PCR shows broad relevance to posttherapeutic monitoring whether after transplantation or other therapeutic modalities. Logically, low or falling transcript levels correlate with successful treatment or continued remission, and elevated or rising transcript levels predict relapse. Consequently, quantitative RT-PCR has emerged as the preferred modality for posttherapeutic monitoring.

Quantitative RT-PCR

Real-time RT-PCR affords sensitive, rapid, and reproducible quantification of mRNA/cDNA transcripts. Although sensitivities are somewhat less than with conventional or nested RT-PCR, the dynamic range of fluorescent detection is much broader, spanning five to six orders of magnitude, with a lower limit of detection of $<0.01\%$ (10^{-4}).[12] Most importantly, real-time RT-PCR permits the serial precision required in clinical diagnostic applications.

Various fluorescent detection systems have been used to quantify *BCR–ABL*. The major chemistries employed are the use of Taqman™ or FRET probes. For both of these, RNA or cDNA standards of known concentration



are used to generate a standard curve [log (copy number) vs. threshold cycle (C_T)], from which the unknown sample quantity is determined and then normalized against an internal reference (ideally *ABL*). The final result is usually reported as a percentage ratio (e.g., *BCR–ABL/ABL*).

Interpretation

The establishment of specific criteria to define molecular response, remission, and relapse has been difficult in light of the on-going evolution, and increasing sophistication, of quantitative technology, without universal technical standardization. Nevertheless, pertinent information can be ascertained.

With respect to monitoring postransplantation, several quantitative indicators emerge. Thus, a modified definition of molecular relapse has been proposed as rising or persistently high levels of *BCR–ABL*, delineated as a *BCR–ABL/ABL* ratio of >0.02% ($\sim 10^{-4}$), in two sequential specimens procured more than 4 months after ASCT.[17]

Although imatinib has shown encouraging results in the treatment of CML, the long-term correlates of clinical outcome remain to be defined. Nonetheless, recent studies suggest that quantitative RT-PCR values correlate with established cytogenetic response criteria and are associated with a differential short-term outcome.[18] In the IRIS study, a ''major molecular response (MMR)'' is defined as a $\geq$3 log reduction in *BCR–ABL/BCR* levels compared to median pretreatment levels. Here, MMR was achieved in 39% of newly diagnosed CML patients after 12 months of imatinib as compared to only 2% of patients on interferon plus Ara-C.[10] Patients with this degree of molecular response to imatinib had a negligible risk of disease progression in the short-term.

One additional advantage of kinetic monitoring with imatinib is that it allows for the early recognition of therapeutic resistance. In this scenario, patients with a suboptimal therapeutic response may undergo screening for causative point mutations (e.g., using allele-specific

Fig. 2 Algorithms for the frequency of therapeutic monitoring in CML: (A) after allogeneic stem cell transplantation (ASCT), (B) with interferon-α, and (C) with imatinib mesylate therapy. [FISH, fluorescence in situ hybridization; mos, months; Ph, Philadelphia chromosome; PCR, qualitative reverse transcription polymerase chain reaction; Q-PCR, quantitative reverse transcription polymerase chain reaction; wk(s), week(s)].

oligonucleotide PCR), the identification of which could prompt implementation of alternative treatment strategies. Preliminary work with microarrays has identified differential gene expression profiles between patients with imatinib sensitivity and resistance.[19] In the future, this technology may predict therapeutic response and thus dictate front-line therapy.

Regardless of the therapeutic strategy, large prospective studies that correlate quantitative RT-PCR data with long-term clinical outcome are keenly anticipated. These investigations will direct the formulation of new guidelines to assist with the future therapeutic monitoring and clinical interpretation of these tests. Until then, a number of algorithms have been proposed for posttherapeutic monitoring (Fig. 2).[20,21] One final caveat in molecular RT-PCR analysis, whether qualitative or quantitative in methodology, is that it cannot assess the clonal evolution of disease. For this assessment, cytogenetic karyotypic analysis is required and must be performed every 6 months throughout the monitoring process regardless of the therapeutic modality employed. The emergence of cytogenetically abnormal (but Ph-negative) clones with the use of imatinib further underscores the need for periodic conventional cytogenetic analysis.[22]

CONCLUSION

The evolution in our understanding of CML has led to the molecular dissection of this disease with the implementation of successful targeted therapy. The molecular laboratory plays a vital role in the diagnosis as well as therapeutic evaluation and monitoring of CML patients. In the future, this may extend beyond merely measuring *BCR–ABL*, as new insights are developed from microarray studies.

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Clarithromycin Resistance Associated Gene Mutations

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Sabine Pereyre
Armelle Ménard
Cécile Bébéar
Francis Mégraud
Université Victor Segalen Bordeaux 2, Bordeaux, France

INTRODUCTION

Resistance to macrolides may occur via different mechanisms. Among them mutations in the 23S rRNA have been recently described. It concerns different bacteria of clinical relevance but most importantly *Helicobacter pylori*, a bacterium for which several detection methods have been proposed. In this review, we will present briefly the basis for the resistance mechanism and the recent development in the field of *H. pylori* diagnosis.

RESISTANCE INDUCED BY RIBOSOMAL MUTATIONS

Macrolide antibiotics inhibit protein synthesis through their interactions with the 50S subunit of the bacterial ribosome by binding near the entrance of the peptide passage channel. The interactions have mainly been mapped by chemical footprinting experiments and X-ray crystallography[1] to the peptidyl transferase region within domain V of 23S rRNA. Positions A2058 and A2059 (*Escherichia coli* numbering) appeared to be essential for macrolide binding. Interaction with domain II of 23S rRNA has also been suggested,[2] but they have no direct interaction with ribosomal proteins.[1]

Three main mechanisms of resistance have been reported: drug inactivation, active efflux, and modification of the target sites by methylation or mutations.[3,4] Resistance conferred by ribosomal mutations was first observed in the single rRNA (*rrn*) operon of yeast mitochondria then obtained in *E. coli*.[3] Reports are now common both in Gram-positive and Gram-negative clinical pathogens (Table 1). This mechanism is responsible for clarithromycin resistance in the vast majority of strains of *Mycobacterium* spp. and *H. pylori*.[3] Similar mutations have also been reported in *Treponema pallidum*, *Propionibacterium* spp., and *Mycoplasma* spp.. Generally, pathogenic species that develop macrolide resistance through rRNA mutations possess only one or two *rrn* operons, such as in the case of *H.pylori* (two operons), *M. avium*, and *M. pneumoniae* (one operon).[3] Resistance in bacteria with multiple *rrn* operons is generally conferred by Erm methylation or active efflux. However, there are recent reports of macrolide resistance in laboratory and clinical strains of *Streptococcus pneumoniae* and *Staphylococcus aureus* which harbor four and six *rrn* operons, respectively.[5,6] Susceptibility of erythromycin decreases as the number of the mutated copies increases,[7] and high-level erythromycin resistance can be achieved only when at least half of the copies are mutated.

The consequence of point mutation in the domain V of 23S rRNA is a decrease of the affinity of the macrolide for its ribosomal target. An absence of binding of radiolabeled erythromycin to ribosomes isolated from *H. pylori* or *M. hominis* resistant strains suggested that the mutations lead to a conformational change in the ribosome at the macrolide binding site.[8,9]

The resistance phenotype conferred by alterations in the 23S rRNA target varies according to the substituted base. There have been a plethora of strains identified with mutation A2058G or A2059G/C (*E. coli* numbering) that confers macrolides, lincosamides, and streptogramin B (MLS_B), and macrolides and lincosamides (ML) resistance, respectively.[3] The A2058G substitution is the most frequent in clinical isolates and leads to the highest minimal inhibitory concentrations (MICs). Substitutions A2058C and A2058U are less common (Table 1). Mutations at positions 2057 and 2611 have also been reported. They destabilize the base pairing G2057-C2611 in the single-strand structure of the central loop but have generally a weak impact on the MICs of macrolides.[3] Mutations at position 2062 were described in mycoplasma laboratory isolates and in *S. pneumoniae* clinical strains,[10,11] and were associated with resistance to 16-membered macrolides and streptogramin combinations. Mutations at positions 2609, 2610, and 2613 have been associated with macrolide resistance in laboratory mutants but have never been reported in clinical isolate. Moreover, a T-to-C transition at position 2717 (*H. pylori* numbering) in domain VI of 23S rRNA conferred a low-level clarithromycin resistance in *H. pylori* clinical isolates.[12] Resistance by ribosomal mutations in domain

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Table 1 23S rRNA mutations that confer macrolide resistance in pathogenic bacteria

Position[a]	Organism	L/C[b]	Mutation[c] (no. of mutated copies)	Phenotype[d]
752	*S. pneumoniae*	L	Deletion A752	M14, M15, Tel
754	*E. coli*	L	U754A	Ery, Tel
2032	*E. coli*	L	G2032A	Cli
2057	*E. coli*	L	G2057A	M14
	P. acnes	C	G2057A	Ery
2057+2032	*E. coli*	L	G2057A+G2032A	Ery, Cli
	H. pylori	C	G2057A (1)+A2032G (1)	M14, M15
2058	*B. pertussis*	C	A2058G	M14, M15
	H. influenzae	L, C	A2058G	M14, M15, Cli
	H. pylori	L	A2058G/C/U	Clr
		C	A2058G (1)	Clr
		C	A2058G (2)	MLS_B
		C	A2058C	ML
	M. abcessus	C	A2058G	Clr
	M. avium	C	A2058G/C/U	Clr
	M. chelonae	C	A2058C/G	Clr
	M. intracellulare	C	A2058C/G/U	Clr
	M. kansasii	C	A2058U	Clr, Azi
	M. smegmatis	L	A2058G (1)	Clr
	M. pneumoniae	L, C	A2058G	ML
	Propionibacterium spp.	C, L	A2058G	MLS_B
	S. aureus	C	A2058G/U (4)	MLS_B
	S. pneumoniae	L	A2058G (2, 3)	MLS_B, Tel
			A2058U (3)	ML, Tel
		C	A2058G (2, 3)	MLS_B
			A2058G (4)	ML, Tel
			A2058U (2)	MLS_B
	S. pyogenes	C	A2058G (4)	Ery, Cli, Tel
	T. denticola	L	A2058G (1, 2)	Ery
	T. pallidum	C	A2058G (2)	M14, M15
2059	*H. influenzae*	L	A2059C	M14, M15, Cli
	H. pylori	L	A2059G/C	Clr
		C	A2059G	ML
	M. avium	C	A2059C/G	Clr, Azi
	M. abcessus	C	A2059C/G	Clr
	M. chelonae	C	A2059G	Clr
	M. intracellulare	C	A2059C/G	Clr, Azi
	M. hominis	C	A2059G	ML, Tel
	M. pneumoniae	L	A2059G/C	ML
	M. smegmatis	L	A2059G (1)	Clr
	Propionibacterium spp.	C	A2059G	ML
	S. aureus	C	A2059G (3)	ML
	S. pneumoniae	L	A2059G (2 to 4)	ML
		C	A2059G (1 to 4)	ML
			A2059C (2 to 4)	ML
2057+2059	*S. pneumoniae*	C	G2057A+A2059G	ND
2062	*M. hominis*	L	A2062G (1)	M16
		L	A2062U (2)	M16
	M. pneumoniae	L	A2062G	M16, S_A–S_B
	S. pneumoniae	C	A2062C (4)	M16, S_A–S_B
2160–64	*H. influenzae*	C	A2160U+A2058G	Clr, Azi
			A2160U+L4	Clr, Azi
			2160GGA→UAU+ L22	Clr, Azi
			C2164G+L4+L22	Clr, Azi

(*Continued*)

Table 1 23S rRNA mutations that confer macrolide resistance in pathogenic bacteria (*Continued*)

Position[a]	Organism	L/C[b]	Mutation[c] (no. of mutated copies)	Phenotype[d]
2609	*E. coli*	L	U2609C	K
2610	*S. pneumoniae*	L	C2610U	M14, Azi, Cli
2611	*E. coli*	L	C2611U	Ery
	N. gonorrhoeae	C	C2611U (3)	Ery, Azi
	M. hominis	C	C2611U + A2059G	ML, Tel
	M. pneumoniae	L	C2611A	M14, M15, Tel
	S. pyogenes	C	C2611U	M15
	S. pneumoniae	L	C2611A (3)	M15, M16, S_B
			C2611G (4)	M14, M15, L, S_B
			C2611U (2, 4)	Azi, Cli
		C	C2611G (3)	M14, M15, Q–D
			C2611G (4)	MLS_B, Tel
			C2611U (4)	M16, Cli
			C2611A	ND
2613	*S. pneumoniae*	L	C2613A (4)	Azi
			C2613U (3)	Azi
2617	*S. pneumoniae*	C	C1617T + L22	Tel
2629	*H. pylori*	C	U2629C	Clr

[a]*E. coli* numbering.
[b]L, laboratory strain; C, clinical strain.
[c]+L4, +L22, associated alterations in ribosomal proteins L4 or L22.
[d]The phenotypes conferred to the different type of macrolide antibiotics are given when these were specified in the original articles. The lack of a notation does not imply sensitivity but indicates that no specific phenotype was reported. M, macrolides; M14, M15, M16, 14-, 15- 16-membered macrolides; L, lincosamides; S_B, streptogramin B; S_A–S_B, streptogramin combination; K, ketolides; ery, erythromycin; clr, clarithromycin; azi, azithromycin; cli, clindamycin; Q–D, quinupristin–dalfopristin; tel, telithromycin; ND, nondetermined.

II of 23S rRNA has been rarely described in laboratory strains of *E. coli* and *S. pneumoniae*.[3,13] An increase of the ketolide telithromycin MIC was described. Recently, mutations in ribosomal proteins L4 and L22 were associated with resistance to macrolides and related antibiotics in laboratory or clinical isolates of *S. aureus*,[14] *M. pneumoniae*,[11] or *Streptococcus* species including *S. pneumoniae*.[5] The mutations are clustered in conserved sequences of L4 and L22 proteins, regions that mediate the binding to 23S rRNA or contribute to the formation of the tunnel wall. As crystallographic studies showed no direct interaction between ribosomal proteins and macrolides,[1] resistance presumably results from an indirect conformational change in the tunnel region that forms a major portion of the macrolide binding site.

METHODS FOR DETECTION IN *HELICOBACTER PYLORI*

Developments in this field are due to the following:

1. The clinical importance of this bacterium which is not only responsible for peptic ulcer disease but also gastric malignancies. *H. pylori* infection has been recognized by the International Agency on Research on Cancer as a type 1 carcinogen and is indeed the first infection to be involved in cancer in humans. Another cancer, gastric MALT lymphoma, can also be cured by eradicating the bacterium.
2. The key role of clarithromycin in eradication treatment: clarithromycin has low MICs, good diffusion, and limited influence of low pH. Nevertheless, clarithromycin resistance may occur and jeopardize the treatment success. The eradication rate of the main treatment combination (clarithromycin and amoxicillin plus a proton pump inhibitor bd for 7 days) decreases from 88% to 18% if the strain is resistant. The prevalence of the resistant strains in adults varies from less than 5% in Northern Europe to more than 15% in Southern Europe. It is even higher (20–25%) among children. The main risk factor for *H. pylori* resistance to clarithromycin is consumption of this drug, essentially for respiratory tract infections.[15]

As resistance is caused by a limited number of point mutations, it was possible to develop methods for their detection. The three major point mutations are A2143G (70%), A2142G (12%), and A2142C (3%) corresponding to A2059G, A2058G, and A2058C respectively in *E. coli*

numbering. However, this last percentage is underestimated because in most of the studies, the A2142C mutation was not sought.[15]

PCR-Based Methods

These mutations can obviously be detected by sequencing, but many other PCR-based techniques have been developed (Table 2). The most widely used is PCR-RFLP with *Bsa*I and *Bbs*I.[8,16] A third enzyme (*Bce*A1) allowing the detection of the A2142C mutation has recently been proposed.[17] However, these techniques have drawbacks, most are time consuming and are subject to the risk of PCR contaminations.

The main progress has been the application of real-time PCR and the fluorescence resonance energy transfer (FRET) chemistry. The first attempt concerning *H. pylori* was made on strains and was published in 1999.[18] Since then, it has been extended to both the detection of the bacterium and its resistance to clarithromycin, directly in gastric biopsies.[19–22] In our study,[21] we only tested sensitivity as biopsies were obtained from patients who had failed a first eradication therapy and were all *H. pylori* positive. Indeed, in only one case out of 200 (99.5% sensitivity) did we not obtain amplification products. The results of FRET–melting curve analysis (MCA) were compared to those of two phenotypical methods of susceptibility testing performed on the corresponding strains obtained by culture: Etest and agar dilution. The results of the two phenotypical methods agreed in 195 cases (98%), and among them the FRET–MCA was concordant in 188 (96.4%). Using agar dilution as the gold standard, sensitivity and specificity of FRET–MCA were 98.4% and 94.1%, respectively. The mutations identified by FRET–MCA were also compared to those obtained by PCR-RFLP performed on a subset of biopsy specimens and a good agreement was found. In only three cases (1.5%) did we find a clarithromycin-resistant phenotype and a wild-type genotype only, suggesting either that mutations in the 23S rDNA were not detected by this method, or that other mechanisms not related to mutations may be involved.

Table 2 Molecular methods for *Helicobacter pylori* testing of clarithromycin resistance

Based on amplification of 23S rRNA gene
– Sequencing
– RFLP (restriction fragment length polymorphism)
– OLA (oligonucleotide ligation assay)
– DEIA (DNA enzyme immunoassay)
– PHFA (preferential homoduplex formation assay)
– INNO-LiPA (line probe assay)
– Mismatch PCR
– DG-DGGE (double gradient-denaturing gradient gel electrophoresis)
– FRET–MCA (fluorescence resonance energy transfer–melting curve analysis)
Based on hybridization
– FISH assay (fluorescence in situ hybridization assay)

Matsumura et al. using a similar biprobe assay reported a perfect sensitivity and specificity (100%) for *H. pylori* detection on 186 biopsies and also a perfect correlation with the PCR-RFLP results in 151 cases. However, few cultures were performed in this study and the A2142C mutation was not reported.[20]

The assay described by Lascols et al. is different for *H. pylori* detection as they used an LC-Red 640 primer as the sensor probe. Sensitivity and specificity of their assay tested on 196 biopsies, in comparison to histology and culture, were 97% and 94.6%, respectively. Regarding clarithromycin resistance determined for 59 of 66 *H. pylori* positive biopsies, the sensitivity and specificity were 91% and 100%, respectively.[22]

In contrast to the previous studies using a biprobe assay, Gibson et al.[18] and Chisholm et al.[19] used a monoprobe assay with SYBR green 1 as the quencher which transfers its energy to a probe labeled with Cy5. For *H. pylori* detection, Chisholm et al. reported a sensitivity of 85% and a specificity of 98% on 121 biopsies tested, 17 being *H. pylori* positive. Sensitivity and specificity for the detection of clarithromycin resistance seem inferior, and few resistant cases were tested.

The FRET–MCA also has the potential of detecting mixed infections, essentially wild type and a resistant mutant, which may not be seen by using phenotypical methods. Indeed, mixed infections represented 21% of the total and three genotypes were even detected in one case. A genotype can be detected when it represents at least 10% of the DNA mixture.

The other advantages of this assay are:

- Obtaining a very quick result (2 hr) with limited manpower which can be compared to the week's delay in obtaining phenotypical results
- Avoiding any handling post-PCR which is an important source of contamination in laboratories
- Avoiding the demanding transport conditions necessary to perform culture and susceptibility testing. Shipment by mail at ambient temperature is convenient.

The limitation of the assay described above[21] is the difficulty in separating the A2143G and A2142G mutations. The melting curves for the two genotypes differ only by 1°C (Fig. 1), which does not allow a reliable distinction in routine testing. Despite the fact that the A2142G mutation is usually associated with higher MICs

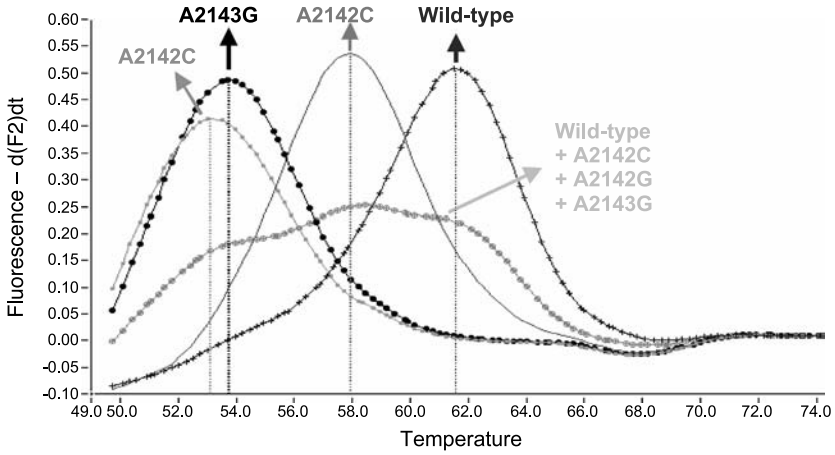

Fig. 1 Melting curve analysis of the 267-bp fragment of the 23S rRNA gene obtained with the LightCycler® assay for the wild-type and mutant strains with the A2142G, A2143G, and A2142C mutations. The melting curve corresponding to a mixture containing equal proportions of wild-type, A2142G, and A2142C is also shown, and three melting peaks with the corresponding T_m can be observed. Values on the *y* axis represent the ratio of the first negative derivative of the change in fluorescence (dF) with the variation in temperature. (*View this art in color at www.dekker.com.*)

than the A2143G mutation, there does not seem to be any clinical relevance as both mutations are highly associated with treatment failure.

The limitation of all FRET–MCA tests is the availability of a special thermocycler which is costly. The first available model used in most of the published experiments is the LightCycler™ (Roche Diagnostics), but several other apparatuses are now coming out on the market and the cost is decreasing. Such thermocyclers can also be used for numerous applications other than the detection of single nucleotide polymorphisms using a biprobe.

An interesting application currently being developed is the detection of *H. pylori* and its possible clarithromycin resistance in stool samples which has the tremendous advantage of being a noninvasive test. The problem of inhibition of Taq polymerase by stool compounds such as vegetal polysaccharides has now been almost solved by using special DNA extraction kits (Qiagen).

Non-PCR-Based Method

The possibility of detecting clarithromycin resistance without performing PCR also exists, by fluorescence in situ hybridization (FISH). This method has been applied to the detection of *H. pylori* and its clarithromycin resistance by Trebesius et al.[23] It consists of an rRNA-based whole cell in situ hybridization using a set of fluorescent labeled oligonucleotide probes. Labeling of intact single bacteria is monitored by fluorescence microscopy. This method allows the detection of *H. pylori* with a 16S rRNA probe labeled with the fluorochrome Cy3 (red) and of resistant mutants with a 23S rRNA probe labeled with fluorescein (green) simultaneously. This method proved to be sensitive and specific when compared to standard methods of culture and susceptibility testing. No discrepant result was noted for 45 cases. In another study, there were 11 discrepant results among 69 cases. These cases corresponded indeed to mixed infections with susceptible and resistant strains. This method has the advantage of being independent of a nucleic acid preparation, it is not prone to inhibition like PCR and is quick. It also allows visualization of the bacteria including coccoidal forms and can be performed on paraffin embedded biopsy samples. A limitation could be the observer-dependent result and sometimes difficulty in reading. It has not been compared yet to the FRET–MCA protocol.

These new methods which provide a result within a few hours are very important for clinical practice because they have the potential to change the management of *H. pylori* infection; indeed, to administer, as a first line therapy, a regimen without clarithromycin in case of clarithromycin resistance instead of an empirical treatment in areas with high levels of this resistance and then to improve the eradication rate.

METHODS OF DETECTION IN THERMOPHILIC *CAMPYLOBACTER* SPP.

Resistance of thermophilic *Campylobacter* spp. to macrolides is not yet a major problem in clinical isolates (<5%), but a trend toward an increase has been noted.

The mechanism also concerns a point mutation in the 23S rDNA essentially in the 2075 position (equivalent to *H. pylori* 2143 and *Escherichia coli* 2059 coordinate), A2075G. The A2074C mutation is seldom found.

We have developed a FRET–MCA similar to the assay described for *H. pylori* but only to be used for detection of the point mutation on isolates.[24] The same probes can be used with different primers.

This assay was applied to 141 thermophilic *Campylobacter* strains (49 macrolide susceptible and 92 resistant). Sensitivity and specificity were 100%; 95% of the cases corresponded to an A2075G mutation.

NEW METHOD OF DETECTION IN GRAM-POSITIVE COCCI

A denaturing high-performance liquid chromatography (DHPLC) technique was recently evaluated in streptococci and staphylococci.[25] This rapid screening method allows automated detection of single base substitution as well as small insertions or deletions employing a combination of temperature-dependant denaturation of DNA and ion pair chromatography. In this method, after PCR amplification of regions of interest in 23S rRNA, L4, or L22 genes, wild-type and mutant PCR products are mixed in an equimolar ratio, heated to denature each strand, and then allowed to reanneal until they form a mixed population of homoduplexes plus heteroduplexes containing the mismatched bases. Under conditions of partial denaturation temperature, the heteroduplexes elute from the column earlier than the homoduplexes because of their reduced melting temperature. Denaturing high-performance liquid chromatography data analysis is then based on a qualitative comparison of peak number and/or shape of a single peak between the sample and reference chromatograms. A good sensitivity was obtained in *S. aureus* mutants as one mutated *rrl* copy mixed with five wild-type copies was detected.

CONCLUSION

The discovery of macrolide (clarithromycin) resistance associated gene mutations has been an important step in the understanding of resistance in many pathogens. At this stage, it also has a practical application for *H. pylori* given its clinical importance and low number of point mutations allowing an easy detection. More progress is expected in the future for bacterial species with mutations at various sites.

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Clostridium botulinum

Miia Lindström
Mari Nevas
Hannu Korkeala
University of Helsinki, Helsinki, Finland

INTRODUCTION

Clostridium botulinum is a bacterium that is widely spread in the environment and in raw foods. *C. botulinum* strains produce lethal neurotoxins during their logarithmic growth. The toxin, when ingested or formed in vivo in humans and animals, causes botulism, a neuroparalytic disease that may lead to death unless treated. The diagnosis of botulism is based on the detection of neurotoxin and *C. botulinum* organisms in a patient. Conventional diagnostic methods for the detection of *C. botulinum* and its toxins are poorly developed. However, the use of molecular tools such as polymerase chain reaction (PCR) methods has greatly facilitated the diagnosis of *C. botulinum* during the last decade.

C. BOTULINUM

Classification and the Microbial Ecology of *C. botulinum*

C. botulinum is an anaerobic gram-positive rod-shaped bacterium that forms heat-resistant spores.[1] The taxonomic denominator is the production of botulinum neurotoxin (BoNT). Based on the serological properties of the BoNT they produce, *C. botulinum* strains are divided into types A–F, with types A, B, E, and F being human pathogens, and types C and D being animal pathogens. Based on their metabolic properties, *C. botulinum* strains are divided into groups I–III.[2] Groups I and II include strains that are pathogenic to humans, whereas group III includes strains causing botulism in animals. Various characteristics of the organisms belonging to different groups are presented in Table 1.

Prevalence of *C. botulinum*

C. botulinum is widely spread in the environment, with group I organisms predominating in the temperate regions of the world, and group II organisms prevailing in aquatic environments in the Northern hemisphere. *C. botulinum* types A, B, and E are more frequently found in the environment than type F. As a consequence of a high prevalence of spores in the environment, raw foods may contain *C. botulinum* spores. Proteolytic types A and B have been frequently detected in fruits, vegetables, and meat, whereas nonproteolytic types B and, E in particular, are frequently present in fish.[3–5]

HUMAN BOTULISM

Human botulism develops as a consequence of BoNT entering the blood circulation and blocking neurotransmitter release in peripheral nerve endings. The clinical manifestation of botulism includes a descending flaccid paralysis with dysphagia, dry mouth, double vision, difficulties in swallowing, dilated pupils, dizziness, and muscle weakness. These are accompanied by paralysis of the more peripheral parts of the body, and, finally, by respiratory muscle paralysis, which may lead to death. In addition, nonspecific symptoms related to different forms of botulism may precede the actual paralysis.

Classical Food-Borne Botulism

The classical food-borne botulism is an intoxication due to the ingestion of BoNT. Thus the first indications of illness before the paralytic condition are gastrointestinal in nature, mainly nausea, vomiting, and abdominal cramps. The typical incubation period is 18–72 hr. The most common differential diagnoses include Guillain–Barré syndrome, chemical intoxication, stroke, or staphylococcal food poisoning.[6] The estimated case fatality rate of food-borne botulism outbreaks worldwide is 20%. During the last decades, a worldwide average of 450 outbreaks of food-borne botulism with 930 cases has been reported annually.[7] More than half of the cases (52%) were because of BoNT type B, whereas 34% and 12% were because of types A and E, respectively. Only on rare occasions is type F toxin associated with human botulism. Countries with a high incidence of botulism include China, Poland, the former Soviet Union, Germany, Italy, Spain, the United States, France, and Yugoslavia. The vehicle foods include most frequently mishandled

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Table 1 Some characteristics of *C. botulinum* strains and botulinum neurotoxin

Feature	*C. botulinum* group I	*C. botulinum* group II	*C. botulinum* group III
Type of neurotoxin produced	A, B, F	B, E, F	C_1, D
Metabolism	Proteolytic	Nonproteolytic	Nonproteolytic/weakly proteolytic
Optimal growth temperature (°C)	35–40	18–30	37–40
Minimum growth temperature (°C)	10	3–3.3	10
Growth-limiting pH	4.3–4.6	5.0	5.1–5.6
Growth-limiting a_w	0.94	0.97	0.98
Heat resistance of spores	$D_{112°C}$ 1.2 min	$D_{85°C}$ 0.3–98 min	$D_{104°C}$ 0.02–0.9 min
Location of neurotoxin gene	Chromosome	Chromosome	Bacteriophage
Species sensitive to neurotoxin	Human (A, B, F), cattle and horse (B)	Human (B, E, F), cattle and horse (B), birds (E)	Cattle and sheep (C, D), horse, mule, and birds (C)

home-prepared foods, but occasionally commercial products are involved.

Infant Botulism

Infant botulism is an infection that may follow the in vivo outgrowth and toxigenesis from *C. botulinum* spores.[8] Infant botulism affects small children under 1 year of age, and is typically because of group I *C. botulinum*. Prolonged constipation, followed by distinctive flaccid paralysis that is manifested by impaired feeding, facial muscle paralysis, and general weakness, is typical. The symptoms of infant botulism may vary from a self-limiting constipation to unexpected death of a child, in which case it is occasionally misdiagnosed as sudden infant death syndrome.[8,9] The case fatality rate is less than 2%.[10] The only foodstuff associated with infant botulism is honey, which may carry a high number of *C. botulinum* spores.[3,11] Dust and other environmental materials seem to be important sources of spores.[8,9]

Other Forms of Botulism

Wound botulism is mainly found among injecting drug abusers using contaminated needles or impure heroin.[12] Wound botulism is an infection that develops when *C. botulinum* spores germinate and grow in profound wounds or abscesses. The clinical picture is similar to food-borne botulism, with the absence of gastrointestinal signs. The median incubation period is 7 days. The estimated case fatality rate is 15%.[7]

The adult form of infectious botulism is rare and resembles infant botulism in its pathogenesis and clinical status.[13] People with altered intestinal flora because of abdominal surgery, prolonged antimicrobial treatment, or gastrointestinal wounds and abscesses may be affected. Because a patient history of ingestion of toxic foods has typically not been found, the diagnosis of classical food-borne intoxication may be excluded.

Inhalation botulism may result from aerosolization of BoNT, and a few human cases have been reported.[14] Iatrogenic botulism with local or generalized weakness is rare and has been reported to develop as a consequence of therapeutic injection of BoNT.[15]

Management of Botulism

Preventive measures against food-borne botulism include proper handling, effective heat processing,[16] and continuous cold storage of foods. The treatment of all forms of botulism basically involves the use of a therapeutic trivalent antitoxin and intensive symptomatic treatment, particularly respiratory support.[17] In wound botulism, the treatment additionally consists of surgical debridement and antibiotic therapy.

DIAGNOSTICS OF BOTULISM AND *C. BOTULINUM*

The diagnosis of botulism is primarily based on the history of eating suspected foods, as well as detecting BoNT in patients and in suspected food samples.[10] The detection of *C. botulinum* cells in clinical and food specimens strongly supports the diagnosis. Electroneuromyography may be used to distinguish botulism from similar neurological diseases.[10]

Conventional Culture Techniques

The culture method for the detection and isolation of *C. botulinum* includes broth culturing in anaerobic media,

such as tryptone–peptone–glucose–yeast extract medium or cooked meat medium, and subsequent culturing on plating media. Typical colonies on egg yolk agar plates show a positive lipase reaction, and a weak β-hemolysis is seen on blood agar. Proper selective media allowing the growth of all strains of *C. botulinum* are not available. Commercial biochemical reaction series have been developed for the identification of anaerobic bacteria, but these tests have been shown to be unreliable in the diagnosis of *C. botulinum*.[16]

Toxin Detection

The only standard method for the detection of BoNT is the mouse bioassay. Two mice are injected intraperitoneally with a sample elution, and, in the case of a positive sample, the mice die with symptoms typical of botulism. The toxin type is determined by seroneutralization of the toxic samples with specific antitoxins. The immunoassay formats reported for the detection of BoNT include radioimmunoassay (RIA), gel diffusion assay, and passive hemagglutination assay (PHA). However, many of these assays provide poor sensitivity or specificity, which decreases the diagnostic value of the methods. An exception is enzyme-linked immunosorbent assay (ELISA), which may reach sensitivity similar to the mouse bioassay. A recently published ELISA format has been validated in the United States.[18] Another method with sensitivity and specificity similar to the mouse assay is the endopeptidase assay, which is based on the specific zinc endopeptidase activity of BoNTs in nerve terminals.[19] Synthetic peptides resembling the soluble *N*-ethylmaleimide-sensitive fusion protein attachment receptor (SNARE) complex proteins involved with neurotransmitter release in peripheral nerve endings serve as substrates for the toxin.

Molecular Detection of *C. botulinum*

PCR and DNA probes provide a sensitive tool for the detection of *C. botulinum*. The greatest sensitivities of PCR protocols for *C. botulinum* in various sample materials vary from 2.5 pg of DNA[20] to 0.1 cfu (or spore)/g food[21] and 10 cfu/g feces.[21,22] A multiplex PCR assay that enables the simultaneous detection of all human pathogenic serotypes A, B, E, and F has provided a marked improvement in the PCR detection of *C. botulinum*.[21] Nested PCR involves several subsequent amplifications and provides an increased sensitivity in the detection of *C. botulinum*, for example, in feces.[22] The disadvantage of PCR detection directly from a sample is the possible detection of dead cells because of intact DNA after cell lysis. This problem is overcome by combining enrichment procedures with PCR.[4] Alternatively, reverse transcription polymerase chain reaction (RT-PCR) may be used to distinguish viable and dead bacterial cells.[23] Quantification may be obtained by combining PCR with the most probable number culture technique,[4] or by real-time PCR.[24]

Molecular Characterization of *C. botulinum*

Molecular characterization of *C. botulinum* may be used to study the genetic diversity of the organism,[4,5] and in tracing the causative agents of botulism outbreaks.[9,25] Pulsed field gel electrophoresis (PFGE) provides excellent discriminatory power and reproducibility, whereas the PCR-based method (randomly amplified polymorphic DNA assay, or RAPD) is less reproducible but can be quickly performed. The application of rRNA gene restriction pattern analysis (ribotyping) has been used to identify bacterial species yielding distinct patterns for groups I and II *C. botulinum*.[4]

The entire genome sequence of *C. botulinum* type A strain ATCC 3502 has been established,[26] which enables the development of DNA microarrays containing the whole genome of the organism. The arrays are anticipated to provide more efficient tools for the genomic analysis of *C. botulinum*.

CONCLUSION

A rapid and reliable diagnosis of botulism is a prerequisite for successful therapy. The only standard method for the detection of BoNT is the mouse bioassay, which leads to ethical concern because of the use of laboratory animals. The lack of appropriate selective growth media renders the culture methods laborious, and nontoxigenic *C. botulinum*-resembling cultures may cause false-positive signals. The molecular detection of *C. botulinum* involves the detection of the *bont* gene, indicating the presence of the organism—but not its toxin—in a sample. Molecular characterization of *C. botulinum* may be applied in tracing the causative agents of botulism outbreaks.

To facilitate the diagnostics of botulism, more advanced methods for toxin detection and isolation of *C. botulinum*, in particular, are required.

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Clostridium difficile

Michel G. Bergeron
Maurice Boissinot
Universite Laval, Sainte-Foy, Quebec, Canada

INTRODUCTION

Widespread use of broad-spectrum antibiotics triggered the emergence of *Clostridium difficile* as an important bacterial pathogen associated with diarrhea and colitis in hospitalized patients. This toxigenic bacterium causes significant morbidity and mortality, and imposes a pronounced economic burden on the health system. Appropriate treatment requires efficient diagnosis, and several techniques have been developed to detect toxins produced by this microbe. However, immunodetection techniques often lack sensitivity, whereas cytotoxicity assays can only be performed in laboratories equipped with tissue culture facilities. Newer and faster methods based on rapid detection of genes encoding the toxins are providing promising simpler approaches.

DISCOVERY AND IMPACT OF *C. DIFFICILE*

C. difficile is an anaerobic, spore-forming, gram-positive rod. This bacterial species owes its name to the difficult circumstances of its first isolation in 1935 from the intestinal flora of healthy neonates.[1] In the laboratory conditions of those days, its slow growth and requirement for culture under anaerobic atmosphere posed some difficulties now essentially resolved by modern techniques developed for the cultivation of anaerobes. Colonization most likely occurs by fecal–oral transmission, and ingested spores of *C. difficile* can survive the passage through gastric acid and germinate into the colon. In the preantibiotic era, pseudomembranous colitis was a rare complication of intraabdominal surgery.[2] However, this life-threatening condition became more frequent with the increased use of antibiotics and, in 1978, *C. difficile* was identified as the etiologic agent of pseudomembranous colitis.[3] This bacterium is now recognized as the cause of approximately 20% of all cases of antibiotic-associated diarrhea. *C. difficile*-associated disease can produce symptoms ranging from mild nuisance diarrhea to fulminant colitis.[2] It has been calculated that patients acquiring this disease during their hospitalization stay >50% longer than the adjusted length of stay for a typical patient whose course was not complicated by *C. difficile* diarrhea.[4] This increases cost by $3669 per infected patient. These authors estimated that the annual cost for treating *C. difficile* diarrhea in the United States exceeds $1.1 billion for hospital care only. Morbidity and mortality associated with *C. difficile* diarrhea are high and reflect the generally poor health of patients at higher risk of acquiring this infection.

PATHOPHYSIOLOGY AND GENOMICS OF VIRULENCE DETERMINANTS

C. difficile's main virulence determinants are two exotoxins: toxins A and B. In animal models, toxin A was shown to be an enterotoxin inducing both diarrhea and inflammation. By contrast, in tissue culture, toxin B was much more cytotoxic than toxin A and was designated as cytotoxin. The genes for toxin A (*tcdA*) and toxin B (*tcdB*) are highly homologous, and the carboxy-terminal regions of both toxins encode a glucosyltransferase specific for the host small GTP-binding proteins of the Rho family. Glucosylation of these small GTP-binding proteins leads to a disorganization of the cytoskeleton and provokes cell death. The toxins also trigger inflammatory response and fluid secretion. Epithelial damages such as necrosis and ulceration can eventually lead to the formation of the characteristic pseudomembranes observed in pseudomembranous colitis.[5] The level of humoral response against *C. difficile* toxins appears to confer a certain amount of protection to the host, which may explain why certain individuals remain asymptomatic carriers of toxigenic strains, others only develop mild diarrhea, and some suffer from severe colitis.[6]

Sequence analysis revealed that both toxin genes are part of a multigene operon located within a 19.6-kb pathogenicity locus (PaLoc) inserted into the chromosome of *C. difficile*. PaLoc genes are conserved in toxigenic strains and are systematically absent from nontoxigenic strains.[7] Restriction enzyme mapping analysis of the PaLoc revealed variant *C. difficile* strains, which differ in length and restriction sites from the reference strain. Twenty groups of such variants have been described and

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defined as toxinotypes I–XX.[8] Although *tcdB* restriction sites are more variable than those of *tcdA*, deletions and insertions are more frequently observed in *tcdA*. In fact, some toxinotypes are associated with a toxin A-negative, toxin B-positive phenotype. These strains, which were first isolated from asymptomatic children, are now increasingly recovered from patients presenting symptoms ranging from diarrhea to pseudomembranous colitis.[9] Therefore the presence of toxin A is not required to cause the disease, and toxin B, by itself, can induce significant damage to human intestines.[10]

EPIDEMIOLOGY AND PREVENTION

Toxigenic *C. difficile* is carried asymptomatically in about 25–80% of infants and in 20% of hospitalized patients, but only in less than 3% of the adult population of developed countries.[11,12] *C. difficile*-associated disease is primarily a nosocomial infection causing 300,000 to 3 million cases of diarrhea and colitis in U.S. hospitals each year.[5] This pathogen is also responsible for community-acquired diarrhea but at a much lower rate. Epidemiological factors influencing this low incidence in the general population are not fully understood, but the number may be underestimated.[2] Risk factors often associated with *C. difficile*-associated diarrhea include old age, length and number of hospital stay, and invasive medical procedures. However, the most important factor appears to be the use of antibiotics. The normal bowel flora possess a certain ability to resist colonization by pathogenic microbes. Alteration of this barrier by antibiotics presumably predisposes to invasion by *C. difficile* and subsequent toxin production. Although virtually all antibiotic molecules have been associated with *C. difficile* infections, the broad-spectrum antibiotics clindamycin, penicillin, and cephalosporin have been the most frequently implicated. In fact, a recent critical review of the 1978–2001 literature describing the association of antibiotics with nosocomial *C. difficile*-associated diarrhea concluded that most studies were flawed due to incorrect control groups, inadequate sample sizes, and imprecise analysis of confounding factors.[13] In their opinion, only two studies were of sufficient quality to reasonably establish a causal relationship between particular antibiotic molecules and *C. difficile*-associated diarrhea, and these molecules were indeed the three listed above. However, this does not exclude other antibiotics from predisposing to the disease.[14] In some institutions, policies limiting the prescription of certain antibiotics appeared to decrease the rate of *C. difficile* colitis and diarrhea in hospitalized patients.[12] However, decision to restrict the use of some therapeutic molecules has to be weighted against the clinical benefits of using such antimicrobial agents.

Patients contribute to disseminate *C. difficile* toxigenic strains in hospital settings where endemic and epidemic situations may occur. To prevent cross-contamination of patients with *C. difficile*, contact precautions must be implemented until diarrhea stops. However, *C. difficile* spores can survive for months in hospital environments, and patients can be contaminated via contact with care personnel or contaminated objects. Moreover, spores can resist several commercial detergents commonly used to clean surfaces in healthcare institutions. Chlorine-based disinfectants appear to be a better choice and may contribute to reduce the incidence of *C. difficile* infections.[15] However, to limit *C. difficile*-associated diarrhea in hospitals, measures must include a combination of rational use of antibiotics and better infection control.[16]

CLINICAL FEATURES AND TREATMENT

C. difficile infection has been shown to cause a wide spectrum of intestinal symptoms including mild to severe diarrhea, chronic diarrhea, colitis, pseudomembranous colitis, and toxic megacolon.[2] Although *C. difficile* is occasionally isolated in extraintestinal pathologies, these cases are infrequent and usually involve a polymicrobial infection. Typically, *C. difficile*-associated diarrhea appears within 1–2 weeks following the administration of an antibiotic. Watery stools may contain mucus and sometimes blood. Patients may present fever, abdominal cramps, and leukocytosis. However, even severe cases may present no diarrhea or just loose stools. Radiographic evidence of distended colon can indicate toxic megacolon. Prolonged or severe disease can be complicated by dehydration problems, bowel perforation, and death. Diagnosis is based on history of recent or ongoing antibiotherapy, appearance of diarrhea or other symptom of colitis, and demonstration of infection by toxigenic *C. difficile*.

Treatment should begin by discontinuing the incriminated antibiotic whenever possible. However, this measure alone will resolve diarrhea in only one fifth of patients. Therefore, in cases with severe diseases or other comorbid conditions, anti-*C. difficile* antibiotics such as metronidazole and vancomycin are the first two drugs of choice for specific *C. difficile* therapy. Treatment of asymptomatic carriers is not recommended because antibiotics may prolong excretion of the bacteria.[12] On discontinuation of anti-*C. difficile* antibiotherapy, approximately 20% of patients experience recurrence of the infection. This situation can be explained by relapses

caused by incomplete eradication of the bacteria due to the persistence of spores in the digestive tract. However, between 32% and 75% of patients suffering from recurrences are, in fact, reinfected by a new strain acquired from the environment.[17]

LABORATORY DIAGNOSTIC APPROACHES

Because many hospitalized patients are asymptomatic carriers, testing patients for *C. difficile* infection is only recommended if diarrheal stools are present.[12] Laboratory diagnosis is performed by detection of toxigenic *C. difficile* by culture, immunodetection of toxin, or cytotoxicity assay.[18]

Stool cultures on selective cycloserine–cefoxitin–fructose agar can yield to *C. difficile* isolation in 2–3 days. Unfortunately, culture is not specific for toxin-producing strains and is seldom used for clinical diagnosis.[5] However, isolated strains provide reliable bacteriological information for epidemiological purposes.

Cytotoxicity assay, which detects cytopathic effects of toxin B on mammalian cells, is the most sensitive and specific test available. It is considered the gold standard test to identify *C. difficile* toxins directly from the stools of infected patients. A filter-sterilized fecal suspension is added into a mammalian cell culture and monitored for the appearance of cytopathic effects caused by disruption of the cytoskeleton. In parallel, a control fecal filtrate supplemented with a specific antiserum neutralizing for clostridial cytotoxin is used to demonstrate the specificity of cytopathic changes. The drawback of this assay is that it is relatively slow (24–48 hr) and requires tissue culture expertise and facilities. Some authors suggest that performing cytotoxin assay directly on stools, as well as by culture followed by detection test for toxin produced in vitro, might improve the detection of patients harboring toxigenic strains.[18]

Enzyme immunoassays (EIAs) for toxin detection are now widely available from several manufacturers. These tests are rapid and simple to perform directly from stool samples. Most assays use monoclonal antibodies against toxin A. However, because some clinical isolates have been shown to produce only toxin B and still cause the disease, more recent EIAs combine the detection of both toxins A and B. However, none of the EIAs is as sensitive as the cytotoxicity assay.[19] This is why in the event that EIA testing is negative and diarrhea persists, it is recommended to repeat EIA testing on a second sample and even a third sample from patients suspected of infection by toxigenic *C. difficile*.[5]

Several sensitive and specific molecular diagnostic methods based on detection of toxin genes directly from feces have been proposed.[20–24] However, most of these tests targeted either only the gene for toxin A, or the gene for toxin B. Therefore strains belonging to toxinotypes altered or even deleted in the primer regions could be missed. In all cases, these assays where developed in classical PCR formats requiring time-consuming and contamination-generating post-PCR manipulations to identify the amplification products. For clinical laboratories, the potential risks of false-positive reactions due to carryover of previously amplified genetic materials render PCR assays relying on agarose gel electrophoresis impractical for analysis of amplicons. In addition, the PCR-inhibitory substances inherently present in feces specimens suppose an efficient sample preparation procedure to remove these inhibitors without having to sacrifice the sensitivity of the assay. Most of these procedures add complex technical steps that are time-consuming. One exception is the cleverly designed immunomagnetic preparation procedure of Wolfhagen et al.[24] However, it had not been tested on many specimens.

Recently, our team published the first real-time PCR assay for the detection of toxigenic *C. difficile* in feces.[25] This test targets both toxin genes and is a closed-tube assay. This means that amplification and detection of nucleic acids are performed in the same tube, therefore minimizing human intervention and reducing risk of contaminating laboratory surfaces with amplified material. The closed-tube assay format is a major improvement, which will permit a more general acceptance of PCR testing in clinical settings. Our test uses a rapid and simple sample preparation protocol requiring hands-on technician time of around 15 min, which is similar to EIA assays used for *C. difficile*. Target sequences for primers and probes were chosen by identifying conserved regions within *tcdA* and *tcdB* gene sequences available in GenBank. The *tcdA* and *tcdB* amplicons are 158 and 101 bp, respectively. The two molecular beacon probes were labeled with different fluorophores, hence allowing multiplex PCR and simultaneous detection of the two toxin genes. The use of two target genes increases assay robustness by maximizing the likelihood of detecting at least one toxin gene if the other presents variations in the primers or probe region. The 45-cycle PCR reaction took 45 min and the fluorescent signal was acquired in real time at each cycle on a Smart Cycler (Cepheid, CA) apparatus. Total time to response was less than 1 hr from reception of samples into the laboratory. The detection limit was 5×10^4 CFU/g of stool. When compared to cytotoxicity testing, the real-time PCR assay demonstrated a specificity and a positive predictive value of 100%, a sensitivity of 97%, and a negative predictive value of 96%. Further

validation of the test in a broader clinical trial is, of course, required. However, such specific, sensitive, and especially rapid identification and detection of toxigenic *C. difficile* may improve the management of patients and facilitate a more rational use of antibiotics.

CONCLUSION

Many difficulties still encountered with *C. difficile* infections are linked to the availability of rapid and simple diagnostic assays. The prevention of *C. difficile* infections is much more complex than just restricting the use of some antibiotic molecules. Almost all classes of antibiotics have been implicated in triggering *C. difficile* diarrhea. Therefore only rational use of all antibiotics for all infections can have optimal impact, and this could only be achieved when clinical microbiology laboratories would have novel molecular diagnostic assays able to provide detection and identification of microbial pathogens within 1 hr from reception of samples.[26,27] Moreover, *C. difficile* toxin infections emerge due to complex interactions between the complicated ''normal bowel microflora,'' host response and general health status, environmental factors varying from hospital to hospital, and the genetic potential of *C. difficile*. It is surprising to realize how little we know about the ecology of the different bacterial species living within our own gut. With the aging population in developed countries and the increasing number of immunosuppressed patients, we are likely to face more opportunistic pathogens such as toxigenic *C. difficile*. Rapid (<1 hr) DNA-based tests used to detect these pathogens should become a very important tool of prevention because they will guide physicians and infection control nurses to respond in an efficient manner to nosocomial infections. The economic impact of such rapid response should be tremendous.

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Clostridium perfringens

Derek J. Fisher
James G. Smedley, III
Qiyi Wen
Bruce A. McClane
University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A.

INTRODUCTION

Clostridium perfringens, a gram-positive, spore-forming rod, is an important cause of histotoxic and enteric disease in humans and other animals. Although ubiquitously distributed in soils throughout the world, *C. perfringens* is also found as part of the normal animal intestinal flora. Like all pathogenic clostridia, *C. perfringens* is renowned for toxin production with an arsenal of over 15 different toxins. The presence of genes encoding the four ''major lethal'' toxins (α-toxin, β-toxin, ε-toxin, and ι-toxin) is used to classify *C. perfringens* isolates into one of the five types (A–E) (Table 1). Type A strains are largely responsible for human gas gangrene and other histotoxic infections, whereas type C strains secrete β-toxin and can cause enteritis nectroticans in undernourished individuals. Although not used in the typing scheme, the *C. perfringens* enterotoxin (CPE) is responsible for *C. perfringens* type A food poisoning, as well as nonfood-borne gastrointestinal (GI) illness. This chapter describes the current status of *C. perfringens* genomics, as well as molecular methods (where available) for diagnosing human infections caused by this bacterium.

C. PERFRINGENS: GENOMIC INSIGHTS

Genome Projects

Early physical maps indicated that the *C. perfringens* genome size varies between isolates (3–3.7 Mb).[1] These studies also mapped the location of chromosomal virulence factors (such as α-toxin and perfringolysin O), the key two-component regulator VirR/VirS, as well as some metabolic and sporulation-related genes.[2] Those physical mapping results have recently been confirmed by genome sequencing projects with two type A *C. perfringens* (strain 13 and ATCC 13124).[3,4]

As typical of clostridial species, the strain 13 genome was found to be AT-rich (71% overall AT content).[4] No high GC content chromosomal regions were detected, suggesting the absence of chromosomal pathogenicity islands.[4] Many sporulation and germination genes with homologues in *Bacillus subtilis* were identified; however, over 80 genes mediating these two processes in *B. subtilis* are absent from strain 13.[4] Genomic analysis of strain 13 identified some potential new virulence factors and putative two-component regulatory systems that could be important for pathogenesis.[4] DNA microarrays and proteomic platforms based on strain 13 have been developed, and their use is now contributing to our understanding of regulatory and metabolic pathways.[5]

Extrachromosomal Virulence Factors

Many *C. perfringens* virulence factors, including toxins such as CPE, β-toxin, ε-toxin, ι-toxin, and β2-toxin, as well as urease, can be encoded by genes located on large (~100 kb) virulence plasmids.[6,7] Interestingly, at least one toxin gene, *cpe*, can reside on either the chromosome or large plasmids (further discussion below).[7] Some virulence genes colocalize to the same plasmid, including the urease and *cpe* genes in various *C. perfringens* types, as do defective *cpe* and ι-toxin genes in type E isolates.[7,8] To date, only one virulence plasmid (pCP13 carrying the gene encoding β2-toxin) has been sequenced.[4] The best studied *C. perfringens* virulence plasmids (other than pCP13) are those carrying *cpe* sequences in types A (discussed below) and E strains.[8,9]

The absence of chromosomal clonality in isolates possessing any of the plasmid-borne toxin genes suggests that these plasmids (or their toxin genes) are mobile. The *cpe* plasmid was recently shown to transfer between *C. perfringens* isolates via conjugation, confirming horizontal transfer for that toxin gene.[10] One insertion element, IS*1151*, has been linked to multiple toxin genes, suggesting that this element may also contribute to toxin gene mobility.[7,9]

Polymerase Chain Reaction-Based Toxin Typing of *C. perfringens*

A rapid multiplex polymerase chain reaction (PCR) assay has been developed for determining the toxin type (A–E)

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Table 1 *C. perfringens* toxin types

Toxin type	Toxin α	Toxin β[b]	Toxin ε[b]	Toxin ι[b]	Genotypes	Diseases[a] Human	Diseases[a] Veterinary
A	+	−	−	−	*plc*	Gangrene	Diarrhea (dogs, pigs, etc.)
					plc, cpe[b]	GI disease (food poisoning,[c] antibiotic-associated diarrhea,[d] sporadic diarrhea[d])	Necrotic enteritis (fowl)
					plc, cpb2[b]		
					plc, cpb2, cpe		
B	+	+	+	−	*plc, cpb, etx*		Dysentery (lambs)
					plc, cpb, etx, cpb2		Enterotoxemia (sheep)
C	+	+	−	−	*plc, cpb*	Enteritis necroticans	Necrotic enteritis (piglets, foals, etc.)
					plc, cpb, cpb2		Enterotoxemia (sheep)
					plc, cpb, cpb2, cpe		
					plc, cpb, cpe		
D	+	−	+	−	*plc, etx*		Enterotoxemia (goats, sheep, etc.)
					plc, etx, cpb2		
					plc, etx, cpe, cpb2		
					plc, etx, cpe		
E	+	−	−	+	*plc, iap/ibp, cpe*[e], *cpb2*		Enterotoxemia (calves, rabbits, etc)

plc=alpha toxin; *cpe*=*C. perfringens* enterotoxin; *cpb2*=*C. perfringens* beta2 toxin; *cpb*=*C. perfringens* beta toxin.
etx=*epsilon* toxin; *iap* and *ibp*=*iota* toxin A and B fragments, respectively.
[a]Diseases listed are inclusive of the toxinotype and are not shown linked to specific genotypes.
[b]Genes encoding these toxins are located on large virulence plasmids (*cpe* can be found on either the chromosome or on a plasmid).
[c]Linked to *cpe*-chromosomal isolates.
[d]Linked to *cpe*-plasmid isolates.
[e]Silent *cpe* gene.

of *C. perfringens.* This PCR assay has largely replaced the demanding classical *C. perfringens* typing procedure, which depended on antibody neutralization of toxins in *C. perfringens* culture supernatants. The most recent version of the multiplex PCR typing assay amplifies different size products from the genes encoding CPE, as well as α-toxin, β-toxin, ε-toxin, β2-toxin, and ι-toxin (Fig. 1).[11] This method is effective using either purified DNA or colony lysates as template. However, care must be taken in using this multiplex PCR for diagnosis, as amplification of a toxin gene does not necessarily correlate with expression of that toxin.[8]

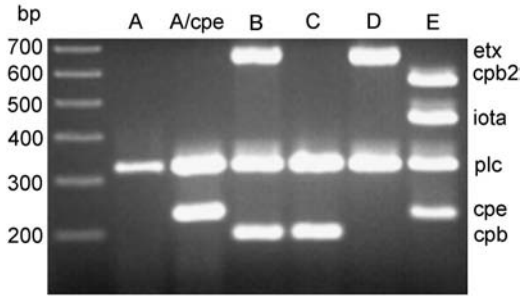

Fig. 1 Multiplex PCR toxinotyping assay for determining the toxin genotype of *C. perfringens* isolates. Representative toxinotypes are located at the top of the gel and the toxin genes amplified are listed on the right of the gel. Primers and PCR conditions were from Ref. [11]. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide staining.

CPE AND GI DISEASE

Disease Summary

CPE-positive strains cause *C. perfringens* type A food poisoning, which currently ranks as the third most common cause of food poisoning in the United States.[12] In addition, enterotoxin-producing *C. perfringens* is an important etiological agent of non-food-borne human GI illnesses such as antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD).[13] The symptoms of CPE-related illnesses range from acute diarrhea and abdominal

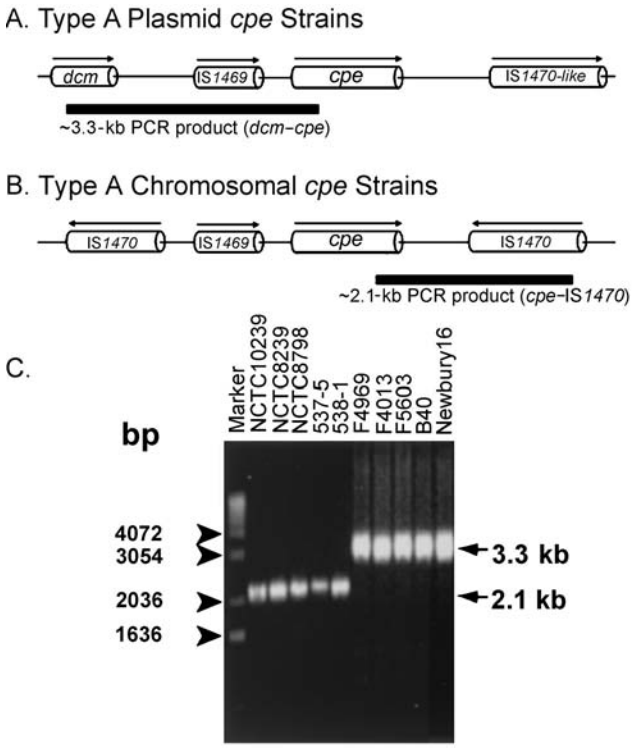

Fig. 2 Duplex PCR genotyping assay for distinguishing between *C. perfringens* type A isolates carrying a plasmid-borne *cpe* gene vs. a chromosomal *cpe* gene. This assay exploits organizational differences between different *cpe* loci to amplify either a PCR product of ~3.3 kb (*dcm–cpe*) from plasmid *cpe* type A isolates (A), or an ~2.1-kb PCR product (*cpe*–IS*1470*) from chromosomal *cpe* type A isolates (B). (C) Representative duplex PCR results obtained using purified genomic DNA as template from five chromosomal and five plasmid type A *cpe* isolates. Primers and PCR conditions were from Ref. [15]. Arrows to the right of gel depict the expected ~3.3- and ~2.1-kb products from plasmid and chromosomal *cpe* type A isolates, respectively. (From Ref. [15].)

cramping (*C. perfringens* type A food poisoning) to more severe and chronic diarrhea (AAD and SD).

Diagnosis

CPE-associated (GI) disease is now typically diagnosed by identifying the enterotoxin directly in fecal samples. CPE enzyme-linked immunosorbent assay (ELISA) and reverse passive latex agglutination (RPLA) tests are available commercially for detecting CPE present in feces, although the RPLA test has a significant false positive rate and the CPE ELISA is not yet licensed for diagnostic use in the United States.

Genetic characterization of *C. perfringens* food-borne and non-food-borne illness isolates has been performed by several groups (e.g., Ref. [14]) using genomic techniques such as ribotyping, restriction fragment length polymorphism (RFLP), and pulsed-field gel electrophoresis (PFGE). These techniques are useful in assessing the clonality of various outbreak and disease isolates. However, because *C. perfringens* is also present in the normal intestinal flora, detection of the *cpe* gene in disease isolates is crucial for identifying *C. perfringens* as the etiological source of GI disease. Multiplex PCR typing of *C. perfringens*[11] can be performed to confirm the presence of *cpe* in disease isolates (Fig. 1, lanes A and A/*cpe*).

As mentioned, the *cpe* gene can be located on either the chromosome or a large plasmid. CPE-positive *C. perfringens* isolates from cases of *C. perfringens* type A food poisoning typically carry their *cpe* gene on the chromosome, whereas *cpe* is usually plasmid-borne in AAD/SD *C. perfringens* isolates.[7] These correlations are useful for molecular diagnostic and epidemiological studies of CPE-based disease (e.g., if a CPE-positive *C. perfringens* disease isolate is shown to carry a chromosomal *cpe* gene, that finding supports identification of a food poisoning case). Alternatively, identifying a plasmid enterotoxin gene in a disease isolate would usually support a diagnosis of AAD/SD.

A recent study by Miyamoto et al.[9] compared the genetic loci of chromosomal vs. plasmid *cpe*-containing type A isolates. This study identified distinct differences in the sequences flanking the *cpe* gene between the two *cpe* genotypes (Fig. 2A and B). An assay recently put forth by Wen et al. exploits these sequence differences by amplifying different size products from each *cpe* genotype (Fig. 2). This genotyping assay can be performed with either isolated DNA or colony lysates as template DNA,[15] with the use of the latter template eliminating at least 2 days from the preparation and isolation of DNA.

This duplex PCR reaction is an ideal presumptive assay for helping to distinguish between food-borne and non-food-borne cases of CPE-associated GI illness. Because the assay provides results within 6 hr of isolating *C. perfringens* colonies from contaminated food or feces, the speed and high-throughput nature of the technique provide a distinct advantage over the classical and more cumbersome techniques of RFLP and PFGE. Limitations of the PCR method include a requirement for a special polymerase (Advantage2® Polymerase Mix; BD Biosciences Clontech, Palo Alta, CA), which is expensive and displays some lot-to-lot efficiency variation (unpublished results). Despite these shortcomings, the *cpe* duplex PCR assay is an extremely useful and time-saving assay for determining the etiological genotype of CPE-positive *C. perfringens* GI disease isolates and should prove useful for clinical laboratories investigating CPE-associated disease.

Management

C. perfringens type A food poisoning is typically self-limiting (the diarrhea removes the enterotoxin from the GI tract), so only the replenishment of fluids and electrolytes is required for treatment. Patients suffering from AAD or

SD are also given symptomatic treatment to restore their fluid/electrolyte balance, but antibiotics such as metronidazole can be prescribed for serious cases.

HISTOTOXIC INFECTIONS

Disease Summary

C. perfringens causes three histotoxic infections: simple wound infections, anaerobic cellulitis, and clostridial myonecrosis or gas gangrene. The most serious of these diseases, gas gangrene, is an aggressive invasive infection that advances rapidly despite antibiotic therapy.[16] Most gas gangrene cases result from major traumatic injuries that allow for the introduction of exogenous or endogenous *C. perfringens* (responsible for 80% of gas gangrene cases) into an ischemic environment.[16] Gas gangrene-related death can occur rapidly and is typically attributed to shock and multiorgan failure resulting from hemodynamic collapse.[17] Studies have demonstrated that α-toxin and perfringolysin O are the primary mediators of gas gangrene, with α-toxin being absolutely required for disease (for a review, see Ref. [17]).

Diagnosis

Clinical diagnosis of gas gangrene is supported by pain at the site of surgery or injury, gas pockets in the tissue (visualized with X-rays), and signs of systemic toxicity.[16] Exploratory surgery is essential for diagnosis and a definitive diagnosis of gas gangrene involves a Gram's stain from tissue smears demonstrating the presence of gram-positive, rod-shaped bacteria.[16] Although α-toxin ELISA and multiplex PCR assays are available, they are typically not used to diagnose gas gangrene because of the rapid progression of the disease and the obvious clinical signs presented by the patient.[18]

Management

Treatment typically consists of clindamycin or tetracycline combined with penicillin.[16] Aggressive surgical debridement including radical amputation (where possible) to remove the infected tissue is mandatory.[17] An alternative supplemental therapy involving hyperbaric oxygen (thought to raise the tissue redox above that required for growth of *C. perfringens*) is also available.[19] Patients with gas gangrene in an extremity have a better prognosis than patients with truncal or intraabdominal gas gangrene because it is easier to remove infected tissues in extremities.[16] Those patients presenting with shock at diagnosis have the highest mortality rate.[16]

ENTERITIS NECROTICANS

Disease Summary

Enteritis necroticans (also known as necrotizing enteritis, Darmbrand, or Pigbel) caused by *C. perfringens* type C isolates is a rare but potentially lethal enteric disease of humans. This illness was first recognized after World War II in Darmbrand, Germany and later in Papua New Guinea.[20] The β-toxin produced by type C isolates is considered as the primary virulence factor based on the relative efficacy of a β-toxoid vaccine.[20] Although the incidence of enteritis necroticans is low, risk factors include reduced intestinal motility and/or low intestinal trypsin levels (the β-toxin is trypsin-sensitive) because of preexisting conditions created by a protein-poor diet, helminthic coinfection, and/or pancreatic disease.[20] The offending type C isolates can be introduced exogenously by ingestion of undercooked meat products (commonly pork).

Diagnosis

Patients typically present with painful, swollen abdomens (with or without bloody diarrhea and vomiting). Symptoms develop 12 hr to several days following the consumption of contaminated foods, and patients may show toxemia in severe cases.[20] On surgical examination, the patient is typically found to have segmental necrotizing enteritis of the proximal intestine.[21] No current assays exist for rapid diagnosis of the disease, and culture diagnosis typically requires samples from the site of infection, along with in vitro toxin assays.[20] The multiplex PCR typing assay could be a helpful, rapid diagnostic test for distinguishing between normal flora type A isolates and type C isolates not normally present in significant numbers in the intestinal flora.

Management

As mentioned, a β-toxoid vaccine has proven useful in high-risk populations (e.g., Papua New Guinea). Therapy for necrotizing enteritis usually includes nasogastric suction, intravenous fluids, and antibiotic intervention (typically penicillin, chloramphenicol, and metronidazole).[20] In severe cases, a laparotomy must be performed to remove damaged tissues.[20] Passive anti-β-toxin adjunctive therapy has not been shown to be effective.[20]

CONCLUSION

This entry was intended to review the genomics of *C. perfringens* and the diseases caused by this bacterium,

including CPE-mediated diarrhea, histotoxic infections, and enteritis necroticans. Although some molecular techniques exist for toxin typing (multiplex PCR) and for the diagnosis of CPE-mediated disease (duplex PCR), similar methods for diagnosing histotoxic infections and enteritis necroticans are currently lacking. With the pending completion of additional genome sequencing projects, improved molecular techniques, and rapidly advancing proteomic and genomic applications, *C. perfringens* researchers will soon gain an increased ability to explore pathogenesis from a molecular perspective. This information should lead to new techniques for diagnosing and treating *C. perfringens*-mediated infections. The authors hope that this brief chapter adequately summarizes the current state of *C. perfringens* genetics/molecular diagnostics and human pathogenesis and regret the reliance on review citations due to page limitations.

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Colony PCR

Gehua Wang
Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba, Canada

INTRODUCTION

The colony polymerase chain reaction (PCR) protocol is one that can be easily performed, and used widely in many fields of molecular microbiology. Various types of samples can be used in colony PCR, and because nucleic acids are relatively stable, the DNA used for colony PCR does not need to be purified or intact. Partially degraded DNA will also provide a suitable target for colony PCR amplification provided that there is enough DNA template to bridge the distance between the forward and reverse primers.

BACKGROUND

PCR is an enzyme-based in vitro method used to perform the following: 1) to amplify specific DNA fragments for molecular diagnosis of heritable disorders; 2) to screen for susceptibility to disease; 3) to identify bacterial and viral pathogens or specific target genes; 4) to biomonitor environmental changes and gene expression; 5) to study the evolutionary process at the nucleotide level. Furthermore, this technique can be used to analyze recombinant vectors to determine both the presence and orientation of the inserted DNA, determine DNA sequence, and examine gene replacement.[1–4]

Purified DNA is most often used as a template in the PCR reaction. However, it is possible to amplify specific DNA sequences without DNA purification by starting with a single living bacterial colony. This technique is known as colony PCR, or whole-cell PCR, and provides a powerful and reliable method for the rapid amplification and isolation of any gene in both gram-negative and gram-positive bacteria or any gene carried on a plasmid. As an example, repetitive element sequence-based PCR (rep-PCR) with different gram-negative bacteria (e.g., *Citrobacter diversus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) and gram-positive bacteria (e.g., *Staphylococcus aureus* and *Streptococcus pneumoniae*) were demonstrated.[5]

In general, broth cultures as well as colonies from agar plates and glycerol stocks can be directly utilized in the PCR mixture. To release the DNA for PCR amplification, the bacterial cell wall can be disrupted by heating. The dH$_2$O-diluted whole-cell sample or overnight culture can also be directly applied to the PCR reaction tube with longer PCR initial denaturing time at 95°C.

DNA TEMPLATE PREPARATION

The DNA template can be prepared from broth culture with an optical density of 0.3–0.4 at 600 nm. Then the optimized whole-cell suspensions from all bacterial samples can be further diluted 300- to 500-fold in dH$_2$O; alternatively, a bacterial colony, 1 mm in diameter or less, grown on an agar plate can be picked with a sterile pipette tip and inoculated into 100 μL of sterile distilled water. This whole-cell preparation is heated at 100°C for 5–10 min in a 0.5-mL Eppendorff tube. These templates are now ready for PCR application, or may be kept at 4°C for up to 1 month.[6] Still, another method involves touching a smaller colony with a sterile pipette tip; this can then be placed directly into a PCR reaction tube for amplification, along with 5–10 min of initial denaturation at 94–95°C.

PCR REACTION

There are four major steps involved in a colony PCR reaction: 1) growing bacteria and preparation of whole cell suspensions; 2) making a PCR master mix and aliquoting this into Eppendorff tubes; 3) performing PCR with suitable cycling conditions; 4) analyzing amplification products on an agarose gel. In general, a reaction mixture of 25 μL contains a final concentration of 200 μM of each deoxynucleoside triphosphate (dNTP), 2.5 μL 10× reaction buffer (100 mM Tris–HCl at pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, and 0.1–1.0 μM of each of the upstream and downstream primers, together with 2.5 units of *Taq* DNA polymerase. A portion (2.5 μL) of the whole-cell preparation of template DNA is also required. The final volume of this mix should be adjusted to 25 μL with sterile water. The PCR cycle should consist of an initial denaturation step at 95°C for 2–10 min (depending on the

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Taq DNA polymerase being used), followed by 30–35 cycles of amplification with denaturation at 95°C for 30 sec, annealing at 50–60°C for 30 sec, and extension at 72°C for 30 sec, ending with a final extension at 72°C for 7 min. Part (5–10 μL) of the PCR product should be loaded onto a 0.5× TBE–agarose gel for analysis. Products from 0.1 to 1.5 kb are easily resolved on a 1.5% gel under UV light after staining with 0.5 μg/mL of ethidium bromide solution for 30 min.

APPLICATIONS

Colony PCR has been found to be a useful technique applicable to both gram-negative and gram-positive bacteria.[5,7] Satisfactory PCR products were obtained while using this method with intact cells, colonies from agar plates, and even liquid cultures which were stored at 4°C for up to 3 months. Moreover, this method has also been used to identify target genes or to distinguish homologous recombination events from nonhomologous ones in yeast.[4] Performing DNA extraction is not recommended for PCR purposes when there are few bacterial cells in the sample because DNA loss is inevitable in the process, which would hinder subsequent PCR amplification.

ADVANTAGES

Colony PCR is a whole-cell-based assay, and thus will save a considerable amount of time, labor, and chemical reagents. The possibility of experimental errors or contamination is also reduced because DNA purification is not required.[3] A comparison study with two DNA extraction methods using mycobacterial isolates indicated that the heating method is more sensitive and no less specific than a conventional chemical method (phenol–chloroform). The loss of DNA during purification and the presence of inhibitory substances are the most likely explanations for the lowered PCR sensitivity results obtained via the chemical method.[8,9] PCR is a very sensitive and rapid method of detecting specific DNA sequences in a wide variety of samples. The most common problem is contamination of reagents with target DNA. However, colony PCR will be performed on bacterial colonies directly obtained from broth culture or plates, which will lower the opportunity of contamination resulting from DNA manipulation or extraction. In addition, no detergents or proteases are used to lyse the bacterial cells, which is also advantageous because these may interfere with the PCR reaction.[3]

SENSITIVITY

The detection sensitivities of colony PCR for *E. coli*, *Aeromonas hydrophila*, *Campylobacter jejuni*, and *S. aureus* were determined by both PCR and plate count methods using agarose gel electrophoresis relative to actual plate counts within the range of 10 to 10^{13} colony forming units per milliliter (CFU/mL). The detection ranges of colony PCR (in CFU/mL) for *E. coli*, *A. hydrophila*, *C. jejuni*, and *S. aureus* were $8 \times 10^{5-10}$, $3 \times 10^{4-11}$, $1 \times 10^{4-13}$, and $3 \times 10^{8-12}$, respectively. However, the capability of detecting the target gene can be reduced by the presence of higher concentrations of nontarget DNA or PCR inhibitors.[10,11] It appears that a range of 10^9 to 10^{10} CFU/mL was most effective for all of the colony PCR assays and only about 10 microorganisms were required for the detection of *Mycobacterium tuberculosis*.[6,8]

PCR INHIBITION OR CONTAMINATION

The use of an internal control is important because impurities or improper nucleic acid concentration may inhibit the PCR reaction, resulting in false negatives. The internal control will give a greater degree of confidence in the validity of a negative amplification result. Every amplification test where inhibitors [such as nontarget DNA, heme, bilirubin, bile salts, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), proteases, and bacterial extracts, etc.] are problematic should have an internal control.[5,11,12] To avoid DNA contamination or carried over false positives, the use of deoxyuridine 5′-triphosphate (dUTP) in combination with Uracil-DNA-*N*-glycosylase (UNG) is recommended. Several other precautions to avoid contamination should be taken, including specific workstations designed for PCR work alone, aseptic techniques, specific pipettes and tips plugged with sterile cotton wool or filter, small aliquots of reagents when performing PCR, and minimizing the number of operations or transfers. A negative control that does not include any target DNA should also be performed with each PCR assay.[7]

LIMITATIONS

Reduced levels of sensitivity have been encountered in the past when the technique was applied to clinical samples, the cause of which has been attributed to the presence of inhibitors. Using wooden toothpicks to transfer colonies may also inhibit PCR reactions in which the PCR mix contains a low concentration of *Taq* DNA polymerase. The character of the inhibitor remains unclear.[13] Even

when the internal positive control does not detect any inhibition in the PCR reaction, the presence of foreign DNA or nontarget DNA could still have interfered with the performance of the PCR, causing a drop in the sensitivity of the test or even leading to a false negative result when it is applied to samples with few target genes present.[10,14] Validations of PCR specificity are recommended, and may be performed with restriction fragment length polymorphism (RFLP) analysis, PCR product sequencing, or Southern blot hybridization.

CONCLUSION

Colony PCR or colony multiplex PCR assays proved to be simple to perform as well as being accurate. It is a very sensitive and reproducible method for direct PCR without the need of DNA preparation. Studies based on *M. tuberculosis* identification using PCR indicated that sonication was the most sensitive method of DNA extraction compared to other protocols such as boiling, nonionic detergents, proteinase K, and freeze-thawing. However, false negative results may occur when viscous specimens are sonicated.[15–18] Afghani and Stutman[9] concluded that boiling is a rapid, sensitive, and specific method of DNA extraction for PCR applications. Unfortunately, accurate estimation of colony PCR sensitivity is difficult. Because the number of bacterial cells used in the PCR to determine the sensitivity of the assay is often adjusted by using the optical density of the suspension, and the actual bacterial cell number per sample may have been higher as a result of the possible clumping of organisms, this will possibly lead to a miscalculated cell count.

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Commercial DNA Extraction Kits

C

Stuart C. Clarke
Stobhill Hospital, Glasgow, U.K.

INTRODUCTION

Genomic DNA is used in a wide range of applications in molecular biology including disease diagnosis, food microbiology, and environmental microbiology. However, DNA analysis in many aspects of medicine and biology rely on the extraction of DNA from complex samples or environments. These may possess numerous contaminants depending on the sample type. In medicine, the extraction of human genomic DNA from tissue samples may be contaminated with commensal bacterial flora. In environmental microbiology, the analysis of water or soil samples may be contaminated by bacterial DNA, plant DNA, and minerals. Interestingly, the analysis of DNA has led to the revised classification of some soil bacteria. Even the complex environment of human blood hampers the detection of infection because of the presence of red blood cells, white blood cells, transient contaminant bacteria, and numerous components of the immune system. Therefore the technique used for DNA extraction is important, as are the steps performed during the process such as cell lysis, DNA recovery, and DNA purification. In medical microbiology, there is a growing interest in molecular methods for the laboratory confirmation of infection and advances in molecular biology have promoted the routine use of techniques such as PCR and DNA sequencing. However, difficulties exist with the extraction of genomic DNA from bodily fluids. Sensitive DNA extraction methods are required but the DNA extracted must be of sufficient quality to be used for the improved diagnosis and surveillance of microbial diseases. In all of the areas above, traditional phenol–chloroform extraction is not usually practical because of the time and safety constraints of the method. Here DNA extraction systems are compared in terms of ease of use, sensitivity, and their ability for automation. An emphasis is placed on their use in clinical microbiology, particularly in relation to the use of whole blood for the diagnosis of microbial infections.

SAMPLES

As described above, there are many areas where purified genomic DNA is required for analysis[1–8] (Table 1). In medicine, samples may include body fluids such as whole blood, serum, CSF, and tissue among others. DNA purified from these sources must be pure, nuclease free, and of good quality (i.e., not sheared). Furthermore, the DNA must be of a quality to provide long-term storage as clinical samples, and indeed others, are often unrepeatable.

MAGNETIC BEAD DNA EXTRACTION METHODS

Some methods for DNA extraction utilize silica-coated magnetic beads onto which the DNA binds and allows extraction, washing, and elution of pure genomic DNA. Such methods include the Bilatest beads DNA extraction kit and Promega bead kit. They are particularly useful for extracting bacterial genomic DNA from whole blood. Typically, a lysis mixture is prepared by mixing magnetic particle suspension with proteinase K and lysis buffer. Premixed lysis solution is then added to the sample and incubated at 55°C to achieve lysis of the red and white blood cells. This is usually performed in a microtitre plate which is then placed on a magnetic separator to allow the metallic bead–DNA complex to sediment. The supernatant is discarded, the DNA washed, and the microtitre placed on the magnetic separator to allow the particle–DNA complex to sediment, and the supernatant discarded. The DNA is then reeluted by adding sterile distilled water and incubating at 65°C.

SILICA MEMBRANE DNA EXTRACTION METHODS

These kits can be used for the extraction of genomic DNA from whole blood or other bodily fluids and are again based on the principle that DNA binds to silica. They are available in single tube or 96-well plate formats. Lysis buffer is added to the sample in order to lyse red and white blood cells and incubated at 70°C. After incubation, ethanol is added, the sample vortexed, and placed on a vacuum manifold to draw the liquid phase through the filter. Washing is repeated and the liquid phase again removed by vacuuming. Elution buffer is added directly to the silica membrane and incubated at room temperature briefly followed by vacuuming to elute the DNA.

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Table 1 Examples of use of DNA extraction methods

Type of DNA required	Sample	Reason	Reference
Human genomic DNA	Paraffin-embedded tissue	Disease diagnosis	[19,20]
Bacterial or parasitical genomic DNA	Feces	Confirmation of bacterial or parasitic infection	[8,21]
Bacterial genomic DNA	Soil	Bacterial quantification	[1]
Viral DNA	Dried blood spots	Confirmation of viral infection	[12,22]
Bacterial DNA	Blood or CSF	Confirmation of bacterial infection	[4,18,23]

FILTER-BASED DNA EXTRACTION METHODS

These kits are operationally similar to the silica membrane-based methods but, instead of the DNA binding to the silica membrane, the DNA is held on the filter by size exclusion. As for the other methods, lysis solution is added to the bodily fluid and left at room temperature for cell lysis to take place. After incubation, neutralization solution is added to the tube and mixed again. Lysate is transferred from the sample tube and dispensed into the corresponding well of a clearing plate placed on a vacuum manifold. The liquid phase is then drawn through the filter into the corresponding well of a collection plate and the filtrate discarded. The DNA remaining in the clearing plate is washed by adding wash solution followed by vacuuming, and again the filtrate is discarded. A new collection plate is placed inside the vacuum manifold and the DNA recovered by adding elution buffer.

AUTOMATION

Many methods in molecular biology require high-throughput sample processing, and therefore manual extraction of DNA extraction is not possible.[5] Although many commercial DNA extraction methods can be purchased in tube format, these still require a significant amount of hands-on time in order to add reagents, remove reagents, and perform centrifugation or vacuum steps.[4,5] Efficient methods of DNA extraction that produce pure, high-quality DNA are crucial to the success of PCR and sequencing reactions and the subsequent treatment of disease.[8,9] Current manual methods of DNA extraction are simple and reliable, and are suitable for extraction of low numbers of samples.[10] As the demand for molecular tests increases, new automated methods of DNA extraction will have to be developed to handle larger numbers of clinical samples.[11] There are a number of DNA extraction kits available, based on a 96-well plate format, which allow integration into the workstation of a robotic liquid handler. This allows a much higher throughput of samples, is less work intensive, and produces PCR-ready bacterial DNA.[4,9,11] A number of commercial robotic systems are available for such purposes from companies such as MWG Biotech, Tecan, Qiagen, Beckman, and Roche.

COMPARISON OF DNA EXTRACTION METHODS

Purified bacterial DNA is required for many procedures in modern molecular biology, but clinical microbiology now routinely utilizes DNA for the laboratory confirmation of various infections.[4,8,12] The infections requiring a laboratory diagnosis are primarily of bacterial, viral, or parasite etiology. As such, infections of public health importance are now often diagnosed by PCR, and even typing information is provided on the organism causing infection. One such example is meningococcal disease. For this infection, rapid, sensitive, and specific methods are required so that case contacts can be managed appropriately. Bacterial DNA can be amplified from clinical samples by PCR to characterize the infecting bacterium.[13–17] As well as dedicated DNA extraction robot systems, a number of commercially available DNA extraction kits can be automated[4,18] and can be modified appropriately to various platforms. Overall efficiency of such methods can be determined by throughput time, DNA yield, and labor intensity.[4] All three main methods of DNA extraction can be automated, but their overall efficiency differs according to manufacturer. Some commercial kits, such as the 96-well plate format of the Promega SV96 kit, allow easy adaptation for use on a liquid handling robot. A large number of samples can then be processed with little or no manual intervention. Such kits are extremely simple to use and can extract up to 96 samples in under 20 min. The kits based on the use of metallic beads are also very simple to automate. Some magnetic bead kits are very efficient and can rapidly extract high-quality DNA from a large number of samples

automatically. Some kits produce more DNA per reaction than others although these may not necessarily be the best for automation and test specificity. Overall, it is difficult to compare methods and decide on the best because it depends on the sample type, downstream method required, and the assay type being performed. However, kits have been compared and automated for the extraction of bacterial genomic DNA from whole blood samples and the sensitivity, specificity, and efficiency of the kits compared. In that study, the Bilatest Beads DNA 2 kit and the Promega Wizard SV96 kit were the most successful kits in all areas[4] and have subsequently been used successfully for the laboratory confirmation of septicemia and meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Heamophilus influenzae*.[18] Ultimately, the choice of kit depends on the needs of users, often whether they prefer a 96-well plate format or a metallic bead system.

CONCLUSION

DNA extraction methods are now becoming an essential component of molecular biology methods for the sensitive and specific amplification of genomic DNA. Numerous commercial kits are now available which utilize three main technologies for the purification of DNA, and these can be used in diverse applications within the specialties of medicine, environmental biology, and food microbiology. DNA extraction methods can also be automated which can provide high-throughput analysis of samples.

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Comparative Genomic Hybridization (CGH)

C

Brynn Levy
Mount Sinai School of Medicine, New York, New York, U.S.A.

INTRODUCTION

Molecular cytogenetics is now considered an integral part of the modern-day cytogenetics laboratory. The ability to identify specific sequences along a chromosome by molecular cytogenetic techniques has been especially useful in 1) defining cytogenetically detected chromosomal material of ambiguous or unknown origin; 2) detecting small submicroscopic rearrangements; 3) rapidly determining the ploidy for specific chromosomes at interphase, e.g., trisomy 21; and 4) mapping particular DNA sequences to specific chromosomes (gene mapping). These specific sequences have been identified by using fluorescently labeled DNA probes that hybridize to the DNA segment of interest. This technique is referred to as fluorescence in situ hybridization (FISH) and forms the fundamental basis of molecular cytogenetics. Since its development in the 1980s, molecular cytogenetics has expanded dramatically beyond two-color FISH. Comparative genomic hybridization (CGH) is one of the more recent innovative molecular cytogenetic techniques that have provided a very unique way to detect chromosomal imbalances in a single-step global-wide scan of the genome. Many factors have made CGH an attractive technique for cancer research, the primary one being that CGH is a DNA-based technique and thus specimen culturing and the availability and quality of metaphase spreads from the specimen are not considerations. This property makes formalin-fixed paraffin-embedded neoplastic tissue and even nonviable tissues, such as that derived from the products of conception, amenable to analysis by CGH. Comparative genomic hybridization has also proved to be an invaluable tool in clinical cytogenetic analysis, especially for samples for which a complete and detailed karyotype could not be obtained by conventional methods. The ability to obtain a genome-wide search for imbalances without any prior information of the chromosomal aberration in question has been particularly useful in clinical cytogenetics. This chapter discusses the technique of CGH and describes its use as both a cancer research tool and a diagnostic technique in clinical cytogenetics.

THE BASICS OF COMPARATIVE GENOMIC HYBRIDIZATION

The development of CGH has provided molecular cytogenetics with a powerful means to scan the entire genome, in a single step, for imbalances in chromosomal material. Comparative genomic hybridization effectively reveals any DNA sequence copy number changes (i.e., gains, amplifications, or losses) in a particular specimen and maps these changes on normal chromosomes.[1,2] Comparative genomic hybridization can detect changes that are present in as little as 30–50% or more of the specimen cells.[2] It does not reveal balanced translocations, inversions, and other aberrations that do not change copy number. Comparative genomic hybridization is accomplished by the simultaneous in situ hybridization of two different genomes, the specimen (test) genome and the reference (normal control) genome. In order to distinguish the two genomes, total genomic DNA from each is labeled with different fluorescent colors and then both are hybridized to normal human metaphase chromosome spreads.[1] The relative amounts of specimen and reference DNA hybridized at a particular chromosome position are contingent on the relative excess of those sequences in the two DNA samples and can be quantified by calculation of the ratio of their different fluorescent colors.[1] Specimen DNA is traditionally labeled with a green fluorochrome such as fluorescein isothiocyanate (FITC) and the normal reference DNA with a red fluorochrome such as Texas Red. A gain of chromosomal material in a specimen would be detected by an elevated green to red ratio, whereas deletions or chromosomal losses would produce a reduced green to red ratio.[1] Figure 1 illustrates the fluorescence capture process as well as how a gain and loss are visualized and then mapped to their source chromosome.

The sensitivity of conventional CGH is in the megabase range, with a theoretical detection limit of deletions estimated to be about 2 Mb.[3] The detection limit of CGH therefore approaches that of a high-resolution karyotype (750–1000 band level) i.e., CGH can detect imbalances around 2–3 Mb in size.[4]

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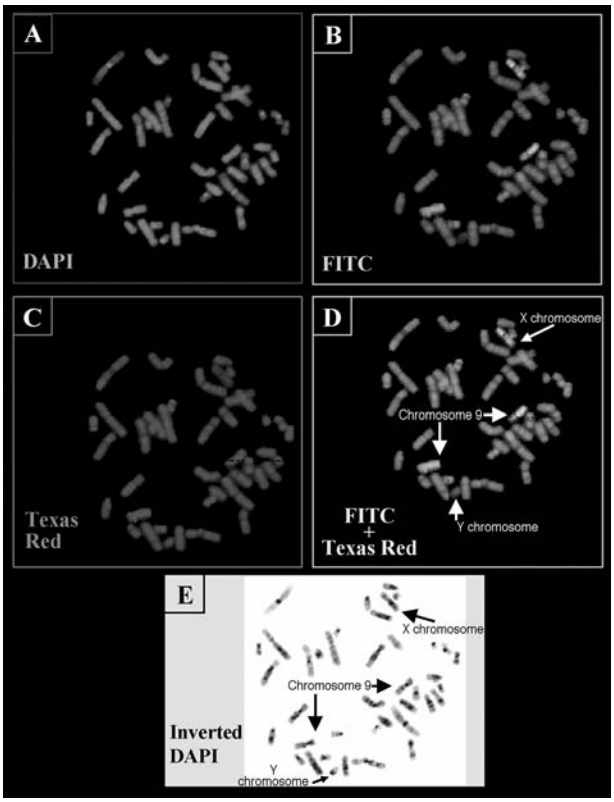

Fig. 1 Fluorescence microscopy utilizing fluorochrome-specific filters to visualize and capture color ratio differences along the chromosomes. A. DAPI image. B. FITC image. C. Texas Red image. D. Combined FITC and Texas Red image revealing an overrepresentation (greater green to red intensity) of the X and 9 chromosomes and an underrepresentation (greater red to green intensity) of the Y chromosome. E. The over- and underrepresented chromosomes are identified by their banding pattern observed in the inverted DAPI image. (*View this art in color at www.dekker.com.*)

Overview of Comparative Genomic Hybridization

The major steps in CGH involve: 1) Preparation of karyotypically normal 46,XY metaphase spreads. Male reference chromosomes are chosen so that both the X and Y chromosomes are available as hybridization targets. 2) Isolation of high molecular weight DNA from specimen (test) and reference (normal) samples. 3) Labeling of specimen and reference DNA with different fluorochromes by nick translation. 4) *In situ hybridization* of equal amounts of labeled specimen and reference DNAs together with unlabeled Cot1-DNA to normal metaphase spreads. 5) Washing off unbound DNA. 6) Counterstaining metaphase spreads with DAPI for chromosome identification. 7) Fluorescence microscopy utilizing fluorochrome-specific filters to visualize and capture color ratio differences along the chromosomes (Fig. 1). 8) Chromosome identification by the inverted DAPI banding pattern (Fig. 1). 9) Quantitation of copy number differences by generating a ratio profile of the specimen to reference DNA fluorescence intensities along each chromosome. 10) Combining the profiles from several metaphase spreads to improve the significance of the result.

COMPARATIVE GENOMIC HYBRIDIZATION IN CANCER CYTOGENETICS

Genetic alterations associated with neoplasia have been well defined in hematological malignancies by both classical and molecular cytogenetics.[5,6] In contrast, there is significantly less information known about the cytogenetics and molecular cytogenetics of solid tumors. This is because of technical difficulties in the production of metaphase spreads from these tumor cells. Karyotype analysis requires viable, proliferating cells that can be arrested in the metaphase stage of the cell cycle. Cytogenetic analysis of these tumors is often hampered as many solid tumor cells fail to proliferate in vitro. For those tumors that do divide and produce metaphase spreads, the quality of the metaphase spreads is often inadequate to allow for recognition of banding patterns. There is also the question of the significance of the cytogenetic data derived from in vitro tumor cell culture as small subclones in vivo may take advantage of the in vitro conditions and thus the nonproliferating cells that constitute the main clone in vivo may escape detection by conventional cytogenetic analysis.[7] In addition, many aberrant chromosomal regions may not be identified because of the highly complex karyotypes of cultured cancer cells carrying both multiple numerical and structural chromosomal abnormalities.

Comparative genomic hybridization allows for direct analysis of genomic DNA obtained from tumor specimens, thus overcoming the problems associated with cell culturing and poor metaphase spread quality. Also, as detection of chromosomal imbalances by CGH is dependent on the aberrations being present in 30–50% or more of cells from which the DNA is extracted,[2] the results derived using this technique reflect changes that are genuinely present in the majority of tumor cells. Comparative genomic hybridization analysis is limited by its inability to provide information about balanced rearrangements, the origins of structural anomalies, or the ploidy status of the cells. However, identification of specific regions of imbalance may be sufficient to provide a location for candidate genes (oncogenes and tumor suppressor genes) causative of the initiation and progression of these tumors.

The use of CGH in cancer genetics has revealed a number of novel recurring chromosomal gains, amplifications, losses, and deletion sites that escaped detection using traditional cytogenetic analysis in various

tumors, including prostate cancer, testicular germ cell tumors, breast cancer, uveal melanomas, small-cell lung carcinoma, gliomas, sarcomas, head, neck, and pancreatic carcinomas, and uterine leiomyomata. The chromosomal aberrations detected by CGH have also provided prognostic information in a number of neoplasms including renal cell carcinomas, bladder cancer, cervical carcinomas, node-negative breast cancer, uveal melanoma, cutaneous melanoma, and prostate cancer. Various international CGH databases have been established including Tokyo Medical and Dental University CGH database (http://www.cghtmd.jp/cghdatabase/index_e.html), the database of Humboldt-University of Berlin (http://amba.charite.de/~ksch/cghdatabase/index.htm), the Progenetix cytogenetic online database (http://www.progenetix.net), and the National Cancer Institute and National Center for Biotechnology Information Spectral Karyotyping SKY and Comparative Genomic Hybridization CGH Database (2001), (http://www.ncbi.nlm.nih.gov/sky). These databases provide a wealth of information on the CGH studies that have been done since 1992.

COMPARATIVE GENOMIC HYBRIDIZATION IN CLINICAL CYTOGENETICS

Clinical cytogenetic studies often yield karyotypes with unbalanced or unrecognizable G-banded cytogenetic material, examples of which include marker chromosomes, unbalanced translocations, intrachromosomal duplications, and deletions. The challenge is to identify the unbalanced material in order to provide the most up-to-date information for genetic counseling. Such information would directly benefit prenatally ascertained cases of unidentifiable chromosomal material, providing couples with a means to make rational and informed decisions concerning the pregnancy. In pediatric cases, such information may provide the parents with a realistic prognosis and be important for the clinical management of the infant. In the past, this required a comprehensive molecular cytogenetic workup using various FISH probes, an endeavor that is both expensive and labor intensive as numerous probes and/or whole-chromosome paints may be required until the origin of the chromosomal material is identified. In addition, the number of commercially available region-specific probes is limited and covers only a fraction of the genome. Comparative genomic hybridization has now been shown to be an effective single-step focused method for the identification of unbalanced aberrant chromosomal material of unknown origin.[8,9] The advantage of CGH over conventional FISH with whole-chromosome paints (wcps) and multicolor FISH is its ability to identify not only the chromosome from which the additional unknown material was derived but also to map the region involved to specific G-bands on the source chromosome (Fig. 2). In addition to being able

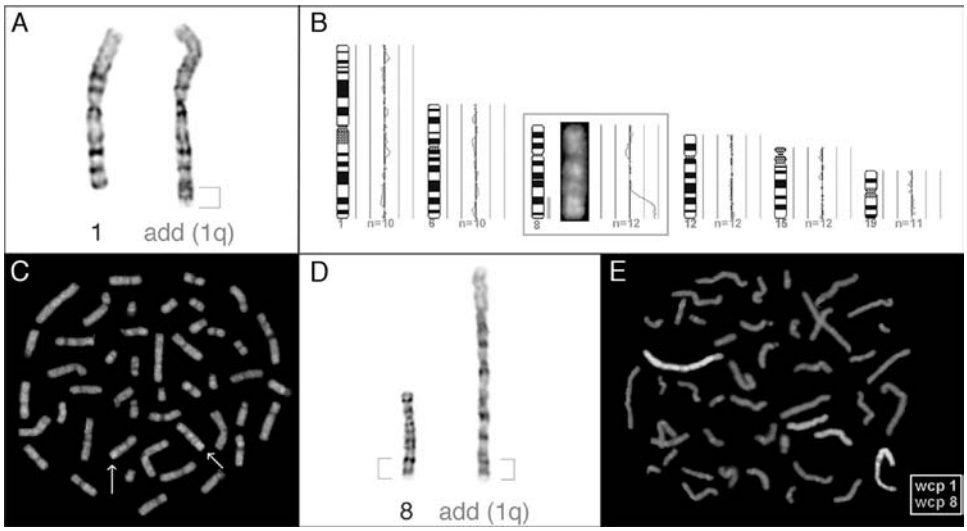

Fig. 2 CGH workup of a patient with multiple congenital abnormalities and additional chromosomal material of unknown origin on the long arm of chromosome 1. A. Partial karyotype showing the G-banded aberrant chromosome 1 with extra material (on the right) and the normal chromosome 1 (on the left). B. Partial ideogram and CGH profiles indicating a gain on the long arm of chromosome 8 from 8q23 to 8qter (orange box). The lines in the CGH profiles represent green/red ratios of (from left to right) 0.5, 0.75, 1.0, 1.25, and 1.5. A ratio of 1.0 represents the balanced state of the chromosomal copy number. C. The gain of chromosome 8 material observed in panel B can be visualized in this panel in the dual FITC/Texas Red image as the green intense regions (arrows). D. Aligning the G-banded extra material on chromosome 1 with its endogenous position on its source chromosome provides visual confirmation of the CGH results. E. Confirmation of the CGH finding by FISH with a chromosome 1 and 8 whole-chromosome paint. (*View this art in color at www.dekker.com.*)

to identify excess and/or missing chromosomal material not resolvable by G-banding, CGH can also be used as a backup method for aneuploidy analysis of specimens that have failed to grow in cell culture. This is especially effective in the analysis of nonviable fetal tissue derived from products of conception which are estimated to have a chromosome abnormality (mainly aneuploidy) approximately 50–70% of the time.[10]

To date, more than 1600 articles have been published on CGH with the greater majority reporting the utility of CGH to identify novel chromosomal imbalances in neoplastic specimens (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?). Less than 10% of CGH papers have dealt with technical aspects and only a limited number have described the application of CGH in a clinical cytogenetics setting. Comparative genomic hybridization has been particularly useful in clinical cytogenetics by facilitating the identification and characterization of unbalanced translocations, deletions, intrachromosomal duplications, and marker chromosomes, including neocentric marker chromosomes.[8,9,11] High-resolution identification of such aberrant material has provided clinical correlation between specific chromosomal regions and various clinical entities such as the duplication 5q phenotype[12] and partial tetrasomy 10p which resulted from an analphoid marker chromosome with a neocentromere.[11] These publications have demonstrated the power of CGH for elucidating karyotype–phenotype correlations as well its potential for providing the groundwork for the discovery of the gene(s) that are responsible for the clinical presentation observed in patients with chromosomal imbalance. The ability of CGH to detect total aneuploidy makes it an appealing diagnostic tool for use in preimplantation genetic diagnosis (PGD).[13–15] Cytogenetic analyses of human preimplantation embryos, generated using in vitro fertilization (IVF) technology, have shown a very high incidence of chromosomal abnormality, with greater than 50% of all embryos identified to have aneuploid cells.[16–18] The majority of chromosome abnormalities detected at this early stage of development are likely to be lethal and their existence may explain a significant proportion of failed IVF cycles. The use of CGH to identify nonaneuploid embryos and selectively transfer them to the mother should theoretically improve the IVF implantation rates and also lower the observed miscarriage rates and thus improve the overall efficiency of assisted reproductive techniques.[15]

CONCLUSION

Comparative genomic hybridization was originally developed as a cancer research tool and rapidly demonstrated its power by detecting novel regions of chromosomal imbalance in a large variety of neoplastic samples. These studies have led to the discovery of many new tumor suppressor genes and oncogenes that play a role in the initiation and/or progression of solid tumors. The application of CGH to prenatal and pediatric samples has also proven extremely beneficial, allowing the delineation of ambiguous and complex karyotypes that could not be defined using classical cytogenetic techniques. In addition, the use of CGH to analyze human preimplantation embryos has provided fascinating scientific data concerning the variety and rate of aneuploidy at this early developmental stage and holds a lot of promise for improving the efficiency of assisted reproductive techniques.

As technical advances improve the capabilities of CGH, future additional applications are likely to include screening for deletions as small as those observed in the microdeletion syndromes such as Prader–Willi and DiGeorge syndromes. This will be made possible by the recent development of newer CGH protocols such as matrix CGH which combines biochip and CGH technologies[19] and array CGH which uses an array of DNA sequences instead of metaphase chromosomes.[20] The use of regular CGH would still remain an attractive and powerful accessory to routine clinical cytogenetic analysis.

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Competitive PCR

M. Pazzagli
C. Orlando
P. Pinzani
University of Florence, Florence, Italy

INTRODUCTION

A reliable approach to quantitative PCR is defined as competitive PCR (c-PCR) and it is based on the addition to the sample of a competitor target (DNA or RNA), acting as an internal standard, which has the same primer recognition sites as the target gene and, consequently, the same behavior during PCR cycling. The PCR amplicons can be then separated by conventional procedures and quantitation is achieved by comparing the signal of the specific target with that obtained from the known concentration of the internal standard (competitor template). This strategy seems to guarantee the independence of the results from any predictable or unpredictable variables that can affect the rate of amplification because the two species are amplified at the same rate and their ratio remains constant during PCR cycling. With this procedure, the parallelism between target and competitor is maintained even after reaching the plateau phase, with an increase in the assay sensitivity.

TECHNICAL DESCRIPTION

The Competitor Templates

Competitive PCR can be adopted for both DNA and mRNA measurements and generally requires the construction of synthetic DNA or RNA competitors.[1,2] DNA competitors have the advantage of better stability and more accurate quantification methods in comparison to RNA competitive templates. Whereas, if DNA is used, competitor quality and quantity can be compared with stable DNA standard preparations, the measurement of mRNA is complicated by the difficulty of obtaining a practicable reference standard for the calibration of the RNA competitor. The use of a DNA competitor for mRNA measurements may simplify the procedure of quantitative reverse transcript PCR (RT-PCR). However, the use of a DNA standard for mRNA measurements, even if it represents an advantage because of its stability and ease of handling,[3] cannot prevent the inaccuracy of RT-PCR which is due to the large variability of reverse transcription efficiency.

In some cases, a DNA competitor can be obtained by selecting the primers on two contiguous exons separated by a small intron, but generally, the competitor must be synthesized ad hoc. Competitors are normally cloned in plasmid vectors, transcribed in vitro to obtain large amounts of specific transcript, and measured precisely to determine competitor concentration.[4]

Synthesis of Competitors

The construction of competitor templates, their exact quantification, and their storage represent the crucial aspects of this technique. Competitors, to act as a functionally appropriate internal standard, must behave during the assay as closely as possible to the template. The efficiency of the enzymatic reactions (reverse transcriptase and PCR) on the two species must be comparable.[5,6] For this reason, the competitor must have comparable dimensions and the same primer recognition sites as the target. However, for the assay to be practical, the separation and the measurement of the two species should be performed in the simplest way possible. Different strategies have been described for the construction of the competitor for c-PCR. Some competitors have been obtained by the incorporation of a new restriction site in the native sequence,[7] and after PCR, the competitor is digested with the relevant restriction enzyme and the electrophoretic bands are then resolved and measured. The main disadvantage of this approach is the unpredictable efficiency of enzyme digestion, which can strongly influence the results of the assay. A variant of this technique is based on the insertion of a small modification in nucleotide sequence of the target (short sequence modification or single-base mutation).[8] After PCR, the competitor can be identified by differential hybridization with specific probes or by electrophoretic techniques [denaturating gradient gel electrophoresis (DGGE) or single-strand conformation polymorphism (SSCP1)]. This approach is quite complex and requires time-consuming and cumbersome post-PCR procedures. Furthermore, the

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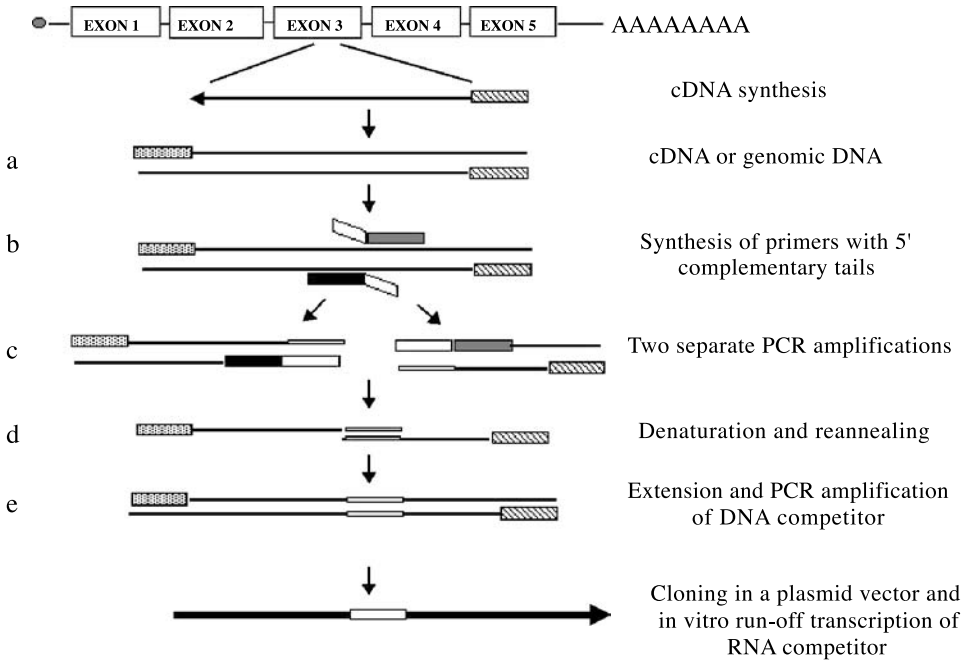

Fig. 1 Construction of competitor templates for competitive PCR or RT-PCR. The figure describes all the steps necessary to the synthesis of an RNA competitor. Steps from a to e represent the procedure to obtain a DNA competitor template.

efficiency of these techniques for discrimination of the two species is extremely variable.

The simplest approach to obtain a synthetic competitor is the introduction of a short deletion or insertion in the center of the competitor. This variation in size, if maintained within an acceptable range (10–15% of the original size), does not modify significantly the amplification rate of PCR. Several methods have been proposed for the construction of this type of competitor. A rapid and efficient method for both DNA and RNA competitor construction is based on a modification of the overlap extension technique[1,2,4] (Fig. 1). Two DNA fragments are amplified in separate PCR reactions, using the designed external primers in combination with respective internal primers containing complementary sequences to each other. The two fragments are then annealed and amplified with the external primers, in the same tube. The resulting sequence is identical to the initial target, with the exception of the added (or deleted) complementary sequence used for the ligation of the two halves of the competitor. With a similar technique, it is possible to generate multiple competitors by ligation of different PCR products (see the chapter on multiplex competitive PCR).

General Rules for Competitive PCR or RT-PCR Assays

In c-PCR, quantification is performed by comparing the final, post-PCR signal of the target with that of known amounts of the competitor. By definition, in c-PCR, the most accurate measurement is obtained when the signals corresponding to competitor and target templates are equivalent (or close to equivalence).[9] Ideally, if the target concentration is in the linear range of competitor/target ratios, a single point could provide an accurate measurement of the target gene.

However, because of the unpredictable target concentration, a variable number of tubes with a constant amount of target and variable competitor concentration must be analyzed in the same run. The number of the dilutions of competitor must be adapted to the requirements of the assay. In DNA assays, three dilutions in a 1 log range can be sufficient,[1,4] whereas for mRNA measurements, sometimes up to five tubes at 10-fold dilutions must be tested to cover the wide range of possible gene expression levels.[10] In a second assay, the range can be restricted for a closer quantification of RNA levels. Finally, an improvement in accuracy of c-PCR can be obtained performing a simultaneous amplification in the same tube of the target gene and a reporter gene.[11]

Competitive PCR for DNA measurements; assay procedure

Some quantitative alterations of DNA, such as the measurement of gene amplification or the study of the replication origin, can be evaluated with the c-PCR

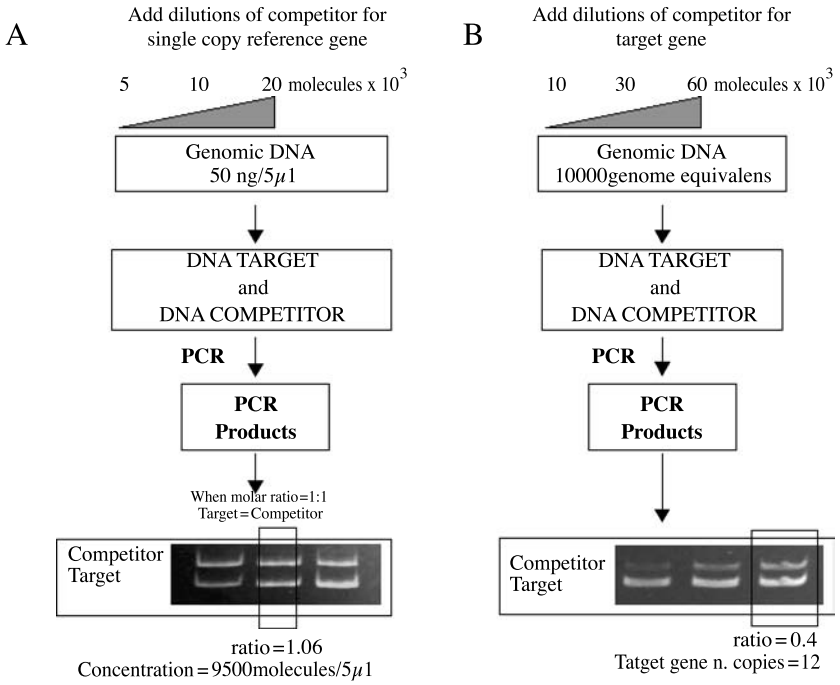

Fig. 2 Example of competitive PCR assay. The assay is performed in two-step (A and B) competitive PCR. In step A, the exact DNA concentration of each samples is accurately measured by reference to a single copy reference gene (i.e., β-globin), while in step B, the exact number of target gene copies was determined. In both PCR steps, we added three dilutions of DNA competitor (single copy reference gene competitor or target gene competitor). The competitor/genomic ratio was evaluated after acrylamide gel electrophoresis of PCR products.

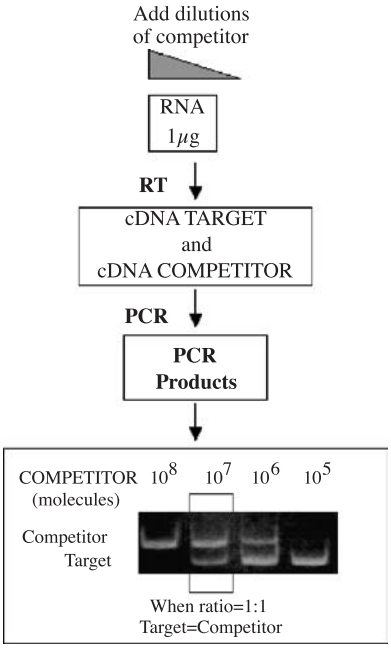

Fig. 3 Example of competitive RT-PCR assay. The assay was performed by the addition of four different dilutions of the specific RNA competitor (from 10^8 to 10^5 molecules) to the unknown sample, prior to the reverse transcriptase reaction (RT). Final quantitation was performed by evaluating competitor/target ratio, as described for DNA measurement.

technology. Because PCR procedures for gene amplification must be able to measure the increase of even a few gene copies, the exact amount of DNA in each sample must be determined more accurately than by spectrophotometric measurement. For this reason, an accurate assay must include a first competitive PCR in which the absolute number of a single copy reference gene is evaluated. This allows the performance of a second competitive PCR in which all samples are measured for gene amplification starting from exactly the same number of DNA molecules.[2–4] An example of the measurement of c-erbB-2 oncogene amplification is reported in Fig. 2. This technique is highly sensitive and allows the measurement in isolated cells obtained by flow sorting,[12] or even in samples with degraded DNA, as in the case of archived samples.[1]

Competitive PCR for RNA measurements; assay procedure

The assay procedure for c-RT-PCR requires the use of an RNA competitor to be added before the reverse transcription according to the scheme reported in Fig. 3. The calculation of the results is the same as for DNA measurements.

SPECIFICITY

Competitive PCR has been developed to overcome the limitations of the traditional PCR methods when used for quantitative measurements of nucleic acids. On the other hand, the PCR approach is maintained and, in particular, the specificity of the assay can be excellent, but, in any case, is related to the primer design and the assay amplification conditions as for the classical PCR methods.

SENSITIVITY

Sensitivity, assumed as the minimal measurable amount of nucleic acids with a reasonable precision, is also excellent and can reach, for DNA gene amplification measurements, also the detection of one gene copy increment. For RNA expression studies, sensitivity is also related to the RT efficiency and to the expression level of the target gene, but single-cell expression studies by c-RT-PCR have been reported.[13]

REPRODUCIBILITY

Coefficient of variation values of 15–20% can be obtained assuming that competitor stability is preserved and quantification of the PCR products is performed with appropriate quantitative devices.[4,14,15]

ROBUSTNESS AND LIMITS

The relative simplicity for the competitor synthesis and the low cost of the final assay make the c-PCR approach as a first choice for quantitative studies of nucleic acids. Stability of RNA competitors, however, can be difficult to maintain. In some cases, the use of a DNA competitor in c-PCR for RNA expression studies has been proposed, but with the limits above reported in ''The Competitor Templates.'' Another important limitation is the high risk of contamination depending on the procedures used for quantification of the PCR products.

CLINICAL APPLICATIONS

The use of c-PCR for DNA measurements has been applied to the study of some quantitative alterations of DNA, such as the measurement of gene amplification[1,2,4] or the study of the replication origin.[16]

The measurement of mRNA molecules with competitive PCR has found wide applications in several fields of clinical laboratory, as for cytokine expression measurement,[17] for the detection of viral nucleic acids for hepatitis C[18] and HIV,[14] and for detection of gene expression in oncology.[19]

CONCLUSION

Competitive PCR is a powerful, versatile, and low-cost technology for quantitative nucleic acid measurements, suitable for both research studies and clinical applications even if some limitations as those above indicated should be carefully evaluated on the basis of the specific experimental design and the available laboratory facilities.

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Corynebacterium diphtheriae—Molecular Detection of Diphtheria Toxin

C

Jennifer K. Spinler
Randall K. Holmes
University of Colorado Health Sciences Center, Denver, Colorado, U.S.A.

INTRODUCTION

Diphtheria is an infectious disease of humans that affects the respiratory tract or skin. It is caused by toxigenic (toxin-producing; *tox*$^+$) isolates of *Corynebacterium diphtheriae* or, rarely, *Corynebacterium ulcerans*.[1,2] A pseudomembrane at the site of infection occurs commonly in respiratory diphtheria and occasionally in cutaneous diphtheria. The major bacterial virulence factor involved in pathogenesis is diphtheria toxin, which blocks protein synthesis and has a lethal dose of <0.1 μg/kg of body weight.[3,4] Diphtheria toxin is encoded by certain corynebacteriophages, and all toxigenic variants of *C. diphtheriae* or *C. ulcerans* are lysogenic for a *tox*$^+$ prophage. Toxin produced locally can spread via the bloodstream and damage vital organs, including the heart, peripheral nerves, kidneys, liver, and adrenal glands.[5] Both toxigenic and nontoxigenic variants of *C. diphtheriae* can cause infections of mucous membranes or skin, but only *tox*$^+$ strains are associated with the harmful manifestations caused by diphtheria toxin. Definitive diagnosis of diphtheria is based on clinical findings compatible with diphtheria plus isolation of *C. diphtheriae* or demonstration of characteristic histopathological findings. Respiratory diphtheria, but not cutaneous diphtheria, is reportable in the United States.

Diphtheria has been nearly eliminated in developed countries by widespread immunization with diphtheria toxoid, an immunogenic but nontoxic derivative of diphtheria toxin.[6] Antibodies raised against diphtheria toxoid by active immunization combine with diphtheria toxin and neutralize its toxic effects. When a clinical diagnosis of diphtheria is made, equine diphtheria antitoxin is administered immediately to the patient as a therapeutic measure without waiting for bacteriological cultures, because delays in administration of antitoxin are associated with greater mortality.[2] Immunization with diphtheria toxoid prevents diphtheria, but it does not eliminate carriage of *tox*$^+$ *C. diphtheriae* in the human population.[7] Diphtheria has the potential to reemerge if immunity is not maintained at a high level in the population. The most devastating recent outbreak of reemergent diphtheria occurred in Russia and the Newly Independent States (NIS) of the former Soviet Union during the early and mid-1990s, resulting in $>150{,}000$ cases and >5000 deaths.[8,9] At least 20 imported cases of diphtheria in adults were diagnosed in Central and Western European countries following exposures that occurred in the epidemic regions.

When *C. diphtheriae* is isolated from a clinical specimen, the most critical issue is to determine rapidly and accurately whether the isolate is *tox*$^+$. This is important to assure that individual patients receive proper treatment, determine if contacts require prophylactic treatment, and assess whether immunization programs or other measures are required to prevent the spread of diphtheria within the population at risk. Toxigenicity testing (originally called virulence testing) on isolates of *C. diphtheriae* can be performed either directly by using bioassays that detect specific toxic effects of diphtheria toxin or indirectly by using molecular methods that detect antigenic determinants of the diphtheria toxin protein or specific nucleotide sequences within the structural gene for diphtheria toxin (*tox*). This chapter summarizes the current status of phenotypic and genotypic tests for diphtheria toxin, with emphasis on test formats that have been used recently by several major diphtheria reference laboratories.

BIOASSAYS FOR DIPHTHERIA TOXIN

Bioassays are based on the ability of diphtheria toxin to cause death, dermonecrosis, inhibition of protein synthesis, or other toxic effects in highly susceptible animals (such as rabbits or guinea pigs) or in cultured cells derived from them. The molecular basis for the toxicity of diphtheria toxin has been well studied (reviewed in Ref. [10]). Diphtheria toxin is secreted by *C. diphtheriae* as a single mature polypeptide of 28,350 Da, containing 535 amino acid residues. Proteolytic nicking of diphtheria toxin and reduction of its two internal disulfide bonds generate fragments A and B. The receptor-binding domain of fragment B binds to the precursor form of a heparin-binding epidermal growth factor-like protein, the receptor for toxin on the surface of susceptible eukaryotic cells. The toxin–receptor complex is endocytosed, and upon

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acidification of the endosome the translocation domain of fragment B undergoes a conformational change, causing it to insert into the endosomal membrane and form a pore through which fragment A is translocated into the cytosol. Fragment A catalyzes the transfer of the adenosine diphosphate ribose moiety from nicotinamide adenine dinucleotide to a posttranslationally modified histidine residue (called diphthamide) on elongation factor 2 (EF-2), thereby inactivating EF-2 and inhibiting protein synthesis. A single molecule of fragment A within the cytoplasm is sufficient to kill a eukaryotic cell.

Because bioassays provide direct measures of toxicity, they have long been accepted as the ''gold standard'' for detecting diphtheria toxin. Because the observed biological endpoints (such as death or dermonecrosis) are not necessarily unique for diphtheria toxin, controls are needed to demonstrate specificity. Typically, duplicate assays are performed in the presence and absence of diphtheria antitoxin, and neutralization of toxicity by antitoxin proves that observed toxic effects are caused by diphtheria toxin. Assays for dermonecrosis following intradermal injections of diphtheria toxin into susceptible animals are more sensitive than tests for lethality following subcutaneous injections, and intradermal tests can detect as little as 50 pg of diphtheria toxin.[11] Several different endpoints have been used to measure toxicity for cultured cells, including cell death, cytotoxicity, inhibition of protein synthesis, etc. Tests on Vero cells in microtiter cultures using a tetrazolium dye to assess cell viability could detect 3 pg of diphtheria toxin per assay, and the results of cell culture assays were in complete concordance with toxigenicity tests performed in animals for a collection of 55 isolates of potentially tox^+ corynebacteria.[12] The advantages of bioassays for diphtheria toxin include high sensitivity and specificity. The disadvantages are that bioassays are labor intensive, costly (especially if rabbits or guinea pigs are required), slow (several days to obtain results), and require facilities for experimental animals or tissue cultures that are not always available in diagnostic laboratories.

IMMUNOASSAYS FOR DIPHTHERIA TOXIN

Immunoassays are based on the ability of specific polyclonal or monoclonal antitoxic antibodies to bind diphtheria toxin. Typically, immunoassays do not distinguish between biologically active forms of diphtheria toxin and biologically inactive forms that retain full or partial antigenicity (such as diphtheria toxoid or partially denatured diphtheria toxin). Immunoassays provide inexpensive and fairly rapid substitutes for bioassays in determining the toxigenicity of clinical isolates of *C. diphtheriae*. Each reference laboratory must perform periodic controls to demonstrate that the results of any particular immunoassay correlate well with the results of standard bioassays for diphtheria toxin.

For many years, the Elek test has been used most commonly in diagnostic laboratories to determine whether isolates of *C. diphtheriae* are tox^+. In Elek tests, a strip of filter paper impregnated with diphtheria antitoxin is applied to the surface of an agar plate containing medium that supports growth of *C. diphtheriae* and production of diphtheria toxin. Isolates of *C. diphtheriae* of unknown toxigenicity are streaked on the surface of the medium at right angles to the strip and parallel to known toxigenic and nontoxigenic control isolates. Toxin produced during bacterial growth and antitoxin from the strip diffuse into the medium, forming precipitin lines where toxin and antitoxin are present at equivalence. Precipitin lines formed by tox^+ unknown and tox^+ control isolates at adjacent positions fuse to give lines of identity. Elek tests are highly reliable when all media, antisera, growth conditions, and bacterial standards are rigidly controlled, but results are likely to be unreliable in laboratories that perform Elek tests infrequently and lack experienced personnel. Incorrect interpretation of nonspecific precipitin lines can produce false-positive results, whereas false-negative results due to low sensitivity of the assay system may occur if test conditions are not carefully controlled. The large epidemic of diphtheria in Russia and the Newly Independent States during the 1990s stimulated interest in developing improved diagnostic tests and increasing the competence of laboratory personnel for identifying *C. diphtheriae* and performing toxigenicity tests. Modifications in procedures for the Elek test improved the reproducibility of results, decreased the amounts of reagents required, and decreased the time required for results from 48 hr to 16–24 hr.[13,14]

Subsequently, a quantitative antigen-capture enzyme immunoassay and a qualitative immunochromatographic strip (ICS) test for diphtheria toxin were developed, both of which offer rapid, sensitive, and specific alternatives to the Elek test for toxigenicity testing.[15,16] The enzyme immunoassay used an equine polyclonal antibody for capture and an alkaline phosphatase-conjugated monoclonal antifragment A antibody for detection of diphtheria toxin. The limit of sensitivity was 0.1 ng of diphtheria toxin/mL, and results available within 3 hr of colony selection agreed uniformly with Elek tests. The ICS test also used equine polyclonal antibody for capture but substituted colloidal gold-labeled monoclonal antifragment A antibody for detection of diphtheria toxin. The limit of sensitivity for the ICS test was 0.5 ng of diphtheria toxin/mL, and results were available within 10 min. Furthermore, when the ICS test was used to compare 850

throat swabs that were inoculated directly into broth for 16 hr or analyzed by conventional culture methods, the concordance for detecting diphtheria toxin by the two methods was 99%, and the sensitivity and specificity of the ICS test for detecting diphtheria toxin were 98% and 99%, respectively. The ICS test has significant advantages over the enzyme immunoassay with respect to ease of test performance, stability of reagents, and documented ability to detect diphtheria toxin production within 16 hr from initial collection of a throat swab from a patient with suspected diphtheria.

GENOTYPIC TESTS FOR DIPHTHERIA TOXIN

A genotypic test is designed to detect the nucleotide sequence for a particular gene. Unlike an immunoassay for a protein, a genotypic test does not require that the gene product be produced. Therefore a genotypic test for *tox* is one step more remote from a bioassay for diphtheria toxin than an immunoassay. Because many tests for specific DNA sequences have high sensitivity, it is possible to develop tests for *tox* that will have few false-negative results. However, the converse is not true, because nontoxigenic isolates of *C. diphtheriae* may contain an inactive allele, a single fragment, or multiple fragments of *tox*. In such isolates, a positive test for the *tox* gene would not agree with the negative result of a bioassay for diphtheria toxin.

Several investigators developed polymerase chain reaction (PCR) tests to detect *tox* in DNA isolated from pure cultures of *C. diphtheriae*.[17,18] The presence of nucleic acid sequences encoding the A fragment of diphtheria toxin agreed well with positive Elek tests for diphtheria toxin production, and the sensitivity of the PCR tests was approximately 50–500 colony forming units (CFU) of a tox^+ isolate of *C. diphtheriae*. A PCR test for detecting *tox* in DNA isolated directly from throat or nasopharyngeal swabs was also developed. It eliminated the time required to culture *C. diphtheriae* and gave results within hours of collecting the initial clinical specimens.[19] Subsequently, a real-time fluorescence PCR assay decreased the limit of detection for *tox* to approximately 2 CFU/sample.[20] This real-time PCR test detected the coding regions for diphtheria toxin fragment A and fragment B in all of 23 tox^+ clinical isolates and 2 tox^+ reference strains of *C. diphtheriae*, and it was superior to routine PCR tests in detecting *tox* in DNA extracted directly from clinical specimens.

The events in Russia and the Newly Independent States during the 1990s provided an opportunity to apply molecular epidemiological methods to an evolving epidemic of diphtheria and showed that most patients were infected by clonally related *C. diphtheriae* isolates that were not highly prevalent before the epidemic.[21] Use of single-stranded conformation polymorphism and direct DNA sequencing methods demonstrated allelic variations in *tox* among clinical isolates of *C. diphtheriae*, but all mutations in *tox* were silent and did not change the amino acid sequence of diphtheria toxin.[22] Therefore the epidemic was not caused by evolution of *C. diphtheriae* strains that produced a variant of diphtheria toxin resistant to neutralization by antitoxic antibodies, and intensive immunization with diphtheria toxoid succeeded in bringing the epidemic under control by the late 1990s.[23]

CONCLUSION

The most important test for new isolates of *C. diphtheriae* is to determine if they are toxigenic, which has important implications for patient care and public health. The ideal test should be simple, sensitive, rapid, reliable, and inexpensive, and it should also correlate well with the biological activity of diphtheria toxin. Bioassays performed in susceptible animals or cultured cells are the gold standard, but unfortunately they are slow, complicated, and costly. Molecular methods based on immunochemical properties of diphtheria toxin or nucleotide sequences of the toxin structural gene serve as surrogates for bioassays and provide indirect assessments for potential toxigenicity of *C. diphtheriae* isolates. Elek tests, based on formation of toxin–antitoxin precipitates in agar during growth of tox^+ isolates of *C. diphtheriae*, require rigidly controlled execution by skilled personnel, although recent modifications have improved their speed and cost-effectiveness. An immunochromatographic strip assay for diphtheria toxin offers great advantages in sensitivity, speed, and applicability for direct use on clinical specimens. Polymerase chain reaction-based methods to detect specific segments of the *tox* gene have been optimized for speed, sensitivity, and reliability, but they do not assess the ability of *C. diphtheriae* to produce the diphtheria toxin protein. Because some isolates of *C. diphtheriae* contain all or part of the toxin gene but do not produce diphtheria toxin, some positive PCR results may lead to false-positive identification of tox^+ *C. diphtheriae* isolates unless bioassays are also performed.

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Cowden Syndrome

Deborah J. Marsh
University of Sydney, Australia

Roberto T. Zori
University of Florida, Gainesville, Florida, U.S.A.

INTRODUCTION

Cowden syndrome (CS, OMIM#158350) can be grouped along with Bannayan–Riley–Ruvalcaba syndrome (BRR, OMIM #153480), Peutz Jeghers syndrome (PJS, OMIM #175200), and juvenile polyposis syndrome (JPS, OMIM# 174900) as one of the autosomal dominant hamartoma syndromes. The estimated incidence of CS is >1:200,000.[1] It is characterized by the appearance of hamartomas (benign developmentally disorganized tumor-like tissue overgrowths) in various organs as well as an increased risk of developing malignancies.

PTEN (*P*hosphatase and *TEN*sin homolog mutated on chromosome *TEN*; location 10q23.3) was identified in 1997 and has alternatively been named *MMAC1* or *TEP1*.[2] *PTEN* encodes a dual specificity phosphatase with homology to the cytoskeletal proteins tensin and auxillin. Using the criteria set by the International Cowden Syndrome Consortium[3] up to 80% of individuals with CS have been shown to have a germline *PTEN* mutation.[4] Genetic scanning in at-risk or symptomatic individuals is available for this syndrome.

CLINICAL DESCRIPTION

Presentation

Cowden syndrome displays a high level of variability in expression, both between and within families; however, it usually presents in the third decade. Hamartomas are derived from all three germ cell layers. Those of the inner organs most commonly involve the breast (70%); thyroid adenomas and multinodular goitres (40–60%); gastrointestinal polyps (35–40%) and can also be seen in the central nervous system and; reproductive tract. The gastrointestinal polyps seen in CS are distinct from those seen in other hamartomatous syndromes, as they are more likely to be of ectodermal and/or endodermal origin.[5] Dermatological manifestations are the most prevalent findings, with palmoplantar hyperkeratosis in 54%, trichilemmonmas (benign tumors of the hair follicle infundibulum) in 99%, and oral papillomatosis in 85% of CS patients (reviewed in Refs. [6,7]). The last two dermatological findings are considered to be the hallmarks of CS. The central nervous system may be the second most common organ system involved in CS, and includes megencephaly or macrocephaly seen in up to 80% of patients and Lhermitte–Duclos disease (LDD).

In addition to these benign findings, patients have an elevated risk for malignant tumors, most commonly in the breast (25–50% in females, and a small percentage of males) and thyroid, especially follicular thyroid carcinoma (3–10%). Cancer has also been reported in CS patients in the brain, gastrointestinal tract, urogenital tract, and skin, but whether there is a true elevated risk in these systems is less well documented.

Cowden syndrome shows partial clinical overlap with the overgrowth disorder BRR syndrome, with intestinal hamartomatous polyps, macrocephaly, and lipomas being seen in both conditions, as well as germline *PTEN* mutation. A number of families described as ''overlap'' families have been reported in which both CS and BRR segregate with the identical germline *PTEN* mutation.[9,10] Bannayan–Riley–Ruvalcaba and Cowden syndromes have therefore been considered to be phenotypic variants of the same condition and have been referred to as the ''PTEN hamartoma–tumor syndrome'' (PHTS).[10] The presence of other genetic and/or epigenetic factors are highly likely to play a role in the determination of phenotype in these conditions.

Clinical Diagnostic Criteria for Cowden Syndrome

The International CS consortium has developed diagnostic criteria for this condition.[3,11] The criterion is less sensitive in children and young adults as findings appear generally in the third decade. Studies of patients who only partially meet these criteria, 'CS-like', or who have multiple primary cancers, have yielded very few germline *PTEN* mutations, thus providing indirect evidence of the usefulness of these criteria.[12,13]

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Clinical Management of Cowden Syndrome

Clinical management of a patient with CS requires comprehensive serial screening with regular follow-up for breast, endometrial, thyroid, renal, and gastrointestinal involvement. Early onset breast cancer is seen in women with CS, with one study reporting an average age of 43.2 years at the time of surgery.[14] Thus self-examination in early adulthood and annual mammography from age 30 years onward is suggested. Annual endometrial examination from age 35 years with suction biopsies and transvaginal ultrasound with biopsy of suspicious areas is recommended. Annual physical examination of the thyroid is recommended from age 18 years, and thyroid ultrasound may be conducted every 1–2 years. Annual urine analysis in adulthood to screen for hematuria secondary to renal cancer is suggested. Annual hemoglobin screening from childhood is reasonable to guard against anemia due to bleeding from gastrointestinal polyps. It is recommend that CS patients undergo a baseline colonoscopy at age 50 years, earlier if symptoms are present, in order to detect hamartomatous polyps [Refs. [5] and [11], and in GeneReviews, Pilarski, Hample, and Eng: *PTEN* Hamartoma-Tumor Syndrome (PHTS), http://www.geneclinics.org].

MOLECULAR DESCRIPTION

PTEN—The Gene and Protein

PTEN consists of nine exons and an open reading frame of 1209 nucleotides encoding a 403 amino acid dual-specificity phosphatase with homology to the cytoskeletal proteins tensin and auxillin. Exon 5 of *PTEN* encodes a classic phosphatase core motif (I/V)HC*XX*G*XX*R(S/T)G at residues 122–132. Three potential tyrosine phosphorylation sites at residues 240, 315, and 336, as well as two potential serine phosphorylation sites at residues 335 and 338, are located in the COOH-terminus. A PDZ binding domain, important for subcellular localization and/or substrate interactions, is encoded by the last four amino acids, ITKV (Fig. 1).

The major endogenous substrate of PTEN is a phospholipid second messenger in the phosphotidylinositol 3-kinase (PI3-K) pathway called phosphatidylinositol 3,4,5-trisphosphate [Ptd-Ins(3,4,5)P_3]. Growth factors such as insulin and epidermal growth factor stimulate PI3-kinase to phosphorylate phosphatidylinositol 4,5-*tris*-phosphate [Ptd-Ins(4,5)P_2] producing Ptd-Ins(3,4,5)P_3. Thus PTEN acts as a 3-phosphatase and antagonist to this process, dephosphorylating Ptd-Ins(3,4,5)P_3 to Ptd-Ins(4,5)P_2. In syndromes such as CS where *PTEN* is mutant, Ptd-Ins(3,4,5)P_3 accumulates, activating the cell survival (antiapoptotic) factor serine–threonine kinase AKT (protein kinase B, PKB). PTEN would therefore seem to act via an apoptotic mechanism, as well as being able to cause cell cycle arrest in cells in G1 phase, possibly via the retinoblastoma protein. PTEN is also likely to have a role in cell migration and invasion as it has been shown to reduce tyrosine phosphorylation of focal adhesion kinase (FAK) (reviewed in Ref. [6]).

PTEN has a processed pseudogene located on chromosome band 9p21 that lacks the initiating methionine, but has greater than 98% homology with the *PTEN* coding region.[15] Further, the 5′ untranslated region of *PTEN* contains multiple CpG islands that are potentially epigenetically regulated by methylation; however, care must also be taken in any methylation studies as the *PTEN* pseudogene also extends into this region.[16]

Identified *PTEN* Mutations

The Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html) has recorded greater than 110 different *PTEN* mutations. In addition to being mutated in the germline of patients with CS and BRR,

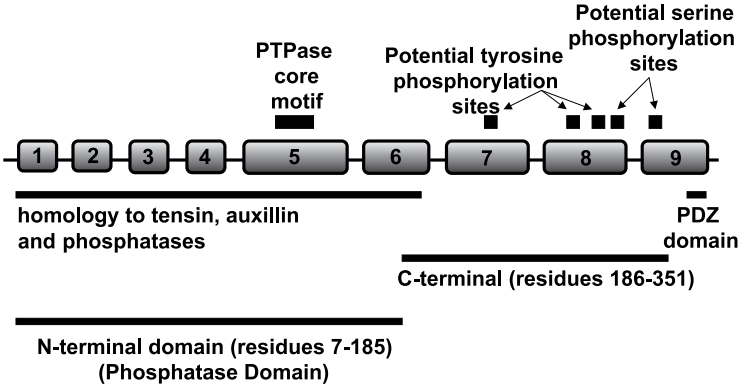

Fig. 1 Schematic of the functional domains encoded by *PTEN*.

constitutive *PTEN* mutation has also been identified in another overgrowth disorder, Proteus syndrome (PS, OMIM #176920). Further, *PTEN* has been described as ''. . .the most highly mutated tumor-suppressor gene in the post-p53 era. . .,''[17] being somatically mutated in a large number of sporadic tumors. Large-scale deletion of the entire *PTEN* gene and flanking region is rarely never reported in classic CS; however, large deletion is seen at a low frequency in both BRR and CS/BRR overlap families.[10,18] *PTEN* mutations have been found to cluster in exons 5, 7, and 8, with 40% of mutations being found in exon 5, which is the largest exon, constituting 20% of the *PTEN* coding region, but also contains the protein tyrosine phosphatase (PTPase) core motif. Recently, some patients with classic CS have been found to have heterozygous germline mutations in the *PTEN* promoter.[18] Some *PTEN* mutations have been shown to lead to loss of phosphatase activity against both protein and phospholipid substrates, whereas others, e.g., G129E, lose lipid phosphatase activity, while retaining phosphatase activity against nonphospholipid substrates.

Genotype–Phenotype Correlations in Cowden Syndrome

PTEN mutations have also been found in up to 60% of patients with BRR[10] and in some patients with hamartomas that carried the diagnosis of other conditions, namely, LDD,[19] PS or PS-like syndromes,[20] and VATER association Proteus.[21] Identical, as well as distinct, *PTEN* mutations have been found in all of these syndromes.

Preliminary genotype–phenotype correlations have been identified in CS. Firstly, in CS families (or CS/BRR overlap families), the presence of a *PTEN* mutation has been correlated with the presence of any cancer, lipomas, or breast fibroadenoma.[10] An association between the presence of a *PTEN* mutation and breast involvement, whether it be benign or malignant disease, has also been observed.[10] A study of a large number of CS families representing multiple ethnicities is required in order to confirm these findings.

Genetic Screening

Who should undergo genetic screening for Cowden syndrome?

Genetic counseling and predictive mutation testing should be offered to all at-risk members of a family with CS as part of their clinical management. As CS is an autosomal dominant condition, children of an affected parent have a 50% chance of inheriting a *PTEN* mutation. Cancer surveillance is indicated only for patients with a germline *PTEN* mutation.

Standard molecular techniques used for *PTEN* mutation scanning

Mutation scanning of genomic DNA using primers designed for intronic regions flanking *PTEN* exons has been employed for standard sequencing of *PTEN*, as well as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), and denaturing high-performance liquid chromatography (DHPLC). One study achieved greater sensitivity of DHPLC for *PTEN* mutation detection by the addition of gc-clamps identical to those used for DGGE.[22]

Because of the presence of the highly homologous *PTEN* pseudogene, any studies conducted using *PTEN* cDNA should exclude amplification of the pseudogene by specific primer design. The recent finding of *PTEN* promoter variants suggests that the promoter region should be considered for analysis in classic CS cases where a *PTEN* mutation cannot be found in the coding, or immediately flanking, regions.[18]

CONCLUSION

Cowden syndrome is a multiorgan system disorder, requiring focused clinical surveillance and regular follow-up. Genetic screening to identify germline *PTEN* mutations is now available and can direct the clinical management of this condition, including family-planning decisions. A number of molecules in the PI3-K pathway in which PTEN functions are currently attracting attention as potential drug targets that may influence future therapies for patients with germline *PTEN* mutation, as well as those with sporadic cancers shown to have *PTEN* involvement.

Online information specifically for patients with CS is available (Burton S, Haidle JL, Hampel H and Eng C: Cowden syndrome. A guide for patients and their families, http://www.vh.org/pediatric/patient/cancercenter/cowden/). Additional detailed reviews recommended for further reading on this subject include Refs. [23,24].

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Creutzfeldt–Jacob Disease M129V Polymorphism

C

Thomas R. Kreil
Hans Peter Schwarz
Friedrich Dorner
Baxter BioScience, Vienna, Austria

INTRODUCTION

The prion protein gene (*PRNP*) encodes the primary structure of a cellular protein, the prion protein (PrP). If present in a partially protease resistant isoform, caused by either mutations in the *PRNP* or an infectious process believed to involve template-directed misfolding of the PrP, the same protein can display the puzzling characteristics of a unique, presumably protein-only, infectious agent. Creutzfeldt–Jakob disease (CJD) and kuru are human transmissible spongiform encephalopathy diseases characterized by the deposition of this aberrant form of the PrP. Susceptibility to accumulation of the pathological isoform of the protein, initiated through either spontaneous or inherited *PRNP* mutation, or exposure to the infectious agent through iatrogenic or alimentary routes, as well as severity and course of disease development are influenced or even determined by the M129V polymorphism of the prion protein gene. For certain applications, it may be possible to exploit this observation for diagnostic purposes to enhance the safety of particular biological medicinal products, such as corneal grafts.

THE PRION PROTEIN GENE, *PRNP*

The human *PRNP* is located on the short arm of chromosome 20 and contains a short 5′ exon, followed by a large intron and then a 3′ exon, which encodes the entire 253 amino acids of the open reading frame of the precursor PrP.[1] Within the gene, three polymorphic regions—i.e., a single octapeptide deletion, M129V, and E219K—have been described, as well as a growing number of spontaneous or inherited mutations associated with the development of CJD.[2]

Investigations of sporadic CJD cases and people infected with CJD through the administration of human cadaveric pituitary gland hormones, such as growth hormone or gonadotropin, have revealed that the codon 129 M/V polymorphism influences susceptibility to disease as a consequence of both mutations within *PRNP*[3] and exposure to infectious material.[4] The major determinant of risk for iatrogenic and sporadic CJD seems to be homozygosity at codon 129.[5]

The same polymorphism can also determine the phenotype of disease as exemplified by the D178N PRNP mutation, which results in fatal familial insomnia (FFI) if M129 is present on the same allele, and in CJD if V129 is present on the same allele, with an additional correlation between codon 129 homozygosity and disease severity in both instances.

Variant CJD (vCJD), a new prion disease first recognized in 1996, is presumed to be caused by oral infection of humans with the bovine spongiform encephalopathy (BSE) agent. To date, approximately 140 cases have been diagnosed, principally in the United Kingdom, where the majority of BSE cases have occurred. All the cases investigated were codon 129 M/M homozygous, which indicates that either other genotypes are not susceptible, or their incubation periods are significantly longer. Experience with kuru, which—as far as we know—is the only other human prion disease acquired orally and was transmitted through ritualistic cannibalism among the Fore people of Papua New Guinea, supports the latter. In the later stages of the kuru epidemic, people carrying the most susceptible codon 129 M/M genotype had practically been depleted from the Fore survivors, and those who were heterozygous or carried the V/V genotype only developed the disease after extremely long incubation periods of 20 years or more.[6]

Determinations of the codon 129 genotype, as typically performed via oligonucleotide hybridization, or recently by a more simple and cost-effective polymerase chain reaction/restriction digestion assay,[7] have revealed approximately 50% heterozygosity, approximately 40% M/M homozygosity, and only 10% V/V homozygosity for healthy Caucasian populations.[7,4] In marked contrast, only around 8% of the Japanese population is heterozygous at codon 129.[8]

THE PRION PROTEIN, PRP

The primary translation product of *PRNP* contains signal peptides of 22 or 23 amino acids at the N- and C-termini,

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respectively, which are both removed during intracellular processing of the nascent PrP. The mature PrP 23–230 is membrane-anchored through a C-terminal glycosyl–phosphatidyl–inositol (GPI) anchor unit, and two asparagine glycosylation sites within the protein can give rise to three glycotypes (unglycosylated, monoglycosylated, and diglycosylated). Structurally, the protein features a fivefold repeat of eight amino acids each (octa-repeats) toward the N-terminus, and a globular domain that contains a short, two-stranded antiparallel beta sheet and three alpha helices in the C-terminal half.[9]

According to the protein-only hypothesis, the hallmark of the infectious process for acquired (iatrogenic or foodborne), sporadic, and inherited prion diseases is recruitment of the cellular PrP to form a self-replicating conformational PrP isomer, ultimately accumulating into aggregated deposits. As soon as this conversion is initiated, different prion strains replicate faithfully upon transmission into new hosts.

Because most available anti-PrP reagents recognize the normal and the abnormal isoform of the PrP, digestion with proteinase K is experimentally used to completely degrade the normal isoform. The same treatment results in a protease-resistant core with an apparent molecular mass of 27–30 kDa (PrP 27–30) for the pathological isoform.[10]

IATROGENIC TRANSMISSION OF CREUTZFELDT–JAKOB DISEASE

Over the last three decades, approximately 270 cases of iatrogenic CJD have been observed.[11] By far, the most frequently involved medical interventions were the surgical use of dura mater grafts (114 cases) and treatment with pituitary-derived human growth hormone (139 cases). In a few cases each, treatment with pituitary-derived gonadotropin, corneal transplantation, or the use of neurosurgical equipment such as stereotactic electrodes was implicated.

With the advent of vCJD, the presence of the pathological PrP and infectivity in lymphoreticular organs such as the tonsils and spleen,[12,13] which are in intimate contact with the circulation, generated concerns about the safety of blood products. Although the concentration of the pathological PrP is higher in the organs of vCJD patients than those with CJD, it needs to be emphasized that infectivity of these tissues is demonstrable in both diseases, and the relative levels have not been determined. For CJD, however, epidemiological data and case studies have provided strong evidence for the absence of any risk for the safety of blood products. For vCJD, neither the pathological PrP nor infectivity has been observed in human blood or plasma despite intensive research,[12,13] and accumulating clinical evidence argues that ''. . . the risk associated with vCJD and BSE is not yet demonstrably worse than the risk from non-vCJD forms of disease, which has been shown to be negligible.''[14] For plasma derivatives, in particular, their manufacturing processes have been shown to be capable of removing significant amounts of prion protein/infectivity in downscaled models,[15–17] should its presence ever be determined in plasma. Taken together, the vCJD risk for blood products can be considered very low as a worst-case scenario, and merely hypothetical for plasma derivatives.

DIAGNOSTIC POTENTIAL OF THE M129V POLYMORPHISM

Although all vCJD cases investigated so far have been found to be M/M homozygous at codon 129, the deferral of such potentially higher risk donors from blood or plasma donation is not feasible based on the already strained supply of these products and the large proportion of the population displaying this genotype. Also, any risk associated with the use of these products remains purely hypothetical at this point, and again there is mounting evidence against any risk being associated with their use.[14] However, for products carrying an identified level of risk and are needed in lower quantities, exclusion based on an identified genetic predisposition for disease development might be an option. Human growth hormone from cadaveric pituitary glands, a product that has historically led to many CJD transmissions, has already been replaced by recombinant versions of the product. Dura mater, another of the products implicated earlier,[11] is no longer in clinical use and has been widely replaced by synthetic fleece or collagen-based products. Corneal transplants have also transmitted CJD,[11] but are still being used because of their unique clinical characteristics. Selection of potential donors based on their PRNP codon 129 polymorphism to exclude homozygous donors who are at the relatively highest risk of developing CJD could add a margin of safety to the use of this product. However, despite the ample number of potential donors, cornea transplants have been in short supply. With the positive results of recent HLA matching studies and a consequent change in medical practice, the supply situation for HLA-matched corneas has been further strained.[18] Against an extremely rare event, losing half the supply of corneas by exclusion of homozygous donors to gain a slight margin

of safety might not seem appropriate. Alternatively, consideration could be given to more effective sourcing strategies, which might allow reassessment of this additional safety measure.

OUTLOOK FOR VCJD

Early estimates predicted that the vCJD epidemic could involve up to 100,000 cases,[19] but these predictions have been revised based on vCJD deaths to date to a maximum of about 500 cases.[20]

Statistical extrapolations from the monthly vCJD statistics for the United Kingdom indicate that the epidemic is already on the decline, at least for people with codon 129 M/M. If experience with the other orally acquired prion disease of humans, i.e., kuru, is predictive, any potential second wave of the vCJD epidemic in other genotypes would be smaller than the proportionate genotype distribution, because not only can the incubation periods be expected to be longer but the susceptibility to disease will be lower as well.[6] These additional cases in codon 129 heterozygous or V/V homozygous people can consequently be expected to occur in the older population corresponding to the longer incubation periods. Although it is not known whether the genotype of those who are affected later in the epidemic would alter the phenotype of vCJD, observations of kuru victims suggest that this will not be the case,[21] and thus the effective surveillance programs now in place would be expected to recognize any second wave of the vCJD epidemic.

CONCLUSION

Certain aspects of prion diseases remain puzzling, even after the Gajdusek 1976 and Prusiner 1997 Nobel Prizes. From a practical perspective, however, light has been shed on many important aspects, such as the diagnosis of disease and risk of transmission by medically important routes.

For growth hormone and gonadotropin, recombinant alternatives have been substituted for earlier versions from cadaveric pituitary glands. Dura mater grafts are no longer in clinical use, having mainly been replaced by synthetic tissues or collagen-based products. The BSE epidemic has been contained, and as a consequence of all the food protective measures, it now appears that the corresponding human disease, vCJD, is also on the decline. Regarding the initial vCJD concern in relation to blood products, available evidence converges to indicate a very low or even absent risk. This is even more apparent for plasma derivatives, which enjoy additional safety margins because of the fortuitous capacity of manufacturing processes to remove any theoretically present prion infectivity. The only identifiable—but very small—risk that remains is for corneal grafts, and genotyping to identify low-risk donors could become an option to enhance product safety as soon as an adequate supply of suitable organs has been established.

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Cryptosporidium parvum

Rebecca A. Guy
Paul A. Horgen
University of Toronto, Mississauga, Ontario, Canada

INTRODUCTION

Cryptosporidium parvum is a protozoan parasite that causes gastrointestinal disease. Infections in humans are usually acute and self-limiting, but can be life-threatening causing debilitating diarrheal disease with high morbidity and mortality rates in malnourished children in developing countries and in immunocompromised patients. *Cryptosporidium* was first documented in mice by Tyzzer in 1907. It was not until 1976 when the first human cases were recognized. Despite the recent recognition of this parasite as a human pathogen, *Cryptosporidium* has in fact a long historical association with humans as evidenced by its discovery in coprolites from ancient Andeans and Peruvians dating back 1000 to 3000 years. Two events helped to establish *Cryptosporidium* as a serious pathogen in humans: the increased diagnosis of *Cryptosporidium* in AIDS patients in the 1980s and the major waterborne outbreak in the city of Milwaukee (United States) in 1993. An estimated 403,000 individuals became infected with *Cryptosporidium* in the Milwaukee outbreak at a cost of $96.2 million in medical expenses and lost productivity. Sporadic waterborne outbreaks continue to occur globally, but on a smaller scale than the Milwaukee outbreak. A recent outbreak in North Battleford (Canada) in 2001 affected over 5000 individuals. Several factors including the ease of dissemination of *Cryptosporidium* and difficulties in monitoring in the environment led to its placement on the Center for Disease Control's B list of biological agents for use in bioterrorism.

The increase in cases and massive waterborne outbreak in Milwaukee spurred a rapid growth in research toward developing effective treatment regimes, methods of detection and inactivation of the parasite in the drinking water supply, genotyping, and understanding the interaction of *C. parvum* with its host. Research efforts have been hampered by the inability of the parasite to complete the entire life cycle in in vitro culture, and by the failure to cryopreserve these organisms or to genetically transform any of its stages. Advances in elucidating the genome will undoubtedly lead to new methods of detection, prevention of infection through novel inactivation strategies, successful drug therapy, and vaccine development.

BIOLOGY OF *C. PARVUM*

Classification

Cryptosporidium is a single-cell organism in the Phylum Apicomplexa. The taxonomy of *Cryptosporidium* is confused and is undergoing revision.[1] Recently, the species *C. parvum* was split in two based on the two major genotypes: *C. parvum* genotype 1 occurs almost exclusively in humans and has been named *Cryptosporidium hominis*;[2] *C. parvum* genotype 2, the bovine genotype, infects both humans and other animals. While *C. parvum* and *C. hominis* are the most commonly associated species of *Cryptosporidium* linked to human disease, several other species including *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium canis*, and *Cryptosporidium muris* infect humans.[1] Recent information on protein and gene structure demonstrate that *Cryptosporidium* is more closely related to the early branching gregarines (such as *Monocystis* and *Gregarina* spp.) than to other members of the Apicomplexa such as *Toxoplasma* and *Plasmodium*, as was originally thought.[3]

Life Cycle

C. parvum has a complex, multistage life cycle, consisting of both asexual and sexual stages within one host.[4–6] The complete cycle is intracellular within epithelial cells. Infection is initiated through ingestion of the sporulated oocyst. Within the small intestine of the host, the oocyst ruptures releasing four sporozoites. Sporozoites rapidly penetrate the plasma membrane of epithelial cells lining the gut and undergo a series of cycles of asexual replication. The eventual production of sexual forms of the parasite leads to the development of sporulated oocysts. Two forms of oocysts are produced. Thick-walled oocysts are hardy, withstanding harsh environmental

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factors until ingested by a new host. Thin-walled oocysts can rupture within the host, leading to autoinfection.[4,6]

Location in Host

Infection is most commonly localized in the lower small intestine; however, *Cryptosporidium* has been found throughout the digestive tract including the pharynx, esophagus, and stomach; in the uterus, urinary bladder, hepatobiliary, and pancreatic systems; as well as in the conjunctiva and upper and lower respiratory tracts. Biliary cryptosporidiosis is the most common extraintestinal manifestation of infection.[4]

CLINICAL DESCRIPTION OF INFECTION

Disease in human hosts greatly depends on the condition of the host, with more severe outcomes in immunocompromised and malnourished individuals.[4] Most patients present profuse watery diarrhea containing mucus, 7–10 days after acquisition of infection. In immunocompetent hosts, the disease is usually self-limiting with a median duration of 9–15 days. Acute diarrhea may be resolved in a few days or may persist for 4–7 weeks. Three major presentations of symptoms are observed: 1) asymptomatic carriage, 2) acute, usually watery diarrhea, and 3) chronic, persistent diarrhea for several weeks. Other symptoms include abdominal cramps, anorexia, nausea and vomiting, fatigue, low-grade fever, and cough. A different picture is seen in immunocompromised patients. Four clinical patterns of infection are observed in AIDS patients with chronic infection as the most common presentation: 1) asymptomatic, 2) transient infection with diarrhea cleared within 2 months, 3) chronic infection, diarrhea for 2 or more months, and 4) fulminant infection with patients passing 2 or more liters of watery stool daily.[4]

TRANSMISSION

The parasite is transmitted through the fecal/oral route by ingestion of oocysts from water, food, or direct person-to-person or animal-to-person contact.[1] Water is the main route of transmission, including drinking water, wells, surface and ground water, rivers, lakes, and swimming pools.[7] Seepage of effluents from humans and cattle into drinking water supplies are the major risk factors for humans. The oocyst wall is highly resistant to environmental stresses. Oocysts can persist for up to 6 months in water and for 2–3 months in soil. Conventional chlorine inactivation strategies used in water treatment utilities are inefficient against this pathogen. Greater success in disinfection is obtained with combined disinfection strategies, such as successive ozone and chlorine treatments[8] or ultraviolet light.[7]

PREVALENCE

C. parvum (*C. hominis*) is distributed worldwide. Prevalence rates in the human population range from 1% to 2% in developed countries and 6% to 60% in developing countries. *C. parvum* is a zoonotic disease, and numerous animals have been shown to carry infections.[9] The greatest potential for zoonotic transmission to humans is from cattle. Estimates of the frequencies of contamination of surface waters with *Cryptosporidium* are from 20% to 96% in North America. Their levels in surface waters are very low, ranging from 0.5 to 5000 organisms in 100 L water.[7] However, these low levels are still a risk for infection as it has been estimated that as few as 10 oocysts can establish infection.[9]

MANAGEMENT

No generalized therapeutic agents are available for treatment of *C. parvum* infection. Administration of oral or intravenous fluids and electrolyte replacement can be used as supportive care until the infection is resolved.

Chemotherapy

The slow advance in therapeutics is attributable to the lack of knowledge of the parasite and its isolated location within the apical membrane of the epithelial host cell, limiting drug access to the parasite.[5] Numerous drugs have been tested for therapeutic effects and none effectively eliminate the parasite. Until recently, paromomycin was the most widely used drug for treatment of *Cryptosporidium*. Nitazoxanide (Alinia) was the first drug to be approved by the Food and Drug Administration (FDA) for treatment against *Cryptosporidium* infection. Its use is currently limited to children between the ages of 1 and 11, with studies in progress to determine safety and efficacy in AIDS patients. Nitazoxanide is available in North and South American and is awaiting approval in Europe.

New genetic information and a better understanding of the intracellular parasite–host interaction will provide clues to novel targets for drug development. Some promising new areas of drug research, targeting biosynthetic pathways and proteins of *C. parvum*, include the shikimate pathway (for synthesis of aromatic compounds, amino acids, ubiquinone compounds, and folate), polyamine biosynthesis, fatty acid synthesis, antitubulin agents, and dihydrofolate reductase (DHFR) inhibitors.[5] In addition to development of new drug targets, the

growing information on the genome will speed testing of existing drugs with established limits of safety in humans. For example, anticancer drugs based on farnesyltransferase inhibitors (FTI), which disrupt protein prenylation, have action against protozoa. Screening of the *C. parvum* genome databases has revealed that this parasite contains protein prenyltransferases and may be a target for FTI inhibition.[10] Improved assays for testing the efficacy of drug action, such as real-time polymerase chain reaction (qPCR) for quantification of live parasites following drug treatment in vitro[11] will undoubtedly accelerate drug development.

Immunotherapy

Immune responses to infection are vital as evidenced by the fact that healthy, immunocompetent individuals resolve infection. An interesting correlation between administration of highly active antiretroviral therapy (HAART) and a decrease in cryptosporidiosis in AIDS patients has been observed. HAART does not directly affect the parasite but exerts its effects through enhancing the immune system of these patients. A picture is slowly emerging of the immune responses during infection. $CD4^+$ T cells play an important role as well as $CD8^+$ (α/β and γ/δ) intraepithelial T cells. Cytokines involved in clearance include INFγ, IL-12, and IL-15.[12,13]

Studies using in vitro and in vivo models demonstrate varying degrees of protection from infection upon administration of polyclonal or monoclonal antibodies that recognize parasite antigens. Targets for antigen selection have been based to a large part on antigenic molecules that are involved in adhesion and entry into the host cell including CSL, gp60/40/15, CP47, P43. Antibodies may also have application in treatment of persistent cryptosporidiosis.[13]

Pathology

Diarrhea during *Cryptosporidium* infection is associated with intestinal malabsorption and enhanced secretion. Infection can lead to blunting of intestinal villi, hyperplasia of crypt cells, and enterocyte brush border damage incurred during parasite invasion.[4] Diarrhea may be a result of increased secretion as a result of ion imbalance following responses to secreted cytokines such TNFα, INFγ, IL-8, and substance P.[12] The list of parasite molecules that may act as specific virulence factors during infection is growing[14] (Table 1). In contrast, protective host cell responses such as host cell-triggered apoptosis and secretion of TGF-β1 and IL-10 may limit epithelial cell damage.[4,12,14]

MOLECULAR CHARACTERIZATION

Genotypes

PCR/RFLP and DNA sequence analysis targeting numerous genes (small subunit ribosomal RNA, adjacent internal transcribed spacer 1, heat shock protein 70, dihydrofolate reductase, and β-tubulin) have been used to analyze variation between genotypes of *Cryptosporidium*.[1] At least eight genotypes of *C. parvum* have been identified.[3] Application of techniques such as microsatellite length polymorphisms, phage display, and strand conformation polymorphism analysis will be invaluable for detecting subtler differences in the population. This

Table 1 Potential virulence factors of *C. parvum*

Putative function	Putative virulence factor	Cellular location
Adhesion	Circumsporozoite-like (CSL)	Apical complex
	Gp60/40/15	Apical complex
	Gp900	Micronemes
	Cp47	Apical complex
	CPS-500	Pellicule
Adhesion, locomotion	p23	Surface
	Trap C-1	Micronemes
Membrane disruption	Haemolysin H4	
Transport	ATP-binding cassette	
Protease	Cysteine protease	Surface
	Serine protease	
	Aminopeptidase	Surface
Multiaction	Heat-shock protein HSP70	
	Heat-shock protein HSP90	

Source: Ref. [14].

will enable tracking of isolates during outbreak situations.[15]

Little is known about the infection characteristics of different species and genotypes in humans and no clear clinical symptoms have been defined for any of the genotypes. New studies in humans and animal models are starting to shed light on the infection characteristics of the different *C. parvum* and *C. hominis* genotypes.[1,15]

Genome

The *Cryptosporidium* genome is ~10.4 Mb, contained in eight chromosomes ranging in size from 0.9 to 1.54 Mb. The *C. parvum* genome contains ~5000 genes, very few introns, less than 1% repeat sequences, and is 68% and 65% AT-rich, for genotypes 1 and 2, respectively.[3,15] Two genome projects, close to completion, are sequencing the genomes from two commonly used isolates of *Cryptosporidium*: strain H representing the human genotype 1 (*C. hominis*) and the Iowa strain representing the bovine genotype 2 (*C. parvum*). A new database, CryptoDB (http://CryptoDB.org), contains the genome data and public sequences and is a very useful tool for obtaining genomic data for *Cryptosporidium*.[16]

Gene Expression Analysis

There is currently no system for transfection of *C. parvum* for gene analysis. A recent study analyzed expression of the *C. parvum* Cpgp40/15 invasion glycoprotein through transfection of the tachyzoite stage of a related Apicomplexan parasite, *Toxoplasma gondii*.[17] The first use of microarrays to analyze gene expression in epithelial cells infected with *C. parvum* demonstrated up-regulation and down-regulation of 236 genes out of a panel of 12,600. These included genes encoding heat-shock proteins, pro-inflammatory cytokines and chemokines, and structural genes, demonstrating considerable changes in host biochemical pathways.[18] In addition, a number of unknown genes were modulated, presenting new avenues of investigation.

MOLECULAR TESTING

Clinical Diagnosis

The gold standard for diagnosis of *C. parvum* is detection of oocysts in clinical samples using microscopy and indirect immunofluorescence (IF). Several enzyme immunoassay kits for detection of soluble stool antigens are available such as the Prospect T *Cryptosporidium* and Crypto CELISA. New and very rapid tests on the market are the ImmunoCard STAT! and ColorPAC solid-phase immunochromatographic immunoassays. Molecular techniques such as PCR are in development and provide a higher degree of sensitivity of detection than microscopy. One clinical trial demonstrated 83.7% sensitivity and 98.9% specificity using microscopy as compared to 100% sensitivity and specificity with PCR.[19] In another study,

Table 2 Molecular assays for detection of *C. parvum*

Assays[a]	Gene targets	Application
PCR	18s rRNA	Human and animal stool
	18s rRNA, oocyst wall	Surface waters
	COWP, Glutamate dehydrogenase	Sewage sludge
	18s rRNA	Soil/manure
	Oocyst wall	Milk, apple juice
PCR-RFLP	SSU rRNA	Shell fish
IMS-PCR	18s rRNA	River water, sewage
RT-PCR	HSP70	River, reservoir, tap water
qPCR	COWP, 18S rRNA, Cp11 rRNA,	Stool
	COWP, oocyst wall	Surface, tap and river water
	COWP	Raw sewage
CC-PCR	HSP70	Raw and filter backwash water
CC-qPCR	18s rRNA	Backwash water
CC-RT-PCR	HSP70	Surface and finished water
NASBA	HSP70	Surface water
FISH	rRNA	Laboratory water

[a]IMS = immunomagnetic separation; RFLP = restriction fragment length polymorphism; qPCR = quantitative or real-time PCR; CC = cell culture; RT = reverse transcription; NASBA = nucleic acid sequence binding assay; FISH = fluorescent in situ hybridization.

the sensitivity of PCR was shown to be comparable to an antigen enzyme immunoassay at 97%.[20]

Detection in the Environment

Intense research effort is ongoing to develop sensitive methods of detection of *C. parvum* (*C. hominis*) in environmental waters and raw and finished water in treatment facilities. Microscopy using IF is the standard used for detection of oocysts in the environment. However, numerous novel methods are being developed to provide rapid, sensitive, and automated detection. Some detection methods are antibody-based, e.g., flow cytometry, quantum dots immunofluorescent detection, and chemiluminescent immunoassays. Other methods are molecular-based[1] (Table 2). One major obstacle in monitoring water is the poor recovery of *Cryptosporidium* oocysts from the large volumes of water ($\geq$10 L) necessary for detection.[7] Another obstacle is that these complex samples contain numerous substances with potential to inhibit molecular-based assays and inhibitors vary with different sample types.[21] Appropriate adaptation of the DNA extraction method allows sensitive quantification of pathogens using assays such as qPCR in complex samples, e.g., raw sewage.[21] New technologies in development, such as electrorotation[22] for detection of live cells and DNA hybridization biosensors,[23] have the potential for extremely rapid screening of low numbers of *C. parvum* and may be the future in detection systems.

CONCLUSION

Considerable progress has been made in understanding *C. parvum* (*C. hominis*) since it was recognized as a serious pathogen of humans. Major technological advances have helped our understanding of this enigmatic pathogen and will undoubtedly continue to provide new insights in our understanding of the biology of this important parasite of humans and animals. With continued research, the complete in vitro culture systems, transfection systems, and cryopreservation methods will be developed, which will further enhance our understanding of *Cryptosporidium* and cryptosporidiosis.

ARTICLES OF FURTHER INTEREST

Giardia lamblia, p. 529
Real-Time PCR, p. 1117
Real-Time Reverse Transcription PCR, p. 1131

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CYP1A2*1F and CYP2D6 Genotyping by Real-Time PCR

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Barbara Messner
Werner Steimer
Klinikum vechts der Isar, Munich University of Technology, Munich, Germany

INTRODUCTION

Pharmacogenetics studies the influence of heredity on the interindividual differences in pharmacological response. In detail, it can be divided into two subgroups: pharmacodynamics and pharmacokinetics. Pharmacodynamics investigates the mechanism of action of the administered drug and its consequences on the body, namely, for example, the structure of receptors or carrier proteins. In contrast, pharmacokinetics analyzes the effect of the body on a drug reflecting, for example, its absorption, transformation, and excretion. The studied proteins are most often drug-metabolizing enzymes such as oxidative enzymes. A major role in drug metabolism is played by the cytochrome P450 (CYP) enzymes, which can be subdivided into several families, e.g., CYP1A2, 3A4, 2C9, 2C19, and 2D6. These enzymes are responsible for the oxidative metabolism of the majority of all therapeutically applied drugs.

In the following, we focus on cytochrome P450 2D6 (CYP2D6) and cytochrome P450 1A2 (CYP1A2).

CYTOCHROME P450 2D6

The CYP2D6 gene is a very polymorphic gene with more than 70 alleles described today.[1,2] This diversity leads to a great variety of activity of the enzyme debrisoquine 4-hydroxylase, which is encoded by the CYP2D6 gene. Many popular drugs such as antidepressants, neuroleptics, or cardioactive drugs, e.g., amitriptyline, paroxetine, haloperidol, metoprolol, etc.,[3] are substrates of CYP2D6. This may lead to adverse drug effects or therapy resistance. Subjects with normal enzymatic activity are called extensive metabolizers (EM). Subjects with increased activity are termed ultraextensive and ultrarapid metabolizers (UM), respectively, and have a prevalence of 1–10% in a Caucasian population.[4] They often do not reach therapeutic drug concentrations and suffer from therapeutic failure. Individuals with low or absent activity run the risk of potentially increased drug concentrations and adverse drug reactions. They are named poor metabolizers (PM) and can be found in approximately 7% of Caucasians.[5] This last group has to be distinguished from subjects with impaired but residual function, called intermediate metabolizers (IM). Substantial ethnic differences are observed regarding the frequency of the different phenotypes.[6]

The high number of polymorphisms with functional consequences results in complex and time-consuming genotyping procedures requiring extensive manpower and making it difficult to employ these methods in a time-critical clinical setting.

Real-time PCR offers substantial benefits in that regard. It is much faster than conventional PCR with subsequent restriction enzyme digestion and gel electrophoresis. Further advantages are lower risk of DNA contamination, facilitated handling, automated data processing, and the lack of toxic reagents such as ethidium bromide.

In different ethnic populations, it is possible to restrict testing to a limited set of alleles without sacrificing appreciable sensitivity of the assay (for details of the different alleles, see Table 1).

In a Caucasian population, six alleles, in detail *3, *4, *5, *6, *7, and *8, cause up to 99% of all PMs.[7] Up to 50–60% of all IMs can be predicted by the additional detection of the −1584 C/G polymorphism of the promoter, which encodes for the *2 and *41 alleles.[8] Duplicated or multiplicated functional CYP2D6 genes account for 10–30% of all UMs.[9] Other UMs may be explained by the *35 allele,[10] but this has been questioned by an in vitro analysis.[11] Taken together, genotyping of less than 10 polymorphisms of the CYP2D6 gene is sufficient to classify the vast majority of subjects in a Caucasian population.

Combining a fast method and a choice of polymorphisms, a comprehensive analysis can be carried out in one working day with a hands-on time of 3–4 hr,[12] employing a long PCR of the CYP2D6 gene on the LightCycler™ as a preamplification step. A preamplification step is necessary to detect both deletion and duplication as well as to avoid confusion from the two pseudogenes CYP2D7 and CYP2D8.[13] Furthermore, the same study reported the detection of *2, *35, and *41 alleles by hybridization probes and melting curve analysis

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Table 1 CYP2D6: Alleles, nucleotide changes, and enzyme activity

Allele	Nucleotide changes	Enzyme activity
CYP2D6*1	None	Normal: wild type
CYP2D6*2	−1584C>G	Normal
CYP2D6*2XN	Multiplication	Increased
CYP2D6*3	2549A>del	None
CYP2D6*4	1846G>A	None
CYP2D6*5	Deletion	None
CYP2D6*6	1707T>del	None
CYP2D6*7	2935A>C	None
CYP2D6*8	1758G>T	None
CYP2D6*35	31G>A	Normal
CYP2D6*41	−1584C	Decreased

Source: Ref. [2].

as well as a real-time PCR for detecting the *5 null allele and duplication either by hybridization probes or by SYBR Green®.[12] The null alleles *3, *4, *6, *7, and *8 were investigated by a conventional multiplex PCR[14] but can also be carried out by real-time PCR. This again improves speed and is performed by use of two primer pairs and five different pairs of hybridization probes and subsequent fluorometric melting point analysis.[15] Two separate real-time PCR reactions were performed in parallel: mutations *3 and *7 were analyzed in one capillary with two hybridization probes, one labeled with LightCycler-Red 640 and the other with LightCycler-Red 705. In a second capillary, mutations *4, *6, and *8 were investigated by dual-color hybridization with LightCycler-Red 640 or 705. In addition, the LightCycler-Red-640-labeled probes of alleles *4 and *6 had different melting points.[15]

Another possibility to verify a deletion, a duplication and, in addition, a multiplication with the definite number of alleles is a quantitative PCR. Newly, a TaqMan real-time PCR was validated for the determination of the accurate gene copy number of CYP2D6.[16] Quantitative amplification data were normalized to albumin as a reference gene with FAM-labeled and VIC-labeled probes in a single-tube assay.[16] (For further information of the used probes, please see Table 2.)

CYTOCHROME P450 1A2

The CYP1A2 gene is not as polymorphic as the CYP2D6 gene. Twenty-five alleles have been described at present.[2] It metabolizes psychoactive drugs such as antidepressants (e.g., amitriptyline, imipramine) and neuroleptics (e.g., clozapine) with variable activity[3] which may lead to treatment resistance. In addition, it may play a role in carcinogenesis. Its expression is inducible by different environmental and dietary substances, such as tobacco smoking, as well as by several polymorphisms. Especially one polymorphism, a C to A transversion of base pair 734 in the noncoding intron 1, called *1F, is discussed to affect the inducibility of the CYP1A2 gene.[17,18]

Table 2 Sequences of the probes used for real-time PCR of CYP2D6

Detected allele	Sequence of probe 5′–3′	Reference
*2/*41	GCACCCAATCCCAGCTAATTTTGTATT-Flu LCRed640-TTGTAGAGACCGGGTTCTT-Ph	[12]
*2XN/*5	TGCTGCCTCCCACTCTGCAGTGCTC-Flu LCRed640-ATGGCTGCTCAGTTGGACCCACGCT-Ph	[12]
*2XN	FAM-CCGGCCCAGCCACCATGG-TAMRA	[16]
*3	TCCCAGGTCATCCGTGCTCA-Flu LCRed705-TTAGCAGCTCATCCAGCTGGGTCAG-Ph	[15]
*4	CGACCCCTTACCCGCATCTCCC-Flu LCRed640-CCCCAAGACGCCCCTTT-Ph	[15]
*6	CCTCGGTCACCCACTGCTCCAGC-Flu LCRed640-CTTCTTGCCCAGGCCCAAGTTGC-Ph	[15]
*7	ACATTCGGAGGTAGGATCATGAGCA-Flu LCRed640-CCCCAGGCCAGCGTGGTCGA-Ph	[15]
*8	CCCAGTTCCCGCTTTGTGCCCTTC-Flu LCRed705-CCCATCACCCACAGGAGTGGTT-Ph	[15]
*35	GTGAGGCAGGTATGGGGCTAGAAGCACTG-Flu LCRed640-GCCCCTGGCCGTGATAGTG-Ph	[12]

Table 3 Sequences of the probes used for real-time PCR of CYP1A2

Detected allele	Sequence of probe 5′–3′	Reference
*1F	GGGCCCAGGACGCAT-Flu LCRed640-GTAGATGGAGCTTAGTCTTTCTGGTATCCA-Ph	[20]

Phenotyping using caffeine showed no difference in CYP1A2*1F metabolic activity between different genotypes in nonsmokers but a significant difference in smokers homozygous for the A allele compared with the other genotypes in terms of a 1.6-fold increased activity.[17] Unexpectedly, another study demonstrated a lower in vivo activity in patients with colorectal cancer than in healthy controls.[19]

The frequency of the *1F allele seems to be higher in Caucasians compared with Japanese. The study which investigated this new polymorphism analyzed 159 healthy Japanese and detected a prevalence of 39% homozygous and 44.6% heterozygous subjects.[18] In contrast, another study examined 236 Caucasians and found 46% homozygous A/A alleles and 44% heterozygous A/C alleles.[17]

In routine genotyping of CYP1A2, only *1F and a second allele, *1D, the deletion of T at position −2464, need to be analyzed because all other CYP1A2 polymorphisms are in linkage disequilibrium with these two alleles.[19]

At present, only one study has described real-time PCR methodology for the detection of CYP1A2*1F. This was performed employing the LightCycler™ instrument and fluorescent hybridization probes and melting curve analysis.[20] (For detailed information of the probes, see Table 3.)

CONCLUSION

The genetics of drug-metabolizing enzymes such as CYP2D6 and CYP1A2 is one major reason for the interindividual variability in drug therapy. Further investigations and large studies are required to elucidate the exact role of the polymorphisms of Cyp1A2*1F and Cyp2D6 in predicting therapeutic drug concentrations and clinical outcome, e.g., therapeutic response and adverse drug effects. Real-time PCR is one option to analyze the patients' genotype in a simple, fast, and reliable way. New functional relevant alleles will probably be found in the future, which can be easily and fast adapted for by this technique at limited cost. Screening prior to therapy is facilitated in a routine clinical setting. Once all major polymorphisms with functional relevance are characterized, microarray assays and gene chips will, in the future, probably offer even faster genotyping at lower cost.

ARTICLES OF FURTHER INTEREST

Cytochrome P450 Enzyme Genotyping—Clinical Applications, p. 323
Fluorescence Resonance Energy Transfer (FRET), p. 491
Long-Distance PCR, p. 735
Multiplex Real-Time PCR, p. 855
Real-Time PCR, p. 1117

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Cystic Fibrosis—Mutation Detection by Microarrays

C

Wayne W. Grody
UCLA School of Medicine, Los Angeles, California, U.S.A.

Carolyn Sue Richards
Baylor College of Medicine, Houston, Texas, U.S.A.

INTRODUCTION

As the most common lethal recessive genetic disease in Caucasians, cystic fibrosis (CF) has assumed a preeminent place in the test menus of molecular diagnostic laboratories. While utilized for diagnosis and prenatal diagnosis since shortly after the causative gene was identified in 1989, total test volume has increased dramatically since the advent of recommendations for general population carrier screening in Europe and North America. However, CF mutation testing remains a formidable technical and genetic counseling challenge owing to the large size of the gene, the large number and variety of reported mutations, the clinical variability of the phenotype, and the uncertainty of genotype–phenotype correlations. With over 1000 mutations currently catalogued, meaningful molecular genetic screening could only proceed with the development of array-based and related technologies. This article will describe the major technologies of this type in current use for CF mutation testing and how they are applied to a genetic disease of such remarkable clinical and molecular heterogeneity.

CLINICAL DESCRIPTION

Cystic fibrosis is often considered the most common serious autosomal recessive disease in Caucasians of northern European ancestry. It is a multiorgan condition, characterized by abnormally viscous secretions from epithelial cells in various tissues, leading to duct obstruction and infections. The most prominent and potentially lethal site for these phenomena is the lung, but CF patients also may suffer from pancreatic exocrine insufficiency, intestinal obstruction (called meconium ileus in the perinatal period), diabetes, biliary cirrhosis, growth retardation and failure to thrive, dehydration because of excessive salt loss in sweat, and sinusitis. In addition, virtually all males with classical CF exhibit a congenital malformation, bilateral absence of the vas deferens (CBAVD), causing infertility.

GENOTYPE–PHENOTYPE CORRELATION

Identification of the CF gene, designated *CFTR* for ''cystic fibrosis transmembrane conductance regulator,'' occurred in 1989.[1,2] It encodes a membrane-embedded ion-channel protein responsible for transport of sodium and chloride. The gene is 230 kbp long, encompassing 27 exons that produce an mRNA of 6500 nucleotides.

Gene sequencing in an initial series of affected patients revealed a recurring deletion, a three-nucleotide mutation of codon 508, which specifies phenylalanine in the protein product.[2] This mutation, now designated ΔF508, has come to be known as the most prevalent (approximately 70% of Caucasian carriers) and ''classical'' CF mutation. It is generally associated with severe disease, including pancreatic insufficiency, although this is not always the case. Since that original discovery, we have come to appreciate the remarkable molecular heterogeneity of CF, perhaps the most dramatic of any Mendelian disorder under study. There are now over 1000 mutations documented in the *CFTR* gene (www.genet.sickkids.on.ca/*CFTR*), and the range of clinical manifestations can be quite wide. Unfortunately, attempts to predict disease severity based on genotype have been frustratingly inconsistent, making genetic counseling for couples at risk rather difficult. Although some mutations are less likely to cause pancreatic insufficiency,[3] there are exceptions to all of the rules. Even the classic ΔF508 mutation may not always produce severe lung disease, even in the homozygous state.[4]

PREVALENCE

Cystic fibrosis is a relatively common recessive disorder in Caucasian populations, having a carrier frequency of about 1 in 29 and a birth incidence of 1 in 2500. Carrier rates in the other major ethnic groups of the United States have been estimated at 1 in 46 for Hispanic Americans, 1 in 65 for African Americans, and 1 in 90 for Asian Americans.[5] As noted, the predominant mutation in

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Caucasian carriers is ΔF508, but most of the other >1000 mutations are extremely rare or even ''private'' (found only in a single family). Perhaps the most accessible population for general carrier screening are Ashkenazi Jews, in whom the high prevalence of the W1282X mutation (45% of carriers) and three others in addition to ΔF508 yields a high screening sensitivity of 97%.[6] At the other end of the spectrum are Asians (and Asian Americans), in whom the disease frequency is low and the mutations are rare and/or not characterized, leading to some controversy over whether these populations should be screened.

DIFFERENTIAL DIAGNOSIS

The characteristic pulmonary and gastrointestinal presentations of CF in its classic form usually allow for accurate diagnosis even in the absence of DNA testing. However, there are atypical and less severe presentations that may mimic other causes of failure to thrive in early childhood, various malabsorption syndromes, and immune deficiencies. Diagnosis can be confirmed by the observation of elevated levels of chloride or sodium in sweat (>60 mEq/L), although sample collection may be difficult in very young infants. Molecular testing is useful to further confirm the biologic basis of the disease, to accurately diagnose individuals with borderline sweat chloride levels, and to provide the family with a precise mutation genotype that can then be used for prenatal diagnosis in future pregnancies.

MANAGEMENT

Whereas in prior decades CF typically resulted in early childhood or adolescent death from end-stage pulmonary disease, aggressive antibiotic therapy with modern agents, supplemented with medical and physical therapies to clear the viscous bronchial secretions, has led to a steady increase in life expectancy, currently extending into the mid- to late 30s (although some patients still die early of meconium ileus or intractable pulmonary infections). More heroic measures, such as lung or heart–lung transplantation, have also become available, and there is great hope for the future (although scant success to date) for gene-replacement therapy.

Because there is still no cure and the disease has a high morbidity, significant mortality, and economic burden, a major thrust of management efforts has been toward prevention. This takes the form of carrier screening to identify couples at risk, followed by prenatal diagnosis for those who wish to avoid the birth or recurrence of an affected child.

GENETIC COUNSELING

As a true recessive trait, *CFTR* mutations in heterozygosity are silent and asymptomatic, and because even their sweat chlorides are entirely normal, CF carriers are unidentifiable unless they have produced an affected child or have undergone mutation testing. Obviously, anyone of reproductive age who has a positive family history of the disease is considered a candidate for carrier testing. But because most CF carriers have negative family histories and only find out they are carriers after the birth of their first affected child, screening of the entire reproductive population has long been discussed as a preventative, public health measure.[7] This is justified because of the high carrier frequency in the general populations of North America and northern Europe. Under current recommendations,[5] couples who are pregnant or contemplating pregnancy are offered DNA-based carrier screening with a standard panel of the 25 most prevalent *CFTR* mutations. If both members are found to be carriers of identifiable *CFTR* mutations, they are referred for genetic counseling to inform them of their 25% risk of producing an affected child and to discuss the options for pregnancy termination. Naturally, such discussions are conducted in a nondirective and noncoercive manner, with full disclosure about the variability of CF symptoms and the uncertain genotype–phenotype correlations.

Couples with a positive family history should ideally first seek mutation testing of the affected index case. If one or both *CFTR* mutations are not detected with the core 25-mutation panel, the case may be referred for extended panel testing or even complete *CFTR* gene sequencing.

MOLECULAR GENETICS

The technical challenge of CF mutation detection derives from the large size of the gene, the myriad possible mutations, and the lack of any mutational ''hotspots'' in the gene, which could be used to limit the necessary targets for analysis. The core panel of 25 mutations designated for population screening in the United States was chosen on the basis of their prevalence (>0.1%) among a large cohort of genotyped affecteds, rather than their convenient location within the gene (on the contrary, they are spread throughout its length).

As more patients and mutations have been studied, the range and complexity of the molecular genetics have continued to grow. For example, some male individuals with *CFTR* mutations may present only with CBAVD and no classical CF symptoms, being ascertained during workups for infertility. Furthermore, one of the more common mutations, R117H, may be associated with either CF or CBAVD, depending on which form of an intronic

repeat polymorphism it is coupled with. When R117H is on the same chromosome (in *cis*) with a run of 5 thymidines (5T) in intron 8 of the gene, it behaves as a CF mutation (albeit usually mild); when in *cis* with the 7T allele it becomes a CBAVD mutation.

An analogous situation has emerged for one of the other mutations in the core panel, I148T, but not until after widespread screening had commenced. Reports from large testing laboratories[8,9] indicated that this mutation was seen in carriers at almost 100 times the expected frequency based on its appearance in affected patients. Rohlfs et al.[8] identified a genetic modifier, a deletion of 6 base pairs, 3199del6, which was an obligate covariant for I148T to behave as a disease-causing allele. In retrospect, these findings are not surprising, as the clinical variability of CF patients with the same primary mutations must be due to the presence of genetic modifiers, both within the *CFTR* gene and elsewhere in the genome, of which we still know all too little.

MOLECULAR GENETIC TESTING

Given the ever-growing list of *CFTR* defects, molecular genetic testing has evolved from relatively simple assays directed only at the predominant ΔF508 mutation[10] to increasingly complex multiplex mutation detection systems. Not all of these are on micro- or miniarray platforms, although the first practical assays for detecting more than a few mutations at a time utilized allele-specific DNA probes bound to solid supports in the form of nylon strips or membranes.[11,12] Originally all such assays were in-house-developed tests (''home brews''), but as the commercial potential of CF testing has exploded with the advent of nationwide population carrier screening,[5] several vendors have come forward with manufactured systems.

The systems available and in use at this time include reverse dot blots (Inno-LiPA CFTR33 Probe Array, Innogenetics; Linear Array CF Gold 1.0, Roche), amplification refractory mutation detection system (ARMS; Elucigene CF29, Orchid), and the oligonucleotide ligation assay (OLA; CF V3.0, Abbott/Celera). More recent additions to the *CFTR* testing arena include the invader assay (Third Wave Technologies) and various microarray platforms (e.g., Nanochip CFTR, Nanogen). Other emerging technologies include matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Sequenom) and real-time PCR (LightTyper, Roche). In addition, a handful of research and clinical laboratories offer complete sequencing of the *CFTR* gene, which can be used to identify rare mutations and polymorphisms and resolve ambiguities not defined by the allele-specific methods.[13] Several of the more popular (and more established) technologies are described in the American College of Medical Genetics (ACMG) *Standards and Guidelines for Clinical Genetics Laboratories*.[14] While these systems include both array-based and nonarray technologies, the remainder of our discussion here will focus on the array-based platforms that are now commercially available.

Reverse Dot Blot

Reverse dot blots entail a hybridization-based technology in which the oligonucleotide probes complementary to the normal and mutant sequences are attached to a membrane in a miniarray format. Patient DNA is subjected to one or more multiplex PCR reactions using biotin-tagged primers for all exons containing mutations of interest, and the resulting biotinylated PCR products (the target) are hybridized to the array (the probes). Both mutant and wild-type alleles are interrogated simultaneously. A streptavidin–peroxidase conjugate complex, which binds to the biotinylated PCR product hybridized to the arrayed oligonucleotide probe, is used to visualize the test results.[12] Current commercially available versions of this technology (Roche, Innogenetics) incorporate the 25 mutations and variants (5T/7T/9T) recommended for population screening. The reverse blot platform has numerous advantages including direct genotyping, nonisotopic detection, and the potential for automation.

Microarray

The true microarray formats are basically sophisticated ASO dot blots on a smaller scale within an electronically controlled hybridization instrument. The current NanoChip from Nanogen consists of 33 mutations, including the ACMG-recommended panel. Each NanoChip consists of 99 test sites (electrodes) attached to platinum wire connections. The negatively charged DNA is electronically guided to a test site where biotinylated samples bind to streptavidin in the site. Following denaturation, fluorescent probes are hybridized to the array and signal is detected after stringent washing procedures. Advantages of this technology include the flexibility of reflex testing, the adaptability of chip design to customization and addition/deletion of mutations in the panel, and the self-contained nature of the constituents. Other manufactured microarrays for *CFTR* mutations in slightly earlier stages of development include those from Motorola Life Sciences, BioArray Solutions, GeneOhm, and Autogenomics. Depending on the density of the array on the manufactured chip, theoretically there would be no limit to the number of *CFTR* mutations that could be addressed, going well beyond the current recommended panel of 25. Indeed, by using an ultra-high-density array of the type

manufactured by Affimetrix, one could theoretically detect all possible mutations and polymorphisms in the *CFTR* gene, both known and unknown. However, the clinical utility of such high-density arrays would need to be examined carefully, because most of the additional mutations will be extremely rare and not well characterized as to their clinical expression. Still, the mutational heterogeneity of the *CFTR* gene and the demand for high-throughput population screening make microarrays an obvious choice of assay platform, even more so in the future than at present.

CONCLUSION

It would probably be fair to say that mutation detection in the *CFTR* gene has been simultaneously one of the greatest successes and one of the greatest disappointments of the molecular genetic testing era. It is perhaps unfortunate, but endlessly instructive, that the first genetic disease targeted for widespread population screening by molecular methods has proven to be so complex at both the genotypic and phenotypic levels. For these reasons, molecular diagnostic laboratories and genetic counselors are still struggling to meet the challenge of delivering and explaining this complex test to large populations of generally healthy people who are not personally familiar with the disorder. Such widespread screening would not even be conceivable without the availability of user-friendly DNA array technologies, and the perceived market afforded by large-population screening has induced manufacturers to develop and market such products as analyte-specific reagents. These products are well adapted to screening for a limited number of *CFTR* mutations (e.g., the standard screening panel of 25 in the United States), but it is clear that a new generation of array techniques will be required if the screening panels are to be expanded appreciably. A number of manufacturers are working on such products, although even if technically robust, expanded mutation panels bring with them yet another level of clinical complexity and genetic counseling challenges. In this way, the CF testing situation provides a foretaste of what we may expect as we embark on molecular genetic screening for even more complex diseases in the future.

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Cytochrome P450 Enzyme Genotyping—Clinical Applications

C

Magnus Ingelman-Sundberg
Karolinska Institutet, Stockholm, Sweden

INTRODUCTION

Interindividual variability in drug metabolism and drug response is extensive. The causes for this are of genetic, physiological, pathophysiological, and environmental origin. Genetic variability is known for drug absorption, drug metabolism, and drug interactions with the receptors. Pharmacogenetics is defined as the genetics behind interindividual differences in drug response and includes studies of variants of genes encoding drug transporters, drug-metabolizing enzymes, and drug receptors. The aim is to understand the genetic factors of importance that explain no drug response in a large part of the population and the occurrence of adverse drug reactions. Today, we have most knowledge about the importance of the drug-metabolizing enzymes in this respect, whereas the research is progressing to yield a better understanding about to what extent genetic variability in drug transporters and drug receptors can explain adverse drug reactions and lack of drug response.

PHARMACOGENETICS OF DRUG-METABOLIZING ENZYMES

Polymorphism of genes encoding drug-metabolizing enzymes is the major known genetic cause for the interindividual variability in drug response.[1–6] Based on the occurrence of mutations in these genes, gene deletions, and gene duplications, the populations can be divided into poor (PM), intermediate (IM), efficient (EM), or ultrarapid (UM) drug metabolizers. The PMs lack the enzyme in question, the IMs are heterozygous for a defect gene, the EMs have two functional alleles, whereas the UMs have multiple functional gene copies on a single allele.[2]

All enzymes participating in the metabolism of drugs in phase I (functionalization) and phase II, where the modified drugs are conjugated to water-soluble end products usually excreted in the urine, are polymorphic. This genetic polymorphism can thus cause abolished, quantitatively or qualitatively altered or enhanced drug metabolism. The evolutionary basis for this variability is adaptation to the environment in the form of dietary habits and genetic drift, the founder effect. Because the genes usually are not essential for survival but of importance for detoxification of environmental constituents including dietary components, no selection pressure is put on the conservation of functionally active genes; subjects lacking one or several types of these genes usually have a normal physiological phenotype. The major part of the interethnic differences in the genetic constitution of these genes is a result of incidental mutations in the genes, amplified in certain areas because of population expansion. The lack of endogenous function of the gene products has thus allowed this extensive heterogeneity with respect to allelic distributions in different parts of the world.

The major influence of interindividual variability of drug metabolism is caused by polymorphism in the cytochrome P450 (*CYP*) genes. These enzymes are responsible for about 75–80% of the phase I-dependent drug metabolism,[1] and this variation often influences the clearance of drugs to a great extent.

CYTOCHROMES P450

The cytochrome P450 enzymes participate in the metabolism of a huge number of xenobiotic chemicals. The polymorphic forms of P450s are many times responsible for the development of adverse drug reactions. It has been described that 56% of drugs which are cited in adverse drug reaction (ADR) studies are metabolized by polymorphic phase I enzymes of which 86% are P450s. One estimates that: 1) ADRs cost the U.S. society about $100 billion; 2) they cause more than 100,000 deaths annually in the United States; and 3) up to 7% of all hospital admissions are due to ADRs (see Ref. [2] for references). In addition, the costs for treating patients with polymorphic forms of P450s are much higher than for those carrying nonpolymorphic alleles.

POLYMORPHISM OF THE P450 GENES

Today, we know about 57 active cytochrome P450 genes (http://drnelson.utmem.edu/CytochromeP450.html, Ref. [7]). All P450 genes in families 1–3 are polymorphic. The functional importance of the variant alleles is, however, different and the frequencies of their distribution in different ethnic groups are tremendously different. On the Human CYPallele Nomenclature Web site at our server

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at Karolinska Institutet (http://www.imm.ki.se/cypalleles), updated information in this area is obtained. About 40% of P450-mediated drug metabolism is carried out by polymorphic enzymes. The rate of hepatic metabolism of a certain drug can differ 1000-fold between the phenotypes. Particularly high penetrance of the genetic polymorphism is seen because of variation in the *CYP2C9*, *CYP2C19*, and *CYP2D6* genes. CYP2D6 is responsible for the metabolism of about 25% of the clinically used drugs, and the polymorphism is relevant for interindividual variability in about 50% of these drugs, i.e., of about 10–15% of all drugs used today (cf. Ref. [8]). Required dosing to achieve the same plasma levels of a drug mainly metabolized by CYP2D6 can differ 10–20-fold between individuals. In Europe, 5.5% of the population are UMs for CYP2D6, and here, no response is often to be expected because of too rapid metabolism. Seven percent of the European population are PMs, where too high plasma levels of the drugs are to be expected at ordinary dosage and a higher frequency of adverse drug reactions is seen. In contrast to the extensive span in metabolic capacity, dosing today is, however, principally only based on the average in the population. By predictive genotyping, a more appropriate initial dosing could be achieved in 50–60 million people only within Western Europe.

About 50% of all drug metabolism in phase I is carried out by CYP3A4. This enzyme is highly conserved and no functionally important variant forms are seen in Caucasians or Orientals. There is a relatively high interindividual variability in CYP3A4 activity, but any genetic origin has to be addressed in future studies.

CLINICAL RELEVANCE OF CYTOCHROME P450 POLYMORPHISM

It is the author's estimate that 15–20% of all drug treatment is influenced by the polymorphism of the cytochrome P450 genes. The clinical importance of the polymorphism of the CYPs is summarized in Tables 1 and 2.

PSYCHIATRIC DISORDERS

Upon treatment with antipsychotic drugs, adverse reactions are more likely to be seen in PMs for CYP2D6. In addition, the costs for treatment of patients are higher and estimates have been made that costs $4000–6000 more per year to treat patients of the variant UM and PM phenotypes.[9] Parkinsonism-like side effects are

Table 1 Relative importance of polymorphisms in human P450s involved in drug metabolism

Enzyme	Estimated fraction of drug metabolism[a] (%)	Substrates	Major allelic variants[b]	Clinical effects of the polymorphism	Significance of polymorphism[c]
CYP1A2	5	Drugs, carcinogens	*CYP1A2*1K*	Less expression and inducibility	+
CYP2A6	2	Nicotine, drugs, carcinogens	*CYP2A6*4* *CYP2A6*9*	Altered nicotine metabolism	+
CYP2B6	2–4	Drugs		Relatively high	+
CYP2C8	1	Drugs	*CYP2C8*3*	Taxol metabolism	+
CYP2C9	10	Drugs	*CYP2C9*2* *CYP2C9*3*	Side effects Drug dosage	+++
CYP2C19	5	Drugs	*CYP2C19*2* *CYP2C19*3*	Drug dosage Drug efficacy	+++
CYP2D6	25–30	Drugs	*CYP2D6*2xn* *CYP2D6*4* *CYP2D6*10* *CYP2D6*17* *CYP2D6*41*	Nonresponse Side effects Drug dosage	+++
CYP2E1	2–4	Carcinogens, solvents, drugs	—	Not shown	—
CYP3A4	45–50	Drugs, carcinogens	*Rare*	Not shown	—
CYP3A5	<1	Drugs	*CYP3A5*3*	Not shown	—

[a]The estimated fraction of responsibility for drug metabolism in phase 1 reactions.
[b]A description of the alleles is found on the human CYP allele nomenclature committee home page: http://www.imm.ki.se/CYPalleles/.
[c]The significance of the polymorphism is based on the number of reports showing impact of the P450 polymorphism on the pharmacokinetics of drugs being substrates for the enzyme in question. The number of + illustrates the relative importance between the different forms of P450.
Source: Ref. [2].

Table 2 Examples of clinical impact of cytochrome P450 pharmacogenetics

Disease	Enzyme	Percentage of dose		Examples
		UMs	PMs	
Depression	CYP2C9			Bipolar disorders and valproate
	CYP2C19		40	PMs and SSRIs
	CYP2D6	200	30	Nonresponders (UMs) and side effects of tricyclics (PMs)
Psychosis	CYP2D6	160	30	Haloperidol and parkinsonian side effects
				Oversedation and perphenazine, thioridazine
Ulcer	CYP2C19		20	Dosing of PPIs
				pH and gastrin changes
Cancer	CYP2B6			Cyclophosphamide metabolism
	CYP2D6	250	60	Nonresponse of antiemetic drugs (UMs)
CV	CYP2C9		30	Warfarin dosing (acenocoumarol)
				Irbesartan and blood pressure response
	CYP2D6	160	30	Perhexiline neuropathy and hepatotoxicity
Pain	CYP2D6			Codeine no response (PMs)
Epilepsia	CYP2C9			Phenytoin pharmacokinetics and side effects

The doses shown for depression and psychosis are weighted as related to the size of samples in all studies published, as reviewed by Kirchheiner et al.[8] The other doses are based on data presented in the text and from Ref. [2]. PPIs: protein pump inhibitors.
Source: Ref. [2].

significantly seen at higher frequency in PMs according to several prospective and retrospective studies.[2] Oversedation is often seen in PMs after treatment with perphenazine, thioridazine, and other antipsychotics, whereas no significant relationship with CYP2D6 polymorphism has been described for tardive dyskinesia, acute dystonia, or akathisia.[10]

Regarding antidepressants, the tricyclic ones are almost entirely metabolized by CYP2D6 and dosage much related to the CYP2D6 phenotype, whereas several of the SSRIs are metabolized by the polymorphic CYP2C19. The kinetics of nortriptyline is much dependent on the number of active *CYP2D6* genes, and the appropriate dosage to receive a therapeutic plasma level varies from 30 and 50 mg in PMs to 500 mg in UMs. One pilot study indicates that the UM phenotype is 10-fold more common in nonresponders of antidepressant therapy than among responders.[11] Treatment with the SSRI drug sertraline, a CYP2C19 substrate, has been found to display adverse drug reactions such as nausea and dizziness, an effect possibly caused by toxic concentrations of the accumulated drug in CYP2C19 PMs, and the pharmacokinetics of citalopram are influenced by the CYP2C19 polymorphism. In addition, the metabolism of valproate, commonly used in bipolar disorders, has been found to be much influenced by the CYP2C9 polymorphism. With respect to both depression and psychosis, Kirchheiner et al.[8] have performed an impressive investigation and concluded that receptor polymorphism hitherto investigated is of no value for prediction of drug therapy, but that dosing of about 50–60% of the drugs used in such therapy to a great extent is dependent on the polymorphism of *CYP2D6* and *CYP2C19*.

ULCER AND GASTROINTESTINAL DISORDERS

Dosing of antiulcer agents to reach a specific plasma level is highly dependent on the CYP2C19 phenotype. Higher efficacy in treatment of ulcer is seen in PMs.[12] Changes in gut pH after treatment of, for example, omeprazole are more pronounced in CYP2C19 PMs. Long-term treatment of proton pump inhibitors leads to a more extensive gastrin release in PMs as compared with EMs. Dosing for long-term treatment is advantageously adjusted to the CYP2C19 genotype/phenotype.

CANCER

Pharmacogenetics has an increased role in cancer treatment.[13] CYP2B6 metabolizes several anticancer drugs, among them cyclophosphamide, and preliminary indications exist for an increased metabolism among subjects carrying a variant allele, *CYP2B6*6*. Tamoxifen is metabolized to its active metabolite by CYP2D6, and a smaller therapeutic effect has been observed in PMs for CYP2D6 and predictive phenotyping/genotyping could be relevant before entering the treatment.[14] The effect of antiemetic drug treatment of cancer patients with drugs

such as the 5-hydroxytryptamine type 3 receptor antagonists tropisetron and ondansetron has been found to be highly related to the CYP2D6 phenotype. Lower plasma levels and higher frequency and intensity of vomiting were seen in subjects carrying more active gene copies of *CYP2D6*.[15]

CARDIOVASCULAR DISORDERS

Both warfarin and coumarols, such as acenocoumarol, as anticoagulants are metabolized by CYP2C9. The maintenance dose of warfarin has been found to differ in many different studies between individuals carrying *2*, *1*, or *0* functionally correct *CYP2C9* alleles.[16] A study indicates that subjects carrying variant alleles required 95 days more to achieve stable warfarin dosing and has increased risk (OR 2.3) for life-threatening bleeding events,[17] findings which are also seen in several other studies. *CYP2C9* genotyping to predict a more safe and individually based warfarin treatment with less side effects might be valuable. In addition, the *CYP2C9* genotype has been described to predict the blood pressure response to irbesartan, and a four- to fivefold higher incidence of side effects has been described in CYP2D6 PMs upon treatment with metoprolol.[2]

The antianginal drug perhexiline has concentration-related hepatoxicity and peripheral neuropathy, and determination of *CYP2D6* genotype has been shown to predict dose requirements and reduce the risk of perhexiline concentration-related toxicity.[2]

PAIN

Codeine and tramadol need to be metabolized by CYP2D6 before pain-relieving effects are observed. No efficacy is thus seen in CYP2D6 PMs. In addition, adverse drug reactions of codeine treatment have been described in CYP2D6 ultrarapid metabolizers when treated with ethylmorphine, oxycodone, and hydrocodone probably because of an extensive formation of morphine.

EPILEPSIA

The CYP2C9 polymorphism influences the pharmacokinetics of phenytoin, and several examples of ADRs have been described in patients with defect *CYP2C9* alleles upon phenytoin treatment like CNS intoxication such as ataxia, diploidia, and other neurological symptoms.

GENOTYPING METHODS

Cytochrome P450 genotyping includes the analysis of single SNPs as well as major gene rearrangements. A major outline of the methods is given in Ref. [18]. Southern blotting including reduction fragment length polymorphism (RFLP) analysis is extremely useful for determination of major differences in gene arrangements, including gene deletion and gene duplications of the various *CYP* gene loci. A desired region of the gene is PCR-amplified and nicktranslated and labeled with ^{32}P, after which hybridization is varied out.[19] Southern blotting is generally extremely informative for the specific locus in question and should be carried out more frequently than is actually seen in the literature. RFLP in combination with gene-specific PCR amplification (PCR-RFLP) can also be used to determine specific mutations in the *CYP* genes, where the polymorphic site must alter or generate a restriction site, resulting in a change in the DNA fragmentation pattern on the gel.[20] Real-time PCR analysis of specific mutations allows rapid and automatized methods for detection of *CYP* single nucleotide polymorphisms (SNPs) and *CYP* gene copy number. Two fluorescently labeled probes are used: one wild-type probe and a mutation probe. During the reaction, amplification of the desired sequence is obtained and can give information after careful optimization of the single mutation present or the number of gene copies. Rapid detection of SNPs within the *CYP* genes can also be obtained using the pyrosequencing technique (Pyrosequencing Inc.). For detection of several SNPs in the CYP genes, DNA Chip array techniques can also be used either by hybridization reactions (Affymetrix Inc.) or by primer extension (CodeLink, Amersham Biosciences). The techniques are developing in a rapid fashion, and several different platforms for detection of CYP gene variants are continuously presented.

CONCLUSION

It is the author's estimate that predictive cytochrome P450 genotyping will improve the clinical efficacy of 15–25% of all drug therapy and reduce the incidence of ADRs by 10–20%. Other aspects that underlie interindividual variability in drug metabolism, such as bad compliance, unfavorable drug–drug interactions, and pathophysiological conditions, must of course remain primary factors of concern in drug treatment. The knowledge in the area of cytochrome P450 pharmacogenetics has, however, now increased to such an extent that it would be possible to put labels on those drugs where we know that the individual response to the drug would benefit substantially by predictive genotyping of the cytochrome P450 gene in question. In relation to the costs for prolonged hospital

visits, etc., the costs for such genotyping are minimal and would increase the health in a substantial part of the population and decrease the costs for the society.

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Denaturing Gradient Gel Electrophoresis (DGGE)

Luciana Cresta de Barros Dolinsky
Universidade Federal do Rio de Janeiro (UFRJ) and Universidade do Grande Rio (UNIGRANRIO), Rio de Janeiro, Brazil

INTRODUCTION

Denaturing gradient gel electrophoresis (DGGE) is a DNA-based method that permits small mutation detection and single nucleotide polymorphism (SNP) analysis. Denaturing gradient gel electrophoresis is the most sensitive mutation detection technique (detection rate close to 100%) and can be used for human hereditary disease diagnosis, large-scale mutation screening, and predictive genetic testing, including prenatal diagnosis and counseling. Denaturing gradient gel electrophoresis can also be used to analyze SNPs in hereditary complex disease traits. As DGGE is usually less expensive and less labor intensive and time consuming than other point mutation detection techniques it is an excellent method to use in medical routine procedure.

DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis is a DNA-based method that permits small mutation detection and SNP analysis with high detection rate (close to 100%)[1] and is less labor intensive and time consuming than other point mutation detection techniques. Despite this, it is easy to reproduce and can be used as a routine method.

TECHNICAL DESCRIPTION

The DGGE method is based on the electrophoretic mobility of a double-strand DNA molecule through linearly increasing concentrations of a denaturing agent (urea and formamide) on a polyacrylamide gel with ethidium bromide staining. As the DNA fragment proceeds through the gradient gel, it will reach a position where the melting temperature (T_m) of its lowest melting domain equals the denaturing agent concentration, resulting in denaturation and consequent marked retardation of the DNA fragment mobility. As the T_m of a melting domain is dependent on its nucleotide sequence, even DNA fragments differing by a single nucleotide in their lowest domain will suffer branching and consequent retardation of their mobility at different positions along the DGGE gel, allowing DNA fragment separation.[2] The choice of the gel denaturant range is based upon the T_m of the fragment to be analyzed, and the electrophoretic runs can be performed at a constant temperature (58°C) that exceeds the T_m of an A–T-rich DNA fragment in the absence of denaturing agents.

Denaturing gradient gel electrophoresis cannot resolve fragments differing by nucleotide changes in the highest melting domain because of complete strand dissociation. This problem is overcome by introducing a GC clamp tail as short as 40 bp to serve as a high T_m domain and prevent complete dissociation of the DNA fragment. The GC clamp tail introduction increases the DGGE mutation detection percentage to close to 100%.[1] It is also possible to split DNA fragments into two segments to allow efficient mutation detection. The melting behavior of a DNA fragment can be simulated by computer software analysis.[3]

The DGGE method is conceptually similar to heteroduplex analysis.[4,5] The PCR–DGGE combination is extremely efficient when applied to heterozygous nucleotide variants because of continuous denaturation and reannealing of single-strand molecules during PCR, allowing for the formation of heteroduplex and homoduplex molecules.[6] The presence of a single nucleotide change within heteroduplexes decreases their melting domain temperature allowing separation from the homoduplexes and easy visual detection of the mutants (Fig. 1). If working with an X-linked disease it is necessary to mix male mutated DNA with a normal male DNA during PCR to ensure heteroduplex formation. To reduce workload, DNA fragments can be amplified in multiplex combinations. A further sequencing of the altered DNA fragment can determine the exact molecular alteration.

SENSIBILITY

Many different techniques such as single-strand conformation polymorphism (SSCP),[7] hydroxilamine and osmium tetroxide (HOT) chemical cleavage,[8] protein truncation test (PTT),[9] and heteroduplex analysis (HA)[4,5] are available to identify single nucleotide variants. However, in many cases, there is not a very high mutation detection rate when we use each one of

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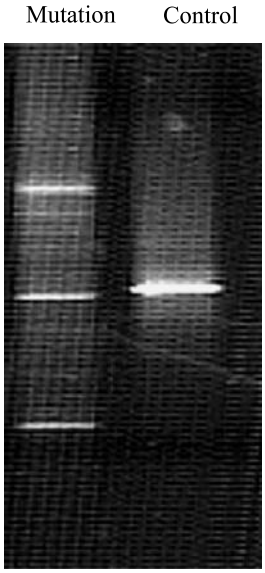

Fig. 1 Small mutation detection in exon 17 of the dystrophin gene.

these techniques principally because of technical difficulties. For example, RNA-based techniques are difficult to use in a diagnostic setting because RNA expression is oftentimes extremely low.

Some laboratories are using haplotype analysis to assess risk status; however, this strategy may be inconclusive because in many cases there is intragenic recombination and high mutation rate. This represents a great obstacle to genetic analysis of patients with small mutations and genetic counseling of their relatives.

Recently, many diagnostic and research laboratories are using denaturing high-performance liquid chromatography (DHPLC)[10] to scan for single nucleotide variations, but even with this method (best detection rate close to 92%) many families remain excluded from the analysis because the detection rate is not 100%. It is important to know that only mutation detection gives absolute certainty about diagnostics and counseling.

Denaturing gradient gel electrophoresis is the most sensitive electrophoretic method (mutation detection rate close to 100%)[1] and, despite this, does not require radioisotope use, is less labor intensive and time consuming, is easy to reproduce, and is usually cheaper than the other routinely used methods for point mutation detection.[7]

CLINICAL APPLICATIONS

Denaturing gradient gel electrophoresis can be used for genetic diagnosis of hereditary human diseases caused by small mutations even when doing diagnosis for X-linked human diseases. It is also possible to analyze SNPs (genetic alterations where at least one allele frequency is higher than 1%) to associate with hereditary complex disease traits.

In many cases, mutation detection can be used in choosing adequate healthcare, as some mutation positions are associated with the worst prognostic. Knowing the exact mutation position makes it possible to establish the prognostic prior to the disease development course and, based on this, to decide which one is the best therapy. Knowing the polymorphism makes it possible to associate some polymorphisms with hereditary complex disease traits for each different population; for example, renin–angiotensin system polymorphisms are associated with high blood pressure in Japanese and African American.[11,12]

Denaturing gradient gel electrophoresis can also be used for predictive genetic testing, including prenatal diagnosis. Knowing a priori if a patient has inherited a familiar mutation with late expression, a polymorphism, or knowing during pregnancy if a baby has inherited a hereditary human disease can help health-care professionals in choosing an adequate strategy to care for the disease, to delay the disease development, or to establish the best life conditions during the disease course.

Denaturing gradient gel electrophoresis is also useful for counseling in an effort to avoid new cases of human hereditary diseases. In X-linked human diseases, for example, carrier couples can alternatively do in vitro fertilization with male embryo implantation to avoid new disease cases. Because mutation detection is close to 100% when using DGGE, we can do prenatal diagnosis and counseling knowing that we will give an answer to the family question and that not one family member will remain excluded from the analysis.

Denaturing gradient gel electrophoresis can also be used to perform large-scale population screening to improve genetic analysis. It is important for this kind of study as some mutations or polymorphisms have a high frequency in one population and a low frequency in another. Analyzing these frequency changes makes it possible to establish specific health-care rules for each different population, principally in hereditary complex disease traits where ethnic factors have an important role in inherited genes.[13]

Denaturing gradient gel electrophoresis is usually cheaper and less labor intensive and time consuming than other routinely used point mutation detection techniques, does not require radioisotope use, is easy to reproduce, and, despite this, has a high detection rate (close to 100%). Considering all these related qualities DGGE can be used as a medical routine procedure for practicing clinicians in hospitals and ambulatory settings.

CONCLUSION

We can conclude that DGGE is an excellent method for small mutation detection principally because it has a high detection rate (close to 100%) and is less labor intensive and time consuming than other point mutation detection techniques.

It can be useful for practicing clinicians in hospitals and ambulatory settings for diagnosis and predictive genetic testing, including prenatal diagnosis and counseling. It can also be used for large-scale mutation and single nucleotide polymorphism screening to improve genetic analysis and to establish adequate healthcare for each different population.

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Differential Display (DD) Analysis

D

Farid E. Ahmed
East Carolina University, Greenville, North Carolina, U.S.A.

INTRODUCTION

Differential display (DD) technique was developed 13 years ago as a systematic method for studying eukaryotic gene expression. The updated method utilizes a combination of three frequently used molecular biology techniques: reverse transcription (RT) of messenger ribonucleic acid [Pol $(A)^+$ mRNA] followed by polymerase chain reaction (PCR) of the resulting complementary deoxyribonucleic acid (cDNA); polyacrylamide gel (PAG) electrophoresis; and cDNA cloning to visualize and compare gene expression patterns between two or more samples. Reverse transcription uses one of three individual one-base fluorescently labeled anchored oligo-dT primers to amplify the 3′ terminal of the mRNA by PCR, instead of the original isotopic labeling method, in combination with one of the various 10–13 arbitrary (random) nucleotide primers. The resulting cDNAs are separated on a denaturing, and sometimes nondenaturing, PAG. A fluorescent scanner views the pattern of bands. The cDNA fragments of interest are retrieved from the gel, purified, reamplified, and either cloned or directly sequenced to identify the differently regulated genes. Finally, confirmation of the differential expression of resulting cDNAs can be carried out by a method such as Northern blotting, RNase protection, or quantitative real-time RT-PCR. Following confirmation, the cloned cDNA probes can be used either to screen a cDNA library for a full-length clone, or more easily to carryout rapid amplification of cDNA ends (5′-RACE).

PRINCIPLES AND GUIDELINES

The genome of higher eukaryotes contains close to 50,000 genes, of which between 10% and 15% are believed to be expressed at a given time in a cell to determine the regulatory mechanisms that control cellular processes controlling our lives such as development and differentiation, homeostasis, response to insult, cell cycle regulation, aging, programmed cell death, and pathological changes such as cancer. Monitoring the pattern of gene expression under various physiological and pathological conditions is a critical step in understanding these diverse biological processes, and comprehending the mechanisms involved allows for needed interventions. Because of the large numbers of expressed genes, powerful tools are needed to characterize the overall pattern of gene expression.[1]

Older methods of identification of differentially expressed genes relied on differential or subtractive hybridization (SH), which although sensitive requires large amount of RNA, is error prone, nonsystematic, laborious, and time consuming, and results are not seen until the end of the process.[2]

The original DD protocol utilized the idea applied earlier for random amplification of fingerprinted genomic sequences. It was published in 1992 by prominent investigators at the Dana-Farber Cancer Institute in Boston, MA.[3] The principle of the method is to detect different types of gene expression patterns using three techniques: 1) RT of DNase I-treated total RNA (to remove any chromosomal DNA) using anchored primers, 12-mer long consisting of a stretch of 11 Ts plus one last non-T base to anchor primers to the pol $(A)^+$ tail of many RNAs. Use of total RNA is preferable to mRNA as it involves less preparatory steps and avoids background smearing;[2] 2) choosing 5′ arbitrary 10 mers that hybridize to cDNA in a degenerate manner for setting lengths of cDNAs corresponding to mRNAs (tags) to be amplified by PCR. For a 5′ primer of arbitrary base sequence, annealing position to cDNA should be randomly distributed from the pol $(A)^+$ tail. Therefore the amplification provided from various mRNAs will differ in length; and 3) employing sequencing gels for isolation of cDNAs. The aim is to obtain a tag of a few hundred bases, long enough to uniquely identify mRNA, but short enough to electrophoretically separate by size. Pairs of primers are selected so that by probability each will amplify DNAs from 50 to 100 (average $\sim$75) mRNAs, as this number can be adequately displayed on one lane of the gel.[3] This method was later on named DD-PCR[1] (Fig. 1), to distinguish it from other DD methods discussed below.

Problems intrinsic to DD were soon encountered such as high noise level due to smearing, misrepresentation of rare messages, bias toward high copy number mRNAs, not revealing differences due to mutational changes, incomplete cDNAs, contamination of purified PCR fragments by unrelated DNA sequences, and additional bands generated by arbitrary primers alone from palindromic

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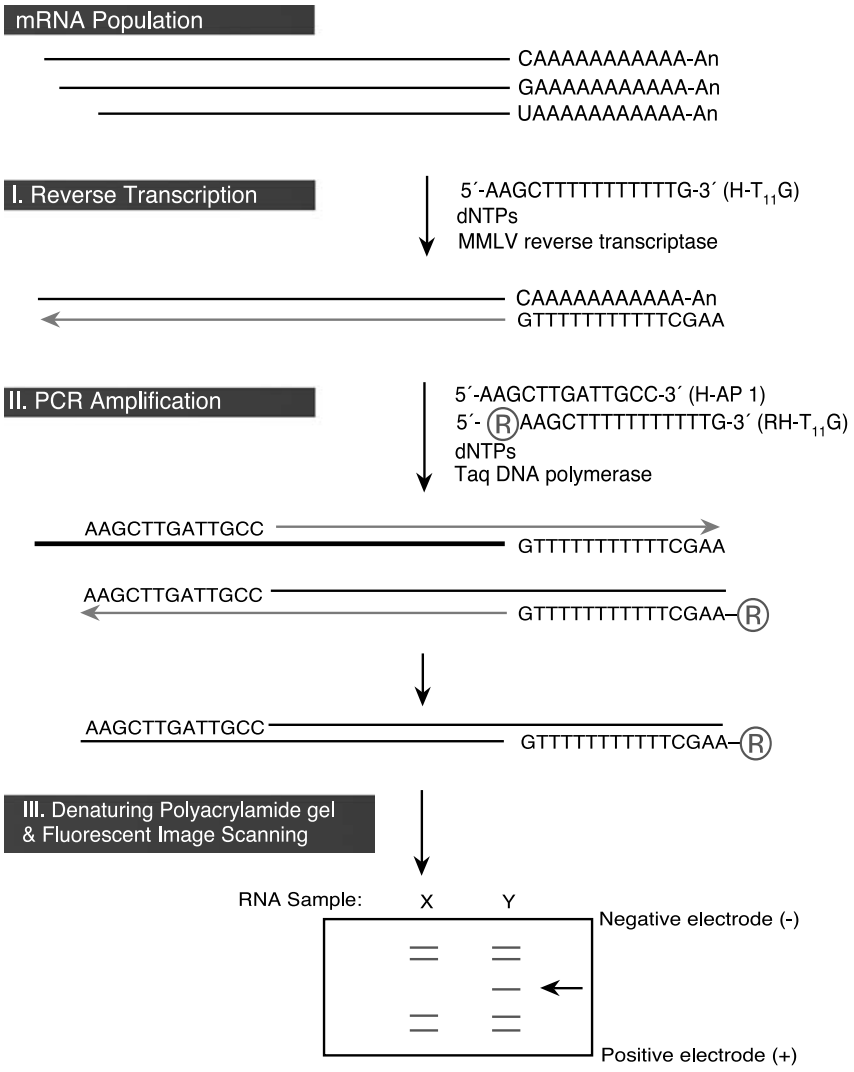

Fig. 1 Schematic of fluorescent DD–PCR method. Total RNA is isolated from cells in culture or tissues. Then, I. A reverse transcription reaction is carried out using one of three specific one-based anchored oligo-dT primers (e.g., H-T_{11}G). II. PCR amplification is performed by using the corresponding fluorescent-labeled anchored oligo-dT primer (RH-T_{11}G; R, rhodamine labeling) in different combination with arbitrary 13-mer primer (H-AP1). III. The amplified PCR products are separated on a denaturing polyacrylamide gel, and the fluorescent-labeled cDNA fragments visualized by a fluorescence scanner. A differentially expressed cDNA fragment in the electropherogram is denoted by an arrow. (Adapted from Ref. [7] with permission.) (*View this art in color at www.dekker.com.*)

sequences within a mRNA molecule; all led to high false-positive rates.[1–7] Therefore many modifications were periodically made to the original protocol to improve these intrinsic problems and to achieve specificity and efficiency as discussed below.

The original primer design was two-base anchored primer, but it resulted in suboptimal amplifications.[2] Three one-base anchored oligo-dT primers differing only at the last 3′ non-T base are now used. This modification cuts down the number of RT reactions needed for each RNA sample and minimized the redundancy and under-representation of certain RNA species due to the degeneracy of the process. Substitution of decamer arbitrary primers with rationally designed 13-mers has increased the accuracy of priming.[5] Furthermore, even longer primers (25–29 mers) and optimal dNTPs concentrations were reported to allow arbitrary priming after an annealing temperature of 40°C in the first PCR cycle, followed by more stringent PCR annealing at 60°C to provide specificity. Additionally, use of hot-start PCR and

other thermostable enzyme mixes suitable for long-length PCR resulted in highly reproducible and representative cDNA bands.[6] Moreover, the introduction of a restriction site at the 5′ end of both the anchored and arbitrary primers facilitated cloning of the cDNAs.[4]

Initially, isotopes labeled oligos, α-[^{35}S] dATP and later on [^{32}P]-end-labeled oligo-dT primer or α-[^{32}P] dATP, were used to autoradiographically detect amplified PCR products on sequenced gels.[2] Introduction of nonisotopic methods using digoxigenin and fluorescent dyes such as tetramethyl rhodamine allowed coupling of the display with digital data analysis, resulting in increased throughput.[2,7] Use of nondenaturing 6% PAG (i.e., without urea) to reduce double bands into a single band was found to reduce band complexity and eliminated several bands of DNA molecules derived from different fragments that occupied the same position in the gel.[8] Differential display–polymerase chain reaction is only capable of determining the 3′ region of the gene, so full-length cDNA can only be achieved either by doing rapid amplification of cDNA ends (5′-RACE) or by probing a cDNA library.[1,7]

Differential display was reported to tolerate a broad range of annealing temperatures and elongation times. However, the major factors that impacted the reproducibility of the method were low concentration of dNTPs and random primers, which made PCR amplifications susceptible to pipetting errors. A final concentration of dNTPs >2 μM and arbitrary primers to 0.2 μM improved the reproducibility of DD.[9] In a recent study utilizing cervical cancer cell line Caski, it was found that—for most primer combinations—fourfold less cDNA and only 25 high-stringency PCR cycling produced reproducible complex band patterns with intensities that reflected 2- to 10-fold differences in expression levels (the most common levels of regulation).[10] To reduce or exclude the bands that are subject to statistical noise from consideration, it has been suggested to start with enough cDNA (i.e., about 30,000 to 100,000 molecules) to obtain 15 ng/mL of amplified product in 25–27 PCR cycles.[11] Although some publications reveal that as little as 1.1-fold amplification is detectable by DD-PCR, the threshold is not precisely known as an upper detection limit may be reached in this technique.[11]

If we consider that there are 15,000 genes expressed in a cell and each fingerprint contains ~70 mRNA species, then in an ideal situation if all mRNAs have the same probability of being displayed in each round, ~600 fingerprints (or 45,000 bands) are required to cover 95% of all mRNAs.[8] For a comprehensive analysis of all mRNA species in a given cell, statistical modeling that predicted at least 240 different DD primer combinations is needed.[3] Recently, an empirical determination of the comprehensiveness of DD-PCR in Chinese hamster ovary fibroblast HA-1 cell line that received (or not) hydrogen peroxide treatment used saturation DNA screening of 324 primer combinations. Results showed that a 100% comprehensive analysis by this technique is not possible regardless of the number of primer combinations. This may be due to a selective resistance to the identification of certain sequences by DD.[12]

Once the first few primer combinations have been tested, the results of the display should be examined keeping in mind that a redesign of the experiment should be made when >5% of the transcripts are differentially expressed, or if no differences exist.[11] It is instructive to keep in mind a few guidelines when performing DD-PCR: 1) drastically different conditions should not be compared; 2) the DD reaction should be repeated for each sample to control for false positives; and 3) multiple samples should be used for each DD experiment in order to provide internal controls.[13]

OTHER DD STRATEGIES AND IMPROVEMENTS TO THE ASSAY

Another strategy to provide RNA fingerprints employed an arbitrary primer instead of an anchored oligo-dT primer in the first step of RT, selecting those regions internal to the RNA that have 6–8 base matches with the 3′ end of the primer. As in DD-PCR, this is followed by arbitrary priming of the resulting first strand of cDNA with the same or a different arbitrary primer, and then PCR amplification. This method is known as RAP-PCR.[14] This method samples anywhere in the RNA, including opening reading frames, and can be used on RNAs that are not polyadenylated (such as bacterial RNA). A limitation of this method too is that rare RNAs will be underrepresented.[1]

Differential display strategies designed to target specific sequences in bacteria, such as highly iterated palindromic (HIP) elements found in half of the genes in the genome of cyanobacteria, provided a convenient global expression strategy for identifying light intensity-regulated genes in bacteria using a limited number of primers.[15] Another area of wide application of DD is a study of scarcely sequenced and complex plant genes, which contain large families of homologous genes.[16]

In order to improve the specificity of DD, attempts were made to replace the 10–13-base arbitrary primers—which hybridize nonspecifically to cDNA templates causing mismatches—with primer sites produced by restriction enzyme digests of double-stranded (ds) cDNAs; thus allowing adapters for priming sites to be ligated into specific regions on the cDNA. These ds cDNAs are then amplified with either anchored oligo-dT

primers combined with ligated primers, or with ligated primers alone, followed by cloning into a vector, amplifying, restriction digest again, sequencing, and identifying the produced fragments. Several of these restriction fragment length polymorphism (RFLP)-based DD strategies, which are variation on the same theme, have been devised, each with its name such as amplification of ds cDNA ends restriction fragments (ADDER), amplified differential gene expression (ADGE), gene calling, ordered differential display (ODD), RFLP-coupled domain-directed DD (RC4D), and total gene expression analysis (TOGA).[1,7] However, RFLP-based strategies require more experimental steps such as second-strand cDNA synthesis, restriction digestion, and ligation of adapter primers over traditional DD, which can lead to error, and their precision over DD-PCR has not been substantiated in controlled experiments.[7]

APPLICATION OF DD TO BIOLOGICAL SYSTEMS

Because of the simplicity, specificity, and versatility of DD, it has been applied to countless applications more than any other genome expression profiling method in different fields of biomedical research as: studying gene expression in many phyla, polymorphism and gene silencing, identifying substrates for RNA-binding proteins, disease diagnosis and prognosis, cell cycle regulation, apoptosis, cancer research, cardiovascular diseases, neuroscience, endocrinology, immunology, and plant science.

A comprehensive compilation of DNA publications in various fields can be accessed through the website of GenHunter® Corporation (http://www.differentialdisplay.com), a company established by inventors of the DD-PCR.[3]

COMPARISON WITH OTHER EXPRESSION PROFILING TECHNOLOGIES

The number of methods employed for gene expression analyses is numerous; however, there are now only a few DD routinely used approaches such as PCR-based, subtractive hybridization (SH), serial analysis of gene expression (SAGE), and microarrays. Additionally, there are variations on the theme that modify or combine features of these basic approaches.

A bottleneck in all variants of DD has been the confirmation of differential expression. Isolation and identification of clones, Northern blots, or RT-PCR analyses are time- and labor-intensive techniques. Therefore several attempts have been made to combine DD with microarrays to facilitate parallel identification of frequently expressed tags simultaneously in many biological systems.[17,18] Furthermore, probes generated by DD from archived experiments can now be reamplified and used as sensitive probes for cDNA microarray studies, revealing more information than was yielded when originally resolved as fingerprints on PAG.[19] The use of fluorescent probes[20] combined with robotics for liquid dispensing and tube handling/rotation, digital analysis, and specialized computer programs for data acquisition, analysis, and storage increases method throughput and facilitates data interpretation.[1,7]

Another strategy combined DD with SH, rationalizing that removing the most commonly expressed mRNA and then displaying the remaining mRNAs might improve displaying.[21]

Table 1 illustrates the advantages and disadvantages of major approaches to gene expression studies. It can be seen that no one method is superior to any other in all parameters surveyed. Ultimately, the correct identification of the gene and its transcript in the hand of the

Table 1 Comparison among major approaches to genome expression profiling

Parameter	DD	SH	SAGE	Microarrays
Year published	1992	1985	1995	1995
Type of system	Open	Open	Open	Closed
Sensitivity	Moderate	High	High	Moderate
Specificity	High	High	High	Moderate
Quantification	Relative	Relative	Absolute	Relative
Previous knowledge of sequence	No	No	No	Yes
Detects novel genes	Yes	Yes	Yes	No
Labor intensity	Moderate	High	High	Moderate
Commercial services	Few	Very few	Few	Many
Cost	Moderate	Low	Moderate	High
Number of publications since inception[a]	3079	1197	386	3233

[a]Based on Medline search via Ovid on 5th of January 2004.

experimenter will attest to the appropriateness of the method to its intended application.

CONCLUSION

Differential display is a systematic, sensitive, and convenient approach that allows many samples to be tested in parallel, and does not require any previous knowledge of mRNA or gene sequence, making it an open system. Initially, DD analysis suffered from a high rate of false positives (or noise) due to the use of short primers and low annealing PCR temperature, and from redundancy in display because short primers may anneal to different parts of the same transcript. However, progressive technical improvements as well as care in experimental design have reduced these shortcomings. Because of its simplicity, sensitivity, reproducibility, and yield to automation, which increases throughput and accuracy, DD, either by itself or in combination with another expression profiling method, has become one of the most widely used methods for studying differentially regulated genes in many biological systems.

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Differential Sequencing by Mass Spectrometry

Christian Jurinke
Christiane Honisch
Dirk van den Boom
SEQUENOM Inc., San Diego, California, U.S.A.

INTRODUCTION

The publication of the human genome sequence in 2001 and the completion of about 140 prokaryotic genomes affirm the success of worldwide genome sequencing projects. The availability of new reference sequence information of various organisms is continuously growing.[1]

Differential sequencing—the systematic comparison of genomic sequences with respect to reference sequences, also called resequencing—represents a central focus of current genome analysis. The exploration of single-nucleotide polymorphisms (SNPs) is one of the most prominent approaches to extract the medical and biological value of genome-sequencing data and to elucidate inter- and intraspecies genetic variations.[2] The analysis of SNPs in the human genome will have a significant impact on the identification of disease susceptibility genes and drug targets and will facilitate the development of new drugs and patient care strategies. Large-scale analysis, detection, and discovery of genetic variability and implementation of dense SNP maps are eminent and push technological development toward high-throughput, cost-efficient applications.

Differential sequencing of infectious agents for fast and reliable identification and typing is an important aspect in the field of molecular diagnostics and epidemiology, including outbreak tracking and classification of pathogens. Fast and highly accurate tools for identification, monitoring, and treatment control are in demand. This article describes the use of mass spectrometry to rapidly identify and localize variable genomic regions based on reference sequences, thus facilitating high-throughput differential sequencing with a gamut of applications.

GENOME ANALYSIS BY MASS SPECTROMETRY: HISTORY AND STATE-OF-THE-ART

Analysis of nucleic acids by mass spectrometry has mainly been accomplished by two common soft ionization techniques—ElectroSpray Ionization (ESI) and Matrix-Assisted Laser Desorption/Ionization (MALDI) mass spectrometry (MS).

The use of MALDI coupled with time-of-flight mass spectrometry (MALDI-TOF MS) has become one of the leading technologies for high-throughput analysis and high-fidelity measurement of nucleic acid sequence variations. Unambiguous detection of known polymorphic sequences has been demonstrated, even in various formats.[3–5]

Early attempts to apply MALDI-TOF to de novo sequencing as an alternative to separation and detection of Sanger sequencing ladders[6] were hindered by physical analyte fragmentation, limited mass resolution, and mass accuracy in the high mass range. Despite several promising biochemical strategies, which generate truncated DNA sequence fragments of a sequencing primer analogous to dideoxy sequencing, routine-read lengths exceeding 100 bp have never been achieved.[7–9]

In addition to the primer extension-based Sanger sequencing approach, several chemical and enzymatic DNA fragmentation approaches have been proposed to generate short-based specifically cleaved MALDI-TOF analytes.

A chemical cleavage approach utilized P3′–N5′-phosphoamidate-containing DNA replacing dCTP or dTTP by their analog P–N modified nucleoside triphospates. Acidic reaction conditions induce base-specific cleavage. However, the required acidic conditions produce unwanted depurination by-products and base loss of adenine and guanine.[10]

A uracil–DNA–glycosylase (UDG)-treatment approach uses strand-separated polymerase chain reaction (PCR) products to generate T-specific abasic sites. Subsequent alkaline and heat treatment induces base-specific DNA cleavage at each T-specific positions.[11] DNA regions of interest require incorporation of dUTP instead of dTTP during PCR. Strand separation is performed by solid-phase separation on streptavidin-coated magnetic beads, which complicates the automatic handling of the assay.

Homogeneous assay formats requiring only subsequent addition of reagents are preferred. Post-PCR in vitro transcription systems combined with base-specific cleavage of the RNA transcripts overcome the issues

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encountered with classical DNA amplification and primer extension reactions.

The current state-of-the-art differential sequence analysis concept uses a Maxim–Gilbert-like approach: base-specific cleavage of nucleic acid amplification products. PCR amplification of the locus of interest is followed by in vitro transcription and base-specific cleavage.[12] This novel comparative resequencing scheme combines a homogenous in vitro transcription/RNAse system with MALDI-TOF analysis of molecular fragment masses—an intrinsic molecule property. No labeling is required. PCR products of up to 1 kb are subjected to in vitro transcription. Subsequent cleavage of the in vitro transcripts by ribonucleases (e.g., RNase A) generates base-specifically cleaved RNA fragments. Sequence-specific mass signal pattern within a mass range of 1000–9000 Da—equivalent to 3–30 nucleotides—are obtained. The result is a characteristic pattern of RNA fragment masses indicative of the original reference sequence.

RNase T1 (a guanine-specific endonuclease) cleaves in vitro transcripts base—specifically at every G-position.[13] An available alternative is RNase A, which cuts specifically at the 3′-end of the pyrimidine residues C and U.

RNA analytes are more stable and less prone to depurination than DNA during the desorption/ionization process in MALDI-TOF MS because of a balancing effect

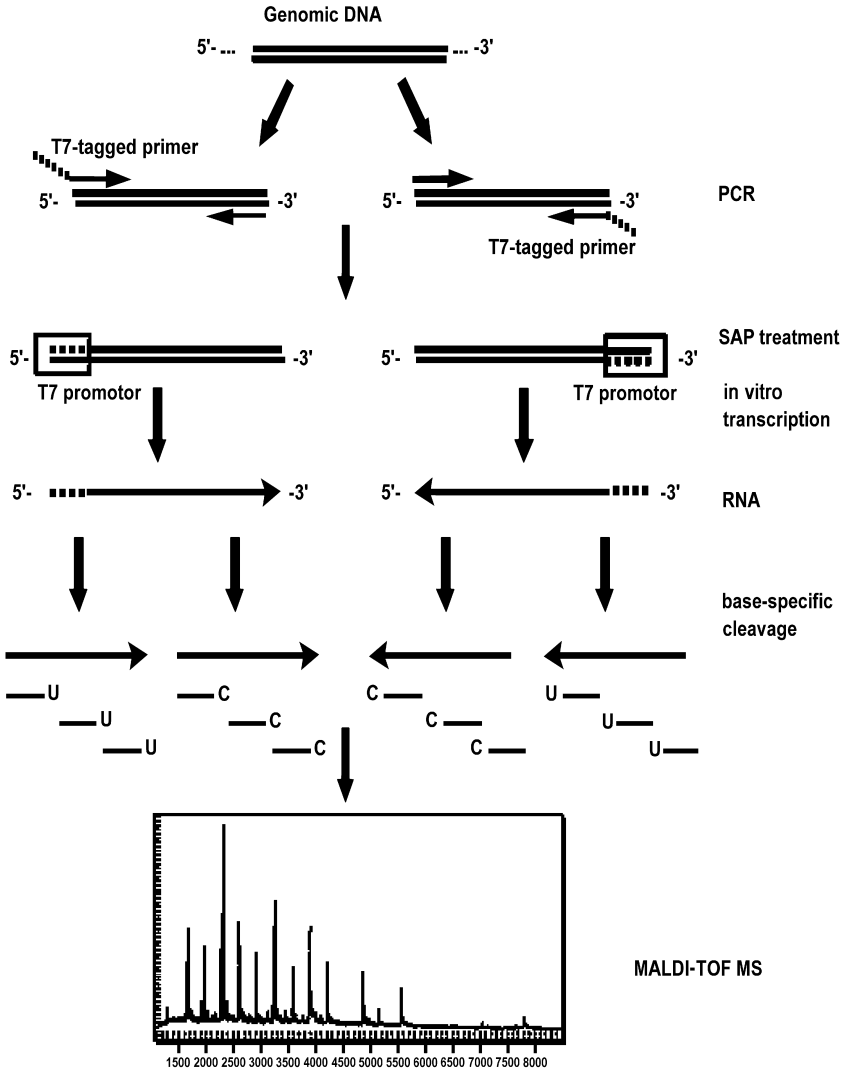

Fig. 1 Base-specific cleavage by MALDI-TOF mass spectrometry. A single-stranded copy of a PCR-amplified target sequence is generated by T7-mediated in vitro transcription and cleaved in four reactions at positions corresponding to each of the four bases. RNA of the forward strand is cleaved at U or C. An A- and G-specific cleavage of the template sequence is facilitated by U or C cleavage of the reverse RNA strand. MALDI-TOF acquisition of spectra of each of the cleavage reactions is followed by comparison to reference sequence derived in silico cleavage pattern.

of the 2′-hydroxy group on the polarization of the *N*-glycosidic bond of the protonated base.

Figure 1 illustrates this robust biochemical scheme for high-throughput differential sequencing. Two PCR reactions of the DNA region of interest introduce a T7-promotor in the forward strand as well in the reverse strand of the amplification product. PCR is followed by shrimp alkaline phosphatase (SAP) treatment to dephosphorylate any unincorporated desoxy-NTPs. RNA polymerase, ribonucleotide, and nuclease-resistant nucleotides are added to the mixture. In vitro transcription generates single-stranded RNA and facilitates further amplification. The RNA is subject to four base-specific cleavage reactions corresponding to each of the four bases. Reactions are driven to completion. This reduces the RNA target to a specific set of RNA compomers in each of the reactions. Analytes are desalted by addition of ion-exchange resin, conditioned, and identified in a single MALDI-TOF MS measurement per reaction. Four sequence-specific mass signal patterns are generated. All cleavage products are consistent with 5′-OH and 3′-phosphate groups, except for the 3′-fragment of the full-length transcript possessing a 3′-OH group.

The experimental set of compomers is used to reconstruct the sequence by cross-comparing the information of the four cleavages to the in silico cleavage pattern of the known reference sequence. Deviations of the pattern indicate sequence changes (Fig. 1).

Figure 2 shows base-specific, cleavage-mediated discovery of a [C/T] sequence change in a 500-bp DNA region of interest. The target region is analyzed by C- and T-specific cleavage of the forward, as well as the reverse strand—equivalent to four base-specific cleavage reactions. Overlays show spectra of the wild-type as well as heterozygous and homozygous mutant samples.

For the wild-type sample [C/C], mass signals of all cleavage reactions can unambiguously be identified based on the reference sequence derived in silico cleavage

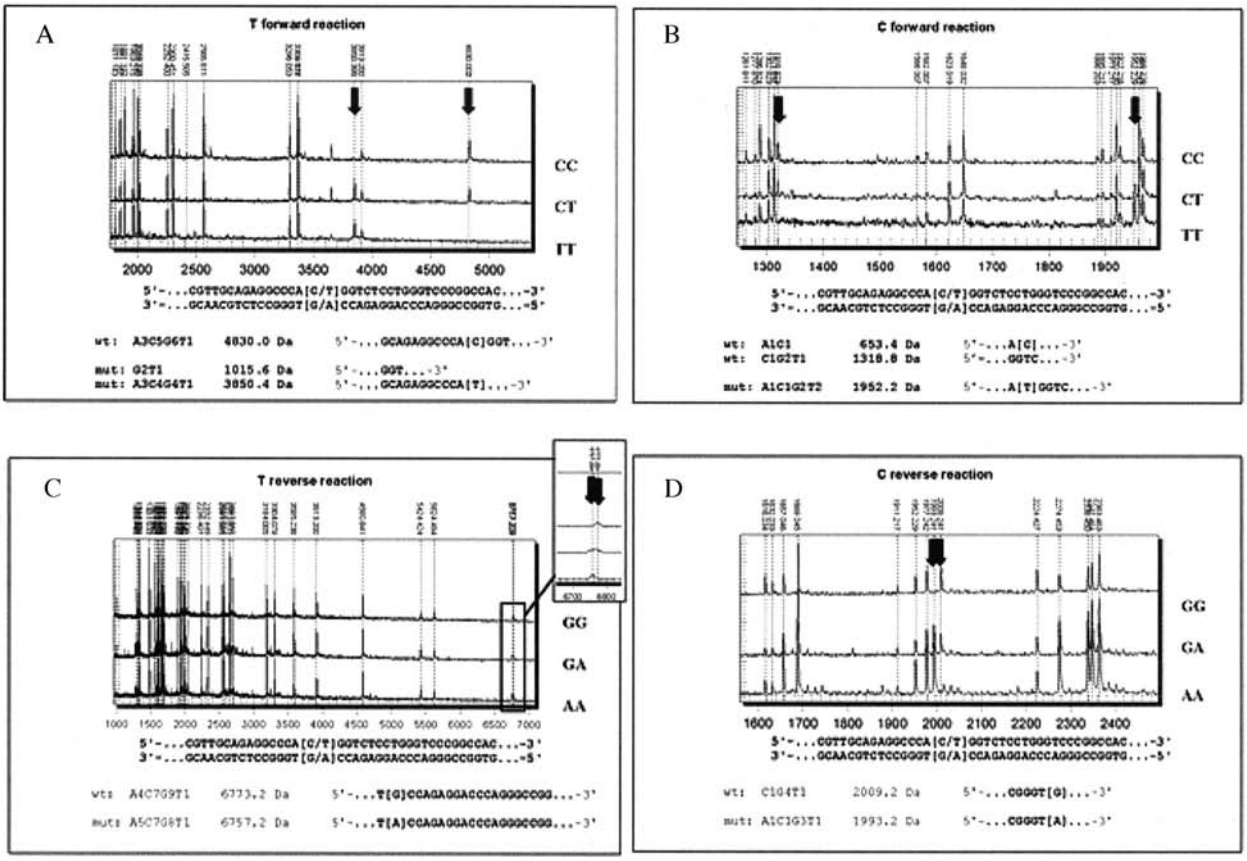

Fig. 2 Identification of a single nucleotide polymorphism by MALDI-TOF MS analysis. Panels A–D show the four base-specific cleavage reactions. Each spectrum reveals the changes resulting from the substitution of a C wt-allele for a T mutant-allele. Spectra of all three different genotypes are overlaid. Changes are indicated by arrows. (A) T-specific cleavage of the forward transcript. (B) C-specific cleavage of the forward transcript. (C) T-specific cleavage of the reverse transcript. (D) C-specific cleavage of the reverse transcript. Spectra are shown covering all detected signals. All signals affected by the sequence change are indicated.

pattern. Deviations from the in silico pattern, detected as a mass shift, the absence of an existing peak, or the appearance of an additional signal, lead to the identification of the sequence variation.

In the T-specific cleavage reaction of the forward transcript, a sequence change from C to T at position 371 of the target region introduces a new cleavage site and splits the 15-bp wild-type fragment into a 12- and a 3-bp fragment. For the mutant [T/T] sample, this results in the disappearance of a mass signal at 4830.0 Da and the appearance of mutant-specific mass signals at 3850 and 1015.6 Da (signal not shown). Spectra containing all of the signals correspond to the heterozygous sample [C/T].

The C-specific cleavage reaction on the forward strand confirms the observation of the identified [C/T] substitution. A cleavage site is removed from a dimer A[C] and generates a 6-bp fragment of 1952.2 Da with the adjacent 4-bp fragment of 1318 Da.

Confirmatory information is generated from the reverse RNA transcript of the target region. Both the T- and the C-specific cleavage reaction generate mass shifts of −16 Da corresponding to an exchange of G vs. A in the affected fragment (Fig. 2).

In conclusion, a heterozygous sequence change can generate up to five discriminatory observations in a mass spectrum by adding or removing a cleavage site, as well as shift the mass of single products by the mass difference of an exchanged nucleotide. Up to 10 observations might be the result of a homozygous sequence change, because not only additional but also missing signals can be utilized for SNP identification.

Mono-, di-, and trimer nucleotides are usually non-informative. They are excluded from the analysis because of coinciding fragments, and their detection is also diminished because of analyte carrier matrix signals within the low mass range.

The combined observations of all cleavage reactions allow for the unambiguous detection, identification, and localization of almost all sequence changes. The inherent redundancy of information from all cleavage reactions substantiates the reliability of the results. Additional supportive information is obtained when signals are correlated across a multitude of samples.

A simulation of arbitrary 500-bp amplicons in the human genome showed that about 99% of all theoretical sequence changes can be detected and characterized.[12]

A homogeneous 384-assay format enables automated processing with liquid handling devices. Nanoliter dispensing onto matrix-coded chip arrays are utilized for automated, reproducible MALDI-TOF measurements. TOF instruments acquire data with turnaround times of 2 sec per sample at a standard 20-Hz laser repetition rate as opposed to hours of analyte separation in conventional sequencing gel electrophoresis. A single MALDI-TOF mass spectrometer can thus scan 2.5 million bp in every 24 hr.

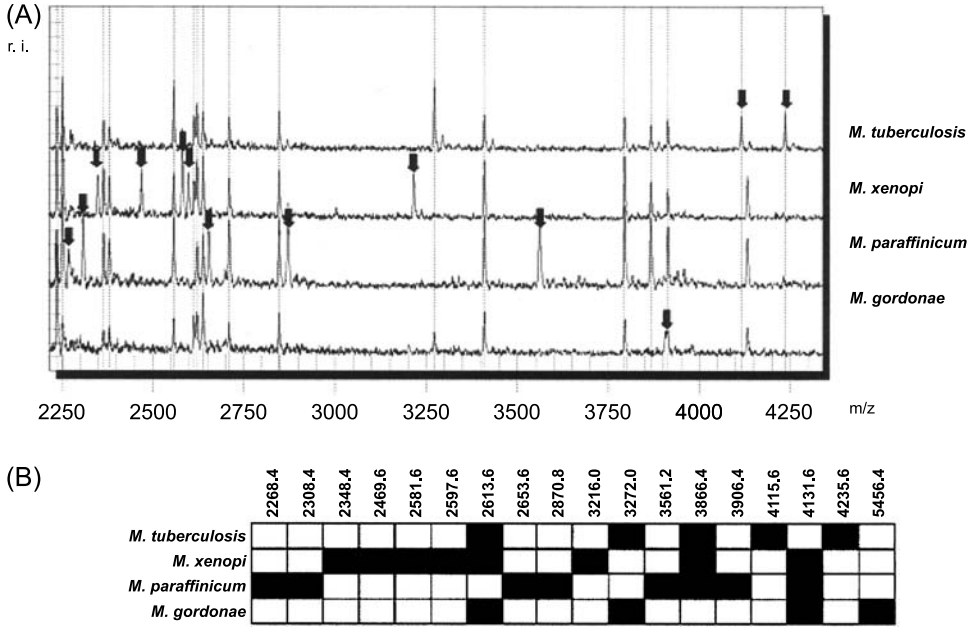

Fig. 3 Pathogen identification by mass spectrometry. (A) Overlay of mass spectra of C-specific forward 16S rDNA fragments of *Mycobacterium tuberculosis* ATCC27294, *Mycobacterium xenopi* DSM43995, *Mycobacterium paraffinicum* DSM44181, and *Mycobacterium gordonae* DSM44160. Identifier peaks are marked with arrow. (B) Barcode of C-specific forward 16S rDNA discriminatory mass fragments.

Real-time quality control of mass spectra results in highly reproducible mass signal patterns facilitating automated peak-pattern interpretation. Signal-to-noise ratio changes can be used as supporting information for sequence changes. A recent software enables automated high-throughput SNP discovery and mutation detection. Time-efficient algorithms calculate the most reasonable explanations for observed mass signal changes, and discover and pinpoint sequence variations based on the information content of the four cleavage reactions.[14]

DIAGNOSTIC APPLICATIONS

MALDI-TOF MS of nucleic acids after base-specific cleavage improves the odds of differential sequence analysis and sets a significant milestone in the field of comparative genomics and genetics. The feasibility of the system exceeds SNP discovery applications. The method facilitates efficient scoring of large numbers of genetic markers in selected populations determining genotypic and phenotypic correlations.

Base-specific cleavage of signature sequences results in species-specific mass signal pattern of the region of interest. These species-specific fingerprints can be utilized to discriminate prokaryotes—bacterial or viral organisms—to the genus-, species-, or strain-specific level and thus facilitate pathogen identification.[15] Figure 3 gives an example of the unambiguous identification and differentiation of four mycobacterial species based on their characteristic mass signal fingerprints of the 16S rRNA gene sequences. All expected signals were identified in the spectra and unique identifier signals marked by arrows could be unambiguously assigned to expected discriminatory species-specific fragments (Fig. 3).

Further expansion of the application portfolio includes the detection of epigenetic modifications, molecular haplotyping, and mutation detection.

Analysis of DNA methylation-mediated gene silencing is seen as a valuable diagnostic tool in cancer research and diagnostics. Qualitative as well as quantitative high-throughput MALDI-TOF DNA methylation analysis relies on bisulfite treatment of genomic DNA, PCR amplification followed by base-specific cleavage. The bisulfite treatment converts nonmethylated cytosine to uracil, while methylated cytosines remain unmodified. As a result, methylated vs. nonmethylated mass signal patterns show significant differences, which allow for differentiation as well as identification of individual sites of methylation in a target sequence. In addition, comparative quantitation of mass signals can be utilized to determine the relative abundance of methylated vs. nonmethylated target gene regions.[16]

Haplotypes are defined as the collection of genotypes found in a single allele or chromosome. Their unambiguous identification provide additional power in the detection of genes involved in common diseases. This can contribute to a better understanding of the complex etiology of diseases. Long-range, allele-specific PCR using nonextendable exonuclease resistant competitor oligonucleotides is performed to isolate desired alleles/chromosomes prior to base-specific cleavage and MALDI-TOF detection. The analysis is not exclusive to known markers, and novel associated subhaplotypes can be discovered resulting in comprehensive haplotype information of the loci of interest.[17]

Additional fields of application include large-scale characterization of cDNAs and alternative splice variants—further steps in the attempt to elucidate the genetic code and individual variations.

CONCLUSION

MALDI-TOF mass spectrometry of base-specifically cleaved nucleic acids is a valuable expansion of the method portfolio in the field of comparative genomics, and opens new routes for diagnostic sequencing applications. MALDI-TOF MS combines the determination of an analyte-specific physical property—the molecular mass—with speed of signal acquisition and high accuracy. This results in a high degree of automation and throughput. The inherent redundancy of observations supporting the discovery of sequence changes is up to five times higher compared to sequencing. This feature should propel MALDI-TOF MS as a gold standard in the fields of SNP discovery, mutation detection, pathogen identification, methylation detection, haplotyping, and cDNA characterization.

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Disease Gene Patents

Jon F. Merz
University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, U.S.A.

INTRODUCTION

This article examines what it means to patent a gene. Numerous ethical concerns have been raised about the effects of such patents on clinical medical practice, as well as on research and development. I describe what kinds of inventions are covered by human gene patents, give examples, and summarize the small body of empirical research performed in the United States that examines the effects of these patents. There is little evidence that early fears about restraining research and constraining the promises of genetic medicine will come to fruition. Nonetheless, there are areas of concern, and policy makers, physicians, and the public should keep their eyes on how genetic patents are being used to ensure that the net social benefits of patenting human genes are maintained.

GENE PATENTS

Nearly 30,000 human genes have been patented in the United States (R. Cook-Deegan, personal communication, 2003). Patents will often be secured in countries throughout the world where the patent owner thinks there may be a viable market. Patents are granted by the U.S. government to inventors for new, nonobvious, and useful inventions and discoveries, and similar standards of patentability are applied around the globe. A patent grants to its owner the right to exclude others from making, using, or selling a patented machine or composition of matter, or from using a patented method, for a period of 20 years from the date of filing a patent application.

A patent grants what is called a negative right—the right to enjoin others from using the invention without permission, and a patent owner may turn to the federal government to use its judicial and police powers to block others from making, using, or selling the invention, and to collect damages from those who infringe. A patent owner generally is not under any legal compunction to ''work'' or license others to use a patented invention, and, as a general rule, a patent even may be used wholly to keep products from coming to market.[1]

Human gene patents result from the cloning and description of the sequence of a gene, the role or function of which is somewhat understood. The U.S. Patent and Trademark Office requires that a patent applicant make a credible assertion of the specific and substantial utility of the genetic invention.[2] Under this kind of rationale, early patent applications by the U.S. National Institutes of Health covering thousands of expressed sequence tags, which are unique nucleotide strings randomly culled out of the genome but have no known function other than as a distinctive marker, were disallowed.

Gene patents cover three distinct types of invention: 1) diagnostics; 2) compositions of matter; and 3) functional use. I will discuss each in turn, with examples and highlights of areas of concern and what is known about each. This overview is centered on U.S. patent law and what is known about how gene patents are being used in the United States. Some of the problems discussed have begun to spill over to Europe and Canada. This is not meant to be a comprehensive international review,[3] but only an attempt to demonstrate the breadth of gene patents, discuss concerns about how they are being used, and summarize relevant empirical data.

First, what we have called disease gene patents *claim* (claims define the scope of patent protection) the characterization of an individual's genetic makeup at a disease-associated locus when performed for the purpose of diagnosis or prognosis.[4] These patents typically cover all known methods of testing, including the use of hybridization, Southern analysis, polymerase chain reaction (PCR), and even DNA chips. Because the fundamental discovery patented is the statistical observation of a genetic difference and a phenotypic difference (such as the occurrence of disease), then any method for testing for that genetic difference can be covered by the patent.[5]

Well-known examples of disease gene patents include those covering genes implicated in breast and ovarian cancers (*BRCA1* and *BRCA2*), colon cancers (*HNPCC* and *FAP*), cystic fibrosis (*CFTR*), hemochromatosis (*HFE*), and a growing number of neurological diseases including late-onset Alzheimer's disease (AD; *Apo-E*), Canavan disease, Charcot–Marie–Tooth disease (*CMT-1A* and *CMT-X*), spinal muscular atrophy (*SMN1*), spinocerebellar ataxia (*SCA1–SCA12*), and others.

There are several characteristics of genes and disease gene patents that demonstrate how the genome is being divided by small patent claims to overlapping genetic

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territory. First, any one gene may have multiple patents claiming the diagnosis of different polymorphisms. Thus several patents have been issued for testing of different mutations in the *CFTR* gene.[6] Furthermore, some diseases (at least the phenotypic expressions of them) are caused by multiple genes, such as Charcot–Marie–Tooth disease.[7] Questions about ownership and access get messy when there are many hundreds of known mutations in multiple causative genes, as exemplified by *BRCA1* and *BRCA2*, for which there are at least a dozen U.S. patents on tests of these two genes.[8] Finally, patents can issue on the same exact molecular test when it is performed for different diagnostic or prognostic purposes. For example, an *Apo-E* test, in which the number of E2, E3, and E4 alleles carried by a patient is assessed, can be performed to: 1) determine whether a patient is at risk for early-onset AD;[9] 2) assess an AD patient's prognosis;[10] 3) determine a course of therapy based on pharmacogenetic receptivity;[11] and 4) assess a patient's prostate cancer risk.[12] In each of these cases, a patent thicket is created, which can lead to difficulties in securing licenses and expenses in paying multiple ''stacked'' royalties to multiple patent owners.[13]

To the best of our knowledge, the owners of the overwhelming majority of issued gene patents have not aggressively enforced their rights against clinical molecular diagnostics laboratories. Nonetheless, a majority of genetics laboratories across the United States report that they have had one or more of the above disease gene patents asserted against them.[14,15] In some cases, these patent owners have been willing to grant a license to laboratories performing a home-brew test. Per-test royalties of which we have become aware include US$2 for the *Δ*F508 mutation of *CFTR* (University of Michigan), US$5 for Gaucher's disease (Scripps Institute), US$12.50 for Canavan disease (Miami Children's Hospital), and, reportedly, more than US$20 for *HFE* (Bio-Rad). In some cases, an up-front license fee has been demanded as well.[16]

Of course, clinical and research laboratories typically pay royalties for the use of patented technologies. For example, the price of widely used PCR machines and reagents includes a premium paid for the exclusivity granted by the patents. In addition, a royalty of about 9% is paid for all testings done by licensed laboratories.[14] The most recent patents enforced against biotechnology companies and testing laboratories are those that claim the extremely broad uses of intronic sequences for generating haplotypes and identifying allelic variation.[17] Disease gene patents vary in significant ways from these more typical patented tools that are used by laboratories for testing a variety of specific disease genes. Critically, because a disease gene patent claims all methods of testing for a specific gene, there is no plausible way of working around these patents and the patents may be used to monopolize a test.

Fortunately, in only a handful of cases have patent owners refused to grant licenses to laboratories to allow them to perform specific tests. Instead, these owners have used the patents to monopolize the testing service and to require physicians and laboratories to send samples for testing to the owner or its limited licensees. Thus, tests for breast and ovarian cancer genes (Myriad Genetics) and a set of neurological disorders (Athena Diagnostics) are generally available from only these commercial laboratories. SmithKline Beecham Clinical Laboratories made a brief attempt at capturing the testing market for hemochromatosis before the business unit was sold to Quest Diagnostics.[16] Myriad has extended its reach beyond the U.S. borders, seeking to enforce its *BRCA* patents in France,[18] Canada,[19] and the UK.[20] The test for Canavan disease, despite being easily included in panel assays that many laboratories can run, was restricted to selected laboratories around the United States by the patent owner.[21]

In these cases, laboratories have been told where patient samples must be sent to have the patented tests performed and how much it will cost. Being compelled to stop providing testing services has serious implications for the ability of molecular pathologists to maintain currency in their field, to treat their patients with comprehensive medical services, to train residents and fellows, to perform research, and to run their laboratories in an efficient manner. Hospital-based laboratories must often eat part of the fixed monopoly costs of the tests, which they are compelled to offer patients but for which health insurance may not cover the full price. Seen in this light, these patents raise the costs of clinical services and restrict physicians' ability to practice medicine.[1,22]

The second broad type of genetic invention relates to compositions of matter (i.e., chemicals and materials), including the isolated and purified gene (cDNA) and all derivative products (e.g., recombinant proteins or drugs, viral vectors, and gene transfer ''therapies,'' and transfected cells, cell lines, and higher-order animal models in which the patented gene has been inserted or knocked out). Examples of recombinant products include insulin and human growth hormone. According to the Biotechnology Industry Organization, there are more than 155 biotechnology drugs and vaccines that have been approved by the U.S. Food and Drug Administration, and more than 370 others in clinical trials.[23]

The primary concern about gene patents is that they will make it more difficult to perform research. In the United States, there is no statutory research exemption, but only an extremely narrow court-defined exemption. As recently summarized by the Court of Appeals for the Federal Circuit in a suit against Duke University,

''regardless of whether a particular institution or entity is engaged in an endeavor for commercial gain, so long as the act is in furtherance of the alleged infringer's legitimate business and *is not solely for amusement, to satisfy idle curiosity, or for strictly philosophical inquiry*, the act does not qualify for the very narrow and strictly limited experimental use defense.''[24] Duke University was not excused from potential infringement of patents covering laboratory equipment simply because the equipment was used solely for research and educational purposes, which are the core of Duke University's business. A strong argument can be made that the research exemption should be much broader, encompassing research aimed at better understanding of the claimed invention, such as how it works and whether it works as taught by the patent, how to improve on it, and how to work around it. Indeed, practically speaking, this may in fact be how patents are most commonly used. The patent law trades a period of exclusivity for disclosure and the teaching of an invention to the trade, and competitors should not have to wait for the period of exclusivity to end before learning from that disclosure and attempting to improve on it.

Little is known about how gene patents are being used, and whether they have a net beneficial or detrimental effect on scientific research and commercial product development. Patents clearly are seen as a necessary stimulus for the infusion of venture and risk capital in the biotechnology industry; less clear is the role patents play in motivating academic researchers. Little data have been generated about the licensing of biotechnology patents. These studies suggest that most genetic inventions are not patented, but when they are, they are licensed on exclusive terms.[25,26] In turn, researchers and firms appear to have developed various strategies to minimize the potential detrimental effects of the patents.[27] Nonetheless, much remains unknown about the effects of these practices on basic research and commercial competition.

Finally, a third and emerging class of gene patents is that claiming the functional use of a gene. These patents are based on the discovery of the role of genes in disease or other bodily and cellular functions or pathways, and claim methods and compositions of matter used to up-regulate or downregulate the gene. For example, a patent that was recently invalidated claimed methods and compositions of matter for the selective inhibition of the *Cox-2* gene, which prevents inflammation and pain. The patent was invalidated because the patentee, the University of Rochester, failed to disclose a chemical entity that would perform such selective inhibition.[28] The patent claimed the mechanism by which two drugs, which later came to market, work—Celebrex, which is comarketed by Pharmacia (of which Searle is part) and Pfizer, and Vioxx, which is marketed by Merck.

A case similar to the *Cox-2* litigation involves a patent awarded to Harvard and MIT and exclusively licensed to Ariad Pharmaceuticals. The patent claims the basic regulation of any gene by reducing the intracellular activity of the transcription factor NF-kB.[29] On award of the patent, Ariad Pharmaceuticals sued Eli Lilly for infringement by their osteoporosis drug Evista and their sepsis drug Xigris, and has asserted the patent against numerous other companies. Eli Lilly's patent applications for these two compounds themselves predate the filing of the NF-kB application.[30] Ariad Pharmaceuticals should have a hard time winning, both because, like the *Cox-2* inhibition patent, the NF-kB patent fails to disclose specific agents for regulating the factor and because the company is trying to assert its patent in a way that would remove from the market chemical entities that predated the discovery and disclosure of the functional pathway by which those drugs work.

Finally, we have the case of Viagra. Pfizer, which has had its erectile dysfunction drug Viagra on the market for several years, recently received a patent claiming the molecular pathway by which Viagra works. The patent claims any selective PDE5 inhibitor used to treat impotence.[31] Immediately on allowance of its patent in late 2002, Pfizer sued Bayer and GlaxoSmithKline for their drug Levitra, and Eli Lilly and their partner Icos for their drug Cialis, both of which were proceeding toward Food and Drug Administration (FDA) approval.[32] The difference between the Viagra case and the *Cox-2* case is that Pfizer actually has and claims a specific class of drugs that work by the claimed functional pathway. Whether this is an adequate basis on which to allow Pfizer to lay claim to all drugs that work by the same molecular mechanism is a fundamental legal question that looms over the pharmaceutical industry.

CONCLUSION

We see that ''gene patents'' cover a broad range of invention. Each type has its own potential uses and marketable products, and each raises potential problems depending on how the patents are used in the relevant marketplace. Much remains unknown, and, indeed, the market is still adapting to these patents. Thus it is extremely important to continue to study and monitor how gene patents are being used, licensed, and enforced to develop policy interventions if deemed necessary.

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Disposable Electrochemical DNA Biosensors

Kagan Kerman
Ege University, Bornova, Izmir, Turkey
Japan Advanced Institute of Science and Technology, Ishikawa, Japan

Mehmet Ozsoz
Ege University, Bornova, Izmir, Turkey

INTRODUCTION

Electrochemical DNA hybridization biosensors have been a major research field for over a decade, because their rapid, simple, and low-cost detection capabilities provide several advantages to clinical, forensic, and environmental monitoring. Basically, DNA biosensors convert the Watson–Crick base-pair recognition event into a readable analytical signal. A DNA biosensor is prepared by the immobilization of a single-stranded (ss) oligonucleotide (probe) onto a transducer surface to recognize its complementary (target) DNA sequence via hybridization. The DNA duplex formed on the electrode surface is called a hybrid. This binding event is then converted into an electrochemical signal by the transducer. The most important advantage of utilizing electrochemistry for transducing hybridization is its suitability for microfabrication. Thus compact, user-friendly, and handheld devices can easily be designed by combining electrochemistry and microfabrication.

THE ADVANTAGES OF USING DISPOSABLE ELECTROCHEMICAL DNA BIOSENSORS

The miniaturization of electrochemical DNA biosensors[1–3] enabled the mass production of disposable sensor strips. These sensor strips have gradually taken the place of the conventional three-electrode system consisting of bulky working, reference, and counter electrodes, and beakers with a large amount of buffer and analyte solution volume, mostly ranging between 1 and 2 mL. However, the disposable sensor strips alone can be described as disposable electrochemical cells, which contain the working, reference, and counter electrodes together on a minimal scale and space. A small droplet of the buffer and an analyte solution of about 20 μL would be sufficient for an electrochemical reaction to take place. Especially, the ''memory-effects'' resulting from the insufficient cleaning of the electrode surface have been completely eliminated with these single-use sensor strips. Thus more reproducible results could be obtained by using a new electrode for each experiment. Such a significant decrease in the required analyte volume, and increase in reproducibility while shrinking the required space in the laboratory, had a revolutionary impact on the field of electrochemical biosensors, so that the term ''on-field analysis'' has become possible with the help of the miniaturization of such electrochemical potentiostats and sensor strips, enabling ''bedside clinical diagnosis'' and ''on-site environmental monitoring.'' Even the diabetes patients themselves have become capable of measuring their own blood glucose levels by using such small handheld electrochemical devices in connection with sensor strips.[4] Nowadays, the biggest challenge facing electrochemists is to develop such a small device, which can perform nucleic acid-based tests with a simple and rapid procedure, as well as high sensitivity and selectivity.

DISPOSABLE ELECTRODE MATERIALS

Nowadays, two important electrode materials are in widespread use for disposable sensor technology: carbon and gold (Au).[5] Especially, carbon is desired because of its rich surface chemistry, low background current, wide potential window, low-cost, and chemical inertness. Disposable carbon electrodes are suitable for various kinds of surface modifications, thus enabling a major number of applications in many fields. However, the electron-transfer rates obtained from carbon electrodes are reported to be slower than those obtained from metal electrodes.[6] Recently, this major drawback has been overcome by the modification of the carbon surface with carbon nanotubes (CNT). Carbon nanotubes enabled enhanced electron transfer on electrode surface owing to their small size providing a larger active surface for easy DNA attachment.[7] Recently, CNT-based screen-printed electrodes (CNTSPE) have been fabricated by Wang and Musameh.[8] Carbon nanotube-based screen-printed electrodes with their well-defined electrochemical activity and mechanical stability are promising candidates for DNA-based testing.

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Highly oriented pyrolytic graphite electrodes (HOPGE) have also been attractive for electrochemical DNA biosensor research.[9–12] The renewal of HOPGE surface is also simple and rapid. A freshly cleaved surface of HOPGE can easily be prepared by contacting a piece of adhesive tape to the graphite surface, and then removing a thin layer of graphite with the tape. Thus the same electrode can be used for several different measurements. DNA and DNA–drug interaction were examined on thin-film mercury-coated HOPGE by Hason et al.[9] Voltammetric microanalysis of DNA adducts with osmium tetroxide,2,2′-bipyridine (Os,bipy) using a HOPGE provided the detection of 140 pg of DNA-Os,bipy after a 5-min accumulation period.[10] Anodic voltammetry and atomic force microscopy (AFM) imaging were performed for the detection of adriamycin and DNA interaction on HOPGE surfaces.[11] Atomic force microscopy surface characterization of the effect of pH and applied potential on the adsorption of DNA on HOPGE has recently been reported by Oliveira-Brett and Chiorcea.[12]

The use of a rigid carbon–polymer composite material as an electrochemical transducer in hybridization genosensors has recently been reported by Alegret and coworkers.[13–15] Graphite–epoxy composites (GEC) have an uneven surface suitable for strong DNA adsorption. Especially, ssDNA was reported to bind strongly to GEC in a way that prevents the strands from self-associating, while permitting hybridization with complementary DNA. Hybridization was detected by monitoring guanine oxidation signal[15] and also through biotin–streptavidin interaction using a streptavidin conjugated to horseradish peroxidase without nonspecific adsorption onto GEC, even when the surface was treated by blocking reagents.[14] Thus screen-printed GEC-based electrodes are also promising candidates for highly specific DNA hybridization detection.

Au is the noble metal of choice for screen printing of disposable sensor strips. Au strips offer a very favorable electron-transfer kinetics and a wide anodic potential range. The main disadvantage would be the limitation of the cathodic potential window.

The disposable electrode designs can be classified into two groups. The most generally used one is the screen-printed (thick-film) electrode (SPE). The second one is the recently developed pencil graphite electrode (PGE). Screen-printed electrode technology has developed rapidly with the help of the recent advancements in microfabrication; however, the PGEs still need to be improved to eliminate the use of external reference and counter electrodes. Whereas SPE is sufficient to perform an electrochemical analysis, a PGE still requires a beaker-type system. The important advantage of using PGE would be that the preparation does not require sophisticated instruments, because commercially available carbon graphite leads constitute the main source of PGEs. A carbon graphite lead with 1-mm i.d. provides a more comfortable electrode system in a beaker as well as a significantly bigger electrode surface with its cylindrical shape in comparison with the planar surface of the traditional Teflon-encased electrodes. A simple mechanical extrusion enables the renewal of the surface of PGE-based biosensors, hence obviating the need for an additional regeneration step and erasing memory effects.

DISPOSABLE DNA BIOSENSORS BASED ON PENCIL GRAPHITE ELECTRODES

Thus these low-cost and easy-to-prepare PGEs have been the ideal choice for several electrochemical DNA biosensor reports. An electrochemical DNA biosensor for the detection of the Factor V Leiden single-nucleotide polymorphisms (SNPs) from polymerase chain reaction (PCR) amplicons using the oxidation signal of colloidal gold (Au) was described by Ozsoz et al.[16] A pencil graphite electrode (PGE) modified with target DNA (Fig. 1A), when hybridized with complementary probes conjugated to Au nanoparticles, responded with the appearance of Au oxide wave at $\sim$+1.20 V (Fig. 1B).

The discrimination against homozygous and heterozygous genotypes has become possible by using specific oligonucleotides, which are full complementary to either wild-type (WT) or mutant (MT) real samples. When the WT probe, which had a complementary DNA base sequence to the WT target, was immobilized on Au nanoparticles, a high Au oxidation signal (Fig. 2a) showed

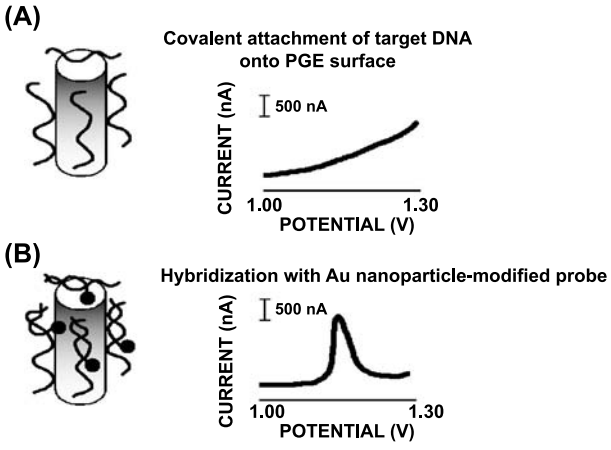

Fig. 1 Detection of hybridization using Au nanoparticle-modified probes. (A) Covalent attachment of target DNA onto pencil graphite electrode (PGE); (B) Au oxide signal appears after hybridization with the complementary Au nanoparticle-conjugated probe.

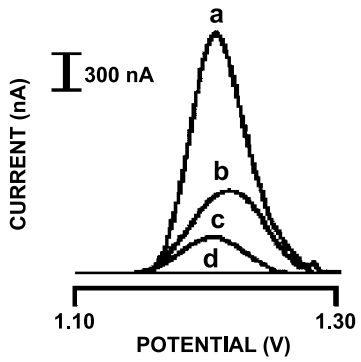

Fig. 2 Differential pulse voltammograms for the oxidation signal of Au at PGE in TBS at (a) wild-type (WT) probe-modified Au nanoparticle after hybridization with WT target; (b) mutant (MT) probe-modified Au nanoparticle after hybridization with WT target; (c) WT probe-modified Au nanoparticle after hybridization with no target on PGE surface; and (d) WT probe-modified Au nanoparticle after hybridization with non-complementary target.

that the PCR product contained WT DNA. When the MT probe was hybridized with the WT target, about one-half of the signal was obtained indicating the presence of SNP (Fig. 2b). Nearly a similar electrochemical response was obtained from the attachment of Au nanoparticle-modified WT probe onto bare PGE (Fig. 2c). No electrochemical signals could be obtained when the noncomplementary target was immobilized on the PGE surface (Fig. 2d).

The intrinsic redox activity of DNA was employed for detecting the duplex formation on a PGE-based biosensor by Wang et al.[17,18] Inosine-substituted probes and monitoring of the electrochemical guanine oxidation signal for hybridization events offer several advantages over the common use of external indicators including the appearance of a new peak, a flat background, and simplicity. Inosine is a hypoxanthine ribonucleoside, one of the basic compounds comprising cells, and also a precursor to adenosine and uric acid. In guanine signal-based electrochemical DNA biosensor schemes, inosine-substituted probes have been intensively used,[19,20] because they are electrochemically inactive. When an inosine-substituted probe-modified electrode is exposed to the target DNA, the guanine signal appears after hybridization. The selectivity of the new device of Wang et al.[17] was demonstrated for the detection of a SNP in the BRCA1 breast cancer gene. Such low-cost, renewable graphite transducers provided an attractive alternative to conventional carbon electrodes used for transducing DNA hybridization. The device offered a greatly simplified operation and held promise for decentralized genetic testing by eliminating the regeneration step and an external electroactive indicator.

DISPOSABLE DNA BIOSENSORS BASED ON SCREEN-PRINTED ELECTRODES

Recent progress in screen-printing technology with PC-controlled, fully automatic, and sophisticated instruments enabled the large-scale fabrication of the SPEs in various electrode layout designs. The printing patterns of conductors and insulators onto the surface of plastic or ceramic planar substrates can be designed and controlled by the software of these instruments. The most suitable substrate material as well as the conducting and insulating ink materials should be chosen for the purpose of the electrochemical test. The most commonly applied conducting inks are the carbon- and Au-based ones. There are mainly four steps involved in the screen-printing process. First, the conducting ink suspension is deposited onto the screen, followed by the loading of the screen mesh with the conducting ink, then the ink is forced through the screen with the help of a squeegee. Finally, the printed pattern is dried and cured. The printing of the insulating layer and the other cover layers, which can vary according to the electrochemical test, is also performed as described above onto the conducting ink-printed surface. However, the overall performance of the SPEs is greatly influenced by the chemical composition of the ink materials and the printing conditions such as applied pressure and temperature.

Screen-printed electrode is the commonly used electrode type in reports not only for clinical but also environmental and food monitoring purposes. As for the clinical applications, Lucarelli et al.[21] have recently reported carbon SPEs for the detection of SNPs related to apolipoprotein E from PCR-amplified samples. The duplex formation was detected by measuring the guanine oxidation signal. The biosensor format involved the immobilization of an inosine-substituted probe onto a SPE and the voltammetric detection of the duplex formation with the appearance of the guanine signal.

Recently, gold electrode-based indicator-free DNA hybridization detection has been reported by using a conventional-type gold electrode.[22] Figure 3 shows the oxidation signal of guanine at about $\sim$+0.73 V by using differential pulse voltammetry on self-assembled L-cysteine monolayer (SAM)-modified screen-printed gold electrode (AuSPE). The electrochemical determination of hybridization between an inosine-substituted probe (Fig. 3b) and native target DNA (Fig. 3a) was accomplished. The indicator-free detection of hybridization on AuSPE is greatly advantageous over the existing carbon-based materials, because of its potential applicability to microfabrication techniques.

Indicator-free, disposable electrochemical DNA-modified carbon SPEs have also been successfully applied to environmental monitoring. The device of Chiti et al.[23]

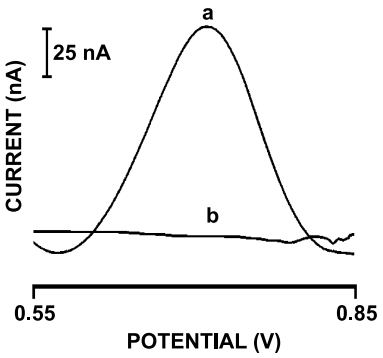

Fig. 3 Differential pulse voltammograms for the oxidation signal of guanine (a) after and (b) before hybridization with target DNA at inosine-substituted probe modified AuSPE.

relied on the intercalative or electrostatic collection of toxic aromatic amines onto an immobilized double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) layer in connection with chronopotentiometric analysis. The anodic signal of the guanine bases of DNA-coated SPEs was affected by structural or conformational modifications of the DNA layer due to the DNA–analyte association. The changes in the guanine oxidation signal were taken as an index for the affinity of the analyte toward nucleic acids. Submicromolar detection limits were obtained for molecules with more than two aromatic rings after a 2-min accumulation.[23]

Indicator-based disposable electrochemical DNA biosensors were also described by using carbon SPEs in connection with a well-described intercalator, daunomycin, as an indicator of the hybridization reaction by Marrazza et al.[24,25] Synthetic probes have been immobilized onto carbon SPEs using adsorption at a controlled potential. The hybrids formed on the electrode surface were evaluated by chronopotentiometric stripping analysis of daunomycin. The DNA biosensor was able to detect 0.2 mg L^{-1} of a 21-base target sequence. The determination of low-molecular weight toxic compounds with affinity for DNA, such as polychlorinated biphenyls (PCBs) and aflatoxin B1, was carried out by monitoring their effect on the guanine oxidation signal.[25] River water samples were also used as real matrices for monitoring of these compounds by using the disposable DNA-modified SPEs.[25]

CONCLUSION

The attractive performance of disposable PGEs and SPEs for the biosensing of DNA hybridization has been reviewed. Such performance compares favorably with that of conventional beaker-type electrodes commonly used for transducing DNA hybridization. The simple and fast utility of these test strips provides the basis of the day-to-day practicality of the biosensor and holds great promise for decentralized genetic testing. An electrochemical device based on such single-use electrodes, thus, is a strong alternative to other optical, gravimetric, or surface plasmon resonance (SPR)-based DNA biosensors, when rapid and cost-effective hybridization assays are concerned. Various electrochemical protocols, such as indicator-free or intercalator-based ones, can be readily combined with a disposable DNA biosensor. However, the integration of a battery-operated electronic microprocessor, as well as the reference and counter electrodes, is crucial for the improvement of PGEs. The excellent performance of the electrochemical self-care devices for blood glucose in connection with disposable electrodes provides an encouraging example for electrochemists to develop a similar device for genetic testing. In the near future, we are looking forward to the entrance of an electrochemical self-care DNA-based device into the booming biotechnology market.

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ARTICLE OF FURTHER INTEREST

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DNA-Binding Fluorophores

D

Mikael Kubista
Jonas Karlsson
Martin Bengtsson
Neven Zoric
Gunar Westman
TATAA Biocenter AB, Göteborg, Sweden

INTRODUCTION

The first cyanine dye, named cyanine because of its blue color, was serendipitously synthesized by C. H. G. Williams as early as 1856. Since the end of the 19th century, the class of cyanine dyes have dominated the field of photography as photosensitizers and are unrivalled even today. The generic cyanine dyes consist of two nitrogen centers, one of which is positively charged and linked to the other center by a conjugated chain of an odd number of carbon atoms (Fig. 1). Symmetrical cyanine dyes commonly contain two benzazole moieties connected by a polymethine chain, whereas the unsymmetrical cyanines usually consist of a benzazole group and a quinoline or pyridine heterocycle, also connected by a methine bridge. The extensive conjugation and the delocalization of charge lead to long-wavelength absorption maxima and large molar absorptivities. Apart from use as photosensitizers, cyanine dyes have been employed as laser materials, photorefractive materials, antitumor agents, and in optical disks as recording media to name but a few applications.

DNA-BINDING CYANINE DYES

In recent years, unsymmetrical cyanine dyes have received considerable interest because of their excellent nucleic acid staining properties. In 1986, Lee et al.[3] showed that the unsymmetrical cyanine dye Thiazole Orange (TO; Fig. 2) exhibits 3000-fold enhancement in fluorescence intensity upon binding to RNA. Later, Rye et al.[4] showed that TO, and the similar dye, Oxazole Yellow (YO) with a benzoxazolium moiety instead of benzothiazolium, have a dramatic increase in fluorescence also upon binding to DNA. Both these studies indicated that the dyes bind in a nearest-neighbor exclusion stoichiometry typical of intercalators.

To obtain stains with high affinity for DNA, Rye et al.[5] developed TOTO and YOYO (Fig. 2), which have two dye moieties connected by a biscationic amine linker. Their affinity for double-stranded DNA is about the square of that of the monomers as a result of bisintercalation and their high positive charge. To enhance the affinity for DNA of monomeric dyes, positively charged substituents (3-propyl trimethyl ammonium bromide) were added in TO-PRO and YO-PRO (Fig. 3).[6]

The absorption maximum of cyanine dyes depends on the length of the conjugated chain and the nature of the heterocyclic moieties. Today dyes are available whose absorptions span a broad range of the visible spectrum (Fig. 3).[6] They typically have several hundredfold enhancements in fluorescence upon binding DNA and a quantum yield in bound state of at least 0.1.

The fluorescence quantum yield of cyanine dyes increases when torsional motion around the methine bridge is restricted, which reduces the probability of nonradiative relaxation from the excited singlet state.[7,8] When the dyes bind DNA, internal rotation is likely to be strongly hindered, which causes the dramatic increase in fluorescence.

The interactions of cyanine dyes with nucleic acids have been thoroughly investigated. Early flow linear dichroism studies indicated that YO-PRO and YOYO intercalate in DNA.[9] Intercalation was also supported by circular dichroism, fluorescence anisotropy, and dye–nucleobase energy transfer measurements. By nuclear magnetic resonance (NMR), the solution structures of TOTO bound to short oligonucleotides have been determined.[10] Frequently, more than one complex was observed. A preferred binding site was bisintercalation in a central CTAG:CTAG binding site with the cationic linker located in the minor groove. The DNA complex is unwound by $\sim 30^\circ$ and extended ~ 2 Å per TO moiety, which is typical of intercalation.

Thermodynamic studies of TO binding to nucleic acids of different base compositions showed that binding has little sequence selectivity.[11] At elevated dye:base pair ratios, a secondary binding mode was observed for both monomeric and dimeric dyes.[9,12] In a spectroscopic study, Nygren et al.[13] determined thermodynamic parameters of TO binding to DNA both as monomer and

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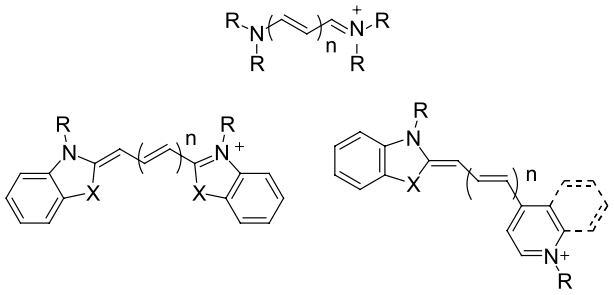
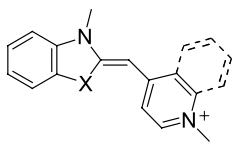


Fig. 1 Generic structure of cyanine dyes (top) and examples of symmetrical (left) and unsymmetrical cyanine dyes (right).



Dye	X	Heterocycle	λ_{max}
BO	S	pyridine	445
TO	S	quinoline	510
PO	O	pyridine	435
YO	O	quinoline	480

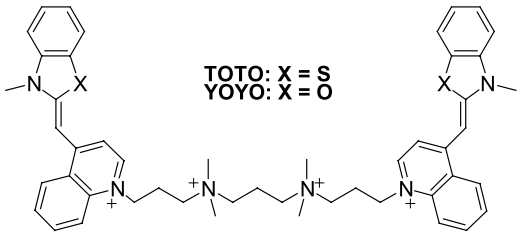
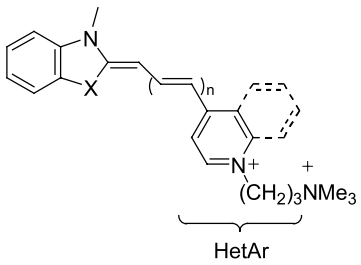


Fig. 2 General structure of the monomeric unsymmetrical cyanine dyes (top), and the homodimeric cyanine dyes TOTO and YOYO (bottom).

as dimer. With several sequences, the dimer complex was found to have lower fluorescence than the bound monomer. By fluorescence polarization measurements of TOTO–DNA complexes, evidence for external binding was obtained.[14] In hole-burning studies, external binding of TO-PRO-3 to DNA was revealed.[15] Atomic force microscopy and viscometry studies support the notion that TO and in particular TOTO bind in more than one mode to DNA.[16] Clearly, the interaction of cyanine dyes with DNA is complex and yet far from fully understood.

Most cyanine dyes have strong affinity also for single-stranded DNA and obtain high fluorescence upon binding.[13,17] This makes them less useful as specific probes for double-stranded DNA. Similar affinity for single- and double-stranded DNA is typical of intercalators of low complexity that mainly gain binding energy through stacking with bases. Minor groove binders, on the other hand, typically show strong preference for double-stranded molecules. In recent years, some symmetrical cyanine dyes have been found to bind in the minor groove. Seifert et al.[18] showed that the symmetrical pentamethine cyanine dye 3,3′-diethylthiadicarbocyanine ($DiSC_2[5]$) (Fig. 4) cooperatively binds as head-to-head dimer in the DNA minor groove forming helical H-aggregates. Mikheikin et al.[19] showed that a series of trimethine symmetrical cyanine dyes, e.g., $DiSC_2$,[3] bind in the minor groove mainly as monomers. These studies suggested that the hydrophobicity of the heterocyclic group is a critical determinant for dimerization. Recently, Sovenyhazy et al.[20] showed that the trimethine cyanine dye TO-PRO-3 (Fig. 4) cooperatively binds in the minor groove of poly(dA–dT)$_2$, although it has previously been considered to mainly intercalate in DNA.

A characteristic of classical minor groove-binding ligands such as Hoechst 33258 and DAPI is its crescent-shaped molecular structure.[21] To improve the preference of cyanine dyes for double-stranded DNA, Karlsson et al.[22,23] designed a series of crescent-shaped unsymmetrical cyanine dyes based on the BO and TO chromophores (Fig. 5). Flow linear dichroism, circular dichroism, and electrophoresis unwinding measurements revealed that these dyes prefer minor groove binding in mixed sequence DNA. Like the intercalating dyes, the minor groove binders exhibit large increase in fluorescence upon binding (Table 1).



Dye	X	n	HetAr	Ex/Em (nm)
PO-PRO	O	0	pyridine	435/455
BO-PRO	S	0	pyridine	462/481
YO-PRO	O	0	quinoline	491/509
TO-PRO	S	0	quinoline	515/531
PO-PRO-3	O	1	pyridine	539/567
BO-PRO-3	S	1	pyridine	575/599
YO-PRO-3	O	1	quinoline	612/631
TO-PRO-3	S	1	quinoline	642/661

Fig. 3 Divalent unsymmetrical cyanine dyes with different spectral characteristics. (From Ref. [7].)

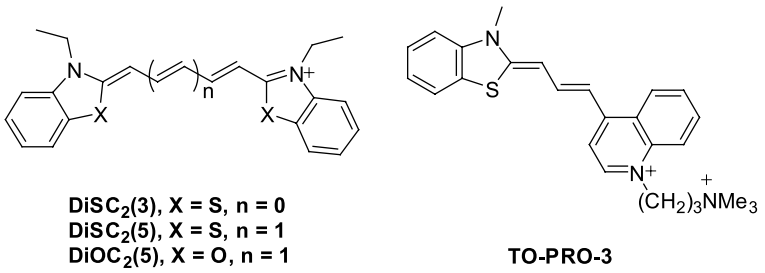


Fig. 4 Symmetrical cyanine dyes (left) and unsymmetrical cyanine dye TO-PRO-3 (right).

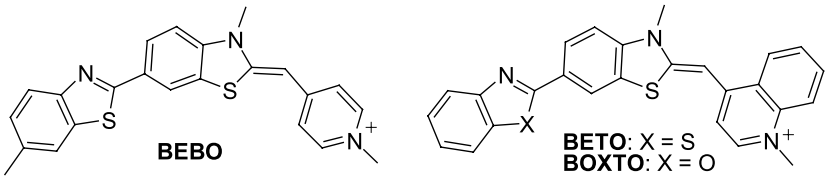


Fig. 5 Minor groove-binding unsymmetrical cyanine dyes.

PROBING POLYMERASE CHAIN REACTION (PCR)

In 1992, Higuchi et al.[25] showed that it was possible to follow the amplification of DNA in real time by adding ethidium bromide to the reaction tube and monitor the increase in fluorescence when the dye binds to the accumulating DNA product. A variety of dyes and probes have been developed for the detection of DNA in homogeneous solution since then. To be useful, the dyes should bind DNA with little sequence selectivity in one dominant-binding mode. They should have strong preference for double-stranded DNA and exhibit large fluorescence enhancement upon binding. Binding should be strong enough to give a signal proportional to the amount of DNA present, but without interfering with the amplification reaction. For the same reason, dissociation kinetics should be fast. Although YO-PRO was used in some early assays,[26] the most common dye is SYBR Green I from Molecular Probes.[27] Its chemical structure has not been published in scientific reports, but its spectral characteristics suggest that it is based on the TO-chromophore, probably with a cyclic substituent (U.S. patents 5,436,134 and 5,658,751). Recently, Bengtsson et al.[28] tested the minor-groove binder BEBO as reporter in real-time PCR (Fig. 6) and found it to compare well with SYBR Green I in all important aspects.

Dyes are excellent reporters in real-time PCR, but they do not distinguish between products. Taqman,[29] Molecular Beacons,[30] Scorpion Primers,[31] and LightCycler

Table 1 Fluorescence and absorbance properties of BETO and BOXTO compared with other minor groove binders and with TO

	$\lambda_{abs.max}$ (nm)	$\lambda_{ems.max}$ (nm)	ϕ_F	ϕ_{bound}/ϕ_{free}	F_{bound}/F_{free}	Reference
Free BETO	487	590	0.015			[22]
BETO-ctDNA[b]	516	561	0.21	14	130	[22]
Free BOXTO	482	588	0.011			[22]
BOXTO-ctDNA[b]	515	552	0.52	50	260	[22]
Free BEBO	448	542	0.011			[23]
BEBO-ctDNA	467	492	0.18	16	245	[23]
Free TO	501	—	0.0002			[13]
TO-ctDNA	508	525	0.11	550		[13]
DAPI-ctDNA	349	456	0.34	18		[24]
Hoechst-ctDNA	356	466	0.42	28		[24]

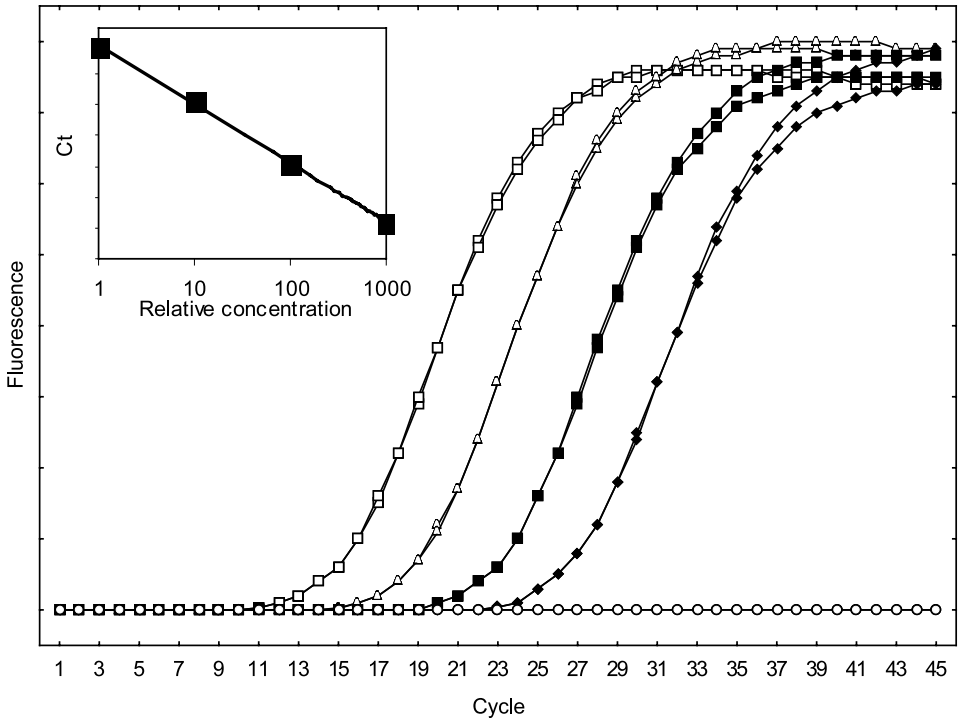


Fig. 6 Real-time PCR amplification with BEBO (0.2 μM) detection. Amplification was performed in a RotorGene with 470-nm excitation and 510-nm detection. Background is subtracted and the amplification curves are normalized. Samples are duplicates of a 1:1, 1:10, 1:100, 1:1000 dilution series of a 222-bp beta tubulin fragment and NTC. Inset: Standard curve (R=0.99955, Slope −3.8, Efficiency 0.83).

hybridization probes are examples of sequence-specific probes that are based on fluorescence resonance energy transfer (FRET) between two dyes tethered to an oligonucleotide. When these probes bind target DNA, the distance between the dyes changes as a result of either probe degradation or conformational change resulting in fluorescence enhancement. Common dyes in these probes are laser dyes with very high fluorescence quantum yields. An interesting alternative to the two-dye probes is the LightUp probe.[32] The LightUp probe has only one dye, which is a cyanine dye, whose fluorescence greatly increases when bound to DNA. Instead of using an oligonucleotide to identify the target sequence, the LightUp probe uses peptide nucleic acid (PNA), for which charged cyanine dyes have negligible affinity. The LightUp probes have been found to be excellent reporters in both real-time PCR and post-PCR applications.[33,34]

CONCLUSION

Fluorescent probes have replaced radioisotopes as labels of nucleic acids. They have important advantages in being nonhazardous and stable upon storage. Moreover, some dyes have different fluorescence in free and bound state, which opens for probing in homogeneous solution, eliminating the need to separate bound and free probes. These dyes were accidentally discovered. Based on detailed structural and thermodynamic investigations of the dyes and their interactions with nucleic acids, we are rapidly learning what governs their properties. This will make it possible to improve the dyes in terms of solubility and sequence nonspecificity, and also to design dyes of different colors. Particularly exciting is the use of the dyes as labels in sequence-specific probes because enhancement in fluorescence can be much more sensitively measured than a change in fluorescence as a result of energy transfer. Also, multiplexing is expected to be much simpler with single-labeled probes.

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DNA Extraction from Mummified Tissues

Yayoi Sato
Chiba University, Chiba, Japan

INTRODUCTION

Nucleic acids extracted from mummified tissues are considered valuable materials for the identification of forensic cases and for the study of ancient human beings in the field of archaeoanthropology and the emergence of pathogens in Paleontology.

After the powerful polymerase chain reaction (PCR) technology was developed, amplification of a minute amount of DNA extracted from various archived tissues could be performed. In the analysis of degraded or ancient DNA, the extraction process represents one of the critical stages. However, significant difficulty in extracting nucleic acids from mummified tissues has been reported due to chemical modification, degradation, the small amounts, inhibition, and contamination over a long period. These problems can make amplification of the nucleic acids very difficult. From a variety of tissue samples including aorta, brain, skin, genitals, ilium. penis, lung, muscle, connective tissue, DNA extraction was carried out using either organic solvents (such as phenol/chloroform) or nonorganic solvents (salt) method. The amount of DNA retained in the mummy is assumed to be minute and degraded, so several modification methods are applied so as to achieve efficient extraction.

Here, several representative protocols for extracting DNA from mummified tissues are presented and the precautions in the analysis are described.

SAMPLES

A variety of tissue samples from mummies have been subjected to DNA extraction, including aorta,[1] brain,[2,3] skin,[4,5] genitals, ilium, penis,[6,7] lung,[8] muscle, connective tissue.[9]

SAMPLE PREPARATION

Samples should be obtained with extreme caution to avoid contamination from other DNA sources. Pre- and post-PCR operations should be performed in separate rooms, and a laminar flow cabinet is used during extraction and purification steps. In each run, there is a negative control (water) used to detect contamination in the reagents.

The surfaces of the biopsy sites are cleaned and tissue samples are obtained from deep sites. After washing with sterile distilled water, tissues are cut into small pieces and then homogenized with phosphate-buffered saline (PBS)[5,6] or pulverized with a mill after lyophilization in the liquid nitrogen.[1,6,8] The sample sizes were 1.6 g, 0.5–1 g, 5 mm^3 size,[2,4,6] and 50 mg, 0.5 g, 1 g,[1,5,6] respectively.

DNA EXTRACTION

DNA extraction from mummified tissues is carried out using either organic solvents (such as phenol/chloroform) or nonorganic solvents (salt) to remove proteins. The amount of DNA retained in the mummy is assumed to be minute and degraded, so several modification methods are applied so as to achieve efficient extraction. The representative methods are described here. The sample size is determined depending on the degree of degradation and the amount of retained DNA in the mummified tissues.

Method 1 (Nonorganic Solvents Method: PUREGENE™ DNA Isolation Kit)

The PUREGENE™ DNA Isolation kit (Gentra Systems Minneapolis, MN) is used to extract DNA from mummified tissues.[1,10]

In this kit, purification of genomic DNA is carried out using ammonium acetate as a substitute for toxic organic solvents in the protein precipitation step. The pulverized tissue is mixed with cell lysis solution containing Tris–HCl, ethylenediaminetetraacetic acid (EDTA), and sodium dodecyl sulfate (SDS), and incubated at 65°C for 60 min. After proteinase K solution (20 mg/mL) was added to the lysate, incubation at 55°C is carried out until tissue particulates have dissolved. RNase A solution (4 mg/mL) is added to the lysate and incubated at 37°C for 60 min. Protein precipitation solution (ammonium acetate) is added to the solution and vortexed vigorously then centrifuged. The DNA is precipitated with 100% isopropanol (2-propanol). After centrifugation, the pellet is

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washed several times with 70% ethanol and then air-dried. Purification is carried out with a Microcon™ YM-100 microconcentrator (Millipore, Bedford, MA). Finally, the DNA is hydrated with sterile distilled water. The extract is quality-checked by agarose gel electrophoresis and determined by a UV spectrophotometer. The E_{260}/E_{280} ratio is recommended to be greater than 1.5, as the success rate of PCR is thought to depend on the purity. When the DNA yield is expected to be low (<1 μg), DNA carrier such as glycogen solution (20 mg/mL) is added.

Method 2 (Organic Solvents Method: Phenol/Chloroform Extraction)

This method has been used conventionally and is still widely used for degraded and aged samples. Many modifications are presented for the kind of organic solvents or reagent of digestion and so on. Here the representative method is described as applied to the extraction of mummified tissues.

5 M GTC digestion method

Guanidium thiocyanate (GTC) is a chaotropic agent commonly used for tissue lysis of archived specimens.[6,7] After homogenization, the pellets are lysed in a 5 M GTC buffer containing 5 M GTC, 0.5% bovine serum albumin, 80 mM EDTA, 400 mM Tris–HCl (pH 7.5), and 0.5% sodium-*N*-lauroylsarcosine at 60°C for 1 hr and then at 37°C overnight. DNA is extracted twice with phenol–chloroform at a 1:1 ratio, followed by chloroform once, and then precipitated by the addition of a 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. The pellets are washed with 70% ethanol and air-dried. They are then dissolved in Tris–EDTA [10 mM Tris–HCl (pH 8.0), 1 mM EDTA] buffer.

Proteinase K digestion method

Samples are put into digestion buffer (10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 50 mM NaCl, 2% SDS) containing proteinase K solution (10 mg/mL) and incubated for several hours at 56°C.[1,8] Additional aliquots of proteinase K are added to achieve complete digestion. Next, the solution is extracted with phenol/chloroform/isoamyl alcohol (25:24:1) by a mild mixer. After centrifugation, the upper phase is transferred to a new tube. The extraction is repeated until the interface is clear. Finally, one more extraction is performed with chloroform to remove phenol, and the aqueous phase is put into another tube. After the addition of 5 M NaCl solution, DNA is precipitated with 100% ethanol. The recovered DNA is washed three times with 70% ethanol and air-dried. It is hydrated with sterile distilled water.

Microconcentrator-Based Method. Samples are put into the solution containing 10 mM Tris–HCl (pH 8.0), 2 mM EDTA, and 10 mM NaCl.[2,8,11] The tissue is broken up into small pieces by vortexing, and then collagenase is added. The tubes are incubated at 37°C with slow agitation for 3 hr. Sodium dodecyl sulfate is then added as well as dithiothreitol (DTT) and proteinase K. Incubation is continued for approximately 20 hr. The solution is extracted twice with water-saturated phenol of neutral pH and once with chloroform/isoamyl alcohol (24:1). It was concentrated by a Centricon 30 microconcentrator.

Silica-Based Method. Extracts of DNA are made from each sample by a silica-based method that is highly efficient in the retrieval of ancient DNA.[5,12]

DNA extraction is carried out with the supernatants in an automated nucleic acid extractor, starting with proteinase K digestion at 56°C for 1 hr. A standard phenol/chloroform extraction is carried out, followed by mixing the samples with a silica powder (glass milk, Dianova). This process produces a binding of DNA to the glass beads in the presence of isopropanol and sodium acetate (2.0 M, pH 4.5). In the last phase of the automated extraction procedure the DNA/glass milk samples are collected on filtration membranes and washed with ethanol. Finally, the DNA is manually eluted from the silica beads with sterile water and run on a 3% agarose gel.

CONCLUSION

The results obtained from the method described above are listed in Table 1.

A PEREGENE™ DNA Isolation kit is useful for extracting DNA from relatively fresh mummified tissues, compared to the archaeological samples, and it presents no hazard.[1,10] For tissues preserved for thousands of years, the phenol/chloroform method may be superior to the kit. Konomi et al.[6] have compared nine extraction methods and concluded that GTC-based methods are more efficient than proteinase K/detergent-based methods for the recovery of both DNA and RNA from mummified tissues. However, proteinase K is a powerful protein lysis reagent and has been widely used. It is not clear which is better for digestion, as both are quite useful.

Although mummified tissues are well-preserved for thousands of years, the integrity of nucleic acids for molecular biological study still remains as an issue.[13] Cooper and Wayne[14] has commented that the extracted DNA from a 2400-year-old mummy was, in hindsight, likely a contaminant both because of its size and nature. Furthermore, it has been shown that more than 99% of DNA isolated from mummified tissues is depolymerized and chemically modified.[2,4,15]

Table 1 DNA extraction from mummified tissues

Sample	Time after death	Extraction method	PCR success rate	Ref.
Aorta	1.5 years old	NO	1/1 (HLA)	[1]
Skin of leg	2400 years old	PC(P)	1/23 (cloned and sequenced)	[4]
Brain	7000 years old	PC(PM)	1/1 (mtDNA)	[2]
Skin of hip, extremities	>500 years old	PC(PS)	8/17 (Y-specific 3.4 kb repeat sequence)	[5]
Genitals, ilium, penis	>800 years old	PC(G)	12/12 (GAPDH), 0/12 (hepatitis viruses)	[6]
Lung	3600 years old	PC(P)	25/48 (*Mycobacterium tuberculosis*)	[8]
Brain	8000 years old	PC(P)	1/1 (mtDNA)	[3]
Genitals	>800 years old	PC(G)	12/12 (GADPH), 2/12 (*M. tuberculosis* complex)	[7]

GAPDH: glyceraldehydes 3-phosphate dehydrogenase; NO: nonorganic method; PC: phenol/chloroform method; (G): GTC digestion; (P): proteinase K digestion; (PM): proteinase K digestion + microconcentrator; (PS): proteinase K digestion + silica.

It is important to prevent contamination, to remove the inhibitors, to achieve efficient recovery, and to confirm authenticity when DNA is extracted from mummified tissues preserved for a long time. The study of ancient DNA poses numerous problems, some of which are presented below.

Small Size of DNA

The DNA recovered from ancient tissues is severely degraded, so it is difficult to amplify successfully more than approximately 200-bp-long fragments.[2,12] In the typing of HLA alleles from aorta tissue of an approximately 1.5-year-old mummy, we have been able to obtain specific PCR fragments sized approximately 100–200 bp long, but have sometimes failed in amplifying a 429-bp fragment used as a positive control.[1]

Low Amount of DNA

DNA yields of approximately 70 ng/μL can be measured for bone samples, and yields of less than 2 ng/μL can be measured for soft-tissue samples.[5] We have recovered 23 ng of DNA per milligram of dried aorta tissue naturally preserved for approximately 1.5 years. Polymerase chain reaction amplification fails when the amount of available DNA is too minute.[16] Both bone and soft-tissue samples are suitable for ancient DNA analysis, but that skeletal remains should be given preference when source materials are severely degraded.

Authenticity of Amplified DNA

It is important to confirm whether the recovered DNA is authentic or not. For example, it should be noted whether it was derived from an authentic human, a modern human, an animal, a pathogen, or some other source. It remains possible that a sample can be contaminated during the experiment. It is therefore necessary for rigid quality criteria to be established to avoid more spurious and unsubstantiated reports.

Marota et al. have reported that DNA would be entirely degraded after slightly more than 800 years, and this result provides an indirect argument against the reliability of claims regarding the recovery of authentic DNA from Egyptian mummies and bone remains.[13]

Inhibitors

DNA extracts from ancient tissue remains often contain an unidentified component that inhibits polymerase activity. Inhibition can result from either reducing sugars[17] or an excess of coextracted microbacterial DNA,[18] the remains of porphirines,[19] degraded nucleic acids,[20–22] and soil components such as humic and fulvic acids, tannins, or iron ions (Fe^{2+}),[23–25] or collagen type.[26] This inhibition may be overcome by the addition of bovine serum albumin and increased amounts of enzyme. Through the purification step by the microconcentrator device described above, the success rate of PCR is increased.

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DNA Storage

C. William Kilpatrick
University of Vermont, Burlington, Vermont, U.S.A.

INTRODUCTION

The successful extraction or amplification of DNA from fossils at least thousands of years old,[1–9] from preserved tissues at human burial sites,[10–16] and from museum specimens,[17–25] clearly demonstrated the potential for the storage of DNA. Spontaneous decomposition of DNA primarily results from depurination/β-elimination[26] and free radical oxidation pathways.[27] In solution DNA will spontaneously degrade to short fragments over a time period of several thousand years at moderate temperatures.[26] Numerous methods have been developed for the storage of DNA or for tissue for the extraction of DNA; however, relatively few studies have been published that examine or compare the ability of these methods to prevent or reduce DNA degradation, especially over periods of times greater than a few months.

MECHANISMS OF DNA DEGRADATION AND LOSS

Degradation or loss of DNA during storage primarily results from depurination/β-elimination,[26] free radical oxidation,[27] action of endogenous nucleases,[28] and binding of DNA to storage tubes.[29] At high ionic strengths, a large proportion of a DNA sample may be bound to the walls of polyethylene and most polypropylene tubes.[29] At moderate or low ionic strength, where DNA is more often stored, most polypropylene tubes bind less than 5% of the DNA; however, some tubes may bind as much as 25% of the DNA and considerable variation was reported among batches from the same manufacturer.[29] Storing short DNA fragments in polypropylene tubes often induces a significant amount of strand separation[30,31] and, at high salt concentrations, leads to the formation of multistranded complexes.[31,32] Polyallomer tubes show no binding or denaturation of DNA under any condition.[29]

In an aqueous solution, DNA is known to degrade by the two-step process of depurination and β-elimination.[26] The depurination reaction is mainly acid-catalyzed under physiological conditions.[33] At sites of depurination, the DNA chain undergoes cleavage by a β-elimination process within a few days.[34] The pH of the storage solution is expected to have a large effect on DNA stability. DNA bases and deoxyribose sugars are also susceptible to degradation by free radical oxidation.[27] Use of specific chelators, such as EDTA, has been found to be extremely effective at reducing free radical oxidation for at least 1 year of DNA stored at room temperature.[35]

Degradation of DNA in a sample may also result from the action of endogenous enzymes, endo- and exo-nucleases, that cleave DNA strands.[28] Temperature, pH, and salt concentration are known to influence enzymatic activity.[36] Nucleases are divalent cation-dependent enzymes and their activity is much reduced at high concentrations of EDTA or diaminocyclohexanetetraacetate (CDTA).

STORAGE OF DNA EXTRACTS

Traditionally, purified DNA is stored in either sterile water or TE (10 mM Tris, pH 7.5–8.0, 1 mM EDTA). In either of these solutions, DNA may be stored for several months at either 4°C or −20°C with little degradation. Extracted DNA stored in TE at temperatures between −70°C and 37°C for 6 months demonstrated little degradation, whereas DNA stored at 45°C to 65°C shows signs of degradation within 24 hr and was completely degraded by 8 days.[37] Repeated freezing and thawing of DNA does not appear to result in degradation. For longer cryogenic storage, DNA should be stored in nuclease-free solutions at −70°C (ultrafreezers) to −196°C (liquid nitrogen).

Alternatively, DNA may be precipitated and stored in ethanol often layered with chloroform at room temperature or 4°C. Although lyophilized DNA stored at −20°C is protected from degradation, problems with rehydration after prolonged storage have been reported.[37] Buffers containing EDTA have been used to store DNA at room temperature with little degradation for periods of time of less than a year[35,38] to over 2 years.[39] High concentrations of EDTA[38] and the addition of ethanol[35] have been found to further enhance DNA stability.

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STORAGE OF TISSUES FOR DNA EXTRACTION OR AMPLIFICATION

Blood samples in vacuum tubes containing EDTA stored for 4 weeks at temperature from 4°C to 37°C yielded high molecular weight DNA, although degradation was evident in samples stored at room or higher temperatures.[40] Interestingly, blood samples stored at 37°C demonstrated less DNA degradation than those stored at 23°C.[40]

CRYOPRESERVATION

Cryopreservation is an effective method of preservation of tissues for DNA extraction and is often reported as the preferred method;[41–43] however, freeze–thaw cycles have been found to produce degradation.[38,44] Numerous noncryogenic methods have been developed and appear to be effective for the preservation of tissues for DNA extraction.[39]

DEHYDRATION

Ethanol or isopropanol has been suggested[45,46] for the storage of tissues for DNA extraction. Tissues stored in ethanol have been reported to yield highly degraded DNA fragments;[44,47] however, much of this degradation appears to occur during the extraction procedure rather than during storage.[39] For short-term storage of up to 1 year, 70% ethanol appears to function as well as 100% ethanol; however, 100% ethanol is recommended for storage exceeding 2 years.[48] Other organic solvents have been examined for DNA preservability of insects and their intracellular symbiotic bacteria.[49] At room temperature for 6 months of storage, acetone, diethyl ether, and ethyl acetate were as efficient as ethanol or isopropanol at DNA preservation, whereas chloroform and methanol were poor preservatives.[49] In addition, acetone was found to be more robust against water contamination than ethanol[49] and may prove to be a better storage solution than ethanol.

Genomic DNA in dried blood spots on filter paper[50] or other media (i.e., Guthrie cards or FTA® cards) stored at room temperature is reported to be stable for at least 7.5 years.[51] This method of tissue storage is widely used in human genetic studies; however, some degradation has been reported.[44,52] The utility of this method of storage has proven effective[53] and recent advancements allow direct PCR amplification without DNA extraction.[54]

LYSIS BUFFERS AND OTHER SOLUTIONS

A number of different ''lysis buffers'' have been developed for the storage of tissues at room temperature.[44,55,56] The various formulations for ''lysis buffers'' generally consist of a Tris buffer at a pH between 7.5 and 8.0, EDTA (chelator), and sodium dodecyl sulfate or *n*-lauroylsarcosine (detergents to lysis cells). These buffers have demonstrated to be effective at room temperature in the preservation of DNA in blood samples for 6 months[44] and tissue samples for 2 years or more.[39]

The use of a dimethyl sulfoxyde (DMSO) salt solution for the preservation of DNA in various tissues has been demonstrated to be effective for storage from 6 months to over 2 years.[39,44,48] This solution of 20% DMSO, 0.25 M EDTA, and NaCl 5% to saturation, pH 7.5[44,48] was found to be the most effective method of noncryogenic storage for the prevention of DNA degradation.[39]

STORAGE OF DNA IN OTHER BIOLOGICAL MATERIAL

Forensic methods of extracting DNA from a wide variety of different types of biological material (feces, hair, nails or claws, bones, teeth, dried skins, formalin-fixed, ethanol preserved specimens) and pathological materials (blood smears, chromosomal spreads, and paraffin-embedded tissues) have provided new sources of DNA for PCR amplification from stored materials. Storage methods for most of these materials have not been evaluated. Absorption of DNA to hydroxyapite is reported to result in a twofold decrease in the rate of depurination of DNA[26] and improves the chance of recovering DNA from old bones and teeth.

Storage of fecal samples in DMSO salt solution was most effective for preserving nuclear DNA, whereas storage in 70% ethanol, freezing at -20°C and drying performed about equally well for preserving mitochondrial DNA and short (<200 bp) nuclear DNA fragments.[57] Four different drying methods of fecal samples were evaluated,[58] and freeze drying and oven drying produced the greatest DNA amplification success.

CONCLUSION

Storage methods for DNA extracts and tissues for the extraction of DNA have been developed that prevent or greatly reduce the primary pathways of DNA degradation. The action of endogenous nucleases and the rates of depurination/β-elimination and free radical oxidation pathways can be greatly reduced by freezing, dehydration, or storage in buffers containing cation chelators, usually EDTA. Extracted DNA is very stable under a variety of cryogenic and noncryogenic storage methods.

Tissues stored for DNA extraction are more susceptible to degradation. Although cryopreservation is the preferred

method by many laboratories, samples are degraded by repeated freeze–thaw cycles. Dried blood spots have proven to be a reliable method to store material for DNA extraction and amplification. A variety of organic solvents, including ethanol, isopropanol, and acetone, have been shown to be effective for the storage of tissue for DNA extraction, although degradation may occur during the extraction process. The use of ''lysis buffer'' or DMSO salt solution is an effective noncryogenic method that greatly reduces degradation that may occur during both the storage and the extraction procedure.

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DNannotator—Annotation Software Tool Kit for Regional Genomic Sequences

Chunyu Liu
University of Chicago, Chicago, Illinois, U.S.A.

INTRODUCTION

Genome annotation is a process of identifying the location and function of genes and many other elements in a genome. Genome annotation documents the true meaning of the genome sequence and serves ongoing biomedical research that is based on genomics information. Public annotation efforts and data sources have some limitations. High-throughput technologies used in many labs produce more and more local data at an increasing rate. Translating discoveries in a local laboratory into genome annotation in a manner that integrates the local data with the public data is another recurrent challenge that requires general software support. DNannotator provides a series of software tools for local batch annotation. The annotation target can be either user's own genomic DNA (gDNA) sequence or public-assembled human chromosomal sequences. The annotation source can be genes, oligos/primers, sequence tag sites (STSs), and single nucleotide polymorphisms (SNPs). Annotation quality can be relatively easily managed as several log data files are provided by DNannotator.

BACKGROUND

Genome annotation is a process that involves procedures in both the wet and the dry lab. The wet lab performs experiments, identifies genes, polymorphisms, and other genomic elements, and studies genes' functions. The dry lab maps those identified elements onto genomic sequences. In this article, we restrict the term of ''annotation'' to dry lab sequence mapping. The terms annotation and mapping can be used alternatively here. To perform an annotation, two basic inputs are required: gDNA sequence data and interesting genomic elements (feature source data) such as genes and markers. Based on the ATGC sequence data, annotations of genes and their regulatory elements can reveal the true functional units of the genome. Annotations of other elements such as genetic markers, oligos, and primers are essential for genetic studies as they are the basis of many genetic experiments.

Ever since the sequence data were obtained from the genome sequencing project, scientists started to perform *large-scale systematic genomewide annotation* on those sequence data. Today, the human genome sequencing is just about finished. The human genomic sequences were assembled by National Center for Biotechnology Information (NCBI) and Celera. The sequence data have been annotated by several public or private institutes using their own tools and data sources. The data can be accessed at Web-based databases, including NCBI's Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606), University of California Santa Cruz's (UCSC's) Genome Browser (http://genome.ucsc.edu/) and Sanger Center's Ensembl (http://www.ensembl.org/), and Celera (http://www.celera.com/). For the public annotation, both gDNA sequences and the genomic feature source data are available. For example, the human chromosomal sequences can be found at NCBI's human genome database; human gene data can be found at either Genbank or Unigene database; SNP data can be accessed at dbSNP.

In the course of many research projects, investigators need to perform *regionwide local annotation*. As lab technologies have improved, especially with introduction of high-throughput experimental methods, many investigators produce large amounts of experimental data and knowledge on genes, regulatory elements, and variants. For example, one ABI 3730 sequencing machine can resequence 1.8-Mb sequence in 1 day. Hundreds of SNPs could be discovered in 1 day. Besides the data generated in the investigators' own lab, many new data can be found in the latest journal publications. Often enough, these new data cannot be found at the time of publication in the existing public genomic annotation databases because of the significant time gap between the time of data deposit to public databases and the time of releasing an updated genome annotation using the corresponding data, assuming all the data are collected by the public databases. Normally, this kind of delay would be more than 3 months. Unfortunately, this assumption about public data collection is not always true either. SNP data are not required to be deposited into any centralized database when a paper is published. It is frequently observed that some SNPs studied or discovered are absent in dbSNP. But dbSNP is the only SNP database used in all public annotation. An effective research design requires efficient

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use of existing knowledge. Hence data produced in an individual investigator's lab and all other data outside of public annotation databases are as valuable as data from public annotation. They need to be integrated with data existing in the current version of public genome annotation. This basic requirement poses an important challenge to current bioinformatics technology because of the following major problems:

1. Heterogeneous annotation target sequence or platform. Because of the human genome sequence assembly, annotation is kept updated at a speed of several months per release, different annotation databases could use different versions of sequence assembly at certain time period. Investigators may want to use different gDNA sequences either for a small regional sequence or a whole chromosome. Sequence assembly is difficult for some genomic regions because of enriched repeat sequences or difficulty of cloning in the public sequencing project. An individual investigator might have better quality sequence assembly of one region than the public assembly. All these different flavors of gDNA sequences created needs of user's local annotation.
2. Enormous amount of data. As described above, an individual lab could generate or collect large amount of data for local annotation.
3. Heterogeneous data formats. Different data source could use very different data format. Preparing input data for annotation tools and formatting output data to meet requirement of data integration and further data mining are not trivial.
4. Annotation quality control. For any intent of feature mapping, the results can be either success or failure. The annotation quality is highly dependent on the sensitivity and specificity of the mapping methods and parameter settings. Any simple bug in the annotation algorithm could cause major defects of the annotation results. The observed problems in public annotation include missing of features which should be mapped, wrong mapping positions, and so on. Because the current public annotation does not provide much log information, it is difficult to detect errors and to find out the causes.

There are a number of tools supporting region-wide local annotation. They can be roughly put into three categories:

1. Use public source data (stored in databases) and some gene prediction algorithm to annotate user's gDNA sequence. Genotator,[1] NIX (Williams et al., http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/), GeneMachine,[2] GAIA,[3] Alfresco,[4] GESTALT,[5] RUMMAGE,[6] and Oak Ridge National Laboratory Genome Analysis Pipeline (http://compbio.ornl.gov/tools/pipeline) provide integrated annotation, including mapping of known or predicted genes and/or regulatory elements by running multiple gene-prediction programs and searching against static public databases. But none of them incorporate methods for SNP mapping, which is essential for positional cloning projects for complex diseases. None of these systems takes source data supplied by the end-user, unless the user can modify the databases in the annotation system.
2. Annotate user's own source data to user's own gDNA sequence. Freeware, such as Artemis,[7] Sequin,[8] and some commercial software such as Vector NTI, provides a good interface to do manual annotation. They do not support batch annotation. Some other programs can be used to assist annotation. For example, BLAST[9] is good at homolog sequence searching. Sim4,[10] est_genome,[11] and Spidey[12] could be used to define the intron–exon structure of a gene. e-PCR[13] can be used to map STSs. However, most of these programs produce data in their own formats which cannot be directly converted into standard format annotation. Therefore, strictly speaking, they are not real annotation tools.
3. DNannotator can use user's own collection of source data (either from public places or generated in the labs) to annotate both user's own gDNA sequence and public chromosomal sequences. DNannotator complements the existing tools mentioned above. It is the first toolkit providing SNP mapping and the only one with the capability to migrate annotations from one sequence platform to another. DNannotator was first described in *Nucleic Acids Research* (2003).[14] It can be accessed at http://sky.bsd.uchicago.edu/DNannotator.htm.

Design and Function of DNannotator

DNannotator takes annotation source data, such as SNPs, genes, primers, etc., prepared by the user, and/or a specified target of genomic DNA, and performs de novo annotation. DNannotator can also robustly migrate existing annotations in Genbank format from one sequence to another given that the new sequence covers the same genomic region. The annotation migration function is useful when we are dealing with different versions of sequence assembly or different scope of a region, e.g., one is small regional sequence and another is whole chromosome sequence. The major functions of DNannotator are illustrated in Fig. 1. The functions of DNannotator are divided into two groups: one for annotation over

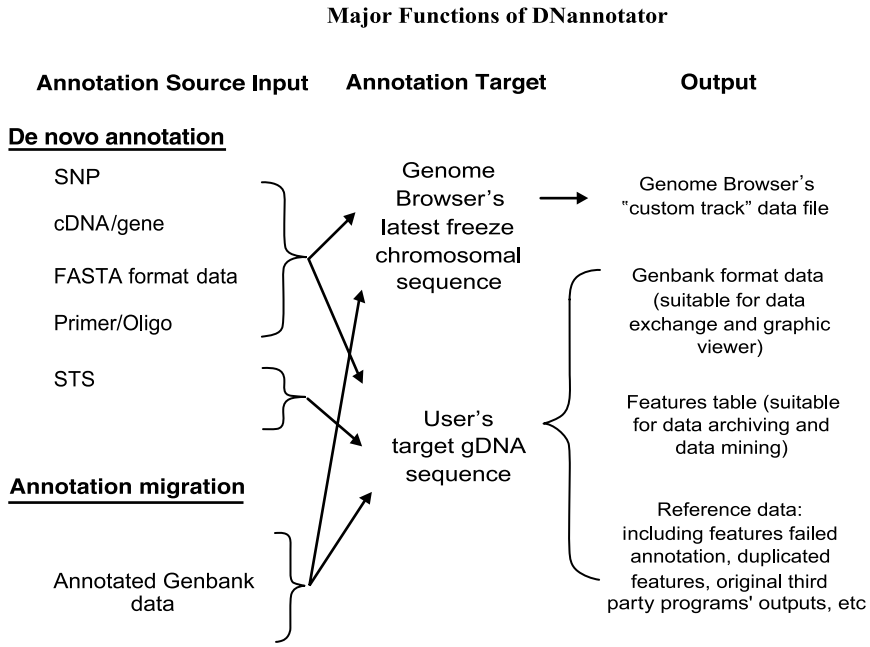

Fig. 1 Major functions of DNannotator. Using input source data of STSs, SNPs, primers, general FASTA, or tab-delimited text data, de novo annotation can be performed on target gDNA sequences through different function modules of DNannotator. A single Genbank format data file can be used as input to perform annotation migration. Similar inputs and outputs are used for the two types of annotation targets.

user's own gDNA sequence and another for annotation over chromosomal sequence (from UCSC Genome Browser's latest version human genome sequence assembly). Both groups can handle similar types of input source data. The input source data can be SNPs, genes, primers/oligos, and STSs. Besides de novo annotation using raw source data, annotated sequence data in Genbank format can be used as input to perform annotation migration, transferring annotated features to another sequence platform—either user's own gDNA or a public chromosomal assembly.

There are four types of optional outputs generated by DNannotator: 1) annotation results in Genbank format; 2) annotation results in tab-delimited text as feature table; 3) reference data reporting features failed at annotation, or features mapped to multiple locations, as well as some sequence analysis raw data such as BLAST's results; 4) besides those, for annotation over a public chromosomal sequence, a GFF format feature data can be provided.

Tab-delimited text output can be imported and managed in a database or spreadsheet and can be combined easily with existing annotation from elsewhere. Genbank format data can be graphically viewed in Artemis (Fig. 2). GFF format annotation results can be viewed in Genome Browser side by side with other annotation provided by Genome Browser (Fig. 3). Reference data can help user to evaluate and improve annotation quality.

MAJOR FEATURES OF DNannotator

DNannotator has four major features: ''local,'' ''batch,'' ''transparent,'' and ''user- and data-friendly.''

1. Local: All functions of DNannotator were designed to handle the user's own local data collection.
2. Batch: All functions were designed to process batch of input source data.
3. Transparent: Log data are provided with the annotation results, so that the annotation quality can be managed. Some major analysis parameters can be adjusted through the Web interfaces.
4. User- and data-friendly: DNannotator not only provides a user-friendly Web interface for data input, but also accepts data on a large scale needed by a research data pipeline. The input data normally can be prepared or organized easily from the lab data or other source; the output data are in multiple optional formats either ready for graphic view, or for databasing, or for further data mining or wet-lab assay design.

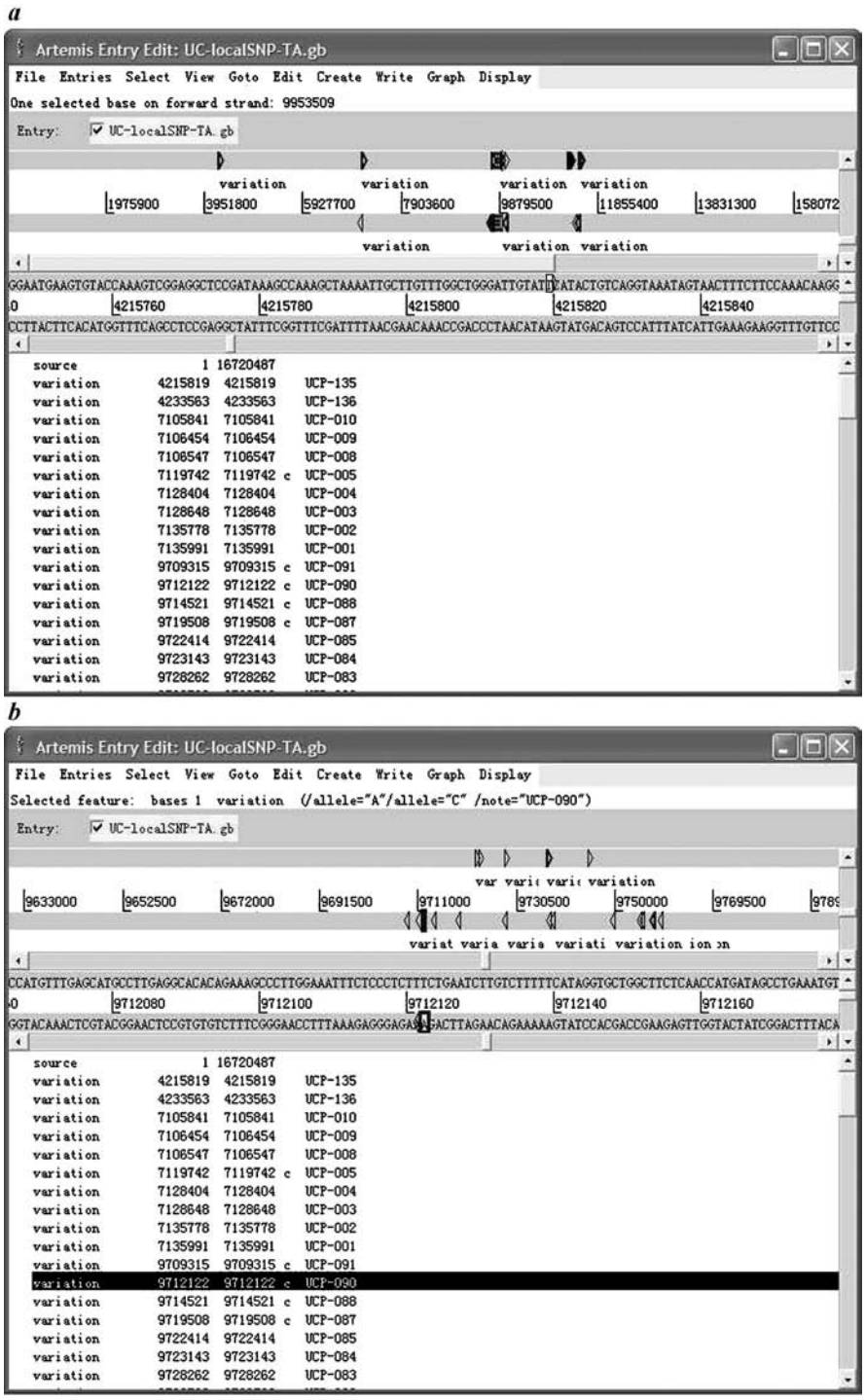

Fig. 2 Graphic view of SNPs annotated onto local sequence assembly. Genbank format output from DNannotator was read in as ''Entry'' by Artemis. One hundred SNPs discovered locally were displayed. (a) Overview of annotations. (b) Zoom-in view of annotations. The window is composed of three panels. Features listed in lower panel, graphic icons in upper panel, and sequence data in middle panel are internally linked to each other. Clicking one of them will activate highlight of corresponding elements in the other panels. (*View this art in color at www.dekker.com.*)

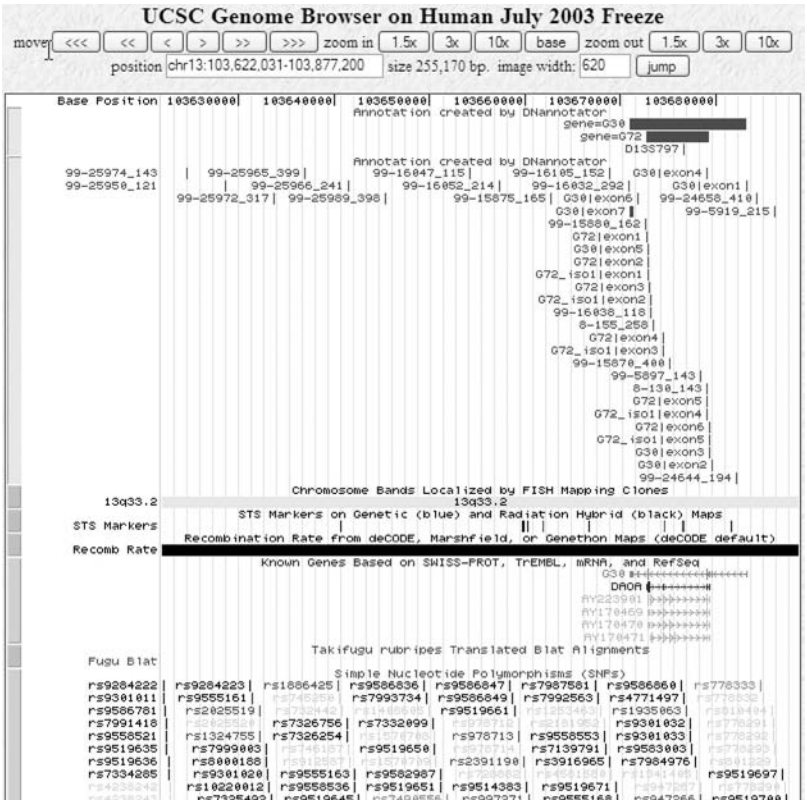

Fig. 3 Viewing annotation created by DNannotator in Genome Browser. The SNPs and genes reported (on small DNA fragment AE014312) by Chumakov et al. were migrated to Genome Browser's latest freeze of chromosome 13 by DNannotator, with an output of custom track data file for Genome Browser, which can be directly viewed side by side with all other annotations provided by Genome Browser. (*View this art in color at www.dekker.com.*)

APPLICATION EXAMPLES OF DNannotator

De novo Annotation

Example 1: from local discovery or newly published data to genome annotation.

In the lab, we sequenced several genes and discovered 100 SNPs. At that time (early 2002), public assembly of human gDNA sequence on 13q32–33 still had a lot of errors. We manually assembled a 17-Mb regional sequence with much better quality, which was later replicated by a subsequent public assembly. DNannotator was then used to annotate all 100 SNPs on this self-made sequence assembly. SNP data were prepared using Microsoft Excel spreadsheet and saved into a tab-delimited text file. DNannotator takes this SNP data file with gDNA sequence in FASTA format and maps all the SNPs to the self-made regional sequence. The Genbank format output is viewed in Artemis as shown in Fig. 2.

Annotation Migration

Example 2: from newly published Genbank data to genome annotation.

Chumakov et al.[15] reported association between G72 gene and schizophrenia. In this paper, 191 SNPs and two novel genes (G72 and G30) on chromosome 13 were reported. Most of these data could not be found in public annotation. Chumakov et al. deposited a series of Genbank format sequence data for chromosome 13 fragments. DNannotator was used to migrate annotations from these fragments to public assembly of chromosome 13 to find out how these SNPs and genes are mapped on human chromosome 13 and how these SNPs and genes related to the other SNPs and genes. As an example, AE014312 is one of the Chumakov's deposit with 250-kb sequence and contains 20 SNPs, 2 genes, and 1 STS. DNannotator takes this input and produced annotation on human public assembly chromosome 13. All features (SNPs, genes, and STSs) were mapped to chromosome 13

in one step. The GFF format data are viewed in Genome Browser as in Fig. 3. In this figure, we can view all annotations provided by Chumakov's group with all the other data created by public annotation.

SUMMARY

DNannotator provides services of batch local annotation which are not available elsewhere. With these tools, user can map SNPs, genes, and STSs to user-specified genomic DNA sequences including the public-assembled human chromosomal sequences. Thus integration of genomic data can be performed easily. DNannotator incorporates the ideals of ''batch,'' ''local,'' ''transparent,'' and ''user and data-friendly'' throughout its tool kits. Lack of these four features is the major shortcoming of many existing bioinformatics tools. We often see new tools handling only a single request, producing outputs without any log information, or ignoring user's own data.

Because of limits of computing power, DNannotator currently only annotates on a single human chromosome per analysis. Therefore the user could meet an obstacle if the source data's chromosomal source is unknown or it is a mixture from multiple chromosomes. We are working toward having biogrid as our computing platform. Once implemented, DNannotator should be able to perform genome-wide annotation.

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Duchenne Muscular Dystrophy

Masafumi Matsuo
Kobe University Graduate School of Medicine, Chuo, Kobe, Japan

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a common inherited disease with a worldwide incidence of 1 in 3500 male births. DMD is a lethal disorder of childhood characterized by progressive muscle wasting. Affected individuals are wheelchair-bound by the age of 12 and succumb to cardiac or respiratory failure in their mid to late 20s.

Dystrophin, the gene defective in DMD was isolated in 1986. Since then, genetic diagnosis of DMD has been done leading to better understanding of the disease process. Based on the molecular pathogenesis of DMD, molecular therapies for DMD have been proposed.

CLINICAL DIAGNOSIS

Patients with DMD show normal growth and development in their early childhood. In DMD, affected boys start to show disturbance of walking and frequently fall because of muscle weakness at 4 to 5 years of age. Patients are shown to have a positive Gower's sign wherein the child climbs up his thighs to extend the hips and push his trunk up. He manages to walk but his muscle strength gradually decreases. He loses the ability to climb up stairs. Lumbar lordosis becomes more exaggerated and the waddling gait increases. Patients usually are wheelchair-bound by the age of 12. Muscle wasting progresses as the patients get older until finally respiratory or cardiac failure develops due to muscle wasting.

Serum creatine kinase (CK) is markedly increased 50 times more than the normal range in infantile DMD. This marked elevation of serum CK is the most important hallmark for the diagnosis of DMD. During the asymptomatic period, elevation of serum CK is the sole sign for DMD. Some DMD patients are accidentally identified due to elevations of AST or ALT, which are commonly examined for liver function, because serum CK elevation is accompanied with elevations of AST and ALT.

GENE DIAGNOSIS

Duchenne muscular dystrophy is caused by mutations of the dystrophin gene.[1] The dystrophin gene is 3000 kb in size and consists of 79 exons encoding a 14-kb mRNA.[2,3] At least eight alternative promoters that are regulated in a tissue- or development-specific manner have been identified on the dystrophin gene. The unusually high incidence of DMD in all human populations could be simply a reflection of the enormous mutation target size of the gene, but the recombination rate is reported to be four times the rate expected for a gene of this size.[4] Nearly two-thirds of mutations identified on the dystrophin gene are deletions or duplications occupying a single or multiple exons, with the rest of the DMD cases having other types of mutations including point mutations. Remarkably, deletion mutations have been localized to two deletion hot spots of the dystrophin gene, the 5′ and the central regions.

Multiplex PCR Analysis

Currently, multiplex PCR analyses that amplify deletion-prone exons are used as the first step for gene diagnosis. Two sets of PCR amplification are used to screen 19 deletion-prone exons (exons 1, 3, 4, 6, 8, 12, 13, 17, 19, 43–45, 47–52, and 60).[5,6] Using this method of examination, one finds that nearly half of the DMD cases are shown to have deletion mutations. Therefore, the rest of the cases need further examination to identify the responsible mutation in the dystrophin gene.

Southern Blot Analysis

To examine the deletion/duplication in every exon of the dystrophin gene, Southern blot analysis is used, using segments of the dystrophin cDNA as probes.[7] Two-thirds of DMD patients are shown to have recombination events of deletions or duplications spread in one or more exons at the genomic DNA level.[8] However, Southern blot analysis not only needs high-quality DNA and radioisotope, but it is also time-consuming.

Detection of Fine Mutation

In DMD cases that have no large recombination event, identification of the causative mutation remains a laborious goal because of the difficulty in detecting a single point mutation in the 3000-kb-sized gene. To

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facilitate the identification of mutations in the dystrophin gene, more than 99% of which is made up of introns, dystrophin mRNA that is 100 times smaller than the dystrophin gene has been analyzed.[9] Analysis of dystrophin mRNA expressed in lymphocytes leads to not only identification of rare genomic mutations, but also to disclosures of nonauthentic alternative splicing.[10–12] In addition, several ways to identify small mutations have been proposed.[13–17] In the advent of recent advances in mutation analysis techniques, more than 90% of DMD cases are shown to have mutations in the dystrophin gene.[18,19]

PATHOLOGICAL DIAGNOSIS

The pathological examination of biopsied skeletal muscle confirms the diagnosis of DMD. Immunohistochemical analyses of normal muscle demonstrate that dystrophin is present along with muscle cell membranes. In DMD, dystrophin is missing from skeletal muscle.[20] Western blot analyses using dystrophin antibody reveals a band corresponding to 427 kDa, close to the predicted size of dystrophin, in extracts of normal muscle tissue, whereas no protein can be detected in DMD.

TREATMENT

For DMD patients, supportive therapies such as rehabilitation or ventilator support are clinically employed but no effective way to improve the clinical course is available. Since the discovery of the dystrophin gene, gene therapy is now considered an attractive way to cure the disease. The main aim of DMD gene therapy is to establish a way to inject constructed dystrophin genes consisting of partial- or full-length cDNA joined to an appropriate promoter. Although much progress has been made in this field of study, we still seem to be a long way from achieving a clinically significant result. As an alternative for gene transfection, molecular therapies have been studied including antisense oligonucleotide treatment[21–23] or translational readthrough treatment using gentamicin.

Antisense Oligonucleotide Treatment

An alternative strategy for DMD treatment is to retard the progression of the clinical symptoms, i.e., to convert DMD into the BMD phenotype. Theoretically, this therapy can be done by changing a frame-shift mutation causing DMD into an in-frame mutation characteristic of BMD by modifying the dystrophin mRNA. Artificial induction of exon skipping with antisense oligonucleotides is a way to make the out-of-frame dystrophin mRNA in-frame. Artificial induction of exon 19 skipping using an antisense oligonucleotides against the splicing enhancer sequence has been reported,[24] and this treatment was shown to produce dystrophin expression in exon-20-deleted DMD myocytes.[23] Disruption of the splicing enhancer sequence to induce exon skipping was further evidenced by the fact that in the nonsense mutation of exon 27 the dystrophin gene resulted in exon 27 skipping, producing an in-frame dystrophin mRNA.[25] In addition, another natural example causing conversion of DMD to BMD was identified in a nonsense mutation in exons 25 and 29.[26,27] Furthermore, BMD has been shown to have a nonsense mutation in in-frame exons.[28,29]

Antisense oligonucleotides against a purine-rich sequence have been used to induce skipping of exons 44, 45, 46, 49, 50, 51, or 53.[22,30,31] In these studies, induction of exon skipping led to the expression of dystrophin in their respective dystrophin-deficient myocytes by correcting the translational reading frame. Recently, double exon skipping of exon 43 and 44 or exon 45 and 51 has been induced.[32] This extends the application of the antisense oligonucleotide treatment to more varieties of deletion mutations of the dystrophin gene.

Phosphorothioate DNA has been the standard choice for the clinical application of antisense technology.[33–37] However, phosphorothioate DNA is associated with a variety of potentially toxic non-antisense effects.[38] In order to develop less toxic antisense oligonucleotides, nucleic acids have been modified in various ways.[39,40] Recently, morpholino modified oligonucleotides were shown to be delivered to muscle cells efficiently.[41] Furthermore, the chimera of 2′-*O*-methyl RNA and 2′-*O*, 4′-*C*-ethylene-bridged nucleic acid (ENA) was shown to induce exon 19 skipping of the dystrophin gene 40 times stronger than the conventional phosphorothioate oligonucleotides.[42]

Translational Readthrough of Stop Codon

Aminoglycoside antibiotics have been suggested as possible therapeutic interventions for treating patients who carry a nonsense mutation because of the ability of these antibiotics to lead translational readthrough of stop codons. To evaluate whether aminoglycosides can be used to suppress the nonsense mutation in a human DMD case, four DMD/BMD cases with various stop codon sequences were tested once daily with intravenous gentamicin at 7.5 mg/kg/day for 2 weeks. However, the full-length dystrophin protein was not detected in posttreatment muscle biopsies.[43] The possible reason for the failure of gentamicin treatment in human cases is the difference in efficiency of aminoglycoside-induced readthrough among the different types of nonsense mutations.

CONCLUSION

Duchenne muscular dystrophy is a fatal disease without any effective treatment. Recent studies opened a door to the establishment of molecular therapy for DMD.

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Echinococcus granulosus and *Echinococcus multilocularis*—Molecular Diagnosis

Donald P. McManus
The Queensland Institute of Medical Research, Queensland, Australia

INTRODUCTION

Echinococcosis (hydatid diseases) is a near-cosmopolitan zoonosis caused by adult or larval stages of dog tapeworms (cestodes) belonging to the genus *Echinococcus* (family Taeniidae). The two major species of medical and public health importance are *Echinococcus granulosus* and *Echinococcus multilocularis* that, respectively, cause cystic echinococcosis (CE) and alveolar echinococcosis (AE). Both CE and AE are serious and severe diseases, the latter especially so, with high fatality rates and poor prognosis if managed incorrectly. Molecular techniques are proving of value in the study of both CE and AE, in particular for investigating genetic variation in the causative organisms, for the detection of parasite nucleic acids in clinical samples, and in the identification of *Echinococcus* eggs. Detecting minute amounts of parasite DNA and mRNA, not only to identify but also to characterize the biological status of parasite material, is becoming recognized as a powerful complementary method to conventional methods such as immunodiagnosis.

CLINICAL FEATURES OF CE AND AE

A comprehensive account of the clinical features of both diseases is available[1] so only a brief description is presented here. Hydatid cysts of *E. granulosus* develop in internal organs (mainly liver and lungs) of humans and other intermediate hosts as unilocular fluid-filled bladders. The cyst (metacestode) of *E. multilocularis* is a tumor-like infiltrating structure consisting of numerous small vesicles embedded in the stroma of connective tissue. The metacestode mass usually contains a semisolid matrix rather than fluid. The initial phase of primary *Echinococcus* infection is always asymptomatic. The incubation period of CE is unclear but probably lasts for many months to years. The infection may become symptomatic if the cysts either rupture or exert a mass-effect. Recurrence may occur following surgery on primary cysts. Cystic echinococcosis has been reported to present for medical attention in subjects ranging in age from below 1 year old to more than 75 years old. The mortality rate is estimated to be 0.2 per 100,000 population with a fatality rate of 2.2%. Over 90% of cysts occur in the liver, lungs, or both. Symptomatic cysts have been reported occasionally in other organs.

Alveolar echinococcosis typically presents later than CE. Cases of AE are characterized by an initial asymptomatic incubation period of 5–15 years duration and a subsequent chronic course. Untreated or inadequately managed cases have high fatality rates. The peak age group for infection is from 50 to 70 years. The metacestode develops almost exclusively in the liver (99% of cases). Parasitic lesions in the liver can vary from small foci of a few millimeters in size to large (15–20 cm in diameter) areas of infiltration.

Early diagnosis of CE and AE can provide significant improvements in the quality of the management and treatment of both diseases. The definitive diagnosis for most human cases of CE and AE is by physical imaging methods such utrasonography. Immunodiagnosis complements the clinical picture while molecular techniques have been recently developed and adapted to advance laboratory diagnosis of AE and CE.

GENETIC VARIATION IN *ECHINOCOCCUS*

E. granulosus comprises a number of intraspecific variants or strains that exhibit considerable variation at the genetic level.[2–4] Conversely, there is very limited genetic variation within *E. multilocularis*[2,3] although recent microsatellite analysis has shown such markers can be used to examine *E. multilocularis* population structure.[5] There are no available data to indicate that either *Echinococcus vogeli* or *Echinococcus oligarthrus*, the other recognized *Echinococcus* species, is variable.

The term strain defines variants that differ from other groups of the same species in gene frequencies or DNA sequences, and in one or more characters of actual or potential significance to the epidemiology and control of echinococcosis. The extensive variation in nominal *E. granulosus* may influence life cycle patterns, host specificity, development rate, antigenicity, transmission dynamics, sensitivity to chemotherapeutic agents, and

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pathology with important implications for the design and development of vaccines, diagnostic reagents, and drugs. For example, the adult parasite of the cattle strain of *E. granulosus* exhibits precocious development in the definitive host with a short prepatent period of 33–35 days, nearly a week earlier than that of the common sheep strain.[6] This complicates control efforts where drug treatment of definitive hosts is utilized as a means of breaking the cycle of transmission, as it necessitates an increase in frequency of adult cestocidal treatment.

A number of well-characterized strains are now recognized that all appear to be adapted to particular life cycle patterns and host assemblages.[2,3] To date, molecular studies, using mitochondrial DNA (*mt* DNA) sequences, have identified nine distinct genetic types (genotypes G1–9) within *E. granulosus*.[2] This categorization follows very closely the pattern of strain variation emerging based on biological characteristics.

TECHNIQUES AND APPROACHES FOR IDENTIFICATION OF *ECHINOCOCCUS* ISOLATES

Genetic variation in *Echinococcus* has been investigated in both the nuclear and mitochondrial genomes. The advent of the PCR has provided a highly sensitive approach that is now widely used for *Echinococcus* identification purposes, including discrimination of eggs. The various techniques applied for studies of this genetic variation have been described in detail.[2] A brief synopsis follows.

RFLP/RAPD Analysis

Earlier studies of molecular genetic variation in *Echinococcus* involved restriction fragment length polymorphism (RFLP) analysis using the conventional Southern blotting approach. It was able to distinguish several distinct strains of *E. granulosus*, and extensive study showed that RFLP patterns were stable within a particular strain. The conventional RFLP procedure was simplified, without loss of resolution or accuracy, by linking RFLP analysis with PCR targeting the nuclear rDNA ITS1 region. The random amplified polymorphic-PCR (RAPD-PCR) has also been used under carefully controlled conditions for distinguishing the four recognized *Echinococcus* species and genetically distinct forms of *E. granulosus*.

PCR-Amplified DNA Sequences

Comparison of the nucleotide sequence of defined DNA segments between organisms provides the most direct and sensitive means of detecting genetic variation. Mitochondrial sequences, particularly fragments of the mitochondrial protein-coding genes, *cox1* and *nad1*, have proved invaluable for *E. granulosus* strain identification.

Mutation Scanning Methods

Mutation scanning methods provide alternatives to DNA sequencing for the high-resolution analysis of PCR-amplified fragments. One such method is single-strand conformation polymorphism (SSCP) that has been used to rapidly screen large numbers of *Echinococcus* isolates. Another useful mutation scanning method is dideoxy fingerprinting (ddF), which is a hybrid between SSCP and conventional dideoxysequencing. The technique has been used for the direct display of sequence variation in the *cox1* gene to type and differentiate all of the *Echinococcus* genotypes examined by their characteristic and reproducible ddF fingerprinting profiles.

Microsatellite Markers

A virtually untapped area for studying diversity in *Echinococcus* is the use of microsatellite DNA. Some microsatellite markers are available for *E. multilocularis*, following the earlier studies of Bretagne et al.[7] who were able to use microsatellite DNA to divide isolates of *E. multilocularis* into three groups: European, North American (Montana), and Japanese. More recently, Nakao et al.[5] isolated two microsatellite loci that were used to demonstrate population-level polymorphisms in *E. multilocularis* adult worms derived from wild red foxes collected from a limited geographical area of Hokkaido, Japan. The provision of microsatellite markers for *E. granulosus* and additional microsatellites from *E. multilocularis* will provide exquisitely sensitive tools for studying the population genetics and transmission biology of *Echinococcus* species.

DNA-BASED IDENTIFICATION OF *E. GRANULOSUS* STRAINS

The various genotypes of *E. granulosus* that have been identified using DNA analysis together with their host range and geographical distribution are presented in Table 1. A brief description of the major findings is presented here and reviewed in Refs. [2,3,8].

Sheep–Dog (G1 Genotype) and Horse–Dog (G4 Genotype) Strains

Discrete horse–dog and sheep–dog forms of *E. granulosus* occur that differ in a wide spectrum of biological criteria.

Table 1 Genotypes/strains of *E. granulosus* categorized by DNA analysis with their host and geographical range

Genotype (strain)	Host origin	Geographic origin
G1 (common sheep strain)	Sheep	United Kingdom, Spain, China, Australian mainland, Tasmania, Kenya, Uruguay, Turkey, Jordan, Lebanon, Italy, Argentina, Brazil, Iran, Nepal
	Cattle	United Kingdom, Spain, Kenya, Tasmania, Jordan, China
	Human	Australian mainland, Tasmania, Jordan, Lebanon, Holland, Kenya, China, Argentina, Spain
	Goat	Kenya, China, Nepal
	Buffalo	India, Nepal
	Camel	China
	Pig	China
	Kangaroo	Australian mainland
	Dog (adult)	Kenya
	Dingo (adult)	Australian mainland
G2 (Tasmanian sheep strain)	Sheep	Tasmania, Argentina
	Human	Argentina
G3 (buffalo strain?)	Buffalo	India
G4 (horse strain)	Horse	United Kingdom, Ireland, Switzerland
	Donkey	Ireland
G5 (cattle strain)	Sheep	Nepal
	Goat	Nepal
	Cattle	Switzerland, Holland, Brazil
	Buffalo	India, Nepal
	Human	Holland, Argentina
G6 (camel strain)	Camel	Kenya, Somalia, Kenya, Sudan, China, Iran, Mauritania
	Cattle	China, Iran, Mauritania
	Human	Argentina, Nepal, Iran, Mauritania
	Sheep	Iran
	Goat	Kenya
G7 (pig strain)	Pig	Poland, Slovakia, Ukraine, Argentina, Spain
	Wild boar	Ukraine
	Beaver	Poland
	Cattle	Slovakia
	Human	Poland, Slovakia
G8 (cervid strain)	Moose	United States
	Human	United States
G9 (?)	Human	Poland

Source: Modified from Ref. [2].

Confirmation of species identity was obtained by phylogenetic analysis (Fig. 1) using data from the complete mitochondrial genomes recently obtained for both strains. Of public health significance is the fact that the sheep strain is infective to humans but, probably, noninfective to horses. The horse strain appears to be poorly infective to sheep and may be noninfective to humans. This is borne out by the DNA data as, to date, the horse strain (G4 genotype) has not been reported in sheep or humans, and the sheep strain (G1 genotype) has not been identified in horses.

It has been recognized for some time that *E. granulosus* is maintained in two cycles of transmission on mainland Australia. One principally involves domestic sheep, whereas the other involves numerous species of macropod marsupials (kangaroos and wallabies). There is interaction between these cycles through a range of carnivores (domestic dogs, feral dogs, dingoes, and red foxes) which are definitive hosts. Early evidence led to their proposed designation as distinct strains but subsequent molecular analysis indicates that only the common sheep strain is present. In biological, epidemiological, and molecular features the common sheep strain can be regarded as homogeneous except in Tasmania where morphological distinctiveness, a significantly shortened prepatency period, and molecular evidence have indicated that a variant of the common *E. granulosus* genotype (designated genotype G2) occurs there. The G2 genotype is also

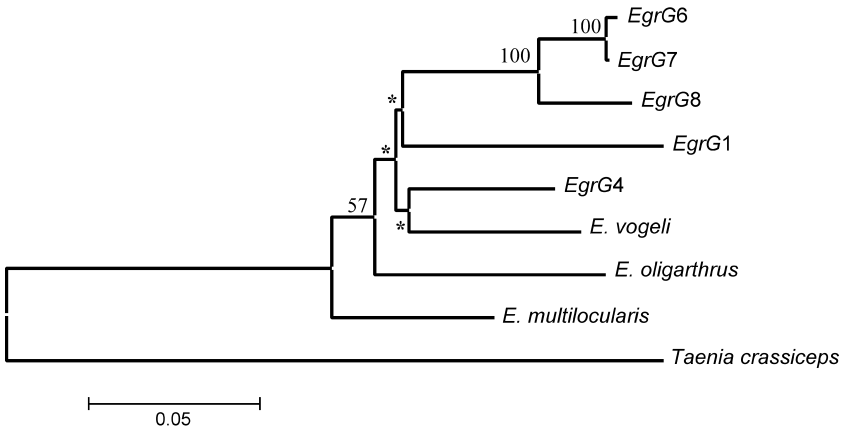

Fig. 1 Inferred relationships among species and genotypes of *Echinococcus*, using *Taenia crassiceps* as an outgroup. (From Ref. [9].) Concatenated sequences of mitochondrial DNA genes [*atp6*, *nad1* (partial) and *cox1* (partial)] were analysed. A distance matrix was constructed from inferred amino acid (aa) sequences (alignment was 451 aa long with 168 variable aa sites 67 aa parsimony-informative sites) using a Poisson correction for multiple hits and the tree constructed using the minimum evolution approach. Five hundred bootstrap resamplings were carried out. Branches with bootstrap support values less than 50% are indicated with an asterisk. EgrG1, EgrG4, EgrG6-EgrG8 are the different genotypes of *E. granulosus*. Units on scale bar: changes per site. The branches indicated by an asterisk were supported by fewer than 50% of the resampled data sets and therefore should be regarded as poorly supported. It is clear that EgrG4, EgrG1, *E. vogeli*, and *E. oligarthrus* are almost equidistant from each other in terms of mt sequences. Furthermore, the *E. granulosus* G1 and G4 genotypes are also almost equidistant from the G6-8 genotype cluster, although there is some structure in this latter group. *E. multilocularis* appears as basal within the genus, but again the branch placing it there is rather poorly supported. Given this, recognition of the sheep–dog (G1 genotype) and the horse–dog (G4 genotype) strains (and possibly also the G6-8 genotypes) as separate species is appropriate. The discrete nature of the two forms is quite clear and the molecular and phylogenetic evidence from this and previous studies suggests the case for reinstatement of their formal taxonomic status as subspecies/species is now proven.

present in Argentina, possibly having been introduced with Merino sheep exported from Australia to Argentina.[10]

Camel–Dog (G6 Genotype) Strain

Two distinct strains of *E. granulosus* have been identified by molecular methods in Kenya. The sheep strain of *E. granulosus* occurs in sheep, cattle, goats, and man, with the camel strain (G6 genotype) infecting camels and occasionally goats. DNA analysis has clearly indicated that the camel–dog strain (G6 genotype) of *E. granulosus* as well as the cosmopolitan, common sheep strain (G1 genotype) also occurs in Iran and China. DNA studies have indicated that the camel strain is infectious to humans and circulates between intermediate hosts including camels and cattle in Mauritania; if the sheep strain is present, it is rarely found there.

As well as Mauritania, molecular studies of *E. granulosus* from Argentina, Iran, and Nepal have reported the presence of the G6 genotype in several human subjects. This has potentially important implications for public health and implementation of hydatid control programs where the camel strain is involved in *E. granulosus* transmission. The camel strain has a shorter maturation time in dogs than the common sheep strain which is the form generally associated with human infection.

Bovine Strain (G5 Genotype)

Until the early 1990s, all surgically obtained human isolates of *E. granulosus* examined by isoenzyme and DNA analysis conformed to the common domestic sheep strain. However, a partly calcified hydatid cyst removed from an 11-year-old Dutch boy typed by PCR/RFLP analysis and *cox1* and *nad1* sequence comparisons with known genotypic sequences showed clearly that the patient was infected, not with the sheep strain, but with the genetically distinct bovine or cattle strain (G5 genotype) of *E. granulosus*. This strain has also been shown to infect humans in Argentina.[11] Thus, in regions where the bovine strain occurs, cattle may act as reservoirs of human infection. As is evident from scrutiny of Table 1, DNA analysis indicates that several *E. granulosus* genotypes (G2, G5, G6, G7, G8, and G9) are infective to humans.

Pig–Dog Strain (G7 Genotype)

Analysis of *E. granulosus* from Polish patients indicated they were not infected with the common sheep strain (G1 genotype) of *E. granulosus*. Instead, the isolates shared similar DNA sequence with the previously characterized pig–dog strain (G7) genotype but exhibited some clear

differences. Accordingly, it was proposed that these human isolates represented a distinct *E. granulosus* genotype (designated G9). Subsequent studies of human and pig isolates from Poland, Slovakia, and Ukraine failed to confirm the existence of this genotype but have provided evidence for the almost exclusive presence of the G7 genotype.[12] Confirmation of the existence of the G9 genotype and identification of the reservoir(s) of human hydatid disease in Poland and other countries in Central and East Europe remain unresolved.

Cervid Strain (G8 Genotype)

The ''cervid'' strain (also called ''sylvatic strain'' or ''northern form'') of *E. granulosus* occurs in North America and Eurasia. The wolf is the principal definitive host, and moose and reindeer (family Cervidae) serve as intermediate hosts; cycles involving sled dogs and domesticated reindeer also occur. Based on its unique *nad1* sequence and ITS1 PCR-RFLP pattern, a cervid strain obtained from Alaskan moose appeared to represent a distinct genotype of *E. granulosus* which was designated G8.[13]

Case-based data have suggested that the course of sylvatic disease is less severe than that of domestic disease, which led to the recommendation to treat cystic echinococcosis patients in the Arctic by careful medical management rather than by aggressive surgery. The first two documented *E. granulosus* human cases in Alaska with accompanying severe sequelae in the liver were recently reported.[14] The results of molecular analysis of the cyst material of one of the subjects supported the identification of the parasite as the sylvatic (cervid) strain (G8 genotype) and not the domestic (common sheep strain), which was initially thought to be implicated in these unusually severe Alaskan cases.[15] The adverse outcomes could have been rare complications that are part of the clinical spectrum of sylvatic CE, an indication that the sylvatic form of *E. granulosus*, especially when affecting the liver, has potential for severe clinical consequences.

DETECTION OF PARASITE NUCLEIC ACIDS IN CLINICAL SAMPLES

Clinical findings require sophisticated investigations to confirm echinococcosis. Usually, primary identification and characterization of echinococcal lesions occur by imaging techniques. However, the diagnostic potential of such techniques is sometimes limited by the atypical appearance of the visualized lesions that may also be insufficient in providing information about the involved species or about the viability of the parasite. Immunodiagnosis is a useful complementary diagnostic tool for the identification of infection and disease.[16] Recent efforts have been undertaken to apply molecular identification methods for direct detection of parasite RNA or DNA in clinical samples by Southern/Northern blotting or PCR.[17] These molecular methods have been used mainly in the clinical context for the primary diagnostic identification of parasite materials in biological specimens resected or biopsied from patients, and also for the assessment of the viability of parasite samples after chemotherapy or other treatment. Detection of parasite nucleic acids in clinical samples from AE or CE patients has been substantially improved by the use of the PCR. Generation of specific primers and their use in PCR and RT-PCR allows the detection of minute amounts of parasite RNA/DNA collected during surgical removal of cyst material or by fine needle aspiration biopsy (FNAB).

CONCLUSION

The range of DNA techniques now available for the study of genetic variation in *E. granulosus* and the molecular epidemiology of cystic echinococcosis is impressive, and much valuable information on the molecular categorization of the different genotypes is now available. Importantly, in many cases, molecular techniques have validated the genetic basis of important morphological and other biological differences that can now be used with confidence as a reliable and simple means of identifying and differentiating between strains and species of *Echinococcus*. The recent publication of the complete sequences of the *mt* genomes of the horse and sheep strains of *E. granulosus*[9] and *E. multilocularis*[18] and *mt* DNA sequences for a number of other *E. granulosus* genotypes[9] has provided additional genetic information that can be used for even more in-depth strain characterization and phylogeny of *Echinococcus* spp. Already, the availability of sequence information has provided a solid molecular basis for revising the taxonomy of the genus *Echinococcus*.[4,19]

Furthermore, the accumulating genetic data may allow insight to several other unresolved questions such as confirming the presence and precise nature of the G9 genotype and its reservoir in Poland, whether it occurs elsewhere, why the camel strain (G6 genotype) appears to affect humans in certain geographical areas but not others, more precise delineation of the host and geographic ranges of the genotypes characterized to date, and whether additional genotypes of *E. granulosus* remain to be identified. In this context, an important recent study by Gonzalez et al.[20] highlights the complexity and genomic organization differences in *E. granulosus*. Based on two *E. granulosus* DNA multiplex-PCR amplification fragments they developed three PCR protocols (Eg9-PCR, Eg16-PCR, and Eg9-PCR-RFLP) for discrimination of

E. granulosus genotypes. They used the approach to identify distinct G1 and G7 genotypes within *E. granulosus* Spanish pig isolates. Sequencing of the *nad1* and *cox1* genes and ITS1-PCR coupled to RFLP confirmed these observations. The Eg9-PCR-RFLP and Eg16-PCR protocols could thus be used as additional methods to discriminate the recognized *E. granulosus* genotypes and they might be especially useful for resolving the issue of the G7/G9 genotypes and human infection in Poland.

Finally, it should be emphasized that as well as proving of value for investigating genetic variation in *Echinococcus* and in the detection of parasite nucleic acids in clinical samples, molecular approaches can be used to identify and discriminate *Echinococcus* eggs (deposited in the feces of dogs and other carnivores) from those of other taeniid eggs in definitive hosts. A PCR-based assay has been developed for detecting DNA of *E. multilocularis* in fecal samples of foxes after isolation of the parasite eggs by a sieving procedure.[21] There is no similar test available yet for *E. granulosus* although one is being developed.[22] The copro-PCR is a valuable method for confirmation of positive coproantigen results by ELISA and for diagnosis in individual animals.

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Ehlers–Danlos Syndrome

Anne De Paepe
Paul Coucke
Fransiska Malfait
Center for Medical Genetics, University Hospital, Ghent, Belgium

INTRODUCTION

The Ehlers–Danlos syndrome (EDS) comprises a clinically and genetically heterogeneous group of connective tissue diseases of which the principle clinical features are skin hyperextensibility, joint hypermobility, and generalized connective tissue fragility. These clinical manifestations are present, to varying degrees, in each subtype of the condition. The latest classification of EDS, the Villefranche nosology, recognizes six subtypes for which major and minor diagnostic criteria were defined. The classic, hypermobile, and vascular type of EDS are the most common, whereas the kyphoscoliosis, arthrochalasis, and dermatosparaxis type constitute very rare conditions. Several EDS subtypes are caused by mutations in the structural genes for type I (arthrochalasis type), type III (vascular type), or type V collagen (classic type) or in genes involved in the processing of type I collagen (kyphoscoliosis and dermatosparaxis type).

EHLERS–DANLOS SYNDROME: CLASSIC TYPE

Clinical Description

Classic Ehlers–Danlos syndrome is inherited as an autosomal dominant trait.[1] The former EDS type I and type II are now reclassified as the ''classic subtypes'' of EDS for which major and minor diagnostic criteria have been established (Table 1).[2]

Major diagnostic criteria for classic EDS are skin hyperextensibility, widened atrophic scarring, and joint hypermobility. Skin hyperextensibility should be tested at a neutral site, meaning a site not subjected to mechanical forces or scarring, such as the volar surface of the forearm. It is measured by pulling up the skin until resistance is felt. The skin is hyperelastic, which means that it extends easily and snaps back after release.

Widened atrophic scarring is a manifestation of tissue fragility and occurs mainly over the knees, elbows, shins, forehead, and chin. It is characterized by splitting of the skin following relatively mild trauma, and formation of ''cigarette-paper scars,'' which are wide and thin scars, often with pigment deposition. Joint hypermobility is general, affecting both large and small joints. It is usually noted when a child starts to walk. It should be assessed using the Beighton scale,[3] the most widely accepted grading system for the objective semiquantification of joint hypermobility. The maneuvers used in this scoring system are listed below.

1. Passive dorsiflexion of the little fingers beyond 90° (one point for each hand).
2. Passive apposition of the thumbs to the flexor aspects of the forearm (one point for each thumb).
3. Hyperextension of the elbows beyond 10° (one point for each elbow).
4. Hyperextension of the knees beyond 10° (one point for each knee).
5. Forward flexion of the trunk with knees fully extended so that the palms of the hands rest flat on the floor (one point).

A score of 5/9 or greater defines joint hypermobility.

Minor diagnostic criteria for classic EDS are smooth, velvety skin, easy bruising, molluscoid pseudotumors, which are fleshy lesions associated with scars over pressure points such as elbows and knees, and subcutaneous spheroids, which are small, hard cystlike nodules, freely movable in the subcutis over bony prominences of the legs and arms. Other minor features include complications of joint hypermobility (e.g., sprains, dislocations, subluxations, and pes planus). Occasional or habitual dislocations of joints, such as the patella, shoulder, and hip, are common and are usually resolved spontaneously or easily reduced by the patient.

Cause and Laboratory Diagnosis

The genes encoding type V collagen, *COL5A1* and *COL5A2*, are known to be associated with the classic form of Ehlers–Danlos syndrome. Type V collagen is a quantitatively minor fibrillar collagen that is widely

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Table 1 Classification of Ehlers–Danlos syndrome

Subtype	Inheritance pattern	Protein	Gene	Methods for laboratory diagnosis
Classic type (EDS type I/II)	AD	Type V procollagen	*COL5A1/COL5A2*	*COL5A1* ''null-allele'' testing Molecular screening for mutations in *COL5A1* or *COL5A2*
Hypermobility type (EDS type III)	AD	Not known	Not known	Not available
Vascular type (EDS type IV)	AD	Type III procollagen	*COL3A1*	Biochemical analysis of type III collagen by SDS-PAGE Molecular screening for mutations in *COL3A1*
Kyphoscoliotic type (EDS type VI)	AR	Lysyl hydroxylase-1	*PLOD1*	Increased ratio deoxypyridoline to pyridoline cross-links in urine by HPLC Measurement of lysyl hydroxylase-1 activity Molecular screening of *PLOD1* gene
Arthrochalasis type (EDS type VIIA /VIIB)	AD	Type I procollagen	*COL1A1/COL1A2* Partial loss or complete skipping of exon 6	Biochemical analysis of type I collagen by SDS-PAGE Detection of complete or partial skipping of exon 6 in *COL1A1* or *COL1A2*
Dermatosparaxis type (EDS type VIIC)	AR	Procollagen-*N*-proteinase	*ADAMTS*-2 gene	Biochemical analysis of type I collagen by SDS-PAGE Molecular screening of *ADAMTS-2*-gene

distributed in a variety of tissues. In skin, tendon, and bone it is present mainly as [α1(V)]2 α2(V) heterotrimers. Type V collagen forms heterotypic fibrils with type I collagen and regulates the diameter of those fibrils.[4] It is thought that the huge amino-terminal propeptide of type V collagen is responsible for this regulatory function.

Biochemical studies on cultured fibroblasts, performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of metabolically labeled collagens produced by dermal fibroblasts, are usually not useful in confirming the diagnosis because type V collagen is synthesized by fibroblasts at low levels, so that quantitation and evaluation of alterations in electrophoretic mobility are poorly reproducible. Rarely, an abnormal electrophoretic pattern for type I collagen is detected because of nonglycine substitution (R134C) in the *COL1A1* gene coding for the proα1(I) collagen chain of type I collagen.[5] A *COL5A1* ''null-allele'' test is available for clinical testing. This test detects a nonfunctional *COL5A1* allele by genotyping polymorphic markers at the genomic and the cDNA level, hence requiring cultured fibroblasts. This test detects a *COL5A1* null-allele in approximately 30% of individuals with classic EDS. Sequence analysis of the *COL5A1* and the *COL5A2* gene is available on research basis only. Approximately 50% of patients with classic EDS have an identifiable mutation in one of these genes. Most molecular defects identified for classic EDS lead to haploinsufficiency of the *COL5A1* mRNA. In approximately one-third of classic EDS patients, nonsense, frameshift, or splice site mutations that introduce a premature termination codon are responsible for a nonfunctional *COL5A1* allele.[6,7] Several mechanisms lead to nonsense-mediated decay of the mutation-bearing mRNA or to failure of the chains to associate. The predicted consequence is synthesis of about half the amount of normal type V collagen. Structural mutations in the *COL5A1* and the *COL5A2* gene, which exert a dominant negative effect, have been demonstrated in approximately 15–20 patients with classic EDS. These structural mutations are most commonly splice site

mutations leading to exon skipping, and remarkably few point mutations that result in the substitution of a glycine in the triple-helical region of the collagen molecule by a bulkier amino acid. A unique point mutation in the *COL5A1* gene that changes a highly conserved cysteine residue to a serine in the C-terminal propeptide of the α1(V) collagen chain has also been identified.[8] A G530S substitution located in the amino-terminal propeptide of the α1(V) collagen chain may be disease modifying in the heterozygous state and disease causing in the homozygous state.[9,10]

Recently, mutations in a noncollagenous protein, tenascin X, have been demonstrated to cause an autosomal recessive condition with great similarity to classic EDS, including hyperextensible skin, hypermobile joints, and easy bruising, but without delayed wound healing or atrophic scarring.[11]

EHLERS–DANLOS SYNDROME: HYPERMOBILITY TYPE

Clinical Description

The hypermobility type of EDS (the former EDS type III) is inherited as an autosomal dominant disorder; it is characterized by severe generalized joint hypermobility, as assessed by the Beighton scoring system, and associated signs, such as recurring (sub)luxations, swellings, and tendinitis. This results in early-onset, chronic, generalized joint pain, which is often debilitating. In particular, temporomandibular and sternoclavicular joints, wrist, shoulder, and patella dislocate frequently. The skin is involved, with smooth, velvety skin, or mild atrophic scarring. Hypermobility type of EDS has to be distinguished from familial articular hypermobility syndrome (FAHS), also called benign joint hypermobility syndrome (BJHS), a separate autosomal dominant condition, which is characterized by severe joint laxity, joint dislocations, and chronic pain, but no skin changes. It is currently under discussion whether BJHS is a mild form of a heritable disorder of connective tissue, and is in fact identical to the hypermobility type of EDS, or whether BJHS represents the upper end of the Gaussian distribution of the ''normal'' range of joint movement. In the absence of biochemical or molecular markers, the relationship between BJHS and hypermobility type of EDS will remain unclear.

Cause and Laboratory Diagnosis

The cause of the hypermobility type of EDS is unknown and hence laboratory diagnosis is currently not available.

EHLERS–DANLOS SYNDROME: VASCULAR TYPE

Clinical Description

The vascular type of EDS (the former EDS type IV) is inherited as an autosomal dominant trait. It deserves special attention because its natural history and prognosis are different from those of the other subtypes. The typical clinical features of vascular EDS comprise a thin, translucent skin with a prominent venous pattern over the chest, the abdomen, and the extremities, increased bruising and bleeding tendency, and proneness to sudden rupture of blood vessels, internal organs, or the gravid uterus. Patients often have a characteristic facial appearance with large prominent eyes, small, pinched nose, small lips, and lobeless ears. Hypermobility is usually limited to the small joints of the hands. Other features include acrogeria, which is characterized by an aged appearance of the extremities, particularly the hands, tendon or muscle rupture, talipes equinovarus, early-onset varicose veins, arteriovenous, carotid-cavernous sinus fistula, pneumothorax or pneumohemothorax, and gingival recession. Family history may be positive for sudden death in a close relative.

A retrospective review of the health history of more than 400 individuals with vascular EDS has delineated the natural history of the disorder.[12] The median age of death is 48 years. Complications are rare during childhood, but 25% of index patients have experienced a first complication by the age of 25 years and 80% by the age of 40 years. Vascular complications and gastrointestinal perforation or organ rupture are presenting signs in 70% of adults with vascular EDS. Such complications are dramatic and unexpected, often presenting as sudden death, stroke, acute abdomen, retroperitoneal bleeding, uterine rupture at delivery, and shock.[13] The average age for the first vascular or gastrointestinal complication is 23 years. Arterial rupture may be preceded by aneurysm, arteriovenous fistulae, or dissection, but may also occur spontaneously. Rupture of the gastrointestinal tract, most of which affect the sigmoid colon, occurs in about 25% of affected individuals. Bowel rupture is usually not lethal. Pregnancy for women with the vascular type has a risk of 12% for death from peripartum arterial rupture or uterine rupture.

Cause and Laboratory Diagnosis

The condition is caused by mutations in the *COL3A1* gene coding for the α1 chain of type III collagen. This homotrimer is a major structural component of skin, blood vessels, and hollow organs. The diagnosis of vascular EDS is based on compatible clinical findings

and confirmed by biochemical testing. Biochemical testing includes analysis by SDS-PAGE of radioactively labeled collagens from skin fibroblast cultures. This is a highly sensitive investigation and probably identifies more than 95% of individuals with structural alterations in the proteins synthesized. It allows one to detect quantitative (reduced amounts of collagen type III) or qualitative (mutant collagen type III with altered electrophoretic mobility) abnormalities of type III collagen. For example, substitution of a glycine residue by a bulkier amino acid destabilizes the helix and delays its formation, causing excessive posttranslational modification and hence an altered electrophoretic pattern. Molecular genetic testing to identify mutations in the *COL3A1* gene is available to patients with a biochemically confirmed diagnosis of vascular EDS. To date, more than 250 *COL3A1* mutations have been identified. These include point mutations leading to substitutions for glycine in the triple-helical region of the collagen molecule, splice site mutations resulting in exon skips, intron inclusion or complex and multiple outcomes, partial gene deletions, and, less commonly, mutations resulting in haploinsufficiency.[14] Vascular EDS is an autosomal dominant disorder, but parental somatic mosaicism for *COL3A1* mutations has been documented.

EHLERS–DANLOS SYNDROME: KYPHOSCOLIOTIC TYPE

Clinical Description

The kyphoscoliotic type of EDS (the former EDS type VI) is a rare, autosomal recessive condition that is characterized by generalized joint hypermobility, severe muscle hypotonia at birth, progressive kyphoscoliosis, which may be present at birth or develop within the first year of life, and scleral fragility with rupture of the globe. Another prominent finding is arterial rupture of medium-sized arteries or aortic dilatation and dissection. The life span of patients is significantly reduced because of these arterial ruptures or because of cardiopulmonary insufficiency as a result of severe kyphoscoliosis. Other features are tissue fragility and atrophic scarring, easy bruising tendency, marfanoid habitus, microcornea, and osteopenia, although without tendency toward fractures.

Cause and Laboratory Diagnosis

This autosomal recessive disorder is caused by deficient activity of the enzyme procollagen-lysine, 2-oxoglutarate 5 dioxygenase-1 (lysyl hydroxylase-1, PLOD), a collagen-modifying enzyme. Deficiency of this enzyme results in a deficiency in hydroxylysine-based pyridinoline cross-links in types I and III collagen. As a result, cross-linked peptides are excreted in urine as by-products of collagen turnover. The diagnosis relies on the demonstration of the presence of an increased ration of deoxypyridinoline to pyridinoline cross-links in urine, quantitated by HPLC, which is a highly sensitive and specific test.[15] Activity of the lysyl hydroxylase-1 can also be measured in cultured fibroblasts.[16] Molecular testing of the *PLOD-1* gene is available on a research basis only. An intragenic duplication caused by an Alu–Alu recombination in introns 9 and 16 is the only common mutant allele, with a frequency of 19% in 35 families with EDS, kyphoscoliotic form.[17]

EHLERS–DANLOS SYNDROME: ARTHROCHALASIS TYPE

Clinical Description

The arthrochalasis type of EDS (the former EDS type VIIA and B) is inherited as an autosomal dominant trait. The clinical hallmark of this type is congenital bilateral hip dislocation.[18] There is also severe generalized joint hypermobility with recurrent dislocations of large and small joints and ligamentous tears. The skin is moderately involved, with skin hyperextensibility, a velvety touch, and poor wound healing with formation of atrophic scars. Other features include easy bruising, muscular hypotonia and delay in gross motor development, kyphoscoliosis and osteopenia with wormian bones and sometimes a history of fractures.

Cause and Laboratory Diagnosis

The cause of the arthrochalasis type of EDS is the result of mutations leading to loss of exon 6, or part of it, of the mRNA coding for one of the α1 chains (EDS type VIIA) or for the α2 chain (EDS type VIIB) of type I collagen.[18] This exon-skipping mutation leads to the loss of the amino-terminal telopeptide, which links the *N*-propeptide to the main, triple-helical domain. This *N*-telopeptide contains the *N*-proteinase cleavage site as well as the critical cross-linking lysyl residue and the cleavage sites for proteinases such as pepsin and α-chymotrypsin. Hence, lack of this segment leads to deficient processing of the *N*-propeptide of type I collagen. The biochemical confirmation of the diagnosis is based on electrophoretic demonstration of pNα1(I) (type A) or pNα2(I) (type B) chains of collagen type I extracted from dermal collagen or harvested from cultured skin fibroblasts. Molecular analysis can be performed to confirm the biochemical

findings and shows complete or partial skipping of exon 6 at the cDNA level of the *COL1A1* or the *COL1A2* gene, respectively, usually caused by splice site mutations at the genomic DNA level.

EHLERS–DANLOS SYNDROME: DERMATOSPARAXIS TYPE

Clinical Description

This rare condition is inherited as an autosomal recessive condition. The clinical picture is characterized by premature rupture of the membranes, extreme skin fragility and laxity, easy bruising, large fontanels, umbilical hernia, short stature and characteristic facies with epicanthic folds, downslanting palpebral fissures, puffy eyelids, blue sclerae, and micrognathia. During childhood and puberty, increased bruising and severe skin fragility predominate the clinical picture. Wound healing is not delayed and initial scar formation is only minimal.[19] In older patients, however, more typical atrophic scarring with pigmentation is seen, probably because of repeated skin tearing and bruising. Joint hypermobility, although not obvious during the first years of life, seem to become more important with age. Fragility of internal tissues is an important feature during childhood, as is illustrated by spontaneous bladder rupture at the age of 5 years in two children with the dermatosparaxis type of EDS (personal observation).

Cause and Laboratory Diagnosis

EDS dermatosparaxis type and the related animal disease dermatosparaxis are recessively inherited connective tissue disorders, caused by a deficient activity of procollagen-I-*N*-proteinase, the enzyme that excises the N-terminal propeptide in procollagen type I, type II, and type III. As a consequence, there is accumulation of pN-procollagen,[20] resulting in polymerization of abnormal collagen fibers that appear thin, irregular, branched and ''hieroglyphic'' in cross section.[21] The diagnosis can be made based on these characteristic clinical, biochemical, and electromicroscopic findings. Molecular confirmation of the diagnosis is available on a research basis. Homozygous mutations in the gene encoding procollagen-I-*N*-proteinase or ADAMTS-2 (a disintegrin and metalloproteinase with thrombospondin-like repeats), an enzyme belonging to an expanding family of zinc metalloproteinases found in vertebrates and invertebrates, have been identified in all reported patients with dermatosparaxis.[22]

CONCLUSION

The Ehlers–Danlos syndrome is a clinically and genetically heterogeneous group of connective tissue diseases. At least six different subtypes are currently recognized, and several fibrillar collagens are involved in the etiology of the condition. Structural mutations in collagen type I, III, and V are causal in the dominant subtypes of the condition, whereas recessive EDS subtypes are associated with defects in enzyme activity involved in the biosynthesis of type I collagen. Developments in the elucidation of the biochemical and molecular basis of EDS, together with increasing clinical experience, add a new dimension to diagnosis, genetic counseling, and management of these heritable disorders. Future research will further increase the understanding of the molecular mechanisms involved and open perspectives for gene therapy.

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Electrospray Tandem Mass Spectrometry for Newborn Screening

Yosuke Shigematsu
Fukui Medical University School of Nursing, Fukui, Japan

INTRODUCTION

Newborn screening programs for inherited diseases are well established in many developed countries. Dried blood spots on screening cards are collected from newborns for early detection of diseases and improvement of their health. Before the recent application of tandem mass spectrometry (MS–MS) to newborn screening, one test on one sample of blood was used to screen a single disease, phenylketonuria (PKU), which was screened originally by a bacterial inhibition assay of phenylalanine.

However, MS–MS enables the sensitive and quantitative analysis of many ionic compounds with high polarity, such as acylcarnitines and amino acids, in a single process. The first proposal of this technology for newborn screening was reported in 1990. More than 30 inborn errors of metabolism (IEM) in the categories of fatty acid oxidation disorders, organic acidemias, and aminoacidopathies can be diagnosed simultaneously by MS–MS measurement of one small punch of a blood spot. In clinical settings, fatty acid oxidation disorders and organic acidemias were known to cause sudden death in infancy, which could be prevented by early recognition of these diseases by MS–MS. The phenylalanine/tyrosine ratio determined by MS–MS was demonstrated to be a sensitive marker in PKU screening among newborns discharged from hospitals less than 24 hr. Thus the use of MS–MS technology has expanded rapidly into newborn screening.

However, most target diseases in MS–MS screening are rather rare and heterogeneous in clinical severity, and the screening's clinical validity, clinical utility, and cost-effectiveness have not yet been fully established. Further careful data collection regarding the sensitivity of MS–MS screening and outcomes of patients in whom disorders are detected is required.

INSTRUMENTATION AND BASIS OF ANALYSIS

Instrumentation for MS–MS screening consists of a triple quadrupole mass spectrometer controlled by a computer system, an electrospray ionization (ESI) interface, and a liquid chromatograph equipped with an autoinjector.

A mass spectrometer is a ''mass'' detector by which gas-phase ions can be analyzed.[1] The masses of ions are recorded as ratios of molecular weight to charge (m/z). Through an ESI device, ionic compounds in the liquid phase can be introduced into MS as gas-phase ions. ESI plays an important role in MS–MS screening by the sequential introduction of sample solutions, which are injected automatically into the continuous flow of a liquid chromatograph at 2-min intervals without chromatographic separation.

A triple quadrupole MS system comprises two mass analyzers, which are separated by a collision chamber that induces the fragmentation of ionic compounds by the introduction of argon gas. Using a computer algorithm, the intensities of the masses of ionic compounds in the first mass analyzer (precursor ions) and those of their fragment ions in the second mass analyzer (product ions) are recorded in synchronized manners. In acylcarnitine analysis, a fragment ion of m/z 85, which characterizes the structure of butylester derivatives of acylcarnitines (Fig. 1), is monitored in the second mass analyzer, whereas the masses of ionic compounds are analyzed in the first mass analyzer. In amino acid analysis, the masses are analyzed synchronously in both mass analyzers to record the intensities of the masses of fragment ions produced from the precursor ions after the loss of 102 atomic mass units (Fig. 2). Thus, without chromatographic separation of the sample mixture, the information on the characteristic fragmentations enables the measurement of acylcarnitines and amino acids with high specificity. Quantification is performed using stable isotope-labeled compounds as internal standards.

Data are recorded by class-specific analysis or target compound analysis.[2] In the latter analysis, called selected or multiple reaction monitoring, a certain transition of the precursor to the product is monitored to analyze a single compound. The degree of fragmentation can be optimized to improve sensitivity by controlling both the amount of argon gas in the chamber and the energy of the compounds entering the chamber and argon. If target disorders in MS–MS screening are restricted, the latter analysis allows for monitoring of the selected compounds.

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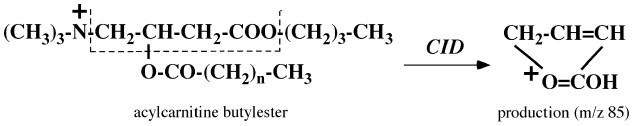

Fig. 1 Fragmentation of acylcarnitine butyl esters in a collision chamber by collision-induced dissociation (CID).

Otherwise, the former analysis, such as the precursor scanning of *m/z* 85 for acylcarnitines, where a certain range of masses of ionic compounds is analyzed sequentially, is more comprehensive and useful to confirm the quality of the processed samples.

PROCEDURES FOR MS–MS SCREENING

Specimen preparation for MS–MS screening requires an extraction and derivatization process,[3] which is performed using 96-well polypropylene microplates in routine analysis.[4–6] One 3.2-mm disk of dried blood spot is punched out into the individual wells of a microplate. To the disk in each well, a methanol solution containing known concentrations of stable isotope-labeled standards is added. The microplate is covered and the samples are mixed. The extract is transferred to a second microplate and dried. The residue in each well is derivatized with butanolic HCl. After removing the excess reagent in each well, the derivatized residue is dissolved in an acetonitrile–water mixture. Then the microplate is sealed and placed in the autoinjector tray for ESI–MS–MS analysis.

In routine analysis under the control of the computer system, intensities of designated ionic compounds of the sample are measured in multiple modes of analysis, and a set of data for the sample is recorded sequentially. The amounts of the compounds as diagnostic markers are calculated using the assumed volume of blood in a punch and the intensities of both the compounds and the respective stable isotope-labeled internal standards. At present, not all internal standards for acylcarnitine analysis are available, and the amounts of some acylcarnitines are calculated using the labeled acylcarnitine with different acyl groups (e.g., deuterium-labeled C_{14}-acylcarnitine for C_{14}:1-acylcarnitine measurement and deuterium-labeled C_{16}-acylcarnitine for hydroxy-C_{16}-acylcarnitine measurement).

For samples with abnormal values for any diagnostic marker, repunched blood spots are analyzed to confirm the initial screening. Then resamplings for newborns with abnormal values are performed to obtain additional clues to the suspected disease and to confirm that the correct specimens have been tested. If the values are very abnormal, a full metabolic workup is initiated immediately.

E

TARGET DISORDERS AND DIAGNOSTIC MARKERS

Target diseases in MS–MS screening are listed in Table 1.

The clinical severity of target diseases, except for PKU and homocystinuria (HCU), is quite heterogeneous.[7,8] Patients with severe forms of the diseases present clinical crises with metabolic acidosis, hyperammonemia, and/or hypoglycemia in the newborn period and may not survive in spite of intensive care. Liver transplantations have been tried for patients with severe forms of organic acidemias and urea cycle disorders. Patients with milder forms may experience intermittent and sometimes life-threatening crises because of catabolic conditions with or without progressive brain dysfunction. Other symptoms of fatty acid oxidation disorders are muscle weakness and pain with high CK values. Some patients with mild forms of the diseases may not show any symptoms without medical intervention.

Long-term therapies for most diseases include low-protein and/or low-fat diets. Depending on the respective diseases, carnitine supplementation, coenzyme administration, avoidance of fasting, and treatment of acute episodes with intravenous infusion of glucose may be required. Medium-chain triglycerides are used for some fatty acid oxidation disorders, and sodium benzoate or alternatives are for hyperammonemia of urea cycle disorders.

Diagnostic Markers for Target Diseases

The cutoffs for diagnostic markers (Table 1) are set empirically in most laboratories based on the data from the retrospective analysis of the patients and the prospective studies. High false-positive rates have been reported in newborns with very low birth weights or those requiring intensive care.[6] To improve the sensitivity for screening, additional markers, such as the ratio of the main marker to the second marker, are proposed.

High levels of free carnitine and low levels of long-chain acylcarnitines in blood spots characterize carnitine

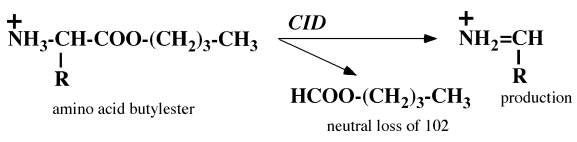

Fig. 2 Fragmentation of amino acid butyl esters in a collision chamber.

Table 1 Target diseases in MS–MS newborn screening

Disease	Primary diagnostic marker
Fatty acid oxidation disorders	
Carnitine transport defect	Free carnitine
Carnitine palmitoyltransferase I deficiency	Free carnitine
Carnitine palmitoyltransferase II deficiency	C_{16}-AC
Carnitine/acylcarnitine translocase deficiency	C_{16}-AC
Very long chain acyl-CoA dehydrogenase deficiency	C_{14}:1-AC
Long-chain hydroxy acyl-CoA dehydrogenase deficiency	Hydroxy-C_{16}-AC
Mitochondrial trifunctional protein deficiency	Hydroxy-C_{16}-AC
Medium-chain acyl-CoA dehydrogenase deficiency	C_8-AC
Short-chain acyl-CoA dehydrogenase deficiency	C_4-AC
Short-chain hydroxy acyl-CoA dehydrogenase deficiency	C_4-AC
Multiple acyl-CoA dehydrogenation deficiency or glutaric aciduria type II	Multiple ACs
Organic acidemias	
Methylmalonic aciduria	C_3-AC
Propionic acidemia	C_3-AC
Multiple carboxylase deficiency	Hydroxy-C_5-AC
3-Methylcrotonyl-CoA carboxylase deficiency	Hydroxy-C_5-AC
3-Ketothiolase deficiency	Hydroxy-C_5-AC
3-Methylglutaconyl-CoA hydratase deficiency	Hydroxy-C_5-AC
3-Hydroxy-3-methylglutaryl-CoA lyase deficiency	Hydroxy-C_5-AC
Isovaleric acidemia	C_5-AC
2-Methylbutyryl-CoA dehydrogenase deficiency	C_5-AC
Isobutyryl-CoA dehydrogenase deficiency	C_4-AC
Glutaric aciduria type I	C_5-DC
Malonic acidemia	C_3-DC
Aminoacidopathies	
Phenylketonuria	Phenylalanine
Maple syrup urine disease	Leucine
Homocystinuria	Methionine
Argininosuccinate synthetase deficiency	Citrulline
Citrin deficiency	Citrulline
Argininosuccinate lyase deficiency	Citrulline
Ornithine transcarbamylase deficiency	Citrulline
Arginase deficiency	Arginine
Tyrosinemia type I	Tyrosine

AC = acylcarnitine; DC = dioylcarnitine.

palmitoyltransferase I deficiency, and the increased ratio of free carnitine to the sum of C_{16}-acycarnitine and C_{18}-acylcarnitine (cutoff of 100) is used as a marker for this disease.[9] Although medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is screened using the cutoff of 0.5–1.0 nmol/mL in most laboratories, a retrospective investigation indicated the diagnostic criterion as a C_8-acylcarnitine concentration of more than 0.3 nmol/mL with a C_8-acylcarnitine/C_{10}-acylcarnitine ratio of more than 4.0.[10]

To detect patients with a mild form of propionic acidemia (PA) and methylmalonic aciduria (MMA), a cutoff of 0.25 for the ratio of the intensity of *m/z* 274 (propionylcarnitine) to that of *m/z* 260 (acetylcarnitine) seems useful.[11] Transient increases in propionylcarnitine have been frequently observed in infants with ABO incompatibility to the mother.[6] In spite of the application of such a marker, some MMA patients with defects in cobalamine metabolism have been missed.[1]

In PKU screening, a high phenylalanine-to-tyrosine ratio is a specific and sensitive marker to detect PKU using specimens collected in the first 24 hr[12] and to distinguish this disease from liver dysfunction and amino acid infusion.[13] The leucine-to-phenylalanine or methionine-to-phenylalanine ratio also may be useful to minimize the false-positive rate in maple syrup urine disease (MSUD) or HCU screening.[6]

Argininosuccinate synthetase (ASS) deficiency, argininosuccinate lyase (ASL) deficiency, and citrin deficiency are detected using high levels of citrulline. The increased argininosuccinic acid concentration, which can be monitored using the designated product ions, is another diagnostic marker for ASL deficiency. Most patients with citrin deficiency have citrulline levels higher than 50 nmol/mL in the neonatal period, together with high values of such amino acids as methionine, phenylalanine, tyrosine, and/or arginine.[11]

Ornithine transcarbamylase deficiency may be screened by the combination of low citrulline and high glutamine levels. Because glutamine is hardly quantified in a routine analysis of MS–MS screening, a second-tier screening method to measure glutamine levels is proposed to reexamine a blood spot with a low citrulline concentration.[14] Similarly, a succinylacetone confirmation test using a blood spot with a high tyrosine level is performed to distinguish tyrosinemia type I from transient neonatal tyrosinemia.[12]

Several organic acidemias share the same diagnostic markers, and urinary organic acid analysis is necessary to distinguish these diseases. Elevated C_5-acylcarnitine due to therapy with antibiotics containing pivaryl ester can be distinguished from isovaleric acidemia by urinary organic acid analysis. High levels of hydroxy-C_5-acylcarnitines because of a maternal 3-methylcrotonyl-CoA carboxylase

(3MCC) deficiency can be also detected by urinary organic analysis of the newborn and the mother.

Prevalence of Target Diseases Detected by MS–MS

MCAD deficiency and PKU are most prevalent among target diseases in Western countries, and the estimated incidence of these diseases is 1:10,000–20,000.[15,16] However, in Asian countries, these two diseases are rather rare, but some organic acidemias and citrin deficiency may be common.[11]

Of 1.0–1.2 million newborns studied in the Neo Gen Screening,[12] the following were detected: 65 patients with MCAD deficiency, 3 with very long chain acyl-CoA dehydrogenase deficiency, 3 with short-chain acyl-CoA dehydrogenase deficiency, 2 with long-chain hydroxy acyl-CoA dehydrogenase deficiency, and 2 with carnitine palmitoyltransferase II deficiency among fatty acid oxidation disorders; 68 patients with PKU, 65 with hyperphenylalaninemia, 12 with MSUD, 5 with ASS deficiency, and 2 with ASL deficiency among aminoacidopathies; and 13 patients with glutaric aciduria type I (GA-1), 15 with 3MCC deficiency, 9 with MMA, and 6 with PA among organic acidemias. Of about 0.18 million newborns in Japan,[11] the following were detected: six patients with PA, two with MMA, three with GA-1, two with multiple acyl-CoA dehydrogenation deficiency, two with MCAD deficiency, four with citrin deficiency, and two with PKU.

EVALUATION OF MS–MS SCREENING PROGRAMS

Clinical validity, such as sensitivity and specificity, may vary depending on different cutoffs among laboratories. In an Australian program, a quite low overall false-positive rate of 0.18% and five false-negative cases in 343,244 newborns were reported.[17] In the North Carolina Newborn Screening Program, an overall abnormal rate of 0.64% in 320,503 newborns was reported, although a majority of abnormal samples were due to tyrosine (0.30%) and propionylcarnitine (0.15%).[18] In our study, an overall false-positive rate of 0.58% in 102,200 newborns included that of 0.39% because of the use of pivalic acid-containing antibiotics.[11]

Clinical utility and cost-effectiveness may also vary depending on the sensitivity and the severity of the diseases. It is reported that MS–MS screening for MCAD deficiency alone appears to be cost-effective; adding the incremental costs for the 13 other disorders still yields a result well within accepted norms for cost-effectiveness in Wisconsin.[19] This report is based on the assumption that, in MS–MS screening for MCAD deficiency, 10% of patients are missed and the false-positive rate is 0.1%. A report from California based on the screening of 32,000 newborns plus other published data indicated that the cost of MS–MS screening per quality-adjusted year of life compared favorably with costs of other accepted screening procedures.[15] However, in this report, discounted treatment costs per case for MCAD deficiency were not calculated. Based on the assumption of a low rate of clinical presentations leading to death and handicap and on the assumption of a 0.25% rate of false-positive results for MCAD deficiency, the cost of false-positive results was estimated as quite high owing to hospital visits, laboratory tests, consultations, and admission.

CONCLUSION

ESI–MS–MS will be introduced into most newborn screening programs around the world because of its ability to deal with a large number of samples and to quantify multiple compounds in one blood spot disk in a single process, and because of the substantial benefit most patients with target diseases and their families can be provided through early treatment. However, it is important to recognize the limitation of this screening in view of the false-negative and false-positive rates. In addition, to patients with some target diseases, informed counseling and therapy based on the evidence cannot be offered at present. Furthermore, unexpectedly high incidences of some target diseases, as compared with those before the initiation of this screening, indicate that this screening may be detecting many patients who will not present the symptoms of the disease. Thus ESI–MS–MS screening programs should be further evaluated in terms of quality control in methodology, selection of target diseases, protocols for differential diagnosis, healthcare systems to follow the patients, and psychosocial burdens on the patients and their families.[20]

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Entamoeba histolytica, Entamoeba dispar

Patrick Gonin
Généthon, Evry, France

INTRODUCTION

Amebiasis is a parasite of high public health importance. It is one of the most important cause of death from parasitic disease in the world. Higher risks are in the countries where the barriers between feces and food/water are insufficient. It is now well established that of the two morphologically identical amoebas, *Entamoeba histolytica* and *Entamoeba dispar*, only the former is pathogenic. Around 500 million individuals in the world are infected with amebiasis caused primarily (90%) by *E. dispar*. *E. histolytica* is a powerful pathogen that uses proteases to destroy hosts' tissues, kill some hosts' cells, and ''phagocyte'' red cells. It may cause amebic colitis, and after breaching the mucosal barrier, reach the liver via the portal tract and induce liver abscesses. Since the first description of this agent, a significant amount of molecular data has now accumulated. This review will describe rapidly the main epidemiological and clinical characteristics of this organism, and focus mainly on the advances made in diagnosis, thanks to *Entamoeba* genomics knowledge.

TYPE OF PATHOGEN

E. histolytica is a nonflagellated protozoan parasite. The parasite life cycle consists of two forms: infective cyst and invasive trophozoite. This latter form can be responsible for liver abscess and amebic colitis, invasion of intestinal epithelium being harmful by direct destruction of tissues. As *E. histolytica* is morphologically undistinguishable from *E. dispar*, differential diagnostic is not easy. *E. dispar* has been described quite a long time ago (Brumpt), but the real scientific evidence of two distinct species is quite recent.[1,2] *E. dispar* has been, for a while, described as the ''nonpathogenic'' form of *E. histolytica* and was supposed to be able to convert to ''pathogenic'' in culture, a contamination artifact of cultures.[3]

CLINICAL DESCRIPTION OF INFECTION SPECTRUM

E. histolytica illness is characterized by chronic diarrhea, which may or may not contain gross blood, and weight loss. However, asymptomatic colonization may occur. Intestinal amebiasis and its complications are amebic colitis, ameboma, toxic megacolon, peritonitis, and cutaneous amebiasis.[4] Extraintestinal amebiasis includes amebic liver abscess, splenic abscess, brain abscess, empyema, and pericaditis.[4]

PREVALENCE

E. histolytica/dispar parasitize approximately 10% of the world population (90% asymptomatic). It has been estimated that there are 40 million amebiasis cases which cause around 110,000 deaths a year.[4,5] Detailed data about the prevalence in asymptomatic individuals in several populations are reviewed by Stanley.[6] The cyst form is resistant for several weeks in a moist environment.[4] Because cysts are resistant to low amounts of water chlorination, special precautions should be taken in endemic zones and known infected households (boil water, wash vegetables and fruits with soap, and if possible, soak for 15 min in vinegar).[4]

MANAGEMENT

Noninvasive *E. histolytica* should be treated using a luminal agent; *E. dispar* infection should not be treated, emphasizing the need for differential diagnostics (see below). Luminal agents include diloxanide furoate, paromomycin, and iodoquinol, with respective treatment duration of 10, 7, and 20 days.[7] Metronidazole is usable as a second choice if luminal agents cannot be used.[4] Invasive amebiasis (colitis, liver abscess) should be treated with metronidazole (10 days). More precise management of invasive form is described in Refs. [8] and [9]. Dehydroemetine might also be considered in fulminant colitis or ruptured amoebic liver abscess.[6]

DIAGNOSIS

Diagnosis has relied for many years on microscopic examination of stools of patients with diarrhea. However, the WHO recommends differentiation of *E. histolytica*

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and *E. dispar*, which is not feasible using this technique.[10] Molecular techniques discussed below have been described, which in many cases are more specific, and allow for differentiation.

MOLECULAR CHARACTERIZATION OF PATHOGEN AND MOLECULAR TESTING

E. histolytica and *E. dispar* genome shotgun sequencings are underway as a joint effort from TIGR[11] and the Sanger Centre,[12] funded by the National Institute for Allergy and Infectious Diseases and the Wellcome Trust, respectively. The genome is around 18–20 Mb in size in 14 chromosomes.[11–13] However, partial genomic data from these organisms are available since the end of the 1980s.[14,15] These data allowed designing molecular assays that are sensitive to detection and, more interestingly, that can differentiate between both.[16,17] Following these pioneering works, other early molecular techniques were designed as well.[18–21] To be readily used in the clinic, molecular detection and differentiation techniques have to be usable on feces, and as any infectious disease diagnostics technique, to be both sensitive and specific (or in fact, have good positive and negative predictive values). As these values depend on prevalence, it is quite clear that a ''good'' technique in a Western country, where *E. histolytica* is not endemic and quite rare, will not be as good as in a developing area, where it is much more prevalent. Moreover, there is a cost issue, regarding techniques such as PCR.

Nowadays, to distinguish between *E. histolytica* and *E. dispar*, there are mainly two alternate means: protein differentiation using monoclonal antibodies[22–25] or genetic differentiation using probes and/or PCR. As a commercial kit, the only available is the Techlab ELISA on stool samples. Numerous PCR techniques have been developed since 1989[18–21,26,27,29–33] (see Table 1 for a comparison). So far, only one real-time quantitative technique has been published, using the Roche Light-Cycler, and thus no internal probe.[26] The authors claim a sensitivity of 0.1 parasite per gram of feces, which is high, with a 100% specificity. Samples were from Vietnam and South Africa, and data are provided allowing for predictive value calculations.

On top of the requirements cited above, a ''good'' PCR technique should be usable on clinical feces such as sodium acetate–acetic acid–formalin (SAF) fixative or frozen samples. As SAF-fixed samples are the easiest to obtain and ship (because they are used for other examinations, and because they have no temperature requirements for shipping), they would be the best. Several authors have tried to design such techniques and investigated the effect of SAF fixative on PCR subsequent detection. It was shown that even if its effect on DNA is indirect, concentrations of formalin higher than 1% seemed to inhibit PCR amplification from 4 days of fixation.[34] This was confirmed by the work of Troll et al.[29] who showed that sensitivity of PCR usually decreased within 2 days in feces stored in SAF fixative. Both teams concluded that the effects of formalin are time-dependent. However, provided that PCR assay is carried out swiftly after fixation, SAF fixative-prepared samples are suitable and have been successfully used in the field by several teams.[35,36]

Several works are underway to design a vaccine, and the completion of *E. histolytica* and *E. dispar* genomes will open new avenues for diagnostics and vaccination.

Significant data have also now been accumulated on the molecular pathways of *E. histolytica* infection (reviewed in Refs. [6] and [4]). Briefly, the amoeba

Table 1 Comparison of several published PCR techniques for *Entamoeba histolytica/dispar* detection/differentiation

Gene target	PCR type	Amplicon size[a]	Specimen	Reference
30 kDa antigen	Classic	100/101	Stools	[16]
SSU rDNA	Classic	876	Stools	[1]
P145/B133	Classic	145/133	Stools	[18]
SSU rDNA	Nested–Hot-Start	900 bp	Stools/cultures	[33]
SSU rDNA	Classic	135 bp	Frozen samples	[32]
P145/B133	Multiplex	132/96	Stools	[28]
SSU rDNA	Classic + hybrid	NI[b]	Frozen samples	[20]
SSU rDNA	Light cycler quant	310	Stools	[26]
SSU rDNA	Classic + hybrid	880	SAF fixed	[29]

Most PCR techniques rely on the amplification of specific regions of the episomal multicopy SSU rDNA gene. Starting specimen is important in the field (freezing not always user-friendly) and because of potential inhibitory problems with SAF-fixed samples. Amplicon size is also important. Techniques yielding amplicons of around 100 bp in size are likely to be much more sensitive than around 1 kb. Only Gonin and Trudel[32] provide an internal control, which allows for inhibition monitoring.

[a]For differentiation: *E. histolytica/E. dispar* amplicon sizes.

[b]NI: Not indicated in paper.

attaches to the intestinal epithelial cell through Gal/GalNAc lectin receptor. The amoebic virulence program is then activated and amoebapores are formed. Amoebic cysteine protease enters into action, which induces cell damage and pIL-1β release. IL-1β then activates NF-κB in distal cells, which promotes proinflammatory cytokines production. IL-8 promotes neutrophils influx which damages the epithelial barrier, and amoebas then cleave extracellular proteins through proteases and invade. In parallel, macrophages arrive and release TNFα, which triggers further inflammation. Once amoebas have gone through the mucosal barrier, the way is open to the liver. However, although inflammation contributes to pathogenesis, innate immunity response seems key in the containment of amoebic colitis, which does not always evolve to liver abscess.

CONCLUSION

Since the discovery of the pathogenic agent of amoebic dysentery, several genomic data have been accumulated. These data have first shown the evidence of two different species: *E. histolytica* and *E. dispar*, and have allowed for a better understanding of pathogenesis, for the design of several laboratory diagnosis and differentiation techniques, and for the investigation of vaccination strategies. However, given the utmost public health importance of this pathogen, relatively few research effort has been devoted till now. The expected rapid completion of both genomes should help researchers and clinicians all over the world to design integrated strategies against this pathogen.

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Epstein–Barr Virus (EBV)

Fritz Schwarzmann
Hans Wolf
Hans Helmut Niller
Universität Regensburg, Regensburg, Germany

INTRODUCTION

In 1958, the British physician Denis Burkitt described a pediatric tumor endemic in equatorial Africa. Burkitt set up the hypothesis of an infectious particle being involved in the pathogenesis. Six years later, particles with herpesviral morphology were detected in cultured cells derived from an African Burkitt's lymphoma. According to the three discoverers, the virus was called Epstein–Barr–(Achong) virus (EBV) by Werner and Gertrude Henle, who did the essential work to describe this virus as a separate entity apart from the morphologically similar Herpes simplex virus. Since 1968, when EBV was first shown to be the causative agent of infectious mononucleosis by Volker Diehl and the Henles, the virus has been related to a number of acute and malignant diseases such as Burkitt's lymphoma, nasopharyngeal carcinoma, or gastric carcinoma. Nowadays, complications because of virus reactivation or transformation become increasingly important.

CLASSIFICATION/TYPE OF PATHOGEN

The Epstein–Barr virus (EBV) belongs to the family of herpesviruses.[1] With respect to morphology, EBV is hardly distinguishable from other family members. Based on pathogenesis, characteristics of replication, and host cell tropism, herpesviruses can be classified into three subfamilies: alpha-, beta-, and gamma-herpesvirus. EBV belongs to the genus *Lymphocryptovirus* of gamma-herpesviruses, which are characterized by a narrow host (cell) range and a slow rate of replication in cell culture.

Depending on the type and the differentiation of the infected cell, EBV follows one of the two alternative strategies to survive: latent persistence or productive replication.[2] During latency, no infectious progeny is released and expression of viral genes is tightly restricted. In comparison, at least 80 viral genes are activated in a timely coordinated manner during lytic productive replication.

CLINICAL SPECTRUM AND PREVALENCE OF EBV-ASSOCIATED DISEASES

Primary Infection and Infectious Mononucleosis

Infection with EBV is widespread and the prevalence of the virus in Western populations is more than 90%. The virus primarily spreads via the oral route (saliva) and at a time, approximately half of the asymptomatic virus carriers shed EBV. In nonindustrialized nations, primary infection takes place between 2 and 5 years of age and is mostly asymptomatic. However, infection later in life increases the risk to develop infectious mononucleosis (IM), which is a transient and self-limiting lymphoproliferative disease characterized by lymphadenopathy, high-grade fever, hepatosplenomegaly, and pharyngitis or tonsillitis.[3] Atypical mononuclear cells and an inverted ratio of CD4+ vs. CD8+ cells are hematological markers of IM. Generally, IM lasts for 4 to 6 weeks, eventually establishing an asymptomatic persistent infection.

Complications of Primary Infection

In rare cases, mostly in children, EBV may cause fatal IM or infection takes a severe chronic active course (SCAEBV).[4] Affected individuals develop life-threatening complications suffering from extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, polyclonal gammopathy, as well as B cell or T cell lymphoproliferative diseases. EBV-specific antibody titers (anti-VCA and anti-EA) are extremely high, indicating uncontrolled systemic viral replication.

Another milder form of chronic active infection (CEBV) affects mainly adults and often starts with IM lasting for months to years.[5] Patients are heavily impaired in daily life by chronic and relapsing mononucleosis-like symptoms often associated with malaise, fatigue, and hepatosplenomegaly. A role in the pathogenesis of EBV for the chronic fatigue syndrome (CFS) has not been proven.

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X-linked lymphoproliferative syndrome (XLPS, Duncan syndrome)[6] is a hereditary immunodeficiency that predisposes affected males to fatal IM characterized by aplastic anemia, hemophagocytic syndrome, dysgammaglobulinemia as well as extensive lymphoid infiltration leading to organ failure. Those surviving primary infection develop different forms of malignant B cell lymphoma later on. The defect has been localized on the X chromosome (Xq25–q27) and specifically to a deficient SH2D1A/SAP gene causing feedback regulation of T cell activation.

Hematological Disorders Associated with EBV

EBV is associated with a number of malignant diseases harboring latent virus.[1] Burkitt's lymphoma (BL), a monoclonal B cell lymphoma with germinal center markers, is endemic and represents the most prevalent tumor in children in Africa. Chromosomal translocations between the protooncogene c-myc and the loci for the immunoglobulin heavy and light chains are characteristic for BL. Association with EBV is variable and depends on the geographical distribution. Whereas endemic BL is positive for EBV in more than 90% of cases, only about 15% are positive in sporadic BL and 30–40% in AIDS-associated BL.

Hodgkin' s disease (HD) is also variably associated with EBV depending on age, geographical distribution, and on the subtype of the tumor.[7] There are at least four subtypes of HD: the ''mixed cellularity'' and ''nodular sclerosis'' types, which are the most prevalent types that are EBV-positive in 32–96% and 10–50%, respectively. The ''lymphocyte predominant'' type seems to be mostly EBV-negative, but the ''lymphocyte depleted'' type is EBV-positive in approximately 50% of cases. AIDS-related cases of HD are EBV-infected in more than 95% of cases.

Immunosuppression significantly increases the risk to develop EBV-associated malignant diseases because of the immortalizing and transforming properties of the virus. More than 90% of posttransplantation lymphoproliferative disorders (PTLD) are related to EBV. PTLD is variable with respect to morphology and clonality. In general, monoclonal tumors are more aggressive than polyclonal ones, likely because of additionally acquired genetic mutations enabling immune escape. Polyclonal proliferation often progresses to oligoclonality or monoclonality without intervention.

AIDS-associated non-Hodgkin's lymphoma (AIDS-NHL) is usually of B cell origin. In contrast to NHL in the course of iatrogenic immunosuppression, there is no clear association with EBV.

Although T cells are not the primary target of EBV, various T cell lymphomas are clearly associated with the virus including T cell lymphocytosis, nasal T cell lymphomas, T cell lymphomas with angioimmunoblastic lymphadenopathic appearance, and NK/T cell lymphoma.[8]

Epithelial Tumors and EBV

EBV is also related to a series of epithelial malignancies.[9] Of these, nasopharyngeal carcinoma (NPC) is the most prevalent tumor in Southeast Asia and is endemic in Southern China and Hong Kong (9 per 100,000), in Northern Africa, and among the Inuits. According to the differentiation of the tumor tissue, NPC is classified into three subtypes: ''squamous,'' ''nonkeratinizing,'' and ''undifferentiated'' NPC. The undifferentiated type of NPC is virtually always associated with EBV. Besides EBV, both the genetic background and diet are cofactors for the development of NPC.

Tumors of the glandular epithelium of the stomach, the salivary gland, the lung, and the thymus are other EBV-related lymphoepithelial tumors. EBV can be detected in 75–100% of the rare lymphoepithelioma-like gastric carcinomas and in approximately 27–35% of gastric stump carcinomas. In addition, EBV is detected in 2–16% of gastric adenocarcinomas.

MANAGEMENT OF EBV-ASSOCIATED DISEASES

Infection with the EBV invariably leads to lifelong persistence. B lymphocytes are supposed to be the primary target cells of EBV in vivo. Memory B cells, which become latently infected, are invisible from an immunological point of view and serve as a lifelong reservoir of the virus.[10] In contrast, activated and differentiating B cell blasts support lytic replication such as differentiating epithelial tissues that have also been demonstrated to be infected in vivo.[11] In immunologically privileged tissues such as lymphoepithelial tissues of the oropharynx, e.g., the parotis,[12] infectious viral particles are shed into the saliva.

Most cases of infectious mononucleosis do not require therapeutic intervention. In cases of clinically severe IM and life-threatening forms of SCAEBV, intravenous application of the nucleoside analog acyclovir or gancyclovir may be necessary to reduce active viral replication.[4] In addition, but not alone, antiphlogistic drugs to reduce the unspecific effects of the cellular immune response may be beneficial. Tonsillectomy is frequently used and was found to reduce symptoms of IM possibly

on cost of later recurrences.[13] In SCAEBV, underlying immune dysfunctions such as insufficient NK cell activity or production of IFN-γ may be treated with various cytokines.

However, chemotherapy cannot access the viruses latently persisting in quiescent B cells nor the viruses in tumor cells. Solid EBV-associated tumors are removed by surgery followed either by chemotherapy, such as in the case of BL and HD, or combined chemotherapy and radiotherapy as for NPC.

Bone marrow transplantation is the state of the art to control the fatal consequences of XLPS, which is known to rely on a regulatory defect in lymphocytes.

Immunotherapy of EBV-Associated Malignancies

EBV-associated monoclonal tumors in otherwise immunocompetent individuals are known to be mostly invisible from an immunological point of view. In comparison, lymphoproliferative diseases after hematopoetic stem cell transplantation or solid-organ transplantation usually arise as polyclonal proliferations of EBV-infected immortalized cells that are subject to immune control. Immediate partial reconstitution of immune surveillance is the first action to take. Without treatment, benign polyclonal proliferations often progress to oligoclonality or monoclonality with greatly reduced chances for successful therapy.

New immunotherapeutic approaches[14] aim at the elimination of B cells through monoclonal antibodies such as anti-CD20, anti-CD21, or anti-CD24. Alternatively, EBV-specific cytotoxic T cells (CTL), which are generated in vitro by cocultivation of donor-derived T cells with EBV-infected B-lymphoblastoid cells, are adoptively transferred in the otherwise immunosuppressed recipients in the case of a developing LPD.

Depletion of B cells and adoptive transfer of T cells are often complemented by the administration of interferons IFN-γ and IFN-α or in vivo blocking of interleukin IL-6 by the addition of anti-IL-6 antibody resulting in remission and prolonged survival of some cases.

Vaccination Against EBV

The development of a vaccine against EBV is still in the preliminary stages. Attempts mainly concentrate on subunit vaccines based on the viral membrane glycoprotein gp350.[15] Gp350 delivered as recombinant protein, as synthetic peptides, or recombinant vectors expressing this antigen offered protection against EBV-induced lymphomas in the Tamarin model. Two human Phase I/II clinical trials, which have been conducted with the gp350 protein, and a live recombinant virus vaccine,[16,17] proved safety and showed sterilizing immunity in more than 50% of vaccinees, with no answer possible on efficacy for disease protection in the rest. Recently, clinical trials with an EBNA3A epitope peptide vaccine were also conducted, showing no adverse reactions.

MOLECULAR CHARACTERIZATION OF THE EPSTEIN–BARR VIRUS

Classification of EBV is based on both phenotypic and genomic sequence variations.[18] Type 1 (type A) and type 2 (type B) EBV strains are distinguished on the molecular level by sequence variations in the viral genes encoding the EBV-associated nuclear antigens (EBNAs). Both types of EBV can be easily identified by PCR.

Characterization and identification of EBV strains has also been reported by Western blot assays based on variations in the molecular weight of the EBNA proteins.

In the context of NPC, a number of sequence variations within the transforming viral LMP-1 gene have been found. Virus strains with a characteristic 30-base-pair deletion have been statistically detected more often in NPC compared to asymptomatic virus carriers. Another strain variation reported to be present in southern Chinese patients with NPC is the ''f'' variant.

In context with severe chronic active infections with EBV, virus strains with a bias to lytic replication and an impaired or even absent capacity to establish latent infection have been reported.[19–21]

In conclusion, molecular characterization of Epstein–Barr viral strains is only of minor importance for routine diagnostics and therapy. The role of distinct EBV strains in pathogenesis is only indicated by circumstantial evidence. Furthermore, molecular analysis of EB viral strains in individuals revealed infection with multiple strains of EBV, with the type of strains in the oropharynx and in the peripheral blood shifting over time.

MOLECULAR TESTING OF THE EPSTEIN–BARR VIRUS

Laboratory testing of Epstein–Barr virus infection involves immunological, hematological, and molecular markers depending on the differential diagnosis required.[18,22]

Serology of EBV is based on the timely coordinated appearance of virus-specific antibodies of the immunoglobulin classes IgG, IgM, and IgA specific for a number of viral proteins. Serology is mainly used to define the carrier status and to differentiate primary infection and

infectious mononucleosis on the one hand from remote infection and cases of virus reactivation on the other hand.

Although high titers of antibodies against early antigens (EA) and the virus capsid antigen (VCA) are suggestive for EBV-associated tumors, an association of EBV with a tumor needs to be confirmed by histological and cytological assays demonstrating a spatial relation of the virus to the tumor cells. Nevertheless, depending on the type and subtype of the tumor, EBV is not present in every malignant cell. This has been suggested to be a consequence of a ''hit-and-run event'' with secondary loss of the virus.

Demonstration of the viral RNA transcripts EBER-1 and EBER-2 by in situ hybridization is the gold standard to show latent EBV infection in tumor tissues, although there is some discussion whether EBV-positive cancer without transcription of the EBERs exist. There, and in cases of oral hairy leukoplakia (OHL), characterized by lytic replication of EBV, DNA in situ hybridization using simple copy genes or the W-repeat fragment may be performed. Because of infiltrating EBV-infected bystander lymphocytes, PCR and Southern blot are less well suited to prove EBV association.

EBV infection of cells can also be demonstrated by immunohistochemical assays. The viral antigen BZLF-1, which is a key protein of the lytic replication of EBV, is particularly useful to demonstrate EBV in cases of OHL. The viral latent membrane protein LMP-1 is used to reliably demonstrate EBV in cases of PTLD and HD in the tumor although not in every EBER-positive cell. In carcinomas such as NPC or in non-Hodgkin lymphomas, LMP-1 is not regularly expressed and therefore EBER in situ assay is more reliable.

Monitoring the viral load over time in the peripheral blood is increasingly used for the adjuvant diagnosis of EBV-associated diseases such as PTLD, HD, and NPC, and is suitable to determine the tumor burden indicative of a relapse of the malignant disease.

CONCLUSION

Epstein–Barr virus is clearly related to a number of acute and malignant diseases. However, pathogenesis is mainly dependent on insufficient immunosurveillance and cofactors such as continuous overstimulation of the immune system, chemicals and diet, and genetic predisposition. Against the background of the high prevalence of the virus within any population, demonstration of EBV infection by serology or by PCR is not sufficient to confirm an EBV-associated disease. Careful analysis of more specific serological markers with respect to particular viral antigens and different classes of immunoglobulins as well as virus load and clonality and physical presence of EBV in the affected tissues and the malignant cells is necessary to discriminate EBV-associated disease from the normal carrier state or opportunistic reactivation. In addition, determination of the cellular immunity against EBV might become an important tool for early diagnosis. In the end, a vaccine against EBV would be desirable to timely treat individuals who are at high risk to develop EBV-associated diseases.

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Escherichia coli Serotypes (Enterohemorrhagic)—Detection by Multiplex PCR

Peter C. H. Feng
Steven R. Monday
United States Food and Drug Administration, College Park, Maryland, U.S.A.

INTRODUCTION

This chapter discusses the gene targets most often used for developing EHEC-specific PCR assays, including the common major and putative virulence genes, and markers that are O157:H7-specific or specific for other serotypes (Table 1). This chapter also includes selected examples of multiplex PCR assays that use combinations of various markers to detect O157:H7 or other EHEC serotypes.

OVERVIEW

Enterohemorrhagic *Escherichia coli* (EHEC) has emerged as an important foodborne pathogen and a predominant cause of hemorrhagic colitis (HC) in humans. This illness, with characteristic symptoms of bloody diarrhea and severe abdominal cramps, may progress into the potentially life-threatening complication known as hemolytic uremic syndrome (HUS).[1,2]

The pathogenicity of EHEC is thought to be attributable to the production of Shiga toxins (Stx), also referred to as Verotoxins, which inhibit protein synthesis by interfering with the functions of the 23S rRNA.[3] Stx1 and Stx2 are most often produced by EHEC strains causing illness, but Stx2 is most often implicated in HUS; hence, it appears to be more important in human infections. There are several variants of Stx2 (Stx2c, Stx2d, Stx2e, Stx2f, etc.),[4] and although many of these are produced by animal or environmental strains, recent evidence suggest that some variants may also cause human illness. It is estimated that there are over 200 *E. coli* serotypes that produce Stx. Collectively known as Shiga toxin-producing *E. coli* (STEC), many of these may be found in the feces of animals or healthy humans and have not been implicated in illness. Therefore EHEC is a small subset of STEC composed of strains sharing the same clinical, epidemiological, and pathogenic features[2] that can be distinguished from STEC via the presence of other virulence traits.

While many EHEC serotypes, including O157:H7, O111:H8, O26:H11, O103:H2, O113:H2, O104:H21, and their nonmotile (NM) counterparts,[5] have been implicated in illness, the O157:H7-type strain is still the predominant pathogen causing most of the EHEC foodborne infections and outbreaks worldwide. The O157:H7 group is clonal and only distantly related to other *E. coli*, except for enteropathogenic *E. coli* (EPEC) of O55:H7 serotype.[6] Enterohemorrhagic *E. coli* strains are serologically and genetically diverse with significant phenotypic and genetic diversity observed even within serologically identical groups, such as with the O157:H7 group.[7] As a consequence, the development of diagnostic assays for EHEC can be complicated. However, these difficulties can be overcome by using polymerase chain reaction (PCR) technology that can differentiate variations within genetic sequences to the level of single nucleotide polymorphisms.

EHEC GENETIC MARKERS

Detection of *stx* genes is fundamental to PCR assays for EHEC, and there are easily a few dozen primers that have been developed for *stx1* and *stx2* genes. However, because there are some homologies between toxins, some primers also detect variant types of *stx*. A study evaluated 14 *stx*-specific PCR primer pairs by using several STEC strains known to produce *stx1*, *stx2*, and/or various Stx variants.[8] All primers specifically detected *stx1* and *stx2*, but varied in the detection of variant forms. However, other primers amplified variant *stx* genes that, when coupled with restriction fragment length polymorphism (RFLP) analysis, distinguished variant Stx types. PCR for *stx* identifies only STEC, hence EHEC detection is more complex and requires testing for other markers. Most EHEC carry the chromosomal locus for enterocyte effacement (LEE) pathogenicity island that encodes several virulence factors, including a type III secretion system that mediates the injection of virulence factors into host cells. Among the LEE-encoded virulence genes are *eae* and *tir* that encodes for intimin and the translocated intimin receptor, respectively, which coordinately interact to mediate bacterial attachment to the intestinal epithelial cells that initiates the development of the characteristic attachment/effacing lesions.[2] Although there seems to be a strong correlation between the presence of the *eae* and

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Table 1 Genetic markers used in multiplex PCR assays for detection of EHEC

Target	Genes
Shiga toxin	stx_1, stx_2, stx_2 variants
Intimin	α-eae, β-eae, γ-eae
Enterohemolysin	*ehxA* (cluster I and II)
Serine protease	*espP*
STEC agglutinating adhesin	*saa*
O157:H7	+92 *uidA*
O157, O111, O113 antigens	*rfb*
H7 flagellin	*fliC*

stx2 genes with the ability of EHEC to cause illness,[9] LEE is also present in EPEC and, therefore, PCR using *eae*-specific primers detect both EPEC and EHEC. Consequently, it is essential that *eae* and *stx* primers are used in conjunction to distinguish EPEC, which do not produce Stx. There are several *eae* alleles (α, β, γ, σ, etc.),[10] which exhibit significant degree of homogeneity, but possess genetic differences that can be exploited to design allele-specific PCR primers to facilitate the identification of EHEC.[10–12] For instance, the β-intimin allele is found in some EHEC serotypes, such as O26 and O111, while the O157:H7-type strain carries the γ-intimin gene.[11]

Other putative EHEC virulence factors are found on a 90-kb EHEC plasmid. One of these is EHEC hemolysin, encoded by the *ehxA* (*ehlyA*) gene produced by O157:H7 and other EHEC serotypes. The *ehxA* gene has been proposed as a detection marker for EHEC; however, its presence in some non-STEC strains and absence in strains causing HUS casts some doubts on its role in pathogenesis. Analysis of 79 STEC strains showed that, while highly conserved, there are two distinct *ehxA* genetic alleles sharing 98% nucleic acid homology.[13] EHEC serotypes such as O157:H7, O26:H11, O111:H8, and O103:H2 maintain cluster I allele, while cluster II is found in EHEC serotypes such as O113:H21 and O104:H21.

Another putative virulence factor on the EHEC plasmid is the *espP* gene that encodes for serine protease. Although the *espP* gene is found in O157:H7 serotype, it is absent in atypical German sorbitol-fermenting O157 strains causing HUS.[7,14] Similarly, the *saa* gene encoding for the STEC autoagglutinating adhesin is found in many strains that do not have *eae* and speculated to be an alternate mechanism for bacterial attachment. However, *saa* is more prevalently found in bovine than human STEC strains,[15] hence the role of these adhesins in EHEC pathogenicity remains to be elucidated.

The *uidA* (*gusA*) gene, which encodes for β-glucuronidase, is expressed by most *E. coli*. One exception, O157:H7, contains the *uidA* gene but produces a nonfunctional enzyme because of a frame-shift mutation in the *uidA* gene.[6] The O157:H7 *uidA* also carries a +92 T to G base substitution that is highly conserved and found only in strains of O157:H7 group.[6] Although not a virulence factor, the +92 *uidA* mutation has been found to be a unique marker and useful for developing O157:H7-specific PCR assays.[16]

EHEC SEROTYPE-SPECIFIC MARKER

Production of the O157 antigen is a distinguishing characteristic of O157:H7 serotype; however, serotyping is problematic because of the cross-reactivity of anti-O157 polyclonal sera with strains of *Citrobacter freundii*, *Escherichia hermanii*, and others. However, serotype-specific genetic markers are specific and provide a viable alternative to serotyping. One such marker is the *rfbE* gene, which encodes for perosamine synthethase that is essential for O157 antigen expression.[17] PCR primers to the *rfbE* locus have been designed that detect O157 strains; however, these primers are not O157:H7-specific and detect other O157 strains that do not produce H7. Similarly, PCR primers specific to the serotype O111[18] and O113[19] *rfb* locus have been designed that detect these serotypes, some of which are EHEC and have been implicated in causing illness.

Production of the flagellar H7 antigen is another trait phenotype of O157:H7 strains. However, as in the case of the O157 antigen, other *E. coli* serotypes also express the H7 antigen. Flagellin is encoded by the *fliC* gene, which genetic analyses have shown to contain polymorphisms that can be distinguished by RFLP analysis of H7 *fliC*-derived PCR amplicons.[20] All O157:H7 and its Stx-producing nonmotile variants have identical H7 *fliC* RFLP profiles that are distinct from that of other H7-producing serotypes, except the EPEC O55:H7 strain, which has not only an identical H7 *fliC* RFLP profile,[20] but also has the H7a,c serological subtype.[21] Genetic studies show that O55:H7 is an ancestral strain of O157:H7, which is closely related and belongs to the same clonal group.[6] Although different, at present PCR primers specific for the H7 *fliC* gene detect all H7-producing strains regardless of its O serotype,[22] and none are specific for the H7 *fliC* allele exclusively found in the O157:H7 and O55:H7 strains.

MULTIPLEX PCR FOR DETECTION OF EHEC

Theoretically, any of the target-specific primers mentioned (Table 1) can be combined to develop multiplex PCR assays for EHEC. However, considerable testing will be required to optimize primer combinations, concentrations, and assay conditions. Some primer sequences may also need to be modified to provide an annealing

Table 2 Selected multiplex PCR assays for EHEC and the combinations of markers used

Genes	Reference
stx_1, stx_2, $eae_{O157:H7}$, eae_{O26}, eae_{O111}	[12]
stx_1, stx_2 ($stx_{2\ variants}$), γ-*eae*, +92 *uidA*, *ehxA* (I and II)	[16]
rfb_{O157}, rfb_{O111}, rfb_{O113}	[19]
stx_1, stx_2, $eae_{O157:H7}$, $eae_{general}$, $fliC_{H7}$	[22]
stx_1, stx_2, ,$eae_{general}$ and rfb_{O157}, rfb_{O111}	[23]
stx_1, stx_2 ($stx_{2\ variants}$) and $eae_{general}$, *ehxA*	[24]
stx_1, stx_2, stx_{2f}, 16SrRNA[a] and stx_{2c}, stx_{2e}, $eae_{general}$, 16SrRNA and stx_{2d}, *ehxA*, rfb_{O157}, $fliC_{H7}$, 16SrRNA	[25]

[a]16SrRNA primers are included to serve as reaction control to ensure the presence of amplifiable template DNA.

temperature that is more suited to all the primer pairs, or to change the size of the amplicons to enable better visualization/detection. Multiplex PCR assays are very useful for characterizations of culture isolates, but when used for testing samples, which most likely contains mixed cultures, the amplicon patterns obtained may not necessarily have been derived from a single bacterial strain.

Selected EHEC multiplex PCR assays and the targets that were successfully combined, optimized, and used to specifically detect O157:H7 or other EHEC serotypes are listed in Table 2. In the following sections, a few of these multiplex PCR assays are presented.

Paton and Paton[23] designed an agarose gel-based, two-reaction system to identify EHEC strains. The first reaction selectively targets *stx*1, *stx*2, *eae*, and *ehxA*, while the second reaction differentiates O111 and O157 strains based on their *rfb* gene. Analysis of 52 previously characterized STEC strains isolated from food, human, or animal sources showed that the assays correctly identified all of the isolates and the determinants carried by each strain with no cross-reactivity to closely related strains. The *stx*2 primers also amplified all *stx*2 variants, except for stx_{2f}, but the *eae* primers were designed for the conserved region of *eae*, and therefore did not identify the specific *eae* alleles carried by the strains.

Another gel-based multiplex PCR described by Feng and Monday[16] used a single PCR reaction to detect *stx*1, *stx*2 (including stx_{2c}, stx_{2d}, and stx_{2e}), and both *ehxA* alleles to identify STEC. However, unlike other assays, this multiplex also incorporated primers to the γ-intimin (*eae*) gene, the only allelic variant expressed by the O157:H7 group, and the +92 *uidA* mutation that has, thus far, only been observed in the O157:H7 serotype. This assay bears the advantage of being able to detect STEC and simultaneously identify EHEC of the O157:H7 serotype. Analysis of 38 strains showed that the assay correctly identified the determinants carried by EHEC, STEC, and EPEC, and also identified all the O157:H7 strains tested. This assay will not identify other EHEC serotypes, which do not possess the +92 *uidA* mutation and may express intimins other than the γ-allele.

Gel-based PCR assays can detect many genetic determinants in one reaction; however, it is time-consuming and provides data that are difficult to quantitate. Furthermore, gel-based results are solely based on amplicon sizes with no means to confirm that the product was derived from specific target amplification or was a consequence of a rare mispriming event. These limitations can be overcome by using real-time PCR (rt-PCR), which enables rapid amplification and real-time monitoring of results, and—when coupled with sequence-specific internal probes—guarantees the amplification of specific targets, which can also be quantified to estimate bacterial load. Reischl et al.[24] used rt-PCR and Fluorescence Resonance Energy Transfer (FRET) technology to develop two assays that amplified and detected sequence-specific EHEC virulence determinants in approximately 60 min. The first assay used a single primer pair to amplify *stx* genes, which were then differentiated into *stx*1 (at 640 nm) and/or the *stx*2 (at 705 nm) genes by toxin-specific, fluorophore-tagged hybridization probes. Melting temperature analysis of probe-hybridized amplicons also allowed the determination of some *stx* allelic variants, except for $stx2_f$, which is genetically distinct and found in STEC isolated from pigeons. The second assay is similar, but uses two separate primer sets and target-specific, fluorophore-tagged hybridization probes to detect the *eae* (at 640 nm) and *ehxA* (at 705 nm) genes. The *eae* primers and probes appeared to be specific to the conserved regions, as the assay did not differentiate *eae* allelic variants. In the analysis of 431 STEC strains, 73 Stx-negative *E. coli*, and 118 other bacterial species, rt-PCR was found to be 100% sensitive and specific for *stx1*, *eae*, and *ehxA*, and had 96% and 100% sensitivity and specificity, respectively, for *stx2*.

Although rt-PCR offers many advantages, reaction kinetics and the limited number of distinct fluorophores presently available limit the number of targets that can be combined in a multiplex format. Furthermore, the cost of the equipment and special order reagents often precludes its current use in routine testing.

CONCLUSION

Most PCR assays developed for the detection of EHEC target the *stx* and *eae* genes and their various alleles. However, because the EHEC group comprises many serotypes that are genotypically and phenotypically diverse, identification of specific strain requires the detection of other putative virulence genes or markers that are unique to O157:H7 or other serotypes. Multiplex

PCR assays, which enable the simultaneous detection of multiple genes, are very useful in the diagnosis of specific EHEC serotypes and the virulence factors carried by these pathogens.

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ESTAnnotator—A Tool for High-Throughput EST Annotation

Agnes Hotz-Wagenblatt
Deutsches Krebsforschungszentrum, Heidelberg, Germany

Thomas Hankeln
Johannes Gutenberg Universität Mainz, Mainz, Germany

INTRODUCTION

The protein-coding genetic information of higher eukaryotic genomes represents only a minor percentage of its total DNA, e.g., 1.5% in the human genome, meaning that genes are usually separated by large stretches of noncoding intergenic DNA. Additionally, eukaryotic genes are organized mosaic-style: rather short exons (with an average size of 150 bp in humans), which form the mature mRNA and contain the essential coding information, are separated by introns, which are spliced out from the primary RNA transcripts and do not contribute to protein-coding. Because of this complex architecture of higher organism genomes, it is notoriously difficult to recognize exons in large stretches of genomic DNA sequences, e.g., obtained in the framework of genome projects, and to identify those exons correctly which altogether make up a complete gene. As a ''shortcut'' alternative to the sequencing and identification of the protein-coding gene repertoire of an organism, Adams et al. introduced a strategy called ''EST sequencing'' (EST = expressed sequence tag). This approach bypasses all complexities of genome structure by focusing only on the transcribed portions of a genome: the procedure starts with the isolation of an mRNA population from a certain tissue. After converting the mRNA molecules into their complementary DNA (cDNA), all resulting cDNA molecules are cloned into suitable vector/host systems. Then, usually thousands of clones are being chosen at random for a DNA sequencing of their cDNA integrates, yielding a catalogue of EST sequences which essentially represents a collection of the transcribed portion of the genome (i.e., the genes). Gathering of sequence information from the protein-coding parts of the cDNA can be optimized by producing 5′EST reads instead of 3′EST reads, which usually cover the 3′untranslated gene regions.

The EST approach is extremely cost-effective and fast, and it gives a good overview of those genes that are active in the tissue used as source for the initial RNA preparation. A dedicated freely searchable database (dbEST), a division of GenBank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html), has been set up to collect EST data from a huge number of diverse organisms (see http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html), and this database currently contains more than 5.6 million human EST reads alone. Despite this wealth of data, EST sequencing is still an essential tool for the discovery of novel genes that have not been identified by genomic sequencing and gene prediction alone.

The production of a gene catalogue from EST data requires several steps of bioinformatical sequence analysis: 1) Because of the single-pass, ''quick but dirty'' sequencing strategy, bad sequence data and EST sequences contaminated with vector or repetitive noncoding DNA have to be removed. 2) Overlapping EST reads have to be clustered to obtain a contig sequence of their underlying cDNA. 3) Both EST singletons and EST clusters have to be annotated by searching for similarity to known genes or proteins already existing in nucleotide and protein sequence databases. Here we describe an EST annotation tool that automates these steps and which was successfully used in categorizing 5000 EST sequence reads during a search for genes involved in differentiation and disease processes in human fetal cartilage tissue.

METHODS AND RESULTS

The basic methodology of generating a gene catalogue by EST sequencing is outlined in Fig. 1. The reader may refer to Refs. 1–3 for details on the EST production process and for the importance of having EST sequence information in the framework of a genome project.

The EST annotation pipeline of ESTAnnotator[4,5] (Fig. 2) can be divided into the following subsections: the preparation of quality-checked input EST sequences (shown in the upper part), the initial analysis of EST reads by database searches (shown at the left lower part), and the clustering of overlapping EST sequences as well as subsequent contig analysis (shown in the right lower part).

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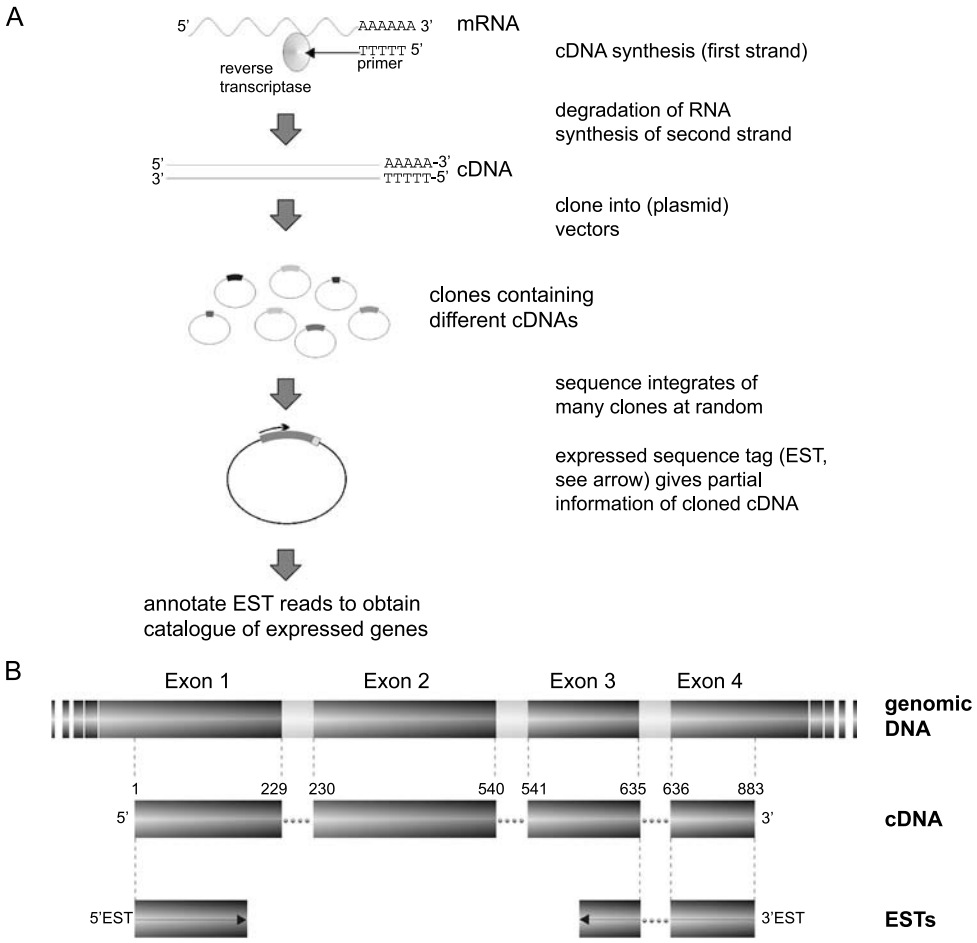

Fig. 1 (A) Outline of an EST sequencing project. Note that the mRNA and cDNA molecules schematically depicted represent the population of all transcribed genes in the analyzed tissue. (B) Positional relationships of exons and introns on the genomic level, the complete cDNA derived from the mature mRNA transcript of the gene, and the partial cDNA sequence information (arrows) present in the 5′ and 3′ EST sequences.

Preparation of Input EST Sequences

ESTAnnotator was designed for processing raw trace files (sequence chromatograms) or sequence text files. As EST sequences are generated by complex cDNA cloning procedures, they are often contaminated with vector and linker sequences. For generating high-quality input EST sequences, vector sequences and naturally occurring repetitive elements are masked by default. If too much sequence information is lost due to quality trimming or masking, no further analysis of the input sequence occurs and the task automatically stops. This was the case for about 2.6% of the EST reads in the cartilage EST project.

BLAST Similarity Analysis

Sequence similarity searching is an important methodology in computational molecular biology. Initial clues to understanding the structure or function of a molecular sequence arise from homologies to other molecules that have been previously studied. Genome database searches reveal biologically significant sequence relationships and suggest future investigation strategies. Database search algorithms are used to compute pairwise comparisons between a candidate query sequence and each of the sequences stored within a database in order to find all the pairs of sequences that have a similarity above a defined threshold. The basic local alignment search tool (BLAST) algorithm is an approximate heuristic algorithm used to compute suboptimal pairwise similarity comparisons. Because of its speed, the BLAST algorithm has become the dominant search engine for biological sequence databases. The EST sequences were run against four different databases using the gapped BLAST program.[6] The search against the NCBI (National Center for Biotechnology Information, Bethesda, USA)

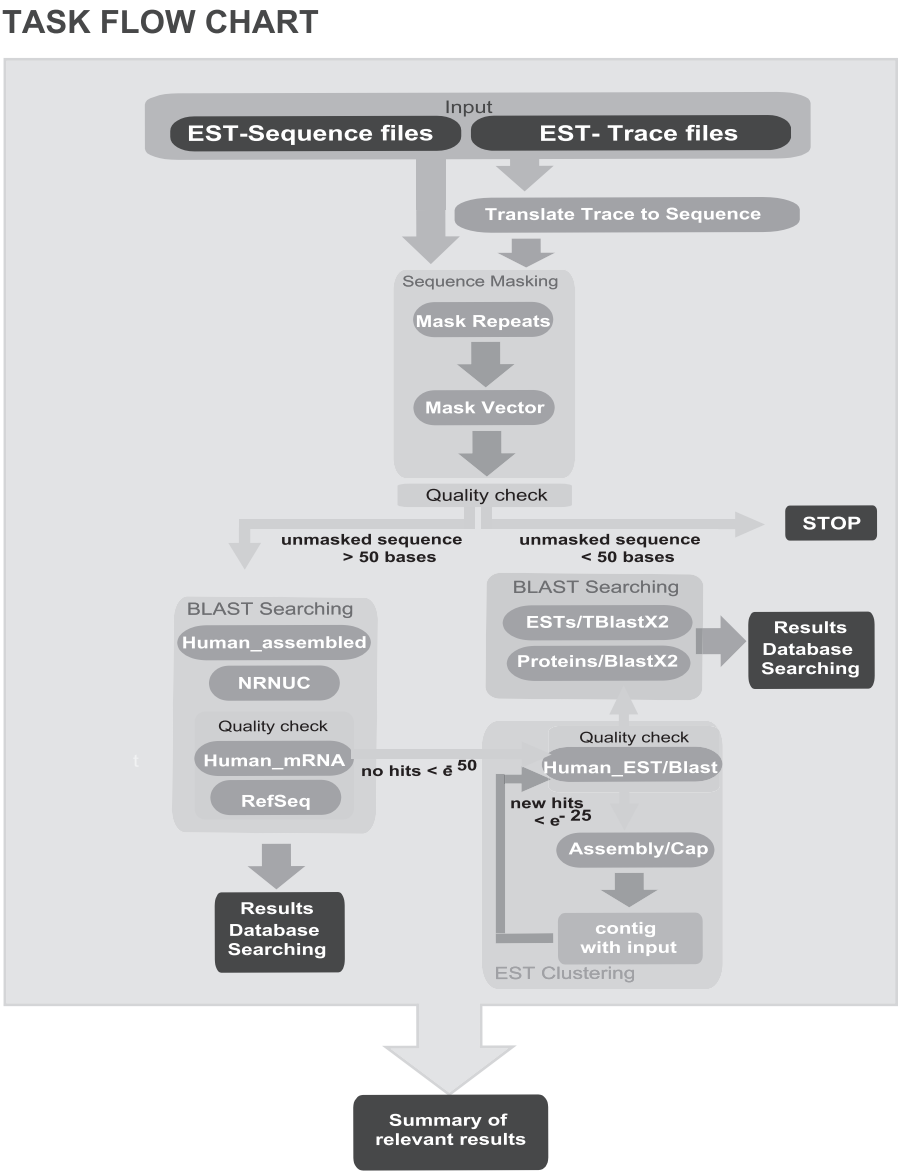

Fig. 2 Flow chart of the ESTAnnotator: programs and rules used for high-throughput annotation of ESTs. (Modified from Ref. [4].) (*View this art in color at www.dekker.com.*)

nonredundant nucleic acid database (ftp://ftp.ncbi.nih.gov/blast/db) should reveal any similar nucleotide sequence deposited in the public databases. For the chromosomal location of the EST within the human genome, another BLAST search was performed against the NCBI human genomic sequence contig assembly database (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens), which contains the nucleotide assemblies of the human chromosomes. For the identification of known, complete cDNA sequences matching our EST reads, additional searches were performed against the NCBI human mRNA database (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/RNA), which contains human ''model mRNAs'' constructed by prediction from genomic sequence data, and the RefSeq database (ftp://ftp.ncbi.nih.gov/refseq/, mRNA part), which contains curated mRNA data from human and other model organisms. If a highly similar mRNA corresponding to our EST read was found in any one of these mRNA databases, no clustering and further database searching were needed. Among the cartilage EST sequences 69.6% showed significant similarity to known genes/mRNAs in the human RefSeq collection, and another 4.8% were found homologous to human model RNAs. Approximately 23% of the cartilage EST sequences could not be identified as known transcripts, but showed significant similarity to genomic regions

and/or other anonymous ESTs.[5] A subset of these potentially novel gene sequences is currently under detailed experimental scrutiny for expression in cartilage tissue using RT-PCR, Northern blotting, and mRNA in situ hybridization.

Using the NCBI human assembly database, a corresponding genomic location could be identified for more than 90% of the EST sequences. This information will be valuable in selecting possible candidate genes from regions of the human genome to which diseases related to malformations of the skeleton have been mapped genetically.

Clustering of Overlapping ESTs and BLAST Analysis at the Protein Level

Expressed sequence tag sequences which could not be reliably assigned to a known mRNA or gene were processed further to finally obtain an annotation. As each gene may have many alternative transcripts that contain exons in different combinations, it is not a trivial task to assign each EST to its progenitor gene. A BLAST search against other human EST sequences from dbEST was therefore used to find homologous, overlapping EST reads in order to extend the original EST sequence by clustering.

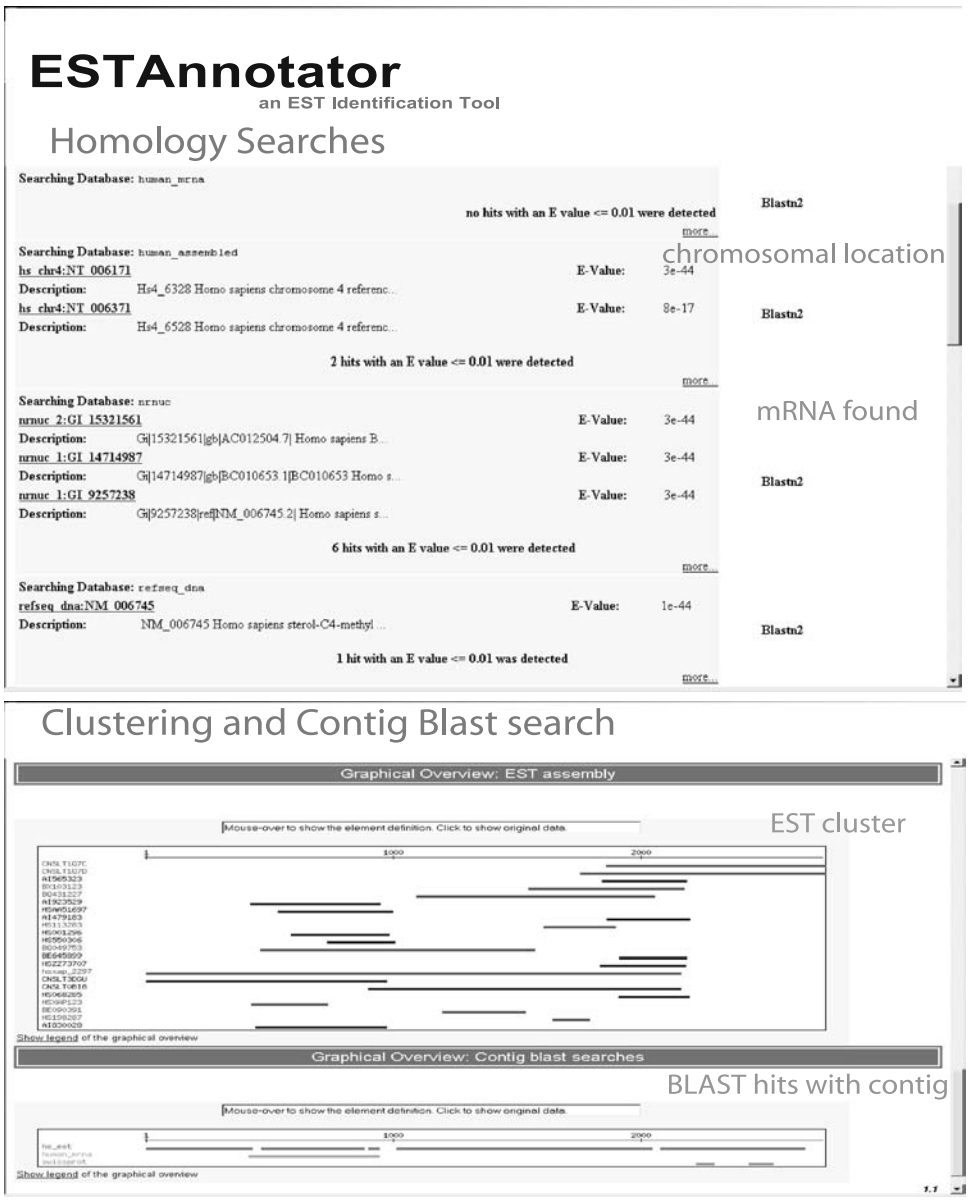

Fig. 3 Parts of the ESTAnnotator report: database search results list and graphical display of the EST contig assembly and protein BLAST hits. (*View this art in color at www.dekker.com.*)

Assembly of such EST clusters, each containing the input EST sequence, was performed by a contig assembly program (CAP),[7] and the resulting consensus sequence of each cluster was saved. The EST cluster consensus sequences (or the input EST sequence alone, in case no cluster was formed) were used for further similarity searching on the protein sequence level in order to detect even remote similarities to known proteins or other anonymous EST sequences in the databases. These searches were performed by BLASTX against the Swissprot protein database (ftp://ftp.ebi.ac.uk/pub/databases/swissprot/) to check for matching, already annotated proteins, and by TBLASTX against all ESTs of all organisms in dbEST to detect similarities to anonymous coding sequences of other organisms.

The ESTAnnotator Report

The final ESTAnnotator report (Fig. 3) is a web page that displays the database ID and the description line of the top three hits of the BLAST search results if their expectation value is below 0.01. Additionally, a link to the original BLAST output is provided. To illustrate the position of the BLAST hits and the clustered EST sequences, corresponding graphical outputs are displayed in the lower part. The alignment information can be accessed by clicking on the hits within the graphical output. By downloading the XML (Extensible Markup Language) report file from the server, the results from the database searches for each EST sequence can easily be parsed into a database file.

WWW Access by the Web Interface to HUSAR (W2H)

Using the W3H task system[8] allowed the immediate integration of ESTAnnotator into the W2H web interface.[9] The ESTAnnotator is available at http://genius.embnet.dkfz-heidelberg.de/menu/biounit/open-husar/.

CONCLUSION

Expressed sequence tag sequencing and annotation are highly useful for identifying the repertoire of genes transcribed in tissues involved in human diseases, and—even in the well-studied human genome—the approach still represents a valuable tool for the identification of novel genes and alternatively spliced mRNAs. The ESTAnnotator facilitates processing and annotation of medium- to large-scale EST datasets. The successive steps of initial EST read quality control, followed first by the identification of ESTs which correspond to already known genes and mRNAs, and then by the clustering and further annotation by database searching of the remaining EST reads have been automated to avoid manual intervention. ESTAnnotator successfully led to the immediate bioinformatical annotation of about 75% of 5000 EST sequences originating from a human fetal cartilage cDNA library.[5] The tool could be further improved by producing a functional classification of the identified cDNAs (e.g., according to GeneOntology criteria; http://www.geneontology.org) together with known splice variants and single nucleotide polymorphisms.

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Ethical Issues Related to Genetic Testing

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Carol Isaacson Barash
Genetics, Ethics & Policy Consulting, Inc., Boston, Massachusetts, U.S.A.

INTRODUCTION

We now know that genes affect virtually all human characteristics and diseases. Experts anticipate substantial potential for developing health care innovations based on advances in genomics.

Ethics (the science of moral principles) and genomics (the science of the function of genes) have been intertwined for centuries. Humans pondered the heritability of traits as long as 6000 years ago with Babylonian engravings evincing the earliest known pedigrees. Aristotle and Hippocrates deduced that most important human characteristics were inherited and transmitted through semen. Thematic in this history is a continued desire to harness genetic knowledge to improve humanity's lot. Genetic testing offers a conduit to that end, with the benefits of testing potentially huge. Gene testing can inform potential parents of the risk of having a child with a genetic disease, permitting them to plan for such, and in many cases permitting them to decide to terminate an affected fetus. Genetic testing of newborns permits immediate treatment to reverse the lethal effects of disease, such as in the case of phenylketonuria (PKU). Other gene tests promise to promote health by indicating the need for increased medical surveillance, a change in lifestyle, or which medications will be safe and effective. Moreover, genetic testing can evaluate the toxicity of chemical compounds and facilitate the development of genetically modified organisms to improve health by removing allergens and adding key nutrients. However, use of genetic testing presents numerous complex ethical issues involving individuals, families, and the society at large.

Concern that use of genetic technologies may create injustices derives both from past injustices[a] the predictive nature of genetic information and the fact that one's genotype is informative not only of that individual but of their blood relatives as well. Potential adversity continues to engage stakeholder organizations around the globe. The two primary ethical concerns arising from the use of genetic technologies are 1) ensuring equitable sharing of the benefits of genetic technology throughout the world, and 2) maximizing the benefits vs. harms in each instance of use. Despite international agreements and recommendations, concerns remain formidable challenges.

WHAT ETHICAL CONCERNS ARISE AND WHY?

Given that the major diseases are known to have genetic components, knowledge of an individual's genotype will be increasingly important to understanding disease development and expression. The mere availability of genetic testing does not ensure that more good than harm will result from use. Ethically appropriate use of gene testing will require not only compliance with practice standards, but physician ability to accurately interpret a test result and manage patient care accordingly, particularly as the majority of tests will predict relative risk (not yes or no) for a specific disease. Although the fund of genomic knowledge is rapidly growing, a clinician's ability to predict health risk based on a gene test result will be in many instances variable and limited. A gene test result will neither indicate *when* a person is likely to become ill nor *how severe* illness will be when it strikes, because *nongenetic* factors significantly influence health status. Determining the medical meaning of a test result will involve synthesizing various factors in addition to the presence or absence of a gene variant, such as an individual's present, past, and anticipated exposures; other biological processes involved; and even one's socioeconomic situation, such as the extent of health benefits and whether one can pay treatments not covered by insurance.

All humans share 99.9% of the same genes. Despite this overwhelming homogeneity in genotype, the 0.1% differences may prove so divisive as to create a genetic underclass of individuals whose genetic lot imposes societal cost burdens. In efforts to ensure that all humanity can benefit from genomic advances, the United Nations Education, Scientific, and Cultural Organization, in 1997, promulgated the Universal Declaration on the Human Genome and Human Rights.[1,2] This document

[a]The American Eugenics Movement spawned forced sterilization laws in many states and mandatory sickle cell testing in the 1970s that led to discrimination.

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commands international commitment to advancing genomics only through the preservation of the fundamental sanctity of human dignity, freedom, and fundamental human rights. Of primary concern to international bioethics committees is that genomic testing improve the world's health rather than increase existing health disparities.[3–6]

The ethical concerns about the use of genetic testing are vast and complex.[b]

The primary ethical issues arising in provision of genetic tests are:

Who owns genetic information?
What constitutes fair use of genetic information?
How can we ensure that genetic tests will be evaluated for accuracy, reliability, and utility before they are commercially available?
Can informed consent, for research and clinical testing, be assured given low levels of understanding of genetics, difficulties in enforcing nondirective counseling standards, when interpretation is required?
Can we ensure that all testing is voluntary?
Should parents always have the right to have their underage children tested?
Can we ensure the privacy of genetic information?
Can we ensure the appropriate handling of biological samples?
Can we ensure the appropriate introduction and use of genetic tests and genetic data?

The bulk of ethics research, recommendations, and policy setting has identified and clarified potential for harm and spurred measures to prevent or mitigate that potential. While most of these measures have yet to be tested, a smattering of high-profile situations illustrate some of the types of ethical quandaries that can arise.

[b]Should genetic tests be regulated differently from other medical tests? Should they be used in locales lacking regulation? How can the use of tests be regulated to ensure that they give factual results? Should regulation exist for all types of genetic defects or just some; for example, are tests for poor eyesight the same as tests indicating a tendency to baldness? Are tests to be used in in vitro embryos to ensure that positive embryos are not implanted? Are tests to be used if no proven disease treatment or strategy to mitigate risk exists, even if individuals desperately want to know? What if one wants to know, not for themselves, but for his/her future offspring, children, or grandchildren? Can we protect individuals from harm when their genetic test result years later means something else, something very negative, which they could not have possibly consented to? For example, if the gene variant associated with malabsorption of codeine, indicating codeine is ineffective or unsafe for the individual, years later comes to be also associated with very high risk for a lethal disease, given certain environmental exposures, and the individual who had a pharmacogenetic test for treatment purposes then has additional information he/she did not expressly consent to, chooses not to know and is psychologically traumatized.

DUTIES AND OBLIGATIONS

What should the states' obligations be, particularly in light of competing needs for limited resources? Mandatory screening adopted with public health rationale does not necessarily mean that everyone benefits from testing. Health care consumers, particularly those in areas using multiplayer systems of care, have widely differing levels of access to tests and interventions. Changes to one's health insurance can create substantial potential for harm if insurers refuse to pay for expenditures associated with a ''preexisting'' (genetic) condition, or impose premium increases that make treatments (even co-pays) unaffordable. Furthermore, the type of genetic test reporting can have devastating consequences. For example, California's newborn screening reporting requirements does not require reporting actual values, but rather only abnormal results. A recent case resulted in permanent injury because the actual values, which showed consistency for congenital hypothyroidism, were not reported. The physician received the results well after the critical point at which harm results if untreated. In court, the state was not held liable.[7] In all cases, beneficence competes with other state demands. Particularly in areas of the world where the state imposes substantial restrictions on individual freedom, the state clearly cannot be relied on assuring ethical principles in practice.

Genomic medicine also challenges physician duties in the context of doctor–patient relationships. Physicians have a duty to protect a patient's confidentiality, but such is not absolute. Doctors are obligated to override patient confidentiality to report infectious disease, and warn people whom their patients have specifically threatened with violence. Whether genetic risks warrant similar exceptions, and if so, when, remains an important clinical concern, although several advisory committees have said that physicians may be permitted to breach confidentiality to warn third parties *only* as a last resort to avoid serious harm. A recent court case involved a daughter who sued her father's physician's estate for failure to warn her about her 50% risk of developing diffuse adenomatous polyposis coli. What ''serious'' harm is, though, is clearly different for different people depending on psychological factors and other situational factors.[7]

There are other questions of physician obligations including, but not limited to, whether a physician is obligated to offer a genetic test, and if so, when and why? In the absence of appropriate indications and established standards of care, physicians have no special duty to offer a specific test. However, physicians have an ethical obligation to be knowledgeable about genetic disorders that occur with increased frequency in certain populations, such as sickle cell anemia in African-Americans, thalassemias in Mediterranean and Southeast Asian

groups, and Tay-Sachs in the Ashkenazi Jewish population. Physicians have an ethical obligation to provide nondirective counseling in support of a patient's voluntary decision making.[8]

VOLUNTARY AUTONOMOUS DECISION MAKING

There is essentially universal agreement at the international level that all decision making about whether or not to undergo a genetic test must be voluntary. The would-be testee should exercise autonomy freely and without coercion. The exceptions involve individuals who are deemed incompetent, and children. Practice standards and policy state that children should be tested only if there is proven medical benefit in doing so. In practice, the benefits of childhood testing depend on the family's ability to access services indicated by a test result. Furthermore, although the law supports parental prerogative, parents, the test deciders for children, may not always have their child's best interest in mind, and ethical dilemmas could easily arise when existing law is insufficient to protect the interests of children against abuses instigated by parents or the state.

The potential to coerce individuals into testing should not to be minimized. Third parties with a financial stake in a genetic test result have tested individuals without their consent, and future instances are quite possible. Recently, the Burlington Northern Santa Fe Railroad tested 35 employees seeking disability compensation for job-related carpal tunnel syndrome for mutations associated with hereditary neuropathy. The testees learned of this by accident, the U.S. Equal Employment Opportunity Commission halted the practice, and a class action suit was settled out of court. In point of fact, the scientific basis for the testing undertaken was inappropriate.[9] Hereditary neuropathies very rarely occur in the population and while carpal tunnel syndrome can be a symptom of the condition, it is one of many that characteristically arise and furthermore arise well before midlife, which is when the workers developed the syndrome.

The government also has a stake in the health of its citizenry. Therefore, it is not implausible that it require genetic testing, particularly to limit its financial burden. In 1992, the state of Colorado considered mandating Fragile X screening believing that large-scale screening, coupled with reproductive counseling, would result in fewer births of affected individuals and thus reduce the state's financial burden arising from the care of such individuals. Studies indicated that the state spends $1,609,852.63 over the lifetime of a person with Fragile X Syndrome, over and above expenditures for ''normal'' children. Extrapolating to a national burden of $280 billion, researchers argued mandated screening could relieve financial burden by 50%.[11]

Other types of state action are equally imaginable. A government might require those on state assistance to undergo pharmacogenetic testing, as a condition of having their medication paid for, to avoid paying both for unsafe and ineffective medications. (Insurers, similarly, might want to as well.) Here, a concomitant ethical concern is that such individuals might unwillingly have personal genetic information they never consented to, and never wanted to know. While the risk that information obtained from a genetic test comes to mean more than one initially consented to exists for anyone undergoing testing, it is particularly problematic in cases where testing is directly, or indirectly, coerced.

GENETIC DISCRIMINATION

A genetic test result can have profoundly adverse effects on an individual, their family members, and on society. A genetic diagnosis might affect a person's ability to perform a job that could not be accommodated for in reasonable ways. For example, an individual with recurrent and untreatable cardiac arrhythmia that leads to loss of consciousness owing to an inherited ion-channel defect would be ineligible to work as a long-distance truck driver. An individual at risk for chronic beryllium disease, given sufficient exposure, may be ''protected'' out of jobs that increase exposure and so, risk of disease. In both cases, individuals could be barred from their preferred occupation, raising the question of how to balance competing rights—an individual's right to self-determination (to choose for themselves which risks he/she is willing to incur), and an institution's right to protect individuals against harm to themselves, and/or the public, including itself (the public from dangerous driving or an individual from increased risk of serious disease). Balancing competing interests is complicated not only by potential for greater good (say, the brilliant chemist with inherited susceptibility for chronic beryllium disease, who, if permitted to work in his risky, but preferred occupation might make a discovery of enormous benefit to society) but also uncertainty about the likelihood of various outcomes associated with a test result. For example, should an asymptomatic individual with a predisposing, but incompletely penetrant mutation for the ion-channeling defect be similarly restricted, as asymptomatic individuals with complete penetrant mutation? Framing the risk benefit question is far from simple.

Determining whether distinguishing individuals' ability to obtain social goods based on genetic information is ever ethically acceptable is likely to remain contentious,

because discrimination is typically covert and so is difficult to identify and remedy. For example, individuals often disclose private information in the context of interpersonal trust, such as friend to friend. Conflict of interest in such disclosures exists when one person has competing obligations, obligations to the friend but also obligations to, say, an insurance agency employer. Information obtained in confidence may then be used against the individual disclosing, if the recipient determines their primary obligation is to an outside entity. Here the disclosure is not entirely consensual, because of the unknown ends. Other types of involuntary disclosures exist as well, as in the case of small rural areas where one's family history may well be public knowledge. Equally, if not more troubling, may be the potential for self-imposed exclusions based on personal beliefs about self-worth and entitlement connected to one's understanding of their genetic test result. Individuals may deny themselves education, marriage, children, etc. because they believe a test result means they are unworthy. Given that we all harbor deleterious mutations, we are all theoretically vulnerable to genetic discrimination at any point in our lives.

INFORMED CONSENT

The ethical constraints that apply to any medical test also apply to genetic testing, although ensuring informed consent complicated by not only the inherent complexity of genetics, but the fact that the patient is not only the individual, but his or her blood relatives as well. Ensuring the genetic literacy of all is a major unmet need. Whether individuals are told all that they need to know may be compromised by practitioner information and level of understanding (also an important as yet unmet need). Whether individuals truly understand the information provided may be questionable, as the consent process itself is not foolproof. Understanding genetic risk is particularly challenging because the tendency to simplify to ensure comprehension can backfire by reinforcing incorrect notions of biological determinism. Conveying information via interpreters is especially challenging from an ethical standpoint for several reasons. Interpreters are not required to be trained in genetics, ethics, or cultural norms associated with nonnative speakers and so may unintentionally use language that conveys nonneutral meanings. Often, children are used to interpret for parents raising a host of issues including patient privacy, the appropriateness of involving children in the intimate issues raised, and the effect of such exposure on a parent's future relationship with the child.

Furthermore, because genetic testing can require family history information, there is potential for harm to the patient (would-be testee), if family members are unwilling to participate. Balancing the privacy rights within families may be contentious. In 1998, a participant in a clinical genetics research study provided her father's family history information, as per the study protocol. But the father blocked researchers' access to his medical record, arguing that he did not consent to disclosure, and disclosure without his consent amounted to a right to privacy violation.[12] The question of whether disclosure of family history information requires the consent of each, and all, family relatives remains uncertain, as does whether consent applies to deceased individuals, and if so, how. Moreover, in the case of identical twins where one wants to know and the other does not, determining whose right merits priority is difficult, particularly in light of the fact that it is highly improbable that both rights could be exercised and adequately guarded.

CONCLUSION

The ethical issues discussed here are by no means the only ones. Despite our best efforts to anticipate and resolve ethical quandaries arising from using genetic technologies, it is likely that unforeseen dilemmas will arise. Despite protective laws and policy recommendations by numerous international organizations, the future impact of such may be substantially challenged.

Medical genomics is here and will be increasingly so in the future. Therefore, ensuring that genetics confers far more benefit than harm is a formidable challenge. Assuring equitable access to the benefits of genetic testing remains an urgent consideration. Ongoing involvement by international organizations will be required to ensure that developing countries are not ultimately harmed and that individuals in such countries can reap the benefits of genetic technologies, as well as engage in policy setting.

Each of us has a stake in how genetic research unfolds and the resulting technologies are applied. The stakes are particularly high because of the magnitude of unknowns involved. High-speed easy chip testing for information A may result in the unanticipated generation and release of information B and C as well. Active involvement by stakeholders may go far in fostering just outcomes. In 2000, the Cannavan's Disease Support Group, which has been instrumental in helping a company develop treatment through raising research funds as well as supplying willing research participants, filed suit against the company for the right to ownership of the research, not to reap financial rewards for profits made from test use, but rather to be actively involved in decisions about the future use of the new knowledge and technology.

The value of a genetic test lies not in the test itself, but rather in what we, as individuals and society, choose to do with that result. What we do with genetic information arguably will have far more impact on health status than the genetic information itself. Whether individuals use test results to alter their behavior to improve their health remains uncertain, especially given evidence that despite high levels of motivation, psychological and other factors have the potential to drive people to act in ways that are contrary to long-term health goals.

In the greater scheme of things, it is unlikely that genetic information will *not* matter. The relevant question is how much it does, or will, matter. Ensuring the just treatment of individuals will involve deciding the appropriate weight of genetic vs. nongenetic factors, lest we reify genetics as the sole arbiter of an ethical decision.

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Fabry Disease

Catherine Caillaud
AP-HP, Institut Cochin, Paris, France

INTRODUCTION

Fabry disease (also known as Anderson–Fabry disease) is an inborn error of glycosphingolipid metabolism caused by a deficiency of the enzyme α-galactosidase A (α-Gal A). It is a lysosomal storage disorder characterized by intracellular accumulation of globotriaosylceramide (Gb3) and related glycosphingolipids, particularly in the vascular endothelium, leading to renal, cardiac, and cerebrovascular manifestations. Fabry disease is an X-linked recessive disorder, caused by mutations in the α-Gal A gene (*GLA*) localized in Xq22. It affects predominantly hemizygous males, but heterozygous females may also have either moderate or severe manifestations. It is a panethnic disease with an estimated incidence of 1:117,000 male births and a reduced median survival rate compared with the general population (50 years for male patients; 70 years for obligate carriers). Fabry disease can be diagnosed reliably in males by the measurement of the α-Gal A activity, and in carrier females by the identification of the *GLA* mutation. Genotype–phenotype correlations are restricted because of the wide variety of mutations involved in the pathogenesis of the disease. Even if enzyme replacement therapy using recombinant α-Gal A has recently demonstrated its efficacy in the management of this severe disease, other therapeutic approaches are also under investigation.

CLINICAL MANIFESTATIONS

Hemizygote Males

The first clinical signs usually occur during childhood or adolescence, but may be delayed until the second or third decade. They predominantly include corneal opacities, acroparesthesias, angiokeratomas, and hypohydrosis, but also symptoms linked to cardiac, cerebral, and renal vascular involvement.[1,2] Acroparesthesias constitute the earliest and the most debilitating symptom of Fabry disease. These painful crises, present in more than 70% of patients, are often misdiagnosed unless other clinical elements provide diagnostic clues. Angiokeratomas are also early manifestations of the disease. They are telangiectases predominantly localized in the umbilical area, on the buttocks, and the scrotum. Hypohydrosis is common, resulting in high-temperature intolerance. Corneal and lenticular opacities are frequent in Fabry disease, such as conjunctival and retinal vascular lesions.

With increasing age, cardiac signs such as left ventricular enlargement, valvular involvement, and conduction abnormalities occur. Cerebrovascular manifestations may include transient ischemic attacks, hemiplegia, thromboses, diplopia, or vertigo. Renal disease due to progressive glycosphingolipid deposition is also one of the major complications in affected hemizygotes. Proteinuria is the first manifestation, followed by a gradual deterioration of renal function. In the third or fourth decade of life, end-stage renal failure may require dialysis and renal transplantation. In contrast with the classical phenotype described above, atypical variants have been described, among which the most common is a cardiac variant exhibiting manifestations limited to the heart (left ventricular hypertrophy).[3]

Heterozygote Females

Heterozygote carriers have often been reported to be asymptomatic, or with attenuated forms of the disease. Corneal dystrophy is present in about 70% and is useful for heterozygote detection. Approximately 30% of heterozygotes have isolated skin lesions and less than 10% acroparesthesias. With age, severe cardiac, cerebrovascular, and renal manifestations may occur, explained by skewed X inactivation. A few carriers exhibit a disease as severe as that observed in classically affected males.[4,5]

DIAGNOSIS OF FABRY DISEASE

Enzymatic Defect in Fabry Disease

α-Gal A: A Lysosomal enzyme

The metabolic defect in Fabry disease is a deficiency of the lysosomal enzyme α-Gal A, which catalyzes the hydrolytic cleavage of the terminal molecule of galactose from Gb3 and other neutral glycosphingolipids (Fig. 1). Therefore undegraded glycosphingolipids accumulate mainly in lysosomes of vascular endothelial and smooth

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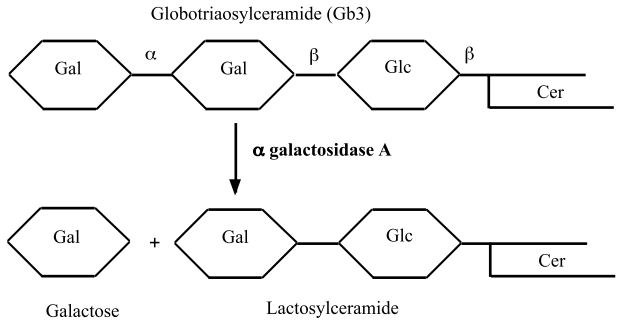

Fig. 1 Degradation of Gb3 or CTH by the lysosomal enzyme α-Gal A.

muscle cells, as well as in epithelial and perithelial cells of most visceral tissues.

The native α-Gal A enzyme is a glycoprotein of approximately 101 kDa with a homodimeric structure. Multiple forms are observed on isoelectric focusing of purified preparations from different tissues, differing in the amount of sialic acid on carbohydrate chains. Transport of enzyme to lysosomal compartments is dependent on mannose-6-phosphate receptors. α-Gal A is an entity different from the isozyme called α-galactosidase B (α-Gal B). This glycoprotein was shown to be an α-*N*-acetylgalactosaminidase, whose activity is deficient in Schindler disease—a recently described neuroaxonal dystrophy.

Direct diagnosis: Enzymatic deficiency

α-Gal A can be specifically assayed by using the synthetic fluorogenic substrate 4-methylumbelliferyl-α-D-galactopyranoside, in conjunction with *N*-acetylgalactosamine in the reaction mixture, to inhibit α-Gal B activity.[6] The assay can be performed in plasma, serum, leukocytes, fibroblasts, or lymphoblasts. This method is simple and reliable, and facilitates routine laboratory diagnosis in hemizygotes by demonstration of a profound α-Gal A deficiency. Some atypical variants may exhibit a residual activity requiring the determination of the Gb3 level to establish their definitive status. In carrier females, the biochemical diagnosis is less reliable because of random X chromosomal inactivation. In many heterozygotes, an intermediate level of α-Gal A can be detected, but activities ranging from zero to normal have been found, even in obligate carriers.

Indirect diagnosis: Gb3 accumulation

The Gb3, also referred to as ceramidetrihexoside (CTH), accumulates in many critical organs of patients with Fabry disease. Conventional methods for Gb3 measurement in plasma or urine sediment require lipid extraction, glycolipid isolation, oligosaccharide hydrolysis, and quantitation of liberated monosaccharides either by gas–liquid chromatography (GLC) or high-pressure liquid chromatography (HPLC).[7,8] These methods are reliable, but tedious and not routinely used. An alternative method using an immunoassay based on the specificity and avidity of the B subunit of *Escherichia coli* verotoxin (VTB) for Gb3 has been developed.[9] More recently, tandem mass spectrometry has also been used for the rapid quantitation of Gb3 by using novel internal standards.[10]

Molecular Basis of Fabry Disease

α-Galactosidase gene

The α-galactosidase (*GLA*) gene is mapped to the long arm of the X chromosome (Xq22.1). It is a 12-kb-long

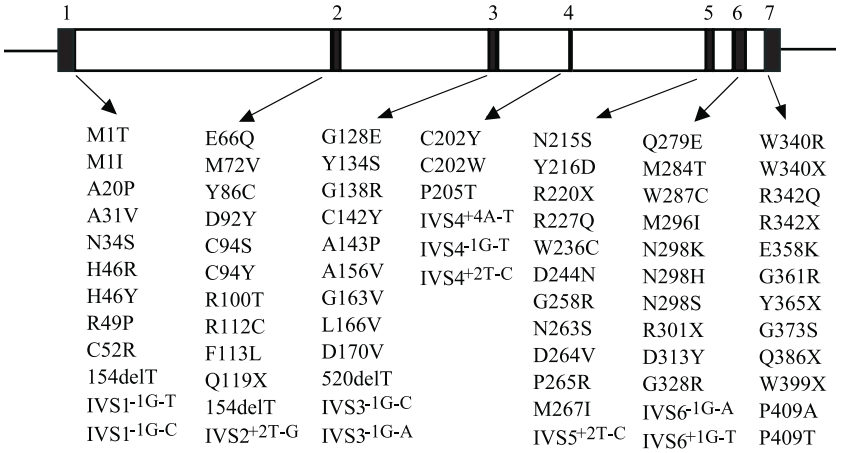

Fig. 2 α-Galactosidase gene representation showing the position of exons and the distribution of some mutations (del = deletion; IVS = intervening sequence).

gene comprised of seven exons, with introns ranging from 200 bp to 3.7 kb (Fig. 2).[11,12] Exon 1 contains the 5′ untranslated region, the sequence encoding the signal peptide, and a sequence for the first 33 residues of the mature enzyme. The full-length cDNA, containing a 1290-bp coding sequence, encodes the 398 residues of the mature enzyme subunit.[13] A particularity of the α-galactosidase cDNA is the absence of 3′ untranslated region. The polyadenylation signal sequence is in the coding region, 12 bp from the termination codon, which is followed by the poly (A) tract.

Mutations in the α-Gal A gene

Identification of mutations in the *GLA* gene can be easily performed by amplification of all seven exons using polymerase chain reaction (PCR) and direct sequencing of the DNA fragments.[14] Gene rearrangements are rare (<5%) in the *GLA* gene, and no whole gene deletion has been detected. To date, over 300 mutations have been identified. Details are given in the Human Gene Mutation Database (see the following web site: http://archive.uwcm.ac.uk/uwcm/mg/search/119272.html). The most frequent lesions are nucleotide substitutions, including missense and nonsense mutations. Small or partial deletions, small insertions, splice mutations, as well as complex mutations (insertion–deletion, inversions) have also been reported. Mutations have been found in all seven exons (Fig. 2). Most of the mutations are private (present in single families). Therefore genotype–phenotype correlations that require clinical information from unrelated patients with the same genotype are limited. Most genotypes are associated with classical forms of the disease. Some missense mutations, initially identified in cardiac variants, have also been found to be associated with classical phenotypes. A model of the human α-Gal A enzyme has recently been obtained, based on the X-ray structure of the highly homologous α-*N*-acetylgalactosaminidase sequence.[15] It will probably further our understanding of the structural basis of Fabry-causing mutations.

Genetic Counseling and Prenatal Diagnosis

Because Fabry disease is transmitted as an X-linked trait, all sons of affected males are unaffected, but all daughters are obligate carriers (Fig. 3). For all heterozygous females, there is a 50% risk of passing the deleterious allele to a son who will be affected, and a 50% risk of passing the mutant gene to a daughter who will be a carrier. Therefore determination of the status of females, either clinically affected or related to affected males, is critical to be able to offer them a reliable genetic counseling. This can be performed more accurately by the characterization of the responsible mutation. In females identified as carriers, prenatal diagnosis can be performed using α-Gal A assay and/or molecular analysis on chorionic villi or cultured amniotic cells, after assessment of the fetal gender.

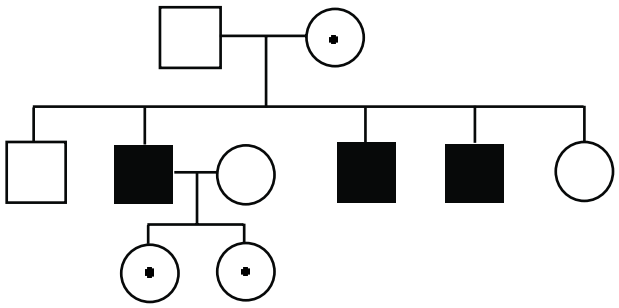

Fig. 3 Pedigree illustrating the X-linked recessive transmission of Fabry disease. (■) Affected male; (⊙) carrier female.

TREATMENT

Symptomatic Treatment

Fabry disease has been managed with palliative measures, most of which are standard interventions for a specific symptom: analgesics for painful crises, valve replacement for valvular disease, anticoagulants for prevention of stroke, and so on. In patients with chronic renal disease, dialysis and renal transplantation are life-prolonging because renal failure is the most frequent cause of death in Fabry disease.

Enzyme Replacement Therapy

Recently, an enzyme replacement therapy has been developed for Fabry disease. Two different recombinant molecules have been obtained: agalsidase β or Fabrazyme® produced in Chinese hamster ovary cells (Genzyme Corporation), and agalsidase α or Replagal® produced in human fibroblasts (Transkaryotic Therapies, Inc.). After preclinical studies in animals, both molecules have been tested in humans in two independent randomized trials.[16,17] Even if studies differed in the enzyme preparation, dose per infusion, and entry criteria, both of them suggested that enzyme replacement therapy was safe and likely to be effective, as demonstrated by the decrease in Gb3 concentrations and the renal histology improvement.[18] The two enzymes have been approved in Europe and other countries and are now available for patients.

Chemical Chaperons

Enzyme enhancement by chemical chaperons has also been proposed in some cases of Fabry disease. This strategy is based on the stabilization of catalytically active but unstable enzymes, either by reversible competitive inhibitors or by substrate analogues. In a cardiac variant of Fabry disease exhibiting a missense mutation associated with a residual α-Gal A activity, intravenous infusions of galactose for 2 years resulted in a significant improvement of cardiac function.[19] This supports the use of chaperon-mediated therapy in Fabry disease and other late-onset enzymopathies with selected mutations.

Substrate Depletion

An alternative treatment for Fabry disease is substrate deprivation, based on the inhibition of the synthesis of the accumulating glycosphingolipid. This approach has been tested in α-Gal A knockout mice using D-threo-1-ethyl-endioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol (D-t-EtDO-P4), a potent inhibitor of glucosylceramide synthase—an enzyme involved in the synthesis of glucosylceramide, which is the precursor of neutral glycosphingolipids.[20] Treatment with D-t-EtDO-P4 resulted in a reduction in renal Gb3. These data suggest that Fabry disease may be amenable to substrate deprivation therapy, in particular if more specific and less toxic components are developed.

Gene Therapy

Gene therapy has been explored in the murine model of Fabry disease by using different viral vectors such as retrovirus, adenovirus, and adeno-associated virus, demonstrating the feasibility of this approach.[21–23] However, deleterious consequences recently described with retroviral and adeno-associated viruses have delayed gene therapy applications in humans for Fabry disease and other genetic disorders.

CONCLUSION

Fabry disease is a complex disorder with a wide clinical variation concerning the severity and onset of symptoms. As it has an influence on the quality of life and the survival rate of both affected males and females, enzyme replacement therapy is a major advancement for this disease. Therefore prompt and accurate diagnosis is now crucial to be able to initiate treatment before irreversible organ damage occurs. Many questions concerning appropriate time for therapy, choice of dose, long-term benefits, and risks still need to be addressed. Novel emerging therapies, such as substrate deprivation, enzyme enhancement by chemical chaperons, or gene therapy, are now in development.

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Facioscapulohumeral Muscular Dystrophy

F

Meena Upadhyaya
David N. Cooper
University of Wales College of Medicine, Cardiff, U.K.

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant, inherited disorder characterized by progressive muscle weakness, including atrophy of the facial muscles, upper arm, and shoulder girdle. Disease progression may eventually involve weakness and atrophy of the pelvic girdle and the foot extensor. FSHD is the third most common form of inherited neuromuscular disorder, after Duchenne and myotonic muscular dystrophies.[1] It is unusual for the disease to become clinically apparent before the age of 10 years. However, when it does occur in fewer than 5% of patients, it is associated with significant facial weakness. The common course of the disease is for symptoms to develop during the second decade of life. Both retinal vasculopathy and high-tone deafness may be seen as part of the clinical spectrum of FSHD.

GENE MAPPING STUDIES

Southern analysis was used to map the FSHD locus to chromosome 4q35[2,3] by using probe p13E-11 derived from the complex locus D4F104S1.[3] Indeed, the FSHD locus is closely linked to the locus D4F104S1 (Fig. 1). However, there are some evidence of genetic heterogeneity in FSHD because about 5% of families manifest an inherited pattern that is unlinked to 4q35. A provisional linkage to chromosome 15 has been suggested, but the gene still remains to be identified.

DNA REARRANGEMENTS DETECTED BY D4F104S1 AND D4Z4 REPEATS

When probe p13E-11 (D4F104S1) is hybridized against genomic DNA digested with the restriction enzyme *Eco*RI, it identifies a 3.3-kb tandem repeat sequence termed D4Z4 (Fig. 1).[4] The variability in the *Eco*RI fragment size is because of the deletion of an integral number of D4Z4 repeats. The D4F104S1 *Eco*RI polymorphism is complex and represents contributions from two separate homologous *Eco*RI polymorphic loci: one located at 4q35, and the other at 10q26. Both polymorphic elements are composed of a variable number of multiple integral copies of the 3.3-kb *Kpn*I repeat unit (Fig. 2). There have been no reports of internal deletions within the body of the repeat itself.[5] There is size overlap between the *Eco*RI D4F104S1 alleles from 4q35 and 10q26. Subsequently, it was established that each of the 10q26-located repeat contains a *Bln*I restriction site (Fig. 2) that is not present on the 4q35-derived repeats.[6] Therefore a complete distinction between 4q35-derived and 10q26-derived fragments can be obtained by double digestion of the genomic DNA with *Eco*RI and *Bln*I; this effectively removes all the 10q-derived repeats. Recently, a *Xap*I restriction site present in only 4q35-derived D4Z4 repeat was identified, and its use complements the *Eco*RI/*Bln*I digests.

In most affected FSHD patients, disease-associated polymorphic alleles range in size from 7 to 38 kb (Fig. 3), which represents the presence of 1–11 D4Z4 repeats compared with the normal allele range of 50–300 kb, representing a D4Z4 copy number greater than 12 repeats.[3,5]

Several 4q35-derived and 10q26-derived D4Z4 3.3-kb repeat units from normal individuals have been cloned and sequenced,[7] and it has been found that the sequence of each repeat unit, whether from the 4q35 or the 10q26 tandem array, is virtually identical and is defined by flanking *Kpn*I restriction sites. Each D4Z4 repeat contains a number of known repeat sequence motifs, including *LSau* and *hhspm*3, a highly GC-rich low-copy repeat. Analysis of the sequence has revealed the presence of a large open reading frame (ORF), which possesses the potential to encode two homeobox-like sequences that have been the subject of much speculation, although no such transcripts have ever been identified.

GENOTYPE/PHENOTYPE RELATIONSHIP

There is an association between the size of the deleted *Eco*RI fragment and the age at disease onset. It has been shown that smaller *Eco*RI fragments are always associated with the most severe form of the disease—a finding not specific to a single ethnic group. Thus the D4F104S1

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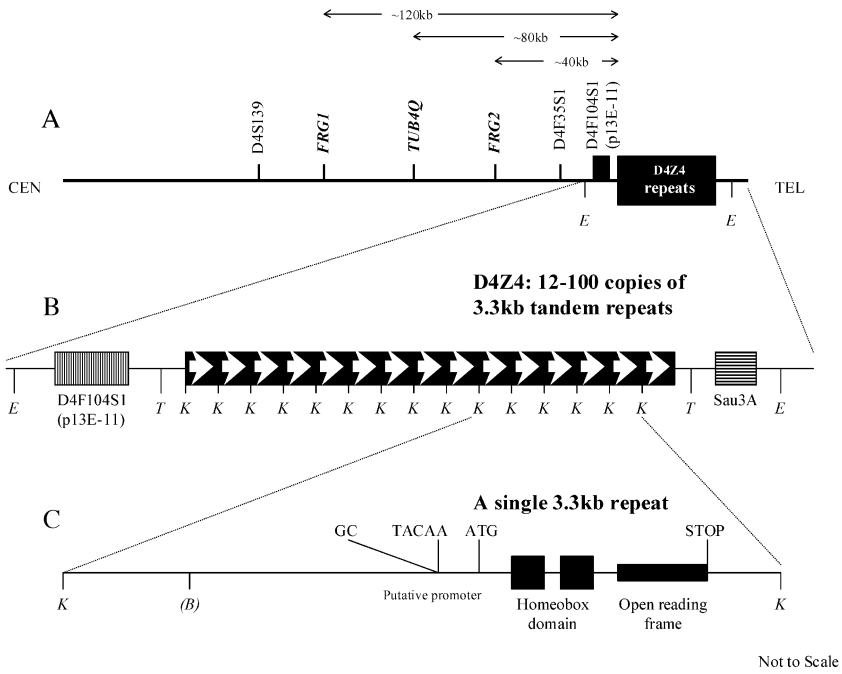

Fig. 1 (A) Restriction map of the 4q35 region. Relative positions of *FRG1*, *TUB4Q*, *FRG2*, D4F104S1 (p13E11), and D4Z4. (B) *Eco*RI fragment detected by probe p13E11 predominantly comprises an array of 3.3-kb tandem repeats, which have a copy number of 12–100 in normal controls and usually 11 or less in FSHD patients. (C) Each 3.3-kb repeat comprises two homeodomains (shaded boxes) encompassing an ORF with an in-frame start codon (ATG) and a stop codon. It encodes the *DUX4* gene. The position of the GC and TACAA boxes in the promoter-like sequence of *DUX4* gene is indicated.

*Eco*RI fragment size range noted in severe childhood cases of FSHD is 10–18 kb; in typical teenage-onset cases, between 18 and 34 kb; and in the oldest late-onset patients, larger than 30 kb.[8] However, it is still impossible to accurately predict the likely severity of disease expression based on repeat number alone. This is because of the high degree of interfamilial and intrafamilial variability of disease expression observed in this disorder, despite the fact that all affected members of a family exhibit the same-sized D4F104S1 allele.

MONOSOMY OF FSHD CANDIDATE REGION

Cytogenetic data indicate that monosomy of the distal 4q35 region, resulting in complete haploinsufficiency of the D4Z4 locus, is not associated with FSHD.[9]

SEQUENCE HOMOLOGY AND GENETIC RECOMBINATION BETWEEN 4Q35 AND 10Q26

There is a high level of sequence homology (98–100%) between D4Z4 repeats from the 4q35 and the 10q26 D4F104S1 loci. DNA sequencing was successful in identifying a unique *Bln*I restriction site present only in each copy of the 10q26-derived repeat units[10] and a *Xap*I site in all 4q35-derived repeats.

The high degree of homology between the 4q35 and the 10q26 D4F104S1 region is thought to have been responsible for interchromosomal exchanges between these two regions. In fact, complete repeat arrays (either of 4q35-derived *Bln*I-resistant D4Z4 repeats, or 10q26-derived *Bln*I-sensitive repeats) may be exchanged between these chromosomal locations. These interchromosomal exchange events are best visualized by pulsed-field gel electrophoresis (PFGE) owing to the large size of the genomic fragments involved; such studies have shown that entire repeat arrays are ''translocated'' in the majority of cases[11] (Fig. 4). It is likely that the sequence exchange is mediated by gene conversion, rather than actual physical translocation. Regardless of the precise mechanism involved, subtelomeric sequence exchanges may also lead to the formation of hybrid arrays containing interspersed 4q-derived and 10q-derived repeat units.

Perhaps surprisingly, these dynamic subtelomeric interchanges do not appear to be associated with the expression of the FSHD phenotype, as these 4q35$\leftrightarrow$10q26 exchanges are evident in about 20% of normal individuals (Fig. 4).[11] It should be emphasized that FSHD only occurs when the greatly deleted D4Z4 repeats (whether

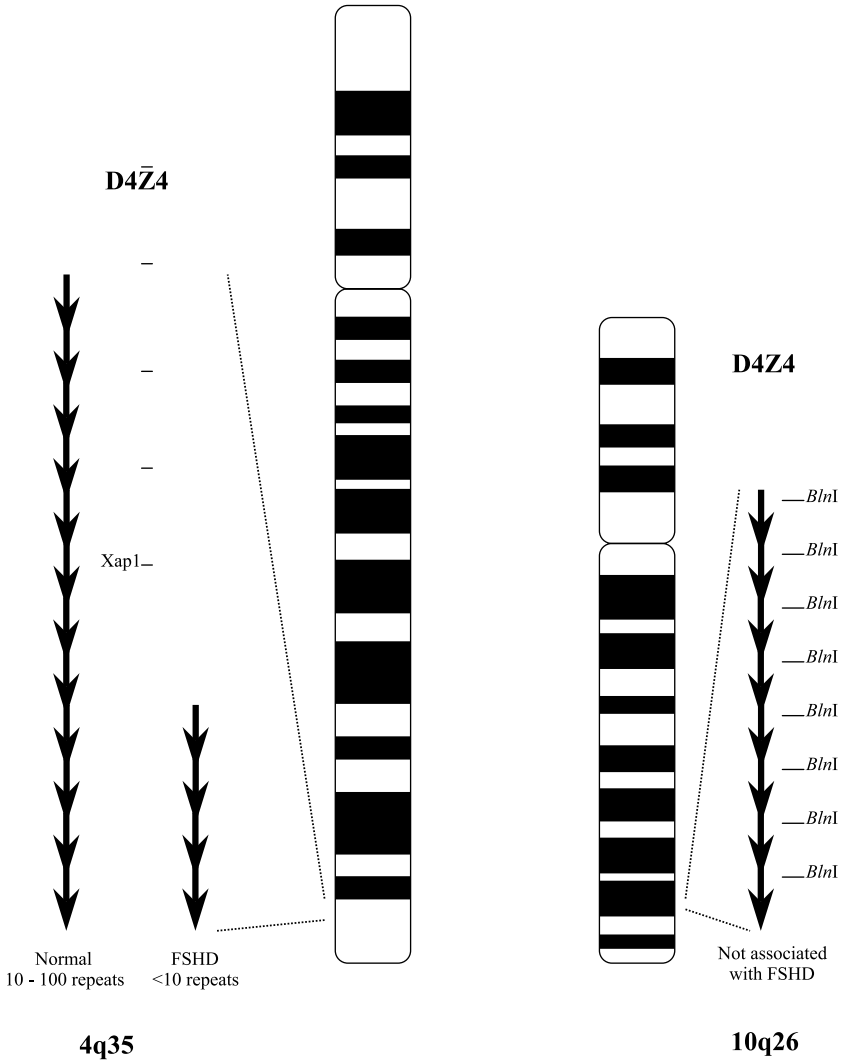

Fig. 2 Location of D4Z4 repeats at 4q35 and 10q26. The 10q-derived D4Z4 repeats differ from the 4q-derived repeats in having an internal restriction site for *Bln*I. Deletion of D4Z4 repeats at 4q35 is associated with FSHD, whereas reduction in the copy number of 10q-derived D4Z4 repeats does not result in any specific phenotype.

they are 4q35-derived or 10q26-derived) are located on chromosome 4.

SOMATIC MOSAICISM

Somatic mutations can occur early in embryonic life and may involve both somatic cells and germ cells. These individuals (gonosomal mosaics) may then be at risk of having affected children. A new mutation often first appears in a mosaic form, usually in a clinically normal person, who then has a constitutionally affected child. Van der Maarel et al.[12] detected somatic mosaicism in 40% of their de novo FSHD families (14% in an unaffected parent and 26% of the de novo FSHD patients themselves). Interestingly, an excess of mosaic-affected males was found in this dataset.

MONOZYGOTIC TWINS

Several studies have reported considerable variation in the clinical expression of FSHD in pairs of affected monozygotic twins.

GENDER BIAS

There appears to be a gender-specific influence on the degree and rate of disease progression in FSHD. The

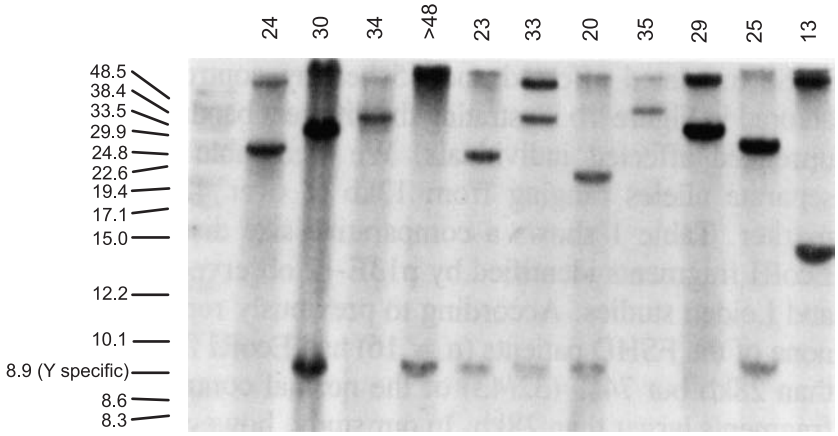

Fig. 3 The length of the smallest *Eco*RI fragment associated with the disease in unrelated FSHD individuals. Ten micrograms of DNA was digested with *Eco*RI, fractionated on a 0.5% agarose gel for 48 hr at 0.5 V/cm, and Southern-blotted onto HybondN (Amersham). The DNA on the membrane was hybridized with radiolabeled DNA probe p13E-11. Lane 1 contains high-molecular-weight markers. Lanes 2–12 contain DNA samples from unrelated FSHD patients. The corresponding smallest *Eco*RI fragment size is written on the top of each lane. The 8.9-kb fragment represents a Y-specific sequence.

age of disease onset is invariably later in female FSHD gene carriers, who are also more likely to exhibit a less severe form of the disease.[13] It has been suggested that female hormonal status somehow confers a mild protective effect. Consistent with this view, disease progression is markedly accelerated in female patients following menopause, which is often associated with a general decline in muscle strength.

MOLECULAR DIAGNOSIS OF FSHD

FSHD is associated with large deletions of the polymorphic D4Z4 repeat array when located at 4q35. The introduction of differential *Eco*RI/*Bln*I double digest has led to a dramatic improvement in molecular diagnostics for FSHD.[14,15] The specificity and sensitivity of this *Eco*RI–*Bln*I double-digest technique for accurate FSHD diagnosis have been well assessed and demonstrated to be high. However, an additional diagnostic complication is created by subtelomeric chromosomal exchanges between the two highly homologous 4q35 and 10q26 D4Z4 repeat loci, which are present in about 20% of the normal population.[11] Diagnostic complexity has been further increased by the recent observation that these subtelomeric exchanges may also result in the formation of hybrid arrays, containing an admixture of 4q35-derived and 10q26-derived D4Z4 repeat units. In addition, the complete loss of the D4F104S1 locus (probe p13E-11) has been reported in FSHD patients. Subtelomeric exchanges may lead to problems during the molecular diagnosis of FSHD in 5% of cases.[11,15] Recent findings using *Xap*I, an additional restriction enzyme, have increased the accuracy of molecular diagnosis for FSHD to 98%.[16]

THE SEARCH FOR THE *FSHD* GENE

Although D4F104S1-associated deletions are closely associated with FSHD in disease expression, the exact nature and location of the *FSHD* gene(s) remain elusive, as does the pathological basis of this association. However, although a number of functional genes have been identified within the FSHD candidate region, including *FRG1*, *FRG2*, and *DUX4*,[17] none of these genes has yet been proven to be the *FSHD* gene.

EXPRESSION STUDIES IN THE FSHD CANDIDATE REGION

Expression studies suggest that a number of genes are dysregulated in FSHD.[18] Genes that are abnormally expressed in FSHD muscles are those involved in myogenesis, cell differentiation, cell cycle control, and response to oxidative stress.

TRANSCRIPTIONAL DEREPRESSION

A 27-bp element within each D4Z4 repeat that specifically binds a multiprotein complex, consisting of YY1, HMGB2, and nucleolin, both in vitro and in vivo, has been shown to mediate the transcriptional repression

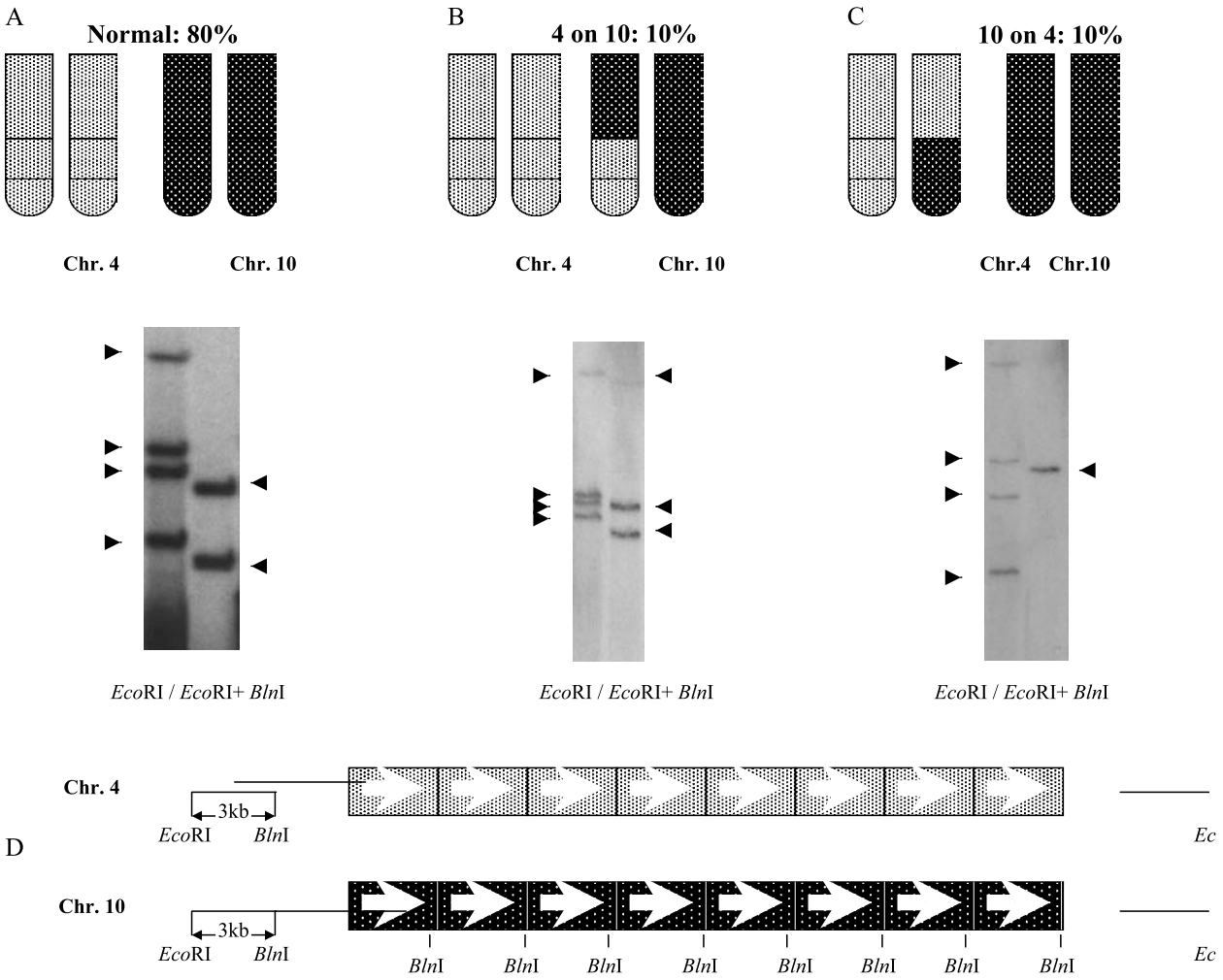

Fig. 4 Subtelomeric sequence exchange between 4q35 and 10q26 in 20% of the normal population. (From Ref. [11].) In the control population, 80% of individuals carry a standard configuration, with 4-type repeats on chromosome 4, and 10-type repeats on chromosome 10. In 10% of individuals, 4-derived repeats are also present on one of their chromosomes 10. Likewise, 10% of the control population carries 10-derived repeats on one of their chromosomes 4. (A) Five microliters of high-molecular-weight DNA digested sequentially with *Eco*RI and *Eco*RI/*Bln*I and hybridized with probe p13E11. In an informative situation, four different-sized *Eco*RI fragments are produced following a single digest: two derived from chromosome 4 and two from 10q. Digestion with enzyme *Bln*I will cleave two chromosome 10-specific fragments. Chromosome 4-specific fragments will be reduced by 3 kb, owing to the presence of a *Bln*I site proximal to the first repeat but distal to the *Eco*RI site (D). However, in 10% of individuals (B), 4-type repeats (*Bln*I-resistant) are translocated to chromosome 10; therefore with *Eco*RI/*Bln*I digestion, three alleles are seen instead of the expected two fragments. Similarly, 10% of control individuals (C) carry *Bln*I-sensitive repeats on one of their chromosomes 4; therefore with *Eco*RI/*Bln*I double digest, one allele (monosomy) is observed.

of genes at 4q35.[19] YY1 is involved in repressing and activating a number of gene promoters. HMGB2 is a member of the family of high-mobility group (HMG) proteins. Nucleolin is involved in chromatin structure formation.

In FSHD patients who possess fewer D4Z4 repeats, the amount of suppressor is greatly reduced; therefore the disease may be seen as being caused by the derepression of the transcription of genes proximal to the repeats. The deletion of repeated 27-bp elements from the subtelomeric region of 4q may act in cis on neighboring tissue-specific genes by derepressing their transcriptional activity, thereby initiating a cascade of events that eventually leads to FSHD. This finding would account for the observation that monosomy of 4q35 does not result in FSHD expression.[9] This elegant study is helping to unravel a new molecular mechanism for FSHD, which may well be found to underlie other genetic conditions.

However, in a recent study, the overexpression of genes in the FSHD candidate region was not reproduced.[20] It is possible that there is overexpression of a 4q35 gene in a small percentage of nuclei in the FSHD muscle fibers.

A polymorphism in the β-satellite repeats located distal to the D4Z4 repeat has recently been identified. This polymorphism, comprising alleles 4qA and 4qB, occurs with nearly equal frequencies in the normal population. FSHD is uniquely associated with 4qA allele.[22,23] Allele 4qA may have additional features that give rise to FSHD either by facilitating the derepression consequent to D4Z4 deletion, or, conceivably, by promoting D4Z4 deletion mutagenesis directly. It remains to be defined how allele 4qA relates to the complex that controls the expression of genes on 4q35. This finding may lead to a better understanding of the instability identified in the FSHD region.

DNA METHYLATION

D4Z4 repeats are hypomethylated not only in FSHD patients with contracted D4Z4 but also in FSHD patients without the contraction of D4Z4 copies.[23]

THERAPY

There is no current treatment specific for FSHD. Surgical intervention may involve scapular fixation and tendon transfers. Albuterol, a β_2-agonist known to increase muscle mass and to cause proliferation of muscle satellite cells, has provided some encouraging evidence of improvements to both muscle strength and mass.[24]

CONCLUSION

Many of the unique and puzzling FSHD disease-associated features need to be taken into consideration when one attempts to deduce the underlying pathological mechanism:

Monosomy of the 4q35 region, which does not produce an FSHD phenotype

High sequence complexity of this region, with its highly repetitive nature and its sequence homologies to loci on other autosomes

Absence of discernible disease phenotype associated with large deletions of the 10q26-located D4Z4 repeat arrays, despite the almost identical sequence of the 4q35-located and 10q26-located ORF

Inverse correlation, which is evident only between chromosome 4-located D4Z4 repeat copy number and clinical severity

Marked variable clinical expression of the disorder and asymmetrical progression of the disease

Discordance in clinical phenotype observed between monozygotic twins

Significant levels of somatic mosaicism observed in both asymptomatic parents and affected individuals

Abnormalities of gene expression in FSHD muscles

Derepression of the transcriptional activity of a number of genes in FSHD muscles

Complete association of the 4qA polymorphic allele with FSHD D4Z4 deletion

Significant hypomethylation of D4Z4 CpG methylation-sensitive restriction sites in FSHD.

The identification of the *FSHD* gene and the characterization of the *FSHD* gene product should enable us not only to offer accurate molecular diagnosis for this disorder and other related conditions, but also to help resolve some of the apparent complexities of the 4q35 FSHD region.

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Factor IX Deficiency

Marian Hill
Gerry Dolan
Nottingham Haemophilia Comprehensive Care Centre, Nottingham, U.K.

INTRODUCTION

Factor IX plays an important role in blood coagulation. Hereditary deficiency of factor IX leads to the bleeding disorder hemophilia B, which is clinically indistinguishable from the more common hemophilia A (Factor VIII deficiency). Hemophilia B is a recessively inherited X-linked disorder with a world-wide incidence of 1/30,000–50,000. Advances in our knowledge of the factor IX protein and its functional activity and in the factor IX gene and the genetic defects associated with hemophilia B have led to major diagnostic and therapeutic advances in this disorder.

FUNCTION OF FACTOR IX

Factor IX circulates in plasma as an inactive zymogen that is activated during coagulation to factor IXa—a vitamin K-dependent serine protease. Activation results from cleavage of factor IX by either factor XIa or factor VIIa/tissue factor in the presence of Ca^{2+}. Factor IXa then forms a complex with factor VIIIa on the cell membrane surface which is responsible for the activation of factor X. This forms part of the series of reactions constituting the blood coagulation cascade that ultimately leads to the formation of a fibrin clot.[1]

CLINICAL FEATURES

Although clinical descriptions consistent with hemophilia have been found in ancient texts, the term hemophilia was not used until 1928, and factor IX deficiency was not described as a cause of hemophilia until the 1950s by Aggler et al.[2] (using the term PTC deficiency) and Biggs et al., who used the term Christmas Factor deficiency after the surname of their index patient.[3] This disorder is now commonly known as hemophilia B. The clinical severity of hemophilia B can largely be predicted from the circulating procoagulant plasma level of factor IX (FIX:C) and these levels remain constant through generations of affected individuals in each family. The severity of hemophilia B can be classified by comparing plasma factor IX levels against a standard that has a value of 1.0 u/mL (100%) (Fig. 1).

Individuals with severe hemophilia B usually have a serious, life-threatening bleeding tendency characterized by spontaneous hemorrhage, excessive bruising/bleeding after trauma, and a requirement for regular replacement therapy. In those with moderate and mild hemophilia, spontaneous bleeding is rare, sequelae of trauma are less serious, and the need for replacement therapy is reduced.

In children with severe hemophilia, the bleeding tendency may first manifest as excessive bruising and hematoma formation when crawling or attempting to walk. When there has been no preexisting history of hemophilia in the family (around 30% of cases), the diagnosis may be made during assessment of the child for nonaccidental injury or after trauma such as circumcision.

The hallmark of severe hemophilia is hemorrhage into large joints (hemarthrosis). The ankles, knees, and elbows are most often affected, and when this occurs, patients may experience premonitory symptoms such as tingling followed by painful swelling of the joint leading to impaired movement. Recurrent hemathroses lead to inflammatory changes in the synovium and destructive changes in cartilage. This may result in total destruction of the joint, marked deformity, and disability. Muscle bleeding may also be an important cause of morbidity such as the classic iliopsoas muscle hematoma, which may cause painful flexion of the hip and compression of the femoral nerve. Bleeding may occur in any part of the body; gastrointestinal, intracranial, and genitourinary bleeds are well-recognized features of hemophilia.

DIAGNOSIS

The initial approach to diagnosis is the laboratory assessment of the coagulation system.

The screening test, activated partial thromboplastin time (APTT), will be prolonged depending on the degree of FIX deficiency and the sensitivity of the reagents used. In those with a prolonged APTT and a family history of hemophilia B, FIX:C is directly assayed using a one-stage

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SEVERITY	PLASMA FIX:C (I.U./mL)	PLASMA FIX:C (%)
Severe	<0.01	<1
Moderate	0.01 – 0.05	1 – 5
Mild	0.05 – 0.4	5 – 40

Fig. 1 Classification of hemophilia B.

or chromogenic assay and the diagnosis is confirmed. In those without a previous history, the deficiency of factor IX must be distinguished from deficiencies of FVIII and XI, all of which may also give a prolonged APTT. When the diagnosis is confirmed, identification of the molecular abnormality of the factor IX gene will facilitate carrier screening in other family members.

MANAGEMENT

Replacement of factor IX is essential for the prevention and treatment of hemorrhage in hemophilia B. Earlier forms of replacement therapy were crude preparations of donated plasma that were often only partially effective. The production of highly concentrated factor IX preparations had a major impact in the management of hemophilia B, facilitating home therapy, early treatment of bleeds, amelioration of joint damage, and a marked improvement in quality of life. However, their use was associated with serious complications:

1. Transfusion-transmitted infection (TTI)

   The use of donated plasma in the management of hemophilia led to the transmission of infectious agents to recipients. Hepatitis B and non-A–non-B hepatitis (later recognized as hepatitis C) were shown to be very common in individuals with hemophilia. In the early 1980s, the catastrophe of HIV transmission through blood products emerged in the hemophilic population, and 60–80% of severe hemophiliacs in Europe and the United States were infected. The risk of TTI was highest with concentrates prepared from larger donor pools. The concentrates that had led to a major improvement in the quality of life of these individuals had also caused the tragedy of transfusion-transmitted infection, which had become the leading cause of death in this population. The risks of TTI have been greatly reduced by measures such as blood donor exclusion policies and virucidal treatment of concentrates.
2. Inhibitor development

   The use of FIX replacement therapy may lead to the development of neutralizing antibodies (inhibitors) against FIX and may render the individual refractory to further treatment. Inhibitors may appear transiently in children with hemophilia but persist in approximately 1.5–3% of severely affected hemophilia B patients. In such cases, hemorrhage may be life threatening or may lead to more severe musculoskeletal complications. There is a strong association between inhibitor development and underlying genetic defect (discussed later). The inhibitors may disappear with immune tolerance strategies and bleeding episodes in inhibitor patients may respond to treatment with recombinant FVIIa.
3. Thrombosis

   Earlier FIX concentrates also contained large amounts of the other vitamin K-dependent coagulation factors: II, VII, and X; the intravenous infusion of these factors: which patients with hemophilia B are not deficient in, led to high plasma levels and thrombotic complications. With the development of high-purity FIX concentrates, thrombosis became far less common.

Recent Developments in Therapy

Recombinant factor IX

The cloning and expression of the factor IX gene led to the development of recombinant factor IX concentrate, which has no exposure to human protein and minimal exposure to animal protein. Pharmacokinetic and efficacy studies have demonstrated satisfactory in vivo recovery although substantially lower than for plasma-derived factor IX concentrate probably because of minor posttranslational differences. Recombinant factor IX concentrate is now widely used.

Prophylaxis

The availability of safer products has also led to a change in therapeutic strategy in that prophylaxis of bleeding is now widely used, particularly in children. This necessitates the intravenous administration of factor IX in doses of 25–40 u/kg twice weekly and leads to a marked reduction in spontaneous bleeding episodes and an improvement in quality of life.[4]

Gene therapy

Hemophilia B (and A) is well suited for gene therapy because it is entirely due to a single gene defect, the therapeutic window is broad, and there are good animal models. Clinical studies on hemophilia B have been

performed using autologous skin fibroblasts transduced with FIX retroviral vector and adenovirus-associated gene transfer by intramuscular and hepatic artery infusion. Results of such studies are moderately encouraging, but major challenges remain particularly in improving and maintaining expression of factor IX and ensuring safety. Several new approaches to gene therapy are in development and promise to make a major contribution to the treatment of hemophilia.[5]

FACTOR IX PROTEIN STRUCTURE

The factor IX gene is primarily expressed in the liver and the factor IX gene product has a multidomain structure (Fig. 2). The first and second domains are a signal peptide and propeptide, respectively. These are cleaved to produce the mature protein that is secreted as a 415 amino acid single-chain peptide. Factor IX undergoes extensive posttranslational modification and the N-terminal domain of mature factor IX contains 12 γ-carboxyglutamic residues; this ''Gla'' domain is important for phospholipid binding. This is followed by two EGF-like domains and an activation peptide (a 36-amino acid region that includes two cleavage sites required for activation of factor IX). The C-terminal catalytic domain is responsible for the proteolysis of factor X to factor Xa, and has substantial sequence homology to other vitamin K-dependent serine proteases. During activation, factor IX cleavage results in a light chain and a heavy chain, held together by disulfide bonds. Structural data is available for factor IX and some of its interactions[6] and this has been extended by molecular modeling studies.[7]

GENETIC DEFECTS IN HEMOPHILIA B

The factor IX gene is located at Xq27.1–q27.2 and the gene was fully cloned and sequenced in 1985.[8] The gene is approximately 35 kb in length and consists of eight exons (a–h), producing a 2.8-kb mRNA encoding a mature protein of 415 amino acids (Fig. 2). The putative promoter region is under the control of a number of regulatory elements.[9] Exon a codes for most of the signal peptide, exon b for the propeptide and most of the Gla domain, and exon c codes the remainder of the Gla domain. Exons d and e code for the two EGF-like domains. Exon f codes for the activation peptide, and exons g and h code for the catalytic domain. The stop codon is followed by a 3′ untranslated region of 1.4 kb with a polyadenylation recognition sequence.

Many different mutations have been associated with hemophilia B and these are found throughout the factor IX gene. They are mainly point mutations, and no common recurrent mutations have been identified. Missense mutations account for approximately 80% of cases of severe hemophilia B; the remaining 20% of cases are due to gross gene deletions, frameshifts, splice junction changes, and nonsense mutations. Almost all cases of nonsevere hemophilia B are caused by missense mutations.[10]

An international factor IX mutation database is available.[10] Over 800 different mutations are reported in the database in association with hemophilia B. In addition to information on point mutations and short additions and deletions, the database also lists tables of gross gene defects and known polymorphisms.

One-third of point mutations occur at CpG dinucleotides, and involve a CG→TG or CA change. The recurrence of some of these mutations in different families supports their role as ''mutation hot spots.'' However, founder effects have also been shown to be responsible for a number of reoccurring mutations in this disorder.[10]

The distribution of mutations between regions of the gene reflects their relative functional importance. Up to half of reported point mutations are in exon h, which codes for the catalytic domain. Similarly, many mutations are found in exon d, which codes for the calcium-binding EGF domain. Hemophilia B has also been associated with changes to 9 of the 12 gamma-carboxyglutamyl residues within the Gla domain, confirming their critical functional role. In addition, substitutions at each of the 22 cysteine residues in factor IX confirm the structural importance of their disulfide linkages.

The risk of development of inhibitors in response to factor IX replacement therapy is determined by the causative mutation and by variations in the immune system of individual patients.

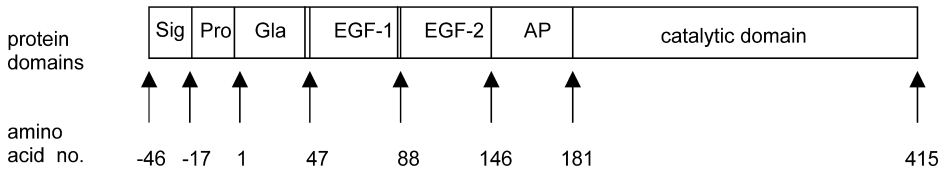

Fig. 2 Illustration of factor IX protein domains. Abbreviations: Sig = signal peptide; Pro = propeptide; AP = activation peptide.

Patients who develop inhibitors are more likely to have null mutations such as deletions and nonsense mutations that prevent synthesis of the factor IX protein. Inhibitors are more rarely found in patients where the mutation causes a loss of function, but not complete absence of the factor IX protein.[11]

Hemophilia B Leyden is a rare form of factor IX deficiency that is characterized by a gradual increase in factor IX levels that begins at puberty. This variant has been associated with a number of different point mutations within an androgen-sensitive region of the factor IX promoter (nucleotide −23 through +13) and several theories have been proposed to explain the recovery seen in such cases.[12,13]

MOLECULAR GENETIC TESTING

Genetic analysis for hemophilia B should be carried out in specialist laboratories and in the context of comprehensive genetic counseling.[14,15] The reasons for referral for genetic testing are:

1. *Assessment of the genetic basis for hemophilia B*

   Nucleotide sequencing can identify a mutation in over 99% of patients tested. In addition to direct nucleotide sequencing, mutation-screening methods have also been used in factor IX analysis.[16] Partial or complete deletions may be further investigated by Southern blotting.

   Although hemophilia B can be diagnosed by measurement of factor IX coagulant activity (FIX:C), genetic analysis confirms hereditary hemophilia and may provide information on the risk of inhibitor development. Knowledge of the genetic cause of hemophilia also facilitates accurate carrier analysis for female relatives.

   Figure 3 illustrates the oligonucleotide primers and conditions used in this laboratory for the amplification of the eight exons of the FIX gene, including splice junctions.

2. *Carrier testing*

   Carrier testing should only be carried out after relevant genetic counseling. Family history may be sufficient to identify some women as obligate carriers (e.g., the daughters of affected males or women with an affected son in addition to maternal relative with hemophilia B). However, for other female relatives, or in cases of sporadic hemophilia with no previous family history (approximately 30% of cases), carrier analysis is required.

   All potential carriers should have their factor IX clotting activity (FIX:C) determined as in a small percentage; this may be low enough to cause a mild bleeding disorder. However, many carriers have normal FIX:C levels. Therefore, genetic analysis is necessary to determine female carrier status and to provide appropriate genetic counseling.

   Carrier analysis is generally carried out by nucleotide sequencing after identification of the causative mutation in an affected relative, although this approach is not suitable for the detection of large deletions.

Where there is a family history of the disorder, linkage analysis using intragenic polymorphisms may be used to

region	primer	SEQUENCE	nucleotide no.	Size (bp)
exon a	1F	CCC ATT CTC TTC ACT TGT CC	2841–3214	376
	1R	GCG TGC TGG CTG TTA GAC TC		
exons b + c	2/3F	GCT CCA TGC CCT AAA GAG AA	9160–9802	645
	2/3R	TGG GTT AGA GGG TTG GAC TG		
exon d	4F	ATC AGA CTC CCA TCC CAA TG	13265–13523	259
	4R	CTT GTT TCA GAG GGA AAC TTT GA		
exon e	5F	CAT GAG TCA GTA GTT CCA TGT ACT TT	20557–20831	277
	5R	TGT AGG TTT GTT AAA ATG CTG AAG TT		
exon f	6F	TTT AAA TAC TGA TGG GCC TGC T	23202–23662	462
	6R	GTT AGT GCT GAA ACT TGC CTA AAT		
exon g	7F	TGC CTA TTC CTG TAA CCA GCA	32918–33162	247
	7R	AGA GCT AGT GGT GCT GCA GAT		
exon h	81F	TTG CCA ATT AGG TCA GTG GTC	33693–34045	353
	81R	GCT GAT CTC CCT TTG TGG AA		
	82F	CCT CAA ATT TGG ATC TGG CTA	33983–34385	403
	82R	GCC CTG TTA ATT TTC AAT TCC A		

Fig. 3 Sequence of oligonucleotide primers that can be used for the amplification of the FIX coding region, including spice junctions. Recommended PCR conditions: 2 mM mixed dNTP; 2.5 U Amplitaq Gold DNA polymerase; 1 × Amplitaq Gold buffer including 2.5 mM $MgCl_2$, 20 pmol/μL each primer, 100-ng template DNA, all amplified using a 35-cycle PCR (30 sec each step) and annealed using a stepdown protocol (five cycles each of 62°C, 60°C, 58°C, 54°C, 52°C, and 50°C).

track a disease-causing allele through a family. There are a number of polymorphisms throughout the factor IX gene that are used for linkage analysis. Intron 1 contains a complex polymorphic region (DdeI polymorphism) with two common variants and several rarer alleles.[17] In addition, there are 11 diallelic polymorphisms that can be used for linkage analysis.[15] All polymorphic sites are in linkage disequilibrium with each other although the frequencies considerably vary with ethnic origin.

Linkage studies are dependent on the availability of samples from family members, and may not be informative in all cases. Carrier analysis by linkage may not be suitable where there is no previous family history.

Prenatal Diagnosis

Prenatal diagnostic testing can be provided for carriers of hemophilia B where a causative mutation has been identified or informative linkage markers determined. Fetal cells are generally obtained by chorionic villus sampling at 10–13 weeks of gestation or from amniocentesis at 16–18 weeks.[14,15]

RESOURCES

OMIM no.	306900
Genecards/GDB ID	GC0XP137318/F9
LocusLink ID	LocusID: 2158
Mutation databases: http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html http://archive.uwcm.ac.uk/uwcm/mg/search/119900.html	
Swiss-Prot	P00740

CONCLUSION

Our knowledge of the key role of factor IX in hemostasis and the structure and function of the FIX gene and protein has greatly expanded. The cloning and expression of the factor IX gene has yielded important advances in both the diagnosis and treatment of hemophilia B. Genetic analysis in this disorder has increased our understanding of the function of the factor IX protein and facilitated accurate carrier analysis. In addition, the production of recombinant factor IX concentrate has greatly improved safety of therapy, and gene replacement therapy has the potential to cure or ameliorate this congenital bleeding disorder.

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Factor X Deficiency

Fabrizio Vianello
University of Padua, Padova, Italy

INTRODUCTION

Factor X (FX) is one of the vitamin K-dependent (VKD) coagulation factors. Factor X occupies a central position in the coagulation cascade and represents the point of convergence between the intrinsic and extrinsic pathways of blood coagulation. Congenital FX deficiency is a rare autosomal recessive disorder which is heterogeneous for both laboratory and clinical expression. Because of the central role of FX in the coagulation cascade, mutations are rare. To date, about 70 missense mutations as well as 12 others including deletions and one nonsense mutation have also been described. The interpretation of the functional effect of missense mutations is facilitated by the availability of crystal structures, sequence alignments, and molecular modeling, and this is effective in identifying structural perturbations in active sites.

FACTOR X FUNCTION

Factor X, the zymogen of the serine protease factor Xa, occupies a pivotal position in coagulation by virtue of the fact that it can be activated through either the contact-activated (intrinsic) pathway or the tissue-factor (extrinsic) pathway. Factor Xa associates with its cofactor, factor Va, on a membrane surface in the presence of Ca^{2+}, to form the prothrombinase complex which activates prothrombin to thrombin[1,2] (Fig. 1). Factor X was identified in 1950 by two groups of researchers, independently, and the name of the two patients affected led to its original designation as ''Stuart–Prower'' factor.[3,4] The characteristics of FX deficiency are a prolonged PT and PTT, a serum abnormality in the thromboplastin generation, and a prolonged Russell's viper venom time.

PREVALENCE

Factor X is one of the rarest of the inherited coagulation disorders. It is transmitted as an autosomal recessive disorder (Fig. 2). The estimated prevalence of homozygous FX deficiency is about 1:500,000, but clinically asymptomatic heterozygotes are likely to be as frequent as 1:500.[5]

CLINICAL DESCRIPTION

Among patients with rare coagulation defects, FX deficiency can be one of the most severe.[6] The onset of hemorrhagic manifestations in profound deficiency is typically seen early in age. In less severe deficiency, patients may experience bleeding at any age. The most frequent sites of bleeding are the mucosal, soft tissue, and central nervous system. Among mucosal bleeding, epistaxis is the most frequent, followed by menorrhagia and gastrointestinal episodes. Soft tissue bleeding such as hemarthroses and muscle hematomas may mimic classic hemophilia. Central nervous system hemorrhages represent life-threatening manifestations of severe FX deficiency. Individuals with mild FX deficiencies may only experience bleeding after trauma or surgery.[7]

MOLECULAR GENETICS

The human FX gene is 22 kb long and is located at 13q34-ter, less than 3 kb from FVII.[8] The sequence of human FX gene is highly homologous to other VKD blood coagulation factors such as FVII, FIX, and protein C, suggesting its origin from a common ancestral gene. The coding region of FX is divided into eight exons, each encoding for a particular domain within protein (Fig. 3). Exon I encodes the signal peptide, exon II the propeptide and Gla domain, and exon III a short linking segment of aromatic amino acids. Exons IV and V encode the two EGF domains, exon VI encodes the activation peptide at the N-terminus of the heavy chain, and exons VII and VIII encode the serine protease domain with the catalytic triad His236, Asp228, and Ser379.[9] Factor X is synthesized in the liver as a single-chain precursor. In plasma, FX circulates as a two-chain glycoprotein of about 59 kDa. The light chain is cleaved from the heavy chain during or after secretion into the circulation. Factor X is composed of a 306-residue heavy chain that is covalently linked by a disulfide bond to a 139-residue light chain. The light chain

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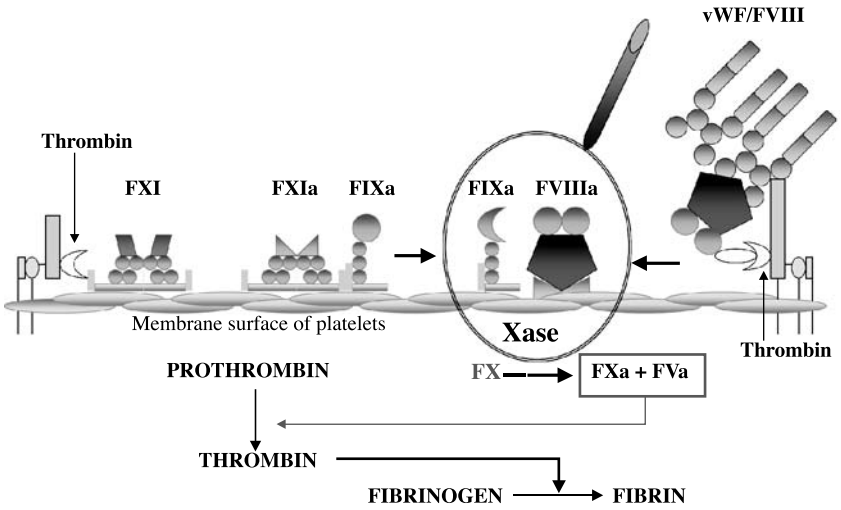

Fig. 1 After binding of thrombin to the platelet surface, FVIII and FXI are cleaved. When FIXa binds to the platelet surface, it forms the ''Xase'' complex together with FVIIIa. The ''Xase'' complex catalyzes the activation of FX. FXa/Va complexes can now produce the burst of thrombin necessary to form a hemostatic clot. (From Hoffmann et al. Thromb. Haemost. **2001**, *85*, 958.) (*View this art in color at www.dekker.com.*)

of zymogenic FX contains three structural domains, each of which possesses distinct functional properties. The heavy chain contains the serine protease domain of 254 amino acids which forms the catalytic triad of the active site.[1,2]

GENOTYPE–PHENOTYPE CORRELATION

Three main groups of FX deficiency can be identified:[9,10] the cross-reacting material (CRM)-negative group in which both antigen and activity are similarly decreased (''hypo'' or type I), while prothrombin time, activated partial thromboplastin time, as well as Russell viper venom time-based assays are prolonged; the CRM-positive group which has normal or near normal antigen levels but factor X activity is decreased (''dys'' or type II); thirdly, the CRM-reduced group in which both activity and antigen levels are reduced but antigen is markedly higher than activity (''hypo–dys'').

Clinical manifestations of FX deficiency are highly variable and usually only develop in homozygotes or double heterozygotes. Factor X levels between 10% and 40% of normal are considered sufficient to prevent

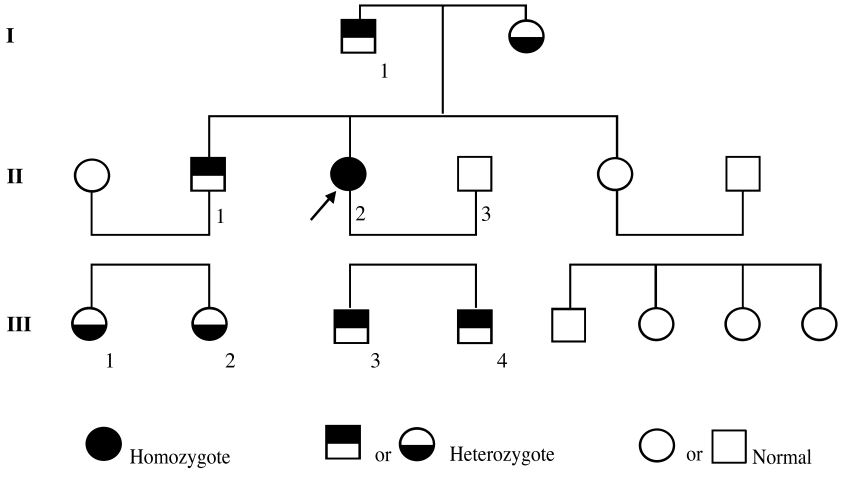

Fig. 2 Family pedigree in a case of FX deficiency. The proband (arrow) showed abnormal PT and PTT values, low FX activity, and normal FX antigen levels. The clinical history was unremarkable. (Original data.)

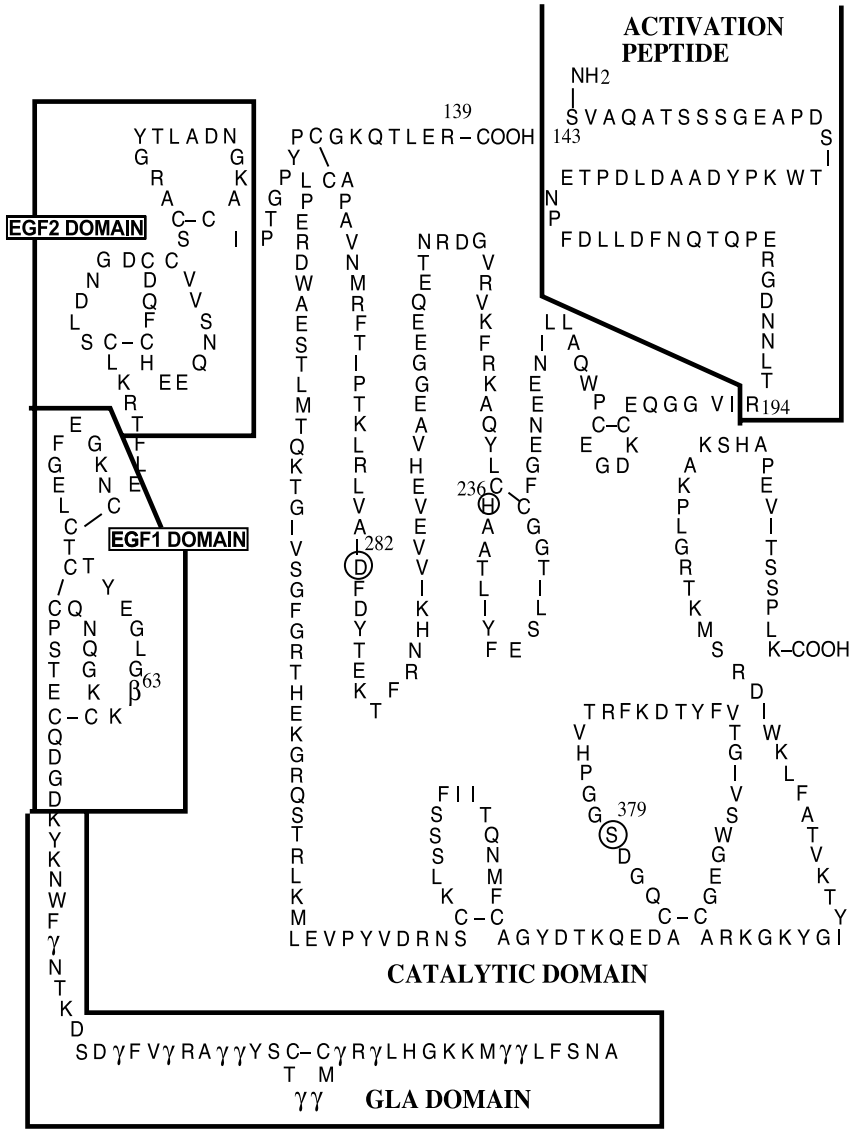

Fig. 3 Schematic representation of the primary amino acid sequence of the two-chain human FX. The form in circulating blood plasma lacking the tripeptide connecting the light chain to the activation peptide is shown. (From Leytus et al. Biochem. **1986**, *25*, 5098.)

bleeding manifestations, and homozygotes may exhibit higher than 10% activity. Thus many individuals carrying a FX mutation may remain undiagnosed or develop hemorrhagic complications only when challenged or because of an additional insult to the coagulation cascade. Although the clinical presentation of FX deficiency correlates poorly with laboratory phenotype, genetic analysis usually provides a plausible explanation for the factor X activity and antigen levels measured in the laboratory. As in other congenital coagulation disorders, FX deficiency usually arises from missense mutations. Amino acid substitutions can significantly change any number of steps in protein formation. Mutations severely impairing the protein structure are frequently associated with a reduction of the antigen as well as the activity level. In these cases, abnormal protein folding may be responsible for an impairment of protein secretion and/or a reduction in protein half-life. Missense mutations responsible for minor structural changes are often associated with an impairment in FX activity in the setting of a normal antigen level. Nonsense mutations and large gene deletions have been reported and are invariably associated with a symptomatic pattern when found in compound heterozygosis with a missense mutation impairing the FX

protein structure.[11] Interestingly, FX knockout mice with a total deficiency in blood coagulation FX present with partial embryonic lethality and fatal neonatal bleeding suggesting the crucial role of FX function.[12] It is likely that genetic mutations that cause total FX deficiency in humans may be incompatible with life as well. Very few symptomatic individuals with total or severe deficiency in the components of prothrombinase have been identified to date.

Missense mutations have been described affecting the different FX protein domains. Mutations in the preprosequence and prosequence, as FX Nice and FX Santo Domingo, have been shown by molecular modeling and expression studies to be responsible for secretion and cleavage problems explaining the type I phenotype.[13,14]

A missense mutation affecting the calcium-binding region of the Gla domain characterize the low activity and normal antigen level in the FX St. Louis.[15]

Mutations affecting the correct formation of disulfide bridges in EGF-1 and -2 domain have been reported, affecting both the function and antigen level of FX-associated mutations.[11] Missense mutations affecting the release of the activation peptide, such as FX Wenatchee I and II, have been shown to be associated with low catalytic activity and reduced FX half-life.[16]

FX Friuli is one of the most well-characterized mutations of the catalytic domain.[17] This variant consists of a $Pro^{343} \rightarrow Ser$ substitution within the heavy chain of FX. Homozygous FX Friuli patients present with a normal antigen level and a normal or near-normal activity by means of RVV assay but a severely reduced (4–9%) function in the intrinsic and extrinsic pathways. Due to the formation of a new hydrogen bond, the tertiary structure of the catalytic domain has been shown to be affected.

A good genotype–phenotype correlation has been demonstrated in an Italian family with FX deficiency, named FX S. Giovanni Rotondo.[18] In this well-characterized variant, one deletion in the region encoding for the activation peptide was described in compound heterozygosis with a missense mutation in the catalytic domain, respectively responsible for the lack of synthesis and/or secretion of FX and for a dysfunctional FX.

The loss of a disulfide bond in the catalytic domain has been shown to be associated with a type I phenotype in FX Padua 4.[19] Interestingly, mutations in this highly conserved residue among other serine proteases, factor VII and protein C, are responsible for a very similar phenotype, suggesting the critical role of this disulfide bond in FX function and secretion.

These few examples of a consistent genotype–phenotype relationship further suggest that the laboratory phenotype is largely a function of the FX gene lesion segregating in the family. Some interindividual variability, even in the presence of identical pathological FX genotypes, is common, which is likely due to the variation between laboratory assays, the contribution of polymorphisms, and possibly also the influence of variation at nonallelic loci.

DIFFERENTIAL DIAGNOSIS

Clinical history in the presence of laboratory prolongation of PT and PTT usually suggests FX deficiency. Specific diagnosis relies on the quantification of FX activity and antigen levels and on the levels of other coagulation factors. Concomitant disease may mimic congenital FX deficiency. Liver disease and vitamin K deficiency can be responsible for acquired FX deficiency, but they more commonly determine abnormalities of other VKD clotting factors. Acquired FX inhibitors have been reported and can be suspected when sample plasma added to normal plasma causes prolongation of PT and PTT. Amyloidosis has been shown to determine FX deficiency by amyloid protein binding to circulating FX.

MANAGEMENT

As FX levels do not correlate with clinical phenotype, therapy of FX deficiency is based on the presence of bleeding manifestations. Transfusion with fresh frozen plasma may be used to treat symptomatic patients. A level of 10–20% U/dL is usually sufficient for adequate hemostasis. The half-life of FX is approximately 40 hr. Plasma is administered with an initial dose of 15 mL/kg, followed by 3–6 mL/kg every 24 hr.

Prothrombin complex concentrates, i.e., FIX, contain a small quantity of FX (about 1 U FX per unit of FIX). Although these concentrates have been shown to be effective in the treatment of bleeding episodes in patients with FX and FVII deficiency, their use has been associated with significant thrombogenicity.

GENETIC COUNSELING

As for other recessive coagulation disorders, consanguineous marriage often explains the presence of homozygous mutations in symptomatic patients. In some cases, compound heterozygotes have been reported. Patients who are severely deficient in FX are severe bleeders and require frequent FX replacement therapy.[8] Therefore, prenatal diagnosis should be performed in pregnant women with at least one family member affected by this coagulation disorder. Knowledge of the underlying mutation is therefore essential to achieve this goal.

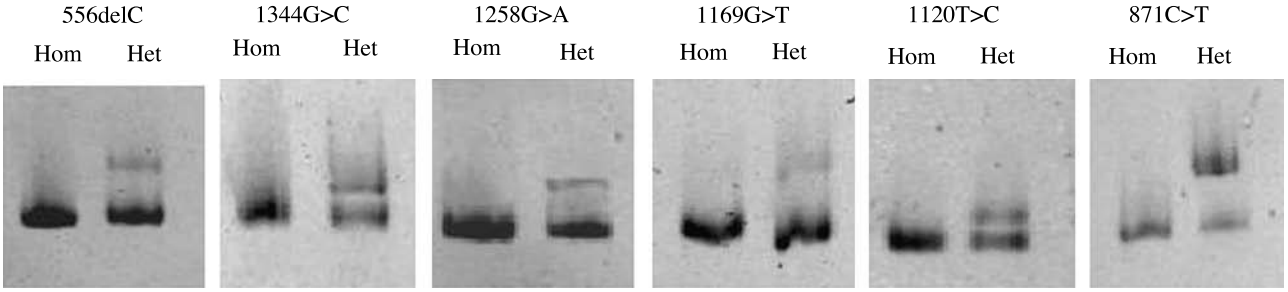

Fig. 4 Conformation sensitive gel electrophoresis of PCR amplification of FX exons 6 and 8. Each picture shows a FX mutation confirmed by gene sequencing. Hom = homoduplex; Het = heteroduplex. (From Ref. [20].)

MOLECULAR GENETIC TESTING

Molecular diagnosis of congenital FX deficiency relies on the detection of gene mutation and on the possible genotype–phenotype correlation. This can be achieved by amplifying the entire coding region of the gene by PCR and then proceeding to sequence the PCR products using the following primers:[17]

Exon 1	5′-GACAACAGCCATCCAAGCTTGGGCGTGGAC-3′
	5′-CTGCCGCCCCTCCAAGCTTCCTGGCGCTGC-3′
Exon 2	5′-GCCTGGGTGAGGGTGACCAG-3′
	5′-AGCGAGGGCGCTGTGGCCTG-3′
Exon 3	5′-AAATCTCTTTTTTCCTTTTAG-3′
	5′-AAATTAGAACAAAAGCAGAAAG-3′
Exon 4	5′-TGATGCCGGAAACAGCTTGCAG-3′
	5′-TGCCACTCTTCAGGGGCATCTG-3′
Exon 5	5′-AGCCTCCATTTCTCCAGCTG-3′
	5′-GTGTCACTGTTACCTGCCAG-3′
Exon 6	5′-TGTGCAGGCTATGGGGAGCCTCTC-3′
	5′-ACAGGTGGTCTCTCCAGCAG-3′
Exon 7	5′-AGTCAGGCAACACCTGTCCACCTG-3′
	5′-TGAAAAGCAGACAGTGACGGTGC-3′
Exon 8a	5′-AACGGATGTGCGAGAGCATGCC-3
	5′-GGCAGGCATCCTCCTGCTTG-3′
Exon 8b	5′-ACCGCAACAGCTGCAAGCTG-3′
	5′-AATCGAGAGACAAACCAGGC-3′

Even with the introduction of automated sequencing system, some intermediate steps can be useful for scanning PCR products with the aim to identify the gene fragment potentially mutated and then confirm the mutation by sequencing. The scanning techniques most commonly used for PCR products are single-strand conformation polymorphism (SSPC), enzymatic or chemical cleavage of mismatched base pairs, and differential unfolding of homoduplexes and heteroduplexes by denaturing gradient gel electrophoresis (DGGE). Recently, conformation-sensitive gel electrophoresis (CSGE) has been successfully used as a simple, inexpensive, and highly reliable method for localizing FX mutations[20] (Fig. 4). The combination of PCR–CSGE–sequencing could be useful in family studies where, after complete characterization of the mutation in a family member by sequencing the entire FX gene, the other relatives are screened by means of CSGE only. Although expression studies are invaluable to detect subtle functional differences in the cases where the mutations are antigen positive (i.e., normal antigen levels), they are less useful where the mutations are antigen negative (as is reflected in the low in vivo plasma levels), as the mutant proteins are likely to be unstable or may not be expressed. With the availability of FX crystal structures, sequence alignments and molecular modeling are effective methods in identifying structural perturbations in active sites.[21] Molecular modeling in combination with energy refinements can be an invaluable tool in the assessment of cases in which protein misfolding is the most likely outcome of a given mutation.

CONCLUSION

With the recent and more accurate studies of the genotype–phenotype relationship, it is now clear that the laboratory phenotype of congenital FX deficiency is largely a function of the FX gene lesion segregating in the family. However, some interesting cases have been reported that suggest more intricate mechanisms as responsible for congenital FX deficiency. In particular, symptomatic patients with autosomal dominant pattern of inheritance have been reported, leading to the hypothesis that the presence of a circulating abnormal FX protein can compete and exert a dominant negative effect by denying the access of the product of the wild-type factor X allele to receptor sites. Larger-scale studies of patients and their immediate relatives will provide more insight into the pathophysiology of congenital FX deficiency.

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Factor XI Deficiency

Pierre Hopmeier
Hospital Rudolfstiftung, Vienna, Austria

INTRODUCTION

In general, factor XI deficiency is regarded as a rare disorder restricted to isolated cases and families. However, it is common in people of Jewish descent, particularly in individuals of Ashkenazi origin. Recently, factor XI deficiency has also been reported in the French Basques population, and the condition may also be very common in persons of African ancestry.

Although factor XI is essential for normal hemostasis, a deficiency state is not always associated with bleeding and may remain without clinical symptoms. If there is a bleeding tendency, it is often differently pronounced between individuals with the same factor XI level.

Compared to hemophilia A or B, the prevalence of spontaneous bleeding, formation of hematoma or hemarthroses is much lower even in severely deficient patients. Also, the association between plasma levels of factor XI and the frequency and severity of bleeding symptoms is much poorer than for a deficiency of factor VIII or factor IX.

BACKGROUND

Plasma factor XI circulates as a homodimer linked by a disulfide bond complexed to high molecular weight kininogen (HK) in noncovalent form at a molar ratio of 1:2.[1] Each monomer is composed of 607 amino acids and has an apparent MW of 80,000. Most, if not all, of factor XI is synthesized by the liver, although mRNA was also demonstrated in platelets and cell lines with megakaryocyte features.[2] Factor XI can be activated by factor XIIa, factor XIa, thrombin, or by autocatalysis through cleavage of a single peptide bond (Arg 369–Ile 370).[3,4]

There is a large body of evidence that under normal conditions, blood coagulation is initiated via the extrinsic pathway. The minute amounts of thrombin formed as a result of exposure of tissue factor will then activate factor XI in a feedback manner, leading to the formation of an amplification loop with continued thrombin generation. Platelets seem to play a central role. They are activated by trace amounts of thrombin, and on the activated platelet surface, thrombin-mediated activation of factor XI is more effective than activation by factor XIa or factor XIIa and directly reflects the occupancy of F XI-binding sites.[3] Furthermore, on the surface of activated platelets, fibrinogen does not inhibit thrombin-mediated factor XI activation, possibly because of colocalization of thrombin and factor XI on a high-affinity binding site.[3] Also, platelet-bound factor XIa, but not factor XIa in solution, is protected from inhibition by protease nexin 2, which is likely to be the most relevant inhibitor of factor XI within the activated platelet environment.[1,3,4]

Because thrombin, particularly in the presence of thrombomodulin, activates thrombin activatable fibrinolysis inhibitor (TAFI), feedback generation of factor XIa is also regarded to be important for the downregulation of fibrinolysis.[5]

In the laboratory, the diagnosis of factor XI deficiency is usually based on the finding of a prolonged activated partial thromboplastin time (APTT). However, common clotting tests for measurement of factor XI generally rely on factor XI activation via the intrinsic pathway and reflect the contribution of factor XIa for initial fibrin clot formation. Several aspects of factor XI activation and function, in particular the interaction with platelets and the effects of maintained thrombin-mediated factor XI generation, are not adequately measured.

GENOTYPE–PHENOTYPE CORRELATION

The lower limit of the normal range for factor XI is commonly given at about 70%. Individuals with levels up to 15–20% are regarded as partially deficient, while individuals with lower levels are severely deficient and represent homozygotes or compound heterozygotes.[6,7] In general, bleeding is negatively correlated with factor XI level.[7] However, only at a very low factor XI level is the risk for excessive bleeding clearly increased, probably because the quantity of factor XI necessary for normal thrombin generation after initiation of coagulation by minute amounts of tissue factor is very small.[7,8] For instance, patients with a factor XI of only about 9% (homozygotes for the type III mutation) still bleed less severely than patients with a factor XI level of about 1% (homozygotes for the type II mutation).[8] On the other hand, it is well established that individuals with a severe deficiency do not always have an increased pro-

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pensity to bleed, whereas some heterozygotes may bleed excessively.[6] Basically, bleeding tendency is unpredictable and may also vary in the same patient.[6,9] Consequently, the inheritance pattern of factor XI deficiency cannot be generally defined as being autosomal dominant or autosomal recessive.

CLINICAL DESCRIPTION

In symptomatic individuals, certain clinical manifestations are common. In women, menorrhagia is frequently reported and there is an increased risk of bleeding after childbirth.[9] Epistaxis may occur, whereas petechiae and bruising are rather unusual.[9] Bleeding can be excessive and frequently manifests postoperatively, in particular after surgical procedures involving tissues with high fibrinolytic activity such as dental extractions, tonsillectomies, prostatectomies, or urinary tract operations. For other surgical interventions such as appendectomies, orthopedic operations, cholecystectomies, or hysterectomies, the risk of severe bleeding is less pronounced.[10] Sometimes the first bleeding episode is observed after circumcision.[9] Bleeding can be immediate,[9] but typically, it is protracted or presents as persistent oozing after surgery.[10]

Antibodies to factor XI are rare and have mostly been described in nondeficient patients as a complication of autoimmune disease.[6] Recently, however, a number of patients who were given plasma replacement therapy were diagnosed with acquired inhibitors to factor XI. They had a mean factor XI activity level of about 1% and were all homozygous for the Glu117stop (type II) mutation, indicating that mutations associated with a very low factor XI level may be risk factors for development of an inhibitor after plasma replacement.[11]

PREVALENCE

F XI deficiency is common in the Jewish population where two mutations, the so-called type II (Glu117stop) and type III mutation (Phe283Leu), prevail. Haplotype analysis indicates that both mutations date back to ancestral founders.[12] In a study performed in Israel, the type II mutation was identified only in Iraqi Jews (allele frequency: 0.0167), which are regarded to represent the original Jewish gene pool in the Middle East. In contrast, both mutations were observed with similar allele frequencies in Ashkenazi Jews (0.0217 and 0.0254, respectively), a main segment of Jews that diverged from the original gene pool and began to migrate to Europe at the time of the Roman Empire.[12] Correspondingly, age estimates based on distribution of allelic variants at a flanking microsatellite marker suggest that the type II mutation dates back more than 120 generations, and that the type III mutation appeared later in history.[13] Recently, in French Basques, a novel mutation (Cys38Arg) with an allele frequency of about 0.005 was reported. Haplotype analysis was consistent with an ancestral founder.[14] Two substitutions, Gln226Arg and Ser248Asn, were identified in an African–American family and subsequently also in population-screening studies indicating that these two variants may be very common in individuals of African origin.[15,16] At present, about 80 mutations have been described in different ethnic groups, the majority being reported in single families or as sporadic cases with unknown allele frequency.

Apart from the Jewish population, inherited factor XI deficiency is generally regarded as a rare condition. However, in a study from the U.K., about 5% of patients with bleeding disorders had factor XI deficiency, many with no known Jewish ancestry.[6,7] Moreover, in our experience, the condition is underdiagnosed. Possible reasons include the fact that factor XI deficiency often remains asymptomatic or is only revealed after hemostatic challenge, and that in APTT assays, results for heterozygote patients may be borderline or within the normal range.[10]

MANAGEMENT

Without hemostatic challenge, patients with factor XI deficiency do not need treatment. In case of dental extractions or surgical intervention, fresh frozen plasma replacement therapy may be required. This can be combined with the application of antifibrinolytic agents. The half-life of factor XI in plasma is about 40–80 hr, and factor XI function is well preserved.[9] For dental extractions, only antifibrinolytic therapy using tranexamic acid from 12 hr before until 7 days after the procedure has also proven to be effective.[6,9] Other products such as fibrin glue or desmopressin have been used in a limited number of patients as well.[9] In general, it is recommended to continue therapy for several days. Factor XI concentrates have been developed but are presently not licensed for use in the United States.

In patients with factor XI inhibitors, the uses of prothrombin complex concentrates and recombinant factor VIIa were successful.[6]

MOLECULAR GENETICS

The gene for factor XI is located on the distal end of chromosome 4 (4q35).[17] It is 23 kb in size and consists of 15 exons (numbered 1–15) and 14 introns (named A–N).

Exon 1 is not translated and exon 2 codes for a signal peptide of 18-amino acids length, which is not integrated into the final protein.[18] Plasma factor XI is the gene product of exon 3–15 and contains a heavy chain of 369 amino acids and a light chain 238 amino acids in length.[5] Platelet factor XI mRNA was reported to be an alternative splicing product lacking exon 5; however, this was not confirmed by other research groups.[2,19] The heavy chain harbors four tandem repeats of 90–91 amino acids, named apple domains (A1–A4). Each one is encoded by two successive exons starting from exon 3.[5,18] Several binding sites are located within these domains. To a large extent, the implicated amino acids have been elucidated.[20–24] The A1 domain carries a binding site for prothrombin as well as binding sites for HK and thrombin.[3] Binding of prothrombin or HK to the A1 domain results in exposure of a platelet-binding site on the A3 domain.[1,24] The interaction of the A3 domain with activated platelets is mediated through binding to GPIb–IX–V complexes within lipid rafts in the platelet membrane.[25] Moreover, a binding site for unfractionated heparin was localized on the A3 domain.[22] Both the A2[20] and the A3 domain[21] were reported to mediate factor IX activation. On the A4 domain, a binding site for factor XIIa was localized.[23] The A4 domain is also essential for factor XI dimer formation because it mediates initial noncovalent dimerization of two factor XI subunits and harbors the Cys321 residue responsible for subsequent dimer stabilization by a single disulfide bond.[26] The light chain is encoded by exon 11–15.[5,18] It contains the active site formed by the catalytic triad (His413, Asp462, and Ser557)[5] typical for most serine proteases, as well as a high-affinity heparin-binding site.[27]

With the exception of three deletions and three insertions,[14,17,28–30] all published mutations associated with factor XI deficiency involving exon 3–15 are single-base substitutions, which are mostly missense mutations. Many are located within binding sites. Therefore one may expect aberrant factor XI molecules to circulate and interfere with normal factor XI function. However, in most instances when factor XI activity and antigen were determined, both were simultaneously reduced.[7] In fact, a defect in secretion was demonstrated for a number of factor XI variants expressed in cell lines, indicating that, in general, circulating factor XI in deficient individuals will be composed of normal monomers.[14,31] However, a few cases of circulating dysfunctional factor XI protein have been reported.[6] Recently, two novel variants with normal expression in cell culture systems were described in several patients. One individual with reduced but appreciable factor XI activity and antigen was compound heterozygous for these mutations. Consequently, all circulating factor XI of this individual must only consist of abnormal monomers. Furthermore, in individuals heterozygous for such mutations, the variant molecules may participate in circulating factor XI forming either homodimers or combine to heterodimers together with normal factor XI molecules.[15,16]

LABORATORY DIAGNOSIS

In clinical practice, e.g., for the planning of operations, diagnosis of congenital F XI deficiency is usually established by APTT-based assays, whereas molecular techniques are mostly restricted to scientific studies. However, there are conditions, such as consumption reactions or the presence of a high titer of lupus anticoagulant, where APTT-based tests may not be suitable and molecular methods are needed to identify a congenital deficiency state. For mutation analysis, genomic DNA is extracted and usually amplified by PCR with primers covering all 15 exons and the intron–exon boundaries. Frequently, primers are designed in such a way that a section of DNA encompassing a number of exons in close proximity to each other is amplified.[31,32] PCR may be directly followed by determination of nucleotide sequences of all PCR products, but frequently a mutation-screening technique such as single-stranded conformation polymorphism (SSCP) analysis is subsequently applied to avoid unnecessary sequencing.[17,33] Restriction fragment-length polymorphism (RFLP)[32] analysis and dideoxyfingerprinting (ddF)[15] have also been used for this purpose. The disadvantage of all of these methods is that the mutation-detection rate may be incomplete. For example, with Fluorescent-SSCP, the detection rate is 80–85% in routine use.[34] Recently, considerable improvement with denaturing high-performance liquid chromatography (dHPLC) has been reported. In one study, the detection rate using this technique was 100%.[34]

CONCLUSION

Congenital factor XI deficiency is probably underdiagnosed, partly because affected individuals often remain without symptoms and because in conventional APTT tests, the results for heterozygotes frequently overlap with the normal range. In general, APTT-based tests are of limited value to assess the risk of bleeding in affected individuals because the association between bleeding propensity and measured factor XI level is poor.[7,16]

Increasing numbers of mutations causing factor XI deficiency are being identified. However, there has been no comprehensive study that focus on whether genetic variants participate in the formation of circulating factor XI only in exceptional cases, or whether this is more

common, and to what extent different factor XI functions are thereby affected. Modified activity tests which better reflect the functional spectrum of factor XI and possibly, mutational analysis, may render factor XI measurement clinically more informative.

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Familial Adenomatous Polyposis (FAP) Syndrome

Nader Hanna
Shaun McKenzie
Heather Pierce
University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.

INTRODUCTION

Familial adenomatous polyposis (FAP) represents the classic paradigm for the hereditary cancer syndromes. It is a highly penetrant autosomal dominant disorder with classic Mendelian inheritance of mutations in the adenomatous polyposis coli (*APC*) gene and characteristic genotype–phenotype correlation. Familiarity with the natural history of the syndrome, inheritance pattern, and the colonic and extracolonic clinical manifestations is essential for early identification of families at risk. A combination of genetic counseling, genetic testing, and aggressive endoscopic surveillance is paramount to management. Surgical management is the cornerstone of therapy as chemopreventive measures continue to be developed.

FAMILIAL ADENOMATOUS POLYPOSIS SYNDROME

Familial adenomatous polyposis syndrome is characterized by accelerated tumorigenesis from normal mucosa to polyps. It occurs in 1:8000 to 1:13,000 live births and shows no racial, gender, or geographical predilection. It has autosomal dominant inheritance with 100% penetrance, and if left untreated, patients affected with FAP will inevitably develop colorectal cancer, typically by the age of 35–40 (mean age 39 years).[1] It accounts for about 1% of all colorectal cancers. Approximately 15%, 75%, and 90% of patients will develop 100 polyps or more (mostly in the left colon) by ages 10, 20, and 30, respectively. These polyps follow the typical adenoma to carcinoma progression seen in sporadic colorectal cancer, with a 5- to 10-year gap seen between diagnosis of polyposis and cancer. Unfortunately, despite the fact that this syndrome is a well-described entity, 59% of patients still succumb to metastatic colon cancer.[2] Of those who do undergo timely colonic resection, upper gastrointestinal malignancy and desmoid formation are the leading causes of death.[3] Patients with FAP are at risk of developing extracolonic tumors, including premalignant periampullary adenomas, desmoid tumors, papillary thyroid cancer, hepatoblastoma, epidermoid cysts, and congenital hypertrophy of the retinal pigment epithelium (CHRPE). The periampullary polyps appear white, smaller, and more sessile than colorectal polyps and usually concentrate in the second part of the duodenum. They are often asymptomatic, and the presence of symptoms suggests malignancy. Periampullary and duodenal lesions tend to occur 10 years after colorectal polyposis, and the average age of onset of periampullary or duodenal carcinoma is 46 years old.[4]

Attenuated flat adenoma polyposis syndrome (AFAP), Gardner syndrome, and Turcot (glioma-polyposis) syndrome are phenotypical variants of the FAP syndrome. They have the same molecular genetic basis as the FAP syndrome. Gardner syndrome is characterized by colonic polyposis along with sebaceous cysts, lipomas, desmoid tumors, fibromas, facial bone osteomas, and impacted or supernumerary teeth.[5] Turcot's syndrome is a genetically heterogeneous condition characterized by CNS tumor formation along with colonic polyposis. Turcot's syndrome usually occurs due to mutations in the *APC* gene or one of the genes associated with hereditary nonpolyposis colon cancer syndrome (HNPCC). Turcot's syndrome due to mutations in the *APC* gene is typically associated with occurrence of medulloblastoma, whereas glioblastoma is the CNS tumor type that is most frequently observed in Turcot's syndrome that occurs due to mutations in HNPCC-associated genes.[6] More recently, an attenuated form of FAP, now known as AFAP, has been described. These patients typically present with less than 100 colorectal polyps developing, on average, 10 to 15 years after those seen with FAP and usually proximal to the splenic flexure.[7] The risk of colorectal cancer is still significantly elevated, with 70% of these patients developing colorectal cancer by the age of 65 if left untreated.[1] Extracolonic manifestations can also occur, most notably gastric polyposis. Moreover, diffuse gastric polyposis can occur prior to the development of colonic lesions, and its presence at endoscopy should prompt exploration into the possibility of this syndrome.[7] Other extracolonic manifestations typical of classic FAP, such as cutaneous lesions, desmoids, and CHRPE, are rare in AFAP.[7]

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GENETIC ALTERATIONS IN FAP

Familial adenomatous polyposis is caused by inheritance of germline mutation in the *APC* gene (chromosomal locus 5q21–q22). Familial adenomatous polyposis is an autosomal dominant disease with extremely high penetrance, as 95% of individuals with FAP have polyps by age 35. Approximately 20–25% of individuals with FAP have the altered gene as a result of a de novo gene mutation[8] and therefore will not have an affected parent. The *APC* gene is a ''gatekeeper'' tumor suppressor gene encoding a large protein which is involved in chromosome segregation, cell adhesion, cell migration, signal transduction, and apoptosis.[9,10] Most FAP-associated mutations in the *APC* gene cause premature truncation of the *APC* protein. Individuals with FAP only have one functional copy of the *APC* gene in each colonic epithelial cell, therefore accelerated tumorigenesis from normal mucosa to polyps and carcinoma occur. The *APC* gene contains 15 exons and 2843 codons. The colonic (including the density of colonic polyposis) and extracolonic phenotypical manifestations of FAP, as well as development of desmoid tumors (10–15% of cases), are related to the location and type (frameshifts and point mutations) of the *APC* gene mutation.[11] A great deal of research has been devoted to the apparent genotype–phenotype correlations for the disease, although due to the variation that occurs among individuals and families with identical mutations in the *APC* gene,[12] these associations are not routinely used as a basis for clinical management strategies.[13] The most common mutation in *APC* is located at codon 1309, and patients with this mutation develop a high number of adenomas at an early age, with an average age at presentation of 20 years. Individuals with mutations in codons 1250–1464 often exhibit profuse polyposis (an average of 5000 polyps).[14] In terms of age at onset of colonic symptoms, individuals with mutations between codon 168 and 1580 present at an average age of 30 years, and individuals with mutations proximal to codon 168 and distal to codon 1580 present at an average age of 52 years.[13] Attenuated familial adenomatous polyposis is most commonly associated with *APC* mutations proximal to codon 168, although germline mutations in exons 6 and 9 as well as in the distal 3′ region of the gene have also been identified in AFAP families.[15] The extracolonic manifestations of FAP also show genotype–phenotype correlations. Desmoid tumors are often associated with mutations at codons 1444 and 1578.[16] Congenital hypertrophy of the retinal pigment epithelium is associated with mutations in codons 463–1387,[17] whereas mutations distal to codon 1444 or before exon 9 are associated with the absence of CHRPE.[16] Mutations downstream from codon 1051 are associated with increased risk of malignant transformation of periampullary adenomas. A retrospective study of 190 patients with FAP showed that the ''Gardner'' phenotype, which includes desmoid tumors, osteomas, and epidermoid cysts, is most often associated with mutations in codons 1395–1493, and that hepatoblastoma and brain cancer were seen only in patients with mutations in codons 457–1309.[18] With recent advances in molecular genetics, these genotype–phenotype correlations may eventually influence the accuracy and effectiveness of genetic testing, screening recommendations, and treatment.

CLINICAL DIAGNOSIS AND GENETIC TESTING

Although the diagnosis of FAP relies primarily upon clinical findings in symptomatic individuals, genetic testing is most often used in the early diagnosis of at-risk family members and in the confirmation of the diagnosis of FAP in patients with equivocal findings. Genetic testing is typically run on DNA extracted from white blood cells obtained from a blood sample. Several DNA-based techniques are available for the detection of *APC* mutations in FAP families (Table 1),[19] including full gene sequencing, mutation scanning by conformation-sensitive gel electrophoresis (CGSE) of exons 1–14 combined with protein truncation testing (PTT) of exon 15, PTT alone, and linkage analysis. Full gene sequencing has the highest estimated mutation detection rate of approximately 95%. Sequence confirmation of familial mutations identified by any technique other than full gene sequencing is highly recommended if the result will be used for predictive testing of at-risk family members. If germline testing reveals a mutation in an individual affected with FAP, the clinical diagnosis is confirmed, and genetic testing of at-risk family members will provide true

Table 1 Molecular genetic testing for FAP

Direct full gene sequencing: Detects APC sequence alteration. Most precise method for mutation detection (~95% sensitivity). Time consuming and expensive
Single-strand conformational polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE): Rely on difference in the movement of a mutated DNA compared with normal DNA during electrophoresis
The in vitro synthesized protein assay (IVSP), also called protein truncation test (PTT): Detects premature truncation of the APC protein. It is the main test for FAP (~80% sensitive)
Confirmation sensitive gel electrophoresis (CSGE) combined with protein truncation testing (PTT): Detects APC sequence alteration and premature truncation of the APC protein (~80–90% sensitive)

positive or negative results. Ultimately, if a mutation cannot be identified, further testing of at-risk relatives should not be pursued, because testing will not be conclusive; a negative result could be a false negative because current testing may not be capable of detecting a mutation even if present. Although it is possible to initiate germline testing with an at-risk family member when an affected family member is not available for testing, this strategy can only yield positive or inconclusive results. In this situation, only the identification of a mutation in a family member can enable a true negative result. Genetic testing should always be done in the setting of pre- and post-test genetic counseling, as endorsed by the American Society of Clinical Oncology in its position statement on genetic testing for hereditary cancer.[20] The process of genetic counseling includes providing risk assessment, educating patients about testing options, discussing the implications for medical management, and providing supportive counseling to the individual and their family. Genetic counseling for FAP should be offered to any patient that has personal or family history features suggestive of FAP, and genetic testing should be offered when results could influence the medical management of that patient or the patient's family members.

As colon screening for those at risk for classic FAP begins as early as age 10, genetic testing is offered to children in FAP families as early as age 8. In AFAP families, colon screening for at risk individuals begins at age 18 years, so genetic testing is generally offered at around 18 years of age. Age-appropriate education should be provided to at-risk children during the genetic counseling process, and children should be involved in the decision whether to undergo genetic testing.

SURVEILLANCE

Examination of the funds at birth to detect CHRPE can identify susceptible family members at young age. In family members of a FAP-positive patient, sigmoidoscopy is recommended annually starting at age 10–12 years to age 24 years, every 2 years until age 34 years, every 3 years until age 44 years and every 3–5 years thereafter. While a true negative genetic test is considered conclusive, some centers recommend interval flexible sigmoidoscopy around the ages of 18, 25, 35, and then per standard American Cancer Society guidelines, if an at-risk individual tests negative.[21] If the genetic test was indeterminate, at-risk individuals should undergo screening sigmoidoscopy every year from 10 to 25 years of age, biannually from 26 to 35, and every 3 years from 36 to 50, and then per ACS guidelines.[21] After colectomy has been performed most authors recommend screening the retained rectum every 6 months or the intestinal pouch every 1–3 years.[22] Screening for AFAP falls under similar guidelines, although screening is usually initiated at a later date, includes upper endoscopy for the gastric polyposis, and requires total colonoscopy because of the predilection for proximal lesions.[1,7]

Screening for extracolonic malignancy has proven less fruitful. Unlike colorectal cancer screening, there has been no reported evidence that early screening or therapy has improved the prognosis of duodenal and periampullary adenomatous disease.[23] Nevertheless, screening for ampullary tumors is also recommended with upper endoscopy every 1–3 years, beginning at age 20–25 years. Screening should be performed with both forward and side viewing scopes with random biopsies taken. In regard to other common extracolonic tumors, annual physical exam is recommended for thyroid disease.[21] Hepatoblastoma screening includes annual liver ultrasound with alpha-fetoprotein level until the age of 6 in patients diagnosed with FAP in infancy.[1]

CHEMOPREVENTION

The discovery of cyclooxygenase enzyme (COX1, COX2) overexpression in colorectal polyps and cancer has led to numerous studies evaluating nonsteroidal anti-inflammatory drugs (NSAiDs) in the treatment of FAP. Numerous studies involving *Sulindac* have shown a decrease in size and number of colorectal polyps (65% and 56%, respectively) in patients with FAP after several months of therapy.[24] Nevertheless, growth resumes as soon as therapy is stopped and there have been no studies to determine the safety of long-term *Sulindac* therapy. There have also been reported cases of colorectal cancer occurring while on *Sulindac* therapy. Studies are now underway to determine whether *Sulindac sulfone*, a metabolite of *Sulindac* without significant COX inhibition, will provide chemoprevention without significant side effects. In 1999, the Food and Drug Administration granted accelerated approval of *Celecoxib* for treatment of colorectal polyposis in FAP based on a 6-month trial that showed reduction in the number of polyps by 28% and the sum of poly diameter by 30.7% from baseline.[25] While these studies are encouraging there remain no long-term prospective studies showing improved prognosis with NSAiD therapy. Furthermore, there have been no studies that suggest that periampulary and duodenal disease responds to either of these two medications.[24]

SURGICAL MANAGEMENT

Once colorectal polyps are found, the only effective management is total proctocolectomy in FAP. Because

colorectal cancer is diagnosed in 5% of FAP patients by 20 years of age, surgery should be recommended at the time of diagnosis. Surgical options for colonic disease include total colectomy with ileorectal anastomosis (IRA), proctocolectomy with ileal pouch-anal anastomosis (IPAA), total proctocolectomy with Brook ileostomy, and total proctocolectomy and continent ileostomy (Koch). It should be noted that for patients with AFAP, IRA is an appropriate therapy.[7]

Duodenal and periampullary disease poses a much more challenging therapeutic dilemma. Endoscopic ablation via snare, argon plasma coagulation therapy, and laser photodynamic therapy are being attempted for mild to moderate disease; however, they also carry significant opportunity for morbidity and are often difficult to tolerate. As only a small proportion of these patients develop carcinoma, it becomes difficult to justify prophylactic resection. This decision is further complicated by the associated significant morbidity of pancreaticoduodenectomy with questionable improvement on prognosis.[23] Consequently, these options are reserved for diagnosed cancer or severe dysplasia.

CONCLUSION

Familial adenomatous polyposis exemplifies the classic hereditary cancer syndrome with Mendelian distribution. Its epidemiology reveals a rare disorder with high cancer-related mortality. Its clinical features are hallmarked by synchronous and metachronous tumor growth at an early age. Understanding the epidemiology, clinical characteristics, and genetics of FAP affords not only evidence-based preventive measures, but also allows for a better appreciation of the behavior of all hereditary cancer syndromes. Screening protocols are available, but not standardized and imperfect. As timely surgery on the primary organ has impacted mortality, the cancer-related deaths associated with extra primary sites continue to pose therapeutic dilemmas. Recent advances in molecular genetics and continued research in its genetic profile exposed an increased genotype–phenotype correlation, which may eventually influence the choice, accuracy, and effectiveness of genetic testing, screening recommendations, and chemoprevention strategies.

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Fanconi Anemia

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Hans Joenje
Gerard Pals
Christian Michel Zwaan
VU University Medical Center, Amsterdam, The Netherlands

INTRODUCTION

Fanconi anemia (FA) is a cancer-prone chromosomal instability disorder with autosomal recessive inheritance. Cells derived from FA patients are hypersensitive to chromosomal breakage by DNA cross-linking agents such as mitomycin C or diepoxybutane, which is used to confirm a clinical suspicion of FA. At least eleven genetic subtypes are distinguished. Eight of these have been connected to distinct disease genes, including the breast cancer susceptibility gene, *BRCA2*. Most FA genes encode orphan proteins with no known molecular function. They are supposed to act in an integrated pathway with BRCA1 and BRCA2 to maintain the integrity of the genome. Molecular diagnosis is feasible in >95% of all cytogenetically confirmed FA patients.

FA: CLINICAL ASPECTS

Fanconi anemia is a rare syndrome (ca. 10 per million newborns) with autosomal recessive inheritance. Clinical symptoms include multiple developmental abnormalities, bone marrow failure, and a high risk of malignancies (Table 1). Clinical suspicion of FA is mostly based on growth delay, skeletal abnormalities (aplastic or hypoplastic thumbs and radii), in combination with bone marrow failure (pancytopenia), which usually starts between 5 and 10 years of age.[1,2] However, the symptoms are highly variable. Therefore, the diagnosis should always be verified using cytogenetic or molecular techniques. Hematopoietic mosaicism occurs in a proportion of patients because of genetic reversion at the disease locus in certain primitive progenitor cells, which may (partially) correct the bone marrow defect and in some cases may lead to an incorrect diagnosis.[3] The bone marrow failure is usually progressive. Most patients die prematurely from the consequences of aplastic anemia, leukemia, or solid tumors.

Some aplastic FA patients may respond to treatment with androgens or steroids. Transplantation with bone marrow stem cells from a related or unrelated donor is currently the only way to correct the marrow failure and reduce the risk of leukemia. Because of their hypersensitivity to cyclophosphamide, which is used in the conditioning regimen for transplants, transplantation centers follow an attenuated protocol for FA patients. Gene therapy trials aimed at correcting the genetic defect in hematopoietic stem cells are ongoing. Like bone marrow transplantation, this treatment could only repair the bone marrow defect and is not expected to change the risk of solid tumors.

GENETIC AND MOLECULAR BASIS OF FA

Fanconi anemia is highly heterogeneous, not only at the clinical level, but also at the genetic level. Laboratory studies using cell lines from 241 unrelated FA patients have revealed as many as 11 distinct ''complementation groups'' or genetic subtypes.[4] For 8 of these groups the disease gene has been identified (Table 2). *FANCC*,

Table 1 Clinical symptoms associated with FA

Congenital abnormalities[a]
Skeleton: Aplastic or hypoplastic radii and thumbs, short stature, small head.
Kidneys: Ectopic, horseshoe.
Other: Heart abnormalities, intestinal atresia, microphthalmia, hypogonadism, abnormal shape of the ears, deafness, skin pigmentation abnormalities.
Hematopoiesis
Bone marrow failure or aplastic anemia occurs typically at 5–10 years.
Cancer risk
Increased risk of AML, mostly occurring at age 5–15 years. At older ages, there is an increased risk of head and neck or esophageal cancer, as well as, in females, cancer of the vulva and vagina.
FA-D1 patients (with biallelic mutations in *BRCA2*) develop malignancies at a much earlier age (15–17 years).
Other symptoms
Endocrinopathy affecting the pancreas (diabetes mellitus) and thyroid; reduced fertility and early menopause.

[a]Congenital anomalies may be absent altogether.

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Table 2 FA subtypes and genes

Subtype	Relative prevalence[a] (%)	Defective gene[b]	Location	Protein (amino acids)	Domain structure
A	66.0	*FANCA*	16q24.3	1455	—
B	0.8	*FANCB*[c]	?	?	?
C	9.6	*FANCC*	9q22.3	558	—
D1[d]	3.3	*BRCA2/FANCD1*	13q12.3	3418	Rad51- and DNA-binding motifs[28]
D2	3.3	*FANCD2*	3p25.3	1451	—
E	2.5	*FANCE*	6p21.3	536	—
F	2.1	*FANCF*	11p15	374	—
G	8.8	*XRCC9/FANCG*	9p13	622	Tetratricopeptide repeats (TPR)[11]
I	1.6	*FANCI*[c]	?	?	?
J[d]	1.6	*FANCJ*[c]	?	?	?
L	0.4	*FANCL*	2p16.1	375	PHD-type zinc finger motif[9,12]

[a]Based on Ref. [4].
[b]For nomenclature rules of FA gene symbols, see http://www.gene.ucl.ac.uk/nomenclature/genefamily/fanconi.html.
[c]These genes remain to be identified.
[d]The proteins defective in these groups act downstream of the monoubiquitination of FANCD2; all the other FA proteins listed act upstream.

-A, *-G*, *-F* and *-E* were identified by complementation cloning;[2,5] *FANCA* was independently found by linkage analysis and positional cloning[6] *FANCD2* by microcell-mediated chromosome transfer,[7] and *BRCA2* by a candidate-gene approach.[8] *FANCL* was recently found through protein association studies.[9]

Most FA proteins assemble into a nuclear multiprotein ''core complex,'' which is required to catalyze a key reaction in the FA pathway: the activation of FANCD2 by monoubiquitination at position K561.[10] An important role for the assembly and/or stabilization of this complex has been assigned to FANCG, which belongs to the family of TPR-containing proteins.[11] FANCL, which carries a plant homology domain (PHD), is a candidate for acting as the ubiquitin E3 ligase in the monoubiquitination of FANCD2.[9,12] Monoubiquitination of FANCD2 is essential for the entire FA pathway because it allows FANCD2 to move into areas of damaged chromatin and to interact with BRCA1, resulting in repair of the damage.[10] BRCA2 and FANCJ are so far the only components of the pathway that act downstream (or independent) of the FANCD2 activation step.[4] The exact nature of the DNA damage response, which when defective causes FA, remains to be defined.

GENETIC COUNSELING

The recurrence risk is as for other autosomal recessive diseases, i.e., 25%. DNA diagnosis is feasible in >95% of the patients (Table 1), allowing early prenatal diagnosis as well as preimplantation genetic screening. As outlined below, prognosis may vary depending on the disease gene and mutations.

GENOTYPE–PHENOTYPE CORRELATIONS

There is wide variability of clinical symptoms, even within complementation groups. The type of mutation in a given FA gene seems to be of more importance for the clinical outcome than the complementation group itself.[13,14] An exception is group D1 (mutated in *BRCA2*, Ref. [8]), which as a group seems to be more severely affected than the non-D1 groups.[15–17] Within group C, the splice site mutation IVS4 + 4A > T, which accounts for over 80% of all FA in Ashkenazi Jewish people,[18] is much more severe than the exon 1 frame-shift mutation 322delG, which is relatively prevalent in the Netherlands.[19] Within group A, null mutations are significantly more severe than missense mutations or microdeletions/insertions that still allow near full-length protein to be made.[13] Group G patients seem to be more severely affected than group A/C patients.[13] There is as yet no definite evidence for any health effects for heterozygous FA gene mutation carriers.

THE DIAGNOSIS ''FANCONI ANEMIA''

Clinical suspicion of FA always needs to be confirmed by a chromosomal breakage test[20] to exclude syndromes

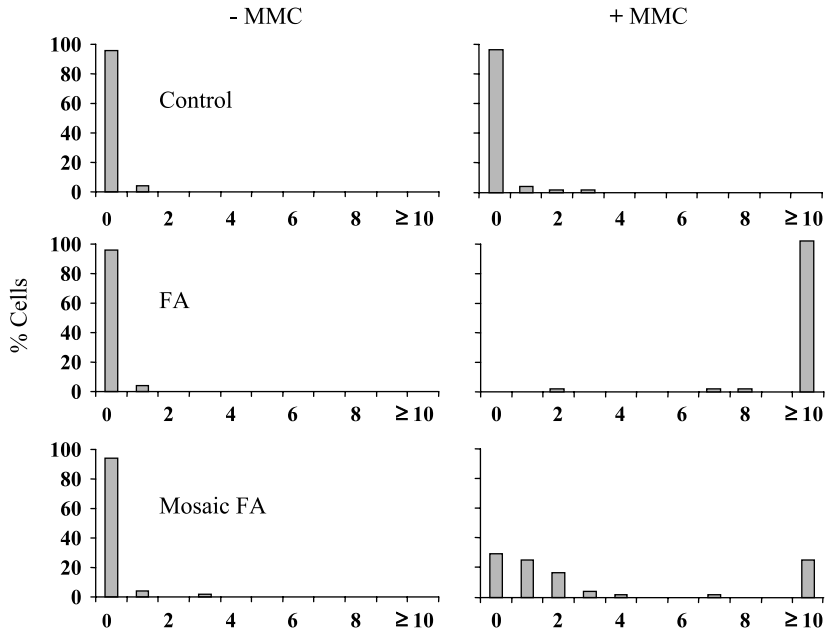

Fig. 1 Mitomycin C (MMC)-induced chromosomal breakage in phytohemagglutinin-stimulated T-lymphocyte cultures distinguishes FA (middle) from non-FA individuals (upper chart). The lower chart represents a mosaic FA patient, showing two types of lymphocytes, one responding as FA and one responding as the healthy control. (*View this art in color at www.dekker.com.*)

with overlapping clinical symptoms. In this test, T lymphocytes in a peripheral blood sample are stimulated to divide and cultured for 3 days in the presence of various concentrations of a cross-linking agent (mitomycin C or diepoxybutane), after which chromosomal aberrations (chromatid breaks and chromosomal interchange figures) are quantified in metaphase spreads. Figure 1 shows results obtained for three individuals, a healthy control, an FA patient, and an FA patient with lymphocyte mosaicism. The latter patient has two populations of T lymphocytes in roughly equal proportions: cells that respond as FA next to cells that respond as healthy control cells. The latter cells, which have originated by spontaneous reversion of one pathogenic allele at the disease locus, tend to increase with time. Therefore, some mosaic FA patients may score negative for the FA diagnosis in this test. Such patients may still be diagnosed by using skin fibroblasts, in which mosaicism has thus far not been noted.[3]

The chromosomal breakage test, originally developed by Auerbach et al.,[20] has been used for both postnatal and prenatal diagnoses and is still considered the standard test for diagnosing all genetic subtypes of FA. However, the test does not seem to distinguish FA from Nijmegen breakage syndrome,[21] which is why further testing is recommended by mutation screening (see below).

Alternative Tests

An alternative way to confirm FA is based on cell cycle analysis using flow cytometry. This test is based on the observation that FA cells (T lymphocytes or skin fibroblasts) tend to become arrested in the G2 phase of the cell cycle.[22] However, this test may fail to diagnose cases with overt leukemia or high levels of lymphocyte mosaicism.

Recently, FANCD2 western blotting has been reported as an alternative procedure to diagnose FA.[23] In this case, growth-stimulated T lymphocytes are tested for the occurrence of the ubiquitinated isoform of FANCD2, which readily reveals FA in cases where this isoform is not detected. This is a very convenient alternative for diagnosing 90% of all FA patients. However, the subtypes FA-D1 and -J, which are defective downstream of FANCD2 ubiquitination, are not diagnosed. In addition, true FA cases with a significant level of lymphocyte mosaicism may not be diagnosed as FA. In negative but still highly suspicious cases, the diagnostic workup may be extended to include additional tests, such as chromosomal breakage analysis in lymphocytes or fibroblasts.[20,24]

MOLECULAR DIAGNOSIS OF FA PATIENTS

Once the diagnosis FA has been established cytogenetically, molecular testing should demonstrate pathogenic mutations in a FA gene. This can be a laborious task, because most of the mutations are private. The following strategy has been developed at our DNA diagnostics laboratory to provide a clinically certified molecular diagnosis of FA. It is recognized that mutations in *FANCA*

account for almost 70% of all FA cases and that about 40% of the mutations in this gene are large deletions; therefore, the search usually starts with a screen for deletions in *FANCA*. However, depending on the circumstances, strategies may differ from case to case, as outlined below.

1. If information is available about the origin or ancestry of the patient a direct test for the following relatively common mutations may provide a shortcut for the classification of the patient:
   FANCA: 1263delF occurs in about 5% of all FA-A cases, whereas Exon11–17del and Exon12–31del occur relatively frequently in Afrikaans-speaking people in South Africa (cited in Ref. [2]).
   FANCC: IVS4 + 4A > T occurs homozygously in 80% of FA patients of Ashkenazi Jewish ancestry[18] and is associated with a severe phenotype. The exon 1 frameshift 322delG, associated with a relatively mild phenotype, is homozygously present in about 50% of FA cases in the Netherlands.[13,19]
   FANCG: L105X is relatively common in FA-G cases in Germany.[25]
2. In the absence of any clue, systematic mutation screening should start with a search for deletions in *FANCA*, because this gene is most often mutated, 40% of all mutations being large deletions. The quantitative multiplex ligation-dependent probe amplification (MLPA) method[26] is used for this initial screen.
3. If negative by MLPA, HPLC under denaturing conditions, followed by direct sequencing of aberrant fragments, is used to screen for mutations in *FANCA*, *-C*, *-E*, *-F*, *-G*, and *-L* simultaneously. Minor sequence variations, such as missense mutations or in-frame microdeletions or -insertions, should be tested for pathogenicity in a cellular transfection assay to check the capacity of the variant gene product for complementing activity (e.g., Ref. [27]).
4. If negative again, *FANCD2* is sequenced. This is done at the level of cDNA, because pseudogenes complicate the interpretation of genome-derived sequences. Seemingly minor alterations should be tested for pathogenicity in a transfection assay.
5. If again negative, *BRCA2* is screened by denaturing gradient gel electrophoresis (DGGE) and sequencing. Minor alterations should be tested for pathogenicity by a complementation test.
6. If negative again, cells from the patient should be tested for their ability to carry out FANCD2 monoubiquitination.[23] If negative, the patient might be defective in *FANCB* or *FANCI*. If positive, the patient may belong to group FA-J or to a new complementation group that is defective downstream of FANCD2 ubiquitination. However, before the latter conclusion can be drawn, *NBS1* should be screened for mutations to rule out Nijmegen breakage syndrome.[21]
7. If the patient has been excluded from all groups for which the disease gene is known, further work to assign the patient to a complementation group (B, I, J, or a new group) is undertaken on a research basis.

CONCLUSION

The genetic basis of FA has been largely resolved. Over 95% of all FA patients have biallelic mutations in one of at least eight disease genes, including *BRCA2*. These genes control a molecular pathway that serves to maintain the integrity of the genome. At least three additional FA genes remain to be identified. The challenge for the immediate future is to clone the missing FA genes and to further define the FA pathway in molecular terms.

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FISH—An Overview

Yao-Shan Fan
University of Miami School of Medicine, Miami, Florida, U.S.A.

INTRODUCTION

Many new techniques of fluorescence in situ hybridization (FISH) have been developed in the past two decades and they are now widely utilized for clinical diagnosis.

FISH TECHNIQUES

The detailed protocols and their applications of the FISH techniques have been described in a recently published book entitled ''Molecular Cytogenetics: Protocols and Applications.''[1]

FISH with Unique DNA Sequences

FISH with unique DNA sequences represents the most basic technique. The DNA segment used as a probe may represent a functional gene or a particular chromosome region or locus. The basic steps of FISH procedure include labeling of DNA probes, preparing interphase or metaphase chromosome slides, in situ hybridization, and visualization with a fluorescent microscope.[2]

Chromosome Painting

Chromosome painting refers to a FISH procedure using probes generated from specific chromosome libraries, Alu and L1 PCR or chromosome microdissection.[3] When the probe contains unique and repetitive sequences from an entire chromosome, whole chromosomes of a homologous pair in metaphases are illuminated with fluorescence (painted). The short or long arm of a particular chromosome can be painted with an arm-specific probe. Using a device containing a 3×8 array, all 24 chromosomes can be painted simultaneously and detected sequentially on a single slide using a standard fluorescent microscope.

Spectral Karyotyping

Spectral karyotyping (SKY) is an automated chromosome painting procedure using a probe mixture containing representative DNA for each of the 24 human chromosomes.[4] The probes are directly labeled with the combinations of five fluorochromes. Through computer classification of the spectra, all 24 human chromosomes can be simultaneously visualized in different colors. SKY has been proven to be a powerful tool for the characterization of complex chromosomal rearrangements in cancer cells and de novo constitutional structural abnormalities. The sensitivity of SKY to visualize DNA alterations is about 1–2 Mb in size.[5]

Multiple Color FISH

Multiple color FISH (M-FISH) is also called multifluor FISH and multiplex FISH.[6] Similar to SKY, the probes are labeled with the combinations of multiple fluorochromes. In contrast to spectral analysis, 24 chromosomes in unique colors are detected by a series of fluorochrome-specific filters with the assistance of computer software.

FISH Following Microdissection (MicroFISH)

To perform MicroFISH, a whole chromosome, a marker, or a particular chromosome band is scraped from metaphase spreads using a micromanipulator.[7] The scraped DNA is amplified by polymerase chain reaction (PCR) and labeled as probes. FISH of such probes with normal reference metaphase chromosomes reveals the composition of chromosomes or the chromosome regions in question. This technique is useful for characterizing structural rearrangements and marker chromosomes. It is also a tool to produce specific painting probes for individual chromosomes, chromosomal arms, or regions.

Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) compares test DNA with normal reference DNA.[8] The test DNA is traditionally labeled with a green color and the reference DNA with a red color. The DNA mixture is then hybridized to normal metaphase chromosomes prepared from a blood culture. By measuring the ratio of green to red color, gains or losses of chromosomes or chromosomal regions in the test DNA can be detected. The size of DNA segments that CGH can detect was estimated to be in the range of 10–20 Mb. CGH has been widely used in the investigations of solid tumors. CGH is also

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useful in the characterization of de novo unbalanced structural abnormalities.

Primed In Situ Labeling

Primed in situ labeling (PRINS) refers to a process of reannealing the short oligonucleotide primers to target sequences in situ, and followed by elongation of the sequences with a *Taq* polymerase and simultaneous labeling of the target sequences with a fluorochrome.[9] PRINS primarily targets short stretches of α-satellite DNA unique to each chromosome. This technique has been used as an efficient alternative tool to detect aneuploidies.

Fiber FISH

This is a procedure of hybridization of DNA probes to extended chromatin fibers (i.e., free chromatin released from lysed cells) on a microscope slide.[10] A modified method is to hybridize probes to unfixed DNA fibers prepared from cells embedded in pulsed-field gel electrophoresis (PFGE) blocks. Fiber FISH has been used for high-resolution gene mapping, and for direct visualization of gene duplication and chromosome breakpoints involved in translocations.

Color Banding

This technique is also called cross-species FISH (Rx FISH) because it utilizes DNA obtained from flow-sorted gibbon chromosomes by PCR amplification.[11] The genome of gibbons has a high degree of homology with human DNA but with extensively rearranged chromosomes. When hybridized with a set of gibbon DNA probes labeled with a combination of FITC, Cy3, and Cy5, human metaphase chromosomes show a distinctive color banding pattern. A high-resolution multicolor banding can also be achieved by using the microdissection-based probes.[12]

Multitelomere FISH

The technique of multitelomere FISH utilizes a device containing 41 subtelomeric probes for all 24 different chromosomes (not including the short arms of acrocentrics).[13] The probes for the short arms and the long arms are dual-labeled with a green and a red fluorochrome, respectively. This allows the detection of submicroscopic deletions or translocations in all subtelomeric regions with a single hybridization.

Simultaneous Immunophenotyping and FISH

This is a simultaneous analysis of cell surface immunologic markers and interphase FISH. The strategy of combining immunophenotyping with FISH enables correlation of chromosome aberrations of interest with cell lineage and differentiation stages of tumor cells, and therefore provides a useful tool for studies of leukemia and lymphomas.[14]

APPLICATIONS

Interphase FISH for Prenatal Diagnosis

Aneuploidies of chromosomes 13, 18, 21, X, and Y account for about 95% of the chromosomal aberrations causing live-born birth defects. The currently used AneuVysion assay kit (Vysis, Downers Grove, IL) includes two sets of multicolor probe mixtures, one for chromosomes 13 and 21, and the other for chromosomes 18, X, and Y. The standardized probes and protocols have been proven to be accurate and very sensitive for prenatal diagnosis of the most common aneuploidies. This technique is particularly valuable for high-risk pregnancies, as indicated by ultrasonography or maternal serum screening.[15]

Prenatal Diagnosis Using Maternal Blood

Fetal nucleated red blood cells passing into the maternal circulation during pregnancy provide a cell source for noninvasive prenatal genetic diagnosis. Analysis of fetal cells by FISH requires relatively pure samples of fetal cells isolated from maternal blood.[16] Many methods including density gradient centrifugation, magnetic activated cell sorting, fetal cell culture, and immunocytochemical staining have been developed for the isolation, enrichment, and identification of fetal cells. An approach of combined cell sorting, immunophenotyping, and FISH appears to improve the sensitivity and specificity of the methods, and thus offers new promise to the future of noninvasive prenatal genetic testing.

Preimplantation Diagnosis of the Common Aneuploidies

A large proportion of patients undergoing in vitro fertilization (IVF) are at the age of 35 years. It was estimated in this group that about 50% of embryos are chromosomally abnormal with aneuploidy being the major contributor. The aneuploidies can be detected by FISH analysis on the first and/or second polar bodies removed from oocytes following their maturation and fertilization. DNA probes for chromosomes 13, 18, and 21 have been used most commonly for FISH studies on polar bodies. Preimplantation diagnosis of aneuploidies

has provided an accurate and reliable approach for the prevention of age-related aneuploidies in IVF patients with an advanced maternal age.[17] Selecting embryos with a normal chromosome complement can also improve the implantation rate in patients with advanced age and in carriers of an altered karyotype.

Studies of Mosaicism

Constitutional mosaicism is the result of postfertilization mitotic error, i.e., a somatic event. Two types of mosaicism, i.e., meiotic vs. somatic, have been defined by molecular studies in determining the origin of the extra chromosome in the trisomic cell line. While meiotic mosaicism refers to the occurrence of a mitotic error producing a diploid cell line in a trisomic conception, somatic mosaicism means a trisomic cell line occurred in a conception that was initially diploid. A generalized mosaicism involves all cell lineages of the conceptus including both the placenta and the embryonic/fetal tissues. A mosaicism occurring only in the placenta is called confined placenta mosaicism. Studies using FISH, CGH, and other molecular techniques have facilitated our understanding of the biological and clinical significance of chromosomal mosaicism in early embryonic/fetal development.[18]

Diagnosis of Microdeletion Syndromes

More than 30 microdeletion syndromes have been described in the past two decades.[19] 22q11.2 deletion, Williams, and Prader-Willi/Angelman are the most common microdeletion syndromes. These syndromes are usually caused by a deletion of a 2- to 4-Mb DNA sequence. The overall incidence of microdeletion syndromes is likely in the range of 1/1000–2000 newborns. FISH using probes for the genes involved in the deletions is necessary for diagnosis of these syndromes. Studies of microdeletion syndromes have led to the understanding of uniparental disomy and genomic imprinting. A combined diagnostic approach has been used for Prader-Willi and Angelman syndromes, i.e., conventional karyotyping to exclude structural abnormalities, FISH to detect microdeletion, and DNA testing for uniparental disomy and mutation.[20]

Detection of Subtelomeric Aberrations in Unexplained Mental Retardation

Genomic alterations in the subtelomeric regions appear to be an important cause of developmental disabilities. In recent studies, subtelomeric aberrations have been detected in 4–6% of the patients with unexplained mental retardation and dysmorphic features or multiple malformations.[21] The technique of multitelomere FISH is considered to be a valuable tool for a definitive diagnosis for such patients.

Identification of Structural Chromosomal Abnormalities

The incidence of constitutional structural abnormalities is approximately 1/200 at birth when chromosomes are analyzed at the level of resolution of 400 bands. Unbalanced de novo abnormalities are present in 1/1000 live birth children, and FISH analysis is necessary for most of these abnormalities.[22] Numerous acquired aberrations leading to gains or losses of chromosomal material have been described in human cancer. It is important to know whether or not a particular chromosomal region or a particular gene is involved in a chromosomal aberration so that a correct clinical diagnosis can be made. Chromosome painting, microdissection, SKY, M-FISH, CGH, and color banding have all been utilized in the studies of structural abnormalities in conjunction with G-banding analysis.

Detection of Specific Gene Rearrangements in Cancer

Over 100 recurrent chromosomal translocations have been identified in human malignant disorders, and many of these translocations involve specific gene rearrangements. The identification of a specific gene rearrangement is not only diagnostic but also important for determining a therapy plan, monitoring treatment, and predicting prognosis. Interphase FISH is highly sensitive in detecting specific gene rearrangements in leukemia and lymphoma, and has shown many advantages over the standard PCR method. Specific gene rearrangements in tumors can be detected by dual color interphase FISH in paraffin-embedded tumor tissues.[23]

Analysis of Gains and Losses of Chromosomal Regions in Tumor

With the interphase FISH approach, chromosomal aneuploidies can be detected in virtually any given tissue or cell source such as touch preparations, sections of frozen tumor, and paraffin-embedded tissue. Similarly, almost all types of clinical specimens can be used for CGH studies of tumors. Many reports have shown gains or losses of

individual chromosomes or chromosome regions correlating with particular tumors, different stages of the tumor, and the prognosis of patients. These studies have yielded extremely important information for our understanding of the biological behavior of tumors.

Detection of Gene Deletion and Amplifications in Tumor Cells

Deletion of tumor suppressor genes such as p53 and RB-1 and amplification of oncogenes such as N-*myc*, C-*myc*, and HER-2/neu can be detected by FISH or CGH studies of tumor tissues. FISH has advantages over Southern blot analysis in terms of speed, technical simplicity, ability to discern heterogeneous gene amplification among tumor cells in the same specimen, and capacity to determine the source of the amplified gene signals. Amplification of C-*myc* has been detected by FISH in many different types of tumors, including medulloblastoma, malignant melanoma, lung cancer, nasopharyngeal carcinoma, ovarian cancer, and prostate cancer. Testing of the HER-2/neu gene in breast cancer has become very important for patient management because of its association with an aggressive disease that can be successfully treated with Herceptin. A combined immunohistochemistry and FISH approach has made the test more sensitive and cost-effective.[24]

CONCLUSION

FISH has been considered a highly accurate test for the diagnosis of microdeletions and for the identification of unknown material in the genome. Using interphase FISH, genomic alterations can be studied in virtually all types of human tissues at any stage of cell division, without the need of cell culture and chromosome preparation. FISH techniques have served as an indispensable diagnostic tool in many areas of medical practice, including medical genetics, maternal–fetal medicine, pediatrics, reproductive medicine, pathology, hematology, oncology, and psychiatrics.

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FISH—Banding Methods and Clinical Applications

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Thomas Liehr
Institut für Humangenetik und Anthropologie der FSU Jena, Jena, Germany

INTRODUCTION

Since their establishment in routine cytogenetics in the mid 1990s, multicolor fluorescence in situ hybridization (FISH) approaches have become indispensable for the identification and description of chromosomal rearrangements. The subsequent exact characterization of chromosomal breakpoints is of superior clinical impact and is the requisite condition for further molecular investigations aimed at the identification of disease-related genes. Thus, various approaches for a differentiation of chromosomal subbands based on multiplex FISH (M-FISH) assays were established. Here an overview is presented on available M-FISH banding methods including their advantages, limitations, and possible applications.

DEFINITION OF FISH BANDING METHODS

Although the G-bands by trypsin using Giemsa (GTG) banding technique is still the starting point of all (molecular) cytogenetic techniques, its technical restrictions are well-known (e.g., only chromosome morphology combined with a black and white banding pattern can be evaluated; for further details, see Ref. [1]). Nonetheless, a quick and cheap overview on eventual changes within the human whole genome can be achieved by that cytogenetic banding approach.

M-FISH methods using all 24 human whole chromosome painting probes simultaneously—such as M-FISH, spectral karyotyping (SKY), combined ratio labeling FISH (COBRA-FISH), and others (reviewed in Refs. [2-4])—reach their limits in the identification of intrachromosomal rearrangements (such as small interstitial deletions, duplications, and inversions without change of the centromeric index) and when exact characterization of breakpoints is required.

These limitations were overcome by the development of new FISH banding methods during the last decade.

FISH banding methods are defined as ''any kind of FISH technique, which provides the possibility to characterize simultaneously several chromosomal subregions smaller than a chromosome arm (excluding the short arms of the acrocentric chromosomes).''[5] In contrast to the standard cytogenetic chromosome banding techniques, giving a protein-related banding pattern, the FISH banding techniques are DNA-mediated.

OVERVIEW ON AVAILABLE FISH BANDING METHODS

Five different FISH banding methods are available at present, which differ in their probe composition as well as in their banding resolution:

1. The cross-species color banding (Rx-FISH) or Harlequin FISH probe set provides the lowest resolution of 80–90 bands per haploid human karyotype and consists of flow-sorted gibbon chromosomes.[6] A set of 110 human–hamster somatic cell hybrids (split into two pools and labeled with two fluorochromes), when hybridized to human chromosomes, leads to about 100 ''bars'' along the genome. This pattern has been called ''somatic cell hybrid-based chromosome bar code.''[7] A combination of the latter and the aforementioned Rx-FISH probe set results in 160-chromosome region-specific DNA-mediated bands in human karyotypes.[7]
2. Spectral color banding (SCAN) was described exemplarily for one chromosome up to the present. Eight microdissection libraries were created along chromosome 10 with the goal to obtain a banding pattern similar to GTG banding at the 300-band level.[8]
3. A chromosome can be characterized as well by a specific signal pattern produced by yeast artificial chromosome (YAC) clones. The first attempts to label each chromosome by subregional DNA probes in different colors were performed by Lichter et al.[9] and Lengauer et al.[10] A YAC-based chromosome bar code was created especially for chromosome 12 but not for the entire human karyotype yet (for review, see Refs. [2] and [3]). A resolution of up to 400 bands can be achieved, depending on the number of the applied probes.

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4. The Interspersed PCR multiplex FISH (IPM-FISH) approach[11] has an approximate resolution of 400 bands per haploid karyotype, mainly dependent on chromosome quality. In IPM-FISH, whole chromosome painting probes are used, which are modified by interspersed PCR, leading to a 24-color FISH painting plus an R-band-like pattern.
5. The high-resolution multicolor banding (MCB) technique is based on overlapping microdissection libraries producing fluorescence profiles along the human chromosomes, and was initially described exemplarily for chromosome 5 in 1999.[12] MCB allows the differentiation of chromosome region-specific areas at the band and subband levels at a resolution of 550 bands per haploid karyotype. As the number of pseudocolored bands per chromosome can freely be assigned using the *isis* software (MetaSystems, Altlussheim, Germany), a resolution higher than that of GTG banding of the corresponding chromosome can be achieved (e.g., up to 10 MCB bands for chromosome 22 equal 800 bands per total haploid karyotype).[2] An MCB set of approximately 140 microdissection libraries covering the entire human genome was described in 2002.[13] A DNA-specific MCB pseudocolor banding pattern on normal human chromosomes is depicted in Fig. 1. A YAC/bacterial artificial chromosome (BAC)-based MCB set turned out to be of lower quality.[14] However, a combination of microdissection-based MCB probes sets with locus-specific or breakpoint-specific probes is very promising.[15] Recently, the simultaneous use of all human MCB libraries in one hybridization step for the characterization of complex karyotypes was performed.[16]

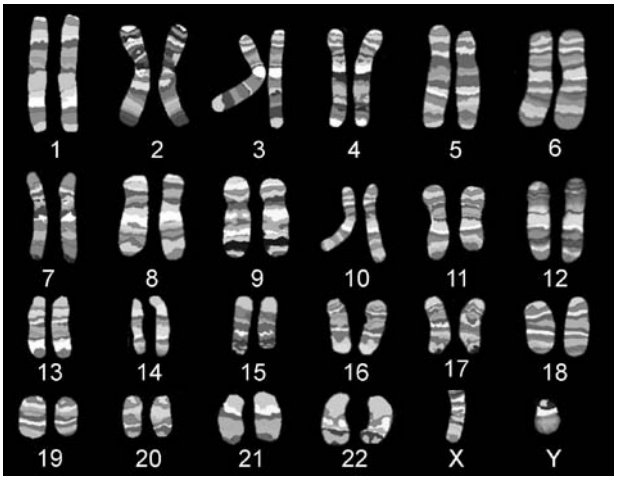

Fig. 1 MCB pseudo-color pattern for all 24 human chromosomes in a ~380-band level. Two homologue autosomes and one gonosome each are presented. The chromosomes depicted here have been put together from 24 different MCB experiments. (*View this art in color at www.dekker.com.*)

ADVANTAGES AND DISADVANTAGES OF FISH BANDING METHODS

FISH banding approaches such as the chromosome bar code technique using YACs, region-specific human–hamster somatic cell hybrids, or nonoverlapping microdissection libraries (SCAN) have, per definition, the disadvantage that unstained and noninformative gaps are left along the chromosome. Such gaps can cause problems, as breakpoints within the unstained gaps cannot exactly be described. Conversely, techniques based on locus-specific probes would theoretically provide the advantage that chromosomal breakpoints could be defined very exactly by the corresponding breakpoint-spanning or flanking clones. However, the coverage of the human genome by nonchimeric clones seems to be presently too low for such a molecular cytogenetic approach (Liehr et al., unpublished data). The IPM-FISH approach, Rx-FISH technique, Rx-FISH combined with somatic cell hybrids, and the MCB method provide the advantage of leaving no noninformative gaps.

Only FISH banding probe sets (1), (4), and (5) are finished for the whole human genome and can be applied to achieve comprehensive information in one single hybridization step. Concerning banding resolution, MCB has the highest and most flexible one. Additionally, according to the question to be studied, it can be chosen if only selected chromosomes or the whole genome shall be hybridized.

APPLICATIONS OF FISH BANDING METHODS

FISH banding methods were used successfully for research in evolution (Rx-FISH; MCB) and radiation biology (MCB), as well as for studies on the nuclear architecture (chromosome bar code; MCB). Moreover, their suitability for diagnostic purposes has been proven in prenatal, postnatal, and tumor cytogenetics (chromosome bar code; Rx-FISH; IPM-FISH, and MCB), indicating that they are an important tool with the potential to partly replace conventional banding techniques in the future (reviewed in Refs. [2], [4], and [5]). Most clinical cases were successfully analyzed by MCB followed by Rx-FISH, whereas the cases studied with the other three approaches are between 0 and <50.

CONCLUSION

The introduction of FISH banding methods was a great step forward for molecular cytogenetic diagnostics. However, none of the mentioned new methods can, for technical reasons, ever be fully informative for itself. The cytogeneticist always has to double check the results obtained in FISH banding with those achieved by other approaches, such as GTG banding, M-FISH or SKY, CGH, or others. Examples for that modus operandi can be found in the literature (e.g., FISH banding and microdissection;[17] FISH banding and CGH;[17–19] FISH banding and region-specific probes;[19–21] FISH banding and locus-specific probes.[5,14,15,22,23] Nevertheless, the goal must be to achieve fully informative cytogenetic results in a minimum of time and with a minimum of FISH experiments. Thus, further developments with respect to probe combinations (such as M-FISH/SKY with FISH banding methods) will be necessary. To perform and evaluate such complex experiments, which then will have to be based on up to nine fluorochromes, further technical developments in microscopy and computer software are necessary.

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FISH—Blood and Bone Marrow

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María D. Odero
Idoya Lahortiga
Isabel Zudaire
María J. Calasanz
University of Navarra, Pamplona, Spain

INTRODUCTION

The development of molecular hybridization techniques such as fluorescence in situ hybridization (FISH) has had a major impact in clinical research. At present, FISH is used routinely to identify recurring chromosomal abnormalities associated with hematological malignant diseases. The high sensitivity of this technique provides valuable tools not only for diagnosis, but also for follow-up and detection of minimal residual disease. The combination of G-banding, FISH, and molecular analyses has provided a powerful approach for classifying these malignant diseases into clinically and biologically relevant subgroups, allowing selecting the appropriate therapy, and monitoring the efficacy of therapeutic regimens. FISH not only provides an excellent aid for the clinical management of patients with cancer, but also constitutes a valuable tool for the characterization of chromosomal rearrangements and has been the first step to further molecular studies. Improvements of the FISH technique, including the development of multicolor FISH to detect several chromosomal changes in a single assay, and FICTION, a technique that permits to correlate phenotypic and genotypic cell features, show that FISH will continue having a major impact on efforts to characterize the genetic changes that give rise to human tumors.

OVERVIEW

In the last 30 years, information provided by cytogenetic analysis has become indispensable for the clinical management of patients with hematological malignancies. Metaphase chromosome analysis of bone marrow (BM) cells enables the entire genome to be screened for evidence of chromosomal changes, which provide the landmarks for the genes involved in leukemogenesis. Up to 80% of patients with acute lymphoblastic leukemia (ALL) and 70% of patients with acute myeloid leukemia (AML) have abnormal karyotypes. A large number of these chromosomal changes are recurrent and have been associated with specific morphological types in both myeloid and lymphoid lineages.[1] As a result, the finding of an abnormal cytogenetic clone may provide the definitive diagnosis. Besides, some chromosomal abnormalities are independent prognostic indicators. Certain karyotypes are associated with a good prognosis, whereas others indicate a poor outcome, leading to the administration of alternative therapies. One illustrative example is AML. In this leukemia, the favorable prognostic subgroup is defined by the presence of leukemic blasts with t(15;17), t(8;21), or inv(16). Unfavorable cases are those with abnormalities involving more than two chromosomes, monosomy 5/5q- or 7/7q-, or rearrangements of the long arm of chromosome 3. The survival rate of patients from this group is less than 20% at 5 years.[2] These patients represent a considerable therapeutic challenge for which no current treatment approach is satisfactory. The patients with a normal karyotype or cytogenetic abnormalities that are not included in these other categories are characterized as having an intermediate risk of relapse.

Conventional cytogenetic analysis is based on the study of metaphase chromosomes by banding techniques. Because it needs tumor metaphases to be analyzed, viable tumor cells have to be obtained from fresh material and cultured. Although highly precise, this technique requires skilled personnel and is time consuming and expensive. These factors have led investigators to seek alternative methods for identifying chromosomal abnormalities. The technique of fluorescence in situ hybridization (FISH) is one such method. At present, FISH is used largely to detect known chromosomal abnormalities using specific DNA probes. The high sensitivity and specificity of FISH, the speed with which the method can be accomplished (virtually overnight), and the ability to provide information from nondividing cells (interphase FISH) have made this molecular cytogenetic technique a powerful tool for genetic analysis.[3] The ability to examine nondividing cells or slowly cycling cells is particularly important in the analysis of some lymphoid neoplasias, in which cytogenetic analysis is often hampered by the small number or absence of mitotic cells. For instance, in chronic lymphocytic leukemia (CLL), G-banding analysis is informative in only a minority of cases, whereas FISH

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can detect chromosomal abnormalities in up to 80% of cases.[4] In a short time FISH, such as the G-banding analysis, has become a screening test to identify recurring chromosomal abnormalities associated with hematologic malignant diseases.

THE FISH TECHNIQUE

The FISH technique is based on the ability of single-stranded DNA to anneal to complementary DNA. In the case of FISH, the target DNA is the nuclear DNA of interphase cells or the DNA of metaphase chromosomes that are affixed to a glass microscope slide. FISH can be accomplished with bone marrow or peripheral blood smears, or other fixed tissue, and modifications of FISH methodology have also permitted the analysis of nuclei extracted from paraffin-embedded tissues or frozen sections from solid tumors. The test probe is labeled by enzymatic incorporation of biotin- or digoxigenin-labeled nucleotides, or directly with fluorochromes, to produce fluorescent signals at the place where the target is located within the nucleus. The use of directly labeled probes has simplified the technique eliminating the probe detection steps. These probes have proven to yield strong signals and are suitable for clinical applications. The cellular DNA and labeled probe DNA are denatured by heating in a formamide solution to form single-stranded DNA. A solution containing the probe DNA is applied to the microscope slide, the slide is covered with coverslips and sealed, and hybridization is allowed to occur by overnight incubation at 37–40°C. Thereafter, the unbound probe is removed by extensive washes, and the slides are processed for probe detection.[3] The slides are typically counter-stained with DNA-binding fluorochromes, such as propidium iodide or 4,6-diamidino-2-phenylindole (DAPI). DAPI staining induces a chromosomal banding pattern that is identical to G-banding; thus DAPI is preferred for the analysis of metaphase cells (Fig. 1).

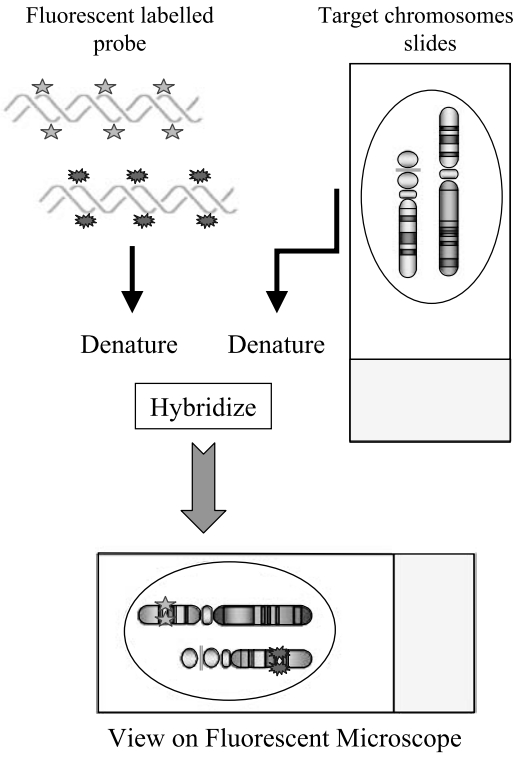

Fig. 1 Schematic illustration of the principles of FISH, showing two-color FISH for detection of two single locus sequences on metaphase chromosomes. (*View this art in color at www.dekker.com.*)

Probes for FISH

Several types of probes can be used to detect chromosomal abnormalities by FISH. These probes are usually divided into three groups: probes that hybridize to unique sequences; probes that hybridize to satellite DNA, tandem DNA repeats that are present at the centromeres of human chromosomes; and probes that hybridize to the whole chromosome, called, for this reason, *painting* probes. An increasing number of locus-specific probes have been established for the diagnosis of leukemias and lymphomas because genetic aberrations in these neoplasias have been shown to predict clinical behavior and outcome better than morphology alone.[5] FISH assays for the detection of diagnostically relevant chromosomal changes in both myeloid and lymphoid neoplasias are commercially available from several companies (http://www.vysis.com/, http://www.cytocell.co.uk/, and http://www.qbiogene. com).

Fluorescence in situ hybridization methods have evolved dramatically during the last years leading to the development of multiple variants. Among those, single- and double-color FISH using satellite and locus-specific probes have special diagnostic applications. These probes can be applied to both metaphase and interphase cells and can detect various types of numerical or structural chromosomal aberrations. For the detection of translocations by conventional interphase FISH (I-FISH), differentially labeled probes flanking or spanning the respective breakpoints can be applied.[5]

To detect chromosomal translocations by I-FISH, there are mainly two types of probes depending on the nature of the involved genes. If both genes involved in a translocation are known, as in the t(9;22)(q34;q11) in chronic myeloid leukemia, differentially labeled probes spanning the respective genes are the most appropriate. The presence of a translocation leads to two fusion signals, one on each of the derivative chromosomes, in

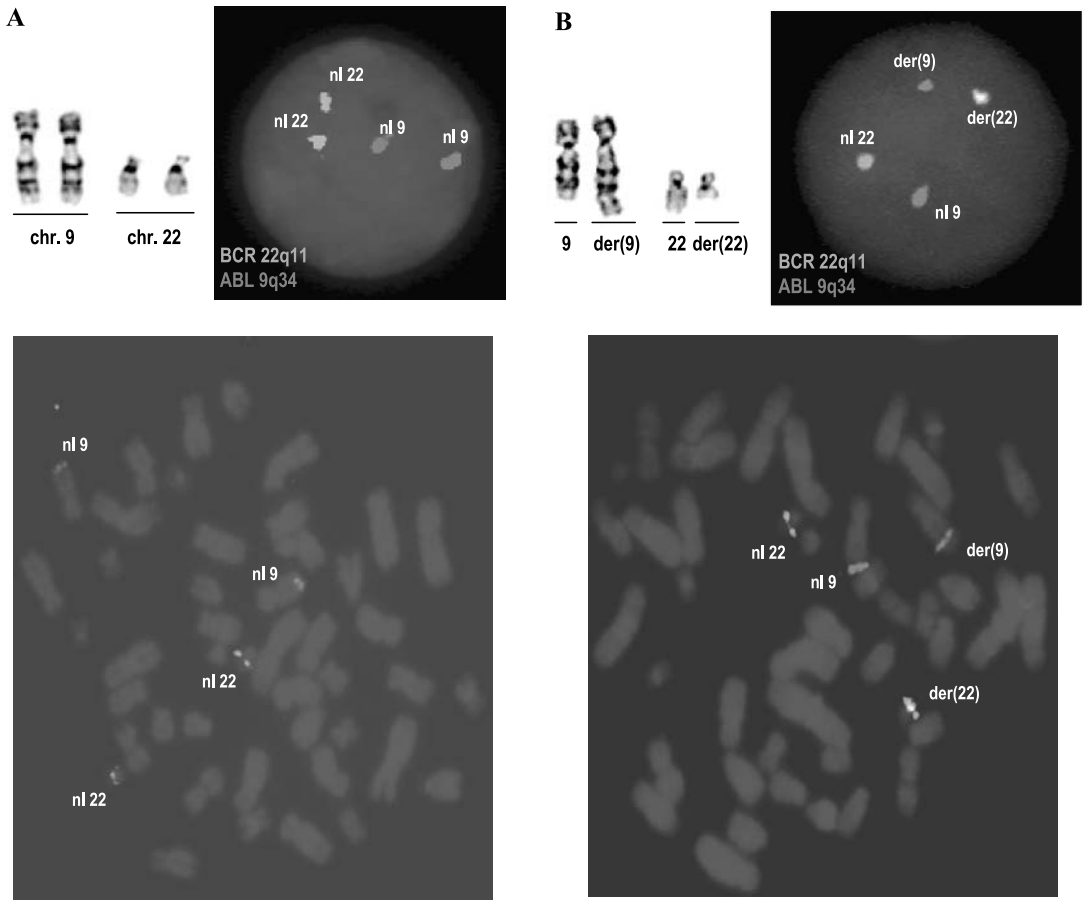

Fig. 2 FISH analysis using the LSI BCR/ABL ES Dual Color Translocation Probe (Vysis, Downer Grove, IL). This probe is a mixture of the LSI ABL probe labeled with SpectrumOrange™ and the LSI BCR probe labeled with SpectrumGreen™. The spanning ABL probe is approximately 650 kb. The SpectrumGreen BCR probe is approximately 300 kb beginning between BCR exons 13 and 14 (M-bcr exons 2 and 3) and extending well beyond the m-bcr region. The probe design allows high sensitivity on the FISH analysis. (A) Partial G-banding karyotype, nucleus, and metaphase from a healthy donor. The nucleus lacking the t(9;22) exhibits a two red, two green signal pattern. (B) Partial G-banding karyotype, nucleus, and metaphase from a patient with CML and a t(9;22)(q32;q11). In the nucleus containing the t(9;22), one green (native BCR), one large red (native ABL), one smaller red (derivative chromosome 9), and one fused red/green signal (derivative chromosome 22) are observed. In some cells, a deletion may occur 5′ at the ABL breakpoint that may reduce the pattern to a single fusion pattern. (*View this art in color at www.dekker.com.*)

addition to the single differentially labeled signals expected from the normal chromosomes (Fig. 2). When a gene locus is rearranged with a wide array of partner genes, the approach would be to use differently labeled probes flanking the recurrent breakpoint regions. In translocations, this would lead to split of the differentially labeled probes, which appear colocalized when the locus of interest is intact.[5] Such assays are, for example, applied for the detection of rearrangements involving the *IGH* locus or the promiscuous gene *MLL*.

For all probes studied, a series of positive and negative controls to establish scoring criteria and calculate the diagnostic cutoff should be established. For I-FISH probes, it is accepted that the diagnostic cutoff should be calculated in at least five healthy donors. For the signal patterns of probes leading to double split and double fusion in case of translocation, the false positive rate is virtually zero.[5]

CLINICAL APPLICATIONS

Fluorescence in situ hybridization techniques have altered the procedures used in many laboratories and promise to have a more substantial impact in the future.[6] FISH is being used increasingly, providing a means of examining the karyotypic pattern of human tumors to the many institutions that do not have access to cytogenetic

Table 1 Applications of fluorescence in situ hybridization

Analysis of tumors
- Detection of numerical and structural chromosomal abnormalities.
- Identification of marker chromosomes (rearranged chromosomes of uncertain origin).
- Detection of early relapse or minimal residual disease.
- Monitoring the effects of therapy.
- Identification of the origin of bone marrow cells following stem cell transplantation.
- Examination of the karyotypic pattern of nondividing or interphase cells.
- Identification of the lineage of neoplastic cells.

Applications in research
- Molecular analysis of chromosomal abnormalities in human tumors.
- Chromosomal localization of genes and DNA sequences.
- Preparation of cytogenetic maps (including determining the order of and distance between sequences).
- Detection of amplified genes.
- Examination of the organization of DNA/chromosomes in interphase nuclei.

laboratory services. A summary of the applications of FISH following Le Beau[3] is shown in Table 1. The application of FISH to the analysis of leukemia samples has improved the conventional cytogenetic analysis. FISH analysis provides more sensitivity and, in some cases, allows the detection of chromosomal abnormalities in samples that appeared to be normal by G-banding. FISH is most powerful when the analysis is targeted toward those abnormalities that are known to be associated with a particular disease. Cytogenetic analysis should be performed at diagnosis because it is important to obtain an overview of the chromosomal abnormalities of the patient at this time. Thereafter, FISH can be used to detect residual disease or early relapse and to assess the efficacy of therapeutic regimens. As mentioned above, FISH allows a large number of cells being analyzed in a short time with high sensitivity.

Detection of Numerical and Structural Chromosomal Abnormalities

In cancer cytogenetics, the application of FISH has resulted in the recognition of new recurring abnormalities in well-characterized diseases, such as leukemias and lymphomas, and in tumors that have been less amenable to conventional cytogenetic studies. An illustrative example is the finding of the t(12;21)(p13;q22) as the most common abnormality in childhood B-cell acute lymphoblastic leukemia, occurring in at least 25% of patients.[7] This abnormality was not detected by the analysis of banded chromosomes because the banding pattern of the involved chromosome bands and the size of the translocated material were so similar. The identification of this rearrangement has important prognostic and therapeutic implications as the translocation has been associated with a favorable clinical outcome.

Fluorescence in situ hybridization is also a sensitive and accurate means of detecting gene loss or deletion and how it has been demonstrated in the diagnosis of microdeletion syndromes. In CLL, genetic studies have identified recurring unbalanced abnormalities with prognostic significance, and FISH has become a diagnostic test to direct and monitor the treatment. Deletions of 13q14 as the sole genetic abnormality identify a group of patients with good prognosis, whereas deletions of 11q23 or 17p13 are important independent predictors of disease progression and survival.[4] However, in patients with multiple myeloma, the presence of chromosome 13 deletions has an adverse prognostic role.[8]

Fluorescence in situ hybridization has also been used to define the commonly deleted segment in recurring deletions, such as the deletions of chromosome 5 that are observed in myeloid leukemias.[9] By using FISH, several probes that fall within the critical region identified can be used to detect the deletions in interphase cells from BM samples from patients with AML, as well as to identify a putative tumor suppressor gene within the commonly deleted segment.

Monitor the Effects of Therapy and Detect Minimal Residual Disease

Posttreatment samples represent a substantial number of all cases analyzed by most clinical cancer cytogenetics laboratories. The detection of chromosomal rearrangements not only provides a diagnostic aid, but also allows the tumor clone to be quantified during follow-up, and for this, FISH provides a rapid means to detect residual leukemia cells. The monitoring should be performed either by G-banding or by FISH. G-banding only provides information about the dividing cells and FISH about both dividing and nondividing cells, so the percentages of malignant cells detected will not be the same. In general, FISH can be used to determine the proportion of normal and abnormal cells in BM and PB and to detect early relapse, although there are some interesting exceptions. An example is the monitoring of patients with chronic myeloid leukemia (CML). Almost all patients with CML carry a t(9;22)(q34;q11) reciprocal translocation that results in the formation of a *BCR-ABL* fusion gene. Treatment with imatinib (formerly STI571) induces major cytogenetic remissions or complete cytogenetic remissions (CCRs) in more than 40% of patients resistant or intolerant to interferon-alpha. Unexpectedly, it has been

reported that targeted therapy of CML with imatinib favors the manifestation of Ph- clonal disorders in some patients.[10] One immediate conclusion is that patients on imatinib should be followed with conventional cytogenetics, even after induction of CCR. Thus, despite the several advantageous features of FISH for the diagnosis of leukemias and other tumors, this technique suffers from technical limitations, being one of the most important that FISH does not provide a global view of the chromosomal aberrations.

The analysis of genetic markers is also routinely used to evaluate the results of BM transplantation. Several groups have shown the applicability of FISH using X- and Y-chromosome-specific probes in detecting host cells in sex-mismatched transplants. This method is particularly useful for samples obtained shortly after transplantation, in which there is no possible cytogenetic analysis because the cell number is very low.

IMPROVEMENTS OF THE FISH TECHNIQUE

A number of recent developments have led to improvements in FISH technology. These include the development of several multicolor fluorescence techniques as well as comparative genomic hybridization (CGH), an approach that allows tumor genomes to be tested for the presence of chromosomal unbalances without the need of metaphases and only requires small numbers of tumor cells to prepare DNA.[11]

Multicolor FISH and SKY

One of the earliest limitations of FISH was the inability to distinguish more than one or two target sequences because of the limited number of fluorochromes available. The introduction of multicolor FISH (M-FISH) and spectral karyotype (SKY) allowed detecting chromosomal abnormalities in individuals with constitutional abnormalities and in tumor samples.[12–14] Simple and complex translocations and relatively large deletions are identified with ease. However, there are some limitations of karyotyping by multiplex FISH of painting probes. This technique is not useful in the detection of paracentric inversions, some pericentric inversions, insertions involving a single chromosome arm, small duplications, and small deletions. Some of the limitations mentioned above can be addressed by using FISH probes. Therefore multiplex FISH is strongest when used in a complementary manner with both conventional cytogenetic and FISH analysis.[15]

Several studies using SKY have shown its power in resolving the full spectrum of chromosome abnormalities in tumors, especially in complex karyotypes, as well as documenting new recurring rearrangements in lymphoproliferative disorders.[16] However, SKY does not seem to be the election choice to detect hidden aberrations in normal karyotypes because only a low percentage of chromosomal abnormalities were detected by this technique. These results, and the fact that this is a laborious and expensive technique, suggest that SKY is not likely to have a clinical impact for the study of normal G-banding cases.

FICTION

An interesting approach that will have a high impact is to combine FISH with morphologic or immunohistochemical assays to examine the cytogenetic pattern of specific cell populations. This is possible because FISH does not require the destruction of the cell structure and tissue morphology. In the future, even more important than I-FISH will be a technique called fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms (FICTION). This method allows the detection of genetic aberrations in immunologically characterized cell populations, even if they present a minority in the tissue studied.[17] Using this approach, the sensitivity of interphase FISH can be even enhanced because only certain cell subpopulations are

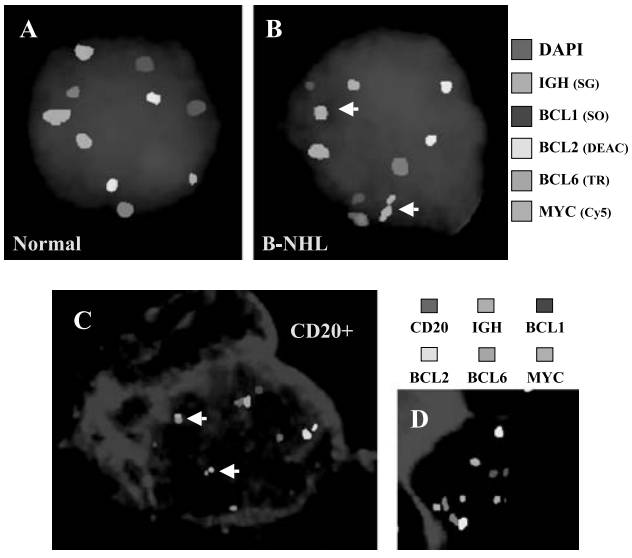

Fig. 3 Multicolor locus-specific assay for the detection of aberrations in NHL. (A) Interphase nucleus of a healthy donor. (B) Interphase nucleus of a patient with NHL with a IGH-MYC fusion due to t(8;14)(q24;q32). (C) M-FICTION assay in the same patient with NHL identified that the translocation was exclusively in the CD20+ B cells. (*View this art in color at www.dekker.com.*)

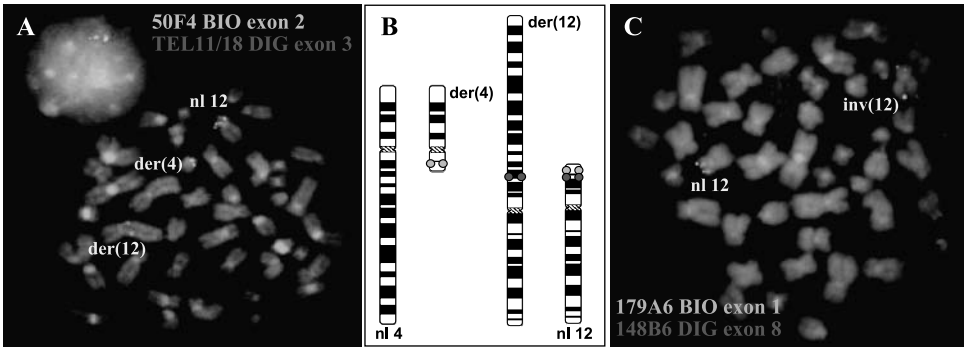

Fig. 4 Examples of FISH analysis of patients with 12p13 rearrangements showing that the gene *ETV6* is involved. (A) Patient with AML-M2 and t(4;12)(q12;p13). The red and green signals both label the normal 12p, whereas the green signal representing the telomeric probe is translocated to the der(4) and the red centromeric probe labels the der(12) chromosome. (B) Simplified representation of this case. (C) Patient with AML-M2 and inv(12)(p13q24). The red and green signals are together on the normal 12p and they are separated on the inv(12) with exon 8 remaining on 12p and exon 1 labeling 12q. (*View this art in color at www.dekker.com.*)

targeted (Fig. 3). The diagnostic importance of FICTION in leukemias and related neoplasms might take significant advantage from the achievements of high-throughput expression studies using, for example, CHIP technology, which aim to identify mRNAs and proteins differentially expressed in biologic and prognostic subgroups of hematological malignancies. By FICTION, the genetic aberrations and the discriminating proteins identified in expression studies could be detected simultaneously on a single-cell level.[17] Thus FICTION could allow a simultaneous and highly sensitive determination, and even quantification, of diagnostic and prognostic changes in leukemias and related disorders at both DNA and protein level.

FISH and Research

The Human Genome Project has stimulated other exciting applications of FISH and multicolor FISH, providing information about sequence-tagged clones placed on NCBI contigs. The BAC Resource Consortium has been working in connecting the cytogenetic and sequence maps of the human genome. This integration has been accomplished by FISH-mapping bacterial artificial chromosome (BAC) clones that contain one or more unique sequence tags. The sequence tags allow each BAC to be positioned on the emerging draft sequence of the human genome. More than 8000 clones have been mapped to date, resulting in at least one clone on average per megabase (Mb) for 23 of the 24 human chromosomes. The addition of these landmarks to the draft genome sequence has aided in the detection and molecular characterization of chromosome abnormalities that cause human disease. Each clone is available as single-colony purified bacterial stock through one of three distributors: BACPAC resources (CHORI) (http://www.chori.org/bacpac), Research Genetics (http://www.resgen.com/resources/index.php3), and the Sanger Center (http://www.sanger.ac.uk/Teams/Team63/CloneRequest).

In several studies, FISH is used in conjunction with G-banding for the accurate location of chromosomal breakpoints, which, in many instances, represents a first step toward the identification of novel tumor-related genes.[18,19] The FISH technique, together with molecular methods, has permitted the cloning of novel oncogenes involved in the pathogenesis of leukemias and lymphomas (Fig. 4). Besides, the subsequent establishment of FISH assays screening the chromosomal abnormalities in the regions of interest has revealed additional cases harboring the same or variant changes.

CONCLUSION

In conclusion, although not fully explored yet, molecular cytogenetic analysis is an invaluable tool in the clinical management of patients with hematological malignancies. Conventional cytogenetic and FISH analysis should be used in a complementary way. With the increasing number of new developments in FISH and molecular biology, it is important to understand the most appropriate techniques to apply to an individual case. RT-PCR offers an alternative to cytogenetics when the presence of the fusion gene is the only indicator required, but, in most cases, G-banding and FISH are the appropriate choice at diagnosis and provide additional important information in the follow-up of the patients. The challenge for the future is to bring these interests together.[15] The development of CHIP technology for a DNA leukemia-specific screening test may eventually replace routine cytogenetics and

molecular screening as we know them today, but until then, cytogenetic analysis remains imperative.[1] The search continues for new recurring chromosomal abnormalities with prognostic significance and the genetic changes associated with them. This is essential to increase further the understanding of the molecular mechanisms of leukemogenesis.

ACKNOWLEDGMENTS

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FISH—Centromere-Specific Multicolor FISH

Thomas Liehr
Institut für Humangenetik und Anthropologie der FSU Jena, Jena, Germany

INTRODUCTION

It is nearly impossible to determine the origin of small supernumerary marker chromosomes (SMCs) by routine cytogenetics. However, fluorescence in situ hybridization (FISH) methods are highly suited for that purpose. Twenty-four-color FISH approaches using whole chromosome painting probes can be used successfully for the determination of such markers' chromosomal origin if the SMC is larger than 17p. Therefore smaller SMCs found in clinical cytogenetics in 0.01–0.05% of cases often could not be characterized in the past. The one-step characterization of small SMCs became possible by recently established methods such as centromere-specific multicolor FISH (cenM-FISH) and related probe sets.

SMALL SMCs

In about 50% of cases with small SMCs, the derivatives are known to originate from chromosome 15.[1] Among the remaining cases, there is: 1) a great variation in chromosomal and parental origin; 2) a possibility of genomic imprinting effects; and 3) homozygosity of autosomal recessively inherited mutations in uniparental isodisomy.[1,2] As great dissimilarities in their clinical outcomes are reported, too, the characterization of prenatally detected—particularly de novo—SMCs is of significant interest for more appropriate medical care and genetic counseling. Characterization of one or more SMCs in a patient should be followed by testing for uniparental disomy (UPD), as UPD can cause clinical signs and symptoms, as well.[3–5]

CENTROMERE-SPECIFIC FISH APPROACHES

To characterize an SMC, whole chromosome painting FISH approaches such as multiplex FISH (M-FISH) or spectral karyotyping (SKY) (overview in Refs. [6] and [7]) are well suited as long as the marker is larger than the short arm of chromosome 17. If it is smaller and including only small chromosome fragments with no euchromatin or nearly no euchromatin, then often no result may be obtained (e.g., Ref. [8]).

For the rapid characterization of such small SMCs, a multicolor FISH technique was established, allowing the unambiguous one-step identification of all human centromeric regions, excluding nos. 13 and 21. The so-called cenM-FISH[9] (Fig. 1) is based on all available centromere-specific DNA probes, labeled in five different fluorochromes. A comparable multicolor FISH, called centromeric multiplex FISH (CM-FISH) approach, was published in parallel.[10]

SMCs are initially detected during GTG banding analysis and can be characterized for the presence of an acrocentric chromosome-derived short arm by nucleolus organizing region (NOR) staining. According to the NOR staining result (NOR-negative or NOR-positive), the origin of SMCs can be determined by application of all human centromeres in one experiment (i.e., cenM-FISH,[9]) or by the acrocentric centromere-specific multicolor FISH (acro-cenM-FISH) probe set,[11] respectively. The latter consists of a probe specific for the acrocentric human p-arms, a NOR-specific probe, a probe specific for Yq12, and all centromere-specific probes for 13/21, 14/22, 15, and 22.[11] These two approaches were successfully applied in about 100 cases with SMCs up to present[9,12–15] (SMC-homepage: http://mti-n.mti.uni-jena.de/~huwww/MOL_ZYTO/sSMC.htm).

In general, neocentromeric SMCs are not stained by any of the two aforementioned approaches; in this case, other techniques for their characterization, such as M-FISH (overview in Refs. [6] and [7]), microdissection of SMCs (e.g., Ref. [16]), or micro-CGH,[16] have to be performed. About 60 cases with neocentromeres are reported in the literature (for review, see Ref. [17]).

After determination of the origin of a small SMC with centromeric DNA, the most important question to address is if there is euchromatic material of the corresponding chromosome on it, or not. This question for partial trisomy can be studied by hybridizing whole chromosome painting or chromosome arm-specific probes (e.g., Ref. [8]). Another possibility is the use of probes, which are able to subclassify chromosomal regions such as multicolor banding (MCB) (e.g., Ref. [16]; Fig. 2). Recently, a probe set specific for the pericentric region of all human

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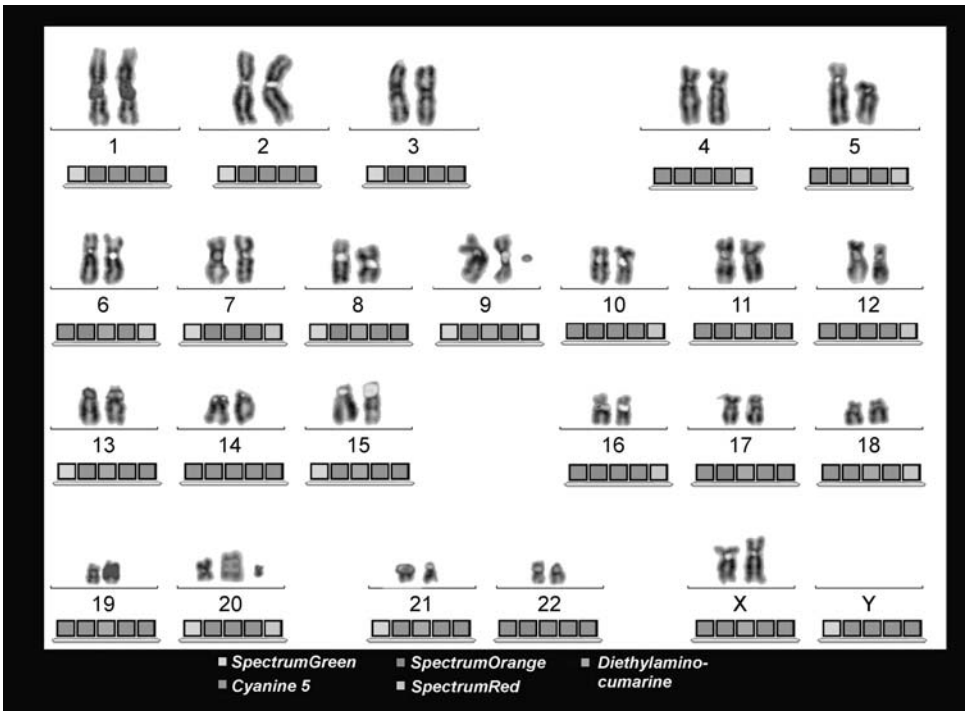

Fig. 1 CenM-FISH result of a case with two small SMCs. CenM-FISH clearly revealed that one SMC was a min(9) and one was a min(20). (*View this art in color at www.dekker.com.*)

chromosomes, the centromere-near multicolor FISH (subcenM-FISH) technique, was introduced to study this question.[18] A chromosome-specific subcenM-FISH probe set consists of a centromere-specific satellite probe, one centromere-near locus-specific probe in the long arm and the short arm (excluding the acrocentric chromosomes), and chromosome arm-specific probes.

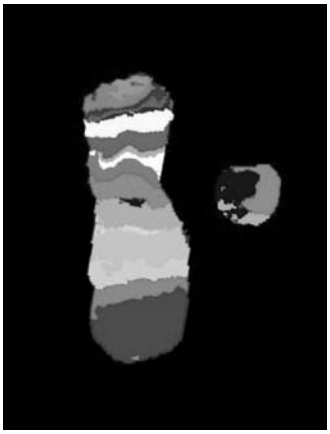

Fig. 2 MCB result of an SMC derived from the X chromosome, which turned out to be a r(X)(p11.21q13.1). (*View this art in color at www.dekker.com.*)

EXCLUSION OF UPD

Different mechanisms for the formation of small SMCs were proposed: trisomic rescue, monosomic rescue, postfertilization errors, and gamete complementation. In addition, speculations that SMCs are due to a transfection of a chromosome derived from a superfluous haploid pronucleus into the zygote are discussed.[19] UPD was reported for the majority of human chromosomes.[4,5] Therefore, in case of identification and characterization of small SMCs, an exclusion of UPD possibly present in the two normal sister chromosomes by molecular genetic methods is reasonable (e.g., Refs. [12] and [15]).

CONCLUSION

In summary, because of the new available molecular cytogenetic approaches, nowadays the detection of a small SMC does not stop after GTG banding, NOR staining, and exclusion of a chromosome 15 origin. The small SMCs can be exactly described after (acro)cenM-FISH or MCB, subcenM-FISH provides information on additional euchromatin, and molecular genetics uncovers eventually present UPD. Thus, a correlation of small SMC morphology and clinics will soon be available, which will be extremely helpful in genetic counseling.

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FISH—Clinical Applications of 24-Color FISH

F

Adewale Adeyinka
Syed M. Jalal
Mayo Clinic and Mayo Foundation, Rochester, Minnesota, U.S.A.

INTRODUCTION

Identification of markers, derivative chromosomes, and analysis of complex karyotypes both for constitutional and acquired chromosome disorders is a labor- and time-intensive effort in a clinical cytogenetics laboratory. 24-Color FISH is an effective and labor-efficient screening method for the entire genome when prior knowledge of the origin of a particular chromosome aberration is not available. This, coupled with its generally high level of accuracy, has made it an important tool in the analyses of prenatal and constitutional cases as well as hematological malignancies and solid tumors.

APPLICATIONS OF 24-COLOR FISH

Multicolor FISH (fluorescent in situ hybridization or 24-color FISH) is a molecular cytogenetic technique that uses fluorescent, combinatorially labeled, chromosome-specific DNA sequences to ''paint'' chromosomes to differentiate and classify nonhomologous pairs[1,2] (Fig. 1). This technique has been successfully applied to human chromosomes as well as to chromosomes of other mammalian species.[3]

The ability to visualize specific human chromosomes using fluorescent probes has been in existence since the 1980s.[4–6] What started as the application of radioactive nucleic acid probes for labeling chromosomes in the late 1960s[7] has now emerged into the possibility to simultaneously scan all of the 24 (22 autosomes and X, Y chromosomes) human chromosomes in defined fluorescent colors. Five fluorochromes are used to assign a spectral signature to each chromosome by combinatorial labeling of one to three fluorochromes for each chromosome using fluorochrome-specific filters and computer software.[1,2] This technique at different points in time has been given different names, e.g., 24-color FISH and Multifluor FISH (M-FISH), but 24-color FISH may be more appropriate. Similar to M-FISH is spectral karyotyping (SKY), another multicolor technique that employs combinatorial labeling but uses, instead of filters, an interferometer to measure emission spectra specific for each chromosome.[1] The details of methods are provided elsewhere[8–10] and are published in this encyclopedia. Since its inception in 1996, 24-color FISH has become an important tool in clinical cytogenetics and has found a wide application in research as well.

Congenital Disorders and Prenatal Diagnosis

Chromosome aberrations substantially contribute to the genetic disease burden. For example, the prenatal frequency of de novo supernumerary marker chromosomes is around 1/2500 and it is estimated that de novo structural rearrangements occur with a frequency from 0.70/1000 rising to 2.4/1000 among mentally retarded individuals.[11,12] In addition, deletions associated with defined phenotypic anomalies have been well documented for almost all major chromosome bands.[13] Balanced reciprocal translocations occur with a frequency of 1 in 600. The risk for unbalanced translocation, which are often associated with phenotypic abnormalities, in the progeny of balanced translocation carriers vary widely from 50% to <1%.[14] Complex chromosome rearrangements, although rare, are well documented; some cases involve as many as 10 breakpoints. The use of in situ hybridization and fluorescent DNA probes for the centromeres, locus-specific probes, and whole chromosome paint probes has facilitated the description of markers, derivative chromosomes, and complex karyotypes in much greater detail for prenatal and postnatal tissues. However, in certain situations when there is no strong clinical evidence pointing to the involvement of a particular chromosome, or when a marker of unknown origin or de novo derivative chromosome is encountered, the choice of individual probes to use is often a matter of trial and error. The introduction of 24-color FISH has now largely resolved this dilemma as each nonhomologous chromosome pair can be viewed in a unique color making it possible to identify and properly classify such chromosome aberrations with ease (Fig. 2). Detailed examples of such application of M-FISH are well documented and the literature is still growing.[9,15–18] For example, translocations between the sex chromosomes, which were beyond the resolution of standard G-banding, were identified by M-FISH in a male suspected Klinefelter syndrome.[15]

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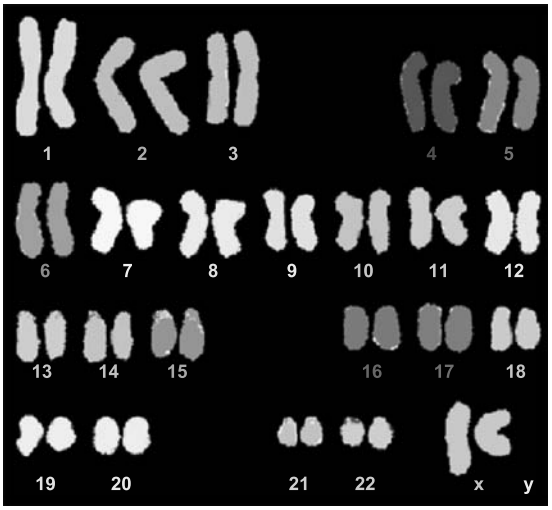

Fig. 1 Normal female karyotype obtained by 24-color FISH analysis. (*View this art in color at www.dekker.com.*)

Cytogenetics is very important for prenatal testing and the use of sensitive methods is important in this field. Another area of reproductive medicine that has created a need for sensitive genetic testing is in vitro fertilization to ensure that viable embryos are selected for this procedure. Twenty-four-color FISH appears to meet these needs. For example, in amniotic fluid specimen for prenatal diagnosis, M-FISH was used to resolve an unbalanced translocation between chromosomes 9 and 15 and subsequently confirmed by whole chromosome painting probes.[15] Also, a supernumerary ring chromosome in another amniotic fluid specimen was identified to be from chromosome 19.[15]

Hematological Malignancies

Over 150 nonrandom and recurrent chromosome abnormalities have been documented for hematological malignancies, including translocations, deletions, duplications, inversions, and aneuploidies. Some of these abnormalities occur in relatively simple karyotypes, whereas others are part of very complex karyotypes that are often beyond the resolution of routine banding techniques. Twenty-four-color FISH analysis has been used to resolve major chromosomal abnormalities in complex karyotypes from hematological disorders and of particular interest is the use of M-FISH to identify the critical anomaly that impacts on diagnosis or prognosis from a complex karyotype in hematological diseases.[17–19] For example, the G-banded karyotype in a male with acute lymphoblastic leukemia was 46,XY,add(1)(q42),add(8),add(14). M-FISH clarified that the add(1) was dup(1), the add(8) was ider(8q)(q10)t(8;22)(q24;q11), and add(14) was der(14)t(3:14). The identification of a t(8;22) was critical to the diagnosis of variant Burkitt's lymphoma involving the translocation of the oncogene c-myc adjacent to the immunoglobulin lambda light chain locus.[9] Poor morphology is another problem often encountered when analyzing chromosomes from bone marrow samples of patients with hematological malignancies. M-FISH is very useful in identifying chromosomes that otherwise would have been classified as markers because of poor morphology. For example, the G-banded karyotype from a bone marrow sample of a 9-year-old boy with unspecified hematological disorder was 45–47,XY,-6,der(9)add(9)(p13)add(9)(q34),-17,-19,-19,-22,+5mar[cp19] with poor morphology. M-FISH detected the cryptic translocation t(12;21)(p13;q21) that was confirmed by locus-specific FISH as TEL-AML1 fusion. This translocation is often missed by standard cytogenetic analysis in children with acute lymphoblastic leukemia (ALL). In addition, M-FISH identified all the remaining markers as der(6), der(9), der(15), der(20), and two normal chromosomes 19.[9]

Solid Tumors

Cytogenetic studies of solid tumors have also benefited from 24-color FISH. Most solid tumors, especially epithelial tumors, are known to be highly aberrant. Numerous marker chromosomes, cryptic translocations, chromosomal insertions, and additions have been properly characterized among the potpourri of aberrations seen in these solid tumors, and it is becoming possible to identify those that may be of pathogenetic importance.[18]

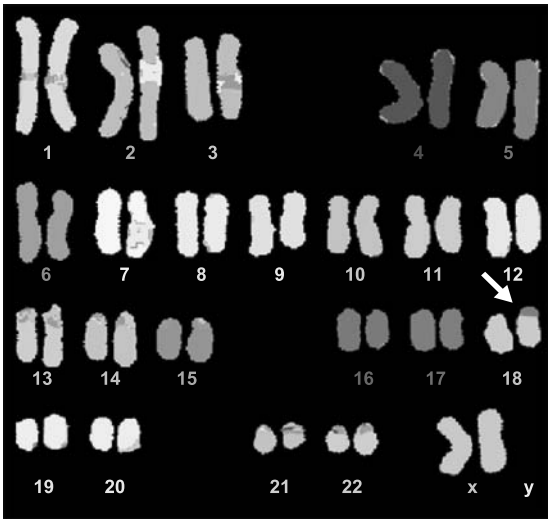

Fig. 2 Identification of a derivative chromosome, der(18)t(17;18) (arrow), using 24-color FISH. Chromosomes 2, 3, 7, and 20 were overlapped by other chromosomes. (*View this art in color at www.dekker.com.*)

CONCLUSION

Twenty-four-color FISH, although comparatively costly and labor-intensive, is an efficient and labor-effective method for scanning the entire genome especially to resolve marker-derivative chromosomes and complex karyotypes. M-FISH should be applied as an adjunct to chromosome-banding methods and other FISH techniques to obtain a comprehensive interpretation of complex and difficult karyotypes.

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FISH—Detection of Individual Radiosensitivity

Alma Kuechler
Klinik für Radiologie, Abteilung Strahlentherapie des FSU Jena, Jena, Germany

Thomas Liehr
Institut für Humangenetik und Anthropologie der FSU Jena, Jena, Germany

INTRODUCTION

In radiotherapy, alongside tumor control, the prevention of severe treatment-related side effects is a major concern. Although the majority of radio-oncological patients tolerate a standard treatment protocol, toxic side effects can be detected in 0.2–10% of the cases.[1,2] The latter can be a result of an increased individual radiosensitivity, caused by a combination of exogenous and endogenous factors including genetic reasons. However, most of the exact underlying mechanisms still remain unknown. Only in few cases increased radiosensitivity is a result of an identified single gene mutation, e.g., in ataxia telangiectasia (AT) or Nijmegen breakage syndrome (NBS) patients, OMIM #208900 and #251260,[3] but in the majority, it is supposed to be modulated by a mixture of different genes.[4]

To have a chance to detect a possible overreaction prior to therapy, the availability of a predictive test system has been the aim for some decades. Although different approaches were tested (e.g., clonogenic cell survival,[5] G2-Assay[6]), there is still no reliable routine assay to identify hypersensitive or less sensitive patients to adjust their therapeutic dose. It is known that chromosomal aberrations are: 1) indicators of a previous exposure to irradiation and 2) can be used to estimate radiosensitivity. Previous studies demonstrated that molecular cytogenetic methods are superior to conventional cytogenetic analysis in detection and characterization of aberrations. At this, the frequency of breaks and the occurrence of specific aberration types reflect individual sensitivity to radiation. Especially complex chromosomal aberrations (CCR) were identified as indicators for increased individual radiosensitivity.

AVAILABLE FISH TECHNIQUES

To detect individual radiosensitivity by fluorescence in situ hybridization (FISH), three approaches are presently available: 1) painting of one up to three different chromosomes in one or different colors, 2) painting of all 24 human chromosomes in different colors (24-color FISH), and 3) FISH-based chromosome banding for the detection of intrachromosomal rearrangements.

ONE- TO THREE-COLOR FISH

Fluorescence in situ hybridization applying one up to three whole-chromosome painting (wcp) probes simultaneously was used to detect radiation-induced chromosome instability in peripheral blood lymphocytes and fibroblasts (e.g., Refs. [8,10,12]). However, in the analyses, the used chromosomes are selected by chance—although a random distribution of chromosomal rearrangements along the chromosomes is up to now still discussed controversially (e.g., Refs. [13,14]). To avoid this problem and to obtain an overview of the whole karyotype, 24-color FISH can be applied using a probe mix of the 24 different human wcp probes.[7,11,15,16]

24-COLOR FISH

This method, first described by Speicher et al.[17] and Schröck et al.,[18] allows the simultaneous visualization of all chromosomes within a metaphase in different specific colors. Nearly all chromosomal aberrations (as reciprocal and nonreciprocal translocations, complex rearrangements, ring chromosomes, acentric fragments, dicentric fragments, or insertions) can be detected and defined in more detail.

The 24-color FISH has been used, for example, for studies on normal peripheral blood lymphocytes irradiated in vitro,[11,15] on radiotherapy-induced residual chromosomal damage in peripheral lymphocytes,[7] on bone marrow cells of Chernobyl victims,[15] and for analysis of chromosomal aberrations after irradiation with ionizing alpha particles. The latter was also used for investigations on the formation of CCR.[19]

Our own studies have focused on the analysis of in vitro-induced chromosomal aberrations in NBS and AT homozygote and heterozygote individuals compared with

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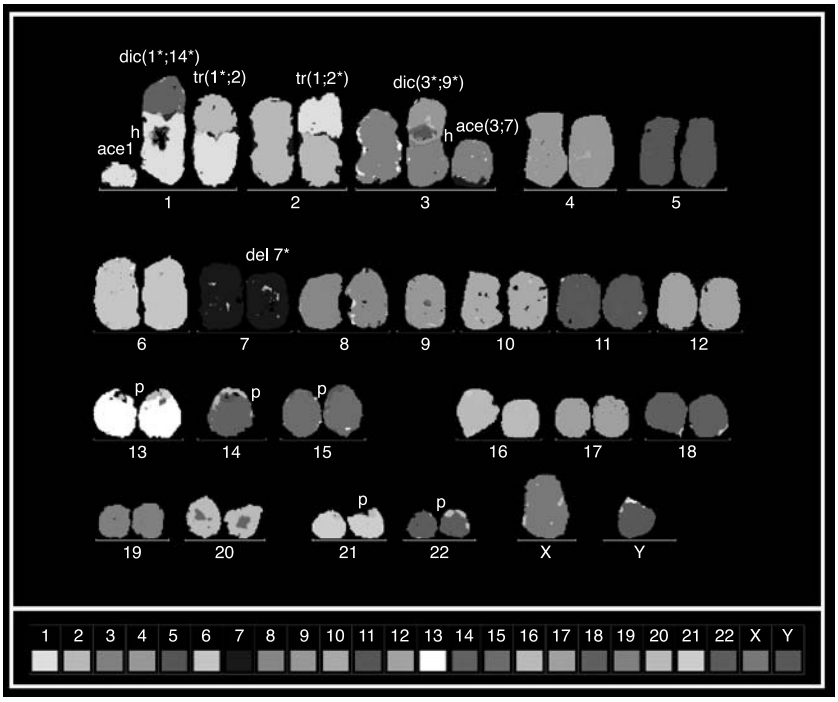

Fig. 1 Example of a 24-color FISH result: karyogram of an NBS-heterozygote person (pseudocolor representation) after in vitro irradiation with 2.0 Gy and scheme of pseudocolors for each individual chromosome. The aberration can be described (nomenclature modified according to the ISCN 1995[25]) as follows: t(1;2),dic(1;14),ace(1),dic(3;9),ace t(3;7),del(7). This result can be summarized as 7 breaks per mitosis. The translocation between chromosomes 3, 7, and 9 forms a complex chromosomal rearrangement. Abbreviations: p = short arms of the acrocentric chromosomes #13, #14, #15, #21, and #22 consisting of repetitive DNA, pseudocolored in a different paint; h = heterochromatic DNA, which is polymorphic and can be present at #1, #9, and #16. (Stars indicate centromere localization.) Images were captured with the ISIS3 digital FISH imaging system (MetaSystems, Altlussheim, Germany) using a PCO VC45 CCD camera (PCO, Kehl, Germany) on an Axioplan 2 microscope (Zeiss, Jena, Germany). (*View this art in color at www.dekker.com.*)

normal reacting controls (Figs. 1 and 2; Ref. 11 and unpublished data). The aim was to demonstrate that a difference in radiosensitivity which was already known between these individuals is also detectable using the 24-color FISH technique. Thus the increased radiosensitivity of NBS and AT patients can serve as a positive control in a predictive assay.

In the following, the criteria used in our own studies are specified to demonstrate that a reliable evaluation requires an experienced cytogeneticist. After the 24-color FISH procedure including hybridization, posthybridization washes, and detection, at least 100 metaphases per irradiation dose and patient/proband have to be acquired. This can be performed filter-based (m-FISH)[17] or spectracube-based (SKY).[18] All captured metaphases have to be karyotyped. For detailed evaluation of each metaphase, it is not enough to rely only on the pseudocolor functions of the used software, but for unambiguous results, one has to check the different fluorochrome channels or hybridization profiles. Then, aberration types and involved chromosomes have to be registered in detail. All occurring aberrations were classified into reciprocal and nonreciprocal translocations, ring chromosomes, acentric fragments, dicentric fragments, inversions, insertions, and complex rearrangements. Translocations, insertions, and complex rearrangements are visible because of a color change along a rearranged chromosome. Dicentrics and ring chromosomes can easily be identified using the inverted DAPI picture. Complex chromosomal rearrangements (CCR) consist, per definition, of at least two chromosomes with three or more breaks.[20]

For further evaluation, the frequency of break events constituting the observed aberrations was estimated as the minimal number of breaks considered to be necessary for producing the aberrations in each metaphase. The total number of break events in each patient/control and irradiation dose was summed up and divided by the number of metaphases analyzed to obtain the average rate of breaks per mitosis (B/M). The radiosensitivity of lymphocytes was expressed as number of radiation-induced B/M and CCR/M after each irradiation dose, subtracted by the 0.0-Gy control value to correct the

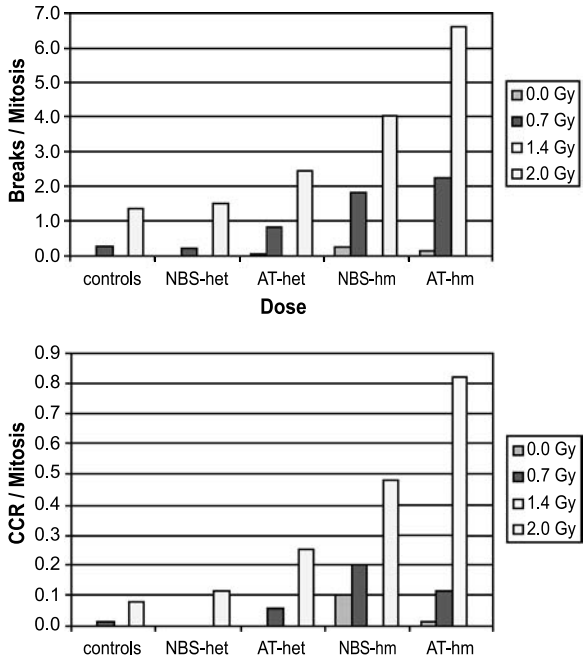

Fig. 2 For the graphic depicted here, four normal controls, two Nijmegen breakage syndrome (NBS), and two ataxia telangiectasia (AT) heterozygotes (NBS-het, AT-het) plus one NBS and one AT homozygote (NBS-hm, AT-hm) were included. The upper diagram shows the average number of breaks per mitosis, while the lower one presents the average number of CCR occurring in each mitosis for each of the five groups. Three different in vitro irradiation doses were analyzed per patient. The applied doses were 0.0, 0.7, and 2.0 Gy; in the AT-hm patient, instead of 2.0 Gy, 1.4 Gy was used. A clear differentiation was possible in both diagrams between normal controls and the homozygote patients, as well as between controls and AT-het. (*View this art in color at www.dekker.com.*)

influence of spontaneous basic aberration frequencies. For further details, see Ref. [11].

The 24-color FISH is a highly informative approach concerning the characterization of radiation-induced chromosomal rearrangements, but is based on a very sophisticated and time-consuming evaluation procedure.

MULTICOLOR BANDING

Fluorescence in situ hybridization methods using all 24 human whole-chromosome painting probes simultaneously reach their limits in identification of intrachromosomal rearrangements (such as small interstitial deletions, duplications, and inversions without change of the centromeric index) and when exact characterization of breakpoints is required. These limitations have been overcome by the development of new FISH-banding methods during the last decade.[21] In the meantime, one of these approaches—the multicolor banding (MCB) technique (or mBAND)[22]—was also used for the analysis of X-ray-induced aberrations.[23,24] However, only a probe set for chromosome 5 was applied, demonstrating that intrachromosomal aberrations are present in a considerable portion in radiation-induced changes.

CONCLUSION

In summary, genetically determined intrinsic radiosensitivity can be detected and quantified by FISH approaches. The 24-color FISH can be used as well as the three-color FISH. FISH is as reliable but more informative than conventional cytogenetic data (e.g., Refs. [8,9]). As stated before, the 24-color FISH approach requires a very sophisticated evaluation. However, the data obtainable by this technique are indispensable to decide whether there exist any hot spots for radiation-induced breakpoints. If so, such hot spots could be characterized in detail by FISH-banding methods,[21] and in the following, locus-specific breakpoint-spanning probes could be applied in the future for quick detection of individual radiosensitivity, possibly even in interphase cytogenetics.

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FISH—Human Sperm Cells

Renée H. Martin
University of Calgary, Calgary, Alberta, Canada

INTRODUCTION

Chromosome abnormalities are amazingly common in humans, leading to infertility, pregnancy loss, and birth of children with mental and physical handicaps. Most chromosome abnormalities have their origin during meiosis; thus information on the causes of chromosome abnormalities and factors affecting their frequency is best studied in human gametes. During the past two decades, there has been an explosion of information on the chromosome complement of human spermatozoa. This has been mediated by the advent of two techniques: human sperm karyotyping using the hamster oocyte system and fluorescence in situ hybridization (FISH) analysis. Rudak et al.[1] first reported the use of hamster ova to reactivate human sperm, allowing the analysis of pronuclear chromosomes. Other laboratories followed with the first publications on the frequency and type of chromosome abnormalities in human sperm.[2–5] Human sperm karyotyping, using the hamster system, provides detailed information on each individual chromosome, permitting analysis of both numerical and structural abnormalities. However, there are significant disadvantages: sperm cells must be capable of fertilizing a hamster oocyte; the technique is very difficult, time-consuming, and expensive; and data yield is small. In fact, only 12 laboratories worldwide have had success with this technique despite many efforts. Fluorescence in situ hybridization analysis with chromosome-specific DNA probes provides a faster, cheaper, and easier alternative for detecting numerical abnormalities (aneuploidy) in human sperm. In addition, sperm cells with defects in fertilization ability and/or motility can be assessed using FISH analysis. This review will outline some of the studies that have been performed on human sperm using FISH analysis.

TECHNICAL DESCRIPTION

Our technique for FISH analysis of human sperm cells has been reported in great detail previously.[6]

IMPACT OF FISH ANALYSIS IN DIFFERENT RESEARCH AREAS

Studies on Normal Men

More than 5 million sperm cells from more than 500 normal men have been analyzed by a number of laboratories from around the world.[7] These studies have included the frequency and distribution of aneuploidy in sperm cells, and the effect of donor age, lifestyle factors, and geographical and ethnic factors.

Frequency and Distribution of Aneuploidy

The frequency and distribution of disomy in human sperm cells is of interest to determine the normal baseline values and to determine if all chromosomes have a similar frequency of nondisjunction or if some are particularly predisposed to nondisjunction.[8] The frequencies of disomic sperm for an individual chromosome are considerably different among the studies. For example, for chromosome 21, the lowest disomy frequency was 0.05%[9] and the highest was 0.95%.[10] It is unlikely that these differences arise only from interindividual variation in disomy. Rather, different approaches in the experimental design, different probes, and scoring criteria used are regarded as being responsible (for a review, see Ref. [11]). This makes it essential for each study to have controls analyzed in the same laboratory.

In a composite analysis of the distribution of disomy frequencies in studies on normal men, the mean disomy frequency for autosomes was 0.15% and 0.26% for sex chromosomes.[7] Our laboratory has determined that most autosomes have a similar frequency of nondisjunction, but chromosome 21 and the sex chromosomes have a significantly increased frequency.[8,12] These results corroborate our earlier studies in human sperm karyotypes.[13] This has also been confirmed by other groups.[14,15] Thus we see aneuploidy for all chromosomes, but chromosome 21 and the sex chromosomes appear to be particularly susceptible to nondisjunction during spermatogenesis.

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Effect of Donor Age

There have been a number of FISH studies addressing the possibility of a paternal age effect on nondisjunction. The great majority of studies has not found a paternal age effect for autosomes.[16–18] However, most studies have demonstrated a significant increase in the frequency of sex chromosomal aneuploidy with donor age[19–21] with approximately a doubling of disomy frequencies in the oldest donors compared to the youngest donors.

Lifestyle Factors

A small number of studies have addressed the possible effects of lifestyle factors such as smoking, air pollution, and caffeine and alcohol consumption on sperm aneuploidy frequencies. Robbins et al.[22] found a significant association between caffeine and alcohol consumption and increased disomy frequencies for some chromosomes. No significant association was noted for smoking. Two studies found a significant association between smoking and aneuploidy for some chromosomes,[23,24] but it is difficult to rule out combined lifestyle factors because smokers also consume more caffeine and alcohol than nonsmokers. Our laboratory studied over 600,000 sperm cells from heavy smokers, light smokers, and nonsmokers (all nondrinkers of alcohol) and found a significant increase of disomy only for chromosome 13, but not for chromosome 21, X, or Y.[25,26] Perreault et al.[26] studied seasonal air pollution and found an association between high levels of air pollution and YY disomy in nonsmoking men. To date, no consistent and clear association has been discovered between disomy frequency and any type of lifestyle factor. More well-controlled studies will be required to uncover any associations.

Geographical and Ethnic Factors

Two laboratories have studied the frequency of sperm aneuploidy in geographically and ethnically diverse populations using the same experimental conditions, DNA probes, and scoring criteria. We found no difference in disomy frequencies for autosomes or sex chromosomes in nonsmoking, nonalcohol drinking Chinese men compared to Canadian men.[12] However, Rubes et al.[23] detected a significant increase in the frequency of sperm disomy in nonsmoking Czech men compared to Californian men. Thus, further studies on different populations are required to determine if sperm aneuploidy frequencies differ in various groups.

POTENTIAL FUTURE APPLICATIONS

Because FISH analysis is simple and provides data on large sample sizes of sperm cells, it has been suggested as a technique for assessing the effects of potential mutagens on human sperm. We and others have studied cancer patients treated by chemotherapeutic agents and determined that these agents can cause a significant increase in the frequency of sperm chromosome abnormalities, particularly during chemotherapy and up to 2 years following treatment.[27–29]

The effects of pesticides have also been studied, as there is increasing concern about potential adverse consequences to reproduction and fertility. Two studies have not shown any association between pesticide use and sperm aneuploidy.[24,30] A third study did find an increased frequency of YY aneuploidy in pesticide factory workers exposed to higher pesticide concentrations.[31]

A number of infertility clinics have advocated the use of FISH analysis in clinical sperm samples from infertile patients. As mentioned above, infertile men certainly have an increased frequency of chromosome abnormalities in their sperm cells. Furthermore, studies have shown that specific men with an increased frequency of sperm chromosome abnormalities have fathered chromosomally abnormal pregnancies, for example, a man with a frequency of XY disomy 14 times higher than controls fathered a fetus with 47,XXY Klinefelter syndrome,[32] and men with an elevated frequency of disomy 21 sperm fathered children with Down syndrome (trisomy 21), shown to be paternally derived.[14] In addition, a number of studies have demonstrated that men with macrocephalic sperm have a very high frequency of sperm aneuploidy and diploidy.[33,34] Thus the addition of FISH analysis of sperm cells might be a useful investigation in male factor infertility, especially in cases known to have a high risk of abnormalities, such as men heterozygous for translocations or men with macrocephalic sperm cells.

CONCLUSION

Fluorescence in situ hybridization analysis of human sperm cells has been used successfully in many studies: from studies of basic mechanisms of nondisjunction, to studies of infertility and effects of mutagens on the chromosome constitution of human sperm. The technique is simple, rapid, and relatively inexpensive. However, it is important to keep in mind that there are some subjective aspects to counting fluorescent signals and each laboratory must develop stringent scoring criteria for test subjects as well as laboratory-based control donors.

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FISH—Q-FISH for Telomere Length Measurement

Predrag Slijepcevic
Brunel University, Middlesex, U.K.

INTRODUCTION

Telomeres are essential structures at chromosome termini required for an accurate segregation of eukaryotic chromosomes. The structure and length of telomeres change after each cell cycle and the nature of this change depends on the cell type. In the case of human primary somatic cells, telomeres gradually shorten after each cell cycle until their structure becomes incompatible with the further progression through the cell cycle, i.e., structurally dysfunctional telomeres activate cellular DNA damage response and can lead to genomic instability. In the case of immortalized human cells or cancer cells, telomere size and structure fluctuate as a result of cell proliferation, but telomeres usually do not shorten because telomerase, an enzyme that synthesizes telomeric sequences, is active in these cells. The importance of telomeres from the clinical perspective is illustrated by at least two major observations. First, there is a correlation between telomere length on one side and either the cell senescence in vitro and possibly in vivo or individual mortality rate on the other side. Second, the enzyme telomerase, which is active in most human cancers, provides cells with the means to indefinitely maintain telomeres and as a result make them immortalized, suggesting that the inhibition of this enzyme could be an effective way to stop cancer cell growth. A major prerequisite for making the above two observations useful in the clinical practice is the capacity to measure telomere length with a high degree of accuracy. At present there are three main methods for telomere length measurement: Southern blot analysis, quantitative fluorescence in situ hybridization (Q-FISH), and flow-FISH. The most informative of these three methods is Q-FISH and this article will describe the principles of Q-FISH and discuss the advantages and disadvantages of Q-FISH in comparison with other methods.

PROBLEMS ASSOCIATED WITH TELOMERE LENGTH MEASUREMENT

The most authoritative recent review on telomere biology and papers describing the key discoveries mentioned above as well as methodological advances are listed in the reference list.[1–6] The classical method for telomere length measurement is based on Southern blot analysis. In a typical protocol, DNA is extracted from a cell sample and the resulting DNA mix is exposed to frequently cutting restriction enzymes, which will produce a series of DNA fragments, and those fragments containing telomeres are called terminal restriction fragments (TRFs). Following gel electrophoresis these fragments are transferred onto a nylon membrane, which is then hybridized with the radioactively labeled telomeric DNA probe. Following autoradiography, telomeres are detected as a smear, the size of which can be measured against appropriate molecular weight markers. This method has some major disadvantages. First, it can only measure the average telomere length and the information about the size of individual telomeres is ignored. Individual telomeres, not the average telomere length, drive genomic instability associated with telomere dysfunction,[7] thus indicating that measuring only the average telomere length may not be appropriate. Second, the presence of degenerate telomeric sequences in subtelomeric regions of chromosomes may interfere with the accuracy of Southern blot-based telomere length measurements, i.e., sequences that do not form part of functional telomeres may also be measured.[8] Third, hybridization efficiency of DNA probes is never 100% because DNA probes used for hybridization and genomic DNA have the same electrical charge and this may cause a charge repulsion between molecules resulting in inefficient hybridization. Therefore, a new method more efficient than Southern blot was required to provide more accurate information about telomere length.

DEVELOPMENT OF Q-FISH

In 1996, Lansdorp and coworkers showed that a modified DNA molecule called peptide nucleic acid (PNA) can be used for quantitative in situ hybridization.[9] The key difference between DNA and PNA is the replacement of the classical phosphate–sugar backbone in DNA by repeating *N*-(2-amynoethyl) glycine units linked by peptide bonds in PNA. Different bases (purines and pyrimidines) are linked to the PNA backbone by methylene carbonyl linkages. Because PNA backbone is not charged, the binding between PNA/DNA strands

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will be much stronger than between DNA/DNA strands, i.e., there will be no charge repulsion between PNA/DNA strands. Therefore hybridization between telomeric PNA probe and telomeric chromosomal DNA will minimize hybridization problems encountered when hybridizing molecules of the same charge, thus leading to higher hybridization efficiency. When the fluorescently labeled telomeric PNA probe is hybridized with metaphase chromosomes then all telomeres are visualized and by using appropriate software, the fluorescence intensity of each individual telomere can be accurately calculated.[8,9] This is a big advantage in comparison with Southern blot, which can only measure the average telomere length. Finally, because of its higher hybridization efficiency relative to DNA, PNA telomeric probe does not bind to degenerate telomeric sequences in subtelomeric chromosome regions.[8] Therefore all three major problems associated with the classical telomere length measurement based on Southern blot analysis (see above) are eliminated by the new Q-FISH protocol, suggesting that this is the most sensitive method for measuring telomere length.

PRINCIPLES OF Q-FISH

As stated above, Q-FISH requires the use of fluorescently labeled telomeric PNA probes. The most commonly used probe is Cy3-labeled $(CCCTAA)_3$. Following hybridization of this probe with the metaphase chromosome preparations, high stringency washes are performed to eliminate background fluorescence that may interfere with the quantitation of telomere fluorescence. Separate images of metaphase chromosomes and telomeres are then acquired using appropriate image-acquisition software packages and the resulting files can be used for the analysis of telomere fluorescence (Fig. 1). The most common software for the analysis of telomere fluorescence is TFL-TELO developed by Lansdorp's group[10] and this software is available free of charge. The software simply combines images of chromosomes and telomeres, and generates a table with the values of telomere fluorescence for each chromosome, i.e., four telomeres/chromosome. In this software each telomere is assigned a different color and, also, p-arm and q-arm telomeres can easily be distinguished. The final product is the set of data containing 184 individual telomere fluorescence values (46 chromosomes $\times$ 4 telomeres). The files generated by TFL-TELO can be exported to any standard software for statistical analysis. Software packages other than TFL-TELO may be available for Q-FISH on a commercial basis.

To ensure reproducibility of measurements by Q-FISH, it is important to minimize sources of variation associated with digital fluorescence microscopy. The first potential source of variation is the length of exposure time required to acquire optimal images of telomeres. Software packages used for image acquisition usually have two options: auto-exposure and manual exposure. In the case of auto-exposure, the exposure time is determined automatically by the image-acquisition software based on the signal strength. In the case of manual exposure desired fixed-time exposure may be selected. Our experience is that if TFL-TELO is used for telomere fluorescence analysis then the appropriate fixed-time exposure selected

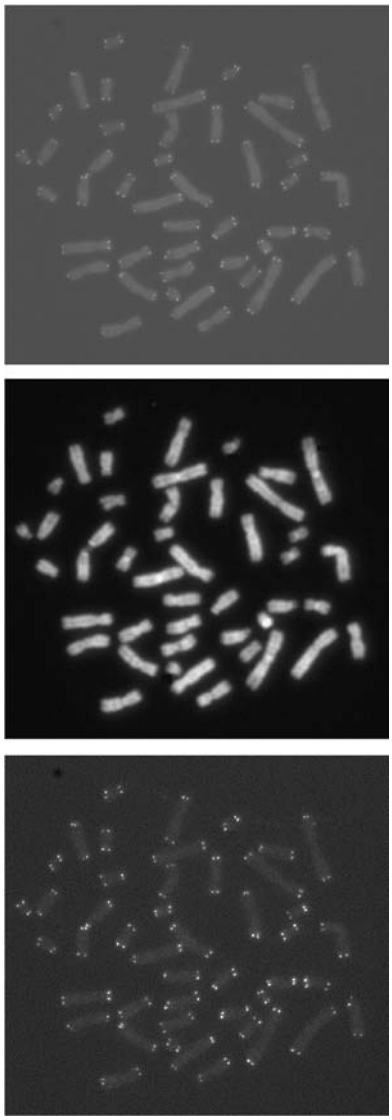

Fig. 1 A human metaphase cell. The top panel shows two-color images in which chromosomes are stained in blue (DAPI) and telomeres in red (Cy3 telomeric probe). The middle and bottom images are separate images of chromosomes and telomeres. (*View this art in color at www.dekker.com.*)

on the basis of trial and error should be used in all experiments. The second source of variation is associated with variability in microscope fluorescence lamp intensity. To avoid inaccuracies due to lamp intensity variations appropriate internal controls must be used. One option is to use fluorescence beads of defined size (i.e., 1 μm) and acquire images of these beads each time a new sample is analyzed. The values of bead fluorescence will then be used to correct the telomere fluorescence intensities of the samples under investigation. In the original Q-FISH protocol another calibration component was used, plasmids containing a defined number of telomeric sequence repeats.[8] This approach allowed for the direct conversion of telomere fluorescence into units of DNA length. Another appropriate internal control is the use of cell lines with known telomere length. In this case multiple images of metaphase chromosomes and telomeres from two cell lines with differing telomere lengths are acquired each time a new sample is analyzed, and these values are then used to generate two telomere fluorescence reference points.[11] The telomere fluorescence of the samples under investigation is then expressed relative to these two reference points.

Numerous studies demonstrated the value of Q-FISH. For example, Q-FISH was instrumental in demonstrating telomere dysfunction in mice lacking the RNA component of telomerase. These mice showed progressive telomere shortening which could only be detected by Q-FISH.[12] In addition, Q-FISH was instrumental in demonstrating 1) the heterogeneity of telomere length in individual human and mouse chromosomes;[8,13] and 2) that the shortest telomeres in the cell, not the average telomere length, drive genomic instability due to telomere dysfunction.[7] Finally, the Q-FISH procedure was recently adapted for telomere fluorescence analysis by flow cytometry and this new protocol was named flow-FISH.[14] Flow-FISH analyses the average telomere fluorescence in interphase cells and the advantage of this technique is that it can identify the average telomere length in a large number of cells with higher accuracy than Southern blot. In addition, flow-FISH is useful in identifying subpopulations of cells with differing telomere lengths, i.e., different subsets of peripheral blood lymphocytes. The most recent modification of flow-FISH is the use of multicolor flow-FISH in which different subsets of lymphocytes are detected by appropriate antibodies which must be attached to fluorochromes different from fluorochromes attached to the telomeric probe.[15] It is important to stress that Southern blot cannot reliably identify subpopulations of cells with differing telomere lengths in a given cell population. Although Q-FISH theoretically can identify subpopulations of cells with differing telomere lengths in a cell population of interest, in reality this technique is limited for this purpose because it relies on the information from only 10 to 15 cells. In contrast, flow-FISH can analyze thousands of cells in a short period of time.

CONCLUSION

Quantitative fluorescence in situ hybridization is the most sensitive method currently available for telomere length measurement. In contrast to the classical telomere length measurement based on Southern blot analysis, Q-FISH 1) identifies telomere length in each individual chromosome; 2) identifies the shortest telomere in the cell; and 3) does not take account of degenerate telomeric sequences present in subtelomeric regions of chromosomes which do not form a functional part of telomeres. The only disadvantage of Q-FISH is that it cannot reliably identify subpopulations of cells with differing telomere lengths and for that purpose it should ideally be combined with flow-FISH.

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FISH—Spectral Karyotyping and Multiplex FISH

Mario A. J. A. Hermsen
University of Oviedo, Oviedo, Spain

Rhona M. Anderson
MRC Radiation and Genome Stability Unit, Didcot, Harwell, Oxfordshire, U.K.

Gerrit A. Meijer
VU University Medical Center, Amsterdam, The Netherlands

Natalia D. Sumption
MRC Radiation and Genome Stability Unit, Didcot, Harwell, Oxfordshire, U.K.

INTRODUCTION

Analysis of the complex mechanisms that initiate neoplastic changes and drive the progression of precursor lesions to cancer is a cornerstone of cancer research. These mechanisms in general consist of a spiral of (epi)genetic alterations followed by altered gene expression patterns, which cause biological changes at the cell and tissue level. Ultimately, these changes lead to clonal outgrowths of cells that, because of genomic instability, are prone to further genetic alterations. In many types of cancer, most of these genomic alterations occur at the chromosomal level, either as numerical changes or as structural rearrangements, making chromosomal instability one of the key factors in tumor progression.

Analysis of *numerical* chromosomal aberrations has proven to be very informative, as can be deduced from the multitude of published studies based on heterozygosity (LOH), comparative genomic hybridization (CGH), and fluorescence in situ hybridization (FISH). However, to fully appreciate the role of chromosomal instability in tumor progression, *structural* aberrations also need to be taken into account.

OVERVIEW

Currently, structural chromosomal aberrations such as exchanges, deletions, and insertions are routinely assayed by G-band analysis because this technique remains the most efficient and cost-effective method for cytogenetic diagnostic purposes. There is a disparity, however, in how informative this type of analysis is according to the tissue of origin of the metaphase cells being studied. In particular, the complexity of structural aberrations in tumor cells coupled with poor quality in terms of chromosome length, morphology, and mitotic index has contributed to the fact that many recurrent and on-going cytogenetic rearrangements in tumor cells remain poorly defined. One approach taken to improve this is the painting of individual chromosomes using FISH-based methodologies, where the chromosomes selected for ''painting'' are determined based on the initial banding assessment. This strategy is practical and affordable for many cytogenetic services, but has the major disadvantage of only being informative for those chromosomes in the rearrangement that were selected for painting.

Major developments over recent years have changed this. In particular, advances in strategies for combinatorial fluorescent probe labeling and technology for the detection of multiple fluorescent dyes in single experiments have made the high-resolution genome-wide analysis of structural aberrations possible.[1] Below, two hybridization techniques are described which ''paint'' all 22 human autosomes and 2 sex chromosomes as unique identifiable colors: multiplex FISH (m-FISH)[2] and spectral karyotyping (SKY).[3] Both techniques are capable of resolving previously unidentified ''marker'' chromosomes (>10 Mb in size) and also subtle cryptic rearrangements. Importantly, compared with PCR and multiple-color FISH, m-FISH and SKY can be used to screen genome wide and do not require prior knowledge of chromosomal breakpoints.

The detailed analysis of structural chromosomal aberrations is therefore no longer restricted to constitutional anomalies, hematological malignancies, and soft tissue tumors, but is now also feasible for the much larger group of epithelial tumors. Preliminary experiments have

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revealed a high number of structural chromosomal aberrations in different types of solid tumors, indicating that such changes could play a much more important role in solid tumor carcinogenesis than thus far assumed.

MULTIPLEX FLUORESCENCE IN SITU HYBRIDIZATION

Multiplex fluorescence in situ hybridization was first described by Speicher et al.[2] and, like SKY, exploits combinatorial probe labeling to uniquely identify each of the human chromosome pairs. Where the two techniques differ is in the mode of fluorescence detection. For m-FISH, this is achieved using single band-pass filters that are specific for each fluorophore employed. Currently, the majority of commercially available probe sets contain five spectrally distinct fluorophores such that each chromosome is labeled down its whole length with one, two, or three different fluorophores. Consequently, only a proportion of the chromosomes is positively hybridized and therefore visible in any one fluorescence channel. Each channel is digitally captured in turn, with modifications to the excitation/emission filters, focal plane, and exposure times necessary between each fluorophore. Cells are also counterstained with DAPI and an image of this is captured to allow the visualization of the whole metaphase enabling the automated delineation of all the chromosomal material in the cell. Once this raw capture sequence has been completed, m-FISH dedicated software processes all six ''raw'' images by registering each image on top of each other (Fig. 1). It is this that produces the final merged or composite pseudo-color m-FISH image, where the color and therefore origin of chromosome represented is determined by the presence or absence of each of the five distinct fluorophores detected on a pixel-by-pixel basis. A combination of optimal FISH biology, hardware, focus, registration of raw images, and signal-to-noise ratio of each of the individual fluorophores used determined the quality of image produced. Therefore standard of analysis is tightly linked to the quality of each of the six raw images initially captured.

There are problems associated with the direct analysis of the final pseudocolor m-FISH image however. A chromosome is determined to be apparently normal if no color junctions are observed down its entire length, thus excluding the chance of detecting inversions, deletions, or homologous pair interactions, unless of course there are

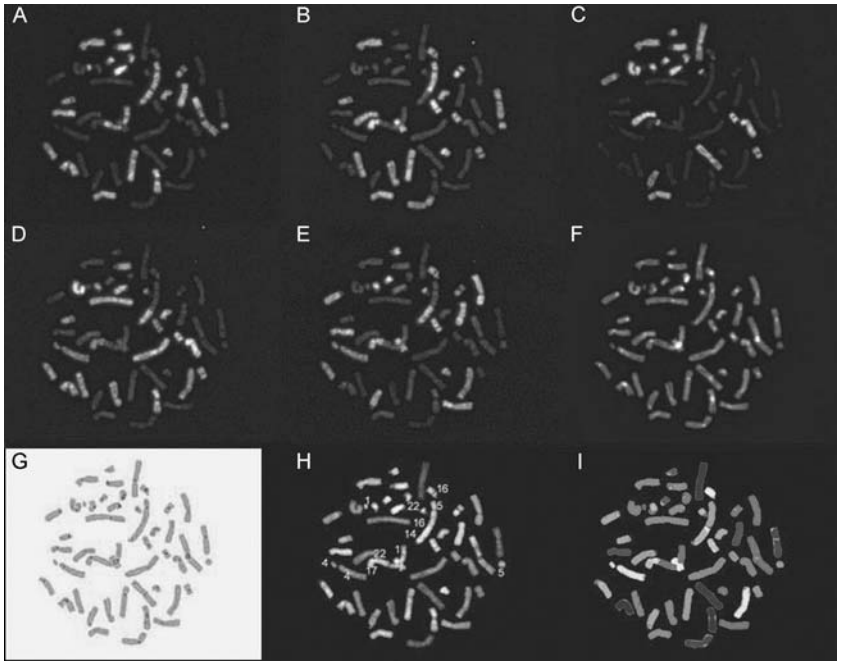

Fig. 1 M-FISH capture and analysis. Each fluorescent channel (A–E) and DAPI (F) is captured separately. Dedicated software processes each of these raw images to produce both a merged (H) and pseudo-color (I) composite. Each cell can be analyzed in detail by exploiting the individual images (A–J). This example shows a complex exchange in a human bone marrow CD34+ cell exposed in vitro to α-particle irradiation ($\sim$1 α-particle/cell). Chromosomes 1;5;14;16;17;22 have repaired as a single complex exchange, plus a deletion in 4p and a frag(4) can be seen. (*View this art in color at www.dekker.com.*)

obvious changes in centromere position or size of chromosome. In addition, depending on the fluorophore combination of chromosomes involved in an exchange, false colors can be produced at the junction as a consequence of flaring, resulting in the suggestion of an additional insertion. Technically though, it is possible to minimize such misclassification through the virtue that m-FISH compiles its pseudoimage via multiple captures. Before describing this in more detail, it is relevant to note that m-FISH is currently being applied for the successful analysis of radiation-induced chromosome aberrations where each of the damaged cells analyzed contains a unique abnormality.[4] Importantly, a number of different laboratories have verified the complexity of chromosome aberrations that can be detected at a single cell level using m-FISH, typically using cells of a similar quality and chromosome length to that seen in tumors.

The procedure of detailed (single-cell) m-FISH analysis is as follows: the DAPI raw image is examined allowing centromere and/or chromosome number to be counted and chromatid-type aberrations (relevant in ongoing genomic instability) to be scored. The chromosomes are karyotyped as enhanced DAPI, and obvious changes in banding pattern, especially involving intrachromosomal or homologous events, are noted. Each fluorescent channel is then assessed in turn looking for discontinuities in color down the length of the chromosomes, before, finally, the composite m-FISH image is assessed. Consequently, because the relative position of observed color junction breakpoints for each fluor is determined separately, the chance of bias from information displayed in the final pseudoimage is limited.

At a minimum, hardware consisting of a fluorescence microscope capable of holding six different epifluorescence filter cubes, camera, and a computer platform that supports dedicated software is required. Overall therefore, irrespective of whether the m-FISH multiplex probe sets are developed locally or obtained commercially, the establishment of an m-FISH facility does represent a significant investment.

SPECTRAL KARYOTYPING

Spectral karyotyping was first described in 1996 by Schröck et al.[3] Metaphase chromosomes are hybridized with the commercially available SKY probe mixture, consisting of 24 chromosome-specific probes. Each probe is differentially labeled with up to four different fluorophores. In contrast to m-FISH, only one image is captured. A fluorescence microscope, using a camera coupled to a spectral color analysis system, analyzes the spectrum from 450 to 750 nm for each pixel in the image. In this way, each (part of a) chromosome can be recognized by its own distinct color spectrum. With dedicated image analysis software, a final karyogram image is created showing all chromosomes with their unique spectral color (or false color if preferred), allowing easy identification of structural changes such as insertions and translocations. In addition, a DAPI image is captured to assess the band position of possible breakpoints (Fig. 2).

Because in SKY analysis the direct spectral image is used, the problem of flaring in the false color image, resulting in the suggestion of additional insertions, can be overcome. Similar to m-FISH, SKY does not detect inversions or duplications (unless there are obvious changes in centromere position or chromosome size) because these do not lead to a translocated color. In addition, small translocated chromosome parts (less than 1–3 Mbp) may be difficult to determine. Notwithstanding these limitations, several spectral karyotyping studies have shown its superior possibilities over conventional cytogenetic analyses. Comparisons showed up to 50% false interpretations with conventional cytogenetic analysis.[5,6] With the help of data from numerical chromosomal analyses, e.g., with array CGH,[7] the interpretation

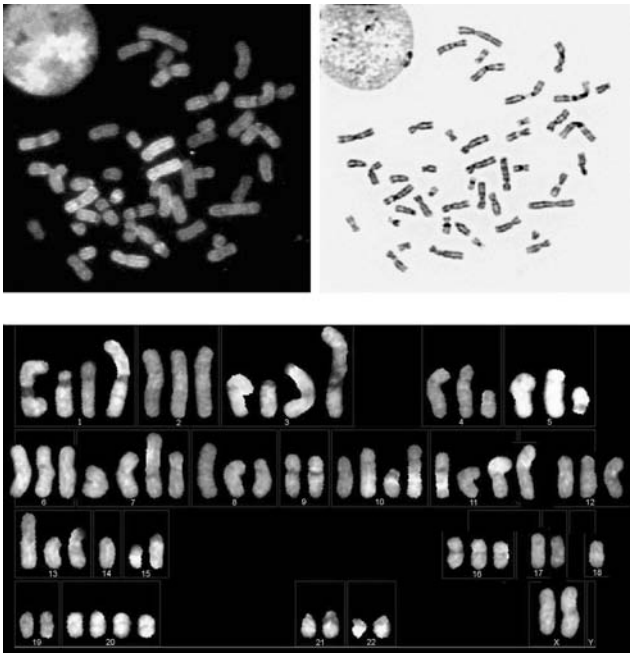

Fig. 2 Spectral and DAPI image of a normal human metaphase (top left and top right, respectively), and a spectral image showing the analyzed karyogram of scc078, an oral squamous cell carcinoma cell line (bottom). The karyogram shows a multitude of unbalanced translocated chromosomes. (*View this art in color at www.dekker.com.*)

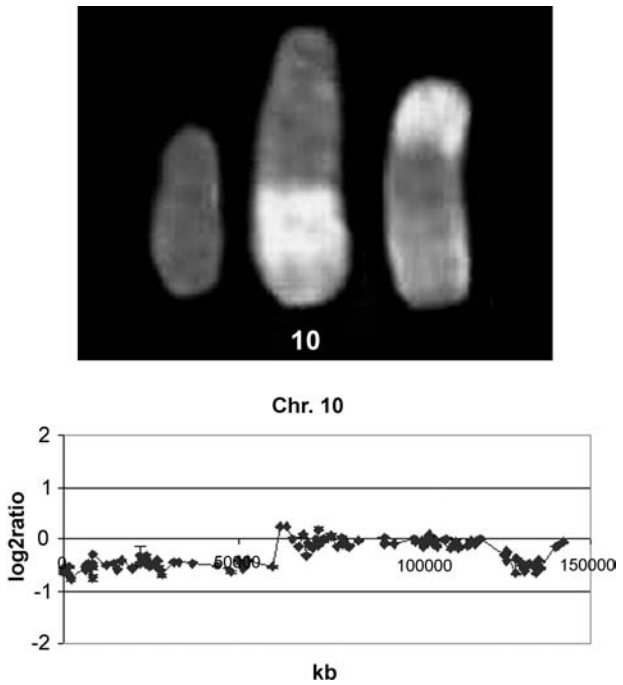

Fig. 3 Complementary information resulting from SKY analysis (left) and array CGH analysis (right) of chromosome 10 alterations in a triploid HPV-transformed keratinocyte cell line, FK18A. The SKY image shows one normal 10 (red), one 10 with a part of chromosome 20 (white) and 11 (green) attached to the q-arm, and one 10 with another part of chromosome 11 attached to the q-arm, and a whole arm 22q replacing the 10p arm. With the help of the array CGH data, it can be confirmed that the 10p arm is only present in 2 copies (in a triploid tumor, this is a loss), that part of the 10q arm is present in 3 copies (the normal number in a triploid tumor), and that the 10qter part, but not the telomere, is again only present in 2 copies. (*View this art in color at www.dekker.com.*)

of chromosome translocations can be improved further (Fig. 3). This illustrates that the respective molecular cytogenetic techniques should be regarded as complementary, providing overlapping and also unique information on the chromosomal abnormalities of tumors.[8,9]

The analysis starts with a background subtraction procedure and contrast improvement for the DAPI image. All chromosomes are checked and joined/overlapping chromosomes can be separated by the user. Subsequent karyotyping is performed by the program, based on the color-spectrum information, which may be corrected in an interactive fashion. Translocations are immediately obvious in the spectral image and may also be visualized in a false color. Using the DAPI image, translocations may be pinpointed to a specific chromosomal band.

A spectral karyotyping system consists of a high-quality fluorescence microscope (e.g., Leica RA) with a special filter set. A high-resolution CCD camera (1280×1024 pixels) and a special device for spectral analysis (SpectraCube™) sit above this. The system is completely integrated with and controlled by a high-performance Windows NT-based computer with dedicated control, imaging, and analysis software (SpectraView Software, Applied Spectral Imaging Inc., Israel) and includes printing and storage devices.

CONCLUSION

The implication of m-FISH and SKY for clinical diagnostics is that previously uncharacterized complex karyotypes and marker chromosomes are now able to be elucidated.[10] In addition, small translocations, as in constitutional anomalies, hematological tumors, and soft tissue tumors, can be identified more easily.[6] By rigorously analyzing all of the information available per cell, the operator not only minimizes error in classification, but also produces reliable and informative data even from those cases with poor mitotic index. Overall, the information gained from m-FISH and SKY, which is relevant for research as well as diagnostics and staging of disease, outweighs the cost and labor intensiveness of this technology.

The impact for health care of studying the role of structural chromosomal changes in cancer development and progression from precursor lesions to cancer is self-evident. Apart from the suffering it causes to individual patients and their families, cancer is one of the major causes of death, whereas cancer diagnosis and treatment consumes a major part of health-care budgets. Because progression of many types of cancer is driven by changes at the chromosomal level, a better understanding of these mechanisms may have a major impact on our ability to identify high-risk patients at an earlier stage. Moreover, knowledge of critical genomic alterations that drive tumor progression may also provide leads for the development of new anticancer drugs.

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Fluorescence Resonance Energy Transfer (FRET)

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Dino A. De Angelis
Memorial Sloan-Kettering Cancer Center, New York, New York, U.S.A.

INTRODUCTION

The phenomenon of fluorescence resonance energy transfer (FRET) was first described by Theodor Förster in 1948. Since then, FRET has become an incredibly useful tool in biology for three reasons: 1) FRET is really sensitive in the range of 100 Å and below, the scale at which transactions between biological macromolecules and complexes occur; 2) the instrumentation is extremely sensitive and is readily amenable to miniaturization, high throughput, and automation; and 3) cell permeable and genetically encodable FRET probes enable the real-time quantitation of dynamic cellular processes in live cells. In this chapter, we introduce the reader to the photophysics underlying FRET and present a brief general overview of the types of biological measurements enabled by the technique. Readers are referred to other chapters in the Encyclopedia for more detailed coverage of specific diagnostic applications of FRET (see for instance ''Molecular Beacons, 5′ Nuclease Oligoprobes'').

FLUORESCENCE

When photons are absorbed by a fluorescent compound (Fig. 1A), within femtoseconds it undergoes a transition from a singlet ground state (S_0) to an excited state which can be S_1 (thick line) or a higher vibrational energy level of S_1 (denoted by the thin lines above S_1). The excitation maximum of a particular fluorescent compound is the wavelength at which this process occurs most efficiently. Within picoseconds, molecules in higher vibrational states relax to the S_1 state (thick line).

Most fluorescent molecules remain in the S_1 state for 1–10 nsec on average, after which they can return to the S_0 ground state through one of a few different relaxation pathways.[1] The radiative relaxation pathway occurs through emission of fluorescent light by the excited fluorophore as it returns to the ground state, indicated by the wavy arrow in Fig. 1A. The light emitted is always of lower energy (longer wavelength) than that of the exciting light.

Several nonradiative pathways compete with fluorescence emission for return to the ground state. These nonfluorescent pathways (labeled NF in Fig. 1A) include internal conversion (a return to S_0 accompanied by release of heat), intersystem crossing (change in the electron-spin orientation resulting in relaxation to a long-lived triplet excited state before decaying to S_0), and collisional deactivation (involving direct encounters between the dye and neighboring solvent molecules). These deactivation pathways are inherent to fluorescent dyes in solution and are present to various extents.

RESONANCE ENERGY TRANSFER

Resonance energy transfer is also a nonradiative pathway, but it requires a stringent set of conditions, unlike the nonfluorescent deactivation pathways mentioned above. In resonance energy transfer, the excited dye is a FRET donor (D) which becomes deactivated via transfer of some of its excited-state energy to a compatible acceptor (A) molecule (Fig. 1B). Because it does not involve the emission of photons from the donor, FRET is more correctly referred to as ''Förster resonance energy transfer,'' or simply RET.[2] Efficient energy transfer from excited D to A requires that 1) the fluorescence emission spectrum of D and the absorption spectrum of A be overlapping (resonant frequencies as shown in Fig. 2A); 2) the distance between D and A be within 10 to 100 Å (1–10 nm); 3) the dipole moments of D and A be approximately parallel to one another. If A is also a fluorescent molecule, FRET results in an increase in fluorescence emission from A (Fig. 1B, indicated by the wavy arrow depopulating A*) in addition to the decrease in fluorescence emission from D. If A is a nonfluorescent molecule, the net result of FRET is a decrease in fluorescence emission from D. In this case, A is a fluorescence quencher and A* becomes exclusively depopulated by nonfluorescent pathways (Fig. 1B, dashed arrow).

DISTANCE DEPENDENCE OF FRET

Fluorescence resonance energy transfer efficiency is inversely proportional to the sixth power of the distance between a donor and acceptor, and therefore is extremely

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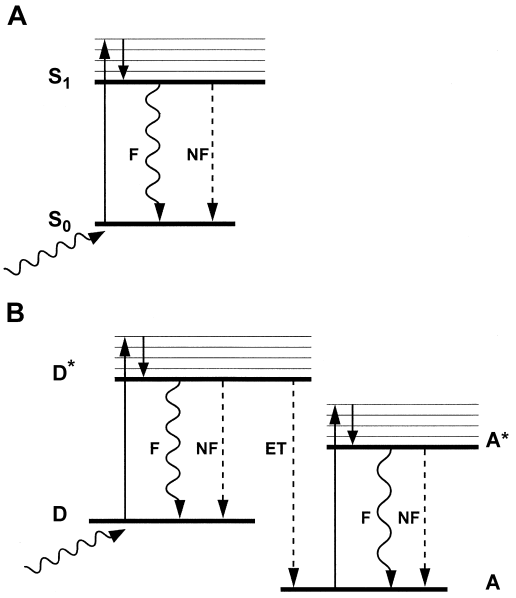

Fig. 1 A) General mechanisms of excited-state deactivation of a fluorophore. Up arrow represents transition from ground state (S_0) to an S_1 excited state (thick line) or higher (thin lines) after photon absorption (wavy upward arrow). Down arrows represent relaxation, first from higher states down to S_1 (short arrow). Relaxation from S_1 to S_0 involves either fluorescence emission (F, wavy down arrow) or nonfluorescent mechanisms (NF, dashed down arrow). B) Mechanisms of excited state deactivation of a donor fluorophore in the presence of an acceptor. Same as A except that energy transfer (ET) is also displayed (longer dashed arrow) which results in less fluorescence emission from the donor excited state (D*) and activation of acceptor molecule from ground state (A) to excited state (A*). Excited state of A can also be depopulated via fluorescent (F) or nonfluorescent (NF) mechanisms.

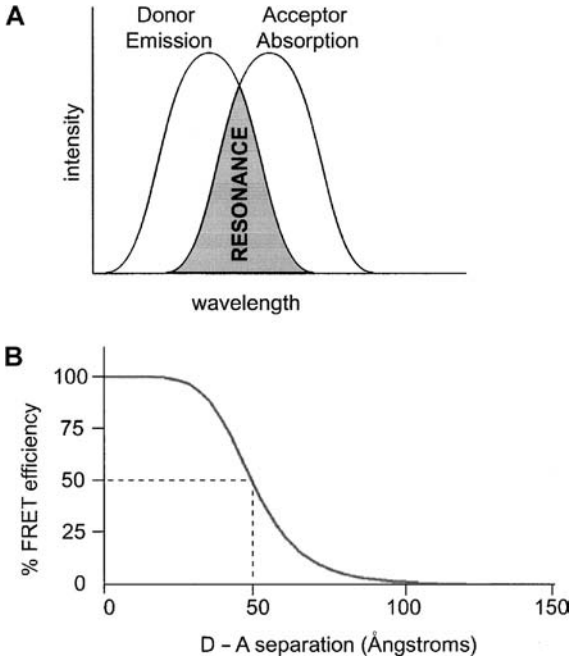

Fig. 2 A) Fluorescence intensity plotted as a function of donor emission and acceptor absorption wavelengths, displaying a spectral overlap required for resonance energy transfer. B) FRET efficiency plotted as a function of the distance between a donor and acceptor pair characterized by a R_0 of 50 Å.

sensitive to their intermolecular separation. This is illustrated in the equation:

$$E(r) = (R_0)^6/[(R_0)^6 + r^6]$$

where E is FRET efficiency, r is the distance separating D from A, and R_0 is the Förster distance. At the Förster distance, 50% of excited D is deactivated via FRET (ET in Fig. 1B), and the remainder is deactivated via fluorescence (F) and other nonfluorescent (NF) pathways. The Förster distance varies between different D–A pairs, typically ranging from 25 to 75 Å (Table 1). R_0 takes into consideration the amount of spectral overlap between the dyes and assumes that donors and acceptors are randomly oriented. For a donor–acceptor pair with an R_0 of 50 Å, doubling the intermolecular separation from 50 to 100 Å results in a 32-fold decrease in FRET efficiency (Fig. 2B).

Distance measurements obtained by monitoring FRET efficiency typically represent an average (rather than absolute) distance between D and A, because of the assumption of random orientation in the calculation of R_0, and the fact that biological macromolecules usually fluctuate between thermodynamically equivalent states. Technicalities aside, the exquisite dependence of FRET on distances of the scale of biological macromolecules (Table 2) underscores the real usefulness of the technique: detecting biological phenomena involving changes in the

Table 1 Förster distances of commonly used FRET pairs

Donor	Acceptor	R_0 (Å)
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46
EDANS	Dabcyl	33
Alexa Fluor 488	Alexa Fluor 546	64
Alexa Fluor 488	Alexa Fluor 555	70
Alexa Fluor 488	Alexa Fluor 568	62
Alexa Fluor 488	Alexa Fluor 594	60
Alexa Fluor 488	Alexa Fluor 647	56
Fluorescein	QSY 7 and QSY 9 dyes	61

Source: Haughland, R.P. Handbook of Fluorescent Probes and Research Products, Ninth Edition, 2002, Molecular Probes Inc.

Table 2 Dimensions of different biological macromolecules in Ångstroms

Molecular feature	Distance in Ångstroms
Carbon–carbon single bond	1.5
1 turn of alpha helix (3.6 amino acid residues)	5.4
Diameter of DNA double helix	22
1 turn of DNA double helix (10 bp)	34
Diameter of a 26-kDa globular protein	40
10 turns of alpha helix (36 amino acid residues)	54
F-Actin filament diameter	60
Eukaryotic plasma membrane thickness	75
Microtubule diameter	250

proximity of two molecules (or two portions of the same molecule) respectively labeled with D and A.

DETECTION OF FRET

A wide array of phenomena can be measured using FRET, and they tend to fall into either of two main categories: 1) homogeneous in vitro assays performed in a spectrofluorimeter or microplate reader and 2) live cell FRET imaging performed by fluorescence microscopy. The basic requirements for the detection of FRET in these two domains are the same: 1) a light source for donor excitation; 2) wavelength filters to spectrally separate excitation photons (originating from the light source) from emission photons originating from both D and A; 3) detectors that can separately register emission photons from D and A as two electrical signals (spectrofluorimeter) or as two digital images (fluorescence microscopy).

IN VITRO: HOMOGENEOUS FRET

Spectrofluorimeters measure the fluorescence properties of homogeneous samples in cuvettes or in multiwell dishes. For a FRET experiment, the light source illuminates at a wavelength intended to excite the donor but not the acceptor. The detectors register photons emitted by both D and A. When D and A are much further apart than the R_0 characterizing the pair, emission from D is much higher than emission from A. The D/A emission ratio is therefore high (excited D deactivates preferentially with the fluorescence emission pathway). In contrast, when D and A are within Förster distance of one another, the D/A emission ratio decreases as D transfers a fraction of its excitation state energy to A, causing less fluorescence emission from D and more fluorescence emission from A. In cases when A is a fluorescence quencher, proximity between D and A simply results in decreased emission from D.

Binding reactions between two molecular entities (receptor–ligand; antibody–antigen; transcription factor–DNA sequence) respectively labeled with a donor and an acceptor can be quantified by FRET.[3] The labeling can be direct (via covalent modification of the molecules of interest) or indirect (i.e., via noncovalent binding of a labeled antibody or other binding moiety). Binding will result in energy transfer from the donor to the acceptor (Fig. 3A).

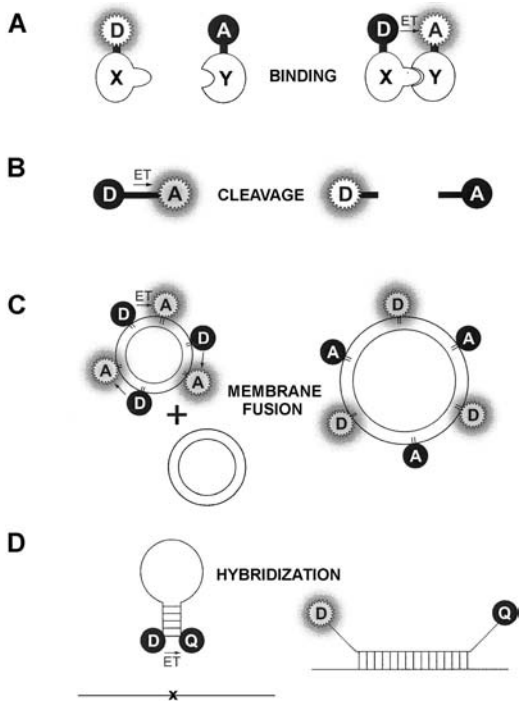

Fig. 3 Types of molecular interactions measured by FRET. A) Binding of two molecular entities X and Y, respectively labeled with donor D and acceptor A results in FRET (ET). B) Cleavage of D- and A-labeled peptide or nucleic acid sequence via protease or endonuclease results in decrease in the FRET signal. C) Fusion of liposomes containing a high concentration of D- and A-labeled phospholipids with unlabeled liposomes results in dilution of D and A and decrease of FRET in the newly fused membrane. D) Molecular beacons oligonucleotide probes adopt a stem and loop structure in the absence of a perfectly matching target DNA sequence, resulting in FRET between donor D and nonfluorescent acceptor Q (quencher). Perfect match results in hybridization of loop sequence to the target DNA and increased separation of D from Q.

Peptides containing a specific protease cleavage site, and labeled with D and A at either termini, can serve as FRET sensors.[4] Fluorescence resonance energy transfer occurs in the intact peptide but incubation with the protease separates D from A causing a decrease in the FRET signal (Fig. 3B). The activity of endonucleases can similarly be detected using a dual-labeled nucleic acid sequence.

Membrane fusion assays can also be performed using FRET, by incorporating phospholipids labeled with D and A moieties into liposomes at relatively high concentrations.[5] Because of molecular proximity, D and A undergo FRET in this liposome population. Fusion of such liposomes with unlabeled liposomes creates larger liposomes in which FRET is decreased due to the increase in average distance between labeled probes via dilution (Fig. 3C).

Molecular beacons are FRET probes that can adopt one of two very different conformations depending on the presence of specific target DNA sequences in a sample (described in more detail elsewhere in this volume, see also Refs. [6] and [7]). The technique is so sensitive that it can resolve single-base pair mismatches. In the absence of a perfectly matching sequence, the molecular beacon adopts a hairpin-like structure (Fig. 3D) which brings the donor- and acceptor-labeled ends of the molecule into close proximity. The acceptor is a quencher (Q) in these probes, therefore very little fluorescence is emitted by the probes in this conformation. In the presence of the exact match, the stem structure opens up, allowing the loop to hybridize to the perfectly matched target and increasing the distance between D and Q.

These are just a few illustrations of diagnostic in vitro assays performed using FRET probes. They are typically easy to set up and can be automated; some are known as ''walkaway'' assays because no further processing of the sample is required following the initial incubation of reagents. The extent of the reaction (whether binding, cleavage, membrane fusion, or other) can be modulated by adding competitors, agonists, antagonists, or other test compounds, either in purified form or from crude extracts, and their effect on the assay can be measured. The kinetics of any assay can be monitored by collecting the emission from D and A continuously or at timed intervals, without interrupting the reaction. In addition, because D–A pairs can be spectrally quite different from one another, several probes can be monitored simultaneously in the same reaction (multiplexing; see Ref. [7]).

In homogeneous FRET, background noise derived from sample autofluorescence can limit the sensitivity of the technique. Two related techniques extend the sensitivity range of FRET measurements by radically reducing noise in the system. Time-resolved FRET (TR-FRET) uses fluorescence donors with unusually long fluorescence lifetimes (400 nsec to 2 msec for chelates of ruthenium and lanthanides compared to 10 nsec for typical fluorescent molecules).[8] Time-resolved FRET delays the collection of light by the detector, allowing any fluorescence that is not derived from the long lifetime donors in the sample—including noise—to decay to zero. The slower decaying donor-specific fluorescence (or FRET signal) can therefore be measured in the absence of background.

Another way to reduce background fluorescence in FRET measurements is by omitting the light source altogether. Bioluminescence RET (BRET) uses a luciferase as a donor which produces light only when the substrate (coelenterazine) is added to the reaction. When bioluminescence is produced in close proximity to the acceptor, energy (not photons) is transferred resulting in an increase in the acceptor fluorescence. As there is no extrinsic illumination to activate sample autofluorescence, BRET can increase the sensitivity of donor–acceptor interactions by an order of magnitude compared to FRET.[9]

IN VIVO: LIVE-CELL FRET IMAGING

Fluorescence resonance energy transfer measurements are not limited to in vitro assays. Cell-permeable FRET

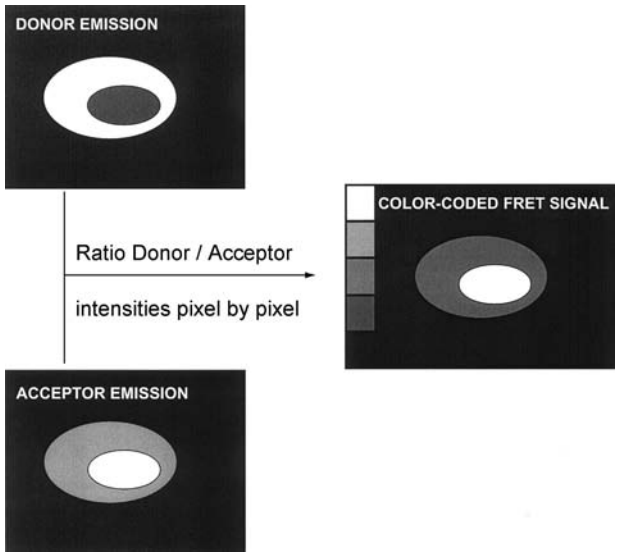

Fig. 4 Fluorescence resonance energy transfer imaging of live cells. Pair of digital images representing collection of light from the donor emission (top left image) and acceptor emission (bottom left image) following illumination of a sample at the donor excitation wavelength. The intensity of pixels from the donor emission image is divided by the intensity of pixels from the acceptor emission image via software and a color-coded map of the ratios is generated (right). In this diagram there is a high FRET signal in the nucleus (low donor intensity/high acceptor intensity).

probes as well as the green, cyan, and yellow fluorescent proteins (GFP, CFP, and YFP), introduced in cells by DNA transfection, have permitted the study of dynamic events involving changes in the proximity of macromolecules in live cells. Sensors based on GFP variants can be targeted to any compartment for which there is a known signal sequence, enabling local measurement of the activity under study. In FRET imaging, light required to excite the donor is focused by a microscope lens onto a live cell sample. Photons emitted from the donor and the acceptor are respectively collected as two digital images (Fig. 4). The intensity levels of the two images are then ratioed on a pixel-per-pixel basis, yielding a two-dimensional map of the D/A emission ratio for the particular time point. The sample can be imaged over time and thus the spatio-temporal features of the FRET signal can be analyzed. By enabling the analysis of phenomena in the range of Förster distances (1–10 nm), FRET imaging goes beyond the limits resolution of light microscopy ($\sim$200 nm).

The binding of two different proteins respectively labeled with CFP and YFP (the pair with the best spectral overlap characteristics for FRET) can be measured kinetically by measuring the amount of FRET they undergo. Protease activity can likewise be monitored in live cells by placing YFP and CFP at each extremity of a cleavable peptide. A powerful class of sensor inserts a conformationally active peptide between a CFP–YFP pair. The peptide can adopt different conformations depending on the environment, thus changing the distance and angle between the fluorescent proteins and yielding differential FRET signals. The detection of fluctuations in intracellular Ca^{2+} concentration was the first demonstrated application of this class of sensors, and the list has grown since (see Ref. [10] for a review).

For microscopy measurements, high levels of cellular autofluorescence can be compensated for by using an unlabeled region of the cell as an internal reference; this background is subtracted from the signal in the region of interest prior to calculating FRET. A more serious problem is photobleaching of the donor—basically, a destruction of the fluorophore caused by overillumination which is common in microscopy because the excitation light scans the same sample area repeatedly. Under photobleaching conditions, the efficiency of FRET undergoes an apparent decrease over time as the donor fluorophore is unable to repopulate its excited state. This is donor dependent, but can be corrected for by establishing the rate of photobleaching in a control sample, and normalizing the FRET signal obtained in the test sample to this baseline.[10] Bioluminescence RET measurements (discussed in ''In Vitro: Homogeneous FRET'') can also be performed in live cells; these experiments do not suffer from photobleaching because all of the light is generated via a bioluminescent reaction.[9]

CONCLUSION

This brief survey has only scratched the surface with respect to the breadth and power of FRET for the analysis of binding, dissociation, hybridization, and other phenomena involving changes in the proximity of biological molecules. Development of FRET-based sensors continues unabated, and so is the development of instrumentation designed to quantify FRET—with a trend toward miniaturization and high-throughput operation to enable diagnostic use of the technology on a genomic scale.

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Fluoroquinolone Resistance-Associated Gene Mutations in *Streptococcus pneumoniae*

George G. Zhanel
Heather J. Smith
University of Manitoba, Winnipeg, Manitoba, Canada

INTRODUCTION

Fluoroquinolones are increasingly used in the empiric therapy of community-acquired respiratory infections likely caused by *Streptococcus pneumoniae* because of the rise in resistance to β-lactams and macrolides. However, fluoroquinolone resistance in *S. pneumoniae* has recently emerged. Fluoroquinolone resistance in *S. pneumoniae* is mediated by two main mechanisms: spontaneous point mutations in the fluoroquinolones' target enzymes (DNA gyrase and topoisomerase IV) and by active efflux. This article focuses on the chromosomal mutations associated with fluoroquinolone resistance in *S. pneumoniae*, their frequency, and the impact of these mutations on clinical outcomes. Automated DNA sequencing methods are usually used to identify fluoroquinolone resistance mutations, but new techniques such as PCR–oligonucleotide ligation assays are currently being evaluated.

HISTORY OF FLUOROQUINOLONES

A novel class of antibiotics, quinolones, originated with the discovery of nalidixic acid (actually a naphthyridine) in 1962.[1] Nalidixic acid displayed good activity against gram-negative aerobes; however, its use was limited because it displayed poor activity against gram-positive organisms, demonstrated poor pharmacokinetics, and had numerous adverse affects.[1] Structural alterations of nalidixic acid resulted in the discovery of the fluoroquinolones, norfloxacin and ciprofloxacin, in the 1980s.[1] Norfloxacin and ciprofloxacin displayed a broader spectrum of activity against both gram-negative and gram-positive organisms and were much more potent than their progenitor antibiotics.[1] Improvements have continued to be made to the fluoroquinolone class via the addition of various molecular substituents to the basic quinolone structure. The newer ''respiratory'' fluoroquinolones: gatifloxacin, gemifloxacin, levofloxacin, moxifloxacin, and the investigational des-fluoroquinolone garenoxacin, demonstrate enhanced activities against gram-positive organisms such as *S. pneumoniae* as well as improved pharmacokinetic properties.[1,2]

FLUOROQUINOLONE CELLULAR TARGETS

Fluoroquinolones function by inhibiting two type 2 topoisomerases: DNA gyrase and topoisomerase IV.[1–3] Type 2 topoisomerases cause staggered single-stranded breaks in both strands of the bacterial chromosome, pass an intact segment of the DNA strand through the break, and reseal the DNA.[1,3–5] Fluoroquinolones bind to the DNA–enzyme complex containing the broken DNA strands forming what is termed the cleavable complex.[3,5–7] DNA repair and replication are inhibited and the resulting release of double-stranded DNA breaks accounts for the bactericidal activity of the fluoroquinolones.[1,3]

DNA gyrase is an essential bacterial enzyme formed as a tetramer of two GyrA and two GyrB subunits (A_2B_2) encoded by *gyr*A and *gyr*B, respectively.[1,3–6] DNA gyrase removes superhelical twists ahead of the replication fork and introduces negative supercoils into DNA, which are necessary for replication initiation. Topoisomerase IV is a tetramer formed of two ParC and two ParE subunits (C_2E_2), encoded by *par*C and *par*E, respectively, that share significant homology with the subunits of DNA gyrase.[1,3–6] Topoisomerase IV's cellular function is the decatenation of sister chromatids during the segregation of replicated chromosomes.[1,3,5]

It was initially believed that the primary target for fluoroquinolones in all gram-negatives, such as *Escherichia coli*, was DNA gyrase, whereas the primary target in all gram-positives, such as *Staphylococcus aureus*, was topoisomerase IV.[1,4] However, it has now been demonstrated that in certain bacterial species, such as *S. pneumoniae*, the primary bacterial target is dependent on the specific fluoroquinolone structure.[1,4,7]

FLUOROQUINOLONE RESISTANCE ASSOCIATED GENE MUTATIONS

Fluoroquinolone resistance ensues from spontaneous point mutations in the quinolone-resistance determining regions (QRDRs) of DNA gyrase and topoisomerase IV. In *S. pneumoniae*, resistance mutations occur most

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Table 1 Fluoroquinolone MIC increase associated with amino acid substitutions in the QRDRs of ParC and GyrA of *S. pneumoniae*

Amino acid alterations		Fold MIC increase from wild type				
GyrA position	**ParC position**	**Ciprofloxacin**	**Gatifloxacin**	**Gemifloxacin**	**Levofloxacin**	**Moxifloxacin**
—	Ser79Phe	4–8	4	2–4	2–8	2
—	Ser79Tyr	4–16	4	2–4	2–8	2
Ser81Phe	—	2	2–8	2–4	2	2
Ser81Tyr	—	2	2–4	2–4	2–8	2
Ser81Phe	Ser79Phe	16	16–32	8–32	16	ND
Ser81Phe	Ser79Tyr	32–64	16	4–8	16–32	16
Ser81Tyr	Ser79Tyr	32–64	8	4–8	8–16	8

ND, no data.
Source: Refs. [3,5,9].

commonly in *gyr*A and *par*C.[1,6–10] Mutations are occasionally reported in *gyr*B and *par*E, but their contribution to resistance appears to be limited and remains controversial.[1,6–8,10]

GyrA Fluoroquinolone Resistance Mutations

The QRDR in GyrA encompasses the region from amino acid 67 to 106.[3] Resistance occurs when amino acids near GyrA's putative active site, Tyr-122, are altered.[3] The specific GyrA amino acids commonly altered in *S. pneumoniae* fluoroquinolone-resistant isolates are Ser-81 and Glu-85.[1,3,4,8,10] These amino acids are near Tyr-122 in the primary sequence.[4] The mutation of Ser-81 to a tryptophan residue reduces the binding affinity of fluoroquinolones for the gyrase complex.[4] Substitutions of phenylalanine and leucine at position 81 are also prevalent.[1,3,4,8,10] Glu-85 can be mutated to either a lysine or glycine residue.[3,4,8,10] Lysine's positive charge and the steric hindrance resulting from the bulkiness of its side chain may reduce the ability of fluoroquinolones to bind to their targets.[8] The result of all the amino acid substitutions aforementioned for Ser-81 and Glu-85 may be the alteration of the quinolone-binding site structure in the DNA gyrase–DNA complex.[4] A reduced binding affinity for the modified enzyme–DNA complex may be the cause of fluoroquinolone resistance.[4]

ParC Fluoroquinolone Resistance Mutations

Similar to GyrA, mutations in the QRDR of ParC alter the quinolone-binding site structure located at the interface of topoisomerase IV and the DNA.[4] The altered structure results in reduced fluoroquinolone binding affinity for the enzyme–DNA complex.[4] The ParC amino acids commonly associated with fluoroquinolone resistance in *S.pneumoniae* are Ser-79 and Asp-83.[1,3,4,8,10] Ser-79 can be mutated to a phenylalanine or tyrosine residue.[1,3,4,8,10] Asp-83 can be substituted by alanine, glycine, asparagine, threonine, or tyrosine.[3,8,10] Ser-52 to glycine and Lys-137 to asparagine substitutions have also been reported, although these do not appear to contribute to increased minimum inhibitory concentration (MIC) values.[8] The MIC increases resulting from the development of *gyr*A and *par*C mutations are displayed in Table 1.

FREQUENCY OF RESISTANCE

Generally in *S. pneumoniae*, *par*C mutations are observed alone in isolates with low-resistance MICs (ciprofloxacin MIC ~4 μg/mL). As the MICs increase, isolates usually have mutations in both *par*C and *gyr*A. The presence of resistance mutations (in ParC, GyrA, and ParC and GyrA) and active efflux based on ciprofloxacin MICs is demonstrated in Fig. 1. The most common mutation in ParC is Ser-79-Phe.[1,10] Of all isolates with a ParC

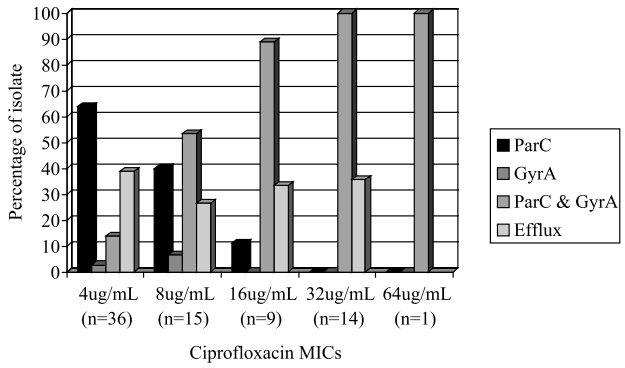

Fig. 1 Resistance mechanisms, QRDR mutations and efflux, identified in 75 ciprofloxacin-resistant *S. pneumoniae* at MICs of 4, 8, 16, 32, and 64 μg/mL (number of isolates per MIC). (From Ref. [13].)

mutation, it has been reported that 50–62.5% have the Ser-79-Phe substitution.[8,10] Likewise, Ser-81-Phe represents 69.2–78.6% of all GyrA changes.[8,10]

CLINICAL IMPLICATIONS

As the prevalence of fluoroquinolone resistance increases, clinical failures during empiric treatment are being reported. Various accounts have begun to emerge of levofloxacin failure during treatment of pneumococcal pneumonia.[6,11] Three of the depicted patients had a history of recent fluoroquinolone treatment.[6,11] The *S. pneumoniae* responsible for this fluoroquinolone failure was subsequently found to have QRDR substitutions in both ParC (Ser79Phe) and GyrA (Ser81Phe/Tyr, Glu85Lys).[6,11] These reports suggest that recent (within the last 3 months) fluoroquinolone therapy must be considered during treatment decisions.[6,11] Two other patients were described who had no history of fluoroquinolone therapy.[11] For one patient, the initial isolate had no detected substitutions in ParC or GyrA; however, following therapy, the resulting isolate had mutations in both ParC (Ser79Phe) and GyrA (Ser81Phe).[11] The other patient's initial isolate was considered susceptible but was found to have a Ser79Phe substitution in ParC.[11] After levofloxacin therapy, the isolate had mutations in both ParC (Ser79Phe) and GyrA (Ser81Phe).

The increasing use of fluoroquinolones as empiric therapy of community-acquired respiratory infections likely caused by *S. pneumoniae*, the rise in fluoroquinolone resistance, and these reports of levofloxacin therapy failure indicate that fluoroquinolone susceptibility testing should be routinely conducted. This could reduce treatment failures in cases in which the infecting isolates have high MICs resulting from mutations in ParC and GyrA. Unfortunately, it has been shown that susceptibility testing is not able to identify isolates containing only a ParC mutation.[7] These are the isolates that are most clinically disconcerting as the acquisition of a second mutation, and the resultant rise to high-level resistance MICs, occurs much more readily than a primary mutation.[7] It has recently been reported that 19% and 60% of *S. pneumoniae* isolates with levofloxacin MICs of 1 and 2 μg/mL, respectively, have ParC mutations.[12] These isolates are considered susceptible and the mutations would not be identified throughout susceptibility testing. Even with routine fluoroquinolone susceptibility testing, these isolates would be considered susceptible, treated with fluoroquinolones, and provide an opportunity for rapid development of highly fluoroquinolone resistant *S. pneumoniae* isolates. Thus, routine fluoroquinolone susceptibility testing should be conducted to reduce treatment failures resulting from *S. pneumoniae* isolates with substitutions in ParC and GyrA and additional testing methods should be developed that can rapidly identify isolates with only ParC mutations.

MOLECULAR TECHNIQUES FOR RESISTANCE DETECTION

Currently Employed Detection Method

The current method used in the detection of QRDR mutations causing fluoroquinolone resistance is automated DNA sequencing. Most studies amplify only the QRDRs of ParC and GyrA, whereas other studies also examine the QRDRs of ParE and GyrB. There are various published primer sets and amplification conditions used in these studies.[2,7,8,10] After purification and quantitation of the amplification products, the sequencing reactions are conducted.[10,12,13] DNA sequencing is performed using an ABI PRISM BigDye Terminator kit and run on an ABI PRISM sequencer.[2,7,10,12,13]

Novel Detection Methods

As previously mentioned, the utility of susceptibility studies for fluoroquinolone resistance mutation detection has been evaluated. Although this method was able to correctly identify isolates without resistance mutations and those with mutations in both ParC and GyrA, it was unable to identify isolates with only ParC mutations.[7] Susceptibility testing as a method of fluoroquinolone resistance detection is not sufficient for clinical use as *S. pneumoniae* isolates with ParC mutations are highly clinically significant.

A novel detection method for QRDR mutations has recently been developed: PCR–oligonucleotide ligation assays. Similar to sequencing studies, this method uses PCR to amplify the QRDRs of ParC and GyrA.[2] Oligonucleotide ligation assays are conducted and the specific mutations are detected colometrically.[2] This method correctly identified the majority (98%) of wild-type isolates and isolates with QRDR mutations in one or both target genes.[2] The substitutions correctly identified by this technique include the most common QRDR mutations (ParC: Ser79Phe/Tyr, Asp83Tyr/Val/Asn; GyrA: Ser81Phe/Ala/Tyr, Glu85Lys).[2] Rare mutations were also reported to be identifiable by this method.[2] The PCR–oligonucleotide ligation assay is much more rapid than DNA sequencing because it requires 12 hr for completion as opposed to 33 hr.[2] This novel, rapid, and accurate method is a good advancement in the detection of fluoroquinolone resistance mutations. However, the robustness of PCR–oligonucleotide ligation assays must be confirmed with

studies of many more isolates to become as accepted as the DNA sequencing gold standard.

CONCLUSION

Fluoroquinolone resistance in *S. pneumoniae* results from spontaneous point mutations in GyrA and ParC. The use of fluoroquinolones in the empiric treatment of community-acquired respiratory infections likely caused by *S. pneumoniae* and resistance to fluoroquinolones is increasing. Treatment failures with fluoroquinolones, such as levofloxacin, have begun to occur. Accordingly, routine fluoroquinolone susceptibility testing should be conducted to identify resistant organisms with substitutions in both ParC and GyrA so that other antimicrobials can be selected for treatment of these isolates. Most importantly, novel, rapid methods of detecting *S. pneumoniae* isolates with ParC mutations alone must be developed to limit the fluoroquinolone exposure of these isolates in hopes of reducing the development of high-level fluoroquinolone resistance.

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Forensic DNA Samples—Collection and Handling

Mark Benecke
International Forensic Research & Consulting, Cologne, Germany

INTRODUCTION

Under dry and cool conditions, DNA is a relatively stable molecule. From a technical and criminalistic point of view, DNA can be collected and stored like most visible biological stains. Crucial steps are examination of evidence including photographic documentation, and storage under dry and cool conditions. Special aids such as sexual assault kits, swabs, drying devices, and filter paper treated with denaturants are available and should be used. However, DNA collection in forensic environments is not a merely technical but also a criminalistic task. Two questions are of special importance: 1) whether a stain is of relevance for the actual crime, e.g., if it could have been left at the scene some time ago by persons who are not related to the crime, and 2) if a stain should be used for extraction straight away, or stored as long as possible for morphological measurements and crime (scene) reconstruction, e.g., the form of blood stains on wallpaper, the exact location of sperm stains on clothing, or the exact location of skin cells found on furniture.

EVIDENCE EXAMINATION

Irrespective of possible chain of custody rules, examination of evidence starts with photographic and/or drawn descriptions of the items received by the forensic biologist. In every photograph, an absolute scale must be visible (millimeters/centimeters; no pennies, no matches). Resolution should be $\geq 2816 \times 2112$ pixels=4 MPixel to allow blowing up of the pictures. Flash light should be avoided because brighter parts of the objects will flash out (become white; a digital artefact).

Biological stains that were detected either by their surface properties (detection by touch: e.g., sperm stains on dark clothing), monochromatic light (e.g., saliva), or regular bright light (e.g., hair or small blood stains) are circled and numbered by use of a water-resistant pen or neon color.

COLLECTION OF BIOLOGICAL STAINS

Swabs

Practically all stains can be collected by rubbing them off with a cotton swab. Stains on fabric should be cut out first. Swabs are soaked with one drop of fresh distilled sterile water. After transfer of the stain to the swabs, they must be dried immediately. Then, they are put in paper containers. The most convenient way to dry swabs is to put them straight into a closed cardboard box at room temperature (Fig. 1). There, they can neither touch neighboring objects nor develop mold.[1]

In professional forensic environments, contamination caused by airflow during the drying process has not been reported to be a problem. Some laboratory manuals ask for drying in closed cupboards (Fig. 2) or under sterile laminar airflow. Cupboards must never tightly close to avoid building up of humidity and mold.

Swabbing is performed by *intense*, *multiple* rubbing of the stained surface to collect a maximal amount of DNA. Inside of oral cavities, the cotton swab is rubbed against the mucous membrane; saliva alone may not contain enough cells.

After complex shooting situations, used bullets can be matched to the victims by swabbing off traces of tissue that remain on the bullet once it enters the body.[2]

Early Swabbing

Swabbing of clothing items, especially of skin, should be performed as soon as possible in forensic and police investigations. For example, DNA typing was possible in the following cases where swabs had been collected early at the scene of the crime. Before swabbing, intelligent criminalistic assumptions concerning the location of the invisible yet possible stains had been made.

- Epithelial cells of an unknown suspect were swabbed off the front side of a collar of a polo-neck pullover. The victim had been stabbed; the stains had not been visible on the collar.

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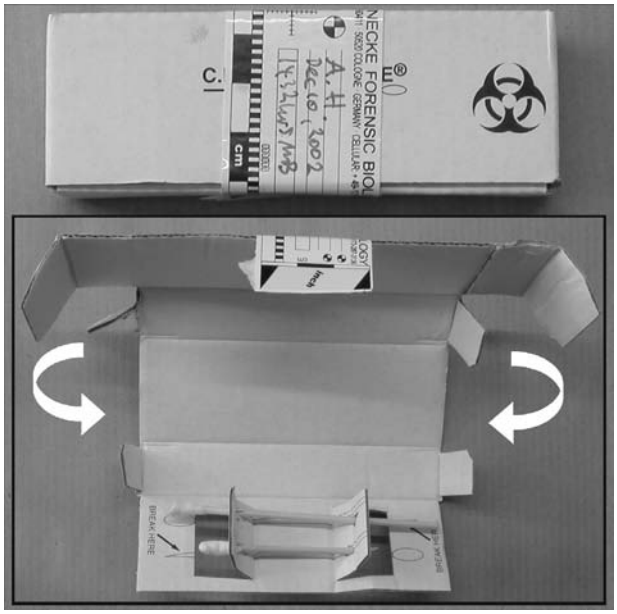

Fig. 1 Cardboard box allows simple and safe drying and storage of swabs. [Swissforensix, Switzerland (patented).] (*View this art in color at www.dekker.com.*)

- DNA contained in epithelial cells that had been transferred by saliva of an offender was swabbed off the skin of an experimental victim that had showered. Amplification of the offender's STRs was successful up to several hours after transfer of his saliva to the skin of the victim.
- In contrast to common belief, corneocytes contain DNA. Therefore, all surfaces that may have been touched by an offender (through grabbing of ropes, wearing of baseball caps, hitting a person, inside of gloves) may be swabbed successfully.[3–5]

Early swabbing is also necessary whenever cells from the top edge of bottles, beer cans, etc. are collected. Collection of the complete bottle or can frequently leads to spilling of its contents and dilution or washing off the cells. If early swabbing is not possible, the liquid must be drained out of the container by drilling a hole in its bottom.

Filter Paper

Liquid blood can be stored on filter paper that is then dried in the same way as cotton swabs. Filter paper that contains denaturants, buffer, and a free radical trap (e.g., FTA paper[6]) will lyse the blood cells and immediately deactivate bloodborne pathogens such as herpes, cytomegalovirus, and HIV.

Filter paper can also be used to store saliva and liquids from decomposed bodies. In automated laboratories, standard-sized filter paper is the preferred option. Pieces can easily be punched out of it by a machine and subsequently processed by a DNA extraction and PCR robot. The advantage of FTA paper over regular filter paper is that it can be used for multiple PCR reactions. Template DNA will stick to FTA paper after washing off the PCR products and can then be reused.

Urine and Feces

Because feces is found especially at scenes of (serial) burglaries, it should be collected irrespective of its repulsive nature. Fresh feces as well as liquid urine should be frozen below $-20^{\circ}C$ to avoid bacterial activity. DNA typing of urine is successful especially if it was excreted in the morning (when the highest number of epithelial cells are found compared to the rest of the day), and from feces after PCR inhibitors are removed. To recover the cells, urine needs to be centrifuged (cells are located in the sediment), whereas stool samples can be extracted straightaway or from swabbings with mini spin columns. The estimated number of up to 6×10^5 pg human DNA/mg stool is never reached in practice because of bacterial and digestive action; nevertheless, up to 170 pg DNA/mg stool were successfully extracted and amplified under case work conditions.[7,8]

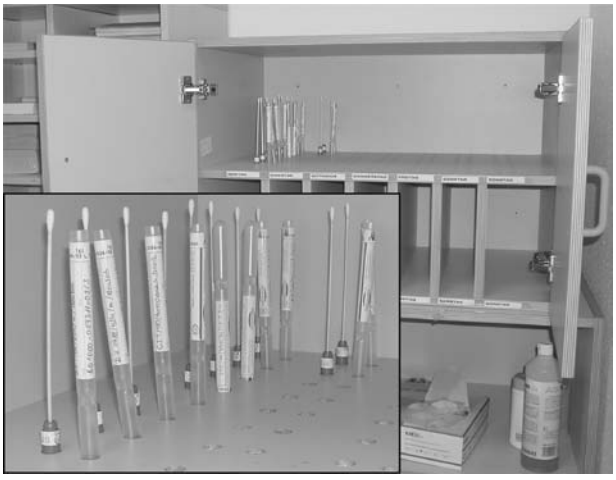

Fig. 2 Some laboratory standard procedures request cupboards to be used for drying of swabs. Sterile conditions are not necessary; contamination is not a problem as long as the swabs do not touch each other or the wall. Note that the cupboard must not be tightly closed to avoid building up of humidity. (*View this art in color at www.dekker.com.*)

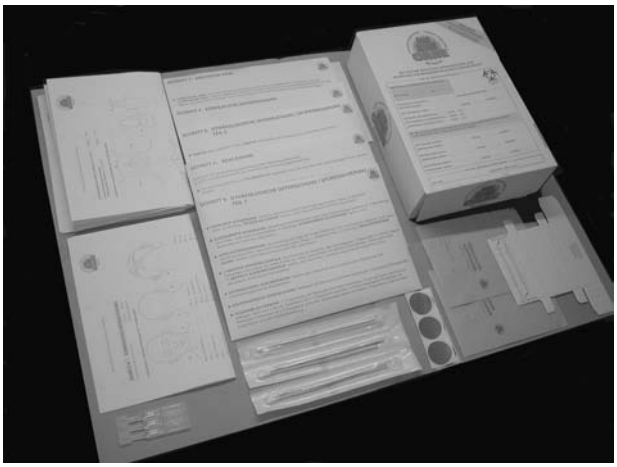

Fig. 3 Sexual assault kit (University of Bern/Swissforensix, Switzerland): Standardized descriptions and checklists are printed on the actual envelopes that contain the collection materials. (*View this art in color at www.dekker.com.*)

Sexual Assault Kits

After sexual assaults, biological stains are often collected in a hospital environment, at home, at a general practitioner's office, or at a police station. To avoid contamination of the samples and to allow full collection following a checklist, sexual assault kits are available. Their use is generally and strongly recommended to guarantee collection of all stains in the best possible way even under highly stressful conditions or in cases where lay personnel has to collect the evidence. The kits consist of prepacked envelopes in a cardboard box, which can be stored and stacked at room temperature (e.g., Sexual Assault Care Kit, University of Bern/Swissforensix, Fig. 3). The envelopes contain swabs, combs for hair (head and pubic), filter paper, sterile distilled water ampoules, large paper bags, and standardized protocol sheets.[1]

Skin Line Prints (''Fingerprints'')

DNA is resistant against many histological stains, including substances used to develop fingerprints (or other skin lines). DNA typing was successful from developed skin line prints after cyanoacrylate (super glue fume) or color reagents such as amido black, leucomalachite green, Hungarian Red, DFO, or luminol had been applied.[9,10]

Developed skin line prints should first be documented with a high-resolution camera (5–12 megapixels) or scanner (1200 dpi minimum). The original skin line prints can then be submitted to DNA storage and extraction like any other biological stain. The stronger the initial fingerprint, the more likely a DNA profile may be obtained.

STORAGE AND EXTRACTION

Dried biological samples should be stored in standardized paper bags (envelopes, brown paper bags) in a dry and cool environment. This will preserve the DNA over months to years. If dry samples need to be stored for more than 2 years, freezing below $-20^{\circ}C$ is recommended. To avoid paper layers sticking to each other in the freezer, the envelopes should be put into plastic bags. Never write on plastic surfaces that become frozen because any type of ink will easily come off. Use paper labels instead.

In temperate parts of the world, DNA was successfully extracted out of clothing and smears on slides that had been stored more than 10 years in dark environments at room temperature. In tropical countries, freezing is always necessary because of the high humidity, which allows bacteria and mold to build up.

Biological stains on glass slides, either embedded (histological tissue samples) or just regular smears (vaginal smears or blood), generally lead to good extraction results. The slides should be stored in standard cases for microscopic slides. Alternatively, they can be fixed with sticky tape inside of a paper envelope. Traces of dust generally do not affect the quality of dry stains but should obviously be avoided.

Insects collected at crime scenes or from corpses should not be dried because museum beetles will frequently destroy the samples within months. The insects should be preserved in 90% EtOH. At room temperature, DNA extraction of such material will then be possible up to several weeks after storage; at temperatures below $-20^{\circ}C$, extraction will be successful for several years.[10–12] Never use formalin to preserve samples; it will degrade the DNA.

Cigarette butts, envelopes with stamps, fingernail clippings, and dried nasal secretions should be stored dry in paper bags, envelopes, or cardboard boxes. Fingernails can be thoroughly swabbed if clipping is not an option.[13–15]

Because telogenic hair and broken-off hair shafts have been successfully used for DNA extraction, hair should be carefully stored, e.g., by attaching one end of every hair with sticky tape to the inside of an envelope or between two layers of filter paper. If hair is collected by the police using sticky tape for fiber collection, all material (fibers, lint, and hair) should remain on the tape until extraction becomes necessary.[16,17]

Extracted DNA Stored in Buffers

Depending on the applied extraction method, DNA stored in TE [10 mM Tris–HCl (pH 7.5), 1 mM EDTA] or similar buffers may be stable for weeks (after Chelex extraction) or months (phenol/chloroform extraction or use of spin columns) in the refrigerator at $+4^{\circ}C$ to $+12^{\circ}C$.

Freezing of extracted DNA in TE buffer below −20°C will preserve the sample for years. Before freezing, it is strongly recommended to distribute the DNA in small aliquots (e.g., 10 μL each) to avoid repetitive thawing and freezing of single samples.

Emergency Buffers

The standard storage buffer for extracted DNA is TE buffer. Before its use, the buffer is autoclaved or cleaned with a sterile filter. It can then be stored at room temperature. Under extreme conditions in the field, if drying of the samples is impossible (because of dust, humidity, chaotic mass disaster environments), TE can be used to collect samples by aliquoting 1 mL TE into sterile 1.5-mL plastic tubes. The collected biological stains can be put inside these emergency containers. DNA must still be extracted as soon as possible, and the samples should be stored as cool as the situation allows.

If more than a few hours are expected to pass before freezing or drying is possible, any solid biological sample in the field should be stored in centrifuge tubes containing aliquots of 95% EtOH. At room temperature, this will preserve the sample's DNA for weeks.[18]

MAIN DESTRUCTIVE INFLUENCES ON DNA

Under the influence of UV light (including sunlight) and acids, DNA contained in biological stains as well as extracted DNA breaks into pieces (degrades). Depending on the intensity of fragmentation, PCR might still be possible. Humidity does not directly affect DNA but will allow mold and bacteria to destroy the sample including the DNA within days. Frequent freezing and unfreezing of stains or extracted DNA will also lead to degradation. Household use of detergents and cleaners does not necessarily destroy DNA.[19] Sperm heads on fabric can survive machine washing at 30–40°C if no bleach was used.

CONTAMINATION

Under conditions of normal case work, contamination is only observed after careless manipulation or purposeful spraying of high (nanogram) amounts of DNA near or directly into open tubes before PCR. Secondary transfer via door handles, etc. is only a problem under extremely careless, unprofessional conditions.[20,21]

Obviously, mixtures of DNA might be present in the samples themselves. Mixtures of epithelial cells with sperm can be separated by differential lysis (separation of sperm from epithelial cells[22]). Other mixtures may show distinctively different peak heights after electrophoretic separation of the PCR products. For example, an object at a scene of crime may have been touched by Person A days before a biological stain (such as blood) of Person B is deposited on the same surface. In that case, a DNA mixture might be present later. It can often be detected by the different peak heights of the STR alleles.

Irrespective of the possible presence of mixtures, swabbing is always recommended if the items cannot be moved, are bulky, or if the stain is located on a person. Subsequent procedures like differential lysis should not be performed before DNA extraction becomes necessary. Generally, once evidence examination is completed, all biological samples should simply be stored cool and dry, and left intact as long as possible.

WITHDRAWAL OF SAMPLES OUT OF STORAGE

If parts of a stored biological sample need to be withdrawn for DNA extraction, forceps and scissors must be wiped with paper towels and 70% EtOH (or methylated spirits) every time they are used. In routine use, cross-contamination caused by wiped, smooth-surface forceps has not been observed. An exception is forceps with grooves. They must be autoclaved before every use because the groves quickly fill up with contaminants.

Still, especially during evidence examination and withdrawal, it is essential to take care of cross-contamination caused by contaminated distilled water, touching the swabs with used gloves, etc. Standard bacteriological procedures are an optimal guide.

Sample Retainment

It is recommended to always retain at least half of a stain in storage. One reason is that extracted DNA in liquid buffers is less durable than the original, dried stain. In addition, the defense should have a chance to reexamine the stain beginning with the original sample, not the extracted DNA. Only if DNA extraction and PCR seem to fail because of low amounts of DNA may stored samples be used up completely. This needs the consent of the prosecutor's (D.A.'s) office. Even in these cases, at least a minute amount of the original material should be stored so that future DNA technologies may be applied later on.

CONCLUSION

Collection of biological stains should be documented by photographs and drawings. Dry and cool storage will allow biological samples to be stored over years.

Extraction of DNA should be performed only if necessary for a current investigation; the original stains

should never be extracted completely. Contamination in the laboratory does not occur if the sampling is performed by trained personnel. Because many surfaces and even stains like fingerprints (skin lines), corneocytes on ropes, telogenic hair, the surface of skin after showering, etc. may contain material that is suitable for DNA typing, intelligent criminalistic decisions have to be made before collecting the evidence.

Intense swabbing and the use of sexual assault kits are simple yet very important procedures that guarantee maximum yield of DNA and collection of biological material even if it is not visible at the moment of collection. Even difficult stains such as feces can be extracted and should be stored frozen whenever possible. Under extreme field conditions, 90% EtOH may be used as a collection and storage liquid.

ARTICLE OF FURTHER INTEREST

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Forensic DNA Typing—Y Chromosome

F

Lluís Quintana-Murci
Institut Pasteur, Paris, France

INTRODUCTION

The human Y chromosome is one of the smallest chromosomes, representing 2–3% of a haploid genome. It is the only chromosome that has no homologue at any state or sex, in contrast to the X chromosome that has a homologue in the female sex. The majority of the Y chromosome (95%), termed nonrecombining Y (NRY), does not undergo recombination during male meiosis; however, two regions, pseudoautosomal regions (PAR1 and PAR2), located at the distal portions of the telomeres, recombine with homologous counterparts on the X chromosome. From a functional viewpoint, the Y performs specialized roles that are crucial for males, and therefore for the whole population, such as sex determination and male fertility. The haploid status of the Y and its exclusive paternal transmission make it a very useful tool in different domains as follows: 1) the estimation of historical patterns of population movements and splitting; 2) forensic applications, such as identification of male DNA in cases with male/female stain mixtures; and 3) genealogical studies and paternity testing, especially for deficiency cases, where the alleged father is deceased and his male relatives need to be tested.

Y CHROMOSOME MARKERS: HAPLOGROUPS AND HAPLOTYPES

One unique feature of Y chromosome markers is that they are inherited as a single block in linkage. Therefore Y-linked variations are largely a result of accumulation of de novo mutations over time.[1] This feature is very useful in high-resolution discrimination of individuals. Large-scale sequencing efforts and the development of rapid mutation detection techniques have accelerated the discovery of Y-chromosome variation. Today, more than 200 biallelic polymorphisms and around 30 multiallelic markers are available,[2–5] most of which are suitable for polymerase chain reaction (PCR)-based genotyping techniques. Biallelic markers mainly include single base pair variants [single nucleotide polymorphism (SNPs)] and also a reduced number of small insertions/deletions (indels), whereas multiallelic markers mainly refer to microsatellites and minisatellites. The main difference between the two sets of markers is their mutation rate: biallelic markers present lower mutation rates (about 2×10^{-8} per base per generation) than microsatellite markers ($2–3 \times 10^{-3}$ per base per generation). Biallelic variants and insertion/deletion polymorphisms almost certainly represent unique molecular events and define stable and deep-rooted branches, known as haplogroups, that can be traced back in time over thousands of years. These haplogroups present either a wide geographic distribution or, in some cases, more regionally clustered.[3,4] The multiallelic markers define the internal diversity of these haplogroups, and the combination of the allelic states of different microsatellite loci are known as haplotypes. Microsatellite haplotypes are very useful for microevolutionary studies and to distinguish subtle genetic differences between populations and/or individuals.[5] The high mutation rate of these markers, which enables a good discrimination between individuals, makes them the most suitable markers in forensic studies.[6] However, this high mutation rate has a disadvantage: the appearance of recurrent mutations. Thus, it is sometimes difficult to distinguish if two individuals are ''identical by state'' (share the same allele but not the same ancestry) or ''identical by descent'' (share the same allele and the same ancestor). This has important repercussions in population genetic studies because, at the population level, the use of these markers alone may lead to an underestimation of genetic distances among populations, thereby distorting genuine population relationships.

THE Y AND THE PAST: HUMAN ORIGINS AND POPULATION DISPERSALS

Y-chromosome studies have shown that Y-linked variation is nonrandomly distributed among human populations, showing lineage profiles that can be strikingly different among different worldwide populations.[2–4] These observations have obviously important consequences in ascertaining the origin of an individual during forensic investigations. From a human evolution viewpoint, Y-chromosome studies have given a major contribution to a better understanding of human origins and population dynamics. Y-chromosome phylogenies support the African origin of our species around 150,000 years

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ago. The most divergent branches of the trees are restricted to African populations, and non-African populations find their roots in branches of African origin.[2–4] These observations support the scenario in which modern humans can be traced back to a single African ancestral population that lived in Africa (i.e., the so-called ''Out of Africa'' model), from where it dispersed throughout the other continents replacing preexisting human species. Substantial continental and local population structure has been revealed, and hypotheses of early human migrations at different times formulated, such as the mode and tempo of the peopling of Asia and subsequent colonization of the Americas and the colonization of the Pacific.[3]

THE Y AND THE PRESENT: FORENSIC AND GENEALOGICAL STUDIES

Sex Assignment

The most obvious use of the Y chromosome in forensics is the sex assignment. The most commonly used test, the Amelogenin Sex Test (AMELY), takes advantage of the homology between the two pseudoautosomal regions of the sex chromosomes and amplifies a segment of the XY-homologous amelogenin gene pair.[7] Although this method avoids the ambiguous nature of a negative result as a result of the failure of the PCR reaction rather than to the absence of Y material, the reliability of the test depends on the assumption that the tested individuals present normal karyotypes and intact sex chromosomes. Nevertheless, individuals presenting a discordance between their sex phenotype and their karyotype (i.e., sex-reversed 46, XX males or 46, XY females) are present at appreciable frequencies in the population and can constitute a source of error in sex assignment tests based only in the AMELY test. It is suggested, therefore, to perform other sex tests in parallel, such as amplification of the *SRY* gene, which will give complementary information to the AMELY test.[8]

Identification and Discrimination of Male-Specific DNA

The analysis of Y-chromosome variation has been extensively used for forensic applications other than sex assignment. They can provide highly valuable information to resolve male–female DNA mixtures in, for example, sexual assault cases. In these cases, samples may present high amount of victims' cells, such as vaginal/anal washings, and sometimes no male autosomal profiles are identified. It has been shown that Y chromosome microsatellite analysis becomes a useful alternative because the use of specific Y-primers can improve the chances of detecting small amounts of the perpetrator's DNA in a background of heterologous female DNA.[9] Another obvious application of the Y chromosome is in paternity testing, especially for deficiency cases where the alleged father is deceased. In this case, the absence of the biological father can be resolved by the analysis of any relative sharing the same male line. However, when comparing the Y-chromosome profile in a paternity test or between a suspect and a sample, inclusion remains an important caveat. Although Y-linked microsatellites can be confidently used for exclusion purposes, nonexclusion can be problematic because 1) all the male relatives of a suspect/alleged father share presumably the same Y haplotype and 2) the Y chromosome of a suspect/alleged father may be present at high frequencies in the population under study, considerably reducing the reliability of the test. In any case, the high level of polymorphism of microsatellite markers offers a significant degree of discrimination between individuals. For example, a study of a German population using a 9-locus microsatellite haplotype, demonstrated a discriminatory capacity between Y-chromosome haplotypes of 97%.[10] However, this may not be the case in other populations with different genetic backgrounds and different population histories, where, for example, male-specific migration processes, founder effects, or genetic drift may have led to over-representation of specific haplotypes in the population.

Retracing Ethnic and Geographic Origins

Another possible information of interest for forensic investigators is that of ethnic origin. Although the highest genetic variation is observed between individuals and not between populations, Y-chromosome polymorphisms may provide the best resolution to ascertain the approximate geographic origin of a suspect.[11] Some haplogroups tend to be restricted to European populations, while others are restricted to sub-Saharan African or East Asian populations.[2–4] Geographic clustering of Y-chromosome haplogroups or haplotypes can be even more restricted. A recent Y-chromosome survey of the British Isles has shown that haplotype frequencies can differ considerably even over relatively short geographic distances.[12] Lineages of recent origin, such as haplogroup R-SRY2627, may exhibit a very limited distribution. This lineage has been observed only among Europeans and is almost restricted to Basque and Catalan populations.[13] In this case, it can be highly informative to predict the origin of the population and for exclusion purposes. However, more populations need to be tested for additional markers in order to create highly discriminative databases that will increase the power of reliability when inferring the geographic origin of a sample.

PRESIDENTS AND TSARS: THE POWER OF Y CHROMOSOMES AND MITOCHONDRIAL DNAs (MTDNAs)

The usefulness of Y chromosome data has been successfully used in some famous and controversial cases, such as U.S. President Thomas Jefferson's paternity of the children of one of his slaves. In 1802, he was accused of having fathered a child, Thomas Woodson, by Sally Hemmings. Also, Sally's last son, Eston Hemmings Jefferson, is thought to be the son of the President, although other scholars give more credence to the hypothesis that they were the sons of Jefferson's sister that fathered Eston. By analyzing several Y-polymorphisms in 14 members of the different male lines involved in the controversy (e.g., those of Thomas Woodson, Eston Hemmings, President Jefferson, and his sister's sons, the Carr's line), the authors found that President Jefferson and Eston Hemmings share the same haplotype.[14] In contrast, this haplotype was absent in Thomas Woodson and the Carr's lines, and its frequency in the general population is very low (0.1%). The authors concluded that President Jefferson fathered his slave's last child, whereas he was not the father of Sally's first child. The possibility that any other President's male-line relative could have fathered Sally's last child cannot be excluded, highlighting therefore the difficulty of being conclusive in these studies, but no historical records support this hypothesis. This study nevertheless illustrates the usefulness of Y chromosome data to disentangle complex hypothesis of alleged paternities and genealogical relationships.

In the absence of male-line comparison, a suitable partner of the Y chromosome is the mitochondrial DNA (mtDNA). This molecule can be highly informative in genealogical studies because, as the Y, it does not recombine and is transmitted through the maternal line without any modification other than naturally occurring mutations. Moreover, analyses of mtDNA in forensics can be even more powerful than Y-chromosome analyses because mtDNA is present at a higher copy number in cells and is more likely to survive prolonged periods than nuclear DNA. The most notorious example of its use in genealogical and forensic studies is the identification of the remains of Tsar Nicholas II and his family. By analyzing the amelogenin gene and autosomal STR variation in nine skeletons found in a grave in Ekaterinburg (Russia), the authors could confirm the presence of a family group, composed of two parents and three daughters and four additional unrelated bodies. To ascertain if the five related individuals corresponded to the Romanov family, mtDNA was analyzed in all bodies and in a living maternal relative of the Tsarina, Prince Philip the Duke of Edinburgh. Indeed, the mtDNA sequence of the putative Tsarina and her three daughters matched exactly that of the Duke of Edinburgh, supporting the hypothesis that their remains correspond to the Romanov family.[15] As to the putative body of the Tsar, his mtDNA sequence presented a very rare sequence heteroplasmy (presence of two alleles at a particular nucleotide position). By analyzing the body of the Tsar's brother and two living maternal relatives, the presence of this rare heteroplasmy was confirmed in the Tsar lineage, providing a powerful evidence supporting the identification of Tsar Nicholas II and his family.[16]

WHAT'S YOUR NAME? WHAT'S YOUR HAPLOTYPE?

In many societies, surnames are inherited patrilinearly, in the same manner as Y chromosomes. This parallel transmission can be used to tentatively associate surnames, or closely related human groups, and specific Y-chromosome haplotypes. For example, by using Y-chromosome microsatellites, it has been shown that half of the individuals with the English surname ''Sykes'' belong to a specific Y-chromosome haplotype, which is not present in non-Sykes samples and, in general, in other UK samples.[17] The presence of other haplotypes among the remaining Sykes samples has been attributed to historical accumulation of nonpaternity. However, further studies have shown that this lineage is present in Baltic States, although it is virtually absent in the British Isles, Scandinavia, and Iceland. This study, although preliminary, may have important forensic and genealogical applications. Increasing the number of microsatellites analyzed in individuals may eventually reach such a level of resolution that surname-specific haplotypes could be observed, with obvious important applications.

The use of Y-chromosome variation has also given insights into genealogies dealing with Jewish identity. According to Jewish tradition, males of the Levi tribe, of which Moses was a member, were assigned special religious responsibilities, and male descendants of Aaron, his brother, were selected to serve as Priests (Cohanim). Whereas in most cases Jewish identity is acquired by maternal descent, membership in the Cohen and Levi castes is determined by paternal descent. By studying a large group of Jewish individuals, which include Israelites, Cohanim, and Levites, it was shown that whereas Israelites and Levites exhibit a heterogeneous set of Y chromosomes, the Cohanim are mainly characterized by a unique haplotype that is present at high frequencies in both Ashkenazic and Sephardic Cohanim.[18] This haplotype, known as the Cohen Modal haplotype, is thought to be a potential signature of Judaic origin.

CONCLUSION

The times when the Y chromosome was considered a chromosome of ''low polymorphism'' have passed away. Today, the Y chromosome phylogeny is robust and rich in variation that has been successfully used in population genetic and forensic studies. Nevertheless, the definition of Y-chromosome variation, through a well-defined battery of markers, in global samples of different human populations remains a task to be accomplished and represents an imperative prerequisite to establish population databases of Y-chromosome haplotypes to be used in forensics. In this context, the precise knowledge of Y-haplotype frequencies in a given population becomes very important when matching the DNA profile of a suspect with the corresponding population database, because this will give the probability of finding this haplotype by chance in the population. The creation of these high-resolution population databases will also increase the probability to deduce the geographic origin of a DNA sample and will shed light on the preliminary associations between certain surnames, or lineages, and Y-chromosome haplotypes with the consequent important forensic and legal applications.

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Forensic Identification—An Overview on Molecular Diagnostic Technology

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Adrian Linacre
Yvonne E. Cruickshank
University of Strathclyde, Glasgow, U.K.

INTRODUCTION

DNA profiling has greatly changed the way in which human identification is performed for the purpose of a forensic investigation. Since the first case in 1985 through to the most recent technological advances, DNA profiling has adapted techniques used in medical and diagnostic research. The use of microarrays and nanotechnology is now becoming part of diagnostic technology, so these same methods will be adapted to forensic science. This chapter outlines the current situation regarding forensic science and diagnostic techniques.

FORENSIC DNA PROFILING

Forensic science, being the application of science to law, has applied many scientific techniques used in other fields of the analytical sciences to address questions raised in a legal context. The application of DNA technology, and in particular the use of the polymerase chain reaction (PCR), has revolutionized the field of human identification. The first use of DNA in forensic science was to help solve a sexual assault in England. Professor Sir Alec Jeffreys coined the term ''DNA fingerprinting''[1] when he was able to link two sexual assaults as committed by the same perpetrator and then to link the crime scene sample to the suspect, Colin Pitchfork. Previous examination of biological evidence used genetic polymorphisms producing different proteins; however, as the human genome is approximately 3 billion base pairs in size and that less than 3% is gene related, greater polymorphism lay in the non-gene-related regions. It was always considered that the DNA from each individual was unique, with the exception of monozygotic twins. Professor Sir Alec Jeffreys noted that there were regions of DNA where the same sequence of DNA was repeated many times. The number by which this repeat motif was found along the chromosome of different people was found to differ. These regions, termed variable number tandem repeats (minisatellites), were exploited in DNA fingerprinting. The field of DNA profiling has now moved to the analysis of hypervariable short tandem repeats (STRs). Short tandem repeats are short regions of DNA, typically 2–8 DNA bases in length, that are repeated polymorphically along the chromosome. The first use of STRs was in 1994 when four separate DNA loci were amplified in one reaction.[2] Within 1 year the multiplex PCR had increased to six independent STR DNA loci.[3] Short tandem repeats are regions of DNA found close to genes, often within introns, in which a sequence of DNA is repeated. The repeat motif is frequently 4 bp in length with the number of repeats varying from 6 to over 30. The number of repeats is known to be highly variable, producing size variations at the loci. The flanking DNA on either side of the STR loci is known to be highly conserved allowing amplification of the locus by PCR using primers made to the flanking DNA. Primers have been designed that will work on all the major ethnic populations.

With increased knowledge of the PCR process the number of loci that could be successfully and routinely amplified increased to a point where currently 10 STR loci form the basis for the National DNA Database (NDNAD) in the United Kingdom. In the United States 13 loci form the Combined DNA Index System, or CODIS. Short tandem repeats are highly abundant in the human genome and are known to be polymorphic.[4,5]

While one STR locus provides little power of discrimination, it is common to analyze 10 STR loci in a single multiplex reaction. Each of the loci examined is independently inherited producing an overall power of discrimination of greater than a billion. This equates to close to identity and meets the current needs of the forensic community.

MITOCHONDRIAL DNA AND FORENSIC SCIENCE

The current situation in forensic science is that there are a number of autosomal STR loci that can be analyzed leading to possible links between a crime scene sample and that taken from a suspect. Mitochondrial DNA has been used in forensic science for human identification and

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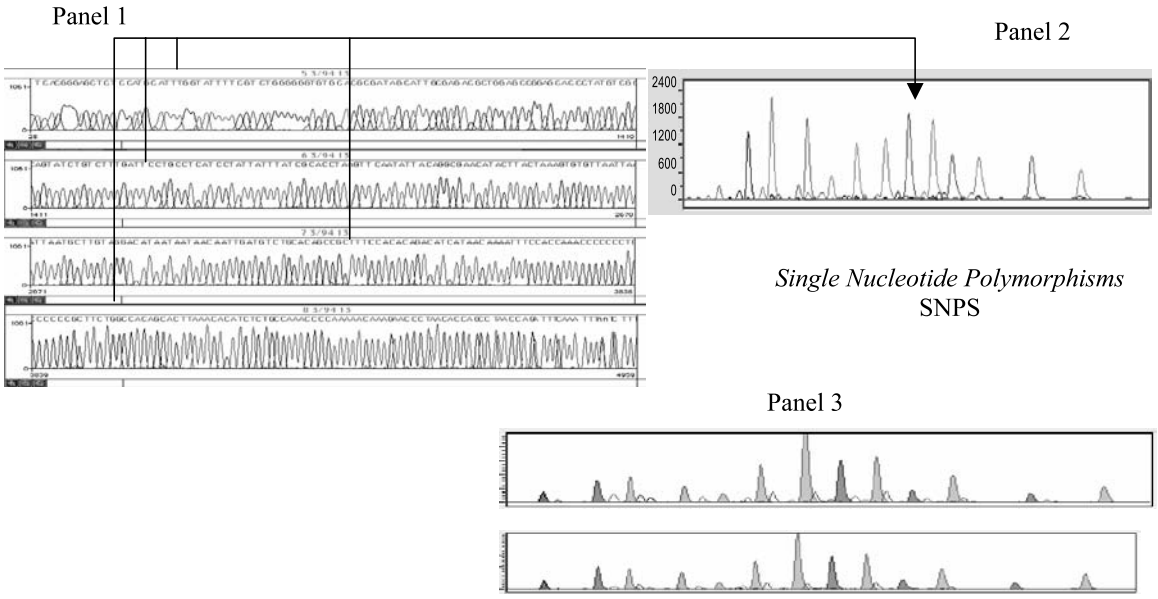

Fig. 1 Example of minisequencing of 12 SNP sites on the human mitochondrial genome. Panel 1 shows a DNA sequence trace from four samples of mitochondrial DNA and highlights single-base changes between one of the samples and the other three. Panel 2 shows the resulting minisequence trace. Panel 3 shows a casework example where the top trace is from a hair found on clothing and the bottom trace is from an alleged victim of a crime. In this case there is a match between the DNA extracted from the hair and the reference sample from the victim. (From work provided by Dr. Gillian Tully for publication purposes and based upon an illustration used in Ref. [10].) (*View this art in color at www.dekker.com.*)

for population studies.[6–8] Within each mitochondrion there is a circular loop of DNA being 16,569 bases in length in humans. There are no STR loci on the mitochondrial DNA, rather, DNA sequence polymorphisms are detected on either side of the point of replication. It is common practice to amplify this fragment of the mitochondrial genome and directly sequence the PCR product.

SINGLE NUCLEOTIDE POLYMORPHISMS

The sequence polymorphisms found within the mitochondrial DNA are termed single nucleotide polymorphisms (SNPs). Single nucleotide polymorphisms are positions within the genome where there is a difference between people at one single base. It may be that at a particular position the nucleotide ''A'' is normally found, but in a minority of people the base ''G'' is present. In such an instance most people will have the base pair A/T with a minority being G/C at the same position. There are a number of SNP sites on the mitochondrial DNA within the loci examined. Single nucleotide polymorphisms were first used in forensic science by the U.K. Forensic Science Service to examine mitochondrial DNA from suspects and from crime scenes.[9] This method examined 12-point mutations known to be variable within the mitochondrial DNA by a technique termed minisequencing. Minisequencing of the mitochondrial DNA polymorphic sites allows rapid screening of the variable DNA bases. In forensic investigations this process is used to exclude all the samples in a case that do not match at the 12 points. An example of an SNP of mitochondrial DNA is shown in Fig. 1.

Detection of SNP by minisequencing requires separation of DNA fragments by either gel or capillary electrophoresis. Much SNP testing for forensic and diagnostic purposes can now be performed using oligonucleotide microarrays.[10] Microarrays (also covered in Chapter 10 of this encyclopaedia) allow the single-base difference to be detected on a silicone chip. The two different versions, either A or G in the case example, are placed on the chip. If A is present then a DNA sequence with T will bind. If G is present a DNA sequence with C will bind. It is possible to detect SNP polymorphic sites on the mitochondrial DNA. An example is shown in Fig. 2.

SNP ANALYSIS IN CODING AND NONCODING DNA REGIONS

Within the chromosomal DNA, SNPs are highly abundant occurring on average 1 base in every 1000.[11,12] As the human genome is now known to be around 3 billion bases

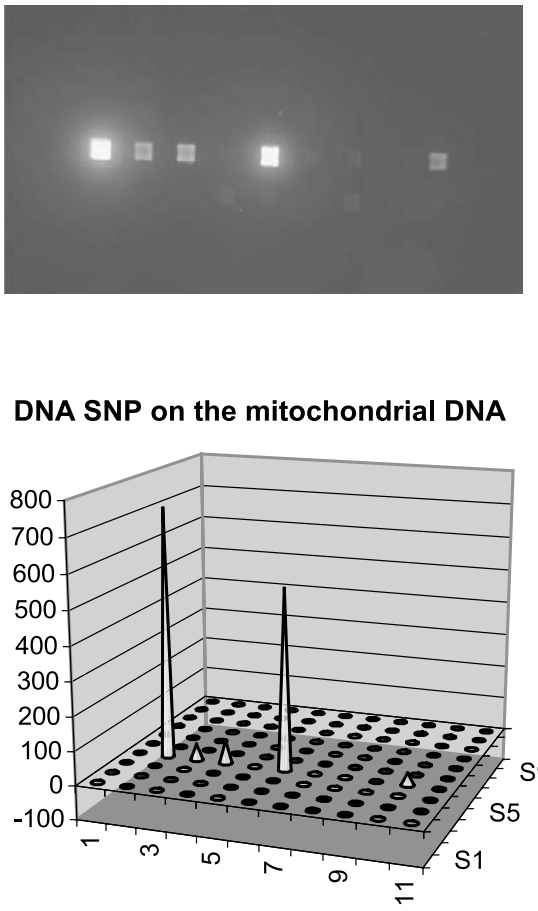

Fig. 2 Analysis of two SNP loci on the human mitochondrial genome. (Taken from research work by Dr. Barbara Llewellyn at the University of Strathclyde.) The top panel shows the results of hybridization of a segment of DNA mitochondrial DNA to oligonucleotide sequences on a silicone chip. The position on the chip where the DNA binds to a complimentary sequence is detected by fluorescence. The bottom panel shows the amount of fluorescence at the two sites. (*View this art in color at www.dekker.com.*)

in size, there are therefore approximately 3 million SNPs within the human genome. To date, the International SNP Map Working Group has identified 1.42 million SNPs distributed throughout the human genome.[13] The SNP loci examined in forensic science are within the 97% of the human genome that is noncoding. Single nucleotide polymorphisms that are present within coding regions of the DNA may have an effect on the protein transcribed by the gene and result in a genetic disorder. As such, SNPs that cause a genetic disorder are of key interest to the medical community. These SNPs may be dominant or recessive and are responsible for a number of key genetic disorders. Additionally, SNPs within the regulator region of genes, such as in promoter sequences, may influence the susceptibility of individuals to particular disorders. The majority of SNPs are considered to have no known effect on the fitness of the organism; however, those SNP loci that affect the fitness of the organism have been the focus of interest to the medical community. The field of medical genetics has recently started to develop a number of diagnostic tests based upon single base-pair mutations in the human genome. A number of genetic diseases are the result of such single base-pair mutations, such as cystic fibrosis and beta thalassaemia.[14,15] In parallel to the investment made in STRs by the forensic community, genetic diagnostics has focused on many such single point mutations. When the mutation becomes a common type, with the rarer allele at more than 1% of the frequency of the common allele, such loci are termed SNPs.[16]

Forensic science reacts to developments in other areas of science, frequently adapting techniques developed for nonforensic purposes. While DNA profiling is no exception, the analysis of STR loci has been highly successful. Tiny bloodstains can be examined and a DNA profile can be produced that has a match probability greater than 1 in a billion to a suspect. There has been much investment in the STR loci, particularly in the formation of DNA databases of felons. However, SNP analysis offers many advantages in the field of diagnostic analysis.

FORENSIC TOXICOLOGY

Forensic toxicology is defined as the study of the adverse effects of poisons on living organisms in relation to the law. Indeed, the most common poisons in today's society are drugs of abuse, controlled under different countries legislations, and the majority of forensic toxicology is concerned with the detection of such drugs within the blood or urine of a person. The analysis determines whether a controlled substance is present, and if so how much is present. In fatalities the quantity of a controlled substance must be known before a conclusion can be made as to the cause of death. To obtain the amount of drug present in the person at the time of death, a backtracking calculation is carried out. In such a calculation there are issues with the interpretation of the toxicology results. This is because one dose may be at a level that is therapeutic to one person but lethal to another. The interindividual differences in response to a drug are due to interindividual differences in drug metabolism, which in turn is a result of genetic polymorphisms in the production of drug receptors and drug transporters. A well-characterized genetic polymorphism occurs within the cytochrome–p450 system where there are subfamilies

of isoenzymes including CYP2C9, CYP2D6, and CYP3A4.[17] Slow, medium and fast metabolizers are the result of the production of different isoenzymes, which is under a genetic control. Single nucleotide polymorphisms to the gene system for the different metabolic systems would allow determination of whether the person had a slow metabolism, hence death resulting in a quantity of drug that would otherwise be therapeutic to a fast metabolizer.

CONCLUSION

One of the greatest advances in forensic science has been the introduction of DNA profiling. To date DNA profiling is performed using microsatellite amplicons (STRs) where hypervariability exists within the number of repeats of a short DNA sequence motif. Greater than 3 million samples have been analyzed by this method on the U.K. National DNA database alone, with currently an increasing number of samples on the U.S. CODIS and similar DNA databases throughout Europe. Due to the great investment in the science of DNA profiling using STR loci there would need to be a great advantage in a new technology. Single nucleotide polymorphisms offer many advantages, particularly in the speed of throughput and option for automation. Approximately 50 SNP loci are required to have the same discrimination power as 10 STR loci, but 50 SNPs can be detected readily and therefore this level of DNA detection can be overcome. One major reason for microarrays not being more common in forensic science is that crime-scene samples are frequently mixed, containing cellular material from more than one person. In such cases, a mix of a person with SNP of type A only and a person who is type G only could not be differentiated from a person who is A and G. Mixtures occur routinely in forensic science thereby adversely affecting the introduction of SNP typing in human identification.

In forensic toxicology the samples are normally from one person only and it may be in application such as this that SNP typing will find a role.

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Fragile X Syndrome

James N. Macpherson
Salisbury District Hospital, Salisbury, Wiltshire, U.K.

INTRODUCTION

Fragile X Syndrome and Fragile Sites

Fragile X syndrome is a relatively common, yet—until recently—poorly understood inherited condition characterized by variable degrees of learning and social disability. First discovered in 1943 (when it was known as the Martin–Bell syndrome), it derives its name from its frequent association with a rare folate-sensitive fragile site (FRAXA) localized near the distal tip of the long arm of the X chromosome (Fig. 1) at Xq27.3, later also confirmed as the site of the gene locus.[1] In fact, there are two distinct conditions associated with expressed site fragility in this chromosomal segment: the FRAXE site is difficult to distinguish from FRAXA by conventional cytogenetics, but high-resolution methods map it slightly distal at Xq28. Some, but not all, properties of the FRAXA locus also apply to FRAXE; the primary discussion in this article will be of FRAXA, but the distinctive characteristics of the FRAXE locus will be pointed out wherever relevant.

FEATURES OF FRAXA AND FRAXE

Clinical Phenotype, Genetics, and Biochemistry

The fragile X syndrome affects males to a greater extent than females: in males, learning and social deficits are moderate to severe, with social impairments usually preventing them from fathering offspring although sperm production is thought to be unaffected. In adult males, visible signs of the condition often include a large head, long face, large ears, and macroorchidism; however, in children, the phenotype is likely to be restricted to developmental delay with perhaps some autistic-like features such as gaze avoidance or hand-flapping.[2] Females with fragile X syndrome vary from apparently normal phenotypes to moderate learning disability, often with heightened anxiety, social withdrawal, or depression.[3] The appearance of fragile X phenotypes in women may be simply a consequence of nonrandom X inactivation, although this has not been proven.

In 1991, the gene responsible for fragile X syndrome was isolated by several independent research groups[4,5] and designated *FMR1* (fragile X mental retardation 1). Most surprising was the discovery of the novel mutational mechanism—the progressive expansion of a tract of trinucleotide (CGG) repeats in the 5′ untranslated region of the gene—culminating in the shutdown of transcription and hypermethylation of the promoter for repeat numbers in excess of around 200. Repeat numbers between around 60 and 200 show pronounced instability at meiosis, with a heavy bias toward expansion; therefore, these behave as ''premutations.'' This process of progressive repeat expansion has been termed *dynamic mutation*[6] and provides an explanation for the unusual inheritance pattern of fragile X syndrome with many nonpenetrant ''transmitting'' males observed in pedigrees. A far greater instability is observed during maternal transmission; although minor instability of premutations may be observed in paternal meiosis, a paternally transmitted premutation rarely, if ever, expands into a full mutation. Rare cases of fragile X syndrome are found to have a point mutation or deletion in *FMR1* instead of a trinucleotide expansion, confirming the loss of gene product as a cause of fragile X pathology.

In addition, in 1991, the existence of the distinct condition of FRAXE was established when it became clear that several ''fragile X'' families (i.e., showing expressed site fragility in Xq27–28 and learning disability) did not have expansions in the CGG repeat tract in the *FMR1* gene. Shortly afterward, these families were found to be segregating an expansion mutation in a GCC repeat tract in exon 1 of another gene, designated *FMR2*.[7] Pedigrees of FRAXE families differ notably from those of FRAXA families in that affected males frequently have children, with the degree of intellectual and behavioral impairment being much less pronounced than for FRAXA. Males with FRAXE mutations do not show the syndromic features typical of FRAXA, and FRAXE is now considered as a nonspecific X-linked mental retardation condition with a much lower incidence than FRAXA. The FRAXE repeat shows a dynamic mutation behavior similar to that of FRAXA, with unstable premutations

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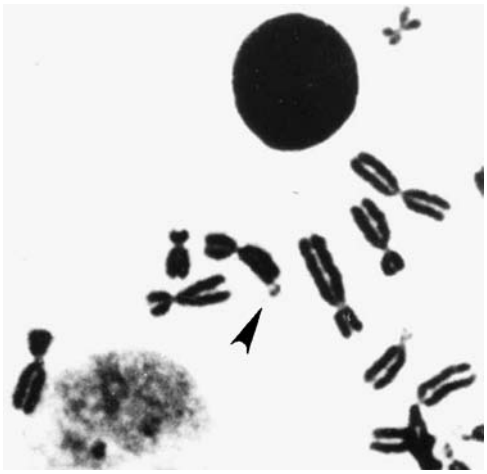


Fig. 1 Fragile X chromosome. (Courtesy of Prof. Pat Jacobs.)

expanding progressively to become full mutations. However, rather surprisingly, there is no obvious homology between *FMR1* and *FMR2* apart from the repeat tract.

The *FMR1* gene responsible for fragile X syndrome comprises 17 exons, is alternatively spliced, and codes for a protein-designated FMRP,[8] expressed in various tissues but especially strongly in neurones and spermatogonia. Although not yet fully understood, the function of FMRP is becoming rapidly elucidated in recent years. It is an RNA-binding protein whose domains also include a nuclear localization signal and a nuclear export signal. It shows affinity for a number of partner proteins in the cytoplasm, where it associates closely with ribosomes. FMRP appears to shuttle in and out of the nucleus, binding a variety of mRNA including that of *FMR1* itself, forming a ribonuclear particle that is transported along neurones to dendrites, where some local protein synthesis appears to take place. One of the key processes influenced by FMRP seems to be the maturation of dendritic spines; these fine protuberances are the principal determinants of synaptic plasticity, which in turn affects learning, memory, and cognition. Abnormal or immature dendritic spines have been demonstrated in both genetically and environmentally induced mental retardation syndromes. Knockout mice deficient in FMRP have been shown to exhibit learning deficits and immature and overabundant dendritic spines.[9]

Population Genetics

The dynamic mutation process creates a situation where new mutations are rarely observed, with all cases of premutations and full mutations in the population having been derived from parents with smaller expansion mutations. It is presumed that the smallest premutations arise from within the size range of 6–59 repeats, which exhibits a stable polymorphism within the general population. In the upper part of this ''normal'' size range, from about 45 repeats upward, the probability of unstable meiotic transmission becomes slightly elevated, but this tendency seems to be greater in some families than others, which creates a broad region of overlap in size between the ''normal'' and ''premutation'' allelic states.[10] This range of sizes, approximately 45–60 repeats for FRAXA and possibly lower for FRAXE, has been termed the ''intermediate range'' or ''grey zone.'' There are substantial difficulties in the genetic counseling of patients found to carry an intermediate-sized allele, especially where the genetic family history is incomplete. The risk of expansion to a full mutation increases progressively with the size of premutation in the mother; the smallest premutation, to date, found to expand to a full mutation in one generation has been 59 repeats.

The main factor believed to differentiate stable intermediate alleles from unstable potential premutations is the presence of interspersed AGG motifs at regular intervals, typically every 9th or 10th trinucleotide, within the CGG repeat tract.[11] These are thought to anchor the repeat sequence and prevent major unequal exchanges leading to expansion. Most normal, stably transmitted alleles are found to contain at least two interspersed AGGs, whereas almost all unstable premutations have pure CGG tracts or a single AGG. Where an AGG is retained, it is almost invariably the most proximal one in the sequence, implying that loss of interspersions and consequent expansion are effected at the distal (3′) end. The evidence is circumstantial because the loss of an AGG leading to acquired instability within a pedigree has yet to be reported. However, the GCC repeat tract at the FRAXE locus appears to be devoid of interspersions even among normal alleles; hence, the presence of interspersed motifs is clearly not the sole mechanism for restricting the mutability of trinucleotide repeat sequences.

Diagnostic Testing

Full mutations at both the FRAXA and FRAXE loci may be readily identified by a Southern blot of a genomic DNA digest followed by hybridization with a specific labeled probe (Fig. 2). Use of a double digest employing one restriction enzyme sensitive to methylation enables the observation of hypermethylation associated with full mutations, thereby providing a more sensitive resolution of full mutations and premutations based on both size and methylation status.[12] Occasionally, mosaics for a full mutation and a premutation are observed; these constitute up to 20% of FRAXA full-mutation patients and may be less severely affected with fragile X syndrome. Care should be taken when interpreting the FRAXE blots using

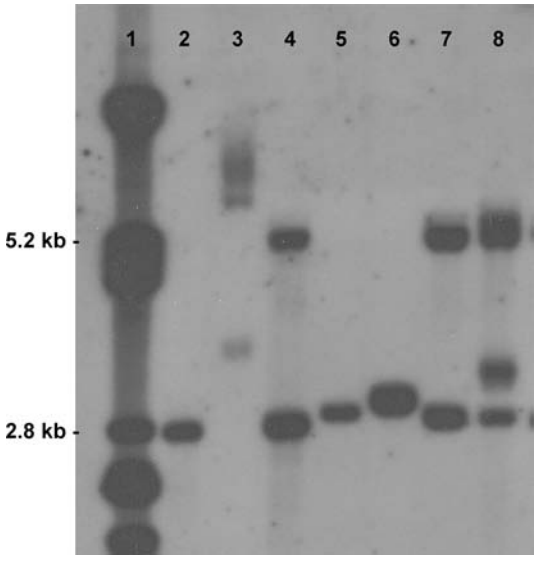


Fig. 2 Southern blot of *FMR1* gene. Digest with *Bst*ZI/*Eco*RI hybridized to probe StB12.3. (From Ref. [12].) Lane 1: Size marker λ*Pst*I digest; lane 2: normal male; lane 3: full mutation/premutation mosaic male; lane 4: normal female (note methylated upper fragment corresponding to inactive X chromosome); lane 5: male with intermediate allele; lane 6: premutation male; lane 7: normal female; lane 8: premutation female.

*Hin*dIII digests because there is a restriction site polymorphism that can mimic full mutations;[13] if this is suspected, a different enzyme digest should be used to confirm the diagnosis. Southern blot analysis is generally performed on samples of peripheral blood but may also be carried out on chorionic villus samples, facilitating reliable prenatal diagnosis of fragile X syndrome.

The high demand for fragile X testing creates a need for a high-throughput exclusion technique, and polymerase chain reaction (PCR) analysis of the repeat sequence using either a radioactive nucleotide incorporation (Fig. 3A) or a fluorescently labeled primer (Fig. 3B) provides rapid and convenient detection of males with a normal-sized allele and of females with two normal-sized alleles.[14] This technique also detects the smaller-sized premutations up to approximately 70 repeats, and enables fairly accurate estimates of repeat size to be made. However, it will not detect the larger premutations or full mutations due to the sharp reduction in efficiency of PCR for long CG-rich repeat sequences. Hence, males who show no PCR product, or females who show just a single allele require further analysis by Southern blot to determine their genotype. It should also be noted that the exclusion of fragile X by this method depends on the assumption that mosaicism for a normal allele and a full mutation is absent or very rare. If there is not enough DNA for conventional Southern blot analysis, it is possible to detect large premutations or full mutations by electrophoresing PCR products, transferring them to a membrane by electroblotting and hybridizing to a $(CGG)_n$ oligonucleotide probe.[15]

An alternative way of diagnosing fragile X syndrome is by in situ FMRP detection using an anti-FMRP antibody, which is detected as a red stain in the presence of FMRP.[16] The technique may be employed using blood cells, but has met with most success using hair root tissues. Clearly, as it is the absence of staining due to lack of FMRP that is indicative of fragile X syndrome, this diagnostic strategy is highly reliant on the efficiency of staining and must, in any case, be followed up by genetic testing of any suspected patients. Furthermore, it will not identify premutation carriers. However, it does have the advantage of detecting the minority of fragile X patients with a null mutation in the coding sequence of the gene rather than a trinucleotide repeat expansion.

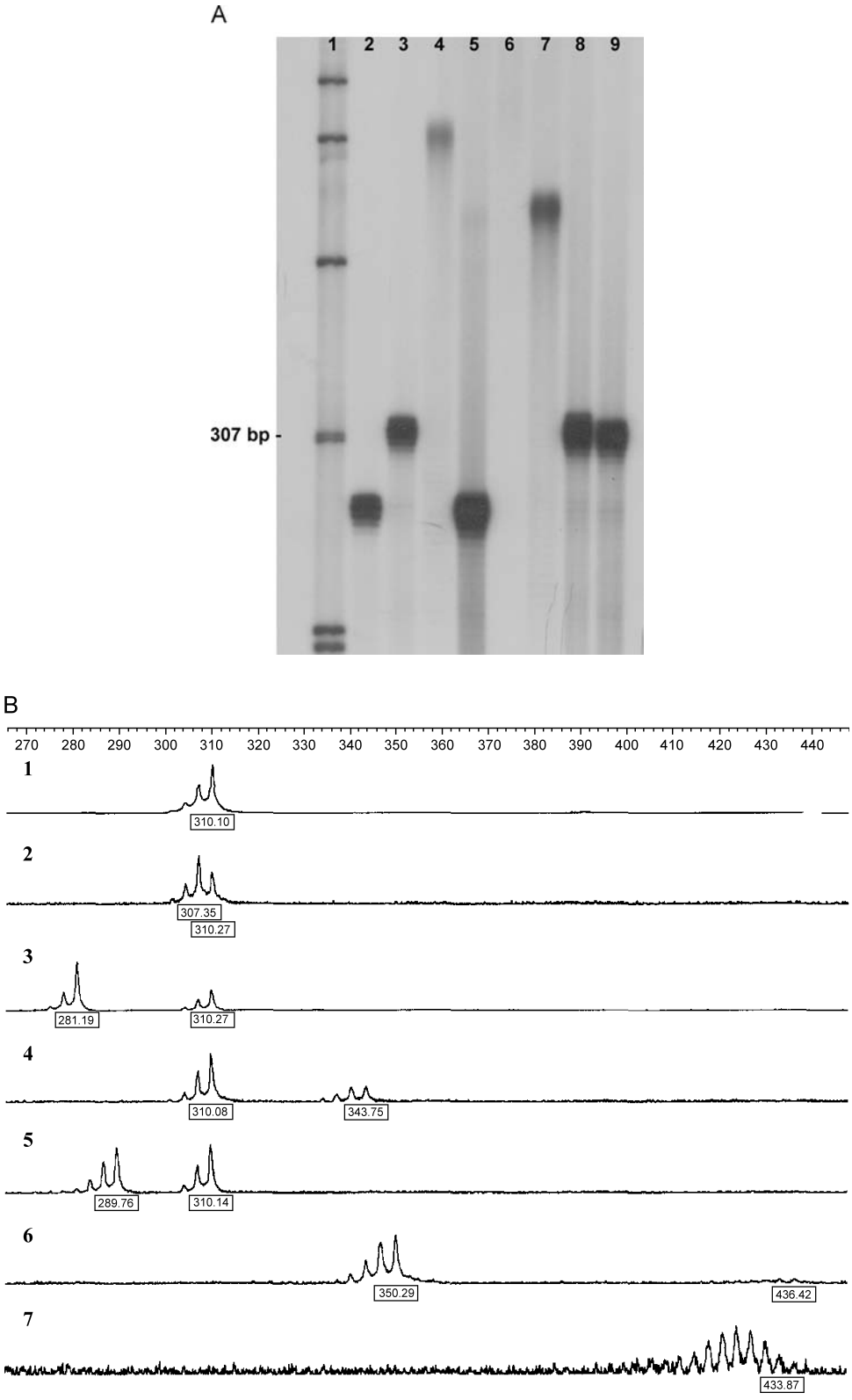
Screening and Prevalence

Estimates of the prevalence of fragile X mutations have shown wide variation. Early screening studies employing cytogenetic fragile-site expression as a diagnostic criterion were subject to errors resulting from nonpenetrance of cytogenetic expression, low-level ''background'' expression leading to false positives, and the pooling of FRAXA and FRAXE site expression—these errors created probable overestimates of the prevalence of *FMR1* mutations. More recent screening studies using molecular testing to identify mutations have arrived at a consensus of 1/4000 to 1/6000 as a prevalence for the FRAXA full mutation;[17,18] although lower than previous estimates, this would still make fragile X syndrome the single most common inherited cause of intellectual disability. There is some evidence of ethnic variation: a few isolated populations have a significantly higher prevalence of FRAXA mutations.[19] The only recorded estimate of FRAXE full mutation prevalence is 1/23,000,[18] but this needs to be corroborated by further studies.

Other *FMR1* Phenotypes

Until recently, it was thought that premutations had no adverse phenotypic consequences. However, two conditions have now been found to be associated with *FMR1* premutation alleles: premature ovarian failure (POF) and fragile X-associated tremor and ataxia syndrome (FXTAS).

POF, defined as the onset of menopause before the age of 40 years, affects some 20% of female *FMR1* premutation carriers.[20] As there is no evidence of POF among full-mutation carriers, it seems likely that this is a causal effect of the premutation rather than any mutation


A
1 2 3 4 5 6 7 8 9
307 bp -
B
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1
310.10
2
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4
310.08
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in an adjacent gene in linkage disequilibrium with *FMR1*. The pathological mechanism by which *FMR1* premutations cause POF is unknown, but a possible biochemical basis for this and other premutation phenotypes has been established by the discovery of elevated *FMR1* mRNA levels and reduced FMRP levels in premutation carriers.[21]

FXTAS is a more recently discovered condition than POF and its properties are still being characterized.[22] However, preliminary studies suggest that it may have a penetrance similar (20–30% of male premutation carriers) to POF, and again no comparable condition has been found in full mutation carriers. It appears that FXTAS may be restricted to male carriers, although studies continue to look for possible equivalent effects in female carriers.

CONCLUSION

The identification of the *FMR1* and *FMR2* genes has immeasurably enhanced the quality of care and counseling available to fragile X families. Many previously unidentified families have been diagnosed, and prenatal diagnosis is routinely available. Although no cure for fragile X is imminent, rapid progress in identifying the pathology of fragile X syndrome and the role of FMRP should be invaluable in the development of appropriate drug therapies and interventions. The mouse model may be a useful vehicle in such an endeavor; however, the gap in physiology between mice and humans should not be underestimated. There remains a pressing need for more accurate risk prediction for alleles in the intermediate and premutation size ranges, which may be possible using advanced sequencing techniques to establish the pattern of AGG interspersions within the repeat tract. Finally, the spectrum of conditions associated with premutations and the etiology of such effects remain to be fully elucidated.

The discovery of the *FMR1* gene and its novel mutational mechanism provided the catalyst for the identification of an abundant class of genes harboring trinucleotide repeats, including those responsible for common genetic conditions such as Huntington disease, myotonic dystrophy, and Friedreich ataxia. Although the first trinucleotide repeat disease to be discovered, FRAXA has not proved to be typical of the genre of such loci: peculiar features include association with fragile sites and loss of gene function due to methylation, extreme parent-of-origin bias in repeat expansion, and pleiotropic phenotypes attributed to premutations. Fragile X syndrome, despite a wealth of intensive study and many significant advances, continues to rank among the most intriguing and enigmatic of all human genetic diseases.

Fig. 3 PCR of the *FMR1* CGG repeat. Method and primers *c* and *f* as in Ref. [14]. (A) Autoradiograph of radioactive PCR gel. Lane 1: Size marker pBR322*Msp*I digest; lane 2: male, 20 repeats; lane 3: male, 30 repeats; lane 4: premutation male, approximately 104 repeats; lane 5: premutation female, 20 plus approximately 73 repeats; lane 6: full-mutation male, no product detected; lane 7: premutation male, approximately 78 repeats; lane 8: heterozygous female, 30 plus 31 repeats; lane 9: homozygous female, 30 repeats. (B) Plots of fluorescent PCR analyzed on ABI377 automated sequencer with Genotyper software. Plot 1: Male, 30 repeats; plot 2: female, 29 plus 30 repeats; plot 3: female, 20 plus 30 repeats; plot 4: female, 30 plus 41 repeats; plot 5: female, 23 plus 30 repeats; plot 6: premutation female, 43 plus 72 repeats; plot 7: premutation male, approximately 70 repeats.

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Gaucher Disease

Deborah Elstein
Ari Zimran
Shaare Zedek Medical Centre, Jerusalem, Israel

INTRODUCTION

Gaucher disease, the most common lysosomal storage disorder, is caused by deficient β-glucocerebrosidase activity and results in progressive accumulation of the substrate in macrophages, leading to hepatosplenomegaly, anemia, thrombocytopenia, skeletal lesions, lung disease, and sometimes neurological involvement. Enzyme replacement therapy (ERT) has proven to be safe and effective. Other strategies including bone marrow transplantation, substrate reduction, enzyme chaperones, and gene therapy have been variously attempted.

BACKGROUND

Gaucher disease was first described in 1882 by a French medical student, Philippe Gaucher, who assumed that the large splenic cells, which today bear his name, were a manifestation of a primary neoplasm of the spleen. More than a century later, Gaucher disease is known as the most prevalent lysosomal storage disorder, caused by an inherited enzymatic defect with consequent accumulation of undegraded glucocerebroside in monocyte–macrophage cells—the ''Gaucher cells.''[1] Nonetheless, neither quantity of residual activity nor genotype can accurately predict the type or degree of severity of the phenotype except in a broad sense. Thus the clinical heterogeneity that marks all forms of Gaucher disease is attributable only in part to the more than 200 mutations within the β-glucocerebrosidase gene;[2] other genetic and/or environmental factors undoubtedly also influence phenotype.

TYPES OF GAUCHER DISEASE

Gaucher disease has classically been divided into three clinical types based on the absence or presence of neurological involvement.

Type I is defined by the absence of neurological involvement and, usually, the presence of the N370S (1226G) mutation on at least one allele. Although pan-ethnic, it is especially prevalent among Ashkenazi Jews (1:850 live births). There is tremendous heterogeneity in the severity of clinical manifestations, ranging from asymptomatic to severely debilitating disease [often defined by the Zimran Severity Score Index (SSI), which awards up to 30 points based on disease features].

Types II and III, both relatively rare and pan-ethnic, are marked by the involvement of the central nervous system.[3] Horizontal supranuclear gaze palsy is pathognomonic for neuronopathic forms.

Type II, the infantile (acute) form, is characterized by several neurological features, including hypertonic posturing, strabismus, trismus, and retroflexion of the head, in addition to massive organomegaly. The onset of symptoms is usually during the first 6 months of life, with progressive deterioration of neurological function until death, usually before the second year.

Type III Gaucher disease has been further subclassified: type IIIa patients exhibit mild to moderate hepatosplenomegaly and slowly progressive neurological deterioration, frequently associated with recurrent myoclonic seizures. Patients with type IIIb exhibit massive hepatosplenomegaly, frequently accompanied by lung involvement, but with minimal neurological signs, usually only the horizontal supranuclear gaze palsy.

Although cardiac involvement is rare in all forms, a unique variant, type IIIc, has been described in Japanese, Spanish, and Arab children, all homozygous for the D409H (1342C) mutation, who evince progressive calcification of aortic and/or mitral valves of the heart that is life-threatening.[4] Homozygosity for this mutation is the single instance of relatively tight genotype–phenotype correlation in Gaucher disease.

MOLECULAR, GENETIC, AND ENZYMOLOGICAL ASPECTS

The genomic area near the β-glucocerebrosidase locus on human chromosome 1q21 includes its pseudogene and several other gene loci. Enzyme and mRNA activities vary among species and tissues, the import of which is unclear. Before the advent of a biochemical assay of β-glucocerebrosidase activity, demonstration of Gaucher

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cells in the bone marrow was used for diagnosis. Since 1970, routine enzymatic assay (pH 4) of leukocytes that hydrolyze 4-methylumbelliferyl-β-glucocerebrosidase to <15% of normal is the gold standard for diagnosis.[1] A limitation of the test is the overlap of activity of heterozygotes with normal values, and the inability of the enzyme level to predict neuronopathic involvement.

Therefore, mutation analysis based on routine polymerase chain reaction (PCR)-based technology has been added as an adjunct for diagnosis because some mutations have been associated with mild, severe, or lethal disease. Primers target the gene fraction of interest and amplify it (but not the pseudogene). Restriction digestion reveals mutations by gel electrophoresis; alternative approaches include allele-specific oligonucleotide hybridization,[2] and mismatched PCR methods by amplification refractory mutation systems[1] or by direct sequencing, if necessary. A new kit assay, the PRONTO™ procedure (Savyon Diagnostics, Israel), detects single-nucleotide polymorphisms in DNA sequences via multistep reactions using biotin-labeled primers, and the final results are read either visually (substrate remains clear or turns blue) or colorimetrically using enzyme-linked immunosorbent assay (ELISA).

Whereas the majority of disease alleles are single-base substitutions resulting in missense mutations, there are also nonsense mutations and rearrangements. Of the more than 200 disease-associated alleles (most of which cluster toward the 3′ end of the gene), fewer than 10 have significant frequencies in various populations, among them N370S (1226G), 84GG, L444P (1448C), IVS 2+1, D409H (1342C), R486H (1604A), and F213I (754T), and the two recombinant alleles, RecNciI and RecTL.[2]

Allele distribution reveals that the common N370S mutation is detected in 71.8% of Ashkenazi Jews (which, in homozygosity, is invariably associated with mild disease), but only in 43.6% of non-Jews;[2] indeed, among non-Jews, the prognostic information of large numbers of private/rare mutations also decreases the usefulness of mutation analysis. Thus genotype–phenotype correlations and genetic and/or environmental modifiers are areas of intense research for improved genetic counseling and open avenues of therapeutic intervention. Currently, chorionic villous sampling and amniocentesis are available for prenatal diagnosis. As a rule of thumb for broad outlines for prediction of phenotype: null/null mutations are incompatible with life, whereas mild/mild mutations generally result in mild type I disease; mild/severe or mild/null mutations often result in symptomatic, nonneuronopathic disease; and combinations of severe/severe or severe/null mutations result in neuronopathic forms.

Mutation analysis for detection of carrier status among Ashkenazi Jews is robust, being nearly 98% reliable.[1]

MALIGNANCY

An increased incidence of monoclonal gammopathies and multiple myeloma has been reported. In a recently completed study of patients in a large referral clinic and from the international registry (ICGG), approximately 4–5% of all patients developed a malignancy.

PREGNANCY

Fertility of both sexes is generally unaffected by the disease; however, pregnancy per se may affect the patient, and, in turn, Gaucher disease may affect the pregnancy. Although many patients experience a rather benign course during pregnancy, delivery, and the postpartum period, patients with more severe manifestations or with a bleeding tendency may not enjoy this outcome; hence, close hematological monitoring is recommended during pregnancy.[5]

VISCERAL SIGNS AND SYMPTOMS

Splenomegaly exists in all patients, and is typically associated with hypersplenism, early satiety, and, in children, growth (height) retardation. Anemia and thrombocytopenia, resulting in fatigue and bleeding tendency, respectively, are among the earliest presenting signs and most prominent features of Gaucher disease. In almost all symptomatic patients, there is some degree of hepatomegaly but only rarely are liver function tests abnormal.

Bone involvement is among the more variable and probably the most debilitating features, particularly the recurrent attacks of ''bone crises'' experienced primarily in childhood. The incidence of these episodes abates with age. The occurrence of pathological fractures, avascular necrosis of the heads of femur and humeri, and compression fractures of the spine are well-documented complications of severe skeletal involvement. Osteopenia and osteoporosis may be important findings even among young adults.

Pulmonary involvement is recognized as an uncommon finding in type I disease; among the neuronopathic forms, it is generally the penultimate cause of death.

THE NEUROLOGICAL CONTINUUM

Although type I is, by definition, nonneuronopathic, and the presence of the N370S allele obviates neurological involvement, nonetheless, recent data are suggestive of a

phenotypical continuum that includes some neurological signs even in patients with type I. Among the earliest anecdotal reports were those of parkinsonism that is marked by early onset, aggressive progression, and refractoriness to conventional anti-Parkinson therapy in patients with very mild type I Gaucher disease.[6]

Similarly, the very-early-onset type II or the ''neonatal form'' has been proposed wherein onset of signs is in the neonatal period, and is in conjunction with other overt abnormalities including ichthyosis, hydrops fetalis, and/or collodion baby.[7]

BIOCHEMICAL AND SEROLOGICAL MARKERS

There is no single biochemical marker for Gaucher disease that correlates perfectly with severity and/or is affected in a predictable manner with therapy. Although acid phosphatase and angiotensin-converting enzyme have long been known to be elevated in most patients, there is a preference to measure chitotriosidase levels,[8] which have been used as a measure of disease severity, to assess the success of therapeutic modalities and to compare treatment regimens.

SYMPTOMATIC MANAGEMENT

Whereas in the past, splenectomy was frequently performed for the management of severe thrombocytopenia and/or for relief of mechanical compression by the greatly enlarged spleen, by removing the main reservoir for Gaucher cells, splenectomy may induce or aggravate liver and bone involvement. Today, splenectomy is rarely indicated.

Total joint replacement is the most important orthopedic intervention, both in the past and in the present, for patients with avascular necrosis of the joints who suffer from severe pain and disability.

A rare but severe manifestation is spinal cord compression caused by the collapse of one or more adjacent vertebral bodies; options of conservative management and surgical decompression have been employed to reduce pain, ameliorate neurological deficit, and correct gibbus formation. Conversely, among children with type III disease where the gibbous develops without destruction of vertebral bodies and with no impingement on the spinal cord, the main concern is compromised diaphragmatic excursion and secondary restrictive lung disease. In these latter cases, a brace may be required, although kyphosis may not be reversible.

BONE MARROW TRANSPLANTATION

Bone marrow transplantation, albeit in limited cases, has resulted in growth spurt in children, reduction of hepatomegaly, and normalization of plasma glucocerebroside levels.[9] Unfortunately, morbidity and mortality of the procedure are high and neurological/cognitive benefit is equivocal. Nevertheless, its success has set precedents for gene therapy and nonmyeloablative stem cells transplantation as potential options.

ERT

ERT[10] with placental-derived enzyme, alglucerase (Ceredase®; Genzyme Therapeutics Inc.), or the recombinant form, imiglucerase (Cerezyme®, Genzyme Therapeutics Inc., MA), has proven to be safe and effective in more than 3000 patients worldwide. Reduction in organ volumes and improvement in hematological parameters have dramatically improved quality of life.

In a recent summary by the International Gaucher Registry, efficacy and safety profiles of ERT in over 1000 patients with 2–5 years of treatment were recorded.[11] For anemic patients, hemoglobin concentration increased to normal or near-normal within a year with sustained response through 5 years. Hepatomegaly decreased by 30–40% over 2–5 years. Splenomegaly decreased by 50–60% over 2–5 years. In patients with bone pain/crises, 52% were pain-free after 2 years and 94% reported no additional crises.

The greatest advantages of ERT may prove to be early administration to children at risk for severe disease, thereby precluding significant visceral and bone involvement.[12]

The effect on bone density is preliminary and complicated by the impact of other genetic and environmental factors. Another option for clinical management of both bone loss and secondary bone pain is biphosphonates, administered in conjunction with ERT, but which may be problematical for children and teenagers.

Infiltrative lung disease secondary to severe Gaucher disease in both children and adults may benefit from ERT. Nonetheless, pulmonary hypertension has been noted in some patients on ERT. Although a causal relationship with ERT has been difficult to prove, treatment withdrawal may be considered in patients who develop progressive primary-like pulmonary hypertension.[13]

Good clinical results have been axiomatic despite a wide range of ERT dosages and frequencies. However, ERT poses significant hardships to patients as it involves repeated intravenous infusions, usually once every 2 weeks, for life. In addition, the high cost limits the number of patients who can avail of this treatment, particularly in

developing countries. Finally, the current formulation is incapable of crossing the blood–brain barrier, thereby circumscribing its value in patients with significant neurological manifestations.

MAINTENANCE REGIMENS

Despite the success of ERT for symptomatic patients, one must ask whether dose adjustments and/or circumscribed ''drug vacations'' may be suggested in individual patients who have had up to 10 years of therapy, particularly those who have achieved near-normalization of major disease parameters. This argument is particularly cogent in light of a report that documents the continuation of the beneficial effects of enzyme on disease parameters after varying periods of withdrawal, with no deterioration among adults.[14] Drug vacations should not be considered for children.

SUBSTRATE REDUCTION THERAPY

Oral substrate reduction therapy (SRT) involves attenuation of the rate of synthesis to achieve a balance with reduced endogenous glucocerebrosidase.

A pivotal trial of SRT was carried out in 28 adult patients (from four centers: Cambridge, UK; Amsterdam; Prague; and Jerusalem) naïve to enzyme therapy, with mild to moderate type I Gaucher disease, who were unable or unwilling to receive enzyme treatment. There was significant reduction in spleen and liver, although amelioration of anemia and thrombocytopenia lagged behind. There were side effects, including diarrhea, abdominal pains, weight loss, tremor, and peripheral neuropathy, which were reversible with dose reduction or withdrawal.[15] A low-dose trial showed dose dependency for clinical improvement but no reduction in severity or frequency of side effects. Finally, using SRT as a maintenance regimen (i.e., switching from ERT or in combination with ERT), at 6 months, there were no clinically significant differences among groups.

SRT, commercially available as Zavesca™ (Actelion Pharmaceuticals, Allschwil, Switzerland; Teva Pharmaceuticals Israel, Netanya, Israel), has received European, Israeli, and American approval for patients with mild to moderate Gaucher disease, for whom ERT is unsuitable or not a therapeutic option, respectively. Because it is a small molecule capable of crossing the blood–brain barrier, this drug may potentially improve neurological features in Gaucher disease and other lysosomal storage disorders, in whom benefits may outweigh risks and discomforts of the drug in its current formulation.

FUTURE IMPROVEMENT OF ERT

Poor delivery of the current formulation of enzyme therapy to the lungs, bones, and brain, and increased convenience call for the development of new generations of enzyme. In this context, the discovery of the three-dimensional structure may provide this possibility and may be used for designing structure-based drugs to restore the activity of defective enzymes.

CHEMICAL CHAPERONES TO INCREASE ENZYME ACTIVITY

The addition of subinhibitory concentrations of *N*-(*n*-nonyl)deoxynojirimycin (NN-DNJ) in fibroblast culture led to increased N370S β-glucocerebrosidase activity that persisted after withdrawal of the putative chaperone, suggesting that NN-DNJ chaperones the folding of mutated protein, stabilizing it for transport to the Golgi apparatus and hence proper trafficking to lysosomes. This modest increase in glucocerebrosidase activity may be sufficient to achieve a therapeutic effect.[16]

GENE THERAPY

Gene therapy is a theoretically curative approach for enzyme disorders and, in fact, Gaucher disease has been among the first targeted by this modality.[17] However, despite the considerable efforts brought to bear, gene therapy is apparently not on the immediate horizon.

ETHICAL AND SOCIETAL CONSIDERATIONS

Although to date only symptomatic management can be recommended for type II disease, ethical issues relating to ERT that are neither life-prolonging nor appreciably improve the quality of life of these babies need to be addressed. Indeed, ethical arguments apply to the emotional outlay in neuronopathic forms, but also to the financial outlay for orphan drugs for nonsevere, non-neuronopathic patients. No evidence-based consensus has been documented to support the extraordinary expense of superhigh doses of ERT for children with neuronopathic forms, and no maintenance trials have been initiated to provide guidelines for tapering/withdrawing therapy once near-normal parameters have been achieved. These cases need to be addressed equitably and ethically.

CONCLUSION

Gaucher disease is caused by an enzymatic defect with consequent accumulation of glucocerebroside. Type I, the nonneuronopathic form, is rather common, with a predilection among Ashkenazi Jews. There is tremendous variability in age of onset, severity, and phenotypical expression in this type. Symptomatic presentation may include hepatosplenomegaly, anemia, thrombocytopenia, and skeletal or lung involvement. Life expectancy is unimpaired. The neuronopathic forms are pan-ethnic with a continuum of neurological and visceral signs and symptoms, and with decreased life expectancy. Diagnosis is performed by assay of β-glucocerebrosidase activity, and molecular analysis of mutations may broadly define genotype–phenotype correlations. ERT has proven to be safe and effective in ameliorating disease symptoms and signs; however, it involves life-long intravenous therapy, is costly, and is incapable of crossing the blood–brain barrier. Thus, other forms of treatment, including bone marrow transplantation and oral SRT, are attractive.

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Gene Dosage—Multiplex Probe Ligation and Amplification

Anders O. H. Nygren
Jan P. Schouten
MRC-Holland, Ltd., Amsterdam, The Netherlands

INTRODUCTION

The multiplex ligation-dependent probe amplification (MLPA) technique provides the means for the quantitative analysis of various changes in gene structure and gene copy number of a large number (40–50) of DNA target sequences in a single reaction. Conventional PCR-based methods for mutation detection such as denaturing high-performance liquid chromatography (dHPLC) and sequencing are not able to detect most exon deletions and duplications because the normal allele that is present is also exponentially amplified. MLPA requires a minimum of only 20 ng human chromosomal DNA and can discriminate sequences differing by a single nucleotide. In MLPA, binary probes containing a sequence-specific part and a universal part are hybridized to their DNA targets and ligated. Each ligated probe is then PCR amplified with a universal primer pair and gives rise to an amplification product of unique size. The relative amount of each amplification product reflects the amount of the target sequence that is present in the nucleic acid sample. As the sequence detected by the probe is only around 60 nt, MLPA is able to detect copy number changes of single exons. The necessary equipment used in MLPA, a thermocycler with a heated lid and a high-resolution electrophoresis apparatus with fluorescent detection, is available in most molecular biology laboratories.

APPLICATIONS OF MLPA

Current applications of MLPA are the following:

1. Detection of unusual copy numbers of single chromosomes (Fig. 1; e.g., Down's syndrome) either from patient material or from small samples (<1 mL) of amniotic fluid, according to Slater et al.[1]
2. Detection of unusual copy numbers of one or more subtelomeric regions. Presence and copy number detection of all chromosome ends can be tested in a single MLPA reaction.[2]
3. Detection of unusual copy numbers of chromosomal regions such as the 22q11 region in DiGeorge syndrome, the ELN gene region in Williams syndrome, and different 17p11.2 region in Smith–Magenis syndrome and CMT1A/HNPP.
4. Detection of unusual copy numbers of specific genes such as the *PLP1*,[3] gene in Pelizaeus–Merzbacher disease (PMD) and the copy number of the *SMN1*,[4] and *SMN2*,[5] genes involved in spinal muscular atrophy.
5. Detection of unusual copy numbers of single exons (Fig. 2). More than 60% of all cases of Duchenne muscular dystrophy are caused by deletion or duplication of one or more exons of the 2.5-Mbp-long DMD gene. All 79 exons of this gene can be screened by performing two MLPA reactions. Depending on the presence of founder mutations in certain populations, deletion of one or more exons of the BRCA1 gene are the cause of 4–30% of all cases of BRCA1-related hereditary breast cancer.[6,7]
6. Detection of gains (e.g., Her2-neu and Myc) and losses (e.g., TP53/p16) of specific genes or chromosomal regions in tumor samples (Nederlof et al., in preparation).
7. With a variation on the ordinary MLPA protocol, both copy number changes as well as methylation status of the Prader–Willy/Angelman region can be determined (Errami et al., in preparation).

A list of commercially available MLPA kits can be found on the www.mrc-holland.com website.

TECHNICAL DESCRIPTION

The MLPA technique permits relative quantification of up to 45 different target sequences in an easy to perform reaction. In MLPA, specially designed oligonucleotide probes are hybridized to their specific DNA sequences in the DNA sample, ligated, and then amplified in a multiplex PCR. Each gene-specific probe consists of two oligonucleotides and each oligonucleotide has a gene-specific part and a universal sequence part used for amplification. The concentrations of oligonucleotide probes and the extended hybridization

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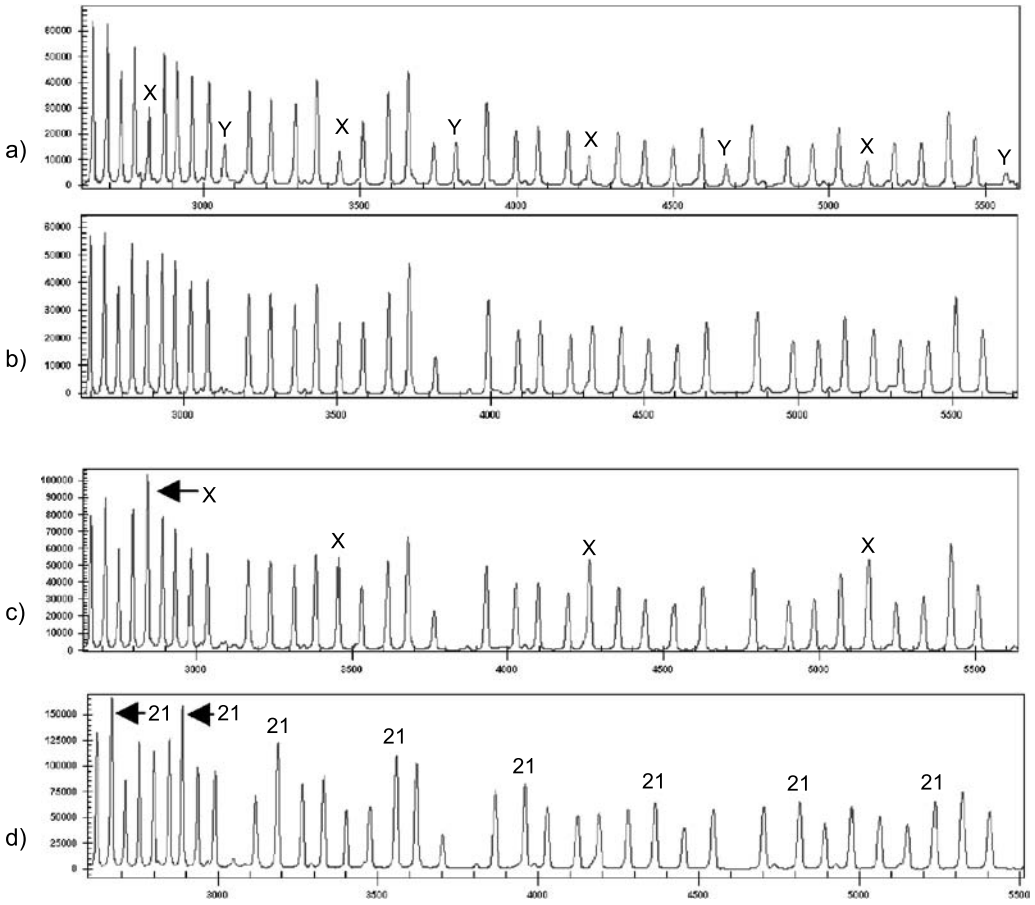

Fig. 1 Detection of trisomies by MLPA. One hundred nanograms of DNA samples were analyzed by MLPA using probe mix P001 (MRC-Holland). A detailed description of the P001 probe mix can be obtained at www.mrc-holland.com. PCR products were analyzed by capillary electrophoresis (Beckman CEQ2000). CE patterns from male (a), female (b), and DNA from a female triple chromosome X (c) and a female with triple chromosome 21 (d). (*View this art in color at www.dekker.com.*)

times are sufficient to ensure practically complete hybridization of target sequences and their corresponding probe oligonucleotides. Only probes hybridized to a perfectly matched target DNA are ligated and then exponentially amplified in the following PCR (Fig. 3). MLPA probes that do not find a target sequence cannot be ligated and consequently will not be amplified in the PCR. Probes that are hybridized to a target but have a mismatch close to the ligation point are not ligated because of the high sensitivity of the thermostable Ligase-65. This allows for the discrimination of closely related sequences such as the *SMN1* and *SMN2* genes both involved in spinal muscular atrophy. Because the relative peak area of each amplification product reflects the relative copy number of the target sequence in the DNA sample, comparison of gel patterns with patterns obtained on a control sample allows absolute copy number quantification. To obtain reproducible results, MLPA currently needs at least 20 ng human DNA, corresponding to approximately 3000 cells or 6000 single-copy target sequences.

Specificity of the Probes

When 40 probes, each consisting of two oligonucleotides, are present in one tube with genomic DNA, not 40 but 1600 different target sequences are theoretically able to hybridize with two oligonucleotides and make them a substrate for ligation. However, the specificity of MLPA is very high and no nonspecific products are generated during the PCR.[8] Control experiments showed that no product was generated when the short synthetic oligonucleotides were omitted or replaced by an identical oligonucleotide with a mismatch at the 3′ end.[5] Several factors contribute to exquisite specificity of MLPA: 1) the need for the two oligonucleotides to anneal in juxtamposition on a target nucleic acid;

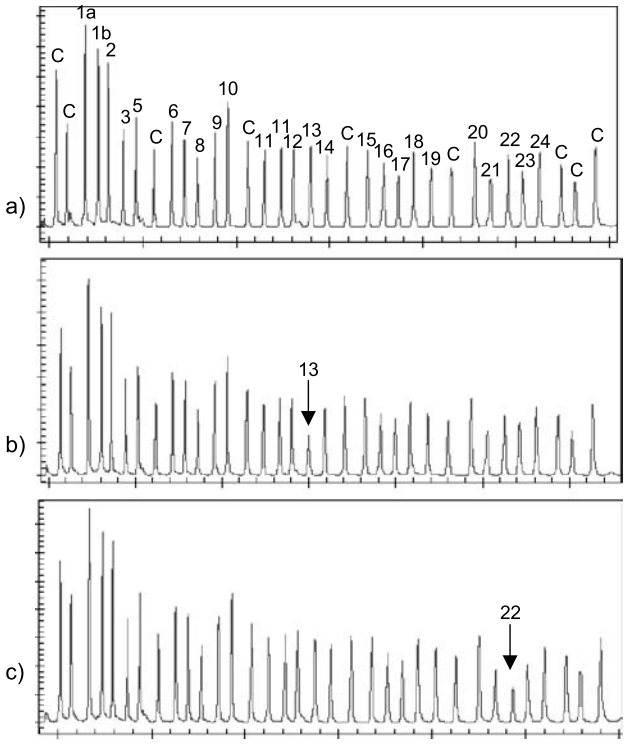

Fig. 2 Detection of exon deletions in the BRCA1 gene with MLPA. One hundred nanograms of DNAs from individuals from diagnosed breast- and/or ovarian-cancer-prone families were analyzed by MLPA using probe mix P002. The P002 mix contains one probe for each of the 24 BRCA1 exons (1–24), one extra each for the exon 1 and the large exon 11 as well as several controls probes (C) specific for different genes on different chromosomes were also designed and used in a single MPLA assay together with BRCA1 probes. A detailed description of the P002 probe mix can be obtained at www.mrc-holland.com. (a) A CE pattern obtained from a control DNA sample containing two copies of the normal BRCA1 gene. (b) A CE pattern obtained from DNA with a heterozygous BRCA1 exon 13 deletion. (c) A CE pattern obtained from DNA with a heterozygous BRCA1 exon 22 deletion. (*View this art in color at www.dekker.com.*)

2) hybridization and ligation are performed at 54–60°C, which reduces unspecific binding and increases specificity; 3) the use of thermophilic ligases requiring NAD (e.g., Ligase-65) that are very sensitive to mismatches next to the ligation site.

Probe Signal and Reproducibility

Twenty-eight probes specific for sequences on chromosomes X, 21, 18, and 13 generated, on average, a 1.44-fold higher signal on the respective trisomy DNA samples as compared with control DNA.[8] This is close to the theoretical 1.5-fold increase. Reproducibility of results in MLPA depends on several factors: 1) the purity of DNA samples, the method of PCR product analysis with capillary electrophoresis being superior to fluorescent detection on slab gels; 2) the software used for peak detection and the method used for normalization of data. Whereas DNA degradation hardly influences the results, the presence of PCR inhibitors, e.g., traces of phenol, does. In general, the standard deviation of results obtained in MLPA varies between 4% and 10% for each probe.

In MLPA the relative signal strength depends on the relative amount of the target sequences present in the sample. In model experiments, it was shown that an almost linear increase of the signal could be obtained when up to fivefold increase of synthetic targets was added to a DNA sample.[8] Addition of higher amounts of synthetic target resulted in an additional nonlinear increase in signal of that probe.

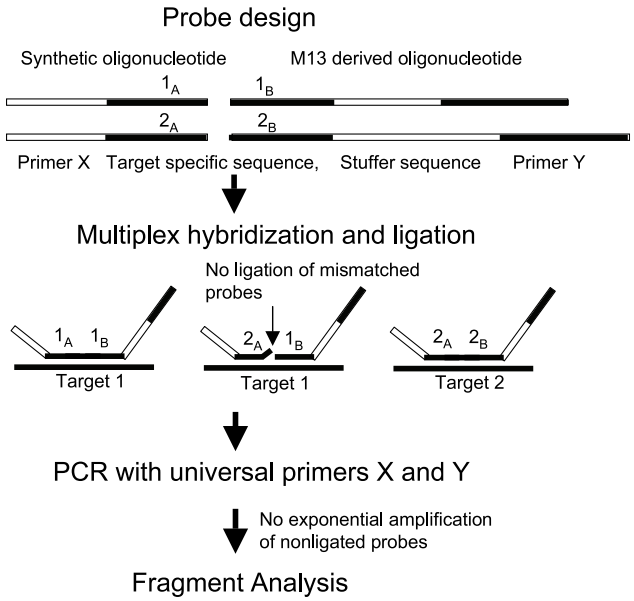

Fig. 3 Outline of MLPA. 1. Probe design: Each MLPA probe consists of two oligonucleotides, one short synthetic and one long M13 derived. The short synthetic oligonucleotide consists of a 5′ universal primer sequence X and a target-specific 3′ sequence. The M13-derived oligonucleotide consists of a 5′ target-specific sequence designed to hybridize in juxtaposition to the synthetic probe, a 3′ universal primer sequence Y and a stuffer sequence that has a different length for each probe. 2. Multiplex hybridization and ligation: One femtomole of each probe oligonucleotide is hybridized to the denatured target DNA. Only perfectly matched probes oligonucleotides are ligated by the thermostable Ligase-65. 3. PCR: Only ligated probes are exponentially amplified by the single primer pair X and Y. 4. Fragment analysis: The amplified fragments are separated and analyzed by capillary electrophoresis.

ADVANTAGES WITH MLPA

1. One technique can be used for many applications. The only difference between different assays is the probe mix used. Hybridization, ligation, and PCR reagents and conditions are identical for the various applications.
2. Multiplex: One reaction provides information on up to 45 targets. For most applications, this is sufficient to answer the specific question asked by the physician.
3. Easy to perform. Large numbers of samples, e.g., 96, can be tested simultaneously.
4. Sensitive. Only 20 ng of human DNA is required. Results do not depend on the amount of sample DNA used.
5. Reproducible. MLPA can detect small differences in copy number such as three vs. two copies of a gene sequence in a complex mixture like the human genome.
6. Requires a target sequence of only 60 nucleotides. Can therefore be used to detect deletions or duplications of a single exon and permits use of DNA of poor quality such as DNA extracted from formaldehyde-treated, paraffin-embedded tissues.
7. Sequences that differ in only a single nucleotide can be distinguished.
8. Low investment. The equipment necessary for MLPA, a thermocycler and DNA sequencing electrophoresis equipment, is present in most molecular biology laboratories. Capillary electrophoresis is preferred but MLPA has also been successfully performed on the older ABI 373 and 377 slab gels.

DISADVANTAGES WITH MLPA

1. DNA samples should be of equal purity. MLPA reactions are more sensitive to contaminants (PCR inhibitors such as small remnants of phenol) than ordinary PCR reactions. Results on DNA extracted from formaldehyde-treated, paraffin-embedded tissues can be good, but requires a DNA sample from healthy tissue that was treated identically for comparison.
2. It is time-consuming and difficult to develop your own probe mix. Each probe requires the design and preparation of an M13 clone, the purification of single-stranded DNA of that clone, and the digestion of that DNA with expensive enzymes. At this moment, MLPA kits are available only from MRC-Holland. It is possible to design a small number of completely synthetic probes, resulting in amplification products with a length between 100 and 130 nt, and to add these to the MRC-Holland probe mixes. Rules and sequences of the PCR primers are available from info@mrc-holland.com.
3. In contrast to Flourescence in situ hybridization (FISH), MLPA as it is performed today cannot be used to investigate single cells. MLPA analysis of DNA samples from cell mixtures will give the average copy number per cell. It will be difficult to detect deletions of a certain gene if the sample from which the DNA was derived contained less than 60% cancer cells.
4. MLPA is not suitable to detect new mutations, although probes can be made to detect specific known mutations. Mutations or polymorphisms exactly at the ligation site will, in most cases, result in a similar decrease in signal as a deletion of the complete target sequence of that probe. Point mutations and polymorphisms within 4 nucleotides of the ligation site will often affect the signal strength. Point mutations as well as small deletions/insertions within approximately 20 nucleotides of the ligation site may affect the probe signal.

PROTOCOL FOR DNA ANALYSIS

The use of a thermal cycler with heated lid (105°C) is essential. The use of 0.2-mL PCR tubes is recommended.

Materials and Methods

Dilute 50–500 ng of target DNA to a final volume of 5 μL with 10 mM Tris–HCl (pH 8.5), –0.1 mM EDTA and denature the DNA for 5 min at 98°C in a thermocycler with heated lid. Mix 1.5 μL probe mix (1 fmol of each probe) and 1.5 μL MLPA buffer (1.5 M KCl, 300 mM Tris–HCl pH 8.5, and 1 mM EDTA) and add to the denatured DNA. Heat the mix for 1 min at 95°C before incubating overnight (16 h) at 60°C. After hybridization dilute the sample to 40 μL with ligase mix (2.6 mM $MgCl_2$, 5 mM Tris–HCl pH 8.5, 0.013% nonionic detergents, 0.2 mM NAD) containing 1U Ligase-65 (MRC-Holland) and perform ligation reaction of the annealed probes at 54°C for 15 min. After ligation inactivate the Ligase-65 by incubating the sample 5 min at 98°C. Mix 10 μL of the ligation mix with 30 μL of PCR buffer (20 mM Tris–HCl pH 8.5, 50 mM KCl, 1.6 mM $MgCl_2$, and 0.01% nonionic detergents). While at 60°C, add 10 μL of a solution containing 10 pmol PCR primers, 2.5 nmol dNTPs, and 2.5 U SALSA polymerase (MRC-Holland) to the mix and perform 33 cycles of PCR (30 sec, 95°C; 30 sec, 60°C; and 1 min, 72°C) followed by a 20-min incubation at 72°C. Finally, a part of this PCR is denatured in 100% formamide, mixed with molecular

weight marker, and run on a sequence-type electrophoresis system.

CONCLUSION

In conclusion, the MLPA method has proved to be very sensitive, reproducible, and capable of simultaneous quantitative analysis of up to 45 genes. Its exquisite specificity overweighs difficulties in probe preparations and holds promise to be a widely used technique in quantitative studies of multiple genes.

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Giardia lamblia

Enedina Jiménez-Cardoso
Hospital Infantil de México Federico Gómez, D.F., Mexico

INTRODUCTION

Giardia intestinalis is the most prevalent parasite in the world; it is acquired through ingestion of food or water that has been contaminated with cysts of *Giardia*. Children are more commonly infected and generally present with diarrhea. Individuals with giardiasis can be asymptomatic or suffer from diarrhea, abdominal pain, anorexia, and emesis. *G. intestinalis* is a peculiar microorganism with genetic characteristics of a primitive eukaryote. In developed countries, *Giardia* is regarded as a reemerging infectious agent because of its increasing role in outbreaks of diarrhea in day care centers. About 200 million people in Asia, Africa, and Latin America have symptomatic giardiasis and there are some 500,000 new cases per year. In Mexico, *G. intestinalis* can be isolated by fecal analysis from 7% to 68% of patients with diarrhea. Reported frequencies of infection in other countries are as follows: United States 1–6%; Panama 10%; Venezuela 9%; Brazil 4–11%; Egypt 4–15%; China 10%; Germany 3–27%; Italy 9%; and Spain 24%. *Giardia* is also one of the most common enteric parasites found in dogs and cats. The presence of human *Giardia* in domestic and farm animals suggests a zoonotic potential for giardiasis. However, at present, it cannot be determined whether cysts isolated from both humans and dogs originate primarily from the human *Giardia* group.

CLINICAL MANIFESTATIONS

Trophozoites of these parasites are located in the duodenum, jejunum, and upper ileum. When symptoms occur, they vary from mild to severe abdominal discomfort, diarrhea, cramping, and bloating.[1,2] Infants may have anorexia, weight loss, or a malabsorption syndrome that resembles sprue.[3,4] When a child is evaluated because of failure to thrive or is immunocompromised, the presence of *Giardia* should be considered.[5] Lactose intolerance may develop in these children and persist after elimination of the parasite.

IMMUNE RESPONSE

Abnormal humoral immune response and hypogammaglobulinemia were demonstrated in patients with severe and prolonged symptomatic giardiasis. The latter condition demonstrates the presence of circulating IgG antibodies. Human serum anti-*Giardia*, confirmed by indirect fluorescent antibody (titer 1:128), was able to kill 98% of the trophozoites in vitro. This immunoactivity was dependent on the classic complement pathway. Using a filter paper assay, serological testing of 135 samples with specific IgG anti-*Giardia* demonstrated a sensitivity of 91% to 95%. The presence of intestinal IgA against *Giardia* was associated with the resolution of infection.[6]

Lymphocyte proliferation was demonstrated by the total number of T cells in the Peyer patches. An increase in T cells was associated with resolution of infection, although the CD4-to-CD8 ratio remained unchanged. Spontaneous macrophage cytotoxicity suggested the importance of these cells in the immune response to *Giardia* infection, because monocytes–macrophages and granulocytes participate in the killing of parasites.

MECHANISMS OF DRUG RESISTANCE IN *GIARDIA*

Many effective drugs are available for treatment, as shown in Table 1. Albendazole has been reported to be as efficacious as metronidazole. Chromosome mapping of a gene expressed in drug-resistant lines revealed that chromosomes could rearrange and that segments could be lost. Further mapping studies indicated that the minor chromosomes observed earlier were partial duplications of major chromosomes with loss of substantial subtelomeric regions from different chromosomal termini. The extensive duplication and rearrangement undergone by the *Giardia* genome includes whole and partial chromosomal duplication, subtelomeric loss followed by duplication, partial duplication under stress, and extensive internal duplication among different chromosomes.

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Table 1 Drugs for treatment of giardiasis

Drugs	Dosage
Adults (nonpregnant women)	
Albendazole	400 mg by mouth once a day for 5 days
Furazolidone	100 mg by mouth 4 times a day for 7–10 days
Metronidazole	250 mg by mouth 3 times a day for 5–7 days
Paromomycin	500 mg (30 mg/kg/day) by mouth 3 times a day for 7 days
Quinacrine	100 mg by mouth 3 times a day for 5 days
Tinidazole	2 g by mouth once
Pregnant women	
Paromomycin	500 mg (30 mg/kg/day) by mouth 3–4 times a day for 7 days
Children	
Albendazole	400 mg by mouth once a day for 5 days
Furazolidone	6–8 mg/kg/day by mouth divided 3–4 times a day for 7–10 days
Metronidazole	15 mg/kg/day by mouth divided 3 times a day for 5 days (maximum=300 mg/day)
Paromomycin	30 mg/kg/day by mouth divided 3 times a day for 7 days
Quinacrine	6 mg/kg/day divided 3 times a day for 5 days (maximim=300)
Tinidazole	50 mg/kg by mouth once (maximum=2 g)
Refractory cases	
Metronidazole	750 mg by mouth 3 times a day for 14 days
Quinacrine	100 mg by mouth 3 times a day for 14 days

Source: Refs. [7,8].

Giardia resistant to metronidazole and furozolidone have been reported. Such resistance can develop via biochemical mechanisms or by abnormalities in the electron transport system within the *Giardia*. In vitro, metronidazole reduced production of toxic radicals by decreased activity of ferredoxin. *Giardia* resistant to furozolidone showed an increase in thiol cycling.

These anaerobic protozoa appear to support novel detoxification mechanisms. In studies on the genetic mechanism of drug resistance, a gene associated with a protein membrane was shown to be deleted from one chromosome.[9]

Albendazole resistance in *Giardia* is associated with cytoskeletal change, rather than with a mutation of amino acid 200 in beta-tubulin. Major chromosomal rearrangements were evident in trophozoites resistant to albendazole, as demonstrated by IFA antitubulin antibodies. These antibodies indicate differences in the cytoskeleton. Using albendazole-resistant *Giardia*, PCR primers were designed to study the sequence of the beta-tubulin gene. Results showed that the beta-tubulin gene did not carry a mutation from the Phe.[10]

DIAGNOSIS OF GIARDIASIS

Common laboratory methods for diagnosis of giardiasis are designed for stool specimens. Samples are collected and preserved in 10% formalin, although fresh stools may be examined with light microscopy. Parasites are observed via wet mount, with trichrome or iron hematoxylin staining. Cysts can also be detected by immunofluorescent antibody labeling. The sensitivity of routine examination of a single stool specimen for cysts is approximately 50% to 70%. Sensitivity increases to 100% when two or three samples are analyzed. The antigen-capture enzyme-linked immunosorbent assay for the detection of *Giardia lamblia*, with monoclonal antibody directed to 66-kDa specific antigen, correctly identifies cases of giardiasis from 67% of stool specimens from affected patients. If the antibody is targeted against the whole antigen, sensitivity increases to 95%.

A capture enzyme-linked immunosorbent assay (CELISA) and counterimmunoelectrophoresis (CIE) were evaluated for their ability to detect human *Giardia* using antibody against the whole trophozoite. The sensitivity and specificity were 100% for both. A complementary study can be performed through a microscopic examination of trophozoites obtained from duodenal material. This test requires a special gelatin capsule that remains in the intestine for several hours.[11]

Fig. 1 Sequence alignment 5′ end 16s rDNA. The alignment of the 290pb of group A, P-1 (Portland M54878), group B (U09491 and Bis/91/Hepu/1279 L-29192), and *G. intestinalis* isolated from symptomatic (1S, 4S, 5S, 6S, 7S, 8S, 9S) and asymptomatic children (1A, 2A, 3A, 4A, 5A, 6A).

```
                    *       20        *       40        *       60
P-1         : CATCCGGTCGATCCTGCCGGAGCGCGACGCTCTCCCCAAGGACGAAGCCATGCATGCCC
L29192      : ......................ATC...............A...C..............
B           : ......................ATC...................A..............
1S          : ............................................................
4S          : ............................................................
5S          : ............................................................
6S          : ......................T.....................C........ .......
7S          : ............................................................
8S          : ............................................................
9S          : ......................T.....................C..............
1A          : ............................................................
2A          : ............................................................
3A          : ............................................................
4A          : ............................................................
5A          : ............................................................
6A          : ............................................................

                    *       80        *      100        *      120
P-1         : GCTCACCCGGGACGCGGCGGACGGCTCAGGACAACGGTTGCACCCCCCGCGGCGGTCCCT
L29192      : ............................................................
B           : ..G.........G..............................................
1S          : ............................................................
4S          : ............................................................
5S          : ..G......C.......G........G.................................
6S          : ..G...........G..G........T.................................
7S          : ..G......C.......G.........................................
8S          : ..G......C.......G.........................................
9S          : ..G...........G..G........T.................................
1A          : ..G......C.......G.........................................
2A          : ..............G..G........T.................................
3A          : ..G.........................................................
4A          : ..G.........................................................
5A          : ..................G.........................................
6A          : ..............G..G.........................................

                    *       140       *      160        *      180
P-1         : GCTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCGCAAGGGCGGGCGCCCGC
L29192      : ............................................................
B           : ............................................................
1S          : ............................................................
4S          : ............................................................
5S          : ............................................................
6S          : ............................................................
7S          : ............................................................
8S          : ............................................................
9S          : ............................................................
1A          : ............................................................
2A          : ............................................................
3A          : ............................................................
4A          : ............................................................
5A          : ............................................................
6A          : ............................................................

                    *       200       *      220        *      240
P-1         : GGGCGAGCAGCGTGACGCAGCGACGGCCCGCCCGGGCTTCCGGGGCATCACCCGGTCGG
L29192      : ............................................................
B           : ............................................................
1S          : ............................................................
4S          : ............................................................
5S          : ............................................................
6S          : ....................................A.........G..............
7S          : ............................................................
8S          : ............................................................
9S          : ....................................A.........G..............
1A          : ............................................................
2A          : ............................................................
3A          : ............................................................
4A          : ............................................................
5A          : ............................................................
6A          : ............................................................

                    *       260       *      280        *
P-1         : CGCGGTCGCGGCGCGCCGAGGGCCCGACGCCTGGCGGAGAATCAGGGTTCGACT
L29192      : ...................................................
B           : ...................................................
1S          : ...................................................
4S          : ...................................................
5S          : ...................................................
6S          : ...................................................
7S          : ...................................................
8S          : ...................................................
9S          : ...................................................
1A          : ...................................................
2A          : ...................................................
3A          : ...................................................
4A          : ...................................................
5A          : ...................................................
6A          : ...................................................
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MOLECULAR METHODS

Molecular methods for the diagnosis of *Giardia* have been described. Polymerase chain reaction (PCR) technology offers alternatives to conventional diagnosis for both clinical and environmental purposes with 98.9% specificity. The PCR using the JW1 forward primer 5′GCGCACCAGGAATGTCTTGT 3′ and the JW2 reverse primer 5′ TCACCTACGGATACCTTGTT 3′ DNA for amplification of 183pb showed a specificity of 100% when used to test samples from children who were immunocompromised or who lacked the usual symptomatology. This is in contrast to 87% specificity when diagnoses were made by common laboratory methods.[12,13] Classification of subgroups of *Giardia* based on ribosomal RNA gene sequence using PCR, and genotype by conventional and real-time PCR/RFPL as well as ribosomal RNA gene sequence using PCR analysis, may facilitate studies of virulence, infectivity, and epidemiology of Giardian infection. Classification of subgroups of *Giardia* based on ribosomal RNA gene sequence using PCR analysis, and on genotype by conventional and real-time PCR/RFPL, may facilitate studies of virulence, infectivity and epidemiology Giardian of infection.

MOLECULAR EPIDEMIOLOGY

The ability to amplify a specific region of the genome has been an important tool in the characterization of different sources of giardiasis and in the identification of DNA polymorphism. This tool allows the definition of a taxonomic and genetic framework for epidemiological and clinical purposes. As an example of such studies, genetic typification of *Giardia* in axenic cultures from children was useful in the characterization of the disease.[14] Epidemiological studies of infected humans and their pets have failed to demonstrate any correlation between *Giardia* infections in these animals and their owners.[15,16]

GENETIC DIVERSITY

According to certain characteristics, two groups of have been described in Europe as Polish and Belgian. In the United States, the designations are groups I and II, and in Australia the groups are called assemblages A and B or genotype A and B.[17–19] Molecular studies have also revealed the existence of genetic differences within assemblage A, named Cluster AI and AII. The latter has been isolated only from humans. Molecular analyses showed that the genetic distance partitioning these two assemblages can be used to delineate other species of protozoa. Nevertheless, at present, two important questions are posed: ''Is giardiasis a disease of animals that is transmitted to humans (zoonosis)?'' and ''What is the taxonomic status of the parasitic protozoan species isolated from humans and animals?'' because molecular analysis shows that the same *Giardia* genotype can be found in humans as well as other mammalian species.

MOLECULAR CHARACTERIZATION OF *GIARDIA* FROM CHILDREN AND ANIMALS

To characterize *Giardia* from humans and animals, fecal samples were obtained from six children with chronic recurrent diarrhea and abdominal pain, seven asymptomatic children, and three dogs. Controls were as follows: *G. intestinalis* group A Portland M54878; group B Belgian U09491; Bris/91/Hepu/1279 L29192; and dog 6 AF199449. DNA isolation was performed according to the phenol–chloroform–isoamilic alcohol method.[20] The gene 16S ribosomal RNA (ssRNA) fragment of 290pb was amplified by PCR using forward primer 5′-CATCCGGTCGATCCTGCC-3′ and the reverse primer 5′-AGTCGAACCCTEATTCTCCGCCAGG-3′, under conditions previously described.[21] The amplification products were purified using Qinquick kit (Qiagen). Double-stranded DNA sequencing was performed using the ABl Prism Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and a set of internal primers. Sequencing alignments of 16S rDNA was performed using clustal W, and a phylogenetic tree was designed using the neighbor-joining method and the phylogenetic distance 3.6 Phillip maximum likelihood (http://www.igbmc.u-strasburg.fr/BioInfo). Analysis of the alignment of DNA sequences from symptomatic (5S, 6S, 7S, 8S, and 9S) and asymptomatic (1A, 2A, 3A, 4A, 5A, and 6A) children with giardiasis showed similarities to genotype group A DNA. However, these 11 sequences showed 37 variable sites when compared with group A (Figs. 1 and 2). Further analysis provided a phylogenetic tree with the following groups: one with samples 3A, 4A, and 5A; a second with 2A, 6A, 6S, and 9S; and a third with 1A, 5S, 7S, and 8S. Their sequences and the phylogenetic distances were very similar in samples from two symptomatic children (1S, 4S), seven axenic cultures

Fig. 2 Sequence alignment 5′ end 16s rDNA. The alignment of the 290pb of group A, P-1 (Portland M54878), group B (U09491 and Bris/91/Hepu/1279 L-29192), and dog6 (AF199449). *G. intestinalis* isolated from symptomatic children and subjected to axenic cultures: IMSS-1, IMSS-2, IMSS-3, IMSS-4, INP-1, INP-3, INP-4 and 2dog, 3dog, 5dog.

```
                    *        20         *        40         *       60
P-1         : CATCCGGTCGATCCTGCCGGAGCGCGACGCTCTCCCCAAGGACGAAGCCATGCATGCCC
L29192      : .....................ATC..............A...C..............
B           : .....................ATC...................A..............
IMSS-1      : ............................................................
IMSS-2      : ............................................................
IMSS-3      : ............................................................
IMSS-4      : ............................................................
INP-1       : ............................................................
INP-3       : ............................................................
INP-4       : ............................................................
2dog        : ............................................................
3dog        : ............................................................
5dog        : ............................................................
dog6        : .....................ATC...................A..............

                    *        80         *       100         *       120
P-1         : GCTCACCCGGGACGCGGCGGACGGCTCAGGACAACGGTTGCACCCCCCGCGGCGGTCCCT
L29192      : ............................................................
B           : ..G.........G.............................................
IMSS-1      : ............................................................
IMSS-2      : ............................................................
IMSS-3      : ............................................................
IMSS-4      : ............................................................
INP-1       : ............................................................
INP-3       : ............................................................
INP-4       : ............................................................
2dos        : ............................................................
3dog        : ............................T...............................
5dog        : ............................................................
dog6        : ..A.........A.........................................A..

                    *       140         *       160         *       180
P-1         : GCTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCGCAAGGGCGGGCGCCCGC
L29192      : ............................................................
B           : ............................................................
IMSS-1      : ............................................................
IMSS-2      : ............................................................
IMSS-3      : ............................................................
IMSS-4      : ............................................................
INP-1       : ............................................................
INP-3       : ............................................................
INP-4       : ............................................................
2dog        : ............................................................
3dog        : ............................................................
5dog        : ............................................................
dog6        : ........................................T.....T....A.......

                    *       200         *       200         *      240
P-1         : GGGCGAGCAGCGTGACGCAGCGACGGCCCGCCCGGGCTTCCGGGGCATCACCCGGTCGG
L29192      : ............................................................
B           : ............................................................
IMSS-1      : ............................................................
IMSS-2      : ............................................................
IMSS-3      : ............................................................
IMSS-4      : ............................................................
INP-1       : ............................................................
INP-3       : ............................................................
INP-4       : ............................................................
2dog        : ............................................................
3dog        : ............................................................
5dog        : ............................................................
dog6        : ..................................................C.A...

                    *       260         *       280         *
P-1         : CGCGGTCGCGGCGCGCCGAGGGCCCGACGCCTGGCGGAGAATCAGGGTTCGACT
L29192      : .................................................
B           : .................................................
IMSS-1      : .................................................
IMSS-2      : .................................................
IMSS-3      : .................................................
IMSS-4      : .................................................
INP-1       : .................................................
INP-3       : .................................................
INP-4       : .................................................
2dog        : .................................................
3dog        : .................................................
5dog        : .................................................
dog6        : ............C.....T...AG.........................
```

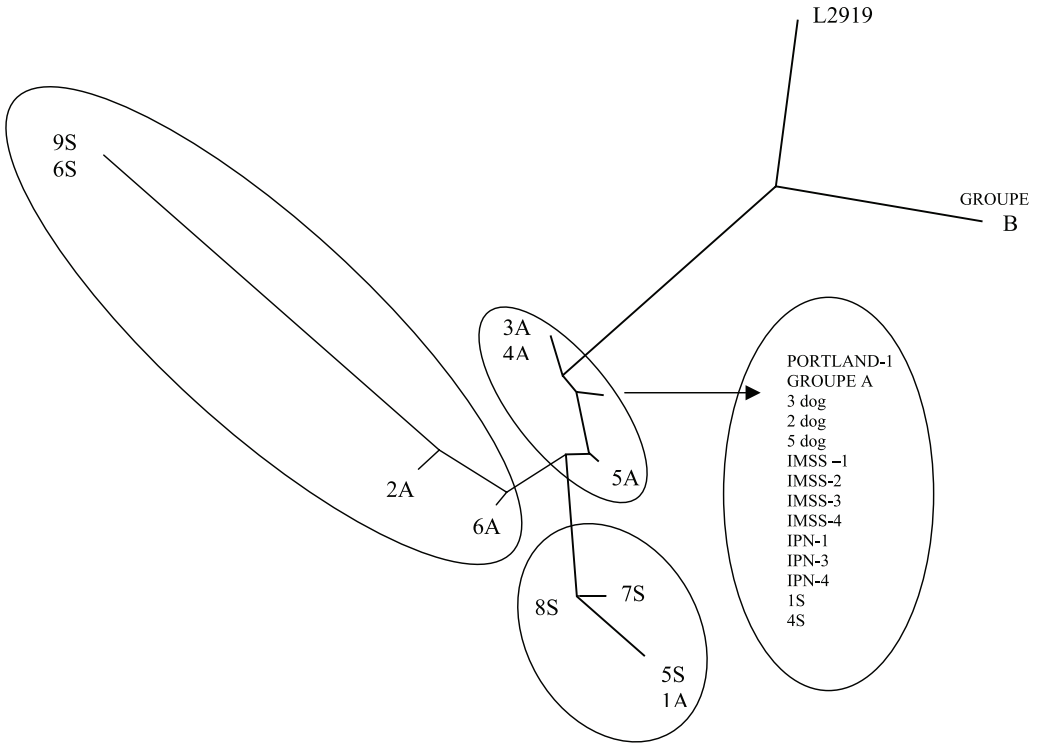

Fig. 3 The phylogenetic tree of *G. intestinalis* from symptomatic (1S, 4S, 5S, 6S, 7S, 8S, 9S) and asymptomatic children (1A, 2A, 3A, 4A, 5A, 6A); axenic cultures (IMSS-I, IMSS-2, IMSS-3, IMSS-4, IPN-1, IPN-3, IPN-4) and *Giardia* in dogs (3dog; 2dog; 5dog) including controls group A, Portland P-1, M54878 and group B (U09491 and L29192 Bris/Hepul/1279).

(IMSS-1, IMSS-2, IMSS-3, IMSS-4, IPN1, IPN2, and IPN3), and three dogs (2dog, 3dog, and 5dog), as seen in Fig. 3. Group B giardias were not found.

ZOONOTIC IMPLICATIONS OF GENOTYPIC CHARACTERIZATION

Previous results clearly indicate that *G. intestinalis* genotype A was isolated from fecal samples from humans and dogs residing in the same environment. Therefore, one can postulate a zoonotic transmission for *Giardia*. This investigation confirms the observation made in molecular epidemiological studies that *Giardia* obtained from infected humans also occurs in many mammalian species.[22] Another investigation demonstrated that 75% of giardias isolated from humans were closely related to those with a canine origin,[23,24] leaving uncertainty about whether the animals were reservoir hosts for humans or vice versa. Finally, when genotypic analysis was made from 11 isolated DNAs, 37 variables were observed that could be divided into three groups. Such differences are possibly due to the host-adapted genotypes or perhaps represent distinct species that could be associated with variable pathology.

CONCLUSION

From a public health perspective, zoonotic risk is associated with genotypes in assemblage A, particularly those in Cluster AI, and less with assemblage B. Finding similar genotypes in different hosts suggests a zoonotic transmission that per se presents important implications for the establishment of a global genetic framework. The ability to accurately identify the *Giardia* genotype is a potential and powerful predictive tool that can support direct evidence of zoonotic transmission. All results suggest that accurate molecular characterization of the parasite with phylogenetic distance should be included in order to determine zoonotic transmission. The genetic diversity only reflects that each major group contains genetically diverse isolates that are not confined to any particular host or geographic location. Giardiasis and its zoonotic potential are important for future cultural, social, and health impact in humans. Currently, no classification schemes exist that can define or detect the pathogenic potential, host range, or virulence in strains that infect man. Studies such as the one presented can determine the kind of *Giardia* transmission in any community.

The potential that giardiasis can be transmitted zoonotically has cultural, social, and health implications.

Therefore, more studies along this line of investigation are necessary to clarify the kind of transmission in every community.

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Glutathione *S*-Transferase Genotype

Richard C. Strange
Anthony A. Fryer
Keele University Medical School, Staffordshire, U.K.

INTRODUCTION

The glutathione *S*-transferase (GST) supergene family is considered a key enzymic detoxicating system that protects cells from endogenous and exogenous chemicals. Indeed, GST peptides are probably expressed in all life forms, implying their importance. However, despite identification of many in vitro substrates, the biological relevance of many of these is uncertain. Indeed, accumulating evidence also suggests a noncatalytic, cell signaling function for the GST.

Although many polymorphic sites have been examined for associations with disease susceptibility there is no clear view as to biochemical consequences of most alleles. We review GST gene organization and allelic sites and consider their possible functions. This forms a basis for selecting diseases potentially influenced by polymorphism.

GST GENES

Two groups of genes encode GST peptides.[1,2] Firstly, the alpha, mu, pi, theta, sigma, zeta, and omega families that encode peptides (molecular weights 24–29 kDa) expressed in cell cytosols, and kappa family those in mitochondria,[1,2] and, secondly, the evolutionarily distinct membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG).[3] The GST demonstrate widespread gene-dependent expression particularly in liver, kidney, testis, bowel, heart, ovary, and skin.

CATALYTIC FUNCTION

Xenobiotics

Much work has focused on the homo- or heterodimeric cytosolic GST enzymes that catalyze detoxication of xenobiotics via conjugation with reduced glutathione. Some substrates such as tobacco-derived carcinogenic benzo[*a*]pyrene diol epoxide result from cytochrome P450-catalyzed activation.[1,2] Importantly, not all substrates are inactivated by conjugation; theta GST utilizes dichloromethane to form reactive *S*-chloromethylglutathione.[1,2]

Response to Drug Treatment

Glutathione *S*-transferase expression has been linked with drug response, particularly resistance to anticancer agents.[2] Glutathione *S*-transferase expression is increased by exposure to various drugs including substrates thought to be inactivated by GSH-conjugation (chlorambucil, nitrogen mustards, melphalan, hydroxyalkenals) as well as nonsubstrates (bleomycin, carboplatin, cisplatin, adriamycin, mitomycin C).[2]

Oxidative Stress

Cytosolic and MAPEG GST demonstrate selenium-independent glutathione peroxidase activities toward numerous products of oxidative stress including lipid and DNA hydroperoxides and 4-hydroxynonenal.[1,3]

Eicosanoid Metabolism

Membrane associated proteins in eicosanoid and glutathione enzymes including leukotriene C_4 synthase, MGST-I, and MGST-II catalyze conversion of LTA_4 to LTC_4 (Fig. 1). Further, 5-lipoxygenase-associated protein (FLAP) with 5-lipoxygenase facilitates conversion of arachidonic acid via 5-HPETE to LTA_4.[1,3] Some cytosolic GST also demonstrate low levels of leukotriene C_4 synthase activity. MGST-II and MGST-III convert 5-HPETE to 5-HETE. Thus the proportions of FLAP to MGST-II/III determine whether 5-HPETE is converted to LTA_4 or 5-HETE. Alpha GST catalyzes the reduction of prostaglandin PGH_2 to $PGF_{2\alpha}$, whereas alpha, sigma, and MGST-I-like-I enzymes catalyze the isomerization of PGH_2 to PGD_2 and PGE_2.[1,3] Alpha, mu, and pi GST also catalyze the inactivation of PGA_2 to PGJ_2. Glutathione *S*-transferase enzymes may also indirectly affect eicosanoid synthesis via effects on the levels of lipid hydroperoxide.

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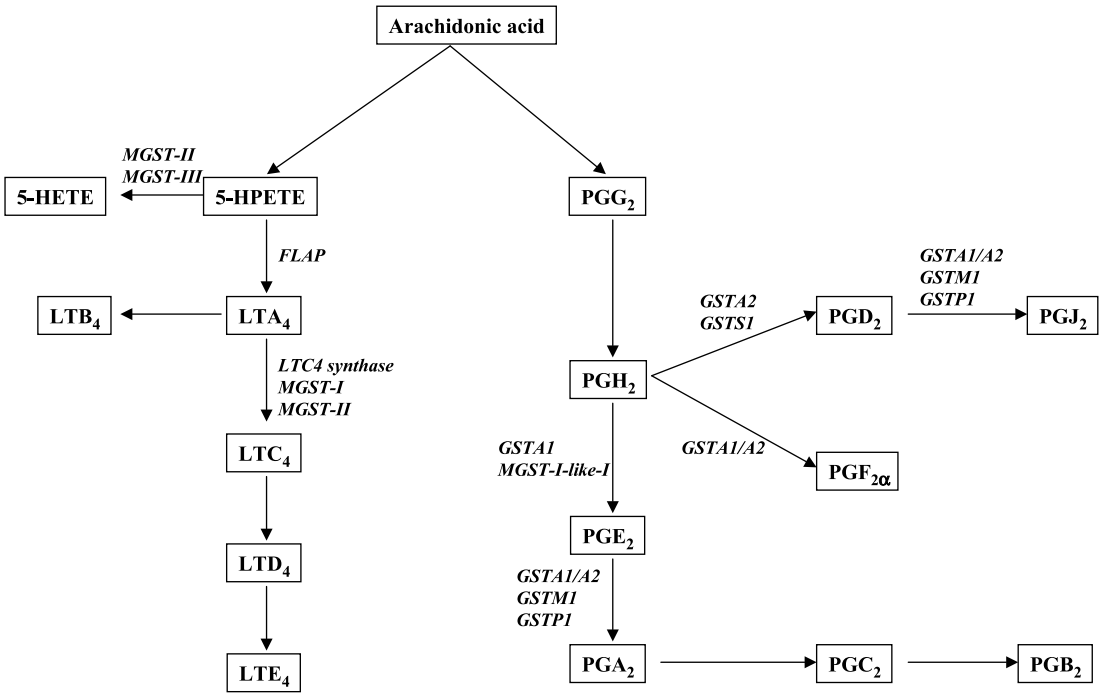

Fig. 1 The role of glutathione *S*-transferases in eicosanoid metabolism. (Based on data described in Ref. [1].)

CELL CYCLE CONTROL

Data showing interactions between GST and peptides involved in signaling pathways suggest a novel, non-catalytic role. Thus under nonstressed conditions, pi class monomers complex with Jun N-terminal kinase (JNK) preventing Jun phosphorylation.[4] Stress results in complex dissociation and restoration of kinase activity. Mu GST influences the heat shock-sensing pathway by inhibiting apoptosis signal-regulating kinase (ASK1) activity.[5] These GST may therefore regulate the mitogen-activated protein (MAP) kinase pathway. Recently, Edalat et al.[6] reported novel interactions between human GST A1-1, GST P1-1 and GST M2-2, and peptides with high sequence similarity to TRAF4-associated factor 1, G protein-coupled receptor MRGX3, tumor necrosis factor superfamily (member 9), and c-Jun N-terminal kinase 3.

REGULATION OF EXPRESSION

Regulation of expression varies between GST classes. In the promoter, response elements to transcription factors including the antioxidant response element, xenobiotic response element, glucocorticoid response element, Barbie box element, and GSTP enhancer 1 have been identified. Motifs for YY1, AP-1, MAF, Nrfl, Jun, Fos, and NK-κB have also been identified in rodents, although not all have been confirmed in human GST.[1,2] Such elements suggest an adaptive response by the GST to cellular stress.

A further mechanism for influencing GST expression is via methylation of CpG islands. In particular, GSTP1 expression appears particularly important in the protection of prostatic cells from oxidant and electrophile stress as methylation in the promoter silences expression allowing genetic damage and progression toward carcinoma.[7] Several studies have shown virtually 100% methylation of the GSTP1 CpG island in prostatic cancer cells.

POLYMORPHISM IN GST

Increasing numbers of GST polymorphisms are being identified using web-based searching although many theoretical single nucleotide polymorphisms (SNP) have not been found in humans.[8]

Alpha Class

Five alpha genes are in a cluster on chromosome 6 with several pseudogenes.[9] They show significant homology making SNP identification and genotyping assay design

problematic. The A1 promoter comprises four SNP in complete linkage disequilibrium.[9] The *B allele (T at position −69, A at position −52) alters an Sp1 binding site resulting in reduced expression. There is no evidence that two nonsynonymous substitutions in A2 (Table 1) alter GST activity. No polymorphisms have been confirmed in A3–5.

Mu Class

The mu genes are in tandem on chromosome 1 and ordered M4–M2–M1–M5–M3 with M3 in reverse orientation.[10] Complete deletion of GSTM1 is common (allele frequency 0.70 in Caucasians). A nonsynonymous substitution in exon 7 (Lys173Asp) is also identified although the differential effect of the A and B alleles on disease risk[1] is likely to result from linkage disequilibrium with a neighboring SNP. Indeed, *GSTM1*A* is in linkage disequilibrium with *GSTM3*B*, a 3-bp deletion which may generate a YY1 recognition motif.[1] The functional effect of an SNP in M4 is unknown. No significant polymorphisms have been identified in M2 or M5.

Theta Class

GSTT1 and T2 are located on chromosome 22.[11] GSTT2 is separated from a homologous pseudogene by D-dopachrome tautomerase. GSTT1*0 is deleted with an allele frequency in Caucasians of 0.40. An SNP (Thr104Pro) in GSTT1 has been described, but little studied. Both the gene deletion and the Pro104 variant demonstrate reduced activity toward haloalkanes. Although three GSTT2 SNPs have been described (Table 1), assay design is hampered by homology with pseudogene GSTT2P. Consequently, there are little data on the clinical effect of these alleles.

Pi Class

Four GSTP1 alleles have been identified: *GSTP1*A–D*[12] (Table 1). The Val^{105} variant, compared to Ile^{105}, confers higher catalytic efficiency for polycyclic aromatic hydrocarbon diol epoxides but lower efficiency for 1-chloro-2,4-dinitrobenzene. The effect of the Ala^{114}–Val^{114} substitution is unclear, although it may enhance the effect of the Ile^{105}–Val^{105} substitution.

Zeta Class

Expressed sequence tag (EST) database analysis has identified three functional substitutions in Z1 (Table 1).[1] The *A allele shows highest activity toward dichloracetic acid and lowest toward fluoroacetate.

Omega Class

Polymorphisms in O1 and O2 are described.[8] O1 is an anomaly as it shows sequence homology and functional activities with non-GST-like proteins including glutaredoxins. Its thioltransferase activity is decreased in both Asp140 (*C) and Asn217 variants, but increased in the *B allele (del115). An A–G substitution in O2 at position 424 gives rise to an asparagine to aspartic acid amino acid change in exon 4. The functional consequence of this is unknown.

MEMBRANE ASSOCIATED PROTEINS IN EICOSANOID AND GLUTATHIONE METABOLISM

Allelic MAPEG genes are candidates for inflammatory pathologies where the relative levels of pro- and anti-inflammatory eicosanoids may be influenced by polymorphism.[13] Indeed, polymorphism in the FLAP and LTC_4 synthase genes has been examined in the context of asthma, where therapeutic modulation of leukotriene synthesis has been used.

GST POLYMORPHISMS: CLINICAL IMPLICATIONS

The wide range of in vitro GST substrates has resulted in them being considered almost universal candidates for susceptibility and outcome in disease.[1]

Tobacco-Related Diseases

GSTM1 and GSTT1 have been particularly studied as risk candidates for tobacco-related cancers.[14,15] While some studies show significantly increased risk for *GSTM1*0* and *GSTT1*0* homozygotes, others have not replicated these findings prompting the use of meta-analysis. For example, analysis using 43 studies indicates that GSTM1 null is not associated with increased lung cancer risk and that there is no evidence for an interactive effect between the genotype and tobacco consumption.[15] Analysis of 31 studies of the influence of GSTM1, GSTT1, and GSTP1 polymorphisms on head/neck cancer risk found modest associations (odds ratio about 1.30) between risk and GSTM1 null and GSTT1 null with greater risk (odds ratio 2.06) associated with combinations of the genotypes.[14] Analysis of six studies also failed to identify significant associations between GSTM1 null and colorectal cancer risk.[16]

Overall, therefore, the concept that GST polymorphisms, by reflecting a detoxication-deficient phenotype,

Table 1 Glutathione *S*-transferase polymorphisms

Class	Chromosome	Genes	Alleles	Nucleotide change	Amino acid change	Functional consequence
Alpha	6p12	A1–A5	A1*A	−69 C	Promoter	Reference
			A1*B	−69 T	Promoter	Decreased expression
			A2*A	335C, 629A	Thr112, Glu210	Reference
			A2*B	335G, 629C	Ser112, Ala210	No change
Mu	1p13.3	M1–M5	M1*A	519G	Lys173	Reference
			M1*B	519C	Asn173	No change
			M1*0	Gene deletion	No protein	No activity
			M1*Ax2	Gene duplication	Overexpression	Increase activity
			M3*A	AAG	Intron 6	Reference
			M3*B	AAG deletion	Intron 6	Altered YY1 motif
			M4*A	2517T	Intron 6	Reference
			M4*B	2517C	Intron 6	No change
Pi	11q13	P1	P1*A	313A, 341C, 555C	Ile105, Ala114, Ser185	Reference
			P1*B	313G, 341C, 555T	Val105, Ala114, Ser185	Substrate-dependent
			P1*C	313G, 341T, 555T	Val105, Val114, Ser185	Substrate-dependent
			P1*D	313A, 341T	Ile105, Val114	No change
Theta	22q11	T1–T2	T1*A	310A	Thr104	Reference
			T1*B	310C	Pro104	Decreased activity
			T1*0	Gene deletion	No protein	No activity
			T2*A	481G	Met139	Reference
			T2*B	481A	Ile139	Not characterized

(*Continued*)

Table 1 Glutathione *S*-transferase polymorphisms (*Continued*)

Class	Chromosome	Genes	Alleles	Nucleotide change	Amino acid change	Functional consequence
Sigma	4q21–22	S1	–			
Zeta	14q24.3	Z1	Z1*A	94A, 124A, 245C	Lys32, Arg42, Thr82	Reference
			Z1*B	94A, 124G, 245C	Lys32, Gly42, Thr82	Decreased activity
			Z1*C	94G, 124G, 245C	Glu32, Gly42, Thr82	Decreased activity
			Z1*D	94G, 124G, 245T	Glu32, Gly42, Met82	Decreased activity
Omega	10q23–25	O1–O2	O1*A	419C, 464+1 AAG	Ala140, Glu155	Reference
			O1*B	419C, 464+1 del	Ala140, del115	Increased activity
			O1*C	419A, 464+1 AAG	Asp140, Glu155	Decreased activity
			O1*D	419A, 464+1 del	Asp140, del115	Not characterized
			O1*E	650C	Thr217	Reference
			O1*F	650A	Asn217	Reduced activity
			O2*A	424A	Asn142	Reference
			O2*B	424G	Asp142	Not characterized
Kappa	Not determined	K1	–			
Microsomal	12p13.1–13.2	MGST-I	*A	598T	3′ noncoding	Reference
			*B	598G	3′ noncoding	Not characterized
	4q28–31	MGST-II	–			
	1q23	MGST-III	–			
	9q34.3	MGST-I-like-I	–			
	5q35	LTC_4S	*A	−444A	Promoter	Reference
			*B	−444C	Promoter	Increased expression
	13q12	FLAP	*A	T	Intron 2	Reference
			*B	C	Intron 2	Not characterized

identify individuals at increased risk of xenobiotic-related cancers is not supported by available data. However, the impact of polymorphism may be more readily observed using alternative endpoints such as formation of DNA adducts. Thus Perera et al.[17] found that in smokers, DNA adducts in white blood cells were significant predictors of lung cancer risk and that adduct levels were higher in individuals with combinations of GSTM1 and GSTP1 genotypes.

Glutathione *S*-transferase variants could mediate risk of nonmalignant diseases in which tobacco is a causative factor. However, the interactive effect of GSTM1 null and cigarette smoking was found to be insignificant in the aetiology of severe coronary artery disease in 868 patients with angiographically characterized disease.[18] Smoking is also associated with disease severity in women with rheumatoid arthritis. We found that smoking was associated with the most severe disease in patients with GSTM1 null polymorphism.[1]

Oxidative Stress-Related Disorders

Oxidative stress is implicated in the inflammatory demyelination that characterizes multiple sclerosis suggesting GST polymorphisms may be associated with disability. In 177 patients with disease duration over 10 years, GSTM3 AA (OR=2.4) and homozygosity for both GSTM1*0 and GSTP1*Ile105-encoding allele (OR=5.0) were linked with severe disability suggesting that long-term prognosis in MS is influenced by GST-mediated ability to remove toxic products of oxidative stress.[1] Exposure to ultraviolet radiation also results in local oxidative stress in skin. Response to such exposure, examined as minimal erythema dose, has been shown to be mediated by GSTM1 and GSTT1 genotype in a gene dosage-dependent manner.[19] Furthermore, nonmelanoma skin cancer has also been linked to these polymorphisms.[1]

Metabolism of Chemotherapeutic Agents

Although there are abundant data on GST expression and drug response, there are relatively little data on the effect of polymorphism on response to treatment. For example, in 148 women with epithelial ovarian cancer who were null for GSTM1 or GSTT1 there was decreased survival and reduced response to primary chemotherapy compared to those with active GSTM1 or GSTT1.[1,2]

CONCLUSION

The classic view that the GST is part of a cellular detoxication system focused on exogenous toxins remains tenable, although the failure to define precise roles for individual GST has hampered assessment of the implications of polymorphism as selection of diseases whose pathogenesis may be affected is subjective. Thus while associations between GST genotypes and clinical phenotype have been reported they cannot be predicted, and it is unclear why some, but not other, links are significant. Further, study reproducibility is a problem, probably because of variable definition of phenotypes and differences in population characteristics. Thus molecular epidemiological studies show that the impact of polymorphism on disease risk and outcome is likely to be most evident in patient subgroups.[1] Such subgroups may be defined by different sets of susceptibility genes and/or environmental exposures and identified by clinical characteristics such as tumor site, age at diagnosis, or gender. For example, the importance of exposure is shown by the chemoprotective effect of cruciferous vegetable-derived metabolites such as isothiocyanates that modulate cytochrome P450 and GST expression.[1,2] Thus GST polymorphisms may influence cancer risk through various mechanisms including interactions with diet-derived compounds. The GST effect may also be exerted via their catalysis of detoxication reactions or by interactions with peptides that mediate signaling pathways.

While most significant associations between GST genotypes and clinical phenotype have not been replicated, a minority have been independently confirmed. For example, GSTP1 Val^{105}/Val^{105} has been associated with reduced risk of airway hyperresponsiveness (odds ratio=0.23–0.38) in three studies in asthmatic adults and children.[20] This genotype is also protective against childhood respiratory illness.[21] The advent of high-throughput genotyping, availability of new SNP, and ease of deriving haplotypes should place an emphasis on large studies incorporating some assessment of reproducibility. Furthermore, assessment of further allelic GST sites associated with binding of peptides involved with cell signaling warrants further investigation.

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Glycogen Storage Diseases

Yuan-Tsong Chen
Deeksha Sarihyan Bali
Duke University Medical Center, Durham, North Carolina, U.S.A.

INTRODUCTION

Glycogen storage diseases (GSDs) are inherited disorders affecting glycogen metabolism. The glycogen found in GSDs is abnormal either in quantity or quality. Liver and muscle are the most common and seriously affected tissues. The liver GSDs usually present with hepatomegaly and hypoglycemia. The GSDs primarily affecting the liver are types I, III, IV, VI, and IX. The most common features of muscle GSDs are muscle cramps, exercise intolerance, fatigue, and progressive weakness. GSDs principally affecting the muscle include GSD type II (lysosomal enzyme deficiency), V, VII, and IX (phosphorylase kinase deficiency).

The frequency of all forms of GSDs is approximately 1 in 20,000–25,000 live births. GSD types I, II, III, VI, and IX are the most common, and they account for approximately 90% of all GSDs.

GSD TYPE I (GLUCOSE-6-PHOSPHATASE OR TRANSLOCASE DEFICIENCY, VON GIERKE DISEASE)

GSD type I is caused by defect in either glucose 6-phosphatase catalytic activity[1–4] (Ia) or a defect in the glucose-6-phosphate translocase[5] (Ib). The stored materials in the liver include glycogen and fat (Table 1).

Clinical Findings

The hallmarks of the disease are hepatomegaly, hypoglycemia, lactic acidosis, hyperuricemia, hyperlipidemia, and growth retardation. Long-term complications include short stature, osteoporosis, gout, renal disease, pulmonary hypertension, and hepatic adenomas. Virtually all females have ultrasound findings consistent with polycystic ovaries.[6] Hepatic adenomas develop in a significant number of patients, and malignant transformation may occur.[7]

Type Ib has a similar clinical course with the addition of neutropenia and impaired neutrophil function resulting in recurrent bacterial infections. Oral and mucosal ulcerations are common, and in some cases, regional enteritis can also occur.[8]

Genetics

Type I GSD is autosomal recessive. The gene for glucose-6-phosphatase (G6PC; OMIM 23220) is located on chromosome 17q21, spans 12.5 kb, and has five coding exons. The gene for glucose-6-phosphate translocase (G6PT1; OMIM 23220) is located on chromosome 11q23 and consists of nine exons. Both types of GSD I are genetically heterogeneous. More than 73 disease-causing mutations are known for type Ia. Some ethnic group-specific and more frequently detected mutations for type Ia—R83C (Jewish), 130X (Hispanic), 35X, G188R, and Q347X (Caucasian), R83H (Chinese), 727 G>T (Japanese)—are responsible for about 78% of the known disease alleles.[9,10]

For GSD Ib, there are about 66 disease-causing mutations known. Two mutations, G339C and 1211delCT, appear to be more prevalent in Caucasian patients, whereas W118R appears to be common among Japanese patients.[10,11] Definitive genotype/phenotype correlation is not apparent for either subtype; however, GSD Ia mutations, 727G>A and G188R, confer specific phenotypes in affected patients when expressed in homozygous state (increased risk for hepatocellular carcinoma and recurrent infections and neutropenia, respectively). Identification of mutations allows prenatal diagnosis of both subtypes.[10,11]

GSD TYPE II (ACID MALTASE OR ACID α-GLUCOSIDASE DEFICIENCY, POMPE DISEASE)

GSD II is caused by a deficiency of lysosomal acid α-glucosidase (GAA; acid maltase), an enzyme responsible for the degradation of glycogen engulfed in autophagic lysosomal vacuoles (OMIM 232300). Three different

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Table 1 Glycogen storage diseases: Distinct types and diagnosis

Disorder	Enzyme defect/deficiency	Inheritance, chromosomal localization, and gene
Type Ia (Von Gierke)	Glucose-6-phosphatase	AR[a], 17q21, Gene: *G6PC*
Type Ib	Glucose-6-phosphate translocase	AR, 11q23, Gene: *G6PT1*
Type II (Pompe) Infantile	Acid α-glucosidase (acid maltase)	AR, 17q25.2–25.3, Gene: *GAA*
Juvenile	Acid α-glucosidase (acid maltase)	AR, 17q25.2–25.3, Gene: *GAA*
Adult	Acid α-glucosidase (acid maltase)	AR, 17q25.2–25.3, Gene: *GAA*
Type IIIa (Cori or Forbes)	Liver and muscle debrancher deficiency (amylo-1,6-glucosidase)	AR, 1p21, Gene: *AGL*
Type IIIb	Liver Debrancher deficiency (amylo-1,6-glucosidase)	AR, 1p21, Gene: *AGL*
Type IV (Anderson)	Branching enzyme (BE)	AR, 3p12, Gene: *GBE1*
Type V (McArdle)	Myophosphorylase	AR, 11q13, Gene: *PYGM*
Type VI (Hers)	Liver phosphorylase	AR, 14q21–22, Gene: *PYGL*
Type VII (Tarui)	Phosphofructokinase	AR, 12q13, Gene: *PFKM*
Type IX	Phosphorylase kinase	X-linked: Xp22.2–22.1, Gene*: PHKA2*; AR, 16q12–13, Gene*: PHKB*; AR, 16p11–12, Gene: *PHKG2*
Type XI (Fanconi-Bickel syndrome)	Glucose transporter 2	AR, 3q26.1–26.3, Gene: *GLUT2*
Type GSD 0	Glycogen synthase	AR, 12p12.2, Gene: *GYS2*

[a]AR: autosomal recessive.

forms of the disease presenting with different ages of onset and clinical severity have been identified.[12,13]

Clinical Findings

The infantile form presents with progressive hypertrophic cardiomyopathy, generalized hypotonia with muscle wasting, hepatomegaly, and macroglossia, followed by a rapid, progressive course. Death usually occurs before the second year of life as a result of cardiorespiratory failure.

The juvenile form presents in early childhood, with progressive muscle weakness including the diaphragm, but no cardiac involvement. Affected patients usually die before puberty from respiratory failure.[12,13]

In the adult form, the presenting symptom may resemble the muscle weakness of limb-girdle muscular dystrophy or a respiratory insufficiency. Definitive diagnosis for GSD type II requires enzyme analysis of skin fibroblasts. In general, enzyme deficiency is more severe in the infantile form than the late-onset juvenile and adult forms.

Genetics

GSD II is autosomal recessive. The gene that encodes acid maltase (GAA) is located on chromosome 17q25.[14] Genetic and biochemical heterogeneity has been observed in GSD II without clear correlation to clinical phenotypes. More than 70 disease-causing mutations have been described,[14] and most are private family mutations. However, some common mutations exist in specific ethnic groups, including Dutch (del exon 18), Asians (C1935A), and African-Americans (C2560T), which could be used for carrier detection and diagnosis.[15]

Major clinical presentation	Enzymatic diagnosis (tissue)	Gene-based diagnosis
Hepatomegaly, hypoglycemia, elevated blood lactate, cholesterol, triglycerides and uric acid	Yes (liver)	Mutation screen
Same as Ia, with additional findings of neutropenia and neutrophil dysfunction	Yes (liver)	Mutation screen
Cardiomegaly, hypotonia, muscle weakness, macroglossia; onset birth to 6 months	Yes (blood, muscle, liver, skin fibroblasts)	Mutation screen and DNA sequencing
Myopathy, variable cardiomyopathy; onset childhood.	Yes (muscle, skin fibroblasts)	Mutation screen and DNA sequencing
Myopathy, respiratory insufficiency; onset adulthood	Yes (skin fibroblasts)	Mutation screen and DNA sequencing
Childhood hepatomegaly, growth retardation, muscle weakness, hypoglycemia, hyperlipidemia, elevated transaminases	Yes (blood, liver, muscle)	DNA Sequencing
Liver symptoms same as IIIa: no muscle symptoms	Yes (liver)	Mutation screen
Failure to thrive, hepatomegaly, splenomegaly, progressive cirrhosis, elevated transaminases	Yes (liver, muscle, skin fibroblasts)	Gene sequencing
Exercise intolerance, muscle cramps, increased fatigability	Yes (muscle)	Mutation screen
Hepatomegaly, mild hypoglycemia, hyperlipidemia, and ketosis	Yes (Liver)	Gene sequencing
Exercise intolerance, muscle cramps, myoglobinuria, hemolytic anemia	Yes (muscle)	Mutation screen and DNA sequencing
Hepatomegaly, mild hypoglycemia, hyperlipidemia, and ketosis	Yes (blood, liver)	DNA sequencing
Hepatomegaly, enlarged kidneys, rickets, renal fanconi syndrome	No	DNA sequencing
Hyperketotic hypoglycemia in infancy	Yes (liver)	DNA sequencing

GSD TYPE III (DEBRANCHER DEFICIENCY, LIMIT DEXTRINOSIS, CORI, OR FORBES DISEASE)

GSD III is caused by a deficiency of glycogen debrancher enzyme (GDE) activity.[16] GDE deficiency impairs the release of glucose from glycogen, but does not affect glucose released from gluconeogenesis (OMIM 232400). The glycogen accumulated has a structure resembling limit dextrin (glycogen with short outer chains).

Clinical Findings

Most patients with type III GSD have enzyme deficiency in liver, skeletal muscle, and heart (type IIIa). However, some patients (<15%) have debranching enzyme deficiency limited only to liver (type IIIb). During infancy and childhood, the disease may be indistinguishable from GSD I. The liver symptoms improve with age and usually disappear after puberty. Overt liver cirrhosis rarely occurs. Hepatic adenomas have been reported, but malignant transformation has not been observed.[17,18]

Definite diagnosis and subtyping requires both liver and muscle biopsies or DNA-based mutation analysis (IIIb).

Genetics

GSD III is autosomal recessive. The gene (AGL) that encodes human debranching enzyme (GDE) is a large single copy gene located on chromosome 1p21, spans 85 kb, and contains 35 exons. There is an extensive clinical and genetic heterogeneity seen in this GSD, with about 35 disease-causing mutations and 16 polymorphisms identified in AGL gene so far.[17,18]

To date, no true frequent mutations have been found except for an ethnic mutation in the Ashkenazi Jewish population (4455delT) and two mutations associated specifically with GSD-IIIb (Q6X and 17delAG).[18] Three polymorphic DNA markers within the gene have been identified. Prenatal diagnosis, carrier detection, and postnatal molecular diagnosis can be offered only to the informative families where linkage has been established or to families with known disease-causing mutation.[18]

GSD TYPE IV (BRANCHING ENZYME DEFICIENCY, AMYLOPECTINOSIS, OR ANDERSON DISEASE)

GSD IV is caused by a deficiency of glycogen branching enzyme activity (GBE1, OMIM 232500) that results in the accumulation of abnormal glycogen with unbranched, long outer chains in the tissues, having poor solubility.[19] Branching enzyme deficiency is a clinically and genetically heterogeneous multisystem disorder with symptoms ranging from classical severe infantile form, with heart, muscle, and liver involvement, to a nonprogressive neuromuscular form.

Clinical Findings

Classical branching enzyme deficiency most frequently presents during the first few months of life, with hepatosplenomegaly and failure to thrive. Progressive liver cirrhosis, with portal hypertension, ascites, esophageal varices, and death, usually occurs before 5 years of age. However, there are patients who have survived without apparent progressive liver disease. The neuromuscular system may also be involved. Severe cardiomyopathy as the predominant symptom has also been reported.[20] The diagnosis of type IV is established by demonstration of abnormal glycogen (an amylopectin-like polysaccharide) and a deficiency of glycogen branching enzyme in liver, muscle, or fibroblasts.

Genetics

GSD IV is autosomal recessive. The glycogen branching enzyme gene (GBE1) is located on chromosome 3p12. Eight disease-causing mutations are known. A point mutation, Y329S, is associated with nonprogressive hepatic form of disease, and it is also found in Ashkenazi Jewish patients with adult polyglucosan body disease. Other point mutations (R515C, F257L, R524X) or deletions are associated with severe infantile phenotype.

GSD TYPE V (MUSCLE PHOSPHORYLASE, MYOPHOSPHORYLASE DEFICIENCY, McARDLE DISEASE)

GSD type V is caused by the deficiency of muscle phosphorylase activity. A deficiency of myophosphorylase impairs the cleavage of glucosyl molecules from the straight chain of glycogen. Separately encoded, tissue-specific distinct phosphorylases are known for liver, muscle, and brain.[21]

Clinical Findings

Clinical symptoms usually appear in adulthood and are characterized by exercise intolerance with muscle cramps that can be accompanied by attacks of myoglobinuria. Serum creatine kinase is usually elevated and increases after exercise. Avoidance of strenuous exercise can prevent major episodes of rhabdomyolysis.

There have been several reports of a fatal infantile form of phosphorylase deficiency.[22] The presenting features were hypotonia, generalized muscle weakness, and progressive respiratory insufficiency.

Genetics

The gene for muscle phosphorylase (PYGM) has been mapped to chromosome 11q13. It consists of 19 exons and there is extensive genetic heterogeneity. A common nonsense mutation R49X in exon 1, which is found in 90% of Caucasians, and a deletion of a single codon in exon 17, found in 61% of Japanese patients. Other common Caucasian mutations (G204S in exon 5 and K542T in exon 14) make DNA-based testing for McArdles disease possible.[21]

GSD TYPE VI (LIVER PHOSPHORYLASE, HERS DISEASE)

The enzyme deficient in type VI GSD is liver-specific phosphorylase. It is relatively rare and a benign form of GSD with no heart and skeletal muscle involvement. Most patients with GSD VI disease present with hepatomegaly and growth retardation. Hypoglycemia, hyperlipidemia, and hyperketosis, if present, are usually mild. The hepatomegaly improves and disappears around puberty. Diagnosis rests on enzyme analysis of the liver biopsy. GSD VI is autosomal recessive. Liver phosphorylase gene (PYGL) has been mapped to chromosome 14q21 and has 20 exons.[23] A splice site mutation in intron 13 has been identified in Mennonite population.

GSD TYPE VII (PHOSPHOFRUCTOKINASE DEFICIENCY, TARUI DISEASE)

Type VII GSD is caused by the deficiency of muscle phosphofructokinase (PFK) enzyme. It is a complex isozyme consisting of three subunits variably expressed in muscle, liver, and platelet tissues.[24]

Clinical Findings

The clinical features are very similar to those in GSD V; however, hemolytic anemia can also occur. Along with glycogen accumulation, there is also an accumulation of fine fibrillar polysaccharide material resembling amylopectin, as what is seen in GSD IV.

An infantile form of phosphofructokinase deficiency has also been reported. These patients present with limb weakness, seizures, cortical blindness, and corneal clouding. Death occurs before 4 years of age from respiratory failure.

Biochemical enzyme analysis or histochemical demonstration of the enzymatic defect in the muscle is required to establish the diagnosis.

Genetics

Type VII GSD is autosomal recessive. The gene (PFKM) is located on chromosome 12q13. Eighteen mutations have been identified in *PFKM* gene. Muscle PFK deficiency seems to be prevalent in Ashkenazi Jews, and two common mutations are found in about 95% of mutant alleles.[25]

GSD TYPE IX (PHOSPHORYLASE B KINASE DEFICIENCY)

A deficiency of phosphorylase b kinase (PhK) is responsible for several forms of GSD that differ both in tissues affected and in patterns of inheritance.[26] The enzyme consists of four different subunits ($\alpha\beta\gamma\delta$), which result in multiple tissue-specific isozymes. The enzyme activates glycogen phosphorylase to enhance the breakdown of glycogen.

Clinical Findings

Patients with deficiency of liver PhK, the most common X-linked form, present in infancy with hepatomegaly, growth retardation, and delayed motor development. The symptoms improve with age, and adult patients have normal stature and minimal hepatomegaly.

Other PhK deficiency variants include an autosomal recessive form (affecting both liver and muscle), an autosomal recessive form (affecting only liver),[26] a mild myopathic form (muscle cramps and myoglobinuria), a severe myopathic form (onset in early infancy), and an isolated myocardial phosphorylase b kinase deficiency.[26,27] Clinical and genetic heterogeneity can be explained by the presence of the four subunits ($\alpha\beta\gamma\delta$) of PhK expressed in tissue-specific manner.

Definitive diagnosis of phosphorylase b kinase deficiency requires demonstration of the enzymatic defect in affected tissues.

Genetics

The genes for α, β, and γ subunits have been cloned.[28] The genes for both muscle and liver α subunit have been mapped to X chromosome (αL at Xp22.2 and αM at Xq12) and the one for β subunit to chromosome 16q12–q13. A number of mutations for different forms of phosphorylase kinase deficiency have been characterized.[26–28] The gene for muscle γ subunit (γM, PHKG1) has been localized to chromosome 7p12.[29] No mutations in this gene have been reported so far. Gene for liver/testis-specific γ subunit (γTL, PHKG2) maps to chromosome 16p12.1 and many disease-causing mutations are known for this gene.[28]

GSD TYPE XI (HEPATIC GLYCOGENOSES WITH RENAL FANCONI–BICKEL SYNDROME)

This rare GSD is caused by defects in the facilitative glucose transporter 2 (GLUT-2), which transports glucose in and out of hepatocytes, pancreatic cells, and the basolateral membranes of intestinal and renal epithelial cells.[30] The disease is characterized by proximal renal tubular dysfunction, impaired glucose and galactose utilization, and accumulation of glycogen in liver and kidney. The affected child presents in the first year of life with failure to thrive, rickets, hepato- and renomegaly, and maybe mild fasting hypoglycemia and hyperlipidemia. The gene for GLUT 2 has been cloned and is localized to chromosome 3q26.1. It comprises 10 exons and there are about 10 mutations identified.[30]

GLYCOGEN SYNTHASE DEFICIENCY

Strictly speaking, glycogen synthase deficiency (Type 0) is not a glycogen storage disease. The patients present in infancy with early-morning drowsiness, fatigue, and

sometimes convulsions associated with hypoglycemia and hyperketonemia.[31,32]

Treatment is avoidance of fasting. This autosomal recessive disease is caused by mutations in the liver glycogen synthase gene (GYS2), which is located on chromosome 12p12.2 (Table 1).

CONCLUSION

GSDs are inherited disorders caused by defects in enzymes or transport proteins involved in glycogen metabolism and glycolysis. Virtually all genes involved in GSDs have been cloned and mutations characterized. Advances in understanding the molecular basis of the various genetic deficiencies, their proteins, and regulation are being used to improve both the diagnosis and clinical outcome. Molecular studies will hopefully lead to the development of successful therapy for the more severe forms of GSD.

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HAART Therapy—Antiretroviral Resistance Genotyping

Kristel Van Laethem
Anne-Mieke Vandamme
Rega Institute for Medical Research and University Hospitals, Leuven, Belgium

INTRODUCTION

Although the introduction of highly active antiretroviral therapy (HAART) in the mid-1990s has resulted in a decrease of the morbidity and mortality in the HIV-1 patient population that has access to treatment, therapy failure still occurs. The surrogate markers for therapy failure, which ultimately results into clinical progression, are CD4 cell count and viral load. As changes in viral load mostly precedes changes in CD4 count, the determination of the viral load is the most relevant laboratory procedure to monitor the short-term in vivo activity of a therapy given to an individual patient. Lack of adherence, lack of therapy potency, pharmacological reasons, drug–drug interactions, and preexisting drug resistance can be factors resulting into therapy failure and rising viral loads.

The evolution of resistance against HIV-1 inhibitors within a patient depends on the generation of genetic variation, reflected by substitutions, insertions, deletions, and recombination events, and on the selection of drug-resistant variants during antiretroviral therapy. The high HIV-1 genetic variability is caused by the error-prone nature of HIV-1 reverse transcriptase, by the absence of any enzymatic proofreading activity, and by the huge rate of HIV-1 replication in vivo. Some of the created genetic variants will result in alterations to the structure and function of the molecules targeted by the antiviral drugs (currently reverse transcriptase, protease, and glycoprotein gp41). These alterations could confer changes in susceptibility to one or more of these drugs. In the presence of therapy, the variants with some level of resistance will gradually overgrow the wild-type variants. Under the continuous selective pressure of inhibitors and with the presence of residual replication as a result of insufficient potency of the treatment, additional mutations will accumulate.

Thus, the main goal of HAART is to reduce the viral load as much as possible to prevent further clinical progression. However, its immediate goal is to reduce the viral load to prevent the formation of variants that result in antiviral resistance to the current therapy and that, because of the phenomenon of cross-resistance within drug classes, ultimately might lead to a limitation or even lack of future treatment options.

METHODOLOGY

Antiviral drug resistance can be determined at the genotypic level by the detection of resistance mutations. Alternatively, it can be determined at the phenotypic level by measuring the ability of an HIV-1 isolate to grow in the presence of a drug, or by measuring the HIV-1 reverse transcriptase or protease enzyme activity in the presence of an inhibitor.

Sequencing is the most commonly used genotypic assay for the monitoring of resistance mutations. It provides information on all nucleotides of the sequenced region. The target sequence is amplified via polymerase chain reaction, and the sequence is determined based on the incorporation of dideoxynucleotides. At the moment, most clinical laboratories perform home-brew sequencing or they use one of the two commercially available sequencing assays [ViroSeq HIV-1 Genotyping System (Abbott) and Trugene HIV-1 Genotyping Kit (Bayer)]. Another option is to send samples to service laboratories (e.g., ViroLogic and Virco).

Presently, only three companies (ViroLogic, Virco, and VIRalliance) and a few national reference laboratories routinely perform replication-based phenotypic assays. These assays start with the amplification of the patient-derived HIV-1 target sequence via polymerase chain reaction. The amplification product is subsequently incorporated into a proviral laboratory clone via ligation or homologous recombination. This generates a stock of chimeric viruses. The viruses are tested for their ability to grow in the presence of different concentrations of inhibitors. The principle of chimeric viruses for the determination of the susceptibility profile is preferred over other phenotypic assays to obtain better reproducibility and automation possibilities.[1,2]

Resistance testing has to be performed on plasma samples before starting, stopping, or changing therapy. It is highly important to monitor the active replicating viral population responsible for the therapy failure, because it is the resistance pattern of this population that can be useful in understanding the observed therapy failure.

An important limitation of current available assays is their need for plasma samples with an HIV-1 viral load exceeding 1000 copies/mL because of the lower

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sensitivity of the cDNA synthesis and amplification procedures of long templates. This is required to achieve reliable results. Otherwise, nonrepresentative variants could be picked up because of stochastic events during the experimental procedures. However, switching therapy early after viral failure, when the HIV-1 viral load is detectable but still very low, results in better chance of response to the salvage therapy. There are only few data that provide the optimal point at which therapy should be changed in terms of long-term clinical outcome, but the short-term risk of any viral replication in the presence of inhibitors is the development of resistance.

Results of the resistance assays represent only the genotypic or phenotypic profile of the majority of quasispecies present in vivo because they have difficulties in detecting minor variants that reflect less than 10–50% of the total viral population.[3] It remains uncertain what the contribution of minor viral variants is to therapy failure.

As genotypic and phenotypic assays start with an amplification procedure, the sensitivity of both tests depends on the efficient hybridization of primers, and this is strongly influenced by the variability at the primer sites. Most of the assays have originally been optimized by using subtype B HIV-1 strains because such strains were responsible for the epidemic in developed countries. However, in recent years, the prevalence of non-B subtypes has been increasing in Europe, and access to therapy has been extended to the developing world where non-B HIV-1 subtypes constitute the largest part of the epidemic.[4] Effort has been made to improve the performance of the assays on non-B HIV-1 subtypes by developing primers at more conserved sites or by the inclusion of backup primers, but no assay can guarantee to reproduce adequate results for all strains.

Both genotypic and phenotypic resistance assays have respective advantages and disadvantages (Table 1). Resistance assays have to be performed in highly specialized laboratory facilities. This particularly holds true for the phenotypic resistance assay because it requires biosafety level 2 or 3 facilities, whereas a genotypic resistance assay can be performed in a dedicated molecular biology laboratory. Because both types of assays require sophisticated technology and knowledge, a strict adherence to current laboratory standards is important; well-trained and experienced laboratory technicians are also essential.

Another practical consideration in the choice of resistance testing is the cost of the procedure. Phenotypic assays are still more expensive than genotypic assays.

Genotypic resistance assays can deliver results within a few days, whereas longer time is required to obtain

Table 1 Advantages and disadvantages of genotypic and phenotypic resistance assays

Advantages	Disadvantages
Genotypic resistance assays	
Have a wider availability, requiring biosafety level 1 facilities	Require plasma samples in general with viral load >1000 RNA copies/mL
Are less expensive	Exhibit difficulties in detecting minor variants
Display a shorter turnaround time (typically 1–2 weeks)	Exhibit a sensitivity that is strongly influenced by virus genetic variability
Detect sentinel mutations that are linked to reduced clinical response but not necessarily to reduced phenotypic susceptibility	The relevance of some mutations remains still unclear
	Some (complex) mutation patterns may be difficult to interpret
Phenotypic resistance assays	
Measure the effective susceptibility to all (also new) drugs	Require highly specialized laboratory facilities, typically biosafety level 2 or 3
Measure effects of mutational interactions	Require plasma samples in general with viral load >1000 RNA copies/mL
	Exhibit difficulties in detecting minor variants
	Exhibit a sensitivity that is strongly influenced by virus genetic variability
	Are more expensive (in general twice as much as genotyping)
	Have longer turnaround time (typically 2–4 weeks)
	The reproducibility range of the assay extends for some drugs above clinically relevant cut-offs
	The clinical relevance of drug susceptibility levels is not always clear

phenotypic resistance results. This turnaround time can be an important factor in some clinical situations, where immediate results are needed, e.g., postexposure prophylaxis and primary HIV-1 infection.

Sequencing has the advantage of detecting all known resistance-related mutations—mutations considered as markers for developing resistance patterns (e.g., K70R or Q151M in the RT gene)—or substitutions that are considered to be associated with a lower genetic barrier to resistance development (e.g., the reversal mutations T215A/C/D/S in the RT gene). Phenotypic assays measure the effective susceptibility, resulting from known, but also unknown, resistance-related mutations. However, they do not detect the presence of the latter two sets of mutations. Although these mutations are associated with reduced clinical response, they are not always linked with reduced phenotypic susceptibility.

In addition, the current phenotypic assays have difficulties in detecting resistance to some nucleoside reverse transcriptase inhibitors. These drugs compete with the natural deoxynucleoside triphosphates (dNTPs) for binding to the reverse transcriptase and incorporating into the growing DNA chain. The phenotypic assays use activated cells with high intracellular dNTP pools that do not always reflect the relevant in vivo cell conditions. The high intracellular dNTP pools lead to resistance levels that cannot be reliably measured because they are within the reproducibility range of the assays.

Another issue in the interpretation of genotypic results is whether the mutations constituting a part of a set of multiple mutations exist on a single clone or are spread across multiple clones of variants. Phenotypic resistance assays resulting in low-level resistance may be caused by a small change in susceptibility of the whole virus population or a mixture of resistant and susceptible virus. The linkage of mutations could have important clinical implications, but at the moment this issue cannot be routinely elucidated with the current resistance assays.

INTERPRETATION

The information provided by genotypic resistance assays may be difficult to interpret. Current knowledge on the correlation between the genotypic and phenotypic characteristics of an HIV-1 isolate is mainly based on in vitro studies of laboratory and clinical HIV-1 strains. The clinical relevance of mutations is also determined from linking mutation patterns with previous therapy history or from linking them with subsequent therapy response. A decade ago, when monotherapy was the standard-of-care, the direct linkage between mutations, therapy experience, and subsequently therapy response was more clear-cut than in the current HAART situation. The potency of new drugs is now investigated in ''add-on'' studies, where the new drug is added to the failing regimen. More recently, this strategy has encountered ethical problems because of the possibility that it could lead into fast resistance development against the new drug. An increasing number of efficiency studies are performed with combinations of the new drug and at least one other suspected active compound. This hampers the understanding of the impact of preexisting resistance and the subsequent viral response to the new drug.

Patients are currently treated with combination therapies that change in time and that result in complex patterns of mutations. The phenotypic effects of mutations and their interactions often cannot be reliably predicted. Some mutations that are developed under the selective pressure of a particular drug can cause resistance toward other drugs from the same class without any previous experience (cross-resistance), and others might reverse the phenotypic resistance level of certain resistance mutations. Phenotypic resistance assays give a direct measurement of susceptibility toward the tested inhibitors that includes the effect of all mutations and their interactions. However, the clinical relevance of phenotypic results can also be difficult to interpret because the threshold at which a specific inhibitor becomes ineffective in vivo has not been determined yet for all available drugs.

The investigation of the correlation between genotype and phenotype has resulted into the development of genotypic–phenotypic relational databases and rule-based algorithms.[5] These systems can be helpful in the interpretation of in vivo resistance. Large datasets containing matched genotypes and phenotypes are already used to predict phenotype from genotype. Rule-based algorithms contain lists of mutations that are: 1) known to confer phenotypic resistance toward a particular drug; 2) known to be part of a mutation pathway toward high-level resistance; 3) and, most importantly, responsible for a reduced clinical response.[6]

At the moment, several interpretation algorithms are available. They give concordant results in most cases. The observed discordances reflect the uncertainty that is still associated with particular set of mutations.[7]

These algorithms should be subject to regular updates as soon as new correlations between genotype and phenotype are established and the clinical relevance of certain genotypes and phenotypes become evident.

From the time antiretroviral drugs were first used in the treatment of HIV-1 infection, it was observed that in many cases therapy failure was associated with the development of resistance mutations at the genetic level and decreases in drug susceptibility at the phenotypic level. Only a few

years ago, after the effect of resistance on subsequent therapy failure was investigated in retrospective studies, the idea to prospectively use the results from genotypic and phenotypic resistance assays in the management of HIV-1 infection was incorporated into the design of clinical trials. In these trials, viral response on therapy guided by genotypic and/or phenotypic resistance results was compared to that during standard-of-care. In some of these prospective trials, access to genotypic or phenotypic drug resistance results led into a short-term, modest viral benefit. In other studies, the effect of resistance testing was attenuated as a consequence of many factors.

The absence of resistance does not always result into therapy response because success is a result of many factors besides antiviral resistance, such as adherence, potency, and pharmacological reasons. It has also been shown that the interpretation of the raw resistance data plays an important role in the successful implementation of resistance testing.[8] It is recommended that the interpretation should be left to experts. These experts can translate the data into recommendations that are useful for clinicians. Another limitation of the use of drug resistance assays in the clinical management of HIV-1 infection is the limited choice of drugs in the treatment of HIV-1 patients who have undergone extensive therapy with multiple failures.[9] Because of the existing cross-resistance within drug classes, a badly managed combination therapy could result into broad cross-resistance toward all available drugs, even if these respective drugs had not been previously used. Whether or not the choice of subsequent therapy is then guided by resistance testing has no longer any relevance, because there remains only limited or no option at all.

Nevertheless, resistance testing has demonstrated its advantages and has been implemented into guidelines.[10–12] It is recommended for primary and recent HIV-1 infection, before initiation of therapy in chronically infected patients if infected within the past 2 years, or in areas with high resistance prevalence, first or multiple therapy failure, and pregnancy.

CONCLUSION

Although resistance testing is widely used in the clinical management of HIV-1 treatment, questions remain. Up until now, the assays have shown a short-term modest viral benefit. Clinicians should value the results obtained with these assays but should, however, not overestimate their importance because there still exists some uncertainty concerning the clinical importance of certain genotypic mutations and phenotypic levels of susceptibility. The decision to use a specific combination of drugs should be made by the clinician, taking into account all clinical, immunological, viral, and pharmacological factors, as well as adherence issues and treatment history.

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Haemophilus ducreyi

Sylvia M. Bruisten
GG&GD Municipal Health Service, Amsterdam, The Netherlands

INTRODUCTION

The gram-negative coccobacillus *Haemophilus ducreyi* causes chancroid in humans. Chancroid is a genital ulcer disease (GUD) that is highly prevalent in underdeveloped countries and in some urban areas of developed countries. It is a sexually transmitted infection (STI) that had an estimated global incidence of 7 million cases in 1997. As is the case with other GUDs, chancroid facilitates HIV-1 transmission. Naturally occurring chancroid is much more prevalent in men than in women and circumcision is known to greatly reduce the incidence. Chancroid manifests itself as a single painful ulcer after infection of damaged skin. Symptomatic diagnosis is complicated because the clinical picture is similar to GUD caused by other pathogens such as herpes simplex virus (HSV) and *Treponema pallidum* (syphilis). *H. ducreyi* is renowned for its problematic detection by culture. Experienced microbiologists are needed and these are mostly lacking where chancroid is highly prevalent. Microscopic diagnosis can only be performed in well-equipped laboratories and the same problem is recognized for amplification methods such as PCR. Nevertheless PCR provides the most sensitive diagnosis of chancroid and should be regarded as the gold standard. Development of new, easy-to-use serological assays is needed for on-site specific diagnosis.

CLASSIFICATION

In 1889, Ducreyi demonstrated the inoculable nature of chancroid pus of genital lesions. The present genus name *H. ducreyi* originated in 1921 from Neveu-Lemaire. *H. ducreyi* is classified in the *Actinobacillus* cluster of the Pasteurellaceae. Synonyms of *H. ducreyi* are *Bacillus ulceris cancrosi* (Kruse 1896) and *Coccobacillus ducreyi* (1921). The *H. ducreyi* genome consists of a single 1.7-Mbp chromosome with 1693 open reading frames (ORFs).[1] One of its virulent strains (35000 HP) has been sequenced completely. Genes with closest homology were found in *Haemophilus influenzae* and *Pasteurella multicoda*; however, there is little long-range conservation of the order of genes or ORFs in the chromosome when *H. ducreyi* is compared to these species.[1] The genes in *H. ducreyi* encoding toxic factors such as hemolysin and the cytolethal distending toxin (CDT) are absent in the genomes of other Pasteurellaceae.[1]

BIOLOGY

H. ducreyi preferentially infects keratinized stratified squamous epithelium. Epidermal microabrasions formed during sexual intercourse or otherwise damaged skin is the portal of entry for infection. After entry into the skin, the bacteria stimulate target cells such as keratinocytes, fibroblasts, and endothelial cells to secrete IL-6 and IL-8.[1] Polymorphonuclear cells are recruited within 24 hr to the epidermis and dermis and form small micropustules. A dermal infiltrate of T cells, macrophages, and some B cells evolves.[2] A tender erythematous papule may develop 4–7 days after initial infection, which can progress to the pustular stage. The bacteria remain extracellular through the pustular stage and resist phagocytosis.[3,4] Pustules often rupture after a further 2–3 days to form painful shallow ulcers with granulomatous bases and purulent exudates.[5] In the absence of effective antimicrobial therapy, the chancroid ulceration can take several weeks to months to resolve. Inguinal lymphadenitis occurs in up to 50% of cases and the lymph nodes may develop into buboes. If not aspirated or drained through incision, buboes can rupture spontaneously.[5]

In the rabbit model, *H. ducreyi* does not grow at temperatures above 35°C, but only in rabbits housed at 15–17°C. In culture the bacteria grow best at 33°C, which probably reflects the fact that in humans only external surfaces are susceptible to infection. Experimental animal models for *H. ducreyi* that most resemble the human in vivo growth are pig and macaque models. A mouse subcutaneous chamber model was developed for studying long-term growth.[6]

VIRULENCE FACTORS

In human experimental models, bacteria are delivered into the skin via puncture wounds using an allergy-testing device.[3] An estimated delivered dose (EDD) of 1 colony-forming unit already results in 50% papule formation.[4]

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Most bacteria localize in the dermis after 48 hr of infection. Several potential *H. ducreyi* virulence factors have been reported in the literature and their possible effects were studied using isogenic mutants by grafting both wild-type strains (i.e., 35000 HP) and mutant strains on each arm of volunteers.[3,4] Factors studied so far include lipooligosaccharide (LOS), pili, soluble cytolethal distending protein (sCDT), copper–zinc superoxide dismutase (SOD), a hemoglobin-binding outer membrane protein (OMP), a hemolysin capable of cytotoxicity, a filamentous hemagglutinin-like protein, and a zinc-binding periplasmic protein.[6]

The LOS of most *H. ducreyi* strains express an epitope that is also present in *Neisseria* strains and that is immunochemically similar to a precursor of a major blood group antigen found on human erythrocytes.[6] Pili are filamentous appendages involved in cell adhesion. The major 24-kDa pilin FtpA subunit shares no homology with other pilins present on the surface of gram-negative bacteria.[6] A gene cluster (*cdt* ABC) codes for the CDT proteins that were shown to block cell division in the G2 phase of the cell cycle. Superoxide dismutase are metalloenzymes that catalyze the conversion of superoxide radicals to oxygen and hydrogen peroxide. Periplasmic Cu–Zn SODs are thought to protect against oxidative cell damage by host immune cells.[6] The hemoglobin binding OMP (108-kDa *HgbA/HupA*) is essential for *H. ducreyi* to utilize both hemoglobin and haptoglobin as a source of heme required for growth. Hemolysins encoded by the *hhdA* and *hhdB* genes are possibly responsible for release of hemoglobin from erythrocytes. Two large genes, *IspA1* and *IspA2*, encode proteins that resemble in part the filamentous haemagglutinin (FhaB) virulence factor of *Bordetella pertussis*. The exact function of the Lsp-A1 and -A2 proteins remains to be determined.[6] Zinc is an important trace element for maintaining structural stability and for catalytic activity of enzymes. The *znuA* gene of *H. ducreyi* encodes a periplasmic protein involved in zinc transport. The isogenic *znuA* mutant exhibited reduced lesions in the rabbit model.

CLINICAL ASPECTS

H. ducreyi is a strictly human pathogen causing chancroid. Probably because initial stages of infection are not painful, patients typically do not seek medical attention until they had ulcers for 1 to 3 weeks, which is probably 3 to 6 weeks after inoculation.[1] The chancroid ulcer has a pale yellow shallow base and diffuse, irregular borders.[7] Three to 25 times more males than females are infected. In men the lesions typically occur on the prepuce and frenulum (Fig. 1), whereas in women the ulcers are usually external and involve the vulva, the labia, and perianal region.[6,7] Extragenital chancroid is rare but may occur by autoinoculation of the inner thighs, breasts, and fingers.[6] A past infection does not confer protective immunity because multiple episodes of *H. ducreyi* infections can occur.

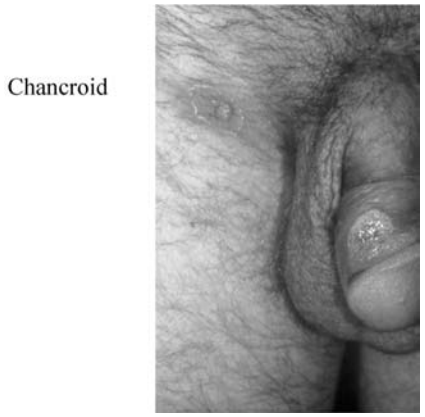

Fig. 1 Chancroid lesions on male thigh and penis shaft. (Courtesy of Han Fennema, M.D., Ph.D.) (*View this art in color at www.dekker.com.*)

Clinical diagnosis of chancroid is unreliable because of similarities of the clinical presentation of different etiologic agents of GUD, the presence of mixed infections, and atypical ulceration due to long-standing disease.[8] Next to *H. ducreyi*, causative pathogens of GUD are herpes simplex virus type 2 (HSV-2) and type 1 (HSV-1) and the bacteria *T. pallidum* (TP), which cause syphilis, and *Chlamydia trachomatis*, which cause lymphogranuloma venereum. In about 20–40% of GUD cases none of these pathogens are found despite use of the most sensitive assays.[9,10] Coinfection of *H. ducreyi* and HSV occurs commonly in the tropics.[6]

DIAGNOSIS

Diagnostic tests in use are culture tests, microscopy, PCR, serology, and antigen assays. Older studies use either culture or clinical diagnosis as the gold standard, but both are inaccurate measures of the true incidence of chancroid.[11]

H. ducreyi is a fastidious pathogen that requires complex media (containing heme) and growth conditions (33°C) for culture. *H. ducreyi* is routinely grown on 5% chocolatized horse blood agar plates, but using two specialized culture media increases the sensitivity.[11,12]

Table 1 Hybridization and amplification test formats for *H. ducreyi*

Target gene for PCR[a]	PCR test format[b]	Number of samples	Sensitivity, % (relative to)	Year of reference
16S rRNA	Nested; one tube	25	100 (culture)	1995[15]
	Colorimetric detection	83	83 (clinical)	
16S rRNA	Single, multiplex;	62	98 (culture)	1996[9]
	colorimetric detection	65	100 (HD PCR)	
16S rRNA	Nested; gel analysis	28	100 (culture)	1998[16]
16S–23S rRNA ISR[a]	Heminested; gel detection	32	96 (clinical)	1998[17]
GroEL; recD	Single; probe detection	5	60 (clinical)	2000[18]
P27 gene (=recD)	Heminested; gel detection	37	100 (culture)	2001[19]

[a]ISR: intergenic spacer region: region between the (rrs)16S and (rrl) 23S rRNA genes.
[b]Described is the type of PCR, either single, nested, seminested or mutiplex. Also the type of detection of the PCR product is given, either by (colorimetric) probe detection or by gel analysis.

Vancomycin (3 μg/mL) should be added to reduce contamination by other microorganisms. Microscopically, the colonial structures have been described as ''schools of fish,'' ''railroad tracks'' and ''fingerprints.''[11] Serological assays were developed that detect either a past infection measuring IgG anti-*H. ducreyi* levels or active infection detecting IgA and IgM levels.[10,13] IgG-based assays are mostly used to perform epidemiological studies.[11] A rapid diagnostic test based on monoclonal antibodies to the hemoglobin receptor HgbA was highly specific, but the sensitivity was too low for on-site diagnosis.[14] The first *H. ducreyi*–specific PCR assays were developed in the mid-1990s (Table 1), and DNA amplification is nowadays considered the most sensitive diagnostic method. Multiplex PCR (M-PCR) was developed in which TP, HSV, and *H. ducreyi* are simultaneously detected.[9,10,20] M-PCR is highly efficient but not commercially available. Nevertheless, PCR should be used as reference standard in evaluations of novel diagnostic techniques.[11]

TREATMENT

In developing countries with poor laboratory skills and facilities, a syndrome-based approach was developed and promoted because it was found to be inexpensive, simple, and very cost-effective. In syndromic management, patients are treated at the first visit with a combination of antimicrobials targeting the local probable etiological agents. The mainstay of chancroid treatment is 4 times daily 500 mg of oral erythromycin for 7 days (WHO website: http://www.who.int/docstore/hiv/STIManagemntguidelines/who_hiv_aids_2001.01/index.htm, accessed May 23, 2003). Other current recommended treatment regimens are single doses of either 1 g of azithromycin, 500 mg of ciprofloxacin, or 2 g of spectomycin.[5] If suspicion of infection with *Neisseria gonorrhoeae* and/or *Chlamydia trachomatis* exists, azythromycin is additionally effective against *H. ducreyi*. Although *H. ducreyi* was initially sensitive to many antibiotics, plasmid-mediated resistance has become an increasing problem worldwide since the 1970s. Resistance was documented for tetracycline, chloramphenicol, sulphonamides, aminoglycosides, and beta-lactam antimicrobial agents.[6] Chromosomally mediated resistance has lately led to decreased sensitivity for penicillin, trimethoprim, and ciprofloxacin.

EPIDEMIOLOGY

Chancroid was endemic in most parts of the world well into the 20th century. In Europe and North America, a steady decline was seen after the discovery of penicillin in 1943.[21] Reduction started, however, with changes toward reduced prostitution and improved sanitary conditions. Nowadays, the prevalence of chancroid is less than 1% in developed countries.[22] The few cases that are detected are usually imported from endemic countries or are associated with commercial sex work (CSW) and the use of crack cocaine and other illegal drugs.[7] Periodic health examinations and treatment of sex workers and other high-risk groups have largely contributed to the near-eradication of chancroid in these European countries.[21] In some cities in the United States, however, especially among high-risk communities, the *H. ducreyi* prevalence can be as high as 20%.[22,23]

Chancroid is still ubiquitous in tropical resource-poor countries located in Africa, Asia, Latin America, and the Caribbean.[6,21] In southern and eastern African countries, the *H. ducreyi* seroprevalence can be as high as 68% among CSW, but this could be overdiagnosis because in Africa the etiology of ulcers is often clinically directed.[24]

In west and central African countries the prevalence of chancroid among STI patients was significantly lower.[8,24]

HIV INFECTION

Chancroid is recognized as an important risk factor for acquiring HIV infection probably because the same type of risk behavior (prostitution, substance abuse) occurs for both diseases.[23,24] *H. ducreyi* infection enhances HIV transmission by several possible mechanisms, such as establishment of a portal of entry, promotion of viral shedding from the ulcer, and an increase in viral load in blood and semen.[25] Increased susceptibility to HIV infection is supported by the finding of recruitment of CD4 cells and macrophages in ulcers.[1] The resolution of treated chancroid lesions is prolonged in coinfected patients, and thus both HIV and *H. ducreyi* may be shed for longer periods even after antibiotic treatment for chancroid.[12] There is concern all over the world that the synergy existing between chancroid and HIV will accelerate the HIV epidemic. In southern and eastern Africa, where the levels of HIV are much higher than in West Africa, high levels of genital ulceration were noted.[24] In West and North Africa (mostly in Muslim countries) circumcision is common practice and this seems to be protective for male to female transmission of bacterially caused genital ulcers (syphilis and chancroid).[24]

PREVENTION

Annihilation of chancroid would be greatly helped by the development of an effective vaccine, but none has so far been developed.[6] Yet eradication of chancroid should be feasible, for one because the treatment of chancroid is cheap and effective.[21] *H. ducreyi* has a short duration of infectivity and can effectively be destroyed using soap and water. To survive in a population, *H. ducreyi* needs sexual networks with frequent contacts and high rates of partner exchange. Institution of preventive measures such as controlled prostitution and education to improve condom use together with regular health checks of sex workers and presumptive antibiotic treatment should all contribute to reduction of chancroid.[21] A concern regarding presumptive treatment is the possible development of antibiotic resistance and the false sense of security among those treated that could impede preventive behavior. This stresses the need for education. Targeting of uncircumcised men who are at higher risk of acquiring and transmitting chancroid may also be very effective.[24]

CONCLUSION

Diagnosis of *H. ducreyi* is performed preferably by PCR, which has the highest sensitivity and specificity. HIV and *H. ducreyi* act synergistically, explaining why there is heightened interest in the biology, immunology, and epidemiology of chancroid. *H. ducreyi* virulence factors are the subject of many studies, also with the aim to develop effective vaccines against chancroid. In addition, without vaccination chancroid may be eradicated by promoting condom use and providing education and accessible treatment facilities to those that are most at risk for recurrent *H. ducreyi* infection.

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Handheld Nucleic Acid Analyzer

James A. Higgins
United States Department of Agriculture–Agricultural Research Service, Beltsville, Maryland, U.S.A.

INTRODUCTION

Since the advent of the polymerase chain reaction (PCR) in the late 1980s, investigators have sought to expand the potential of this technology to that of on-site, ''bedside'' diagnostics, in which clinical parameters can be determined in real time, without the need for transport of samples to a remote testing facility. Discoveries of clandestine bioweapons programs in Iraq and the former Soviet Union in the early 1990s led the U.S. Department of Defense to sponsor development of nucleic acid-based instruments suitable for detecting a variety of pathogens at both front-line and rear-echelon locations. As we enter the mid-2000s, both of these research initiatives have begun to yield commercial handheld instruments, capable of performing PCR and RT-PCR in real time; future devices promise to offer direct detection of target nucleic acids without the need for amplification. Other instrument packages look to provide the end-user with both sample preparation/nucleic acid extraction and detection in one portable instrument. This entry reviews existing handheld nucleic acid detection instrumentation, discusses their advantages and disadvantages, and previews newer devices and platforms expected to continue to make this area of medical diagnostics particularly innovative and exciting.

THREAT AGENT DETECTION: IMPETUS FOR HANDHELD NUCLEIC ACID ANALYZER DEVELOPMENT

Successful performance of PCR obviously depends on the ability of the thermal cycling platform to maintain accurate gradations of temperature in the manifold or block containing the reaction tubes. The heating and cooling apparatus necessary to provide these temperature changes places constraints on the size and weight of the thermal cycler. ''First-generation'' thermal cyclers, such as the Perkin Elmer 480 model instrument, while reasonably light in weight and small in footprint, were not designed with field use in mind. However, during operations in the Persian Gulf in 1990–1991, field laboratories for detection of threat agents were deployed and operated by both the U.S. Army and the U.S. Navy. Polymerase chain reaction on conventional thermal cyclers, with agarose gel electrophoresis used to confirm amplification products, was used on a regular basis by these laboratories with a high degree of success, despite the trying conditions associated with performing molecular assays in a desert environment. By the mid-1990s the U.S. Army adopted PCR-based assays as a critical component of the field-deployable 520th Theatre Area Medical Laboratory (TAML), which was designed to provide rapid threat agent detection in battlefield conditions.

Just as Western defense and public health personnel were coming to grips with the disclosure of the size and scope of the Iraqi bioweapons program, it was revealed that the Soviet Union had a clandestinely operated sizeable, well-funded program of its own. Among the pathogens selected for weaponization were smallpox, plague, tularemia, and anthrax. The likelihood that U.S. and NATO troops may encounter such agents heightened the importance of on-site diagnostic techniques and, consequently, research in this area was prioritized by the Department of Defense, primarily under the auspices of the Defense Advanced Research Projects Agency (DARPA).

Research on portable nucleic acid-based detection devices was considerably aided by the discovery in 1991 that the 5′–3′ exonuclease activity of *Taq* polymerase, the enzyme mediating PCR, could be harnessed to provide real-time monitoring of the reaction via the hybridization of a fluorogenic oligonucleotide probe.[1] In 1995 researchers at Genentech, Inc., and Applied Biosystems reported the first use of a commercial probe expressly designed for real-time PCR, the ''TaqMan®'' probe.[2] With this assay system, fluorescence generated by the probe could be detected by the appropriate instrumentation and the user provided with a graphical depiction of the accumulation of the PCR amplicons over the length of the reaction. The TaqMan assay was notable for being conducted in the same reaction tube from start to finish; there was no need for postreaction manipulations. Probe design and synthesis was relatively straightforward and a TaqMan-based protocol for the detection of the foodborne bacterium *Listeria monocytogenes* was published in 1995.[3] These results gave impetus to the design and evaluation of TaqMan probes against a variety of bacterial and viral threat agents, such as orthopoxviruses.[4]

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Fig. 1 The Smiths Detection BioSeeq™ instrument weighs 6.5 lb (2.9 kg) and measures $12 \times 8 \times 2$ in. ($30.4 \times 20.3 \times 5.0$ cm) and can run for up to 1 hr on the power provided by eight alkaline ''C'' batteries. There are six independently programmable reaction modules and data are displayed to the user via a small inset screen; the instrument can also be linked to a laptop computer for more involved assay programming and data display. (Photo courtesy of P. Emanuel, U.S. Army, Edgewood, MD.) (*View this art in color at www.dekker.com.*)

The implementation of fluorogenic probes freed the user from the need to conduct electrophoresis to determine whether or not a PCR assay was successful, and had obvious implications for reducing dependence on a laboratory infrastructure. However, the need for power sources and optics capable of monitoring the fluorescence produced by a real-time PCR assay was a major impediment to the design of portable thermal cyclers. For example, the first commercial platform dedicated to real-time PCR, the Perkin Elmer/Applied Biosystems 7700 model Prism® instrument, appeared in 1996–1997, but was too heavy (140 kg) for field use. U.S. Department of Defense-sponsored research sought to address this issue, and in 1997 researchers at the Lawrence Livermore National Laboratory (Livermore, CA) succeeded in fabricating a battery-powered, suitcase-sized real-time thermal cycler capable of using TaqMan probes: the MATCI, or Miniature Analytical Thermal Cycler Instrument.[5] Central to the operation of the device was its use of silicon ''thermalcycler'' units to mediate the heating and cooling of the associated 25-μl volume plastic reaction tube; this allowed for a one cycle per minute cycling speed. The MATCI provided sufficient temperature fidelity to allow for differentiation between sequences, with a high degree of homogeneity (such as poxvirus hemagglutinin gene), on the basis of a single nucleotide substitution.[6]

The MATCI could only accommodate one reaction tube at a time; the next iteration of the technology, the Advanced Nucleic Acid Analyzer (ANAA), which appeared in 1998, could perform 10 reactions simultaneously, and detection of as few as 500 cells of *Erwinia* was accomplished after only 7 min of thermal cycling.[7] The ANAA was a suitcase-sized instrument and researchers at the Lawrence Livermore National Laboratory were interested in advancing the miniaturization of the system to a handheld format; this led to the fabrication of the Handheld Advanced Nucleic Acid Analyzer (HANAA), arguably the world's first handheld, real-time thermal cycler instrument. The HANAA could perform four reactions simultaneously, with the use of two light emitting diodes (at 490 and 525 nm, with a combined emission of over 1 mW of power) allowing for monitoring of two dyes (e.g., FAM in one channel and JOE in the other).[8] The HANAA weighed less than 1 kg, and several sequential reactions could be performed, powered off a 12 V/3.5 A battery pack.[9] Reactions of up to 25 μl in volume were conducted in customized plastic tubes, and an improved silicon/platinum thermalcycler unit ($22 \times 6.6 \times 2$ mm) permitted ''traditional'' cycling parameters (e.g., 95°C for 0 sec, 55°C for 10 sec, and 72°C for 15 sec for 40 cycles) to be conducted in less than 20 min; strongly positive samples could generate positive results by 13 min of assay time.[10]

Prototype HANAAs were distributed to a variety of testers and feedback was favorable enough to result in licensing of the technology to Smiths Detection, Edgewood, MD; the result was production of the BioSeeq™ handheld real-time thermal cycler, available for purchase in 2003 (Figs. 1 and 2). The BioSeeq can accommodate up to six independently programmable reactions, each with a volume of 25 μL. In their evaluation of the BioSeeq during an 8-month beta testing phase, Emanuel et al.[11]

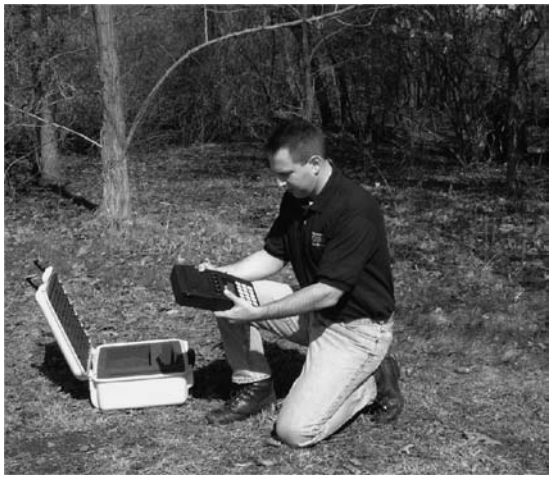

Fig. 2 Demonstration of the use of the BioSeeq™ instrument in the field. (Photo courtesy of P. Emanuel, U.S. Army, Edgewood, MD.) (*View this art in color at www.dekker.com.*)

used TaqMan probes directed against the *Francisella tularensis tul4* and *fopA* genes. Detection limits for the BioSeeq were 200 and 300 fg, respectively; these are approximately 100 and 150 genome equivalents of *F. tularensis*. In comparison, the detection limit of the assay when performed on the ABI 7900 real-time thermal cycler instrument was 50 fg, approximately 25 genome equivalents. Assay specificity, determined by testing a panel of 27 bacterial species and strains, including other possible threat agents, was the same for both targets on both instruments. When DNA extracted from tissues of mice infected with the *F. tularensis* LVS strain via aerosol exposure was assayed on the ABI 7900 and the BioSeeq, results were comparable, indicating that the handheld instrument was successful in analyzing the DNA in reaction volumes half the size of those of the ABI 7900 (which uses a volume of 50 μl). Custom reagents for a variety of threat agents are expected to be available for the BioSeeq in the Winter of 2003/2004, and the device is projected to sell for $25,000.

OTHER DOD-SPONSORED THREAT AGENT DETECTION PLATFORMS

In 2004, another real-time, handheld nucleic acid-based detection platform will become available for use by U.S. government agencies: the Razor, manufactured by Idaho Technology, Inc. (Salt Lake City, UT; Fig. 3). The Razor builds on the experience of Idaho Technology's suitcase thermal cycler, the Ruggedized Advanced Pathogen Identification System (RAPID), which was designed in collaboration with personnel from the U.S. Air Force. The RAPID is essentially a ''combat'' version of the LightCycler® (which is licensed by Roche, Inc., for sale to non-U.S.-government laboratories) and has been used extensively for the detection of threat agents in the United States, Europe, and the Middle East. The RAPID is capable of using prepackaged, freeze-dried real-time PCR and RT-PCR reagents, and this convenience has been carried over to the Razor, in which reactions are performed in plastic pouches containing the freeze-dried reagents of interest. The end-user simply uses a conventional syringe to inject sterile water into the pouch, reconstitutes the reagents, injects template, and proceeds with the assay; up to 12 reactions can be done in one pouch. The Razor, which weighs 8 lb (3.6 kg) and can analyze the entire 12-sample pouch in approximately 22 min, is designed for field use off a battery power source. The instrument's software is preloaded with thermal cycling protocols for detection of a variety of threat agents, and results are presented to the user either via plus/minus scoring on the integral LCD display (Fig. 3), or via a more comprehensive graphical display on an attached laptop computer.

Fig. 3 The Idaho Technology, Inc., Razor instrument is an 8-lb. (3.6 kg) handheld real-time thermal cycler that performs the reactions in specially formatted plastic pouches (foreground) that contain freeze-dried reagents for up to 12 samples. The reagents are reconstituted via injection of sterile water through syringe ports on either end of the pouch. Inset: results of an assay are displayed to the user as plus/minus scoring on an LCD display panel. (Photo: James Higgins; inset: Matt Scullion, Idaho Technology, Inc., Salt Lake City, UT.) (*View this art in color at www.dekker.com.*)

HANDHELD NUCLEIC ACID ANALYZERS FOR CLINICAL USE

Simultaneously with DoD-sponsored research, a number of private companies have pursued the development and fabrication of handheld nucleic acid analyzers with an eye toward capturing what is expected to be a very lucrative and burgeoning market in ''point-of-care'' (POC) diagnostics.

Convincing physicians and clinical laboratory directors that POC nucleic acid analyzers, much less molecular methods per se, are worth embracing has traditionally been difficult.[12] Sample processing, particularly of matrices with a high concentration of PCR inhibitors (such as blood), continues to be a major hurdle. Also, firms hoping to market POC diagnostics must convince physicians and laboratory directors that the sometimes higher costs of their assays can be justified by improved sensitivity and specificity over existing methods. Nonetheless, according to *Small Times* magazine author Marlene Bourne, currently 21 companies are working on molecular diagnostic devices involving analysis of nucleic acids; at least 12 appear to be interested in the POC market (''Bantamweights vs. giants: Is a lab-on-a-chip brawl a mismatch?,'' *Small Times*, October 31, 2003).

While an exhaustive recounting of these projects is beyond the scope of this entry, some of the more interesting platforms are worthy of mention.

Clinical Microsensors (CMS), a division of Motorola, was founded in 1995 to exploit technology, developed by researchers at the California Institute of Technology, for ''bioelectronic'' detection of nucleic acid hybridization events. In late 1998 CMS had produced a prototype handheld instrument, powered by a 9-V battery, that could accommodate a ''DNA chip'' fabricated to contain 14 gold electrodes with DNA probes attached to their surface. The device promised a degree of sensitivity sufficient to detect DNA in wastewater, sludge, and blood, all very challenging sample matrices.[13] In 2000 CMS unveiled a desktop instrument, the 4800 eSensor™, which is now in commercial release; this can assay from 1 to 48 eSensor chips at one time. According to a December 2003 conversation with Motorola scientist Tim Tiemann, development of a handheld analyzer that would provide onboard sample processing and nucleic acid purification, as well as incorporating eSensor-based detection technology, is actively ongoing.

Fig. 4 Image of a prototype of Nanosphere's Verigene Mobile device, which will allow the user to perform sample processing and nucleic acid detection via hybridization assay in one handheld instrument suitable for the clinical diagnostic market. Inset: the company currently markets the portable Verigene ID system, which provides detection of nucleic acids via a proprietary, nanoparticle-mediated hybridization chemistry. (Photo courtesy of Jamie Abrams, Nanosphere, Northbrook, IL.) (*View this art in color at www.dekker.com.*)

One company has begun production (in June 2003) of a handheld nucleic acid analyzer suitable for use in a clinical diagnostic setting; this is the Verigene™ ID instrument (Fig. 4) which exploits the nanoparticle detection method developed in the laboratory of Chad Mirkin at Northwestern University, Illinois.[14] The initial Verigene ID assay is for detection of SNPs associated with coagulation disorders; future assays are planned for infectious agents and other clinical parameters.

Other firms developing POC diagnostic platforms based on array-mediated detection of nucleic acids include Nanogen, Affymetrix, and Caliper Technologies; all of which have experience in the fabrication of large, benchtop instruments. In addition to clinical tests, it is anticipated that handheld devices will also be useful for other situations in which molecular detection is particularly helpful, such as in monitoring crops and agricultural products for the presence of genetically modified organisms (GMOs); testing of environmental samples for the presence of viral, bacterial, fungal, and protozoal pathogens; and analysis of food for the presence of both pathogenic and spoilage-associated microorganisms.

For the devices mentioned above, the chemistry mediating detection of the nucleic acids of interest may or may not involve PCR and nucleic acid hybridization; for example, NuGen Technologies, Inc., (San Carlos, CA) is relying on a proprietary method called exponential single primer isothermal amplification which may allow for detection of very low quantities of target nucleic acids and can be adapted for use in microarray or microfluidic formats. Another interesting amplification chemistry is the loop-mediated isothermal amplification (LAMP), in which *Bst* DNA polymerase is used to mediate autocycling strand displacement DNA synthesis.[15]

COMBINING SAMPLE PREPARATION AND ANALYSIS IN ONE PLATFORM

The ideal handheld nucleic acid-based analysis device would incorporate both sample processing and real-time analysis and detection in one instrument. The technical challenges are formidable but some progress in fabricating such a device is underway. Nanosphere's proposed Verigene Mobile instrument (Fig. 4) promises to combine microfluidic purification of the sample and subsequent nucleic acid detection via hybridization with probe-bound nanoparticles, all in one handheld device. The Verigene Mobile will be a dedicated POC instrument suitable for use by personnel who do not have intensive training in molecular biology-based assays; the sample will be deposited into a cartridge which is in turn inserted into

the device for automated processing, with results presented to the user in real time.

CONCLUSION

By Winter of 2004 at least three handheld nucleic acid analyzers—the Smiths Detection BioSeeq, the Idaho Technology, Inc. Razor, and the Nanosphere Verigene—will be available for use. The first two instruments will rely on PCR-mediated amplification of the DNA or RNA template, whereas the Verigene relies on its proprietary nanoparticle-mediated hybridization format to detect target nucleic acids. Other devices that promise the same degree of portability may become available in 2004 and offer the end-user the ability to extract DNA or RNA from a variety of sample matrices and volumes. With continuous advances in highly active fields of research such as microfluidic systems, micro-total analysis systems (μTAS), nanotechnology-based ''lab on a chip'' devices, and microelectromechanical systems (MEMS), the future looks very exciting for the advent of novel handheld molecular biology-based devices suitable for use in the field and at the point of care.

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Haploid Analysis (Monosomal Hybrid Technique)

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Robert E. Pyatt
Emory University, Atlanta, Georgia, U.S.A.

INTRODUCTION

With the excitement and knowledge gained from the completion of the Human Genome Project, our understanding of underlying genetic determinants in monogenic and complex diseases promises to exponentially increase over the coming years. These developments should also prove to be a boon for the field of molecular diagnostics and improve our ability to diagnose and treat genetic diseases. A number of technologies, which are routinely applied toward mutation detection in the clinical laboratory setting including single-strand conformation polymorphism, denaturing gradient gel electrophoresis, denaturing high-performance liquid chromatography, and direct sequence analysis, exist today. The sensitivity of mutation detection in all of these methods is hampered by the presence of the wild-type allele, which can obscure the mutant, making it difficult to differentiate between the two. Monosomal cell hybrids can be created through the disruption of cell membranes between two cells in contact, which leads to the fusion of the membranes and the formation of a hybrid cell. When hybrids are formed between human and rodent cells, the resulting progeny will retain a random assortment of human chromosomes, which can be specifically selected for. By utilizing hybrids that are monosomal for any specific human chromosome in diagnostic testing, the sensitivity of these existing techniques can be improved by allowing each chromosome to be analyzed independently.

MONOSOMAL HYBRID TECHNIQUE

Figure 1 depicts the basic technique used for monosomal hybrid generation.[1–3] Donor cells, or human cells in which the separation of chromosomes to a haploid state is sought, are either low-density peripheral blood cells or a transformed lymphoblastoid cell line. Rodent cell lines—typically mouse[3–9] or hamster[2,10–12] cell lines—that have been engineered to allow for the selection of human–rodent fusion progeny are commonly used as recipient cells. Both cell types are initially incubated together in a low volume to promote membrane-to-membrane contact between donor and recipient cells. After a short time, an agent is introduced, such as polyethylene glycol (PEG)[1,2,6,7,10–12] or electricity,[3–5,8,9] which disrupts cell membrane integrity and promotes fusion. Cells are then washed and allowed to recover from this traumatic treatment for 1–2 days in normal culture media before selective conditions are applied.

Only a small percentage of cells will stably combine into donor–recipient progeny after fusion treatment and the remainder of unfused cells must be eliminated to prevent culture overgrowth. Although many different rodent cell lines are available as recipients, most base their methods of selection on similar biochemical principles. Nucleotides can be synthesized either from basic building blocks by the de novo pathway, or by recycling of preformed bases through the salvage pathway. In the salvage pathway, the enzyme hypoxanthine–guanine phosphoribosyl transferase (HPRT) catalyzes the addition of hypoxanthine or guanine to 5-phosphoribosyl-α-pyrophosphate, forming inosine or guanosine monophosphate, respectively. In recipient cell lines mutated for this enzyme, the salvage pathway is blocked and renders them dependent on the de novo one for growth.[2–12] Alternatively, pyrimidine salvage can also be blocked by mutating the thymidine kinase (*TK*) gene, which blocks the phosphorylation of thymidine, leading to the formation of thymidine monophosphate.[1]

After fusion, cultures are shifted to a medium containing hypoxanthine, aminopterin, and thymidine (HAT). Aminopterin is a competitive inhibitor of dihydrofolate reductase, which blocks the synthesis of tetrahydrofolate and, consequently, the de novo synthesis of purines. With both synthesis pathways blocked, unfused recipient cells are eliminated, whereas fusion daughter cells can use hypoxanthine and aminopterin in the salvage pathway if they have successfully retained the pertinent genetic material from the human donor cells. Because the gene for HPRT is located on the X chromosome and the *TK* gene is located on chromosome 17, these methods select directly for progeny containing one or more of these chromosomes, and indirectly for all other human chromosomes retained along with them. Hybrids containing chromosome 2 can be directly selected for using recipient hamster cells in which pyrimidine de novo synthesis is blocked and growth without uridine is impaired.[2] Although other strategies for selection exist, such as temperature-specific defects in protein synthesis,[2] selection systems based

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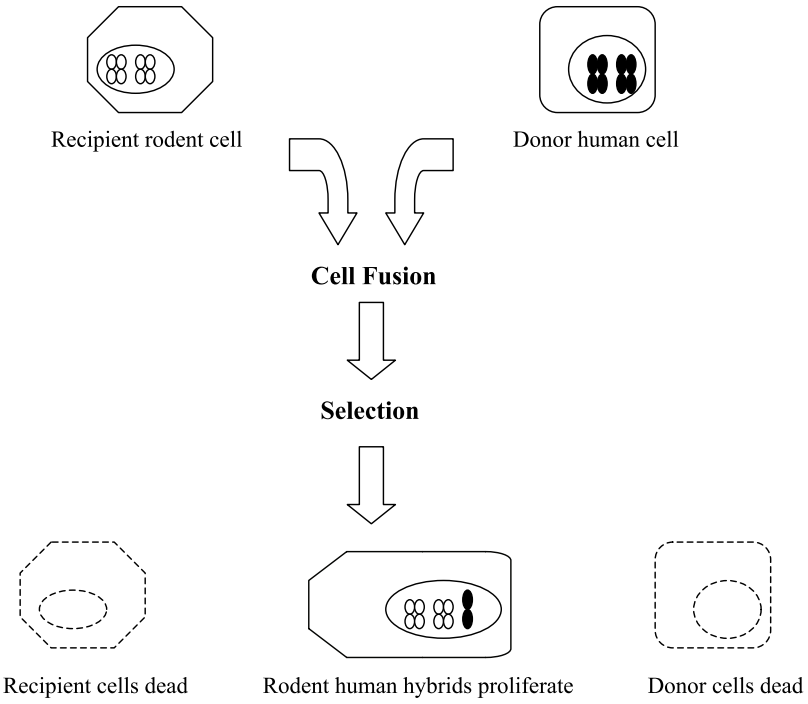

Fig. 1 Procedure for the creation of monosomal human–rodent hybrids.

on pathways of nucleotide synthesis are, by far, the most common.

Unfused human donor cells must also be removed from hybrid cultures. Chemicals such as oubain, a disruptor of sodium–potassium pumps,[1] or geneticin, an aminoglycoside related to gentamicin, can be used because human cells are sensitive to their actions.[3] However, the most common strategy exploits the adherent growth characteristics of recipient cells. Lymphoblasts and lymphoblastoid cell lines grow in suspension, but fusion progeny will retain the adherent quality of their recipient parents. Unfused donor cells can then simply be removed from cultures by repeated washes.[7,10–12]

After the specific selection criterion is applied, fusion progeny then proliferate to form colonies that are isolated and characterized for their specific human chromosomal complement. During the process of cell culture following fusion, hybrids will eliminate human chromosomes not directly required in the selection process. Over time, human chromosome retention becomes more stable and hybrids can be passaged without further losses.[2] This then has the effect of creating a series of hybrids containing the chromosome directly selected for along with a random assortment of other human chromosomes. Panels of highly polymorphic microsatellites or cytogenetic probes can be then employed to determine the number and allele of each chromosome within a particular hybrid clone. Figure 2 shows such a strategy employed for the analysis of a series of hybrids derived from the fusion of a lymphoblastoid cell line from an individual with trisomy 21 and Ade-C Chinese hamster ovary cells.[10–12] The recipient cells are deficient for the glycinamide ribonucleotide formyl transferase, an enzyme located on human chromosome 21 that blocks purine de novo synthesis. Hybrids containing human chromosome 21 can then be directly selected for by culture in purine-free media.[10–12] Multiple microsatellites are often analyzed to identify markers that are heterozygous and can, therefore, be used to distinguish each individual chromosome in a hybrid series. The original trisomic donor for the series of hybrids shown in Fig. 2 possessed three alleles of 166, 170, and 172 at the dinucleotide microsatellite D21S215 and, consequently, this marker was informative for chromosome 21 content in each hybrid. Although the direct selection methods eliminate nonfused recipient cells and any fusion progeny not containing at least one copy of the selected chromosome, hybrids can still retain more than one copy of that chromosome. In this series, fusion resulted in one hybrid clone retaining one copy of donor chromosome 21 (Fig. 2, row D), one hybrid with two copies of chromosome 21 (Fig. 2, row C), and a third hybrid retaining the complete trisomic set from the donor (Fig. 2, row B). Because the utility of fusion hybrids lies

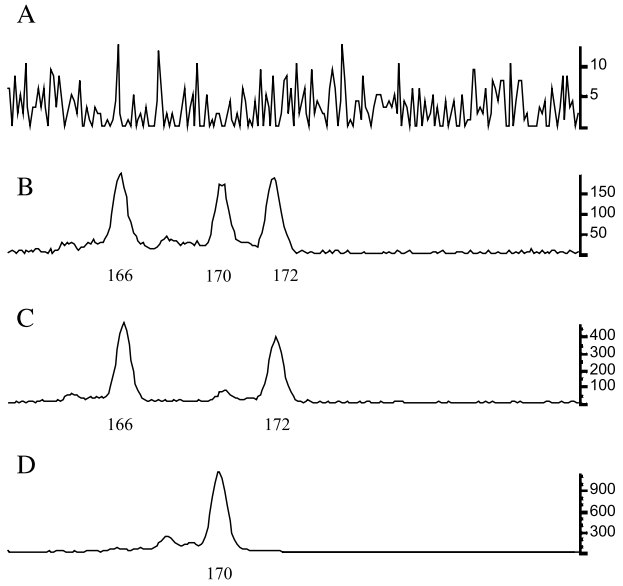

Fig. 2 Genotype analysis of hybrids at the microsatellite D21S215. DNA from a series of human–hamster hybrids (rows B–D) and the hamster recipient cell line (row A) were amplified at this dinucleotide marker using fluorescent-labeled primers to identify the human chromosome 21 content in each hybrid.

in their retention of a single copy of one human chromosome, the characterization of all hybrids must be conducted in an exhaustive manner to accurately define appropriate monosomal substrates for further genetic analysis. This is in addition to the random assortment of other human chromosomes retained along with the selected chromosome, which should also be defined.

UTILITY OF HYBRIDS IN DIAGNOSTIC TESTING

Hybrids are of greatest utility in diagnostic testing when traditional mutation detection methodologies have been ineffective or have proven inconclusive. Recently, the Molecular Pathology Laboratory at Ohio State University described the use of monosomal hybrids in the identification of large genomic alterations in individuals with hereditary nonpolyposis colorectal cancer (HNPCC).[8] Hereditary nonpolyposis colorectal cancer is an autosomal dominant cancer syndrome characterized by an increased risk for colorectal tumors and cancers at a distinct set of other anatomic sites including the stomach and endometrium.[13] The pedigree examined included two first-degree relatives—a deceased proband and her mother—with multiple instances of cancer, including colorectal cancer and many of the extracolonic tumors associated with the HNPCC cancer spectrum. The colorectal tumor from the proband exhibited microsatellite instability, which suggested an underlying defect in mismatch repair; however, direct genomic sequencing of the mismatch repair genes *MSH2*, *MLH1*, and *MSH6* failed to identify a pathologic mutation. Altered Southern blot hybridization patterns for the *MSH2* gene were observed for multiple restriction enzyme digestions of genomic DNA from both individuals compared with normal controls. This suggested the presence of a large genomic deletion in the 5′ portion of that gene, but attempts to define the deletion

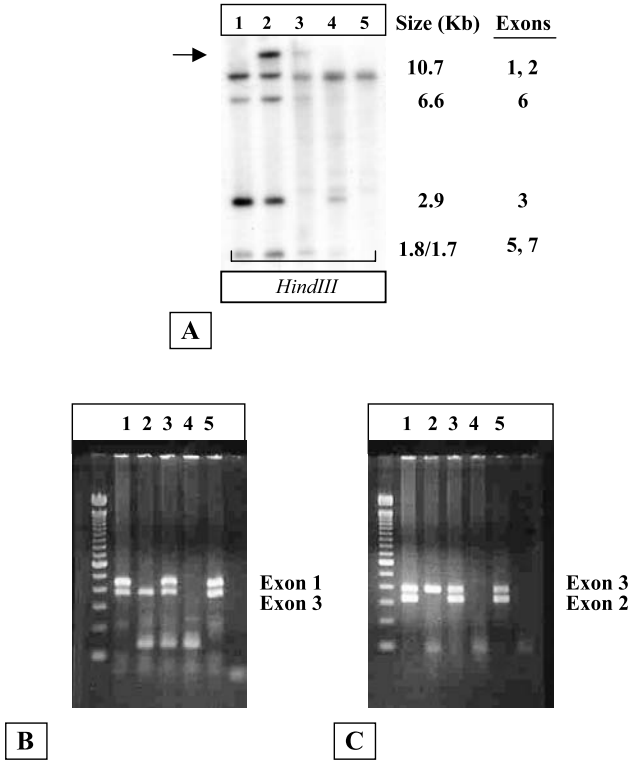

Fig. 3 Southern blot analysis and multiplex PCR reactions of mouse–human chromosome 2 monosomal cell hybrids. *Hin*dIII digests of a normal control (A; lane 1), proband (A; lane 2), two monosomal cell hybrids with one allele each of MSH2 (A; lanes 3 and 4), and the mouse fusion recipient cell line (A; lane 5) were hybridized with the same *MSH2* cDNA probe. The size of each fragment is presented in kilobases (kb) along with the corresponding exons, and the arrow illustrates a new fragment seen in digests of affected family members. Multiplex PCR reactions using primers to either exons 1 and 3 (B) or exons 2 and 3 (C) were then conducted with normal controls (B and C; lanes 1 and 5); the monosomal cell hybrid with the unique *Hin*dIII hybridization pattern (B and C; lane 2); the monosomal cell hybrid with the wild-type pattern (B and C; lane 3); and the mouse fusion recipient cell line (B and C; lane 4). (From Ref. [8]. Blackwell Publishing.)

breakpoints by long-range polymerase chain reaction (PCR) using genomic DNA proved inconclusive.

Hybrids monosomal for chromosome 2 were then created using lymphocytes from the mother of the proband to directly examine each allele of *MSH2*. Southern blots were repeated, and although one allele of *MSH2* from the donor demonstrated the wild-type hybridization pattern (Fig. 3A, lane 4) also seen in normal controls (Fig. 3A, lane 1), the second *MSH2* allele (Fig. 3A, lane 3) presented the same new fragment of approximately 11 kb as seen in the genomic DNA from the proband (Fig. 3A, lane 2). *MSH2* exon-specific PCR reactions for exons 1 and 3 (Fig. 3B) and exons 2 and 3 (Fig. 3C) showed that the allele with the altered hybridization pattern failed to amplify at exons 1 and 2 (Fig. 3B and C, lane 2), whereas the allele with the wild-type pattern amplified at both exons (Fig. 3B and C, lane 3). With the further identification of the deletion breakpoints, carrier testing could then be conducted on siblings of the proband to determine their relative risk for tumor development.

The literature contains a number of other case reports where monosomal cell hybrids were utilized as successful templates for traditional mutation detection techniques. Hybrids have also been used to identify deletions and splice site alterations in mismatch repair genes by use of PCR, reverse transcription (RT) PCR, and direct sequencing in two series of HNPCC patients in which conventional mutation detection techniques had failed using genomic DNA.[3,4] Similar methodologies were also used to confirm homozygosity for a single nucleotide substitution in the *CSA* gene in a patient with autosomal recessive Cockayne syndrome.[6] Hybrids have been used in cytogenetic cases to identify the translocation breakpoints in a patient with multiple myeloma using chromosome painting[7] and the parent of origin for a Robertsonian translocation involving chromosome 21 in an individual with Down syndrome by fluorescence in situ hybridization.[10] Finally, monosomal hybrids were used to demonstrate the presence of two SMN1 copies on one chromosome in a carrier of autosomal recessive proximal spinal muscular atrophy (SMA).[9] Molecular diagnosis of SMA carriers is typically performed through a quantitative PCR assay for SMN1 copy number,[14] and an individual with two SMN1 copies on one chromosome and none on the second chromosome would appear normal by the dosage assay but would be a carrier in reality.[9]

CONCLUSION

Although hybrids have demonstrated great potential in enhancing the sensitivity of existing mutation detection strategies, their conventional use in diagnostic laboratories has failed to fully materialize. This is due primarily to the time required for the fusion protocol to yield sufficient genetic material for use in molecular or cytogenetic assays and the high cost of the procedure. The HNPCC case described above required almost a year for the generation of sufficient DNA from hybrids for Southern blotting and PCR, which included two independent blood draws from the donor after the first fusion attempt failed. Cell culture is itself an expensive practice, and both the need to culture a large number of fusion clones to ensure the capture of each chromosome of interest and the duration of culture needed to amplify a hybrid from a single cell greatly increase the cost of this technique. The development of specific selection techniques would allow the direct capture of every human chromosome and reduce the total number of hybrids that are collected and analyzed, but this technique will always be somewhat hindered by biological limitations of cell culture.

Because the genetic material from hybrids is destined for molecular or cytogenetic analyses, there is always some concern that cross-reactivity of human PCR primers or cytogenetic probes with recipient DNA could complicate the interpretation of diagnostic results. The human *MSH2* probe hybridized to the blot in Fig. 3 demonstrates specific hybridization to a fragment of 10.7 kb from the mouse recipient cells, which corresponds to the fragment containing exons 1 and 2 in humans. Because the *MSH2* allele in lane 3 presents with a new restriction fragment due to the deletion of exons 1 and 2, the 10.7-kb fragment corresponding to those sequences should be missing, clearly confirming their deletion. However, because the human probe cross-hybridizes with the murine genomic DNA in this area, confirmation of the deletion had to be conducted using highly specific PCR primers, as shown in Fig. 3B and C.

Matsui et al.[15] have evaluated the cross-hybridization of human spectral karyotyping probes to mouse chromosomes, and although no specific hybridization was observed, they noted a weak, generalized background of staining over the mouse material. Consequently, further evaluation of cross-reactivity between cytogenetic and molecular probes and both mouse and hamster genetic materials is suggested. The use of recipient-only controls in all diagnostic assays is a must. Additionally, the possibility of interspecies chromosomal rearrangements in hybrids and the subsequent effects on results from diagnostic assays must also be considered in each case.[1]

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HBV Quantification

Annemarie Berger
J.W. Goethe University, Frankfurt, Germany

INTRODUCTION

For hepatitis B virus (HBV), which is not cultivable in vitro, serological assays permit an accurate diagnosis and follow-up of acute or chronic infection.

Qualitative serological assays for the detection of HBV antigens (HBsAg and HBeAg) and antibodies (anti-HBs, total and IgM anti-HBc, and anti-HBe) are also widely available and standardized.[1]

While active HBV infection is defined by the presence of hepatitis B virus surface antigen (HBsAg) in plasma, the secretory version of HBV core protein, HBeAg, serves as a marker for viral replication and can easily be detected by enzyme immunoassay (EIA). However, the absence of HBeAg in serum or plasma does not exclude viral replication.[2,3] Reasons may be the relatively low sensitivity of current HBeAg tests or mutations in the precore region of the HBV core gene.[4] Nevertheless, there is a strong correlation between the detection of HBeAg and the HBV DNA level.[3]

OVERVIEW

Attempts have been made to use the quantitative determination of HBeAg for the monitoring of antiviral therapy. In patients who respond to interferon-α therapy, a decline in the HBeAg concentration of more than 90% is observed by week 12 of therapy, whereas nonresponders do not demonstrate such important declines in HBeAg levels.[5] However, the clearance of HBV DNA from serum may precede the disappearance of HBeAg by several months.[6]

Hepatitis B virus DNA quantification is used for monitoring of antiviral therapy, for determination of infectiosity, and, in combination with serological markers, for the resolution of unusual profiles, i.e., isolated anti-HBc reactivity, as well as for cases in which the presence of HBV mutants is suspected.

Methods for the detection of viral genome (nucleic acid testing, NAT) have considerably extended the diagnostic repertoire of virological laboratories (VL) in recent years, proving superior to conventional techniques in many circumstances. In addition to qualitative analysis, quantitative NAT—initially used mainly for research purposes—has now firmly established itself in routine diagnostic virology.[7–9]

TEST METHODS

Molecular biology-based assays are invaluable tools for the management of chronic viral hepatitis. Assays employing target—namely, polymerase chain reaction (PCR) and nucleic acid sequence-based amplification (NASBA, TMA)—or signal amplification techniques—branched chain signal amplification (bDNA) and the hybrid capture system (HCS)—are now commercially available for the quantification of viral burden in infected patients (Table 1). Different assays have different sensitivities and ranges of linearity. More recently, ''real-time'' PCR techniques have been developed. The principle is to detect amplicon synthesis and to deduce the amount of viral genomes in the starting clinical samples during, rather than at, the end of the PCR reaction. These methods are theoretically more sensitive than classical target amplification techniques and are not prone to carryover contamination. Their dynamic range of quantification is consistently wider, making them particularly useful for quantifying the full range of viral loads observed in untreated and treated patients.

Whatever the technique used, software is used to calculate the threshold cycle (CT in ABI Prism, Cp in LightCycler) in each reaction with which there is a linear relationship with the initial amount of DNA. In each run, parallel processing of a panel of quantified standards is used to establish a standard curve for quantification (for further review, see Ref. [10]).

MANAGEMENT OF HBV-INFECTED PATIENTS

No molecular biology-based assays are necessary for the diagnosis of acute hepatitis B, which is based on serological testing. Chronic hepatitis B is defined by HBsAg persistence in serum for more than 6 months. In this setting, HBV DNA detection–quantification is necessary to determine whether or not HBV is replicating.

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Table 1 Commercially available HBV DNA quantification assays

Assay	Test principle	Quantification	Dynamic range
HBV Hybrid Capture II (Digene, Gaithersburg, MD)	Hybridization with RNA probes, detection after signal amplification with multiple conjugated antibodies	Comparison of the chemiluminescence emission with a standard curve generated simultaneously	1.4×10^5–1.7×10^9 copies/mL Ultrasensitive version: 4.7–5.7 $\times 10^7$ copies/mL
Versant HBV DNA (Bayer, Ludwigshafen, Germany)	Hybridization with capture and extender probes and preamplifier probes. Detection after signal amplification with branched DNA (bDNA) probes and conjugated probes	Comparison of the chemiluminescence emission with a standard curve generated simultaneously	Version 2.0: 7×10^5–5.7×10^9 copies/mL Version 3.0: 1.5×10^3–1×10^8 copies/mL 350–1.7 10^7 IU/mL
Cobas Amplicor HBV Monitor (Roche, Basel, Switzerland)	PCR-based target amplification, coamplification with an IS, WT, and IS-specific hybridization of the amplified products. Quantification is based on calculation of the OD values of IS and WT	Calculation of the copy amount in comparison of the OD values of WT and IS	300–200,000 copies/mL 52–3.4 $\times 10^4$ IU/mL
Cobas Taqman HBV (Roche)	Real-time PCR-based target amplification. Coamplification with an IS. Simultaneous detection of the fluorescence signals with specific labeled probes during the amplification	Comparison of the WT signal to the IS signal	Sensitivity: 6 IU/mL Range: 30–1.1 $\times 10^8$ IU/mL 35–6.4 $\times 10^8$ copies/mL
RealArt HBV TM PCR (Artus, Hamburg, Germany)	Real-time PCR-based target amplification. Coamplification with an internal control (=amplification control). Simultaneous detection of the fluorescence signals with specific labeled probes during the amplification	Comparison of the WT signal with a standard curve generated simultaneously	0.4–4 $\times 10^8$ IU/mL

IS = internal standard; WT = wild-type.

In the presence of HBeAg, the diagnosis of replicating chronic hepatitis B can be made whatever the viral load. Chronic hepatitis due to precore HBV mutants presents as hepatitis B e antigen (HBeAg)-negative chronic hepatitis B with generally lower replication levels than HBeAg-positive patients. HBeAg-negative chronic hepatitis B (CHB) represents a late phase in the natural course of chronic HBV infection that develops after HBeAg loss and seroconversion to anti-HBe. It is usually associated with mutations in the precore region of the HBV C gene inducing a stop codon that inhibits the production of HBeAg.[11] The diagnosis of HBeAg-negative CHB is based on HBsAg positivity, HBeAg negativity, and mainly on increased aminotransferases (ALT) and serum HBV DNA levels. Although the cut-off level of serum HBV DNA has not been definitely determined.[12] It has been suggested that an HBV DNA load of less than 10^5 copies/mL is associated with an ''inactive carrier state.''[13]

The inactive HBsAg carrier state is characterized by detectable HBsAg and anti-HBe in serum, undetectable HBeAg, low or undetectable levels of HBV DNA, normal ALT, and minimal or no necroinflammation; although inactive cirrhosis may be present if transition to an inactive carrier state occurred after many years of chronic hepatitis.[14,15]

Persistence of significant levels of viremia that are not detected by many hybridization assays of low sensitivity may be observed after anti-HBe seroconversion. The precise monitoring of viremia levels using more sensitive assays and the search for HBV mutant strains are warranted in patients undergoing antiviral therapy.

The correlation between HBV DNA serum levels and severity of liver disease is rather low. Therefore histological examination of liver biopsy material is still the best way of assessing the severity of chronic hepatitis B and establishing the prognosis. Nevertheless, active HBV replication is associated with a significant risk of

disease progression. This risk is low in the absence of detectable HBV DNA.[13]

The major issue is the value of serum HBV DNA quantification to assess the antiviral response to therapy and in monitoring disease progression. Serial quantitations of HBV DNA levels during antiviral therapy—although more cumbersome than HBeAg testing—permit the early identification of nonresponders, thus avoiding ineffective and expensive therapy.[6,16]

ESTIMATION OF INFECTIVITY

Another important reason for HBV viral load determination is the assessment of the infectivity of hepatitis B carriers. Without intervention, more than 90% of HBeAg-positive female chronic HBV carriers transmit the virus to their infants;[17] of these, 85–90% develop chronic HBV infection—in most cases asymptomatic—themselves, thus perpetuating the infection in high-endemicity settings. Immediate postpartum immunization of the infant can efficiently prevent transmission. It has to be considered that the level of viremia present in maternal serum can only be approximated with the HBeAg assay. Recent quantitative evaluation of sera for HBV DNA using molecular hybridization technology has shown that wide fluctuations in the concentration of virus exist in HBeAg-positive carriers. Vertical transmission is rarely documented with maternal HBV DNA levels below 10^7 geq/mL (5 pg/mL).[18,19] In a recent study, no cases of transplacental transmission of HBV were observed with maternal HBV DNA levels of 6.0×10^5 geq/mL or lower. In mothers with HBV DNA levels of $10^6/10^7$ and 10^8 geq/mL, 2% and 22% of the children, respectively, had HBsAg detected in the blood within 24 hr of birth (=transplacental transmission).[20]

HBeAg-positive physicians will not be allowed to exercise exposure-prone procedures. This is inadequate, as HBeAg-negative mutants of HBV may reach high titres. Hepatitis B virus transmission from four HBeAg negative surgeons to patients has been documented.[21] A more reliable estimate of the infectivity can be obtained by testing serum concentrations of HBV DNA.[22]

The European consensus panel[23] now proposes a maximum HBV DNA level of 10^4 geq/mL, but does not supply scientific data that provide support for proposing this level.[24]

CONCLUSION

The quantification of viral genome has, within a few years, become an integral part of the clinical management of patients suffering from infection with HBV. The modern molecular biological techniques have evolved from being elusive and research tools into widely used and, meanwhile, rather reliable virological routine diagnostic tools.

Besides providing prognostic information on individual cases, genome quantification plays a most important role during monitoring of the patient's response to antiviral treatment.

Further, hitherto less well-established uses of VL testing include the assessment of an individual's infectivity, be it in the health-care setting (transmission risk of blood-borne viruses) or in infected pregnant women.

Practical problems still waiting to be resolved are virus strain-related differences in quantitative results and a relatively low degree of intra- and interassay as well as interlaboratory reproducibility. The establishment of internationally accepted standards against which all working reagents could be calibrated, using a common standard unit of measurement, IU, would overcome this major problem.[25] Further work is required to fully standardize assays and quantification units, improve automation, and better define clinically relevant thresholds.

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HCV Genotyping

Alexander C. Haushofer
General Hospital of St. Pöelten, St. Pöelten, Lower Austria, Austria

Harald H. Kessler
Medical University Graz, Graz, Austria

INTRODUCTION

Hepatitis C virus (HCV) is a small, enveloped Flavivirus with a positive strand ribonucleic acid (RNA) genome of approximately 9400 bases in length. The genome comprises one large open reading frame flanked by untranslated regions (UTRs), a highly conserved one at the 5′ end, and another one which shows high heterogeneity at the 3′ terminus. Substantial nucleotide sequence variations between various HCV isolates were found throughout the entire HCV genome. The genetic variation of HCV is thought to reflect sequence errors because of a nonproofreading RNA-dependent RNA polymerase. Many different but closely related HCV variants are thus generated within one HCV-infected individual called quasispecies.

OVERVIEW

Hepatitis C virus genotypes may differ by as much as 33% of their nucleotide sequence distributed over the entire viral genome. Different HCV isolates are distinguished based on their genetic similarity in genotypes, which further separate into subtypes. All HCV isolates fall into phylogenetically related clusters called subtypes. Subtypes can be classified into several major types (sequence similarities of 65–75%).[1] Eleven genotypes as well as more than 90 subtypes have been identified.[2,3] Despite a high degree of sequence conservation in the 5′UTRs, genotype-specific differences exist in this region. Based on sequence variations in the 5′UTR and the NS5 region, Simmonds et al.[4,5] proposed a classification of HCV types and subtypes confirmed by phylogenetic tree analysis, which is currently most often used. Therapeutical management of chronic HCV infection has been based on the HCV genotype as recommended in Consensus Statements published by the European Association of the Study of the Liver and the National Institutes of Health.[6,7]

Nucleotide sequencing of the entire HCV genome followed by composition of a phylogenetic tree has been considered as ''gold standard.''[8] This approach, however, is cumbersome and regarded as impractical for routine clinical laboratory settings.[9] The 5′UTR region has been shown to be more convenient for genotyping in routine diagnostic laboratories.[3] Moreover, this region is usually chosen as target sequence for detection of HCV RNA, thus allowing direct analysis of amplification products in a genotyping assay. The less conserved NS5B region, which contains a subtype-specific motif and which is standard for epidemiological applications, may also be chosen.[2] For serological (ELISA-based) typing methods, type-specific antigenic properties of several epitopes encoded by the NS4 and core regions are used.[10] The core and NS3 may offer additional possibilities for HCV genotyping; however, their high variability renders the respective methods less sensitive, especially if only one probe/subtype is used.[11]

METHODS FOR GENOTYPING

Hepatitis C virus genotypes can be determined in routine laboratory settings by direct methods based on molecular typing and by an indirect method based on serological typing[12–14] (Table 1).

Direct Methods

Sequencing

The TruGene™ HCV 5′NC Genotyping Kit. The TruGene™ HCV 5′NC Genotyping Kit (Bayer Healthcare Diagnostics, Berkeley, CA) is based on a semiautomated terminator cycle sequencing technique of PCR products from the 5′UTR region conveniently retrieved from the Cobas Amplicor™ HCV Test (Roche Molecular Systems, Pleasanton, CA) or the Cobas Amplicor™ HCV Monitor Test (Roche). For the sequencing procedure, the CLIP™ sequencing technique (Bayer), allowing both directions of the amplification product to be sequenced simultaneously within the same tube, is employed. Electrophoresis and data analysis are carried out with the automated

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Table 1 Methods for HCV genotyping

Test name	Manufacturer	Method	Gen. region	Routine lab
TruGene™ HCV 5′NC genotyping kit	Bayer	DM; terminator cycle sequencing	5′UTR	Yes
Celera HCV genotyping assay	Celera; distributed by Abbott	DM; real-time PCR	5′UTR	Yes
Inno-LiPA HCV II (Versant)	Innogenetics; distributed by Bayer	DM; reverse-hybridization	5′UTR	Yes
RFLP analysis	Home-brew	DM; restriction fragment length polymorphism	5′UTR	No
Gen-Eti-K DEIA	Sorin Biomedica	DM; PCR and DNA enzyme immunoassay (EIA)	Core	Yes
HTA/HMA-Heteroduplex tracking/mobility assay	Home-brew	DM; electrophoresis of hybridization products of RT-PCR products with ST-specific probes	Core/E1	No
SIA-3 or SIA-6	Murex Diagnostics	IM; serotyping	NS4	Yes
RIBA HCV SIA	Chiron Corporation	IM; serotyping	NS4 and core	Yes

Gen. region = genomic region of the HCV used for genotyping; Routine lab = suitable for routine diagnostic laboratory settings; 5′NC = 5′ noncoding region; DM = direct method based on molecular typing; 5′UTR = 5′ untranslated region; E1 = envelope 1; IM = indirect method; NS4 = nonstructural 4 region.

OpenGene™ DNA sequencing system (Bayer) that is combined with the automated Long-Read Tower™ for the separation of the sequenced fragments. Data are acquired with the GeneLibrarian™ module of the GeneObjects™ (Bayer) software.

Multiplex real-time polymerase chain reaction-based genotyping

The Celera HCV Genotyping assay (Celera Diagnostics, South San Francisco, CA; distributed by Abbott Laboratories, North Chicago, IL) is based on a multiplex real-time PCR technique. This assay is performed on the ABI Prism 7000, a real-time cycler (Applied Biosystems, Foster City, CA), and the analysis software used is SDS v1.01. Per sample, three reactions using three different fluorescent dyes for simultaneous detection are necessary: reaction 1 detects genotypes 1a and 1b; reaction 2 detects genotypes 2a, 2b, and 3; reaction 3 detects genotypes 4, 5, and 6.

Genotype-/subtype-specific hybridization

The Inno-LiPA HCV II (Versant; Innogenetics, Ghent, Belgium; distributed by Bayer) assay is based on the reverse-hybridization principle of RT-PCR products derived from the 5′UTR.[3] Biotinylated RT-PCR products (RT-PCR products from Cobas Amplicor™ HCV Test; Roche) are hybridized to a selection of 19 specific oligonucleotide probes immobilized on nitrocellulose membrane strips. The RT-PCR products hybridize to a probe matching the sequence of the isolate and allowing discrimination at the subtype level. HCV genotypes/subtypes are identified by comparing the pattern of positive (colored) lines with a special interpretation chart.

Restriction fragment length polymorphism

Sequences amplified by PCR from the 5′UTR are cleaved by restriction endonucleases for identification of different HCV genotypes/subtypes. Initially, enzymes *Hae*III–*Rsa*I and *Hin*fI–*Mva*I are employed for cutting the PCR fragments from the 5′UTR which is followed by cleavage using *Bst*U1 or *Scr*FI. HCV genotypes/subtypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5, and 6 can be identified.[15]

DNA enzyme immunoassay

The DNA enzyme immunoassay (DEIA) (Gen-Eti-K DEIA; Sorin Biomedica, Saluggia, Italy) is based on a combination of nested PCR (core region) and DNA enzyme immunoassay.[16] Amplification products are hybridized to type-specific oligonucleotides. The hybrids are detected by a standard ELISA technique which employs monoclonal antibodies reacting with double-stranded DNA.

Heteroduplex tracking assay

The heteroduplex tracking assay (HTA), also called heteroduplex mobility analysis (HMA), is based on the fact that the genetic relationship between isolates can be determined by the relative migration of the heteroduplex on gels.[17] This assay involves hybridization with probes from known HCV subtypes to RT-PCR products generated

from homologous sera or from unknown samples and agarose gel electrophoresis of hybridization products.[18,19] The formation of a heteroduplex band on a gel indicates genotype and subtype.

RT-PCR with genotype-specific primers

Okamoto et al.[20] described a polymerase chain reaction (PCR)-based assay using primers for the hepatitis C core gene. This assay is just able to identify subtypes 1a, 1b, 2a, and 2b. The primary PCR with consensus primers is followed by a second-round PCR with type-specific primers. HCV subtypes are determined by the size of the amplification products generated. The original assay has been modified by Widell et al.[21] by adding another second-round PCR for identification of genotype 3.

Indirect Method

Serotyping assay

The HCV serotyping assay (SIA-3 or SIA-6 Murex HCV Serotyping assay, Murex Diagnostics Ltd, Dartford, U.K.) is based on the detection of genotype-specific antibodies directed to epitopes encoded by the NS4 region of the HCV genome. Eight (SIA-3) or 24 (SIA-6) NS4-encoded genotype-specific branched peptides are used in a competitive way to discriminate between genotypes 1–3 and 1–6, respectively.[10] With the RIBA HCV SIA (Chiron Corporation, Emeryville, CA), eight serotype-specific HCV peptide antigens, five of which are from the NS4 region of HCV subtypes 1a, 1b, 2a, 2b, and 3, and three of which are from the core region of types 1 and 2, are immobilized. Genotyping is achieved by an algorithm.[22]

Comparison of hepatitis C virus genotyping methods

The Inno-LiPA HCV II (Innogenetics), probably the most frequently used assay, has been well characterized in earlier studies.[23,24] In comparative studies, the Inno-LiPA HCV II assay (Innogenetics) and the TruGene™ HCV 5′NC Genotyping Kit (Bayer) show concordant results on the genotype level in 100% and 99.5%, respectively.[25–28] The overall accuracy for the discrimination of subtypes, however, was found to be lower for both, the Inno-LiPA HCV II assay (Innogenetics) and the TruGene™ HCV 5′NC Genotyping Kit (Bayer) with 95.5% and 85.9%, respectively, when using the TruGene™ HCV 5′NC Genotyping Kit (Bayer) or the Inno-LiPA HCV II assay (Innogenetics) as reference test for the alternative assay.[27] As recently reported, the TruGene™ HCV 5′NC Genotyping Kit (Bayer) and the Inno-LiPA HCV II assay (Innogenetics) gave concordant results on the subtype level in 90.9% of samples.[29] Accuracies for the discrimination of subtypes were 76% and 91.9% for the TruGene™ HCV 5′NC Genotyping Kit (Bayer) and 74% and 85.8% for the Inno-LiPA HCV II assay (Innogenetics), respectively, in comparison to sequence analysis of the NS5B region.[25,26] Sandres-Sauné et al.[30] showed a concordance of 94%, regarding subtypes 1a or 1b, when comparing the TruGene™ HCV 5′NC Genotyping Kit (Bayer) and a noncommercial assay using the NS5B region. The insufficient sequence polymorphism of the 5′UTR has been suggested as the major problem preventing correct discrimination of HCV subtypes. For the discrimination of subtypes 1a and 1b, it was shown that the polymorphism at position 99 of the 5′UTR may not always be linked to subtype 1a or 1b sequences detected in the core region.[23] This was also demonstrated in two comparative studies which focused on the performance of the Inno-LiPA HCV II assay (Innogenetics) versus that of NS5B sequencing with a concordance of 80% and 94%, respectively.[30,31] This limitation is common to all HCV genotyping procedures based on the analysis of the 5′UTR.[25]

Mixed HCV infections with different HCV genotypes/subtypes may occur in patients with intravenous drug abuse, hemophilia, and those on hemodialysis. The Inno-LiPA HCV II assay (Innogenetics) shows a high sensitivity for detection of minor sequence variants[11] and may be useful for detection of mixed infections involving different HCV genotypes.[8,32] This assay was consistently able to detect as little as 1–2% of the viral population (minor genotype) compared with restriction fragment length polymorphism (RFLP) (which may detect 5–30%) in a recent study.[33]

The TruGene™ HCV 5′NC Genotyping Kit (Bayer) or sequencing of the NS5b region is only possible if the serum HCV level exceeds 600 IU/mL.[30] To ensure accurate results, especially for low HCV levels, optimal blood collection systems containing a liquid nucleic acid stabilizer capable of stabilization nucleic acids within the blood samples even at room temperature for at least 96 hr are recommended.[34] After centrifugation, aliquots should immediately be frozen at −70°C.

In a comparative study, the TruGene™ HCV 5′NC Genotyping Kit (Bayer) and the DEIA (Sorin Biomedica) showed concordant results on subtype level in 91%.[9] However, serological methods, while easier to perform, lack sensitivity and specificity in comparison to molecular assays.[10,13,14]

Restriction fragment length polymorphism analysis based on the 5′UTR may be more sensitive than HCV serotyping but less sensitive when compared with the Inno-LiPA HCV II assay (Innogenetics).[33,35]

CONCLUSION

Today, tests for HCV genotyping usually employ the 5′UTR. All of them have been more or less found to be reliable for genotyping in the routine laboratory setting. Incomplete or incorrect subtyping may be avoided by using less conserved parts of the HCV genome, such as the core or the NS5B region. Those inherent problems of the 5′UTR are, however, not crucial for clinical purposes because therapeutic implications are only based on genotype analysis.

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HCV Quantification

Alexander C. Haushofer
General Hospital St. Pöelten, St. Pöelten, Lower Austria, Austria

Harald H. Kessler
Medical University Graz, Graz, Austria

INTRODUCTION

HCV is a small, enveloped human flavivirus with a positive-strand ribonucleic acid (RNA) genome of approximately 9400 bases in length. The genome comprises one large open reading frame flanked by untranslated regions (UTRs), a highly conserved one at the 5′ end, and another one which shows high heterogeneity at the 3′ terminus; the secondary and tertiary structures are essentially preserved and have essentially regulatory functions.

Since the discovery of the hepatitis C virus (HCV), 70–80% of former posttransfusion or sporadic chronic non-A, non-B hepatitis cases have been determined as HCV-induced hepatitis. Acute HCV infection is often asymptomatic and approximately 70–80% of cases progress to chronic hepatitis. This may lead to cirrhosis in 20% of these patients, subsequent to hepatocellular carcinoma, which has an incidence of 1–4% per year. Factors associated with disease progression following HCV infection include viral genotype, alcohol consumption, and viral load.

OVERVIEW

High viral loads are associated with decreased rates of response to interferon therapy,[1] and a decrease in the HCV load during the early phase of treatment (2–12 weeks) has been shown to predict effective treatment responses.[2]

Molecular techniques can be labor intensive and time consuming with in-house methods.[3] In this review, currently used assays for HCV virus load testing based either on target amplification [the HCV (Cobas) Amplicor Monitor 2.0 test (Roche Diagnostic Systems, Branchburg, NJ); the HCV Superquant (National Genetics Institute (NGI), Culver City, CA); nucleic acid sequence-based amplification; the NASBA-QT test (Organon Teknika, Boxtel, The Netherlands); and real-time detection polymerase chain reaction (RTD-PCR) test applying 5′-nuclease PCR/TaqMan PCR (Perkin Elmer Corp./Applied Biosystems, Foster City, CA; Cobas TaqMan HCV Test, Roche Diagnostic Systems)], or on signal amplification [branched DNA (bDNA) assays; the VERSANT HCV RNA 3.0 assay (Bayer Diagnostics, Berkeley, CA)], or on immunological detection of free HCV core antigen [the trak-C test (Ortho-Clinical Diagnostics, Inc., Raritan, NJ)] are presented (Table 1).

General Considerations

Six key performance characteristics of quantitative analytical assays can have important effects on the result, use, and interpretation of these assays: linearity, accuracy, precision, tolerance limit, sensitivity, and specificity.[4]

QUANTITATIVE HCV ASSAYS

The Amplicor HCV Monitor 2.0 and Cobas Amplicor HCV Monitor 2.0 Test (Roche Diagnostic Systems)

Based on reverse transcription polymerase chain reaction (RT-PCR), the Amplicor HCV Monitor test (Roche Diagnostic Systems) was developed.[5] Recently, an improved version 2.0 was introduced, which achieved an equivalent quantitation of each genotype over the quantitative range (5×10^2 to 5×10^5 copies of RNA/mL).[6,7] HCV RNA is extracted from plasma by chaotropic salt and is then precipitated by isopropanol. Both RT and PCR of RNA are accomplished in one tube using the recombinant thermostable DNA polymerase (*r*Tth), which offers both reverse transcriptase activity, forming a cDNA from the RNA target sequence of the 5′ UTR, and polymerase activity for amplification of the cDNA under appropriate conditions. An internal quantification standard shows the same primer-binding sites as the target but with a changed internal sequence, which allows binding to special detection probes. This allows quantification after a series of dilutions at the end of the assay. To meet the needs of routine diagnostic laboratories, PCR amplification and detection of amplified

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Table 1 Commercial quantitative HCV assays

Test name	Manufacturer	Method	Linear range
(Cobas) Amplicor HCV Monitor 2.0 test	Roche Diagnostic Systems	MM; RT-PCR; Cobas Amplicor—semiautomated PCR	5×10^2 to 5×10^5 IU/mL; 6×10^2 to 7×10^5 IU/mL (with CE certification)
Superquant assay	NGI[a]	MM; multicycle RT-PCR; Southern blot	4×10 to 2.0×10^6 IU/mL
NASBA-QT test	Organon Teknika	MM; nucleic acid sequence-based amplification	5.5×10^2 to 5.5×10^6 IU/mL
Cobas TaqMan HCV Monitor	Roche Diagnostic Systems	MM; real-time homogeneous PCR, 5′–3′endonuclease activity of *Z05* polymerase; Cobas TaqMan 48 or 96	3×10 to 2×10^8 IU/mL; DL: 1×10^1 IU/mL
Versant HCV RNA 3.0 test	Bayer Diagnostics	MM; bDNA	3.2×10^3 to 40×10^6 copies/mL[b]
trak-C Test	Ortho-Clinical Diagnostics	IM: HCV core AG ELISA	DL: 50×10^4 IU/mL

MM = molecular method; IM = immunological method; IU = WHO international HCV RNA standard; DL = detection limit.
[a]Test only available through the National Genetics Institute.
[b]A total of 5.2 HCV RNA copies/IU.

products (Amplicor HCV Monitor 2.0) have been semiautomated with the Cobas Amplicor (Roche Diagnostic Systems).[8,9] AmpErase® (uracil-*N*-glycosylase, or UNG) is used, which destroys dUTP-containing amplicons from previous amplifications and provides safety from contamination of amplicons of previously amplified samples. In contrast to signal amplification techniques, the amplified target is quantified.

The Superquant Assay (NGI)

The HCV Superquant assay (NGI), which is based on a multicycle RT-PCR method with an internal control, followed by Southern blot detection for the quantification of HCV RNA, shows a relatively large dynamic range from approximately 40×10^1 to 2.0×10^6 IU/mL. A cDNA is produced from extracted HCV RNA. This cDNA is then amplified in four separate PCRs with inclining cycles. The PCR products are visualized by Southern blotting, and subsequent quantification is accomplished by densitometry, comparing the product band intensity of the wild-type HCV to the internal control product band intensity.[10] The Superquant assay utilizes robotic instruments for agarose gel electrophoresis, vacuum transfer Southern blot hybridization, and immunostaining.[11]

The NASBA-QT Test (Organon Teknika)

Like RT-PCR, in the NASBA-QT test (Organon Teknika), the target nucleic acid is first amplified and then the amplification products are quantified in comparison with three different synthetic internal standard RNA calibrators.[12] This isothermal amplification technique uses RNA, not DNA. Instead of a precipitation of RNA by alcohol used in RT-PCR, the extraction is performed by adherence of RNA to silica particles. The amplification of target RNA involves the coordinated activities of three enzymes—reverse transcriptase, RNase H, and T7 RNA polymerase—in four steps: extension, degradation, DNA synthesis, and cyclic RNA amplification. RNA calibrators are added in three concentrations to control efficiency in extraction and enzymatic amplification. Quantification results from the ratio of signals of the specimen HCV RNA divided by the signal of the appropriate internal calibrator.

The RTD-PCR Test (5′-Nuclease PCR or TaqMan PCR)

Morris et al.[13] described the application of a fluorogenic probe-based (TaqMan probes) PCR assay (TaqMan; Applied Biosystems) for the detection of HCV RNA in serum and plasma. This assay allows the direct detection of specific PCR products by monitoring the increase in fluorescence of a dye-labeled oligonucleotide probe. TaqMan PCR uses the 5′–3′ endonuclease activity of *Taq* DNA polymerase to digest a probe labeled with a fluorescent reporter and quencher dye, and inclining PCR cycles result in exponential amplification of PCR products and fluorescence activity. Takeuchi et al.[14] described the

establishment of a real-time detection system for real-time quantitative HCV PCR—the ABI PRISM 7000 instrument (Applied Biosystems). This instrument combines thermal cycling, fluorescent detection, and application-specific software in a single instrument.

Now, the Cobas TaqMan HCV test, a real-time homogenous quantitative PCR using TaqMan technology, and the Cobas TaqMan 48 or 96 analyzer are introduced for routine diagnostic laboratory. In this quantitative assay, the recombinant *Thermus* specie Z05 polymerase is used, which offers reverse transcriptase, polymerase, and endonuclease activity. Roche Diagnostic Systems states a detection limit of 10 IU/mL and a high dynamic range between 30×10^1 and 2×10^8 IU/mL.

The bDNA Test

Chiron Corporation has developed a bDNA signal amplification for viral load detection of hepatitis C.[15] The pelleted virus is lysed with proteolytic agents and detergents to release RNA and then is added to wells of a microwell plate. RNA is captured by hybridization with a set of specific synthetic oligonucleotide capture probes immobilized to the plate. A set of target probes hybridizes to both the viral RNA and the preamplifier probes (the backbone of the branched complex). The capture probes and the target probes bind to the 5′ UTR and core region of the HCV genome. The amplifier probe subsequently hybridizes to the preamplifier, forming a bDNA complex. By a series of hybridizations, multiple bDNA amplifier molecules and alkaline phosphatase-labeled probes are then hybridized to this immobilized complex. Detection is achieved by a chemiluminescent substrate. The emitted signal directly correlates to the amount of HCV RNA present.

The HCV Core Antigen ELISA—trak-C Test

Recently, as alternative to molecular quantitative detection of HCV load, a quantitative immunoassay for the measurement of total hepatitis C nucleocapsid core antigen—the trak-C test (Ortho-Clinical Diagnostics, Inc.)—in the presence or absence of antibodies to HCV in human serum or plasma was introduced.[16]

Comparison of Assays

In comparison with the HCV Superquant assay, only available through NGI and not accessible to other laboratories, quantification with the Cobas Amplicor HVC Monitor 2.0 test (Roche Diagnostic Systems), suitable for routine laboratory settings, has been shown to be linear up to viral loads of 8.5×10^5 IU/mL by appropriate dilution.[17] Results of both tests correlated significantly. Moreover, Konnick et al.[18] demonstrated a good agreement between the Cobas Amplicor HVC Monitor 2.0 (Roche Diagnostic Systems) and the Superquant (NGI) assay results.

Through some technical improvements in probe design and background reduction, bDNA Version 3.0, the Versant HCV RNA 3.0 test (Bayer Diagnostics), now shows a broader range of 5.2×10^2 to 8.3×10^6 IU/mL for quantification of HCV RNA[19] compared with the previous range of bDNA 2.0 (Chiron Corporation) of 2×10^5 to 5×10^7 copies/mL.[15] Later, the reportable range was further improved (3.2×10^3 to 40×10^6 copies/mL; conversion factor: 5.2 HCV RNA copies/IU). Beld et al.[19] found a good correlation for viral load (expressed in international units per milliliter) obtained from the Amplicor HCV Monitor 2.0 test (Roche Diagnostic Systems) and the Versant HCV RNA 3.0 test (Bayer Diagnostics).

Zanetti et al.[20] demonstrated a good correlation of the trak-C test (Ortho) with the Cobas Amplicor HCV Monitor 2.0 test (Roche Diagnostic Systems) except for HCV RNA levels $<5 \times 10^4$ IU/mL, where the trak-C test was negative due to a lower sensitivity compared to the Cobas Amplicor HCV Monitor 2.0 test (Roche Diagnostic Systems). An additional new quantitative assay based on PCR (the LC × HCV Quantitative test; Abbott, Chicago, IL) is being evaluated.

Sample preparation is currently thought to be the major weakness in the molecular detection of HCV RNA. Conventional sample preparation protocols are usually time consuming, labor intensive, and sensitive to contamination producing false-positive results. Therefore, besides ready-to-use sample preparation kits, automated specimen preparation systems such as the Cobas Ampliprep (Roche Diagnostic Systems) designed for Cobas Amplicor PCR and, in the future, for Cobas TaqMan 48 and 96 analyzer were developed and brought to the market. It could be demonstrated that quantitative test results were comparable between a combination of the Cobas Ampliprep with the Cobas Amplicor HCV Monitor 2.0 test (Roche Diagnostic Systems) and the Cobas Amplicor HCV Monitor 2.0 test (Roche Diagnostic Systems) alone within ± 0.5 $\log_{10}$.[21] Standardization for any commercially available assay or method is essential for providing reliable information for appropriate patient management, especially if, following the recommendations of EASL Consensus Panel,[22] a clinically relevant threshold of 2×10^6 copies/mL is used as a guideline for determining the outcome of treatment with interferon plus ribavirin in patients infected with genotypes 1, 4, and 5, or defining the duration of treatment. The NIH Consensus Statement[23] defined early viral response (EVR) as a minimum of 2 $\log_{10}$ decrease in viral load during the first

12 weeks of treatment, which is predictive of sustained viral response (SVR) and should be a routine part of monitoring patients with genotype 1.

CONCLUSION

Several well-documented assays are nowadays available for quantitation of HCV load. Standardization for any commercially available assay and, in the future, automated sample preparation systems are essential for providing reliable information. For an independent comparison of different assays, the World Health Organization (WHO) First International HCV RNA Standard has been introduced to permit normalization of reported viral titers in international units.[24] Shiffman et al.[25] demonstrated for the Amplicor HCV Monitor 2.0, the Superquant assay, and the Versant HCV RNA 3.0 test (Bayer Diagnostics), using the international unit standard, that approximately 90% of results was within 1 $\log_{10}$ unit. Participation in proficiency programs, such as Quality Control of Molecular Diagnostics (QCMD), should be mandatory in routine laboratory settings.[26]

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Helper-Dependent Adenoviral Vectors

Philip Ng
Baylor College of Medicine, Houston, Texas, U.S.A.

INTRODUCTION

The adenovirus (Ad) has been extensively exploited as a gene therapy vector because of its ability to efficiently transduce a wide variety of cell types from many different species independent of the cell cycle to direct high-level transgene expression. First-generation Ad vectors (FGAd) typically have the viral early region 1 (E1) replaced by the therapeutic transgene. First-generation Ad vectors are replication deficient and require E1 complementing cells for propagation. However, the majority of the Ad genome remains intact leading to low-level expression of viral genes in the transduced cells. This is directly cytotoxic and also leads to an adaptive cellular immune response against the transduced cells consequently resulting in transient transgene expression and long-term toxicity, thus rendering these vectors unsuitable for many gene therapy applications where long-term transgene expression is desired. In an attempt to further attenuate Ad, vectors have been engineered with deletions or mutations in the viral E2 and E4 genes in addition to deletion of E1. Despite the potential offered by these multiply deleted Ad vectors (also called second- or third-generation Ad vectors), the majority of the viral coding sequences still remain and therefore so does the potential for their expression. The advantages of multiply deleted Ad over FGAd remain controversial as some studies show them to be superior in terms of reduced toxicity and enhanced longevity of transgene expression whereas others do not. Significant improvement in the safety and efficacy of Ad-based vectors came with the development of helper-dependent adenoviral vectors (HDAds, also referred to as gutless, gutted, mini, fully deleted, high-capacity, Δ, pseudo) which are deleted of all viral coding sequences. Helper-dependent adenoviral vectors retain the advantages of FGAd including high efficiency in vivo transduction and high-level transgene expression. However, owing to the absence of viral gene expression in transduced cells, these HDAds are able to mediate high-level, long-term transgene expression in the absence of chronic toxicity. In addition, because the vector genomes exist episomally in transduced cells, the risks of germline transmission and insertional mutagenesis leading to oncogenic transformation are negligible. Moreover, the deletion of the viral sequences permits a tremendous cloning capacity of ~37 kb allowing for the delivery of whole genomic loci, multiple transgenes, and large *cis*-acting elements. A summary of the current state of HDAd is presented here and a more comprehensive discussion can be found elsewhere.[1] Discussions regarding Ad and early-generation Ad vectors can be found elsewhere in this volume.

PRODUCTION AND CHARACTERIZATION

Because HDAds are deleted of all viral coding sequences, a helper virus is required for their propagation. The first efficient and currently most widely used method for generating HDAd is the Cre/loxP system developed by Graham and coworkers in 1996[2] (Fig. 1). In this system the HDAd genome is first constructed in a bacterial plasmid. Minimally, the HDAd genome contains the expression cassette of interest and ~500 bp of *cis*-acting Ad sequences necessary for vector DNA replication (ITRs) and packaging (Ψ). Because efficient packaging into the Ad capsid requires a genome size between ~27.7 and ~38 kb, ''stuffer'' DNA is usually included in HDAd. To rescue the HDAd (to convert the ''plasmid form'' of the HDAd genome into the ''viral form''), the plasmid is first digested with the appropriate restriction enzyme to liberate the HDAd genome from the bacterial plasmid sequences. 293 cells expressing the site-specific recombinase Cre are then transfected with the linearized HDAd genome and subsequently infected with the helper virus. The helper virus bears a packaging signal flanked by loxP sites, the target sequence for Cre, and thus following infection of 293Cre cells, the packaging signal is excised from the helper viral genome by Cre-mediated site-specific recombination between the loxP sites. This renders the helper viral genome unpackagable but still able to undergo DNA replication and thus trans-complement the replication and encapsidation of the HDAd genome. The titer of the HDAd is increased by serial coinfection of 293Cre cells with the HDAd and the helper virus and the HDAd is finally purified by CsCl ultracentrifugation. Subsequent improvements of this original system permit efficient and simple large-scale HDAd production with yields of >10,000 vector particles per coinfected producer cell and with helper virus

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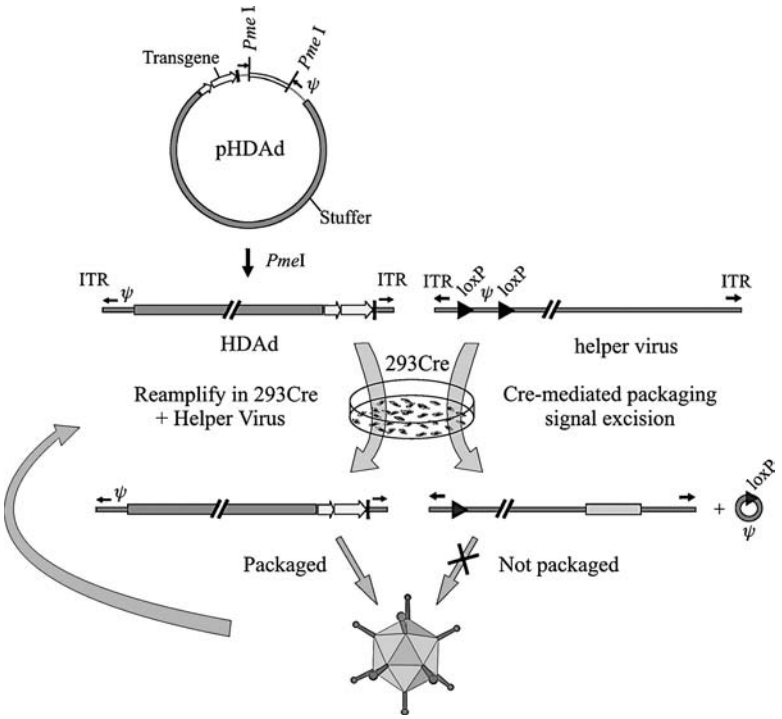

Fig. 1 The Cre/loxP system for generating HD vectors. The HDAd contains only ~500 bp of *cis*-acting Ad sequences required for DNA replication (ITRs) and packaging (ψ), the remainder of the genome consists of the desired transgene and non-Ad ''stuffer'' sequences. The HDAd genome is constructed as a bacterial plasmid (pHDAd) and is liberated by restriction enzyme digestion (e.g., *Pme*I). To rescue the HDAd, the liberated genome is transfected into 293Cre cells and infected with a helper virus bearing a packaging signal (ψ) flanked by loxP sites. Cre-mediated excision of ψ renders the helper virus genome unpackagable, but still able to replicate and provide all of the necessary *trans*-acting factors for propagation of the HDAd. The titer of the HD vector is increased by serial coinfections of 293Cre cells with the HDAd and the helper virus. (*View this art in color at www.dekker.com.*)

contamination levels of <0.02%.[3] Detailed methodologies for producing HDAd are described in detail elsewhere.[3,4]

IN VIVO APPLICATIONS

Liver Transduction

The liver is an attractive gene therapy target because the fenestrated endothelium permits exposure to intravenously delivered vector, hepatocytes are well suited for secretion of therapeutic proteins into the circulation for systemic delivery, and it is the affected organ in many genetic disorders. Helper-dependent adenoviral vectors are particularly attractive vectors for liver-directed gene therapy because of their ability to efficiency transduce hepatocytes following intravenous injection.

To evaluate the utility of liver-directed, HDAd-mediated gene therapy in a large animal model, three baboons were intravenously injected with 3.3 to 3.9×10^{11} vector particles per kilogram of a HDAd expressing human α_1-antitrypsin (hAAT).[5] Human α_1-antitrypsin antagonizes neutrophilic elastase and is abundantly expressed in hepatocytes and at a lower level in macrophages and α_1-antitrypsin-deficient patients have shortened life expectancies because of emphysema. Expression of hAAT persisted for more than 1 year in two of the three animals (Fig. 2). Maximum levels of serum hAAT of 3 to 4 μg/mL were reached 3 to 4 weeks postinjection in these two baboons and slowly declined to 8% and 19% of the highest levels after 24 and 16 months, respectively. The slow decline in hAAT expression was attributed to the fact that the baboons were young (7.5 and 9 months old) when injected, and that the decrease in hAAT concentrations was correlative to the growth of the animals. The third baboon had significantly lower levels of serum hAAT which rapidly declined to undetectable levels after 2 months. This baboon had generated anti-hAAT antibodies thus accounting for the low level and rapid loss of serum hAAT. No abnormalities in blood cell counts and chemistries were observed in these three baboons at any time, starting 3 days postinjection. In contrast, hAAT expression lasted only 3 to 5 months in all four baboons

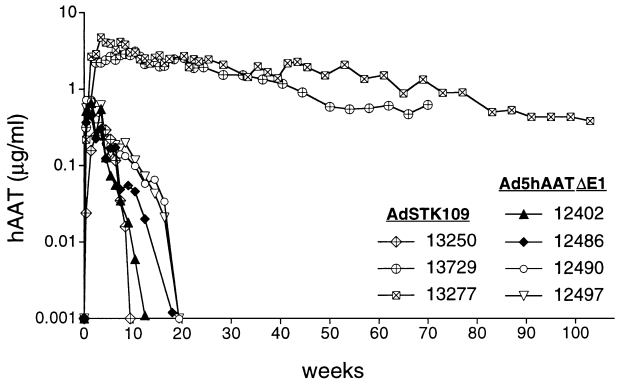

Fig. 2 Serum levels of hAAT in baboons following intravenous administration of the HDAd AdSTK109 or the FGAd AdhAATΔE1. Baboons 12402 and 12486 were injected with 6.2×10^{11} particles/kg of AdhAATΔE1. Baboons 12490 and 12497 were injected with 1.4×10^{12} particles/kg of AdhAATΔE1. Baboons 13250, 13729, and 13277 were injected with 3.3×10^{11}, 3.9×10^{11} and 3.6×10^{11} particles/kg, respectively, of AdSTK109.

injected with an FGAd expressing hAAT (Fig. 2). This was shown to be due to the generation of a cellular immune response against viral proteins expressed from the vector backbone of the FGAd resulting in the elimination of transduced hepatocytes. These experiments convincingly demonstrated that HDAds were superior to FGAd with respect to duration of transgene expression and hepatotoxicity in a nonhuman primate.

The potential of liver-directed, HDAd-mediated gene therapy was investigated for the phenotypic correction of hypercholesterolemia in the apolipoprotein E-deficient ($apoE^{-/-}$) mouse model.[6] Apolipoprotein E, a 34-kDa plasma glycoprotein, is a component of all plasma lipoproteins except low-density lipoprotein (LDL) and plays a major role in lipoprotein catabolism by acting as a ligand for the LDL-receptor (LDLR) and the LDLR-related protein for transport of excess cholesterol from the peripheral tissues to the liver for excretion. The $apoE^{-/-}$ mouse is an excellent model for cardiovascular disease because they develop severe hypercholesterolemia and atherosclerotic lesions similar to those found in humans. Chan and coworkers investigated correction of hypercholesterolemia in $apoE^{-/-}$ mice with either a FGAd or a HDAd expressing apoE.[6] Injection of $apoE^{-/-}$ mice with FGAd resulted in an immediate rise in plasma apoE levels and a concomitant drop in plasma cholesterol levels to within normal range. However, this effect was transient, as apoE levels rapidly declined to pretreatment levels by day 28 and plasma cholesterol levels increased after 28 days, returning to pretreatment levels by 112 days. In contrast, a single injection of HDAd resulted in immediate lowering of plasma cholesterol to subnormal or normal levels for the rest of the natural lifespan of the animal (2.5 years). Plasma apoE levels reached ~200% wild-type and remained at supraphysiological levels for >4 months, only to decline slowly to wild-type levels at 1 year and remained at 60–90% physiological concentrations for the lifetime of the animals (2.5 years). Analysis of total plasma cholesterol revealed normalization of the plasma lipoproteins back to a predominately HDL pattern seen in wild-type mice. Aortas in all mice, examined at 2.5 years after treatment with HDAd, were essentially free of atherosclerotic lesions in contrast to saline-injected mice whose aortas were completely covered with lesions. Significantly, this study demonstrated that a single injection of HDAd encoding apoE could confer lifetime protection against aortic atherosclerosis. Toxicity studies revealed that whereas injection of FGAd resulted in significant chronic hepatotoxicity, no such evidence of damage was observed following injection of HDAd. In summary, this study demonstrated the tremendous potential of HDAds for gene therapy; a single injection of HDAd resulted in lifelong expression of a therapeutic transgene and permanent phenotypic correction in a mouse model of a genetic disease without long-term toxicity.

Muscle Transduction

Duchenne muscular dystrophy (DMD) is a lethal, X-linked, degenerative muscle disease with a frequency of 1 in 3500 male births caused by mutations in the dystrophin gene. Dystrophin is an essential structural component of the skeletal muscle cell membrane, linking intracellular actin filaments with the dystrophin-associated proteins (DAPs) in the sarcolemma. Dystrophin deficiency results in instability of the muscle cell membrane causing muscle fiber degeneration. The length of the dystrophin cDNA (14 kb) precluded its inclusion into most gene therapy viral vectors but following the development of HDAds with large cloning capacity, gene transfer of the full-length dystrophin cDNA became feasible. Gilbert et al.[7] demonstrated that a single injection of a HDAd carrying two copies of the full-length human dystrophin cDNA resulted in transduction of 34% of the fibers of the total tibialis anterior (TA) muscle in neonatal *mdx* mice, a genetic and biochemical mouse model for DMD. The amount of dystrophin produced in these muscles was five times that in normal human muscle. However, only 7% transduction was achieved following injection into the TA muscle of adult *mdx* mice. In these transduced adult fibers, the amount of dystrophin produced was only 10% of the amount in normal humans. However, the high levels of transduction were transient and a humoral immune response was mounted against the foreign human dystrophin protein in the *mdx* mice. Importantly, such a response was not observed in immunodeficient SCID mice suggesting that sustained expression could be achieved in the absence of an immune response to the

transgene product. Indeed, injection of a HDAd expressing the full-length murine dystrophin cDNA into the tibialis anterior muscle of neonatal *mdx* mice resulted in sustained expression for at least 1 year in 52% of the muscle fibers. The treated muscle showed restored dystrophin–glycoprotein complex to the sarcolemma, significant improvement in isometric force production, resistance to high stress muscle contraction damage, and improved muscle histopathology.[8] This study demonstrates the tremendous potential of HDAd-mediated DMD gene therapy.

Brain Transduction

The brain is an attractive target for gene therapy to treat CNS structural and functional deficits such as aging-related memory loss, Parkinson's disease, Alzheimer's disease, or amyotrophic lateral sclerosis. Zou et al.[9] compared the efficiency, toxicity, and persistence of HDAd and FGAd vector-mediated gene transfer into the hippocampus or lateral ventricle of the brain in 20-month-old rats. Transgene expression peaked 6 days post-injection for both vectors. In the hippocampus, transgene expression from the FGAd decreased rapidly after day 6, being significantly lower than HDAd-injected hippocampus by day 16, and undetectable by day 183. In the ventricle, transgene expression from FGAd was significantly lower than HDAd by day 33 and undetectable by day 66. In contrast, transgene expression from the HDAd remained relatively stable with expression remaining >60% of peak levels on day 183. Overall, expression from HDAd was significantly higher than from FGAd at all time points after 6 days. First-generation Ad vectors induced substantial inflammatory and immune response which were significantly higher than those induced by HDAd at all time points after 3 hr. While both vectors induced a rapid increase in the proinflammatory cytokine that peaked at 3 hr postinjection, by 3 days, the level was significantly lower in the HDAd-treated brains than the FGAd-treated brains.

Lung Transduction

The lung is another attractive target for gene transfer primarily because of the desire to develop gene therapy for cystic fibrosis (CF). Cystic fibrosis is caused by recessive mutations in the CF transmembrane conductance regulatory (CFTR) gene which encodes a membrane chloride channel present in the epithelium of the lung and other organs. Morbidity and mortality in CF patients is due to lung disease characterized by inflammation, obstructive mucus, and persistent infection. Helper-dependent adenoviral vector efficiently transduces airway epithelial and submucosal cells in mice following intranasal administration, and in contrast to FGAd results in negligible inflammation, with transgene expression lasting for up to 15 weeks.[10] Moreover, intranasal administration of a HDAd bearing the CFTR gene resulted in expression of CFTR in the airway epithelial cells of CFTR-knockout mice and could protect the lung from bacterial challenge.[11] These studies suggest that HDAd-mediated CF gene therapy would benefit CF patients by reducing susceptibility to opportunistic pathogens.

ACUTE TOXICITY

In addition to long-term, chronic toxicity mediated by viral protein expression from the vector backbone, systemic administration of FGAd also results in acute toxicity. This acute toxicity occurs immediately following vector administration and is characterized by high-level inflammatory cytokine production, consistent with activation of an acute inflammatory response.[12–15] While the precise mechanism responsible for this acute response remains to be determined, it appears to be mediated by the viral capsid in a dose-dependent manner with potentially severe and lethal consequences. Indeed, the death of a partial OTC-deficient patient, whose clinical course was marked by systemic inflammatory response syndrome (SIRS), disseminated intravascular coagulation, and multiorgan failure, was attributed to acute toxicity from the administration of a second-generation (E1- and E4-deleted) Ad vector.[16] Because the viral capsid of early generation Ad and HDAd are identical, it has been hypothesized that HDAd would also provoke an identical acute response. Indeed, this was confirmed in nonhuman primates in which dose-dependent acute toxicity, consistent with activation of the innate inflammatory immune response, was observed following systemic administration of HDAd.[17] It is important to note that robust activation of the acute inflammatory response is also observed in mice given systemic Ad. However, unlike primates, lethal SIRS does not develop in rodents, even at high doses which are lethal to primates. This may reflect species-to-species differences in the quality of the innate immune response or sensitivities of the end organs to pathologic sequelae. This likely accounts for the plethora of studies reporting negligible toxicity in mice given high-dose HDAd and underscores the importance of safety and toxicity evaluations in larger animals for all gene therapy vectors.

CONCLUSION

Helper-dependent adenoviral vectors possess many characteristics that make them attractive vectors for gene therapy of a wide variety of genetic and acquired diseases. However, acute toxicity provoked by the viral capsid

currently hinders the clinical application of this otherwise promising technology. Clearly, studies to elucidate the mechanism(s) of Ad-mediated activation of the innate inflammatory response are needed. Perhaps with a clearer understanding of this phenomenon strategies can be developed to minimize, if not eliminate, this acute toxic response. If this can be accomplished, then HDAd should be able to provide sustained, high-level transgene expression with no further long-term toxicity.

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Hemoglobinopathies, Structural

H

Lewis P. Rubin
Women & Infants Hospital and Brown Medical School, Providence, Rhode Island, U.S.A.

INTRODUCTION

The hemoglobinopathies resulting from mutations in the α- or β-globin gene clusters are the most common single-gene disorders in humans. An estimated 7% of the world population carries one or more globin gene mutations[1] and over 300,000 persons each year are born with a severe hemoglobinopathy. Although most of the more than 700 identified hemoglobin variants are clinically inconsequential, several different single nucleotide substitutions lead to critical amino acid substitutions that cause hemolytic anemia, i.e., sickle cell disease (SCD), unstable hemoglobins, or hemoglobin tetramers that have altered oxygen affinity.

These variant hemoglobins have featured prominently in the development of molecular medicine and genomic and proteomic diagnostics. Pauling's investigations on hemoglobin in sickle cell anemia were the first demonstration of a causal link between an inherited alteration in protein molecular structure and a disease. In 1985, SCD also was the first genetic disease involving a point mutation in a single-copy gene to which diagnostic Southern blotting was applied, i.e., hybridization of genomic DNA with allele-specific oligonucleotide probes.

Heterozygosity for clinically significant hemoglobin variants (e.g., HbS, HbC, and HbE), as is the case for thalassemias and glucose-6-phosphate dehydrogenase (G6PD) deficiency, confers advantage in malarial endemic regions. This hypothesis was first formulated by Haldane, and the complex interrelationships among genetics, ecology, and culture on the frequency of sickle cell disease (SCD) were later established by the anthropologist Livingstone.[2] Subsequent research on genetic drift and gene flow supports the proposition that an introduction of agriculture several thousand years ago promoted malarial endemicity and exerted evolutionary selection on hemoglobinopathies.[3] In historic malarial belt high-risk populations, different globin chain abnormalities (hemoglobin variants and thalassemias) often interact to produce marked hemolytic anemia or red blood cell (RBC) sickling. Structural hemoglobinopathies that cause anemia also complicate pregnancy by increasing risk of intrauterine growth restriction, preterm birth, low birth weight, and perinatal mortality.[4]

OVERVIEW

Tetrameric hemoglobin consists of two different globin chain pairs, each containing one heme molecule. Balanced expression of α-globin and non-α-globin chains is necessary for normal hemoglobin synthesis and RBC function. During development, three embryonic hemoglobins are produced: Hb Gower-1 (ζ_2/ε_2), Hb Gower-2 (α_2/ε_2), and Hb Portland (α_2/γ_2). By week 8, fetal hemoglobin (HbF, α_2/γ_2) comprises at least 50% of the hemoglobin present. A δ- to β-globin chain switch by 12–16 weeks after birth results in a shift to the predominant adult hemoglobin, HbA (α_2/β_2). This phenomenon of postnatal hemoglobin switching precludes clinical consequences of β-globin chain variants before or at the time of birth. Individuals with clinically significant hemoglobin variants, and those that produce disease when coinherited with α- or β-thalassemias, tend to become symptomatic only after the decline in HbF levels after the first months.

Diagnostic testing for hemoglobinopathies generally is performed for clinical evaluation and population (newborn) screening. In couples considering pregnancy, pregnant women, or conceptuses, laboratory evaluation for hemoglobinopathies can confirm a provisional diagnosis, e.g., SCD, determine the cause(s) of a hematological abnormality, e.g., anemia or microcytosis, permit genetic counseling for prospective parents, or make a fetal diagnosis.

LABORATORY METHODS (PROTEIN AND DNA ANALYSIS)

The initial step in investigation of a suspected hemoglobinopathy is to obtain a complete blood count (CBC). Inspection of the blood smear red cell morphology is particularly useful in detecting an unstable hemoglobin, SCD, or thalassemia. A reticulocyte count is indicated whenever there is hemolysis, which may occur with unstable hemoglobins or SCD.

Proteomic diagnosis using electrophoretic separations is the foundation for analysis of hemoglobin variants. Presumptive diagnosis of an abnormal hemoglobin

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frequently can be made based on characteristic electrophoretic mobility and clinical setting. Nevertheless, each electrophoretic technique has distinct pitfalls. For routine clinical laboratory diagnosis, the most widely used method is alkaline (pH 8.6) cellulose acetate electrophoresis. It is capable of separating HbA, HbF, HbS, HbC, and several less common hemoglobin variants. However, similarity of overall surface charge causes comigration of several other hemoglobins. Of practical importance, HbE, HbA_2, HbC, HbE, and HbO are not well separated and HbS cannot be distinguished from the less important HbD or HbG. Identification of these hemoglobins is assisted by acidic (pH 6.2) citrate agar electrophoresis.

Thin gel isoelectric focusing (IEF) yields better resolution and quantification of certain hemoglobins. In IEF, hemoglobins migrate in a pH gradient to a position of zero net charge (isoelectric point). Because IEF is relatively simple, fast, and adaptable for automation, it has become the most common method used in mass newborn screening programs.

Increasingly, electrophoretic methods are being replaced or supplemented by high-performance liquid chromatography (HPLC) and capillary zone electrophoresis. Cation-exchange HPLC combines advantages of mobile phase pH and ionic strength gradients for quantitative separation. It is a method of choice for quantification of HbA_2 and HbF and identification of certain variant hemoglobins.[5] Appropriate column selection permits separation of more than 45 hemoglobin variants and detection of as little as 0.1% of total hemoglobin in 0.5 μL of whole blood.[6] Capillary zone electrophoretic methods are also sensitive, rapid, and highly selective.[7] Combination with IEF permits identification and quantification of HbS, HbC, HbE, HbO-Arab, and HbD-Punjab.

Careful electrophoretic technique also permits visualization of the split HbA_2 band. This finding is helpful to distinguish α-chain variants, e.g., the clinically innocuous HbG-Philadelphia, from β-chain variants such as HbD-Punjab. HbA_2 can be quantified by cellulose acetate electrophoresis followed by elution and spectrometry, by microcolumn chromatography, or, most accurately, by HPLC.

Accurate diagnosis in complex or indeterminate phenotypes usually requires DNA analysis. Several point mutations for hemoglobin variants can be recognized by the change in susceptibility to cleavage by specific restriction endonucleases, e.g., HbO-Arab with *Eco*RI. The sickle cell (HbS) mutation eliminates sites for *Mnl*I, *Dde*I, and *Mst*II, allowing detection by restriction fragment length polymorphism (RFLP) analysis. Recently, PCR-based assays for detection of single nucleotide polymorphisms (SNPs) have offered a simple, low-cost genotyping method for diagnosing homozygous HbS and other SCD syndromes.[8] Mutation detection by gene chip (microarray) analysis has been developed for diagnosis of β-thalassemias. This high-throughput, sensitive methodology will likely be adapted for hemoglobinopathy diagnostics.

The discovery that fetal cell-free DNA can be isolated from maternal plasma[9] holds the promise in the near future for rapid, ultrasensitive, and noninvasive detection of many fetal hematological disorders. This method already has become a standard clinical laboratory technique for genotyping fetuses at risk for Rh isoimmunization and erythroblastosis fetalis (see Chapter ?).

Hardison et al.[10] have constructed a Web-accessible, updated relational database on the genomic sequence changes leading to hemoglobin variants and thalassemias called *HbVar* (http://globin.cse.psu.edu/globin/hbvar/). *HbVar* is searchable by mutation genomic location, sequence alterations, biochemical and hematological effects, pathology, population frequencies, and references.[11]

HEMOGLOBIN C

The HbC β-globin mutation (β^6 Glu → Lys) has similar consequences to those induced by the sickle cell disease (HbS) mutation (β^6 Glu → Val); that is, a (−)-charged glutamic acid is replaced by a (+)-charged residue. Homozygosity for HbC causes severe hemolytic disease. Blood smears of affected individuals show frequent target cells (because of increased RBC surface area to cell volume ratio) and polychromasia. Haplotyping studies indicate that HbC mutation had a unicentric origin in sub-Saharan Africa. Recent evidence also supports the Haldane hypothesis for a protective effect of HbC heterozygosity against severe malarial infection.[12]

The HbC band is identifiable by alkaline cellulose acetate gel electrophoresis and confirmed by acidic gel electrophoresis. Of note, HbC and HbA_2 coelute on diethylaminoethyl (DEAE) cellulose column chromatography, a common procedure for quantifying HbA_2. Homozygous HbC disease also can be differentiated from HbC/β-thalassemia by cation-exchange HPLC. HbC and HbS-Oman (see below) coelute on HPLC, but the presence of distinctive sickled ''Napoleon hat cells'' on a blood film will suggest HbS-Oman. DNA analysis will confirm the specific mutation(s).

Detection of HbC by DNA analysis has been more difficult than for HbS because no known restriction endonuclease site is abolished or created by the mutation. However, newer allele-specific PCR amplification techniques permit rapid identification of the HbC mutation.

HEMOGLOBIN CONSTANT SPRING

Hb constant spring (HbCS) is an abnormal hemoglobin characterized by a mutation of the α2-globin gene termination codon that produces an elongated α-chain. HbCS occurs in South China, Southeast Asia, and the Mediterranean region. The carrier frequency is estimated to be 5% in northeast Thailand and Laos and it is the most prevalent nondeletional α-thalassemia in Southeast Asia.

Asymptomatic heterozygous HbCS is difficult to detect by standard electrophoresis, as HbCS levels are less than 1% of total hemoglobin. In contrast, the homozygous state causes hemolytic anemia; HbCS levels are often 6–8% of total hemoglobin and Hb Bart's (see Chapter ?) is about 1–2%. The diagnosis should be suspected in individuals (especially from high-risk ethnic groups) who have mild anemia, minimal microcytosis, nucleated RBCs, marked basophilic stippling on blood smear, low (<2%) HbA_2 levels, or persistent Hb Bart's. Southern analysis (e.g., reverse dot blot format) using allele-specific probes for the α2-chain termination mutants will confirm the diagnosis. More recently, simplified PCR assays for HbCS have been developed.[13]

HEMOGLOBIN D

Hemoglobin D-Punjab (HbD-Los Angeles) is common worldwide and is the most frequent abnormal hemoglobin in the Xinjiang region of China. HbD is a β-chain variant (β^{121} Glu → Gln) resulting from a G → C substitution. The same nucleotide is altered in HbD-Punjab and HbO-Arab (see below). When HbD (or HbO-Arab) is coinherited with HbS, HbS polymerization is facilitated leading to severe sickling.

By IEF, HbD may be difficult to distinguish from other β-globin variants (e.g., HbS) that have similar electrophoretic mobility or from γ-globin variants. HbD is a slow-moving species on cation-exchange HPLC. It elutes soon after HbA_2 and may interfere with accurate integration of the HbA_2 peak. The presence of HbD can be confirmed either by PCR or direct sequencing.

HEMOGLOBIN E

The HbE mutation (β^{26} Glu → Lys; GAG → AAG) occurs almost exclusively in Southeast Asia, Bangladesh, northeast India, and Sri Lanka. It is the most common abnormal hemoglobin in this region and reaches a carrier frequency of at least 50% at the border of Thailand, Cambodia, and Laos. HbE has become more frequently encountered in Europe and North America with increased Southeast Asian and Bengali immigration. The protective effect of HbE against severe malaria may be related to an unidentified membrane abnormality that renders most of the RBC population relatively resistant to invasion by *Plasmodium falciparum*.[14]

Individuals with HbE trait or homozygous HbE have hypochromic microcytosis and a modest anemia, but HbE/β-thalassemia can cause severe, transfusion-dependent anemia.[15] In fact, globally, HbE/β-thalassemia is the most prevalent form of β-thalassemia major. In Southeast Asia, HbE may also be coinherited with one of several endemic α-globin abnormalities (including HbCS) and cause complex thalassemia syndromes.[13]

In high-risk populations, the England–Fraser discriminant function [MCV − RBC − (5 × Hb) − 3.4] may be helpful in identifying individuals carrying HbE.[16] HbE is distinguished by characteristic electrophoretic mobilities on alkaline and acid gels, properties on anion/cation-exchange HPLC, and mild insolubility. Of note, coinheritance of HbE with α-thalassemia may confound routine diagnostic investigations because the hemoglobin interactions can lead to reduced HbE levels and altered hematologic features.

Newborn screening programs need to incorporate strategies to distinguish the relatively benign homozygous HbE state from HbE/β-thalassemia. Hemoglobin quantitation by HPLC is helpful in this regard; newborn infants with HbE/β-thalassemia have lower HbE percentages and different HbF/HbE ratios compared to HbE homozygotes. DNA analysis using multiplex allele-specific PCR will confirm the presence of HbE and distinguish the homozygous HbE from HbE/β-thalassemia genotypes.

HEMOGLOBIN O

The HbO-Arab substitution is β^{121} Glu → Lys that results from a point mutation in the same codon (β^{121}) as the HbD mutation. This amino acid residue is a contact point for hemoglobin polymer formation. As the case for HbD, HbO-Arab is not clinically consequential unless coinherited with HbS. HbO-Arab occurs in North Africa, the Near East, Balkans, and Mediterranean, i.e., the historical extent of the Ottoman Empire, and gene flow may have followed Sudanese contingents in the Ottoman military.

Homozygous HbO-Arab individuals are clinically asymptomatic, but laboratory evaluation will reveal a mild compensated hemolytic anemia. HbO-Arab/HbS compound heterozygotes, often Near Eastern or African-American, have clinical and laboratory manifestations of SCD.

Accurate diagnosis of HbS/HbO-Arab individuals requires electrophoresis at both alkaline and acidic pH; HbO-Arab has similar electrophoretic mobility to HbC,

HbE, and HbA_2 on cellulose acetate at alkaline pH, and it migrates closely to HbS on citrate agar at acidic pH. With the introduction of newborn hemoglobin screening, HbO-Arab is increasingly identified at birth.

HEMOGLOBIN S

Sickle cell disease was the first human genetic disorder in which a causative mutation was identified at the molecular level, the β-globin chain substitution β^6 Glu → Val. When deoxygenated, HbS polymerizes, causing sickling and leading to anemia, acute chest syndrome, stroke, splenic and renal dysfunction, vaso-occlusive painful crises, and susceptibility to bacterial infections. SCD is the most common hemoglobinopathy in the United States, with 8–10% of African–Americans carrying the sickle hemoglobin (HbS) gene. Haplotype analysis has revealed several origins for the HbS mutation in Africa, Asia, and Southern Europe. Haplotype is correlated to some extent with disease severity: individuals with the ''Indian/Arabian haplotype'' have the mildest course, whereas those with the ''Bantu haplotype'' often exhibit severe SCD.

Neonatal screening for SCD has been driven by the demonstration that daily oral penicillin administration prevents early morbidity and mortality from pneumococcal septicemia.[17] This is the single instance where newborn screening has been prompted by evidence from a randomized, double-blind, placebo-controlled clinical trial.

In France, a combination of IEF and more sensitive, quantitative cation-exchange HPLC has confirmed the status of 99.7% of newborn infant dried blood spot hemoglobinopathy screening samples. Exceptional cases required reverse-phase HPLC for γ- or α-globin chain variants or confirmatory testing by DNA.[18]

Digestion of genomic DNA from uncultured amniocytes with *Mst*II results in fragments larger than those produced by some other enzymes and therefore has been particularly useful in prenatal diagnosis of SCD. These genotyping methods are being supplanted by PCR amplification of the relevant segment of the human β-globin gene, followed by hybridization of the amplicon with allele-specific oligonucleotide probes, and, most recently, by direct PCR amplification using allele-specific primers.

A related HbS variant, HbS-Oman, contains the classic HbS β^6 Glu → Val mutation as well as a second mutation in the same β-chain, β^{121} Glu → Lys, i.e., the HbO-Arab mutation. This HbS-Arab double mutation can produce particularly severe sickling. Deoxygenation of HbS-Oman RBCs in vitro induces polymerization and a characteristic ''yarn/knitting-needle'' cell deformation.

CONCLUSION

Hemoglobinopathies are the most common class of single-gene disorders worldwide. Structural hemoglobinopathies have been paradigms of ''molecular diseases'' in which codon and resultant amino acid changes produce a dysfunctional protein.

Familiarity with the performance characteristics of different methods of hemoglobin variant analysis is basic to accurate diagnosis. Hemoglobin electrophoresis is useful for screening a small number of samples, but the bands are relatively wide, many hemoglobin variants overlap, and quantitation by densitometry is inaccurate at low concentrations. IEF and HPLC-based methods offer specific advantages. HPLC offers excellent sensitivity and specificity plus the attribute of quantification, important for accurately discriminating phenotypes where a hemoglobin variant and β-thalassemia are coinherited.

The increasing adoption of newborn screening programs for hemoglobinopathies, initially driven by presymptomatic identification of homozygous HbS disease and other double heterozygous forms of SCD, has permitted frequency analysis of phenotypes containing hemoglobins F, A, S, C, E, O, and D. Most newborn screening programs currently perform IEF on eluates from the newborn dried blood spots. Some programs use HPLC or cellulose acetate electrophoresis as an initial screening method.

The introduction of PCR-based techniques has significantly improved accuracy, reliability, and throughput of structural hemoglobin diagnosis. In the United Kingdom, the reported total error rate for PCR-based antenatal diagnosis of hemoglobinopathies has been 0.41%, comprising a 0.10% laboratory error rate and a 0.31% nonlaboratory error rate.[19] DNA analysis is available on a clinical basis for the mutations associated with each of the hemoglobin variants discussed in this article. In light of the high prevalence of hemoglobinopathies, diagnostic genomics for hemoglobin variants is a current priority in developing countries.

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Hereditary Breast and Ovarian Cancer Syndrome

Nader Hanna
Heather Pierce
University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.

INTRODUCTION

Hereditary breast and ovarian cancer syndrome (HBOC) is an inherited autosomal dominant disorder because of germline mutation in *BRCA1* and *BRCA2* genes. Inheritance of *BRCA* mutations confers increased lifetime risk for breast, ovarian, and other malignancies. Individuals with suspected HBOC should undergo thorough pedigree analysis, education, and counseling in regards to genetic testing, disclosure and interpretation of the results, and determination of cancer risk. Comprehensive review of surveillance strategies, chemoprevention, surgical prophylaxis with its limitations and potential adverse effects, and the need for lifelong follow-up and screening for other malignancies is essential.

OVERVIEW

Five to ten percent of breast cancer and 7–10% of ovarian cancer demonstrate a pattern of autosomal dominant inheritance. Hereditary syndromes with breast and/or ovarian cancer are summarized in Table 1. Mutations in the *BRCA* genes account for only 2–3% of all breast cancers. A single copy of the mutated gene is inherited, and mutation of the second wild-type allele of the gene, through acquired genetic events, is required for loss of function and tumorigenesis. Accumulation of additional somatic mutations in other tumor suppressor genes or oncogenes accounts for the phenotypic differences and explains the increasing age-related penetrance of tumorigenic phenotype in HBOC syndrome. *BRCA* genes act as tumor suppressor and DNA repair genes. The *BRCA1* gene is located on long arm chromosome 17 (17q12–21) and encodes an acid nuclear phosphoprotein. It is responsible for breast cancer in 45% of families with multiple breast cancers and up to 90% of families with both breast and ovarian cancers. The *BRCA2* gene is located on the long arm of chromosome 13 (13q12–13) and accounts for breast cancer in 35% of families with multiple breast cancers. The lifetime risks for breast, ovarian, and other *BRCA1*- and *BRCA2*-associated cancers in HBOC syndrome are summarized in Table 2.

CLINICAL FEATURES IN HEREDITARY BREAST AND OVARIAN CANCER SYNDROME

BRCA mutations occur in approximately 20% of families with inherited susceptibility to breast cancer. Personal and family characteristics associated with an increased likelihood that a *BRCA* mutation will be identified are listed in Table 3. Breast cancers in *BRCA1* carriers often have medullary features, are more likely to be poorly differentiated with high mitotic rates and S-phase fraction, lack an in situ component, have low estrogen and progesterone receptors score, and node-positive. *BRCA2* tumors are more histologically heterogeneous and more likely to be estrogen–receptor-positive. For *BRCA1* carriers, breast cancer survival rates are similar to those for sporadic cancer patients when controlling for stage. The lifetime risk for second primary breast cancers in *BRCA*-mutation carriers with breast cancer is 40–60%, with 5-year risk estimates of 22–31%. The lifetime risk for male breast cancer in *BRCA2*-mutation carriers has been reported to be approximately 6.3%.[1] The average age of onset of ovarian cancer is 48–54 years in *BRCA1* carriers and 62 years in *BRCA2* carriers, similar to onset ages in sporadic cases. Ninety-four percent of *BRCA1* ovarian tumors have serous histology (60% in sporadic cases).

GENETIC TESTING AND COUNSELING IN HEREDITARY BREAST AND OVARIAN CANCER SYNDROME

Genetic testing for HBOC has become generally accepted as part of standard clinical practice and should be offered to any patient that has personal or family history features suggestive of HBOC (Table 3) and when genetic testing results could influence the medical management of that patient or the patient's family members. Clinical laboratories employ a variety of molecular techniques to detect germline mutations in *BRCA1* and *BRCA2* in a peripheral blood sample (Table 4). The sensitivity of molecular testing for *BRCA* cancer-predisposing mutations is

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Table 1 Syndromes associated with hereditary breast and/or ovarian cancer

Hereditary cancer syndrome	Associated tumor suppressor genes or oncogenes	Associated malignancies
Hereditary breast and ovarian cancer	*BRCA1*	— Breast, ovarian, colon, and prostate cancer
	BRCA2	— Breast, ovarian, prostate, pancreatic, bile duct and gall bladder, stomach cancer, and malignant melanoma
Li–Fraumeni	*P53*	— Soft-tissue sarcomas, breast cancer, brain tumors, acute leukemia, and other epithelial and mesenchymal tumors
Cowden's disease	*PTEN*	— Breast cancer, thyroid cancer, and colonic neoplasms
Peutz–Jegher	*STK11/LKP1*	— Colorectal cancer, ovarian stromal tumors, uterine and cervical cancer
		— GI tract polyposis and cancers, breast, testis, cancers
Ataxia telangiectasia	*ATM* (autosomal recessive)	— Lymphoma, leukemia, breast cancer, and other solid malignancies
Hereditary diffuse gastric cancer	*CDH1*	— Gastric cancer and lobular breast cancer in females
Hereditary nonpolyposis colorectal cancer	Mismatch repair genes (*MSH2*, *MLH1*, *PMS1*, *PMS2*, *MSH3*, *MSH5*, *MSH6*)	— Colorectal, endometrial, ovarian, stomach, uroepithelial, hepatobiliary, brain, and small bowel cancers
Gorlin (nevoid basal-cell carcinoma)	*PTCH*	— Basal-cell carcinoma, medulloblastoma, ovarian calcifications and fibroma, and leiomyosarcoma
Multiple endocrine neoplasia type 1	*RET* oncogene	— Parathyroid hyperplasia, gastrinoma, and pituitary neoplasms

dependent on the method used for analysis and the a priori risk of the person tested to have a mutation in either gene based on the person's cancer history, family history, and ethnic background. No currently available technique can guarantee the identification of all cancer-predisposing mutations in the *BRCA1* or *BRCA2* genes.[2] The molecular method with the highest level of sensitivity in situations where a familial mutation has not yet been identified is DNA sequencing. Mutation-specific clinical testing is utilized primarily for the three Ashkenazi Jewish founder mutations, which include 185delAG and 5382insC in *BRCA1* and 6174delT in *BRCA2*, as well as in families in which a mutation has previously been identified. Clinical interpretation of the results of genetic testing is divided into three categories: positive (a deleterious mutation is detected), negative (no mutation is detected), and uninformative (a variant of unknown clinical significance is detected). The only truly informative

Table 2 Estimated lifetime risks of *BRCA1*- and *BRCA2*-associated malignancies

Gene	Associated malignancy	Risk to age 70 in mutation carriers
BRCA1[1,19–21]	Female breast	56–87%
	Ovary	15–54%
	Prostate	3.33RR (95% CI 1.78–6.20)
	Colon	4.11RR (95% CI 2.36–7.15)
BRCA2[1,21–25]	Female breast	56–87%
	Ovary	10–23%
	Male breast	6.3%
	Prostate	4.65RR (95% CI 3.48–6.22)
	Pancreas	3.51RR (95% CI 1.87–6.58)
	Gall bladder/bile duct	4.97RR (95% CI 1.50–16.52)
	Stomach	2.59RR (95% CI 1.46–4.61)
	Malignant melanoma	2.58RR (95% CI 1.28–5.17)

Table 3 Personal and family history features suggestive of HBOC

Personal features	—	Breast cancer before age of 40
	—	Bilateral breast cancer
	—	History of both breast and ovarian cancer
Family features	—	Two or more family members <50 years of age with breast cancer
	—	Both breast and ovarian cancer in the family
	—	Breast cancer in one or more male family members
	—	A family member with known mutation in breast cancer susceptibility genes
	—	Ashkenazi Jewish background

genetic test results are a positive result or a negative result in a family in which a deleterious mutation has previously been identified. A variant result or a negative result in a family in which no mutation has been identified is of limited clinical value because it is unknown whether a detectable deleterious mutation is responsible for the cancer diagnoses in the family.

Adequate pretest and posttest genetic counseling should be provided to patients that undergo genetic testing to obtain full-informed consent. Assessment of an individual's risk for hereditary cancer through genetic counseling and genetic testing has been shown to improve patients' risk comprehension, facilitate more informed decisions regarding cancer prevention, and assist in the identification of at-risk family members.

Pretest genetic counseling is a complex, time-consuming, and frequently emotionally charged process. In its most basic form, pretest genetic counseling for *BRCA1* and *BRCA2* testing should include 1) basic information about breast cancer; 2) basic information about hereditary cancer; 3) information about hereditary breast cancer, including clinical management options for families with hereditary breast and ovarian cancer syndrome (HBOC); 4) possible outcomes of genetic testing for HBOC; 5) potential benefits, limitations, and risks of genetic testing for HBOC; and 6) alternatives to genetic testing for HBOC. In addition to these requirements, the session should include discussion of the potential risk for insurance or employment discrimination, confidentiality issues, and the importance of sharing test results with at-risk relatives. Adequate posttest genetic counseling should include discussion of test results and options for clinical management, plans for sharing test results with at-risk family members, and additional genetic-assessment options if the test results are uninformative.

SCREENING IN HEREDITARY BREAST AND OVARIAN CANCER SYNDROME

Although overall population screening reduces breast cancer mortality by 25–40% in women between the ages of 50 and 70 years, no data are available on the outcomes of interventions to reduce risk in HBOC syndrome. No data are available to demonstrate that surveillance for ovarian cancer in high-risk women reduces mortality[3] because transvaginal ultrasound and CA-125 lack sensitivity and specificity. However, recommended screening in *BRCA* gene mutation carriers includes monthly breast

Table 4 Molecular techniques for genetic testing of *BRCA1* and *BRCA2*

Technique	Features and limitations
DNA sequencing	— Highest overall level of sensitivity
	— May detect variants of unknown clinical significance
	— Intronic mutations, genomic rearrangements may be missed
	— Higher cost, longer turnaround time than most other techniques
Exon scanning (CSGE, SSCP, DGGE)	— Rapid, low-cost initial screening technique
	— Requires sequencing to confirm the exact nature of the mutation
Protein truncation testing (PTT)	— Useful for detecting loss-of-function mutations
	— Change-of-function mutations may be missed
	— Requires sequencing to confirm the exact nature of the mutation
Southern blot	— Can screen for large genomic rearrangements
	— Will not detect point mutations or small deletions and insertions
Allele-specific oligonucleotide (ASO)	— High level of sensitivity for mutation-specific testing
	— Rapid, low-cost technique
	— Only screens for specific known mutations
Recombination-specific PCR	— Can detect specific genomic rearrangements
	— Only screens for specific known rearrangements

self-examination starting at age 18, semiannual clinical breast examination and annual mammography starting at age 25, and starting at age 30–40, semiannual transvaginal ultrasound with color Doppler, serum CA-125, and pelvic examination. Several studies have shown that in women with germline *BRCA1* and *BRCA2* mutations, breast cancers are likely to occur as interval cancers[4] and that standard mammography is more likely to be negative than in women at low or moderate risk.[5] Recent data suggest that magnetic resonance imaging (MRI) of the breast may be much more sensitive than mammography for detecting cancer in mutation carriers.[6]

CHEMOPREVENTION IN HEREDITARY BREAST AND OVARIAN CANCER SYNDROME

Women who desire risk reduction intervention for breast cancer have the option of chemoprevention with tamoxifen therapy (20 mg/day for 5 years) or prophylactic surgery. The National Surgical Adjuvant Breast and Bowel Project prevention trial in healthy women 35 years and older with a 1.7% or greater cumulative 5-year risk for developing breast cancer demonstrated short-term reduction in risk of developing estrogen–receptor-positive breast cancer by 49%. The utility of tamoxifen for breast cancer risk reduction in women under the age of 35 years is unknown and current evidence-based data regarding tamoxifen breast cancer risk reduction in *BRCA* gene mutation carriers are insufficient. Women with *BRCA1* mutations who develop breast cancer have estrogen–receptor-negative tumors in approximately 80% of cases, whereas women with *BRCA2* mutations who develop breast cancer have estrogen–receptor-positive tumors in 80% of cases. Therefore, the use of tamoxifen for breast cancer prevention in *BRCA1*-mutation carriers cannot be routinely recommended, whereas it appears to be a reasonable choice for risk reduction in *BRCA2*-mutation carriers based on current evidence. The incidence of contralateral breast cancer in *BRCA*-mutation carriers was shown to be reduced by 50% with tamoxifen use,[7] by 60% after chemotherapeutic treatment of the first breast cancer,[7] and by 58% after bilateral oophorectomy.[7,8]

The use of oral contraceptive pills (OCP) as chemoprevention in ovarian cancer has been extensively studied. It has been estimated that there is an overall risk reduction of approximately 40%[9] and that more than half of all ovarian cancer in the United States could be prevented by the use of oral contraceptive pills for at least 4 or 5 years.[10,11] However, the role of chemoprevention against ovarian cancer in HBOC syndrome is unknown. There have been conflicting reports on the impact of OCP use on breast and ovarian cancer risk in *BRCA*-mutation carriers.[12–15]

PROPHYLACTIC SURGERY

Prophylactic surgery in HBOC syndrome includes prophylactic bilateral mastectomy and/or complete bilateral salpingo-oophorectomy (including the entire fallopian tubes and infundibulopelvic ligaments). Prophylactic bilateral mastectomy with reconstruction option is the safest measure for prevention of breast cancer. In *BRCA*-mutation carriers, prophylactic bilateral mastectomy was associated with an 85–100% risk reduction for breast cancer.

In HBOC syndrome, prophylactic oophorectomy is recommended at age 35 or after childbearing is completed.[3] Among mutation carriers, prophylactic oophorectomy has been shown to reduce the risk of breast and ovarian cancer by 53–68% and 85–96%, respectively. Ten to fifteen percent of women with *BRCA1* or *BRCA2* mutations who underwent prophylactic oophorectomy were found to have occult ovarian carcinoma.[16] A residual risk of primary peritoneal carcinoma and peritoneal carcinomatosis has been documented in 2–11% of women who undergo prophylactic oophorectomy.[17] The majority of women (86.4%) who had prophylactic oophorectomy were satisfied because surgery effectively reduced their anxiety about ovarian cancer, with the benefit of anxiety reduction outweighing the potential adverse events of surgery.[18]

CONCLUSION

Identifying HBOC syndrome has clinical implication for both the clinician and the individuals diagnosed, as they have an increased risk of several malignancies. Management strategies to address the increased cancer risk include genetic counseling and testing, targeted surveillance, chemoprevention, and prophylactic surgery. Genetic testing in high-risk family members is strongly recommended. The available current data indicate superiority of surgical prophylaxis compared with surveillance in reducing cancer risk in carriers of *BRCA* mutations. Surgery limitations and related adverse effects on morbidity and quality of life should always be discussed in extensive preoperative counseling. For evidence-based medical recommendation, more prospective data with appropriate surveillance protocol, longer follow-up, and quality-adjusted overall survival as endpoints are needed.

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Hereditary Diffuse Gastric Cancer Syndrome

Nader Hanna
University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.

INTRODUCTION

Hereditary diffuse gastric cancer (HDGC) syndrome is an autosomal-dominant inherited cancer susceptibility syndrome with high penetrance for gastric cancer caused by germline truncating mutation in the E-cadherin (*CDH1*) tumor-suppressor gene. Male carriers have a 67% lifetime risk for gastric cancer; female carriers have a 83% lifetime risk for gastric cancer and a 39% risk for breast cancer. The syndrome is characterized by early, diffuse, multifocal submucosal involvement, rendering endoscopy and random biopsy inaccurate. Once a carrier is identified, prophylactic total gastrectomy is the only effective treatment.

OVERVIEW

Despite a declining incidence of, and mortality from, gastric cancer worldwide, it remains the second largest cause of cancer-related death. Most gastric cancers occur sporadically without evidence of an inherited cancer predisposition. Familial clustering of gastric cancer is observed in approximately 10% of cases. However, hereditary gastric cancer caused by inheritance of germline mutations accounts for only 1–3% of all gastric cancers and is seen in HDGC, hereditary nonpolyposis colon cancer syndrome (HNPCC), Peutz–Jeghers syndrome, Cowden's syndrome, and some kindreds affected with Li–Fraumeni syndrome and familial adenomatous polyposis (FAP; Table 1). Compared with other malignancies such as colorectal cancer, the genetic events that contribute to the tumorigenicity of gastric cancer are rather undefined. However, it is believed that complex interactions between dietary factors, *Helicobacter pylori* infection, and genetic predisposition contribute to the pathogenesis of gastric cancer, account for phenotypic differences, and explain the increasing age-related penetrance of tumorigenic phenotype.

BIOLOGICAL BASIS OF HDGC SYNDROME

HDGC is an autosomal dominant disorder with high penetrance, caused by germline mutations in the *CDH1* gene encoding for E-cadherin. E-cadherin is a 120-kDa calcium-dependent transmembrane glycoprotein localized in lateral cell–cell contacts and enriched in the zonula adherens junctions.[1] The intact function of this adhesion molecule is crucial for the establishment and maintenance of epithelial tissue polarity, structural integrity, and cellular differentiation.[1,2] It has five tandemly repeated extracellular domains that mediate intercellular adhesion through homophilic cellular interactions, and a cytoplasmic domain that is responsible for the adhesive function of E-cadherin to the actin cytoskeleton through a complex with α, β, and γ catenins.[2] E-cadherin mutations have been described for breast, gastric, endometrium, ovary, and thyroid carcinomas. However, frequent somatic mutations in *CDH1* have been particularly implicated in the carcinogenesis of gastric carcinomas (in 40–83% of sporadic diffuse-type gastric cancer, but not in sporadic intestinal-type gastric cancer.[3,4]) and infiltrative lobular breast carcinomas.[5,6] In diffuse gastric carcinomas, the predominant mutations are exon skippings causing in-frame deletion, in contrast to premature stop codon mutations identified in lobular breast cancer. Germline mutation of *CDH1* gene in gastric cancer was originally discovered and reported in three Maori families in 1998.[7] The characteristics of the 18 families reported in the literature to have HDGC syndrome are summarized in Table 2. Both copies of the gene must be mutated to result in loss of function and carcinogenesis. A single copy of the mutated gene is inherited, and mutation or loss of promoter methylation of the second wild-type allele of the gene, through acquired genetic events, is required for loss of function and tumorigenesis. The accumulation of additional somatic mutations in other tumor-suppressor genes or oncogenes accounts for the phenotypic differences, and explains the increasing age-related penetrance of tumorigenic phenotype.

CLINICOPATHOLOGICAL FEATURES

HDGC was defined, during the First Workshop of the International Gastric Cancer Linkage Consortium (IGCLC) held in 1999, as any family that fits the following criteria: 1) two or more documented cases of diffuse gastric cancer in first-degree/second-degree relatives,

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Table 1 Hereditary syndromes associated with gastric cancer

Hereditary cancer syndrome	Associated genes or oncogenes	Associated malignancies and their penetrance
HDGC	*CDH1*	Gastric cancer (70%) Lobular breast cancer in females (39%)
Hereditary nonpolyposis colorectal cancer (HNCC; Lynch syndrome)	Mismatch repair genes (*MSH2*, *MLH1*, *PMS1*, *PMS2*, *MSH3*, *MSH5*, *MSH6*)	Colorectal cancer (70–85%) Endometrial cancer (40–60%) Ovarian cancer (12%) Stomach, uroepithelial, hepatobiliary, brain, small bowel cancers
Peutz–Jeghers	*STK11*, *LKP1*	Colorectal cancer Ovarian stromal tumors Uterine and cervical cancer Gastrointestinal tract polyposis and cancers, breast and testis, cancers
Cowden	*PTEN*	Thyroid cancer Breast cancer Gastric cancer
Li–Fraumeni	*p53*	Leukemia Breast cancer Gastric cancer
FAP	*APC* (adenomatous polyposis coli) *DCC/DPC/JV18* *p53* *KRAS*	Colorectal carcinoma (100%) Ampullary carcinoma (30%) Desmoid tumors (10%) Gastric cancer

with one at least diagnosed before the age of 50 years; or 2) three or more cases of documented diffuse gastric cancer in first-degree/second-degree relatives, independently of age of onset.[14] All gastric carcinomas from documented germline mutation E-cadherin carriers have been of the diffuse or linitis plastica type. The penetrance of germline *CDH1* mutations is estimated to be approximately 70%.[7] The average age of onset of gastric cancer is 38 years, with occurrence in the 20s not unusual. The lesions are multifocal, spread submucosally without forming grossly visible exophytic mass, and are not associated with precancerous morphological or pathological abnormalities, rendering screening endoscopy and early diagnosis very difficult. Clinical diagnosis was often at an advanced incurable stage. Lobular breast carcinoma,[8,10,15] colorectal cancer, and prostate carcinoma[7,9,11] have also been documented in mutant E-cadherin gene carriers and may be a part of the malignant spectrum in HDGC. The estimated cumulative risk of gastric cancer by age 80 years was 67% for men [95% confidence interval (95% CI), 39–99] and 83% for women (95% CI, 58–99). For women, the cumulative risk of breast cancer was 39% (95% CI, 12–84). The combined risk of gastric cancer and breast cancer in women was 90% by age 80 years.[15]

MANAGEMENT STRATEGIES

Individuals with suspected HDGC should undergo thorough pedigree analysis, education and counseling in regards to genetic testing, disclosure and interpretation of the results, and determination of cancer risk. A comprehensive review of surveillance strategies, chemoprevention, surgical prophylaxis with its limitations and potential adverse effects, and the need for lifelong follow-up and screening for other malignancies is essential. Genetic testing and counseling guidelines for HDGC are provisional at this time and are only provided on an investigational basis. These guidelines are likely to change to parallel the ongoing scientific discovery of the genes responsible for the syndrome. Molecular techniques employed to detect germline mutations are summarized in Table 3. No currently available technique can guarantee the identification of all cancer-predisposing mutations in the *CDH1* gene. The molecular method with the highest level of sensitivity in situations where a familial mutation has not yet been identified is DNA sequencing. Mutation-specific clinical testing is utilized primarily in families in which a mutation has previously been identified.

Table 2 Characteristics of the 18 families reported in the literature to have DHGC syndrome

Ethnic background	Mutation	Location and type of mutation	Age at diagnosis of DGC (years)
Maori[8]	G1008T	Exon 7 (splice site)	14–67
Maori[8]	C2095T (Q699X)	Exon 13 (silent)	30
Maori[8]	2382–2386insC	Exon 15 (frame shift)	16–35
Maori[9]	C190T (Q64X)	Exon 3 (nonsense)	22–28
White[9]	A(49-2) G	Exon 2 (splice site)	34–69
White[9]	G59A (W20X)	Exon 2 (nonsense)	27–50
White[8]	G70T (E24X)	Exon 2 (nonsense)	37–46
White[10]	372–377 delC	Exon 3 (frameshift)	15–58
White[11]	C187T (R63X)	Exon 3	33–69
White[8]	G586T (G196X)	Exon 5 (nonsense)	31–55
White[8]	1487del7	Exon 10 (frameshift)	31
White[8]	1588insC	Exon 11 (frameshift)	40–63
White[11]	1711insG	Exon 11 (frameshift)	30–68
White[11]	C1792T (R598X)	Exon 12 (silent)	23–43
African–American[8]	G(1137+1) A	Intron 8 (donor splice site)	29–58
Korean[12]	A731G	Codon 244 in exon 6 (missense)	30–63
Korean[12]	T1460C	Codon 487 in exon 10 (missense)	42–49
Japanese[13]	G185T	Codon 62 in Exon 3 (missense)	46–72

There are no effective screening regimens or surveillance modalities. Upper endoscopy with biopsy, endoscopic ultrasound, and/or chemoendoscopy appears to have no value in the detection of early and potentially curable multifocal submucosal gastric cancer. Surveillance endoscopy was unable to detect early cancer found in prophylactic total gastrectomy specimens. Surveillance modalities that can identify carriers who would benefit from early prophylactic gastrectomy are being evaluated. These include endoscopic surveillance with optical coherent tomography and molecular marker analysis of gastric lavage fluid.

There are no known nonsurgical prevention strategies for *CDH1* carriers at this time. However, prophylactic treatment with demethylating agents, such as 5-azadeoxycystidine, has been suggested based on the observation that some tumors demonstrated methylation of the *CDH1* promoter as the ''second hit.''[16,17] A recent report also suggests that E-cadherin methylation is an early event in gastric carcinogenesis and is initiated by *H. pylori* infection.[18] Another potential strategy is early and annual screening and complete eradication of *H. pylori* infection in *CDH1* carriers, similar to the recommendation of the Maastricht 2-2000 Consensus

Table 3 Molecular techniques for genetic testing

Technique	Features and limitations
DNA sequencing	Highest overall level of sensitivity May detect variants of unknown clinical significance Intronic mutations, genomic rearrangements may be missed Higher cost, longer turnaround time than most other techniques
Exon scanning	Rapid, low-cost initial screening technique Requires sequencing to confirm the exact nature of the mutation
Protein truncation testing (PTT)	Useful for detecting loss-of-function mutations Change-of-function mutations may be missed Requires sequencing to confirm the exact nature of the mutation
Southern blot	Can screen for large genomic rearrangements Will not detect point mutations or small deletions and insertions
Allele-specific oligonucleotide (ASO)	High level of sensitivity for mutation-specific testing Rapid, low-cost technique Only screens for specific known mutations
Recombination-specific polymerase chain reaction (PCR)	Can detect specific genomic rearrangements Only screens for specific known rearrangements

Table 4 Long-term complications after total gastrectomy

- Esophagojejunostomy anastomotic stricture (10–15%)
- Lactose intolerance: occurs in approximately 50% of patients
- Malabsorption and steatorrhea: occur in 66–100% of patients due to relative pancreatic insufficiency, bacterial overgrowth, and rapid intestinal transient time
- Postprandial fullness and dumping syndrome: occur in 20–30% of patients due to rapid emptying of hyperosmotic carbohydrates into the small bowel and the release of enteric hormones
- Iron and vitamin B_{12} deficiency
- Weight loss: approximately 10% (average of 4–7 kg) of the preoperative weight (mainly body fat) during the first year

Report, stating that patients with a family history of gastric cancer should undergo *H. pylori* eradication therapy.[19] Several analyses showed an association between *H. pylori* seropositivity (especially CagA-positive strains) and gastric cancer, particularly in younger patients and non-cardia adenocarcinoma.[20,21] The increased prevalence of *H. pylori* infection and *H. pylori*-associated precancerous changes has also been observed in first-degree relatives of sporadic gastric cancer patients.[21] In a cluster of familial gastric cancer, genetic abnormalities were found in first-degree relatives only in the presence of *H. pylori* infection.[22] Successful eradication of *H. pylori* improves the secretion of vitamin C into gastric juice, which may increase protection against gastric cancer. A randomized, placebo-controlled trial in people at high risk for developing gastric cancer showed that effective anti-*H. pylori* treatment and dietary supplementation with antioxidants were statistically significant in promoting the regression of precancerous lesions.[23]

The optimal clinical management of E-cadherin germline mutation carriers is unclear and controversial, especially in regard to the role of prophylactic gastrectomy. Prophylactic total gastrectomies in five patients (ages 37, 39, 40, 41, and 47 years) with a known E-cadherin germline mutation were first reported in 2001.[24] All five patients had negative endoscopic evaluation, yet all postgastrectomy specimens demonstrated occult microscopic foci of intramucosal signet ring cell adenocarcinoma in various regions of the stomach. Huntsman et al.[25] reported similar findings in another five subjects (ages between 22 and 40 years). At present, the International Gastric Cancer Consortium (IGCC) recommends testing for E-cadherin alterations in patients with diffuse gastric cancer and suggests prophylactic total gastrectomy in carriers of E-cadherin germline mutations.[14] Complete removal of gastric mucosa should be confirmed intraoperatively on frozen section by a demonstration of esophageal and duodenal mucosa at the proximal and distal margins, respectively. Prophylactic gastrectomy also has the potential benefit of anxiety reduction and perhaps improvement of the overall quality of life in these mutation carriers. However, there are several concerns for adopting a universal policy of prophylactic gastrectomy in *CDH1* mutation carriers. Prophylactic gastrectomy would result in a nontherapeutic surgery in 30% of these carriers. The biological behavior of these early submucosal multifocal lesions is not known. The age at which prophylactic surgery should be recommended is not clear. Although prophylactic gastrectomy at a young-enough age reduces the risk of gastric cancer development, its effect on the overall life expectancy is unknown and unproven. Carriers of the *CDH1* gene are at increased risk for developing other malignancies and require regular screening. Gastrectomies, in general, are associated with operative mortality of 5% or less and a morbidity rate of 35–40%. Significant long-term sequels (Table 4) can have a significant negative impact on quality of life. Clinical studies have shown that patients with jejunal pouch reconstruction have better food intake, fewer postprandial symptoms, less weight loss, and a chance for a better quality of life. However, it is expected that prophylactic total gastrectomy in these otherwise healthy young individuals would have lower morbidity and less negative impact on quality of life.

CONCLUSION

Identifying HDGC has clinical implications for both the clinician and the individuals diagnosed with this hereditary form of cancer, as they have an increased risk of gastric cancer and other malignancies. Management strategies to address the increased cancer risk include genetic counseling and testing, targeted surveillance, chemoprevention, and prophylactic surgery. Genetic testing in high-risk family members is strongly recommended. The available current data indicate the superiority of surgical prophylaxis compared with surveillance in reducing gastric cancer risk in carriers of *CDH1* mutations. Surgery limitations and related adverse effects on morbidity and quality of life should always be discussed in extensive preoperative counseling. For evidence-based medical recommendation, more prospective data with appropriate surveillance protocol, longer follow-up, and quality-adjusted overall survival as endpoints are needed.

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Hereditary Hemochromatosis

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Ronald T. Acton
University of Alabama at Birmingham, Birmingham, Alabama, U.S.A.

James C. Barton
Southern Iron Disorders Center, Birmingham, Alabama, U.S.A.

INTRODUCTION

Hereditary hemochromatosis (OMIM #235200) is an autosomal recessive disorder of iron metabolism that affects 1/200 to 1/400 Caucasians of central, northern, and western European descent. Some Hispanics also develop hemochromatosis that is phenotypically and genotypically similar to that in Caucasians. Other iron overload disorders in Caucasians, most of which are uncommon, share some clinical and biochemical features with ''classic'' hemochromatosis, although they are associated with mutations in other genes. Iron overload in other racial/ethnic groups also differs phenotypically and genotypically from ''classic'' Caucasian hemochromatosis.

In some persons with hemochromatosis, excessive iron accumulation in parenchymal cells can lead to hepatic cirrhosis, primary liver cancer, arthropathy, diabetes mellitus, hypogonadism, and cardiomyopathy, if untreated. Diagnosis is usually based on detection of elevated serum transferrin saturation in the absence of other demonstrable cause. Iron overload is characterized by elevation of serum ferritin concentration or hepatic iron concentration, or by quantitative phlebotomy to achieve iron depletion. Complications of hemochromatosis can be prevented by early diagnosis and simple, effective treatment to remove excess iron and maintain low normal body iron stores (therapeutic phlebotomy). Because hemochromatosis is common, easily diagnosed, and readily treatable, it has become a public health concern in a large fraction of the world's population.

GENETIC FEATURES OF HEMOCHROMATOSIS

Some features of hemochromatosis are displayed in Table 1.[1–3] Hemochromatosis is linked to human leukocyte antigen (HLA) class I genes within the major histocompatibility complex (MHC) region on Ch6p.[1] Mutations in a nonclassical MHC class I gene (*HFE*), which maps ~4 Mb telomeric to HLA-A at 6p21.3, account for most cases in Caucasians[4] (Table 2). ''Classic'' hemochromatosis is associated with homozygosity for the *HFE* C282Y mutation. C282Y often lies within an ancestral HLA haplotype A*03-B*07.[1,6] This haplotype is the predominant hemochromatosis-associated haplotype in northwestern European countries.[6] This is attributed to the origin of C282Y on an A*03-B*07 haplotype in northwestern Europe and its early dissemination by Vikings.[6] Modification of the ancestral chromosome by recombination and admixture as a result of geographic migration explains the occurrence of chromosomes bearing C282Y in association with different HLA haplotypes.[6] Thus some nonancestral haplotypes also occur with significantly increased frequencies in hemochromatosis patients in various countries in Europe and in descendants of Europeans.[6]

C282Y and H63D, the most common *HFE* mutations detected in Caucasians, occur with frequencies of 0.01–0.14 and 0.05–0.22, respectively.[7] Frequencies of these mutations vary among racial, ethnic, or national groups, and are positively correlated with the prevalence of hemochromatosis.[7] In various Caucasian populations, 60–100% of hemochromatosis cases are attributable to homozygosity for C282Y.[4,8] Some patients are compound heterozygotes for C282Y and H63D, or are H63D homozygotes.[4,8] Other persons with a hemochromatosis phenotype have ''atypical'' *HFE* genotypes, i.e., are heterozygous for C282Y or H63D, have uncommon *HFE* mutations, or do not have a detectable mutation in *HFE* coding regions (Table 2).[4,8,9] Differences in case ascertainment criteria could partly explain dissimilarities in the geographic distribution of hemochromatosis patients with ''atypical'' *HFE* genotypes.[8,9] Missense mutations in other genes that control iron absorption could also account for hemochromatosis phenotypes in some cases.

The severity of iron overload in Caucasians with hemochromatosis is variable, and is correlated with the particular *HFE* genotype; for example, persons with the genotype C282Y/C282Y usually have more stored iron at diagnosis than persons with the genotypes C282Y/H63D or H63D/H63D.[8] Persons who inherit C282Y on the ancestral haplotype characterized by HLA-A*03,-B*07 may have more severe iron overload, on the average, than those who lack this haplotype.[10] Some investigators have suggested that ''mutations at a second HLA-linked locus,

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Table 1 Features of hemochromatosis

Variable	Clinical feature
Affected populations	Caucasians of European descent
Prevalence in populations	1/200–1/400
Prevalence in liver biopsy specimens	0.034, 0.046
Serum transferrin saturation with iron	Usually elevated
Serum ferritin concentration	Often elevated
Primary target organs of iron overload	Excess iron is deposited in almost all organs and tissues in severe cases, especially liver
Pattern of iron deposition	Primarily parenchymal cells
Complications of iron overload	Hepatic cirrhosis, primary liver cancer, diabetes mellitus, hypogonadotrophic hypogonadism, arthropathy, cardiomyopathy, hyperpigmentation, abnormalities of ascorbic acid (vitamin C) metabolism
Pattern of inheritance	Autosomal recessive
Associated genes	Mutations in *HFE*
Prevention or treatment of iron overload	Phlebotomy

Source: From Ref. [3].

Table 2 *HFE* mutations associated with hemochromatosis

Location	Nucleotide change	Mutation designation
5′	−7 T→C	–
Exon 2	?	V68X
Exon 2	84 C→T	H28H
Exon 2	157 G→A	V53M
Exon 2	175 G→A	V59M
Exon 2	187 C→G	H63D
Exon 2	189 T→C	H63D
Exon 2	193 A→T	S65C
Exon 2	196 C→T	R66C
Exon 2	211 C→T	R74X
Exon 2	277 G→C	G93R
Exon 2	del277	G93fs
Exon 2	314 T→C	I105T
Exon 2	370del22	–
Exon 2	–	V68δT
Exon 3	317 A→C	Q127H
Exon 3	502 G→C	E168Q
Exon 3	502 G→T	E168X
Exon 3	506 G→A	W169X
Exon 3	527 C→T	A176V
Exon 3	Cytosine deletion	P160δC
Intron 3	IVS3+1 G→T	–
Exon 4	673 G→A	R224G
Exon 4	814 G→T	V272L
Exon	829 G→A	E277K
Exon 4	845 G→A	C282Y
Exon 4	845 G→C	C282S
Exon 4	848 A→C	Q283P
Exon 5	989 G→T	R330M
Intron 5	IVS5+1 G→A	–

Source: From Ref. [5].

rather than recombination between A*03 and C282Y, could explain some regional disease associations with non-A*03 haplotypes.''[11] Conversely, other investigators suggest that ''another haplotype containing HLA-B*07 and HLA-Cw*0702 contains an element affording protection against hemochromatosis.''[12] Locus heterogeneity also has been proposed as an explanation of the observations that the distribution of *HFE* genotypes in patients deviates from that expected under Hardy–Weinberg equilibrium.[4] It is possible that multiple MHC haplospecific regions contribute to the hemochromatosis phenotype.[8] For example, a modifying gene in the region of D6S105 on Ch6p may affect the severity of iron overload in hemochromatosis.[13] Among hemochromatosis patients homozygous for C282Y, those with D6S105 allele 8 homozygosity had significantly higher hepatic iron indices than those heterozygous or nullizygous for D6S105 allele 8.[13] In contrast, an analysis of microsatellites between HLA-A*03 and *HFE* in C282Y homozygotes and their siblings revealed no evidence of modifier genes in this region.[14] Polymorphisms in the promoter region of the tumor necrosis factor-alpha (TNF-α) gene, also located within the MHC region, have been implicated in the development of iron overload or expression of its complications by some[15] but not other investigators.[16]

In addition to Ch6p alleles, the expression of iron overload in individuals with hemochromatosis is associated with sex, age, intake of iron and other dietary factors, coinheritance of forms of hereditary anemia that stimulate iron absorption, and the severity of physiological and pathological iron and blood loss.[1,2,11] At present, only short-term longitudinal studies to ascertain the development of iron overload and its complications in C282Y

homozygotes have been reported.[17] Therefore debate regarding the penetrance of iron overload and associated complications in cohort analyses of C282Y homozygotes is unresolved.[18] Until long-term studies are conducted, estimates of penetrance will remain controversial.

ROLE OF *HFE* IN IRON METABOLISM

The first indication that class I MHC molecules are involved in cellular iron metabolism were observations that beta 2-microglobulin knockout mice develop iron overload.[19] Subsequently, HFE protein was observed to have sequence and structural homology with MHC class I proteins,[4] but lacks a functional peptide binding groove and interactions with T cells.[20] HFE lacks a peptide-binding groove analogous to classical class I MHC proteins because of the greater proximity of its $\alpha 1$ and $\alpha 2$ helices. Moreover, C282Y, the predominant *HFE* mutation associated with hemochromatosis, encodes a tyrosine for cysteine substitution that results in loss of the disulfide bond in the $\alpha 3$ domain.[21] Without this disulfide loop, the molecule cannot fold properly, does not associate with beta 2-microglobulin, and is not expressed on the cell surface.[21] Wild-type and H63D HFE proteins form stable complexes with the transferrin receptor (TfR), whereas the C282Y mutant protein does not associate with TfR[22] The TfR-binding site of HFE is located on the C-terminal area of the $\alpha 1$ domain helix and in an adjacent loop.[22] This region of HFE is distinct from the peptide-binding site of classical class I MHC proteins. HFE binds TfR at or near the site where diferric transferrin binds. Although HFE competes with transferrin for binding sites on TfR, it is unclear if this is the mechanism whereby HFE regulates iron metabolism. In cells expressing HFE, TfR levels are increased and ferritin levels are decreased.[23] In cultured cells, expression of HFE reduces the uptake of ferritin from transferrin, suggesting that HFE has a role in regulating transferrin-mediated iron uptake. There are other proteins known to have a role in regulating iron absorption and metabolism. The relationship of their interaction with HFE is less well understood or is unreported.

EARLY DIAGNOSIS OF HEMOCHROMATOSIS: PHENOTYPING VS. GENOTYPING

Early diagnosis of hemochromatosis permits treatment of iron overload and thereby prevents premature death due to hepatic cirrhosis (and primary liver cancer) and diabetes mellitus complications.[1,2] Preventing iron overload may also reduce the frequency or severity of arthropathy, hypogonadotrophic hypogonadism and other endocrinopathic disorders, and cardiac abnormalities.[1,2] The cornerstone of case detection in medical care and in population screening has been the measurement of serum transferrin saturation.[1] The severity of iron overload is estimated by quantification of the serum ferritin concentration, and by measurement of iron stores in liver biopsy specimens. An estimate of excess body iron stores can also be determined after the completion of iron depletion by therapeutic phlebotomy, although this does not provide data useful in prospective case ascertainment.

After the cloning of *HFE* and observations from early reports of significant associations of the mutations C282Y and H63D with hemochromatosis in Caucasians, there was hope that *HFE* genotyping could be used to identify persons at risk for developing iron overload. This would allow early intervention that could prevent or reduce the morbid conditions that often develop in persons with severe iron overload. Although genotyping is useful to detect hemochromatosis in western Caucasian ethnic groups and family members of affected individuals,[24] there are still too many unanswered questions regarding factors that affect clinical penetrance to recommend universal population genotypic screening. However, *HFE* genotyping is a useful test that can augment phenotyping with serum transferrin saturation and serum ferritin levels.

With the recent discovery of mutations in genes associated with other forms of hemochromatosis there is now the possibility that these mutations when present with *HFE* mutations can enhance or modify the risk for ''classical'' hemochromatosis (Table 3). In addition to ''classical'' hemochromatosis three other forms of hemochromatosis have been described. Juvenile hemochromatosis (OMIM #602390) is a relatively rare severe autosomal recessive disorder with an early age of onset.[25] Clinical symptoms usually appear in the second to third decade of life. There may be two forms of this disorder. Mutations in *HAMP*, a gene located on chromosome 19q13 (OMIM #606464) that codes for hepcidin, have been observed associated with one form of severe juvenile hemochromatosis in Italian families and in individuals with, ''altered indicators of iron status.''[26,27] Mutations in *HAMP* have been observed in subjects with iron overload who are also heterozygous for C282Y or S65C.[27–29] These data suggest that the onset or severity of iron overload may be ''modified'' by the presence of *HAMP* mutations in C282Y homozygous or heterozygous individuals.

The other form of juvenile hemochromatosis is linked to a gene on chromosome 1q21 (OMIM #608374). Mutations in the transcript unit *LOC148738* now referred to as *HJV* have been reported associated with juvenile hemochromatosis in several families.[30] This gene appears to modulate hepcidin expression.

Mutations in *TFR2*, which maps to 7q22 and codes for transferrin receptor 2, have also been reported associated

Table 3 Non-*HFE* mutations associated with hemochromatosis[a]

Gene	Location	Nucleotide change	Mutation designation
FPN1	Exon 3	C→A	A77D
	Exon 3	190 T→C	Y64N
	Exon 5	430 A→C	N144H
	Exon 5	431 A→C	N144T
	Exon 5	485–487 del TTG	V162del
	Exon 5	734 A→C	N144H
	Exon 6	744 G→T	Q248H
HAMP	Exon 2	93del G	–
	Exon 2	175 C→G	R59G
	Exon 2/ Intron2	IVS2+1 (−G)	Met50del
	Exons 2/3	IVS2+7 G→A	–
	Exons 2/3	IVS2+56 G→A	–
	Exon 3	166 C→T	R56X
	Exon 3	208 T→C	C70R
	Exon 3	212 G→A	G71D
HJV	Exon 3	220del G	V74fsX113
	Exon 3	NR	C80R
	Exon 3	253 T→C	S85P
	Exon 3	295 G→A	G99R
	Exon 3	295 G→?	G99V
	Exon 3	302 T→C	L101P
	Exon 3	391–403del	R131fsX245
	Exon 3	445delG	D149fsX245
	Exon 3	503 C→A	A168D
	Exon 3	509 T→C	F170S
	Exon 3	516 C→G	D172E
	Exon 3	573 G→T	W191C
	Exon 3	615 C→G	S205R
	Exon 3	NR	I222N
	Exon 4	749 G→T	G250V
	Exon 4	806–807insA	N269fsX311
	Exon 4	NR	I281T
	Exon 4	863 C→T	R288W
	Exon 4	954–955insG	G319fsX341
	Exon 4	959 G→T	G320V
	Exon 4	NR	R326X
	Exon 4	NR	C361fsX366
	Exon 4	1153 C→T	R385X
TFR2	Exon 2	64 G→T	V22I
	Exon 2	251 C→T	A75V
	Exon 2	84–88 ins C	E60X
	Exon 4	515 T→A	M172K
	Exon 5	741 C→G	I238M
	Exon 6	750 C→G	Y250X
	Exon 9	1154 C→A	A376D
	Exon 10	1391 A→G	R455Q
	Exon 16	594–597 del	AVAQ594→597 del
	Exon 17	2069 A→C	Q690P
	Exon 18	2255 G→A	R752H

NR= No report.
[a]These data do not include gene mutations that cause heritable forms of anemia associated with iron overload.

with a form of hemochromatosis (OMIM #604250) in several racial and ethnic groups.[31–36] The mutations originally reported in Sicilian families[31] were considered to be rare based on other investigators failure to observe them associated with iron overload in several racial/ethnic groups.[37,38] However, mutations in *TFR2* when present with *HFE* mutations could also modify the penetrance of an iron overload phenotype.[36]

Mutations in the ferroportin 1 gene (*FPN1*), located on chromosome 2q32, are associated with an autosomal dominant form of hemochromatosis (OMIM #606069) in several racial and ethnic groups.[39–48]

Although it is likely that there will be cases of iron overload due to various combinations of mutations in the genes just described, this possibility has not been extensively explored. The frequency in which mutations in these genes appear in various racial/ethnic groups varies. Thus, it is likely that the combination of mutations found associated with iron overload will also vary by racial/ethnic group. This will have to be considered in the selection of cohorts to assess these possibilities. Current large multiethnic screening studies in diverse geographic regions may provide answers to these questions and predict more precisely who will develop iron overload.[49]

MUTATION DETECTION

The mutations herein described have been detected by a variety of methods—all of which have been utilized to identify genes associated with other disorders. Mapping of putative genes involved in iron overload for the most part has been conducted by multipoint linkage analysis, followed by positional cloning and sequencing of the area of interest.[4,30] After a chromosome region is defined and the candidate gene sequenced, a comparison of sequences from patients and controls may reveal missense, nonsense, and splice site mutations or deletions, which results in a nonfunctional product or failure to synthesize the product. Once the sequence of the gene is known, primers of the various regions can be generated and used in PCR, followed by denaturing HPLC to detect mutations.[50] Once a given mutation has been identified, subjects can then be genotyped by use of PCR-SSP or PCR-SSCP, PCR-RFLP followed by gel or capillary electrophoresis, mutagenically separated PCR, heteroduplex analysis, PCR followed by dot blot analysis, mass spectrometry based on single nucleotide extension, and microchip arrays.[51,52] Primers specific for several mutations can often be used together in multiplex PCR, allowing for rapid comprehensive genotyping.[52] Most of these assays are robust. For example, the analytic sensitivity and specificity for detecting C282Y homozygosity are 98.4% and 99.8%, respectively.[53]

CONCLUSION

The increase in knowledge about hemochromatosis has raised the interest of health care providers and the public in this common yet frequently undiagnosed disorder. It is now clear that ''classic'' hemochromatosis is only one of many distinct iron overload disorders. However, it is possible to diagnose hemochromatosis and detect iron overload with the genotypic and phenotypic assays currently available before significant organ and tissue damage occurs. As more is learned about the genetics and environmental modifiers of hemochromatosis and iron overload, the morbidity associated with this disorder should be significantly reduced.

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Hereditary Hemorrhagic Telangiectasia (Rendu–Osler–Weber Disease)

Carlo Sabbà
Anna Cirulli
Ginevra Guanti
Università di Bari, Bari, Italy

INTRODUCTION

The Rendu–Osler–Weber disease or hereditary hemorrhagic telangiectasia (HHT) is a disease characterized by epistaxis, mucocutaneous telangiectases, and visceral arteriovenous malformations (AVMs) particularly in the brain, lung, gastrointestinal tract, and liver. Hereditary hemorrhagic telangiectasia is transmitted as an autosomal-dominant disorder whose phenotypic penetrance is age-dependent and nearly complete by age 40. Mutations in two different genes identify HHT type 1 and HHT type 2, respectively: endoglin (MIM#131195; XM_055188) located on chromosome 9q33–q34 and activin-like kinase type 1 (ALK-1) or ACVRL1 (MIM#601284; Z22533) on chromosome 12q13. The existence of a third locus has been predicted. Hereditary hemorrhagic telangiectasia type 1 is considered a more severe form of the disease with an earlier onset of epistaxis and telangectasia and an occurrence of pulmonary arteriovenous malformations higher than in HHT-2 subjects.

The heterogeneous clinical setting renders the diagnosis difficult, thus explaining why HHT was once considered a rare disease. Moreover, frequent misdiagnosis has led to an underestimation of the actual prevalence of HHT which currently is 10–20/100,000. Extensive experience is necessary with both the various clinical expressions of the disease and the instrumental diagnostic possibilities for a correct diagnosis. In fact, a single biological test is not yet available and diagnosis is made following the clinical criteria: recurrent epistaxis, mucocutaneous telangiectases, autosomal-dominant inheritance, and visceral involvement. The diagnosis is considered definite if patients demonstrate at least three of the above criteria, suspected in the presence of two criteria, and excluded if only one criterion is present.

Recent progress in the field of genetics has permitted the identification of many gene mutations, thus, facilitating the characterization of the at-risk members of the same family.

PATHOPHYSIOLOGIC AND GENETIC FEATURES

The first step in the telangiectasia formation consists a focal dilatation of postcapillary venules with predominant stress fibers in pericytes along the luminal border. As the venules enlarge, they become convoluted and form excessive layers of smooth muscle cells without elastic fibers and connect directly with the dilated arterioles.[1]

The molecular basis consists of a defect of the TGF-β signaling pathway which, in normal conditions, plays an important role in vascular remodeling and maintenance of vessel wall integrity.[2] The genes responsible for HHT, endoglin and ALK-1 represent a coreceptor and a type 1 TGF-β receptor, respectively. In particular, endoglin is a homodimeric transmembrane glycoprotein expressed predominantly in endothelial cells and is composed of disulfide-linked 90-kDa subunits with large extracellular domains and serine/threonine-rich cytoplasmic regions without consensus signaling motifs. Thus far, numerous mutations of *ENG* gene have been described and these mutations tend to be unique and family specific.[3,4]

ALK-1 has the characteristics of a Type 1 serine–threonine kinase receptor; in vitro, it binds to activin or TGF-β types 1, 2, and 3, but the natural ligand in vivo is unknown. ALK-1 mutations include insertions, deletions, and nonsense mutations and, *as in endoglin*, several of these mutations give rise to an unstable protein. Both ALK-1 and endoglin have been suggested to trigger HHT according to the haploinsufficiency model.[5–7]

The exact mechanism by which mutations of endoglin and ALK-1 induce the diffuse angiodysplasia is poorly understood. The transmission of the TGF-β signals from the cell surface to the nucleus requires a complex system of receptors and cytosolic Smad proteins. Endoglin modulates this transmission, forming a complex with TGF-β1 and TGF-β2 receptors on the endothelial cells. Upon association with type 2 TGF-β, the receptor endoglin is phosphorylated. The association with TGF-β2 receptor results in an increased phosphorylation of type

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1 TGF-β receptor which, in turn, phosphorylates Smad2. This activated complex associates with Smad4 and, entering the nucleus, regulates the transcription of the target genes.

A balance model for the role of ALK and endoglin in angiogenesis has been proposed. Activation of ALK-1 leads to the endothelial cell (EC) resolution phase where angiogenic factors, such as the vascular endothelial growth factor (VEGF), are repressed; when the TGF-β1 signal is mediated by ALK-5, it transforms ECs into the activation phase of angiogenesis. The ALK-1 pathway is disrupted in HHT type 2, leading to the dominance of the ALK-5 signaling route, thus stimulating the activation phase of ECs and resulting in arteriovenous connections between dilated venules and arteries. In HHT type 1 a reduced dosage of endoglin may give a significant decrease in TGF-β1 concentration, impairing the ALK-1 and ALK-5 pathways. Therefore, having a higher sensitivity to TGF-β1 than ALK-1, ALK-5 pathway predominates and induces the arteriovenous connections.[2] According to the proposed model, the overexpression of several vascular growth factors, such as VEGF, would result from an aberrant angiogenic signal in HHT and might participate in the formation and enlargement of the vascular malformations. In fact, in our recent study, we demonstrated increased levels of VEGF in the serum of HHT patients, in agreement with the VEGF stimulation synthesis proposed in the murine model.[8]

Recently, ALK-1 signaling has been demonstrated to have a role in arterialization and remodeling of arteries so that the arterioles rather than the venules are the primary vessels affected by the loss of an ALK-1 allele. Thus, contrary to the current view of HHT as a venous disease, the arterioles rather than the venules are the primary vessels affected by the loss of an ALK-1 allele.[9]

Genetic Testing

The search for mutations is time consuming and expensive because of genetic heterogeneity and the fact that mutations in either gene tend to span the entire coding sequence and are usually family-specific. Direct sequencing, SSCP, heteroduplex analysis, Southern blotting and RT-PCR are the techniques more frequently utilized for mutation screening. Considering that any endoglin mutation will determine a reduction by half in the detection of its cell surface expression, Abdalla et al.[6] have developed a mutation detection strategy based on the flow cytometry quantification of surface endoglin on newborns' HUVECs and on adult patients' activated monocytes followed by quantitative multiplex polymerase chain reaction (QMPCR). As HHT-2 patients show no reduction of steady-state endoglin expression, this method eliminates the necessity of screening for both HHT genes and allows the detection of large deletions and duplications involving whole exons, which would not be identified by the aforementioned screening procedures. Once the mutation has been identified, screening of the ''at-risk'' members of the family is important to identify the carriers of the gene alteration in order to potentially prevent or timely treat more serious sequelae. With this regard, the report of intracranial hemorrhage in neonates with family history of HHT[10] suggests the usefulness of prenatal diagnosis in order to ensure appropriate medical care for the mutation carriers at childbirth.

CLINICAL FEATURES

Nose

Recurrent epistaxis represents the most common and, frequently, the initial symptom of the disease, affecting more than 90% of HHT patients. It begins by the age of 10–20 years and becomes more severe over time. These episodes may be so mild as to never suspect the presence of HHT, or so severe as to require iron supplementation and multiple transfusions.[1] Although nose bleeding usually does not represent the prevalent cause of death in HHT patients, it significantly compromises the quality of life of the affected persons,[16] thus justifying the multiple efforts to find a definite therapy for this symptom.

Epistaxis treatments include cauterization, septal dermatoplasty, laser ablation, and transcatheter embolotherapy, but unfortunately, results have been controversial. Medical therapy includes estrogens, inducing a metaplasia in the nasal mucosa, and antifibrinolitic drugs.[1] Antifibrinolitic drugs are administered as a nasal ointment[12] or orally, leading to less-frequent epistaxis episodes.[13]

Skin

Mucocutaneous telangiectases are usually found later in life with regard to epistaxis and affect the lips, tongue, palate, fingers, face, conjunctivas, trunk, arms, and nail beds. Bleeding from these telangiectases is possible, but rarely clinically important. The therapeutic options, usually considered for esthetic reasons, include topical agents and laser ablations.[1]

Lung

Pulmonary arteriovenous malformations (PAVMs) represent a direct connection between a branch of the

pulmonary artery and a vein through a thin-wall aneurysm. Pulmonary arteriovenous malformations occur in 20–60% of the HHT population and prevalence varies according to the type of gene mutation.[14] The direct right-to-left shunts originating in PAVMs are responsible for dyspnea, fatigue, cyanosis, and polycytemia. Paradoxical embolization occurs when emboli (thrombotic or bacterial) originating in the venous circulation bypass the normal filtering system of the pulmonary capillaries, enter the arterial circulation, and occlude arteries in the various organs. Stroke, TIA, and brain abscesses represent the most frequent complications and often the initial manifestation of PAVMs.

Pulse oximetry and chest X-rays are deemed insufficient as screening procedures, whereas the echo-bubble is considered more accurate as it demonstrates the presence of microbubbles in the left atrium after an intravenous injection of agitated saline in four to five cardiac cycles. Angiography is regarded as the gold standard and remains fundamental for those patients requiring transcatheter embolotherapy which represents the best therapeutic approach.[1,15] Recent studies have shown that the echo-bubble remains positive after embolotherapy, even in patients without residual PAVMs visualized with angiography. This may reflect the presence of residual PAVMs that are too small to visualize using angiography.[16]

Brain

Cerebral arteriovenous malformations (CAVMs) may affect about 5–10% of HHT patients. Generally, the neurological symptoms in HHT depend on PAVMs, but it is not uncommon that seizures or paraparesis masks the presence of CAVMs.[1] A subarachnoid hemorrhage is the most severe complication of CAVMs, so severe as to justify screening procedures.[7] Angio-MRI and angiography represent the main diagnostic techniques. Current therapy consists of neurovascular surgery, embolotherapy, and stereotactic radiosurgery.[1]

Digestive Tract

Telangiectases of the digestive tract are similar in size and appearance to those of the nasal and oral mucosa and may affect the esophagous, stomach, duodenum, small bowel, and colon. The actual prevalence of digestive telangiectases is still unknown because of the difficulty of performing endoscopy of the entire digestive tract and because nonsymptomatic telangiectasic lesions are probably present in a larger proportion of patients than those currently diagnosed.

Hemorrhaging from gastrointestinal (GI) telangiectases tends to appear in the fourth or fifth decade of life, affecting 13–33% of HHT patients.[18] However, this is probably an underestimation of the problem as most episodes of hemorrhaging are attributed to epistaxis rather than to a genuine GI bleeding.

While the endoscopic diagnosis of GI telangiectases in the upper digestive tract and colon is relatively uncomplicated, the investigation of the source of small bowel bleeding is more complex. Recently, the wireless-capsule endoscopy has been performed to study HHT patients with obscure and uncontrolled GI bleeding.[19]

The management of the GI bleeding in HHT patients is difficult, especially when the telangiectases are multiple and diffused throughout the entire digestive tract. Photocoagulation or laser procedures are effective in the short term, but less satisfactory over longer periods. Pharmacological therapy with estrogen and progesterone has been demonstrated to reduce the need for transfusions in HHT patients with GI bleeding, but the mechanism responsible for this effect is unknown as yet.[1]

Liver

Vascular malformations in HHT predominantly consist in shunts from the hepatic artery to the hepatic veins; direct connections between portal and hepatic veins are less frequent. The typical clinical manifestations depend on the predominance of such shunts. High-output heart failure results from an anastomosis between the hepatic artery and hepatic veins; portal hypertension is caused by shunts from the hepatic artery to the portal vein; portosystemic encefalopathy is the result of portal to hepatic vein shunting. Finally, anicteric cholestasis is probably due to a hypoperfusion of the peribiliary plexus.[20]

Recently, the abdominal CT multislice scan has been performed in a prospective study revealing that 74% of HHT patients have liver AVMs, most frequently asymptomatic. Doppler ultrasonography has been proposed as the ideal tool for the screening and follow-up of HHT patients with liver involvement. Selective angiography of the hepatic artery is considered the gold standard for diagnosis of hepatic shunting although abdominal CT multislice scan has been demonstrated to be very accurate, safer, cheaper, and better tolerated by patients.[21]

Treatment options for liver involvement in HHT are rather limited. Segmental embolotherapy of the branch hepatic arteries has led to controversial results because of severe complications, such as liver necrosis. Liver transplantation has been performed with good results.[20]

CONCLUSION

In the last few years, the understanding of HHT has expanded rapidly and several diagnostic and therapeutic protocols have been proposed. On the other hand, the management of the disease is extremely complex because multiple organs are involved. The management of HHT

patients should aim at controlling local and systemic symptoms and preventing complications. Epistaxis greatly influences the quality of life of HHT patients and it should be treated especially when the episodes begin to be very frequent and severe. Oral tranexamic acid administration and estrogen therapy in women significantly reduce the frequency of the episodes and ameliorate the quality of life of these patients. Septal dermatoplasty should be proposed when severe epistaxis leads to life-threatening anemia.

In the follow-up of affected persons, the lung and the brain are of particular concern because each may contain clinically silent lesions that can result in sudden morbidity and mortality. Echo-bubble is widely recognized as the most accurate technique to screen PAVMs. Cerebral screening by MRI should be performed at least once during the lifetime of patients, preferably in childhood. Presymptomatic intervention by embolotherapy for both PAVMs and CAVMs may positively influence outcome.

The systematic screening of liver involvement has not been generally suggested because the hepatic shunts are frequently asymptomatic, but considering the high frequency of these lesions and the possibility of severe complications, adult patients should undergo abdominal echo-color-Doppler examination before being included in a follow-up program. Lastly, the screening of the GI telangiectases should be performed in the presence of a severe anemia only partially explained by recurrent epistaxis. Endoscopy permits the diagnosis and treatment of telangiectases located in the upper digestive tract and in the colon with laser coagulation, whereas the examination of the small bowel depends on the availability of capsule endoscopy which, however, cannot be considered yet as a screening procedure because of its high cost. In conclusion, at present, HHT management requires a multidisciplinary approach and the availability of several diagnostic and therapeutic options. Multispecialistic cooperation is needed to develop a specific biological test for HHT diagnosis in addition to new therapeutic approaches for the disease, such as gene replacement.

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Hereditary Nonpolyposis Colorectal Cancer (HNPCC) Syndrome

H

Shaun McKenzie
Heather Pierce
Nader Hanna
University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.

INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, also known as Lynch syndrome, is due to germline mutation in mismatch repair (MMR) genes. It represents a highly penetrant autosomal dominant disorder with classic Mendelian distribution. Although it lacks a pathognomonic phenotype characteristic often seen in some hereditary cancer syndromes, there are some fundamental clinical characteristics that should raise suspicion to the possibility of this diagnosis. A combination of genetic counseling, genetic testing, and aggressive endoscopic surveillance is paramount to management. Surgery remains the definitive therapy in HNPCC-affected patients, but the appropriate role for prophylactic resection remains to be delineated.

OVERVIEW

Sporadic colorectal cancer accounts for 75% of all cases and mostly occurs in persons over 50 years of age. Inherited genetic mutations account for 5% of cases, whereas familial colorectal cancer occurs in 20% of cases where there is no identifiable hereditary syndrome, suggesting genetic contribution and/or common exposures in these families. Hereditary nonpolyposis colorectal cancer accounts for 2–5% of all colon cancers. It has autosomal dominant inheritance pattern with gene penetrance for colon cancer of approximately 70–85%. Hereditary nonpolyposis colorectal cancer gene carriers also have increased risk for malignancy at several extracolonic sites (Lynch II or cancer family syndrome), primarily endometrial (5–10% of all endometrial cancer cases), gastric, hepatobiliary, pancreatic, renal pelvis, ovarian, brain, and small bowel carcinoma.[1] The risk of endometrial cancer is 40–60%, and the risk of all other extracolonic cancers is less than 10–15%. Furthermore, two other named familial syndromes are associated with HNPCC. The combination of HNPCC and cutaneous lesions such as adenomas, eptheliomas, carcinoma, keratoacanthomas, and epidermal cysts is known as Muir–Torre syndrome. The combination of HNPCC and CNS lesions, specifically glioblastoma multiforme, is known as Turcot's syndrome.

Hereditary nonpolyposis colorectal cancer results in accelerated tumorigenesis from polyp to cancer in 1–2 years. Males have overall increased risk, with early age of onset about 45 years. HNPCC-related colorectal cancer accounts for 15–20% of all colorectal cancers diagnosed before the age of 45. Clinicopathological features of colorectal cancers in HNPCC are summarized in Table 1. Multiple colon cancer develops both synchronously and metachronously in patients with less than total abdominal colectomy[2] (about 40% within 10 years). In patients treated with total colectomy and ileorectal anastomosis, the risk of rectal cancer is 1% per year for the first 12 years making annual endoscopic examination of the rectal stump mandatory.[3] In HNPCC syndrome, endometrial and ovarian cancers have a similar mean age at onset (during the fourth decade) to colorectal cancer and usually precede that of sporadic gynecologic malignancy by 15 years. The risk of endometrial cancer (60%) in mutation-positive women exceeds that of colorectal cancer (54%).[4] Ovarian cancer occurs 3.6–13 times more frequently than expected in the general population.[4]

GENETIC ALTERATIONS IN HNPCC

Hereditary nonpolyposis colorectal cancer occurs due to inherited germline mutations in one of at least five mismatch repair (MMR) genes including *hMLH1* at 3p21.3, *hMSH2* at 2p22–p21, *hMSH6* at 2p16, *hPMS1* at 2q31–33, and *hPMS2* at 7p22. The products of these five genes all participate in a multimeric DNA mismatch repair complex. Germline mutation of MMR genes results in the accumulation of somatic errors within the genome at an accelerated pace, leading to an increased risk and early age at onset of malignant neoplasm. A molecular hallmark of HNPCC is a high level of microsatellite instability (MSI), which is a variation in the length of short repeat

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Table 1 Clinicopathological features of HNPCC-associated colorectal cancers

Characteristic clinical features:[14]
– Early age of onset about 45 years
– 70% of tumors are proximal to splenic flexure
– Approximately one-third of all tumors occurring in the cecum
– Synchronous and metachronous lesions
– One in five patients diagnosed with HNPCC develops rectal cancer as the index colorectal lesion
– Improved survival compared with sporadic colorectal cancer
Distinct pathological (but not pathognomic) features:[14]
– High grade: mucinous and poor differentiation
– Prominent peritumoral lymphocytic infiltration
– Crohn's disease-like reaction
– Tumor infiltrating lymphocytes
– High level of microsatellite instability (MSI)

DNA sequences that results from this generalized genomic instability. High levels of MSI are found in the DNA from colorectal tumor tissue, but not in the adjacent normal colorectal mucosa, in approximately 90% of individuals with germline mutations in an HNPCC-associated gene, although high levels of MSI are also detected in about 15% of sporadic cases of colorectal cancer.[5]

Mutations in *MLH1* and *MSH2* account for the majority of cases of HNPCC.[6]

Mutations in *MSH6* are thought to account for up to 10% of HNPCC families and are associated with higher frequency of endometrial cancer. Mutations in *PMS1* and *PMS2* have been reported to be associated with only a few HNPCC families.[7] Although the majority of mutations identified in *MLH1* and *MSH2* are small genomic insertions or deletions that result in a truncated protein product, it is estimated that up to 20% of families meeting the Amsterdam criteria may have large deletions or other genomic rearrangements in *MLH1* or *MSH2* that are not detectable by conventional testing techniques.[8]

CLINICAL DIAGNOSIS AND GENETIC TESTING

Diagnosis of this syndrome requires evaluating a combination of clinical criteria including a thorough family history, histological tumor characteristics, and consideration of genetic testing. The International Collaborative group for HNPCC initially developed the Amsterdam Criteria to help identify potential HNPCC patients.[9] However, 60% of families with a known germline mutation do not meet the criteria for the disease,[10] and only 40–80% of patients meeting the criteria have an identified germline mutation.[11] The revised Amsterdam criteria were developed to include extracolonic malignancy.[12] These criteria increased sensitivity by 12%,[13] but the identified MMR mutations were found in only 5–50%.[11] Subsequently, the Bethesda guidelines (Table 2) were developed to help identify patients who should undergo microsatelite instability analysis.[15]

Strategy for molecular testing for HNPCC varies among clinical centers, but generally is performed as a multistep process. At some centers, MSI testing and immunohistochemistry for MLH1 and MSH2 proteins are performed on colorectal tumor tissue from an individual putatively affected with HNPCC prior to consideration of germline *MLH1* and *MSH2* testing, as it may help more accurately target individuals who are likely to carry germline mutations.[16] This strategy has been endorsed by the American Gastroenterological Association in its position statement on genetic testing for hereditary colorectal cancer.[17] Tumors are considered to have high MSI when two or more of a panel of two mononucleotide (Bat 25 and Bat 26) and three dinucleotide repeats (D5S346, D2S123, and D17S250) show differences between the length of the microsatellite repeat regions amplified in tumor and normal DNA. Once high level of MSI is detected in tumor DNA, germline testing (typically performed on peripheral lymphocytes) by sequencing, conformational-sensitive gel electrophoresis (CSGE), or single-strand conformation polymorphism (SSCP) analysis for mutations in *MLH1* and *MSH2* is considered. Loss of MLH1 or MSH2 protein

Table 2 Bethesda guidelines for testing of colorectal tumors for MSI

– Individuals with cancer in families that meet the Amsterdam criteria
– Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers
– Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age less than 45 years, and the adenoma diagnosed at age less than 40 years
– Individuals with colorectal cancer or endometrial cancer diagnosed at age less than 45 years
– Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age less than 45 years
– Individuals with signet-ring-cell-type colorectal cancer diagnosed at age less than 45 years
– Individuals with adenomas diagnosed at age less than 40 years

Source: From Ref. [15].

staining by immunohistochemistry may direct which gene to target for germline analysis. Individuals with low or absent levels of MSI in tumor DNA are unlikely to harbor germline MMR gene mutations, and germline testing is usually not pursued. If germline testing reveals a mutation in a putatively affected family member, the HNPCC diagnosis is confirmed, and genetic testing of at-risk family members will provide true positive or negative results. If no mutation is detected, or if a variant of unknown clinical significance is detected, further genetic studies for large genomic rearrangements may be pursued, or segregation analysis in families carrying the same variant may be initiated. Ultimately, if a mutation cannot be identified, further testing of at-risk relatives should not be pursued, because testing will not be conclusive; a negative result could be a false negative because current testing may not be capable of detecting a mutation even if present. If MSI testing is not possible in the affected individual because of the absence or condition of the tumor specimen, or if the first three conditions of the modified Bethesda criteria are met, AGA guidelines support consideration of initial germline testing in the affected individual. Although it is possible to initiate germline testing with an at-risk family member when an affected family member is not available for testing, this strategy can only yield positive or inconclusive results. In this situation, only the identification of a mutation in a family member can enable a true negative result.

Genetic testing should be offered when results could influence the medical management of that patient or the patient's family members. It should always be done in the setting of pre- and posttest genetic counseling, as endorsed by the American Society of Clinical Oncology in its position statement on genetic testing for hereditary cancer.[18] The process of genetic counseling includes providing risk assessment, educating patients about testing options, discussing the implications for medical management, and providing supportive counseling to the individual and their family.

SURVEILLANCE

Once a diagnosis of HNPCC has been made, or the diagnosis is indeterminate based on genetic analysis, aggressive screening protocols should be implemented. Although screening programs in average-risk people prevent death from colorectal cancer, no data are available regarding the effectiveness of surveillance in hereditary colorectal cancer syndromes, as people with genetic risks are excluded from colorectal screening trials and a randomized trial with a no-screening arm for patients with genetic risk is not feasible. Observational studies performed on families with HNPCC and FAP suggest a stage shift toward earlier stages and a probable reduction in colorectal cancer mortality among screen-detected cancers. In HNPCC, current recommendation for surveillance includes full colonoscopy starting at age 20–25 years (or 5 years younger than the youngest age at diagnosis in the family) and repeated every 1–2 years.[19] Annual colonoscopy should ensue at age 40. Rectal stump surveillance with annual sigmoidoscopy should continue in patients after subtotal colectomy. This level of screening has been shown to decrease the risk of colorectal cancer by greater than 50% and 65% fewer colorectal cancer deaths.[20,21] Screening for extracolonic malignancy in Lynch II syndrome has not proven as beneficial as that for colorectal cancer because of lack of sensitivity for the available screening modalities. Nevertheless, recommendations exist. For endometrial and ovarian cancer, annual transvaginal ultrasound with endometrial aspirate cytology and CA-125 is recommended starting at age 25–35 and repeated every 1–2 years.[19] While upper GI endoscopy and genitourinary ultrasound with urinalysis and cytology are not recommended in the entire HNPCC population, they may be helpful and are recommended in pedigrees that show a propensity toward malignancy at those sites.

CHEMOPREVENTION

The possibility of chemoprevention in these patients is an attractive area of research. Epidemiological studies have shown that chronic ingestion of nonsteroidal antiinflammatory agents (NSAiDs) reduces the incidence of colorectal cancer and that continued use of oral contraceptive pills (OCPs) decreases the incidence of both ovarian and endometrial cancer.[22] Three separate studies have shown no benefit of oral calcium supplementation decreasing cancer risk in HNPCC patients.[23] No studies have evaluated the role of OCPs in women with HNPCC.

SURGICAL MANAGEMENT

Once colorectal polyps are found, the only effective management is total abdominal colectomy with ileorectal anastomosis. In HNPCC, endometrial and ovarian cancers have a similar mean age at onset to colorectal cancer; therefore prophylactic total abdominal hysterectomy with bilateral salpingo-oophorectomy should be considered at time of colon surgery if the patient is postmenopausal or has completed her family. The success of polypectomy during colonoscopy and the fact that 15–30% of mutation-positive carriers may not develop colorectal cancer have fueled the debate over

prophylactic surgery vs. rigorous endoscopic surveillance and endoscopic polypectomy. Therefore, decision and timing for prophylactic subtotal colectomy should be individualized and based on the histological characteristics of the polyp(s), compliance with surveillance program, and patient's personal preferences.

CONCLUSION

Hereditary nonpolyposis colorectal cancer is the most commonly occurring familial colorectal cancer syndrome. Knowledge of the clinical and pathological characteristics of the syndrome and the possible association with extracolonic malignancies is crucial for identifying patients and families that would benefit from genetic counseling and testing. Management strategies to address the increased cancer risk in carriers include targeted surveillance and prophylactic surgery. Surgery limitations and related adverse effects on morbidity and quality of life should always be discussed in extensive preoperative counseling. Chemoprevention may prove helpful but remains in a state of discovery.

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Heteroduplex Analysis (HA)

Philip L. Beales
Institute of Child Health (UCL), London, U.K.

INTRODUCTION

The Human Genome Project has now provided a reestimate of the total number of genes within the human genome, and as specific links are made between genes and disease, the need for simple and cost-effective gene mutation detection becomes increasingly important. Nonetheless, the choice of test undertaken for any given screen presents a challenge, and in doing so, a number of considerations should be taken into account: 1) the level of throughput required; 2) the sensitivity and specificity permissible; 3) the resources available; 4) the speed with which a result is needed; and 5) cost.

Direct sequencing, for long considered the gold standard of mutation detection, is still costly and time-consuming; however, in terms of simplicity, heteroduplex analysis (HA) is unsurpassed in its application and analysis. This chapter will review the technique, its applications, and the latest developments which keep HA at the forefront of scanning technologies.

HETERODUPLEX ANALYSIS

More than 90% of disease-related mutations are caused by ''microlesions'' [single-base substitutions or small insertion/deletions (indels)] rather than large lesions (such as rearrangements or large indels) (Human Gene Mutation Database[1]). Given this overrepresentation of microlesions, it is paramount that mutation detection methods are sensitive to single-base substitutions. The principle of heteroduplex analysis relies on the formation of heteroduplex species formed when there is a complementary sequence mismatch in a double-stranded DNA fragment. The electrophoretic mobility of a heteroduplex is altered relative to a homoduplex and in general tends to retard migration of the fragment. Clearly, if an alteration is homozygous no heteroduplex species will be formed and therefore it will be necessary to mix wild-type and mutant fragments together prior to the denaturation and annealing steps to generate four species: a wild-type homoduplex, a mutant homoduplex, and two heteroduplexes. The occurrence of heteroduplex fragments was, in fact, first reported as a PCR artefact.[2]

Bhattacharyya and Lilley[3] predicted the formation of two forms of heteroduplex depending on the type of underlying mutation. A small insertion or deletion produces a ''bulge'' as a result of a relatively large stretch of unmatched bases in the duplex (Fig. 1). Such ''bulges'' have been directly visualized on electron microscopy.[4] In contrast, a ''bubble'' will form at the site of a base substitution. The net effect on migration of a ''bulge'' heteroduplex is much greater relative to a homoduplex, owing to the bending of the DNA at the site of the bulge. In fact, the larger the sequence mismatch, the greater the bend and, consequently, the greater is the degree of retardation. In contrast, the ''bubble''-type heteroduplex produces only subtle perturbations of structure, owing to less bending of the DNA and hence relatively minor migration defects. Nonetheless, the migration of these species can be further influenced by factors such as the length of fragment and even the nature and position of the mismatch.[5]

Heteroduplex Analysis Matrices

At its earliest inception, the sensitivity of HA was limited by the availability of suitable gel matrices. Slab–gel systems are limited to agarose and polyacrylamide. The improved MDE gel solution (Cambrex) is a polyacrylamide-like matrix with a high sensitivity for DNA conformational changes. The gel's unique structure allows DNA separation on the basis of both size and conformation, thus increasing the probability of detecting sequence differences from as low as 15% achieved in standard polyacrylamide gels to approximately 80%.

A number of modifications to the matrix have been reported to improve the gel's ability to enhance fragment retardation. For example, the use of mild denaturants which exaggerate the effects of the mismatch forms the basis of conformation-sensitive gel electrophoresis (CSGE).[6–8] Ganguly et al.[9] have adapted the technique for use with fluorescently labeled fragments which can then be resolved using the ABI 377 (Applied Biosystems) DNA sequencer platform. They labeled PCR products with either 6-FAM, HEX, or NED prior to heteroduplex formation.

An array of matrices is now available for use with capillary electrophoresis such as cellulose derivatives and hydrophilic polymers substantially improving the resolution of HA.[10–12] One of the earliest reports to describe

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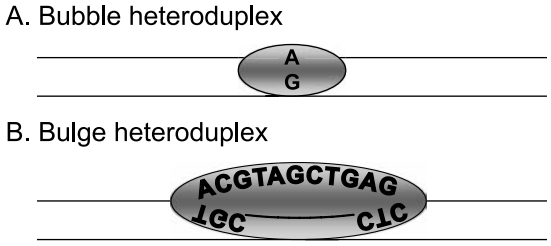


Fig. 1 Types of heteroduplex. (A) ''Bubble-type'' heteroduplex forms in the presence of one or more base substitutions. (B) ''Bulge-type'' heteroduplex forms with larger lesion such as insertion or deletion. (*View this art in color at www.dekker.com.*)

the adaptation of HA to capillary electrophoresis employed an entangled polymer matrix under nondenaturing conditions.[13] Rozycka et al.[14] adapted the technique for use with a single capillary fragment analyzer (ABI 310, Applied Biosystems) and were able to achieve high sensitivity in products of <350 bp. Such systems utilize replaceable linear polyacrylamides (LPAs) offering lower viscosity, high resolution, and rapid replacement of matrix thus permitting multiple reuse of the capillary. To attain high-throughput coupled with a greater resolution, Gao and Yeung[15] described temperature-gradient capillary electrophoresis. They exploited the lower melting temperature of heteroduplexes relative to homoduplexes and applied a temperature gradient to partially denature the DNA fragments during electrophoresis. By covering the entire range of melting temperatures of duplexes, they were able to achieve a high sensitivity for single-base substitutions set in a 96-well format. Our group, in an effort to raise the level of throughput without compromising sensitivity, designed a modification called multiplex capillary heteroduplex analysis (MCHA) using the 96-capillary MegaBACE1000 DNA Analyzer platform (Amersham Biosciences).[16] We described an additional level of throughput achieved by multiplexing up to six differently labeled (6-FAM, HEX, or TET) or sized products (up to 494 bp) in a single well. This coupled with 96-sample capability per run allows up to 576 samples in 1 hr or as many as 3000 samples in a day. Multiplex capillary heteroduplex analysis is extremely effective at detecting known alterations (specific) but has proven to be a useful scanning tool for the detection of novel mutations (Fig. 2).

Sensitivity of HA

The sensitivity of heteroduplex analysis has consistently been reported to be high when utilized alone or together with another complementary technique such as single-strand conformation polymorphism (SSCP). Rosetti et al.[17] compared conventional horizontal gel HA with SSCP using known mutations from four different genes. Heteroduplex analysis detected 93% of mutations compared with 67% using SSCP; however, when combined, all mutations were revealed.

A comparison of HA, SSCP, and DGGE used for detecting apolipoprotein mutations in a large cohort of patients with hypercholesterolaemia revealed HA and DGGE to be equally as effective but superior to SSCP.[18]

Recently, Crepin et al.[19] designed an assay combining HA and an SSCP variation they called mutation detection gel analysis (MDGA) for the detection of multiple mutations in MEN1 using fluorescently labeled PCR fragments. Heteroduplexes were resolved on a 0.5 × MDE gel and for MDGA, single-stranded products on an Elliomut gel (Elliotek, Paris), both electrophoresed as a slab using an automated sequencer (ABI 377). Peaks were compared with controls and abnormal profiles subsequently sequenced to reveal the true nature of any underlying mutation. They demonstrated that each mutation cast a specific and reproducible pattern dependent on the base change. Heteroduplex analysis detected 88.5% of mutations and 100% when combined with MDGA. The authors questioned the specificity of the tests and suggested this be remedied by reducing the fragment length to less than 400 bp. They concluded that even if the positive predicted value of the combined test does not reach 100%, the negative predictive value does, a point of critical importance in genetic testing.

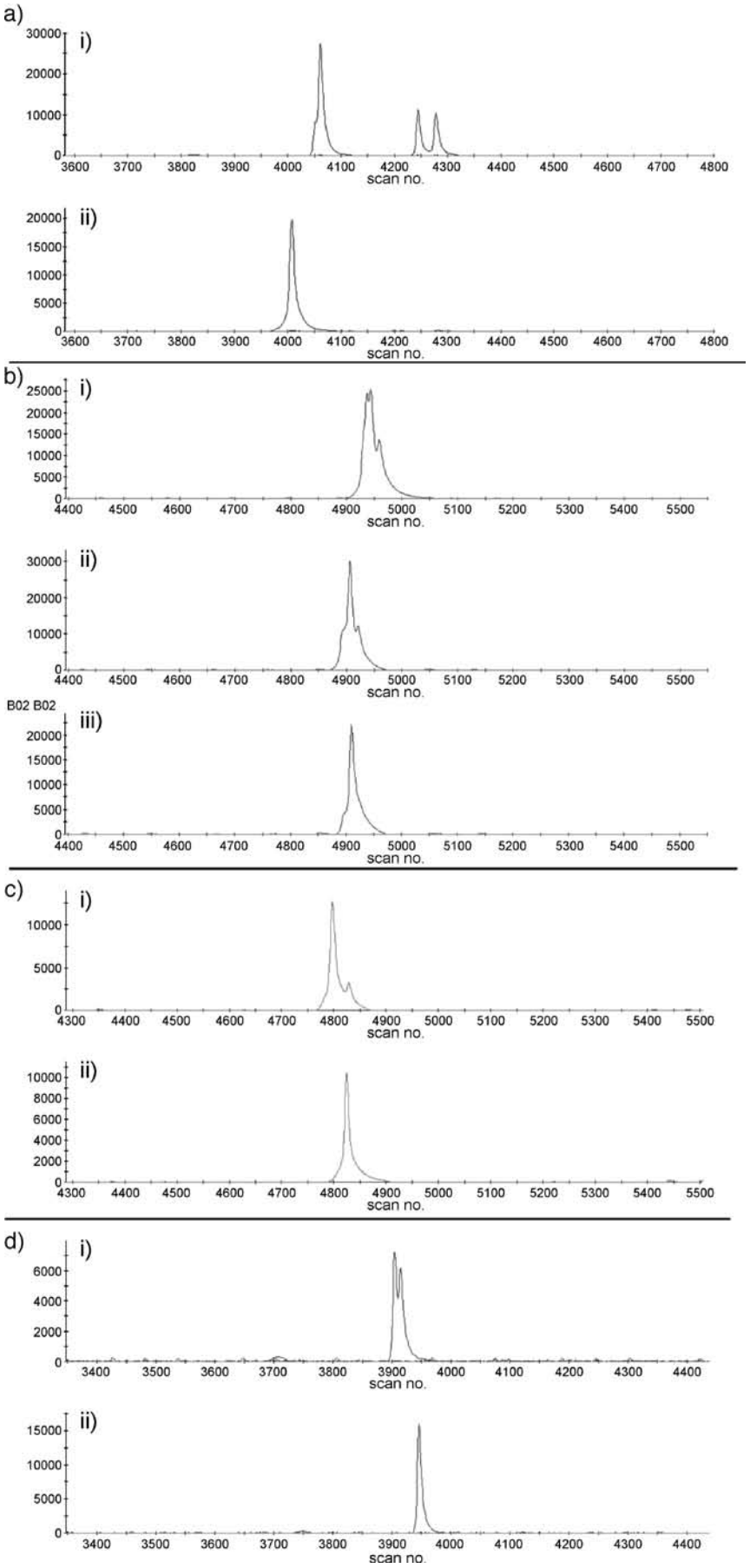
The study of Hoskins et al.[16] which tested 12 sequence changes (2 deletions and 10 substitutions) in four Bardet–Biedl syndrome genes concluded that HA when adapted for use on a 3% nondenaturing capillary injected matrix (LPA) gave outstanding sensitivity (100%) alone. Furthermore, they calculated the sample cost to be at least one-tenth that for direct sequencing.

Factors Influencing Sensitivity

A number of factors are now known to influence the ability to detect single-base substitutions, which include the length of the fragment, the position of the mismatch within the fragment, the GC content, and the nature of the mismatch.

Fragment length

Very short (<150 bp) or very long (>600 bp) fragments are considered unsuitable for HA. Rozycka et al.[14] showed a reduction in sensitivity as the fragment length increased above 350 bp. Hoskins et al.[16] using a capillary gel were able to detect mismatches in fragments of up to 500 bp with no deterioration in sensitivity.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Position of mismatch

Mismatches within 50 bp of the product start and end are difficult to detect.[14] Therefore, attention to the design of amplification products is paramount.[16]

GC content

The GC content of the fragment in question and the sequence flanking the change can influence the ability of HA to detect the mismatch. This is thought to be due to the three-dimensional conformation adopted by the double-stranded DNA which in turn is dependent on the GC content. The relationship of the GC-rich sequence in the region surrounding the mismatch will also influence detection rates especially if the mismatch is buried in the heteroduplex structure compared with a more superficial location.

Nature of the mismatch

The presence of a mismatch, e.g., a C>T within a double-stranded fragment, will generate four species: a wild-type homoduplex (composed of C:G), a mutant homoduplex (T:A), and two heteroduplexes, one containing a C:A mismatch and the other a T:G. Highsmith et al.[5] designed a DNA ''toolbox,'' a set of molecules of varying length and GC content containing all possible mismatches at a particular location, to determine which factor had the greatest effect on the ability to detect a single-base substitution. They reported that the factor with the greatest effect was the nature of the nucleotide change; the order of detection being G:G/C:C>A:C/T:G>A:A/T:T. They concluded that this hierarchy of resolution was in agreement with the measured thermodynamic stability of oligonucleotides with different base-pair mismatches.[20–22] It was presumed that the larger the distortion due to several unstacked bases, the greater the differential migration in the gel compared with fully complementary strands.

In an analysis of all mutations recorded in the Human Gene Mutation Database (HGMD), Krawczak et al.[23] reported that 60% were transitions. One reason for the predilection toward transitions in any set of alterations is the relatively high incidence of CG>TG and CG>CA substitutions caused by deamination of methylcytosine. Methylated cytosines most commonly occur in CpG dinucleotides and are susceptible to deamination favoring the formation of thymine (CG>TG). CG>CA substitutions occur if, following deamination of a methyl-cytosine in the antisense strand, there is a miscorrection of G>A in the sense strand. In an update to the study of Hoskins et al.[16] (unpublished) these types of mutation accounted for 21% of substitutions and 33% of transitions consistent with Krawczak et al.'s findings. The majority of the remaining transitions were A>G substitutions, with five of seven adenines preceded by another adenine. The local DNA sequence environment may therefore influence the sensitivity of techniques such as MCHA.

ADAPTATIONS

The use of denaturing HPLC systems to resolve heteroduplexes from homoduplexes has been reported for many gene mutations and has been discussed in another chapter. Heteroduplex analysis has been adapted for the detection of specific mutations in numerous conditions including von Willebrand's disease, PKU, hemochromatosis, and rifampicin resistance in tuberculosis.[24–27] As discussed above, test samples which are heterozygous for a mutation provide for a straightforward preparation of DNA heteroduplexes without the need for annealing to a reference sample, an additional step which can be time-consuming. For single-base substitutions the conformational change is often relatively small, but to enhance this difference for specific mutations a special fragment, termed a universal heteroduplex generator (UHG), has been developed.[28] This synthetic sequence is similar to genomic sequence but contains specific substitutions or indels at nucleotide positions corresponding to and contiguous with known mutation sites within the genomic DNA. A PCR-amplified test fragment is mixed with the UHG, denatured, and then slowly cooled to form a heteroduplex which is then electrophoresed on a suitable gel (Fig. 3). If the test fragment contains a point mutation then the heteroduplex will contain both a bulge and bubble, and, consequently, will migrate much more slowly than a heteroduplex without the mutation or a homoduplex.

Fig. 2 Peak traces of multiplex capillary heteroduplex analysis (MCHA) depicting profiles for different categories of mutations. a) A 2-bp deletion in *BBS2* × 4. i) Homozygous patient sample mixed with control DNA giving four species: a mutant homoduplex, a wild-type homoduplex, and two heteroduplexes; ii) control DNA homoduplex. b) A G→C substitution in *BBS4* × 15. i) Patient sample; ii) patient sample mixed with control DNA; iii) control DNA homoduplex. c) *BBS4* × 16 SNP. i) Heterozygous sample mixed with control DNA; ii) control DNA homoduplex. d) A T→A substitution in *BBS2* × 4. i) Patient sample mixed with control DNA; ii) control DNA homoduplex. (From Ref. [16].) (*View this art in color at www.dekker.com.*)

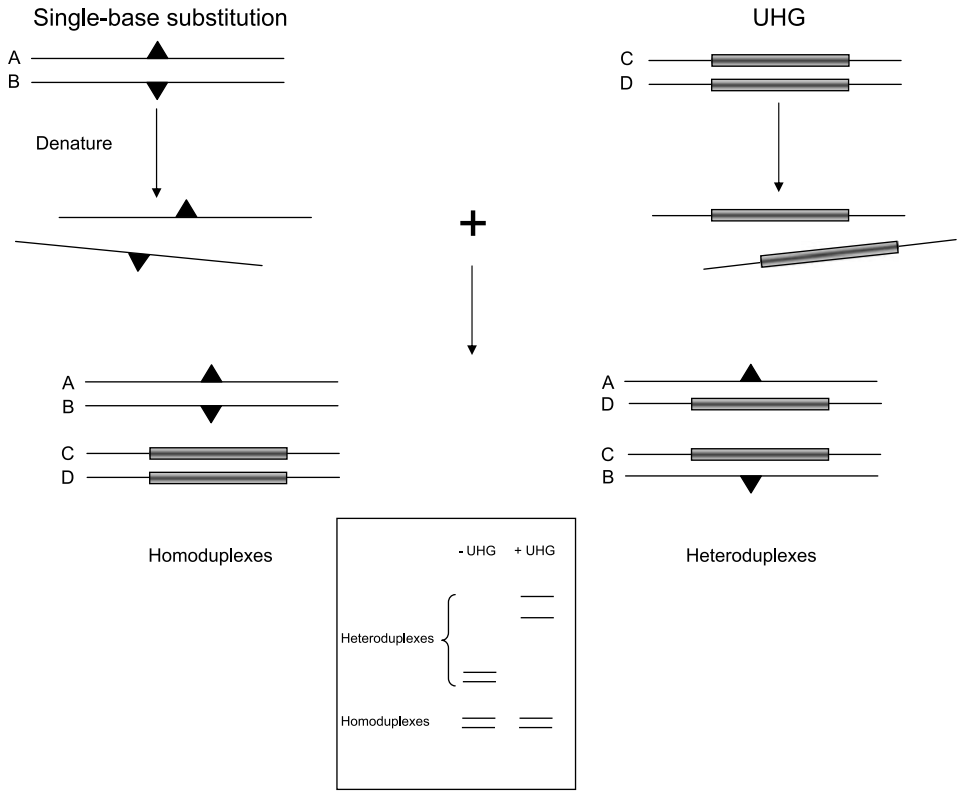

Fig. 3 Schematic of the principle of the universal heteroduplex generator (UHG). The artificial mutation enhances the bubble effect of an existing mutation resulting in greater separation on gel electrophoresis.

CONCLUSION

Despite continuing improvements to direct sequencing methodologies, SSCP and HA remain the most popular scanning techniques. Heteroduplex analysis, although similar in principle to SSCP, is considered to be the simpler and quicker of the two techniques. The relative sensitivities, although initially low, have now been substantially enhanced by the development of new gel matrices and, more recently, the adaptation to fluorescent capillary platforms (e.g., MCHA) enabling mutation detection rates close to 100%. The cost of HA compared with direct sequencing for scanning purposes is much more economical even when abnormal resulting fragments are subsequently sequenced to reveal the nature of the underlying mutation. Developments such as the universal heteroduplex generator allow the technique to be tailored to specific mutations.

Already a number of reports have considered the use of microchip-based electrophoresis in resolving heteroduplexes. Footz et al.[29] described a simple but sensitive method for specific mutation detection for *BRCA1* and *BRCA2*. They utilized a proprietary microfluidic chip (Micralyne, Edmonton, AB) consisting of a defined network of microchannels in glass, similar to conventional capillaries. The chip was filled with a commercially available sieving matrix (POP-6, Applied Biosystems) and loaded with 200–300-bp test PCR products. They experienced very high sensitivity rates for known mutations and reported rapid run times. Each run utilizes a very small quantity of PCR product (250 pL) and virtually no optimization is required. Clearly, similar efforts to increase throughput in a microchip-based system are on the horizon.

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HLA Typing by PCR-Based Techniques

J. -M. Tiercy
University Hospital, Geneva, Switzerland

INTRODUCTION

Tissue compatibility is determined by genes of the major histocompatibility complex, known as the human leukocyte antigen (HLA) system in humans. The HLA region comprises 12 genes that are characterized by an extensive degree of polymorphism. Initially detected by serology, HLA diversity has been found by molecular typing techniques to be much larger than anticipated, with over 1500 HLA alleles currently known and more to be disclosed. Three major polymerase chain reaction (PCR)-based methodologies are now used routinely for HLA genotyping: PCR sequence-specific primers (SSPs), PCR sequence-specific oligonucleotide probe (SSOP) hybridization, and direct sequencing of PCR products. The multigenic system, high heterozygosity rate, intralocus and interlocus sharing of sequence motifs, and the growing number of new alleles all make unambiguous HLA allelic assignments a complex issue in molecular diagnostics. The choice of the typing method will be influenced by the clinical setting, scaling of typing samples, emergency, and level of resolution required. Laboratories are facing several challenges: different clinical transplant programs call for different resolution levels, unambiguous allele assignments may require the use of more than one molecular technique, efficient quality assurance systems must be set up to control commercially available reagents, and updates should cope with newly identified HLA alleles.

HLA POLYMORPHISM

HLAs are encoded by 12 genes located in the MHC on chromosome 6. The multigenic HLA system comprises the most polymorphic loci of the human genome, with a total of 1572 alleles currently identified in worldwide populations (Fig. 1).[1] HLA molecules are structurally homologous cell surface glycoproteins, which are classified into class I (HLA-A, HLA-B, and HLA-C) and class II (HLA-DR, HLA-DQ, and HLA-DP) antigens as a function of their structure, tissue distribution, and characteristics in peptide presentation to T-cells. The biological function of HLA molecules is to present pathogen-derived peptides to T-lymphocytes. Because of their biological role as antigen-presenting molecules,[2] HLA diversity has been maintained in human populations to allow the widest possible range of foreign peptides being presented to T-cells. Peptide–HLA complexes are recognized by clonally distributed T-cell receptors (TCRs). TCRs are also able to recognize allogeneic HLA molecules at a high frequency, so that 1–10% of the peripheral blood lymphocytes of an individual can respond to a nonself HLA antigen. Thus immune responses against incompatible HLA antigens represent a major barrier to organ and hematopoietic stem cell (HSC) transplantation.

HLA antigens were initially discriminated by serology (i.e., complement-dependent cytotoxicity assays using alloantisera from multiparous women immunized against paternal HLA antigens). However, it was soon realized that serologically defined antigens could, in fact, be split into subtypes (alleles): first, by in vitro cellular assays, and, second, by DNA sequencing analyses (Fig. 1).[1,2] For example, the 21 DR serotypes are now subdivided into 342 alleles with widely different frequencies in different populations. A centralized repository (http://www.ebi.ac.uk/imgt/hla) of the sequences of all HLA alleles named by the World Health Organization (WHO) Nomenclature Committee for Factors of the HLA System is a very useful tool for clinical laboratories. Early recognition of the limitations of serological typing led to the rapid development of DNA typing techniques combining PCR and the knowledge of sequences of the polymorphic exons of HLA genes. HLA genotyping can be viewed as a parallel analysis of multiple regions of variation (single nucleotide polymorphism, or SNP) and the complexity of the analysis resides in the high number of SNPs in coding regions and the sharing of SNPs among HLA alleles.

Major applications of HLA genotyping concern clinical transplantation programs (i.e., solid organ and HSC),[3,4] HLA–disease associations (e.g., narcolepsy, ankylosing spondylarthritis, Behçet's disease, Birdshot retinochoroidopathy, rheumatoid arthritis, insulin-dependent diabetes mellitus type I, and coeliac disease), and vaccination protocols (e.g., melanoma-specific antigens). HLA polymorphisms are also widely used as informative markers in population genetics studies.[5]

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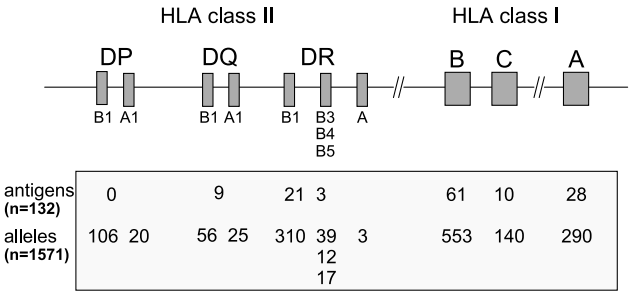

Fig. 1 Schematic representation of HLA class I and class II loci in the MHC that comprises >200 genes on the short arm of chromosome 6. The corresponding number of antigens (as defined by serology) and alleles (as defined by nucleotide sequence) is indicated for each locus. (From Ref. [1].) About 10% of the alleles assigned by the Nomenclature Committee are characterized by silent substitutions. Three HLA class I genes (A, B, and C) located at the telomeric end of the MHC encode, respectively, for the heavy chains of HLA-A, HLA-B, and HLA-C antigens that pair with β_2-microglobulin. (From Ref. [2].) Polymorphic residues are essentially located in the α_1-domains and α_2-domains encoded by exons 2 and 3, respectively, which form the peptide-binding site. HLA class II antigens (DR, DQ, and DP) are heterodimers encoded by an α-chain and a β-chain gene that colocalize at the centromeric part of the MHC. Essentially all of the polymorphisms are located in exon 2 (α_1-domain) of β-chain genes, whereas the *DRA* gene is non-polymorphic, and DQA1 and DPA1 loci exhibit a low level of polymorphism. The HLA-DR subregion presents an additional complexity level with the presence of a second *DRB* gene in most haplotypes: DRB3, DRB4, or DRB5. The nonclassical polymorphic HLA genes—*HLA-DM*, *HLA-DO*, *HLA-E*, *HLA-F*, *HLA-G*, *MICA*, and *MICB*—that code for molecules implicated in the immune response are also located in the MHC. (*View this art in color at www.dekker.com.*)

LEVELS OF RESOLUTION

Three different levels of resolution for HLA typing are usually recognized (Table 1). Low-resolution typing, also referred to as *generic* typing or *two-digit* typing, corresponds to the identification of broad families of alleles that cluster into serotypes (e.g., A*02), and is the equivalent of serological typing (A2). High-resolution typing (four-digit typing) allows the discrimination of individual alleles within each serotype (e.g., A*0201). Intermediate resolution provides information on a limited number of alleles that could be present in a given individual, excluding all other known alleles. In clinical transplantation setting, low-resolution HLA typing is required for solid-organ transplant programs, whereas high-resolution typing is required for HSC transplantation, particularly for unrelated donor matching.[3,4] For HLA–disease association studies, some diseases require low-resolution typing only (B27 for ankylosing spondylarthritis, and B51 for Behçet's disease), whereas other diseases rely on high-resolution typing (DQB1*0602 for narcolepsy).

THE TECHNIQUES

Restriction Fragment Length Polymorphism

In the early age of molecular typing, restriction fragment length polymorphism (RFLP) allowed to discriminate not only the serotypes, but also some groups of alleles subdividing serotypes, particularly for DRB and DQA1/DQB1 loci.[6] However, most polymorphic restriction sites were located in noncoding regions and the technique, although very useful in the late 1980s, was not resolutive enough to cope with the continuously growing HLA allelic polymorphism and was too cumbersome for routine clinical use.

PCR-SSOP

The combination of PCR technology and hybridization with SSOPs was first applied to HLA class II typing[7,8] because of the limitations of DR serology and of the better knowledge of allelic polymorphism at DR/DQ loci. The procedure relies on the locus-specific amplification of the genomic DNA segment comprising the polymorphic sites of HLA alleles, exon 2 for HLA class II genes, and exons 2+3 for class I genes. Amplified DNA is then immobilized on a solid support (dot blot), usually a nylon membrane, and then hybridized with a battery of SSOPs (direct hybridization). The higher the number of probes, the better is the resolution level. The method is well suited for large-scale typing, as many DNA samples can be tested in a single hybridization step, but it requires simultaneous hybridization with a large number of probes for which each melting temperature has to be optimized.

Alternatively, probes are fixed on a solid support, a membrane,[9] a microtiter tray,[10] or microbeads, and a single DNA sample can by genotyped in a single hybridization step. Ideally suited for rapid HLA typing as required for clinical transplantation, the method has now been developed by a number of commercial companies that have automatized the hybridization/washing/reading steps, thereby allowing high-throughput typing. Usually 50–100 probes per locus are used for intermediate/high-resolution typing.

PCR-SSP

The procedure relies on the specificity of primer extension that is matched or mismatched with the template at its 3′

Table 1 HLA nomenclature and levels of resolution

HLA	Definition	Resolution
A2	Refers to the A2 antigen defined by monospecific/polyspecific antisera	Low
A*02	Any of the 64 known A*02 alleles (A*0201–0264)	Low
A*0205/0208/0214	Any one of these three alleles; other A*02 alleles are excluded	Intermediate
A*0201	Allele defined	High
A*0232N	Allele with a substitution in the coding sequence that leads to a stop codon (null allele)	High
DRB1*030101 and DRB1*030102	Two DRB1*0301 alleles that differ by a silent substitution in exon 2	High
A*24020102L[a]	A*2402 allele with low expression due to a mutation in intron 2 sequence	High

[a]The first two digits refer to the serotype (A*24), the third and fourth digits define substitution(s) in the coding sequence (A*2402), the fifth and sixth digits describe synonymous substitution(s), and the seventh and eight digits refer to substitution(s) in intron or 5′ or 3′ sequences. N and L mark alleles with no or low surface expression, respectively.

end. A combination of two primers designed for each of the two polymorphic sequence motifs in cis allows the identification of an allele or a group of alleles that are characterized by these two motifs. The presence or absence of the two motifs in cis is usually detected by gel electrophoresis,[11] but other detection methods have been developed.[12] The method is rapid and ideally suited for a small number of samples. The method can be applied for low-resolution typing, using primer combinations that detect all alleles within a given serotype, as well as for high-resolution typing. Depending on the HLA phenotype, a combination of 300–400 different primer mixes is necessary to determine the HLA-A/B/C/DRB1/DRB3/DRB4/DRB5/DQB1 alleles in a given individual.

Direct Sequencing

The gold standard remains the complete sequencing information of HLA alleles. In routine practice, usually only the most polymorphic exons are sequenced: exon 2 (270 bp) for class II, and exons 2+3 (540 bp) for class I genes. A number of sequencing protocols have been published so far (e.g., Ref. [13]). These are based either on locus-specific PCR and sequencing of both alleles at once, which is faster but implies possible ambiguities in sequence interpretation, or on group-specific PCR that allows to separate the alleles, which is more time-consuming but avoids ambiguities. Protocols validated through the 13th International Histocompatibility Workshop are available at the following web site: http://www.ihwg.org/components/sbtover.htm.

Conformational Analyses

Discrimination of HLA alleles can also be achieved by analyzing variations in DNA conformation (secondary structures of PCR products) by polyacrylamide gel electrophoresis. Single-strand conformation polymorphism or heteroduplex analyses have been implemented by using a reference strand in duplex formation, which increases the resolution power of the technique.[14] The reference strand conformation-mediated analysis (RSCA) method, as a high-resolution technique, can be applied for donor selection in the HSC transplantation setting.

Microarrays

A few pilot studies, based on known polymorphisms, have demonstrated the feasibility of genotyping HLA-B alleles by SSOP hybridization with 137 probes[15] immobilized on glass supports, or five HLA-A exon 2 polymorphic sites using ligase detection reaction.[16] Direct SSOP hybridization with >9000 test samples spotted on a glass slide has been reported.[17] Therefore the development of microarrays in the field of HLA typing is still in its infancy.

THE CHOICE OF THE METHOD

Clearly, two parameters will determine the choice of the methodology: the level of resolution required (low, intermediate, and high) and the clinical setting (i.e., organ or HSC transplantation, HLA–disease associations, bone marrow donor registry screening, and population genetics studies). For solid-organ transplantation where the method has to be fast and of low resolution, PCR-SSP or reverse PCR-SSOP hybridization should be an adequate technique. For high-throughput typing, such as for bone marrow donor registry recruitment or population genetics studies, reverse PCR-SSOP hybridization is well suited, whereas for final stem cell donor matching (high resolution), PCR-SSP/SSOP or RSCA combined

with direct sequencing should provide the highest level of resolution.

NEW HLA ALLELES

Approximately 50–100 new alleles are discovered each year and most of these are classified in a given serotype based on sequence homology only. Programs for organ sharing and bone marrow donor searches are still largely based on the serological types defined by microlymphocytotoxicity assays. Through international workshops and collaborative studies, data that link individual alleles defined by molecular typing and serotypes are now available.[18] New variants are not expected to have a significant impact in clinical transplantation because of their extremely low frequency and because of the linkage disequilibrium between HLA loci. However, it is important to be able to discriminate such rare alleles, particularly in patients with hematological disorders for whom unrelated HSC transplantation is the best therapeutic option. The functional relevance of new alleles that differ in exons coding for domains of the HLA molecules that do not directly interact with TCRs is unknown. Because of new polymorphisms, past allele assignments may become outdated. Updates of previous typing results based on newly identified alleles can be performed based on stored typing patterns.[19]

CONCLUSION

Although HLA genotyping remains a challenge in molecular diagnostics, PCR-based HLA typing techniques have dramatically improved the quality of patient–donor matching and have contributed to the success of transplantation programs. For just a few diseases, HLA alleles are diagnostically useful; but for several autoimmune disorders, high-resolution HLA typing has provided important insights into pathological mechanisms. A still open question is whether to drop out serology and rely only on DNA typing. Although a few laboratories have already made that choice, a frequently mentioned argument in favor of maintaining serology is the need to monitor cell surface expression of HLA antigens and detect possible null alleles that would be mistyped as functional alleles by molecular typing because the mutation is located in a region that is not tested by the technique (intron or nonpolymorphic exon sequence motif). In kidney transplantation, where cytotoxicity crossmatch remains the gold standard, monitoring the expression of HLA antigens at the cell surface by using serology should be helpful. The diversity of the HLA system is so high that clinical laboratories must continuously cope with new polymorphisms, adapt quality assurance systems for reagents and algorithmic softwares, and ensure that appropriate interpretations are provided in case of typing ambiguities. Depending on clinical application, such ambiguities need to be resolved, sometimes at the expense of maintaining several molecular techniques running in parallel.

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HLA-DQA1 Typing Using DNA Microarray

H

Dat D. Dao
Laurie R. Lewis
Houston Advanced Research Center (HARC), The Woodlands, Texas, U.S.A.

INTRODUCTION

Genes of the major histocompatibility complex (MHC) encode human leukocyte antigen (HLA) molecules and have been implicated in the genetic background of many autoimmune and infectious diseases. Among the three classes of HLA molecules, class II genes, primarily the DR and DQ loci, are thought to be functionally significant in disease susceptibility; however, associations have been demonstrated with the class I and class II regions as well.[1] The human genome project has stimulated research on the relationship between specific HLA alleles and disease susceptibility, creating a need for high-throughput HLA typing. To demonstrate the methods of developing a microarray system for high-throughput HLA typing, DQA1, being the least complex molecule of class II HLA genes, was selected as a model. Understanding experimental conditions and accuracy of data analysis to distinguish allelic single-base changes will provide a foundation for the development of a DNA microarray to detect base changes in other HLA genes of interest.

MHC AND HLA

The MHC is a genetic locus which spans 4 Mb in the p21 region of chromosome 6 and is responsible for controlling the immune response. Encoded in this region are several classes of proteins referred to as HLA, which are divided into three groups: class I, class II, and class III. The HLA class I molecules are designated HLA-A, HLA-B, or HLA-C and are encoded by genes of the MHC, which participate in antigen presentation to cytotoxic T ($CD8^+$) cells. The HLA class II molecules are designated DP, DQ, or DR and are encoded by genes of the MHC, which participate in antigen presentation to helper T ($CD4^+$) cells. The HLA class III region is located within the HLA region because its components are related to the functions of HLA antigens, or are under control mechanisms similar to the HLA genes. HLA class III contains loci responsible for complements, hormones, and intracellular peptide processing and other developmental characteristics.[2] HLA class I and class II genes play an important role in the communication between cells, determining whether transplanted tissue or bone marrow is histocompatible (accepted as self) or histoincompatible (rejected as foreign), and are thought to be functionally significant in disease susceptibility.

The expression of all MHC loci is codominant; both the set of alleles inherited from one's father and the set inherited from one's mother are expressed on each cell. In most cases, the entire linked MHC, called a haplotype, is inherited intact, without recombination.[3] Research has indicated that individuals with certain allelic genotypes may be at higher risk for developing diseases affecting the immune system. Because both alleles contribute to the phenotype equally, it is important to investigate the genotypes in disease association studies rather than alleles on their own.

There are three standard levels of HLA typing: low, medium, and high. Low-resolution typing defines broad families of alleles. Medium-resolution typing helps determine that the individual has one of the many alleles within a low-resolution group and eliminates others. High-resolution typing is an effort to definitively state which allele resides at a locus for an individual.[4] Solid organ transplant programs work well with low-resolution typing, whereas bone marrow programs require high resolution.

The HLA system has the greatest degree of polymorphism in the human genome. As of August 2003, there were over 1600 alleles recognized at the classical loci (Table 1).[5] Mutations and polymorphisms are constantly being identified, making HLA typing for donor and recipient matching in transplantation increasingly complex and creating the need for analytical tools to detect genetic patterns potentially related to disease. The completion of the human genome project has given scientists the ability to identify genomic variation in cells and tissues quantitatively, simultaneously, and automatically under a variety of conditions using high-throughput microarray analysis.[6]

MICROARRAY TECHNOLOGY

Microarray technology can be used to distinguish between DNA sequences that differ by as little as a single nucleotide polymorphism (SNP). Microarrays generally

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Table 1 Genotypical alleles (as of August 2003)

HLA class I								
A	**B**	**C**	**E**	**F**	**G**	**H**	**J**	**K**
282	537	135	6	2	15	0	0	0
HLA class II								
DRA	DRB	DQA1	DQB1	DPA1	DPB1	DMA	DMB	DOA
3	418	24	55	20	106	4	6	8
HLA-DRB								
DRB1	DRB2	DRB3	DRB4	DRB5	DRB6	DRB7	DRB8	DRB9
342	1	39	12	17	3	2	1	1
Other non-HLA genes								
MICA	MICB	MICC	MICD	MICE	TAP1	TAP2	LMP2	
56	0	0	0	0	6	4	0	

It is not feasible to publish a comprehensive list for each loci within the HLA system, as new members of allele families are being added constantly. The most current accepted alleles at any loci can be found using the following web site link: http://www.anthonynolan.com/HIG/data.html.

work as follows: 1) DNA ''probes'' complimentary to the gene of interest are bound to a solid substrate (i.e., glass); 2) DNA ''targets'' (i.e., sample) are added and hybridize where the sequence is complimentary to the probe and anneal to form a DNA duplex; and 3) hybridized targets are detected using a reporter system [i.e., enzyme-labeled fluorescence (ELF; Molecular Probes, Eugene, OR)]. A good model system for HLA typing using DNA microarray technology is the HLA class II gene—*DQA1*.

HLA-DQA1

Class II genes are composed of both α-chains and β-chains in the HLA-D region and are responsible for 644 different alleles. The HLA-DQ molecules, consisting of two polymorphic polypeptide chains, account for 24 of the known class II alleles and are thought to be involved in the pathogenesis of several human diseases.[7] The HLA-DQA1 region of the *HLA-D* gene, located near the centromere, is responsible for assisting in the distinction of intruder cells from the body's own cells, and is one of many locations where the donor and recipient are required to have identical alleles. The DQA1 locus tends to be an ideal area of basic human identification. Because of the small number of known alleles associated with DQA1, substantially less than other areas, it makes a good model for that of other areas. This type of array serves as a model for typing other important gene regions in the HLA complex and can also be used to generate arrays for the detection of single-base changes in other genes of interest.

HLA-DQA1 TYPING (LOW RESOLUTION) USING DNA MICROARRAY

Oligonucleotide Probe Design and Synthesis

Design oligonucleotide probes by looking for an area of the sequence that matches the order for the selected allele but for no other group.[8] A combination of sense and antisense sequences can be used to formulate the microarray for low-resolution HLA-DQA1 typing (Fig. 1). When developing and choosing optimal probes, take into consideration: 1) desired probe length: the optimal probe length is 12 bases, which provides a high level of discrimination between alleles and allows hybridization to take place at room temperature; 2) placement of the mismatched base(s): optimal placement of the mismatched bases is near the center of the sequence; 3) suitable G/C content: G/C content should range from 42% to 58%; and 4) free energy values: values of free energy should be greater than $-3.1\ \Delta G$ so that the duplex formations are kept to a minimum. Manual design is not very successful. The best results are obtained by using

a

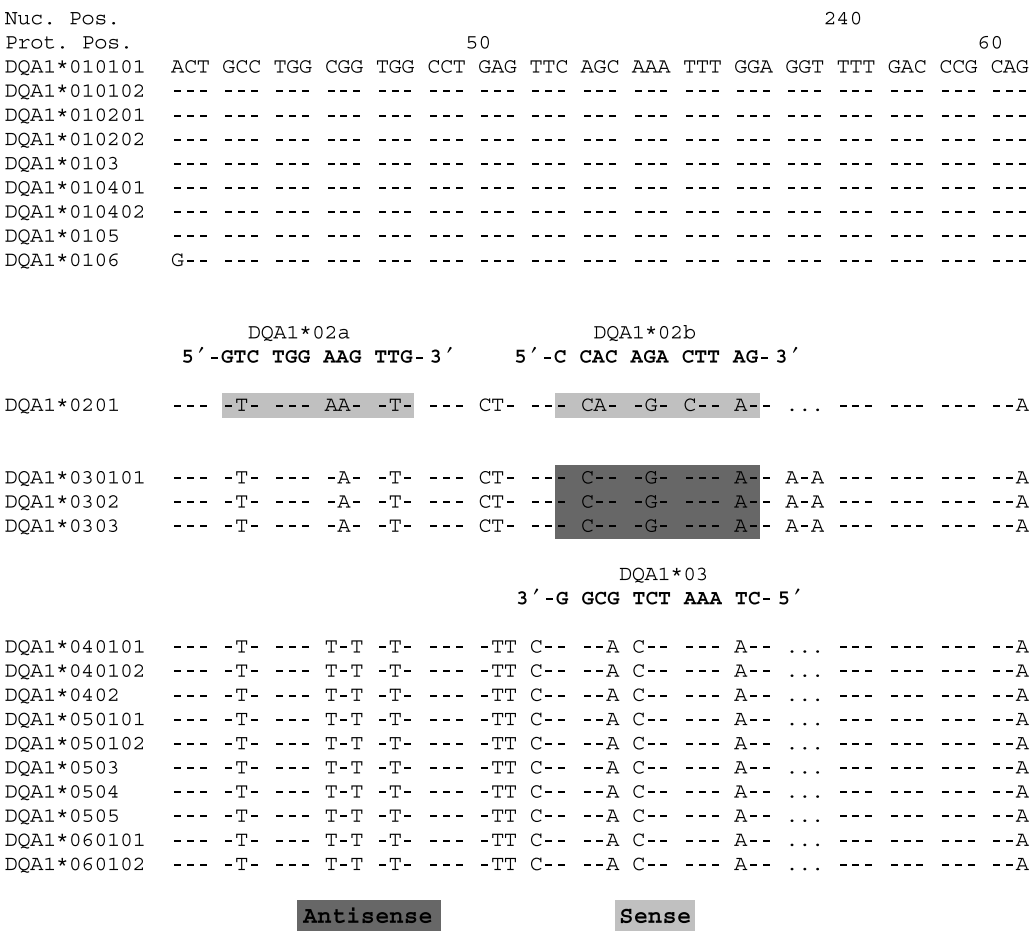

Fig. 1 (a) Low-resolution probes for the typing of HLA-DQA1*02 and HLA-DQA1*03 alleles: a region of the *HLA-DQA1* gene showing variations in the sequence for each allele. Probe sequences are shown by either a light bar (sense probe) or a dark bar (antisense).

Fig. 1 *(Continued next page)* (b) Low-resolution probes for the typing of HLA-DQA1*01 alleles: a region of the *HLA-DQA1* gene showing variations in the sequence for each allele. Probe sequences are shown by either a light bar (sense probe) or a dark bar (antisense). (c) Low-resolution probes for the typing of HLA-DQA1*05, HLA-DQA1*04/HLA-DQA1*06 alleles: a region of the *HLA-DQA1* gene showing variations in the sequence for each allele. Probe sequences are shown by either a light bar (sense probe) or a dark bar (antisense). (*View this art in color at www.dekker.com.*)

software that implements (1)–(4) above and searches out the ideal placement of the probe along the sequence of the gene. Produce amine-modified probes by means of a standard phosphoramidite and segmented synthesis strategy for the simultaneous synthesis of a large number of oligonucleotides.

DNA Microarray Preparation

An aliquot of synthesized amine-modified oligonucleotide probes should be diluted to the desired concentration (10 μM) and attached to a glass slide. The slides should be dried at room temperature and washed with distilled deionized water before use.

Preparation of Target

Prepare DNA targets by polymerase chain reaction (PCR) amplification of the 189-bp region of the *HLA-D* gene (DNA isolates) using the 5′ biotinylated primer sequences (forward primer: 5′CACCCATGAATTTGATGGAG; reverse primer: 5′TCATTGGTAGCAGCGGTAGAGTTG). PCR is performed in a total volume of 50 μL consisting of 10 mM Tris–HCl, pH 8.0, 50 mM KCl, 0.01% gelatin,

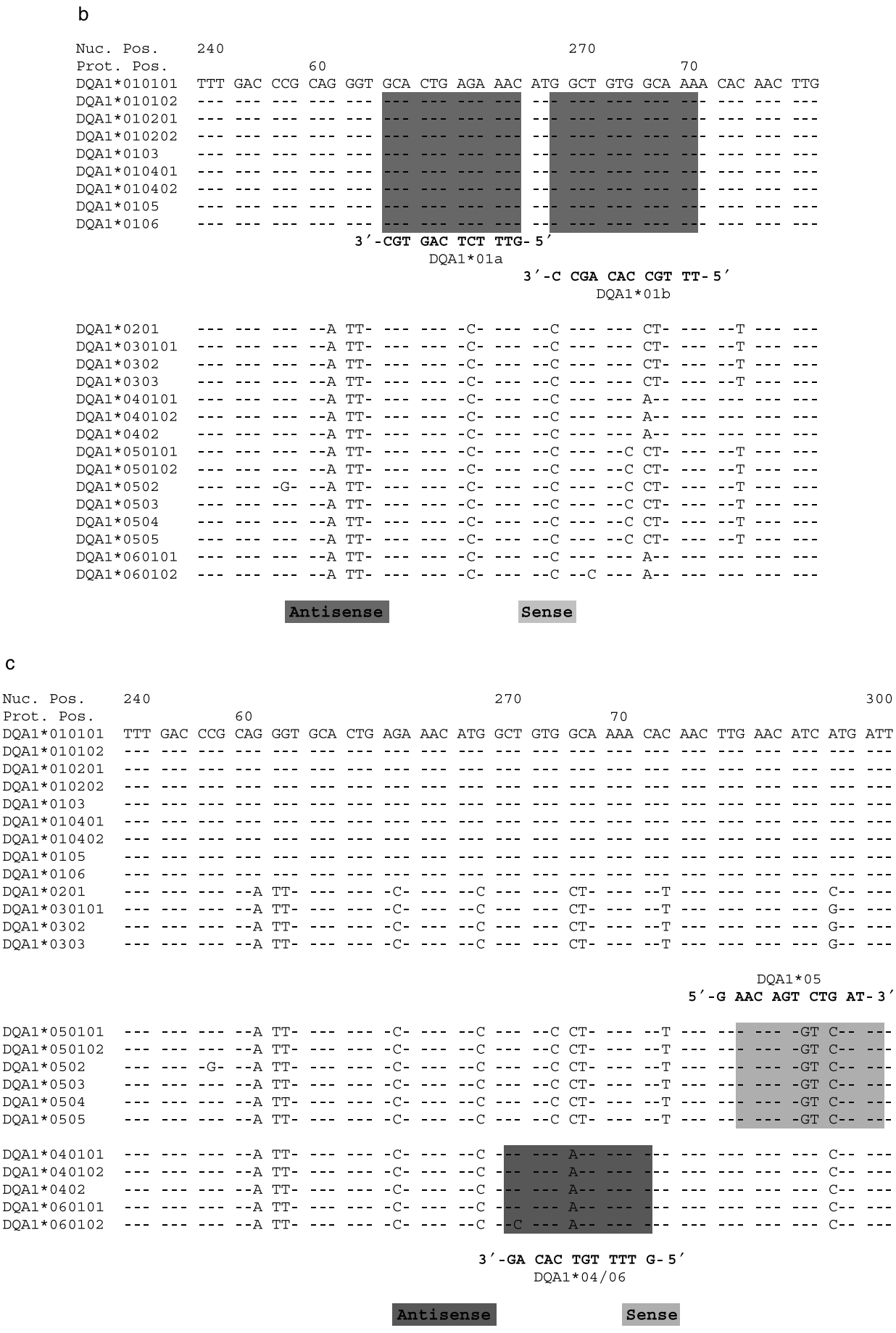

Fig. 1 (*Continued*).

Table 2

Probe #	AA	# Allele	DQA1 Alleles	Probe Sequence
1	67–71	6	0101, 0102, 0103, 0104, 0105, 0106	3′-CGT GAC TCT TTG-5′[a]
2	24–28	6	0101, 0102, 0103, 0104, 0105, 0106	3′-C CGA CAC CGT TT-5′[a]
3	55–59	1	0201	5′-GTC TGG AAG TTG-3′[b]
4	55–60	1	0201	5′-C CAC AGA CTT AG-3′[b]
5	35–39	3	0301, 0302, 0303	3′-G GCG TCT AAA TC-5′[a]
6	68–72	2	0401, 0601	3′-GA CAC TGT TTT G-5′[a]
7	24–28	4	0502, 0503, 0504, 0505	5′-G AAC AGT CTG AT-3′[b]

[a]Antisense probe.
[b]Sense probe.

100 μM dNTP, 1.5 mM $MgCl_2$, 20 pmol of each primer, and 2.5 U of AmpliTaq Gold DNA Polymerase (Roche Molecular Systems, Branchburg, NJ). The amplification involves a 10-min activation time at 94°C, followed by 30 cycles at appropriate denaturation, annealing, and extension temperatures (i.e., 94°C for 30 sec, 54°C for 1 min, and 72°C for 30 sec) with a 5-min extension after the last cycle.

Hybridization

Resuspend cDNA targets in 5× SSC/0.1% sodium dodecyl sulfate (SDS) and denature at 99°C for 2 min. Add 50 μL of solution to a prewetted glass slide (2× SSC, 0.1% SDS), cover with a glass cover slip, and place in a moist chamber and incubate at 65°C for 12–18 hr. Then wash in 2× SSC/0.1% SDS for 2 min, 1× SSC, 0.2× SSC, and 0.05× SSC sequentially for 1 min each before scanning.

Perform hybridizations in 6× SSC, 0.3% SDS, and 5 μL of the amplified DNA. Heat-denature then wash slides for 15 min at room temperature in 3.3 M tetramethyl ammonium chloride (TMAC).

Detection of Fluorescence

Incubate slides with 50 μL of streptavidin–alkaline phosphatase conjugate (1:1500 dilution in phosphate-buffered saline/0.2% bovine serum albumin/0.1% Tween-20) for 2 hr at room temperature. After two 10-min washes

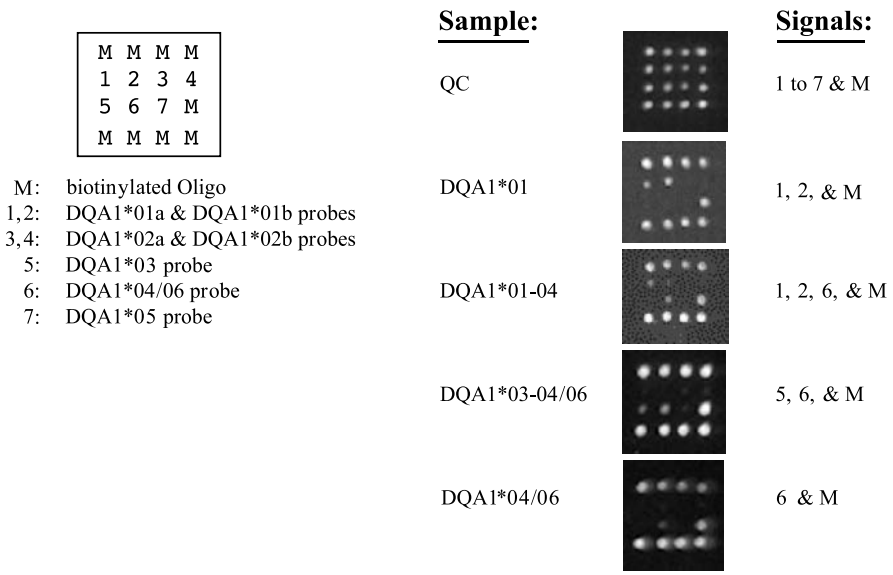

Fig. 2 Low-Resolution Typing HLA-DQA1: (Left) DNA microarray illustrating positions of probes for alleles 01, 02, 03, 04/06, and 05 in the HLA-DQA1 gene. On the bottom left is probe nomenclature for the corresponding probe numbers on the DNA microarray. (Right) Samples were tested on the array in a blinded fashion. The spot diameter is 120–130 μm, with 500 μm spacing. Alleles found by DNA chip analysis are displayed by positive probe signals. All photographs were taken with the use of a CCD camera under a UV light source.

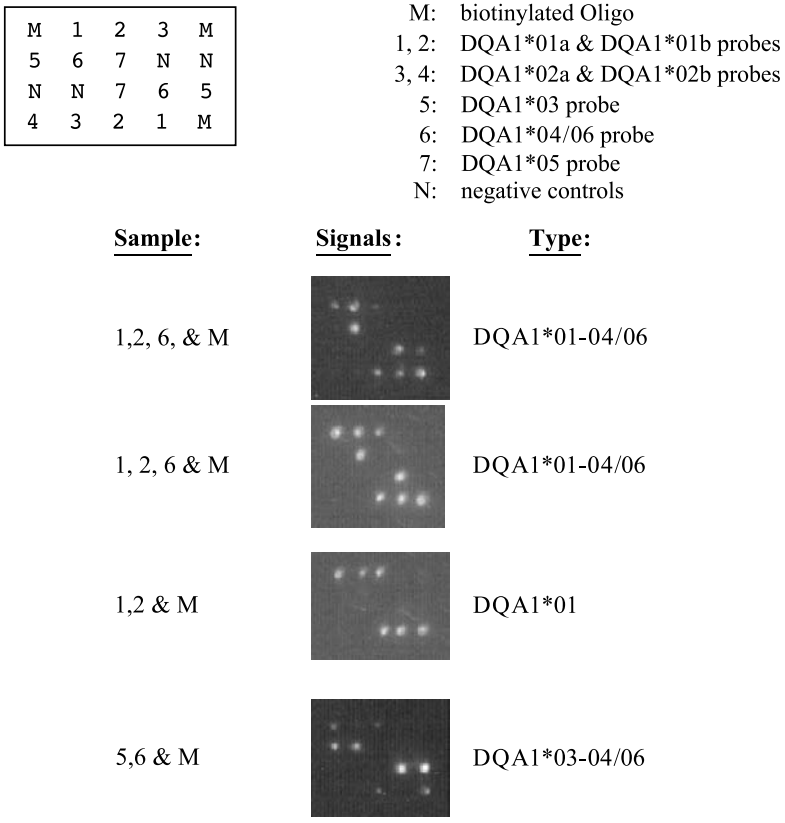

Fig. 3 HLA-DQA1 typing of cervical carcinoma and control samples using DNA microarray: (Top) DNA microarray illustrating positions of probes for alleles 01, 02, 03, 04/06, and 05 in the HLA-DQA1 gene. On the right is probe nomenclature for the corresponding probe numbers on the DNA microarray. (Bottom) The same samples used for ''gold standard''were tested on the array. The spot diameter is 120–130 μm, with 500 μm spacing. Alleles found by DNA chip analysis are displayed by positive probe signals. All photographs were taken with the use of a CCD camera under a UV light source.

in 2× SSC/0.1% SDS, add 50 μL of ELF substrate (Molecular Probes) to the slide and cover with a coverslip. Place in humidity chamber protected from light and incubate overnight. Results are obtained by viewing the slides with an ultraviolet (UV) transilluminator or with the use of a charged-couple device (CCD) camera over a UV light source.

HLA-DQA1 Typing Study

A rapid and accurate test that distinguishes one allele from another was the focus of this investigation. To distinguish one allele from another, short oligonucleotide probes 12 bases in length attached to a glass slide were utilized (Table 2). Each probe represents a different low-resolution allele in the DQA1 region. Details of the application of these inkjet microfabricated arrays for HLA-DQA1 low-resolution typing of DNA samples from HLA class II reference panel (UCLA Tissue Typing Laboratory, CA) can be found in Haddock et al.[8] HLA-DQA1 microarrays were fabricated at the Houston Advanced Research Center, The Woodlands, Texas, using microspotting quill pins. Biotinylated polymerase chain reaction (PCR) products were allowed to hybridize to the prefabricated glass slides and hybridization signals detected using enzyme-labeled fluorescence (ELF) with the aid of a charged-couple device (CCD) camera.

Low-resolution alleles were successfully typed using a 189-base pair region of the HLA-DQA1 gene by observing easily recognizable positive hybridization patterns. In Fig. 2, detection of homozygous as well as heterozygous samples was accomplished by viewing probes fluorescing under UV light. All samples were obtained from UCLA's Standardized DNA Reference Panel for HLA class II. Each DNA sample was labeled with a letter or letters, which corresponded to the key provided in the Standard Reference Material. The results were compared to the key to verify that the HLA typing of the sample was the same. This type of array is important because it encompasses the use of short oligonucleotide probes that provide a high level of discrimination between alleles. Furthermore, this type of

array serves as a model for typing other important gene regions in the HLA complex. It can also be used to generate arrays for the detection of single-base changes in other genes of interest. In order to test the above samples for HLA-DQA1 using microarray technology, a 4×5 DQA1 array was generated as illustrated in Fig. 3. DNA isolated from cervical cancer patients and normal controls were used for HLA DQA1 typing. The results indicated low-resolution typing of DQA1 of both homozygous and heterozygous individuals can be done using DNA microarray.

Quality Control

To verify the working capabilities of each probe in the array, a mixture of 20–22mer prefabricated target DNA containing species complementary to each wild type and mutation probe should be developed and used to validate DNA microarrays for each batch of chips prior to use for typing.

CONCLUSION

It has been shown that microarray technology for identification of HLA alleles can be used for HLA typing;[7,9] however, specificity of signal detection, accuracy of data analysis, optimization of experiment conditions, and ever-increasing identification of new HLA alleles being identified remain as obstacles. The future of using DNA microarrays in HLA typing lies in companies' active development of technology that can compensate for these obstacles. To date, no solid platform for HLA typing using microarrays is available commercially. However, promising development is underway. TeleChem International, Inc., NGS-ArrayIt, Inc. Division (Sunnyvale, CA) has developed Next Generation Screening™ (NGS™) technology, an exciting new technology that uses ''patient-on-the-chip'' microarray technology as a very cost-effective means of HLA analysis. BioCore Co., Ltd. (5th Floor, Heasan Building, 108-1, Yangjea-dong, Seocho-gu, Seoul, South Korea) is in the process of commercializing an HLA chip that can identify the HLA-DR genetic locus type. In the mean time, one way researchers can get around the inherent obstacles is by knowing the alleles and frequencies that are present in the populations they are studying. Web sites listing the frequencies of HLA alleles (www.allelefrequency.net) and their linkage to a particular disease in worldwide populations (Anthony Nolan Trust: http://www.anthonynolan.com/HIG/data.html) have been established.[10] Networking of accumulated HLA data could be the key in helping investigators successfully make use of DNA arrays in HLA typing and propelling the understanding of the association of disease susceptibility, clinical manifestation of disease screening, and diagnostic development.

ACKNOWLEDGMENTS

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HPV Typing—Comparison of Different Molecular Assays

Antoinette A. T. P. Brink
Chris J. L. M. Meijer
VU University Medical Center, Amsterdam, The Netherlands

Adriaan J. C. van den Brule
PAMM Institute, Eindhoven, The Netherlands

Peter J. F. Snijders
VU University Medical Center, Amsterdam, The Netherlands

INTRODUCTION

Several current developments in the field of human papillomavirus (HPV) research show the increasing need for HPV typing. First, the number of known HPV types is still increasing. Although novel HPV types are often isolated from malignancies, their transforming potential can only be determined from a combination of in vitro and epidemiological studies, the latter including typing. Second, many HPV infections consist of multiple HPV types and in those cases, typing is necessary to determine the contribution of the individual HPV types. Third, there is an increasing interest in HPV vaccination, which entails studies of geographical distribution of HPV types and their associated diseases as a basis for vaccination programs. Moreover, HPV typing is of utmost importance to monitor the efficacy of HPV-based vaccines, the latter being type-specific.

A large range of HPV typing methods is available, all with respective advantages and limitations. In this article, we will describe briefly the most important current HPV typing techniques and give recommendations for their respective applications.

HUMAN PAPILLOMAVIRUS TYPES

Human papillomaviruses (HPVs) are associated with various benign and malignant epithelial proliferative diseases. Over 100 genotypes of HPV are recognized.

An HPV isolate is described as a novel type if the nucleotide sequence of its E6, E7, and L1 genes differs more than 10% from that of any other HPV type. Based on tropism, a distinction can be made between *cutaneous* HPV types that infect the epidermis and *mucosal* HPV types that infect the epithelia of the anogenital or the aerodigestive tract.

In vitro studies showed that a group of phylogenetically related *mucosal* HPV types has oncogenic potential: their E6 and E7 proteins interfere with cell cycle regulation by mediating degradation of p53 and pRb proteins, respectively. These oncogenic HPVs, designated *high-risk* (HR) HPVs, are an important causal factor in carcinogenesis of the uterine cervix.[1] Epidemiological studies have shown that HR-HPVs include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 and probably also types 26, 53, and 66.[2] Nononcogenic or *low-risk* (LR) genital HPVs are phylogenetically distant from the HR-HPVs. They include HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108. Their E6 and E7 proteins are less competent in interfering with p53 and pRb functions, and they can cause benign proliferations such as condylomata acuminata (genital warts). For a number of mucosal HPV types, the risk has not been determined yet.[2]

Cutaneous HPVs belong to other phylogenetic subgroups than mucosal HPVs. Some of them play a role in benign epidermal proliferation, e.g., HPV 1 which is causally related to verruca vulgaris (warts). In addition, associations between cutaneous HPVs and malignant skin diseases were discovered (reviewed in Ref. [3]). For example, squamous cell carcinomas (SCCs) from patients with the rare hereditary disorder epidermodysplasia verruciformis (EV) are associated with a particular group of 22 phylogenetically related cutaneous HPVs which are therefore designed EV-HPVs. They include HPV 5, 8, 9, 12, 14, 15, 17, and 19–25. These types were also detected in other cutaneous malignancies such as SCC in individuals without EV and basal cell carcinomas (BCC). The prevalence of HPV in skin tumors is especially high in immunosuppressed individuals.

Human papillomavirus typing methods can be divided in target-amplification methods, i.e., methods involving polymerase chain reaction (PCR), and non-PCR techniques. In the following section, technical aspects of the different currently available HPV typing methods are

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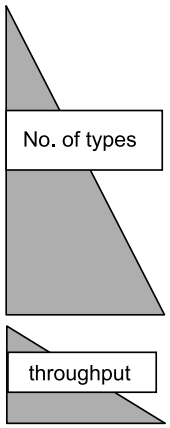
Table 1 HPV typing methods and their applications

Typing resolution	Method	Clinical specimens: Cervical smears in preservation medium[a]	Cervical smears in PBS	Archival cervical smears (slides)	Biopsies snap-frozen	Biopsies formalin-fixed
Group-specific (e.g., HR vs. LR)	• Hybrid Capture 2[4]	+[b]	—	—	—	—
	• Consensus PCR+blot/ EIA (cocktail probes)[12]	§[c]	+	§	+	+
Distinguish known types	• Type-specific PCR+blot/EIA[6,17]	§	+	§	+	+
	• Type-specific PCR real-time	§	§	§	§	§
	• Consensus PCR real-time[16]	§	§	§	§	§
	• Consensus PCR-RFLP[15]	§	+	§	+	+
	• Consensus PCR-RLB/LiPa[6,7,10] or microarray[11]	§	+	§	+	+
Find novel types	• PCR+sequencing[13,14]	§	+	§	+	+
	• PCR+RFLP[15]	§	+	§	+	+



[a]Media preserving the integrity and morphology of cells, e.g., Sample Transport Medium (Digene), PreservCyte (Cytyc).
[b]+=can be used on sample ''as is'' or on crude extract (proteinase K treatment/freeze–thaw/boil).
[c]§=needs additional extraction/purification of DNA.

described. Table 1 gives an overview of the different techniques, sorted to typing resolution and throughput if applicable. In addition, the suitability for various clinical specimen types is indicated.

DESCRIPTIONS OF METHODS

Nonamplification Methods

The best known technique in this category is the commercially available, FDA-approved Hybrid Capture 2 (HC2) method.[4] It detects 13 genital hr-HPV types by a mixture of full-length RNA probes; hence it is suitable only for group-specific detection and not for high-resolution typing. Hybridization of one or more of the probes to HPV DNA present in heat-alkaline-denatured clinical samples is detected by peroxidase-labeled antibodies that recognize the RNA/DNA hybrid and visualized by chemiluminescence. The nonamplification nature of HC2 greatly decreases the risk of false positives by reaction product carryover. It is self-evident that the analytical sensitivity of this method is lower than that of PCR-based methods (Table 1). Some cross-reaction of the HC2 probes with HPV types not represented in the probe mix, including some nononcogenic HPVs, have been described.[5]

Polymerase Chain Reaction-Based Methods

Consensus primer polymerase chain reaction

Most of the PCR-based HPV typing methods rely on the use of consensus primers that amplify a broad spectrum of HPV types, followed by detection with type-specific probes. The consensus primers may contain mismatches that are accepted under low-stringency PCR conditions as in the GP5+/6+ system.[6] Alternatively, the consensus primers may be degenerate as in the MY09/11[7] and CPI/II[8] systems or both degenerate and containing inosine residues at ambiguous base positions such as the IU/IWDO[9] and SPF[10] primers.

Typing of consensus polymerase chain reaction products—endpoint measurement

Typing of PCR products was traditionally performed by means of dot blotting or Southern blotting and hybridization with type-specific oligonucleotides. More recently, reverse hybridization techniques were introduced. These methods rely on the hybridization of labeled consensus PCR products to HPV type-specific oligos immobilized on filters. Examples are reverse line blot (RLB) analysis following MY09/11[7] or GP5+/6+[6] consensus PCR or a line probe assay (LiPa) following SPF PCR.[10] Detection of the hybridized PCR product is performed by a colorimetric reaction[7,10] or by chemoluminescence,[6] the latter allowing repeated usage of the same filter.[6] Instead of filters, glass microarrays of HPV type-specific probes can also be used.[11] A schematic representation of the principle of reverse hybridization is given in Fig. 1. These methods are far less laborious than normal hybridization methods when it comes to detection

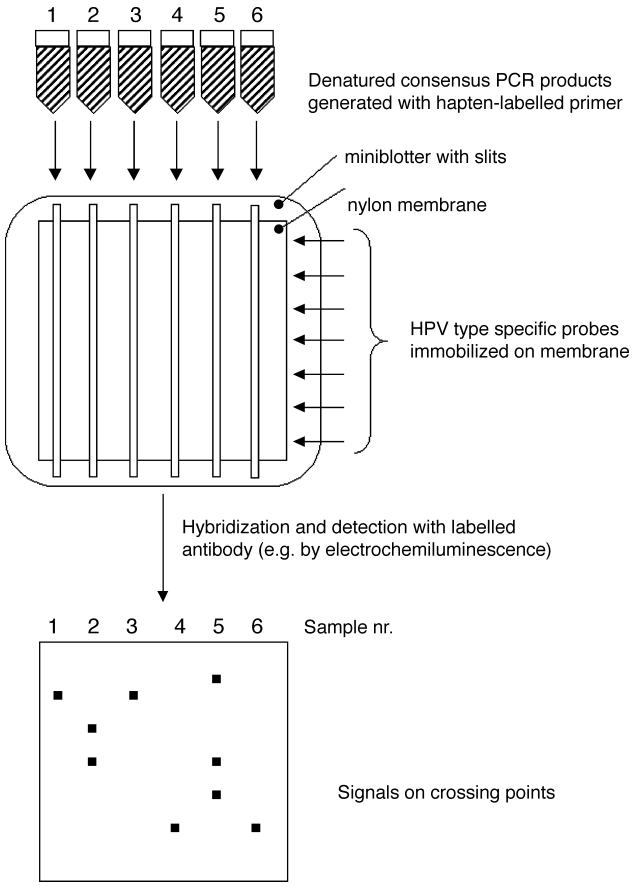


Fig. 1 Reverse hybridization methods: RLB.[6] Schematic representation of the principle. HPV type-specific probes are attached to a membrane using a miniblotter with slits. Subsequently, the membrane is rotated 90° in the miniblotter. Consensus PCR products are generated using hapten-labeled primers. After denaturation, the single-stranded PCR products are pipetted into the slits of the miniblotter. If a particular HPV type is present in a sample, hybridization will occur on the crossing point of the sample slit with the respective HPV probe line. Subsequently, hybridization is detected using a labeled antibody directed against the hapten and visualized using chemoluminescence.

of multiple HPV infections. For group-specific detection of HPVs without high-resolution typing, an enzyme immunoassay (EIA) can be applied conveniently using cocktails of, for example, HR- or LR-HPV probes.[12]

Two nonhybridization typing methods following consensus PCR are sequence analysis of the PCR product[13,14] and restriction fragment length polymorphism (RFLP) analysis.[15] RFLP implies the digestion of consensus PCR products with restriction endonucleases and comparison of the digestion pattern with those of known HPV types. These techniques are useful if unknown types of HPV are present in the specimens, but they have several drawbacks as compared with hybridization methods. For example, RFLP and sequence analysis are not suitable for the detection of infections with multiple HPV types: these will usually give an uninterpretable mix-up of digestion/sequence patterns. In addition, these two methods are less suitable for high-throughput analyses because they are relatively laborious. Finally, RFLP and sequence analysis are less sensitive than hybridization methods because more PCR product is needed to generate a positive signal.

Typing of consensus polymerase chain reaction products—real-time measurement

As opposed to typing after consensus PCR, it is also possible to type during the reaction, in real time. Various real-time PCR techniques are available, the best known being molecular beacons, the fluorescent 5′ exonuclease assay (e.g., TaqMan), and fluorescence energy resonance transfer assays (e.g., LightCycler).

At present, the only real-time assay that allows consensus HPV-PCR with simultaneous typing is a molecular beacons assay.[16] However, the multiplicity of this technique is limited because the current generation of real-time thermocyclers does not allow for more than six differentially labeled probes. Hence for typing of multiple HPV infections, reverse hybridization methods are preferred over real-time assays.

Type-specific polymerase chain reaction

If one is looking for a particular HPV type, type-specific PCR can be applied (described in Ref. [6,17] among others). Care should be taken when designing primers because they may still react with other types if chosen in well-conserved regions. Confirmation of the specificity of type-specific PCRs, as with consensus primer PCRs, is mostly performed by (regular or reverse) filter hybridization or by EIA, but can also be performed in real-time. A great advantage of real-time assays is the possibility to quantify the HPV in the specimen. However, to obtain reliable quantification data, DNA extraction is usually necessary, thus increasing the workload.

Analytical sensitivities and specificities of the assays

In general, the sensitivities of HPV typing assays are dependent on the reaction that precedes the typing step. The sensitivity of the only nonamplification technique described in this article, the HC2 method, is 5000 copies of the HPV genome per reaction well according to the manufacturer. All other methods described here consist of a PCR step followed by a typing step. These methods have a much higher analytical sensitivity than HC2, with a slight variation between the methods (reviewed in Ref. [18]). In general, less than 100 HPV genome equivalents in a reaction tube are sufficient to generate a positive PCR signal and to enable typing.

It is self-evident that specificities of the HPV typing assays rely on the specificities of the respective probes for their corresponding HPV types under the respective hybridization conditions. In addition, PCR-based methods depend on the specificity of the amplification primers.

WHAT TO CONSIDER WHEN SELECTING AN HPV TYPING METHOD

Study Design and Desired Typing Resolution

For mucosal HPVs, a simple detection of HR-HPV without typing may be sufficient in many study designs. These include population-based screening studies for cervical cancer, in particular, the identification of women with abnormal cervical cytology who are at risk of progressive cervical intraepithelial neoplasia (CIN) disease[19] and the follow-up after treatment of CIN.[20] The method of choice in such studies would be hybridization with a HR probe cocktail, either after consensus PCR or using HC2.

Other studies may call for high-resolution HPV typing. These include studies of possible relations between HPV type and clinicopathological aspects such as histology (e.g., mucosal HPVs in adenocarcinomas, SCCs, and small-cell carcinomas of the uterine cervix; cutaneous HPVs in different cutaneous diseases) and patient survival. Furthermore, HPV typing can be helpful to determine whether two HPV-associated lesions within the same patient are pathogenetically related (e.g., a cervical carcinoma and an earlier detected CIN lesion).

Especially in the light of the current interest for HPV vaccination (reviewed in Ref. [21]), it is important to study geographical distribution of HPV types and their associated diseases as a basis for vaccination programs. Once vaccination has started, HPV typing remains necessary to monitor the changes in prevalence of the type(s) represented in the vaccines.

Considerations Regarding Clinical Specimens

Finally, the type of clinical specimens available will determine the choice of the typing method. The majority of the techniques mentioned can be performed with any type of clinical material (Table 1). An exception is the HC2 test (Digene), the manufacturer of which recommends the use of a dedicated specimen collection medium for cervical and vaginal swabs. These samples can also be used for PCR, but only after DNA isolation because organic solvents in the Digene- and other cell-preserving media will inhibit PCR. In archival material (smears on slides or formalin-fixed, paraffin-embedded material), the DNA is usually degraded to fragments <200 base pairs. This renders them unsuitable for PCR-based methods that generate products of >200 base pairs.

CONCLUSION

As seen in the previous paragraphs, the aim of the study and the clinical material available will determine the choice of the HPV typing method. In general, consensus PCR followed by reverse hybridization is very sensitive and gives the most extensive typing information for many kinds of clinical specimens, including those containing multiple infections.

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HTLV-1

Shimeru Kamihira
Nagasaki University, Nagasaki City, Japan

INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) was discovered by Poiesz et al. as the first causative retrovirus of human cancer. Since then serological and clinicopathological studies have clarified the causative relationship between the virus and other diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis, arthropathy, and polymyositis. However, notably, most individuals infected with the virus show no clinical features of the infection (healthy carriers). Accordingly, HTLV-1 quantitative detection by real-time polymerase chain reaction (PCR) would be significant in understanding not only the infectious status but also the pathological roles of the causative virus.

TYPE OF PATHOGEN

HTLV-1 belongs to the family Retroviridae. HTLV-1 is morphologically a type C retrovirus and mainly infects CD4 T-cells, and the proviral genome is known to be randomly integrated into the human genome with a different genomic integration site in each infected cell. The provirus consists of *gag*, *pol*, *env*, and *pX* genes. HTLV-1 is causatively implicated in the pathogenesis of human neoplastic, degenerative, and inflammatory diseases such as adult T-cell leukemia (ATL), HAM/TSP, uveitis, arthropathy, and polymyositis.[1-3] But, notably, most individuals infected with HTLV-1 are persistently asymptomatic throughout life.

CLINICAL DESCRIPTION OF INFECTION SPECTRUM

Although HTLV-1 has been demonstrated to infect several kinds of cells other than T-cells in vitro, the virus is thought to usually infect CD4 T-cells in vivo. Each T-cell infected with the virus is believed to harbor one copy of the provirus. Accordingly, the quantitative detection of the HTLV-1 proviral load by real-time PCR would not only mean the existence of HTLV-1 infection but would also provide a surrogate marker for HTLV-1-infected cell count.[4] In particular, in ATL, this indicates that the proviral load corresponds to tumor burden of ATL cells including minimal residual disease (MRD) in remission state because ATL cells usually harbor one proviral genome monoclonally integrated within the same genomic integration site of clonal ATL cells. Thus, detection of the proviral load by real-time PCR is relevant and sufficient to clinically screen HTLV-1-associated disorders and to evaluate the HTLV-1 pathological status by monitoring the infected cell count in peripheral blood, body fluids such as cerebrospinal fluid, and lymph node suspension cells.

PREVALENCE

The prevalence of HTLV-1 infection varies in different parts of the world, whether endemic to those areas or not.[5] There are two main endemic areas worldwide, Southwestern Japan and the Caribbean basin, ranging from 5% to nearly 30% seropositivities. Other parts of the world, such as Central and West Africa, Melanesia, the Middle East, and India, have also been reported to be endemic. Even in the endemic area of Nagasaki district located in Southwestern Japan, the infection rate differs, being only 1–3% in the urban area of Nagasaki and being 15–30% in rural areas in the Goto Islands located in the East China Sea about 100 km from Nagasaki. Most ATL or HAM/TSP have broken out in the endemic areas.

The three principle routes of HTLV-1 transmission include mother-to-child transfer by breast milk, sexual intercourse, and blood product transmission. However, nobody knows why the endemic areas are restricted to certain areas of the world. ATL develops 30–60 years after infection with HTLV-1 during childhood. The lifetime risk in a carrier of HTLV-1 is estimated to be about 5% in ATL and less in HAM/TSP.

MOLECULAR CHARACTERIZATION OF THE PATHOGEN

The reason HTLV-1 causes the completely different neoplastic and nervous degenerative disorders, namely ATL and HAM/TSP, remains to be elucidated. The proviral

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genome consists of *gag*, *pol*, and *env* genes, which are commonly characteristic of all retroviruses. Interestingly, the HTLV-1 genome contains a specific sequence in the 3′ site of the genome designated as the *pX* gene, encoding alternatively a 40-kDa protein (p40 tax). In ATL, p40 tax proteins are thought to play a pivotal role in the early event of tumorigenesis. Because the proteins can transactivate some cellular oncogenes/suppressor genes, growth factor genes, and cell surface receptor genes, such as c-*fos*, c-*myc*, *egr-1*, *p53*, *Bax*, c-*Jun*, *IL-2R*, *IL-1*, *IL-6*, *GM-CSF*, *TGF-β1*, *PTHrP*, and *TNF-β*.[3] Tax, then, has the potential to immortalize lymphocytes infected by HTLV-1 in vitro. These tax-immortalized cells are usually still IL-2-dependent, so that other cellular events such as *p53* and *p16* mutations are required to transform these cells. In fact, most ATL demonstrate either *p53* or *p16* mutation in clinical samples.

However, in degenerative and inflammatory disorders such as HAM/TSP and uveitis, slow viral infection, cell-mediated immunity, and an aberrant cytokine network are possible pathogenic mechanisms.[6] Recently, circulating $CD8^+$ cytotoxic T-cells specific for HTLV-1 *pX* have been notably found in patients with HAM/TSP. Furthermore, susceptibility to HAM/TSP is considered to be determined by host immunogenetic factors such as HLA haplotypes linked to HTLV-1 responsiveness.

MOLECULAR TESTING

PCR technology can quantify extremely low levels of rare (usually 1% or less) infected T-cells. The infectious status in HTLV-1 indicates that the provirus DNA sequence is integrated within the host genome from infected T-cells, so the proviral load by real-time PCR can disclose viral pathologies related to the disorders, including cell burden of the infected cells.[4,7] The technology to detect PCR products in real time has been available for more than about 7 years. Now, there are two main real-time PCR routine methods for quantitative detection of HTLV-1 proviral load: the TaqMan and Perkin-Elmer/Applied Biosystems Sequence Detection System (Applied Biosystems, Foster, CA, USA) and the LightCycler Technology (Roche Diagnostics GmbH, Mannheim, Germany). Here, we review the performance of quantitative HTLV-1 provirus detection by real-time PCR, including our experiences using the LightCycler Technology with specific probes.

Target for Detection

The detection target is mainly a part of the *pX* gene of the HTLV-1 provirus integrated within the host genome, which is highly conserved even in defective viruses. However, despite a high degree of homology among the HTLV-1 strains, deletions or mutations in the *pX* region have been described especially in patients with HAM/TSP. As specimens for quantifying the proviral load, fresh or frozen peripheral blood mononuclear cells (PBMCs) are used most often. After being collected by Ficoll–Conray gradient centrifugation, the genomic DNA is processed by the phenol–chloroform method. The genomic DNA is stored at 4°C until use. Genomic DNA doses applied to PCR assay should be precisely quantified using the dsDNA PicoGreen Quantification Kit (Molecular Probes, Eugene, OR, USA) to make precise results.

Primers and Probes

The primers and probes for the HTLV-1 provirus target should be theoretically optimized and sequences should be modified for each system. In almost all assay systems the primer pairs and probes have mainly been selected within the *pX* region of the provirus, which is the most stable to prevent false-negative results.[7–10] These designs have also been supported by our experiences with HTLV-1 Southern blot hybridization (SBH) analysis in 300 ATL cases, nearly 30% of which had defective viruses, but always conserved the *pX* region including the primer sequences and probe sequences. However, there are several reports that some assay systems especially for healthy HTLV-1 carriers use the primers designed to detect the *gag*, *pol*, or *env* regions of the provirus.[11] In particular, the *pol* gene among them has been described to be more adequate for attempts to qualify a broad spectrum of HTLV-1 strains.

Now, the primers and probes in our assay system by real-time PCR are described as follows.

Primers

Sense: 5′-CCCCCTTCCGAAATGGAT; antisense 5′-CCGGGGTGGCAAAAAATC.

Probes

Reporter probe with fluorescein isothiocyanate (FITC): 5′-GAGGGTGTACAGGTTTTGGGGC-3′-FITC; quencher probe with LC Red 640: LC Red-5′-GGAGT-CCGGGGTCTGGAAAAGA-3′ (phosphorylated at the 3′ end).

Conditions for Real-Time PCR

PCR conditions for HTLV-1

PCR conditions for quantitative HTLV-1 detection should be optimized according to the differences of primer

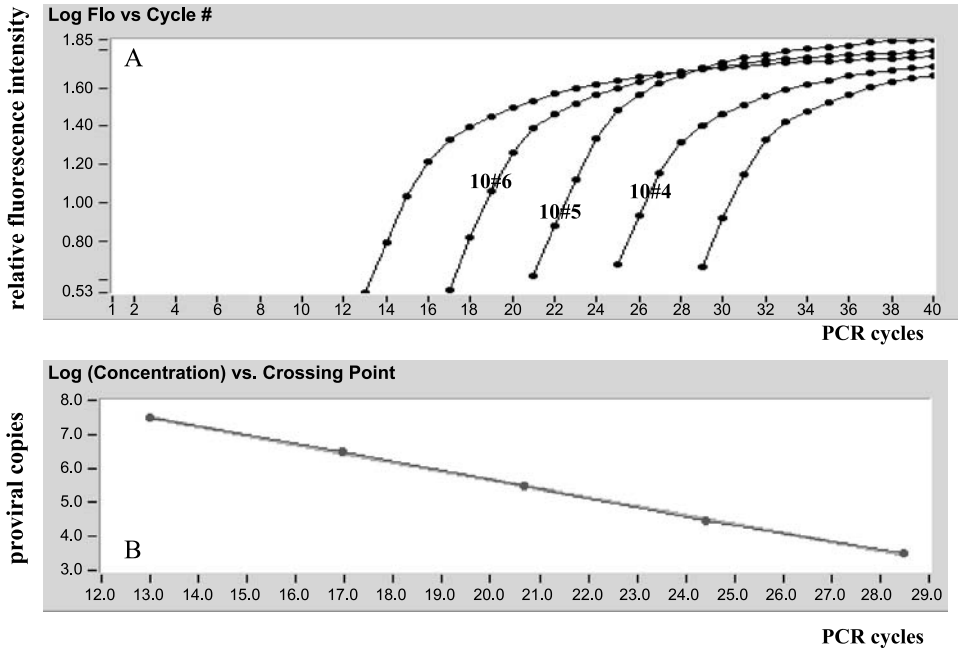

Fig. 1 Generation of an HTLV-1 proviral DNA standard curve. (A) Amplification plot of a serial dilution of the HTLV-1 pBR322 vector plasmid. (B) HTLV-1 standard curve generated by the C_t values in (A) for a known amount of template molecules. (*View this art in color at www.dekker.com.*)

designs and PCR technology applied. In our laboratory, the PCR reaction has been carried out using the LightCycler Technology assay instrument. For each test sample, 30 ng of genomic DNA is used as a template for amplification using LightCycler-FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics GmbH) in the LightCycler PCR instrument, and then the amplified products are automatically measured according to the manufacturer's instructions. The reaction conditions are 95°C for 10 min for activation of the Taq polymerase and then 50 cycles of 10 sec at 95°C (denaturation) followed by 5 sec at 64°C (annealing) and by 10 sec at 72°C (extension).

Standard curve and proviral load interpolation

For routine quantification of DNA or RNA, two general methods of data analysis exist: absolute quantification and relative quantification. Absolute quantification determines the exact template copy number, usually by relating the PCR signal to a standard curve. So it is important to know what kind of a template for a standard curve is used. In general, two kinds of control template—a plasmid vector inserting the region of HTLV-1 genome, and an HTLV-1-infected cell line monoclonally integrating one copy of the provirus for generation of a standard curve—are used. Then, unknown copy numbers are calculated by interpolation from such plasmid control regression curve and reported as copy equivalents per 10^{4-6} PBMCs or percent. In our case, an HTLV-1 standard curve is generated using serial dilutions as the template for the real-time PCR from 10^7 to 10^1 copies of the proviral DNA derived from a clone in pBR322 vector as follows. A 9-kb proviral DNA fragment is cloned into the pBR322 and propagated. After digestion with *Eco*RI, the insertion is purified and quantified with the PicoGreen dsDNA Quantification kit. Molecular concentrations are calculated and the purified insert is used for the generation of the standard curve (Fig. 1).

The copy numbers in the samples are estimated by interpolation from the plasmid control regression curve and an internal control of β-globin to correct for sample DNA quality differences. Copy numbers were reported as copy equivalents per 10^4 PBMCs, mainly lymphocytes.

Assessment of Detection by Real-Time PCR Assay

Accuracy and reproducibility

The HTLV-1 proviral copy load is thought to range widely from 100% to 0.1%, or less of the PBMC fraction in clinical practice. Accordingly, the assay is needed to have a broad dynamic measurable range of at least 5 $\log_{10}$, especially with the low proviral load of 0.1–0.01% or less. Indeed, the performance of real-time HTLV-1 assay

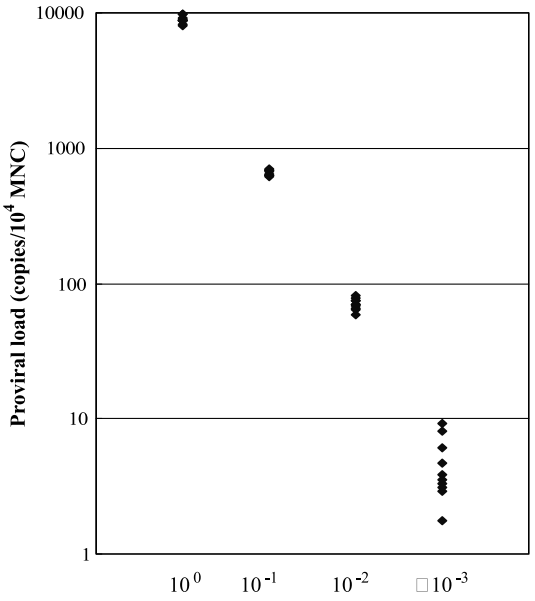

Fig. 2 Accuracy and reproductivity within run depend on different levels of proviral load. (*View this art in color at www.dekker.com.*)

systems developed is reported to be acceptable in terms of specificity, low detection limit (one copy per reaction), broad linear dynamic range, and reproducibility to be used both for qualitative detection and quantitative follow-up. Our real-time PCR system has also shown to be accurate and reproductive at different cell concentrations of 10^0–10^{-3} equivalent to about 100–0.1% infected cells/10^4 PBMCs in which 10^4 ATL cells harboring one proviral copy were diluted with normal lymphocytes, as shown in Fig. 2. The mean copies/10^4 PBMCs and coefficient values (CV) at each concentration were 8913±570, 655±32, 70±7, and 5±2, and 6.4%, 4.8%, 9.6%, and 51.8%, respectively. The between-day precision using the PBMCs from a seropositive healthy individual is reproducible with a CV of 15.9% and a mean of 189 copies. Now, Table 1 represents the practical data of the proviral load by real-time PCR in asymptomatic healthy carriers and patients with HAM/TSP, giving probably the approximate values.

Trials in clinical samples

A quantitative real-time PCR assay has been practically used to measure the HTLV-1 proviral load in PBMCs, subsequently making a diagnosis of persistent HTLV-1 infection and HTLV-1-associated disorders, and monitoring cell burden, namely, the numbers of lymphocytes or ATL cells harboring the HTLV-1 genome within their genomic DNA. These assays have been described to be excellent for diagnostic sensitivity and specificity in many articles cited using a Medline search. Our PCR assay also has given us a reasonable outcome [i.e., all of the seronegative samples were undetectable, whereas the seropositive samples were all positive in 108 cases without ATL (healthy carriers) and in 59 cases with overt ATL]. The HTLV-1 proviral load was 301±339 copies (mean±SD) per 10^4 PBMCs in healthy carriers, and ranged from 1.72×10^2 to 1.53×10^4 copies in ATL in parallel with ATL cell counts. Of the 108 healthy carriers who had no clinical manifestations without a clonal band by the HTLV-1 SBH test, the proviral load, in combination with the percentage (%) of ATL-like flower cells defined by PB smear morphology, enabled the carriers to be subgrouped into three categories [7] as shown in Fig. 3. However, in 7 of 59 ATL cases characterized by the SBH test for HTLV-1 integration status, the proviral load and ATL cell counts by morphology were discrepant. Interestingly, all of these cases showing discrepancies had two or three bands by the HTLV-1 SBH analysis using the *Eco*RI restrictive enzyme which has no cleavage site within the proviral DNA sequence. The ratio of the proviral load to ATL cell number was closely equivalent to the band number obtained by the SBH analysis, indicating that ATL cells from these cases harbored multicopies within a single ATL cell.

These cross-sectional data from each real-time PCR assay system for HTLV-1 quantification have been generally reported to be concordantly stable and significant, but the true usefulness of the ''quantification'' of

Table 1 Summary of the practical data of the proviral load by real-time PCR in asymptomatic carriers and patients with HAM/TSP in different studies

	Nagai et al.[8]	Gabet et al.[9]	Manns et al.[10]	Dehee et al.[11]
Asymptomatic carriers	120 copies/10^4 PBMCs (1.2%)	4068 copies/ 1.5×10^5 (2.7%)	1076 copies/10^5 lymphocytes (1.1%)	4532 copies/10^6 PBMCs (0.45%)
HAM/TSP	798 copies/10^4 PBMCs (8.0%)	ND	4861/10^5 lymphocytes (4.8%)	105,160/10^6 PBMCs (10.5%)

Values in parentheses represent percentage of cells infected with HTLV-1.

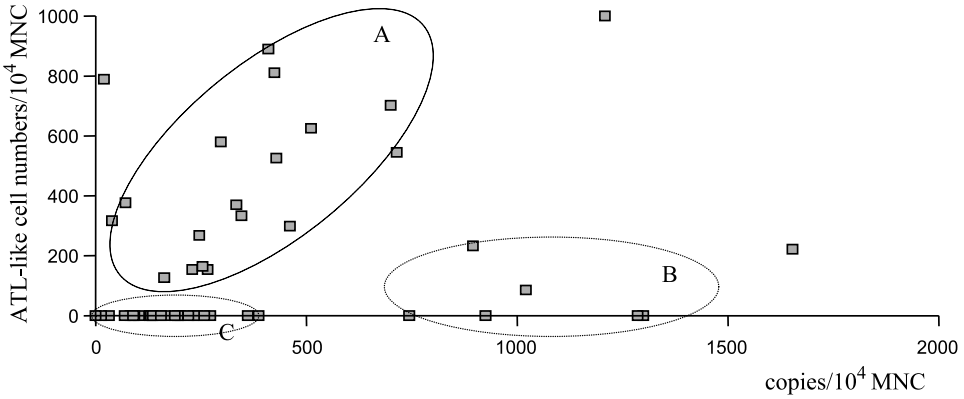

Fig. 3 Twin plot graph showing the correlation between the copy number by the proviral load and ATL-like cell counts in healthy carriers. The points corresponding to individual cases tend to fall into three subgroups A, B, and C. (*View this art in color at www.dekker.com.*)

the HTLV-1 proviral load has to be confirmed on a large series with a long-term follow-up of the carriers and the patients.

CONCLUSION

A real-time PCR assay for HTLV-1 using suitable primers and probes is relevant for quantitatively detecting the provirus of HTLV-1 in healthy carriers and patients with HTLV-1-associated disorders to understand the pathological roles of the virus.

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Human Herpesvirus (HSV) 1 and 2—Resistance Testing

Florence Morfin
Emilie Frobert
Danielle Thouvenot
Claude Bernard University-Hospices Civils de Lyon, Lyon, France

INTRODUCTION

Herpes simplex virus (HSV) type 1 and type 2 are very common. After primary infection, these viruses have the ability to establish latent infection and may be responsible for frequent recurrent infections. They are most often associated with mild mucocutaneous infections and rarely with serious diseases affecting the central nervous system, the eye, and the newborn. These viruses may also be responsible for serious complications among immunocompromised patients.

HERPES SIMPLEX RESISTANCE HISTORY

Acyclovir (ACV) is the gold standard for the treatment of HSV infections. This drug is a nucleoside analogue of guanosine that has to be phosphorylated three times. The first phosphorylation is achieved by the thymidine kinase (TK) encoded by the virus; this step is important as it allows ACV to become active only in infected cells. The second and third phosphorylations are carried out by cellular thymidylate kinases. Acyclovir triphosphate is a competitive inhibitor of viral DNA polymerase and is a DNA chain terminator.[1]

Herpes simplex virus strains resistant to ACV have been reported since 1982.[2] They were most often recovered from immunocompromised patients previously treated with ACV.[3] Herpes simplex virus resistance to ACV may be associated with a mutation in one of the two viral enzymes involved in ACV mechanism of action: TK and/or DNA polymerase. Mutations occurring in the viral gene encoding TK are the most frequent and 95% of ACV-resistant isolates present a TK-deficient phenotype.[4] These mutations leading to resistance occur spontaneously during viral replication and resistant viruses are then selected by antiviral treatment. Few cases of resistance have been associated with a mutation in the viral DNA polymerase gene.[5] This mechanism is rarely involved as a functional DNA polymerase is essential for viral replication unlike viral TK.

CLINICAL DESCRIPTION AND EPIDEMIOLOGY

Among immunocompetent patients, resistance to ACV is rare, with a prevalence below 1%[6] and most often detected in the course of recurrent genital herpes. In this situation, the observed prevalence ranged from 3.5%[7] to 8.6%,[8] and in most cases, clinical course was unchanged.

Prevalence of ACV resistance among immunocompromised patients is about 5% according to several published reports recently reviewed by Bacon et al.[6] But this global incidence has to be specified according to the type of immunosuppression as resistance may be detected in up to 25% of allogeneic bone marrow transplant patients presenting with an HSV infection.[9]

Among immunocompromised patients, resistance is associated with clinical failure after 7 to 14 days of treatment. Lesions may present as large, ulcerated, and extensive. These infections are often serious and may become chronic.[10] After healing, recurrent infections are most often associated with an ACV-sensitive strain, but some cases of recurrences due to an ACV-resistant virus have been reported.[11–14]

MANAGEMENT OF RESISTANT INFECTIONS

The first way to manage ACV-resistant HSV infection is, when possible, to decrease immunosuppressive treatments.[15] An increase of antiviral dose should also be considered.[16]

Several alternative antiviral drugs are also available. Penciclovir is a nucleoside analogue of guanosine and is very similar to ACV. Indeed, most ACV-resistant HSV isolates are also resistant to penciclovir. Foscarnet, a pyrophosphate analogue, and cidofovir, a nucleotide analogue of cytidine, act directly on viral DNA polymerase without previous activation by viral TK and both these molecules are active on viruses resistant to ACV because of a mutation in the TK gene.[17,18] Acyclovir-resistant management strategies usually recommend the use of

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foscarnet as a first line treatment and cidofovir in case of failure of foscarnet.[16] However, in clinical practice, both of these drugs may be associated with a significant level of toxicity.

Cross-resistance between ACV and foscarnet has been documented in immunocompromised patients.[19–21] Nevertheless, foscarnet-resistant strains sensitive to ACV have also been reported.[21] Acyclovir-resistant strains, cross resistant or not to foscarnet, have always been shown to be sensitive to cidofovir.[9,22] In addition, resistance to cidofovir, never reported so far in clinical strains, has only been described in strains selected in vitro.[23]

PHENOTYPIC DETECTION

In vitro evaluation of HSV susceptibility to antiviral drugs is based on the multiplication of a virus in the presence of increasing concentrations of antiviral drug. These techniques allow the calculation of the antiviral drug concentration leading to a 50% inhibition of viral replication (inhibitory concentration 50%, IC50). In order to discriminate between susceptible and resistant strains, IC50 thresholds have to be defined for each virus and antiviral drug pair. These values are usually determined according to the mean value obtained for susceptible viruses: three to five times the mean value of ACV-susceptible HSV for example. But these thresholds are arbitrary and the best way is to check the evolution of IC50 values of sequential isolates from a patient.

GENETIC CHARACTERIZATION

Herpes simplex virus TK is involved in 95% of HSV resistance to ACV. It is a 376-amino acid protein, encoded by a gene of 1128 bp (UL 23). It exhibits an ATP binding site (amino acid 51 to 63) and a nucleoside binding site (amino acid 168 to 176) and six regions conserved among herpesviridae TK (amino acid 50 to 66, 79 to 91, 162 to 178, 212 to 226 and 281 to 292). Figure 1 presents the localization of mutations reported in ACV-sensitive and ACV-resistant HSV clinical isolates, in relation to the localization of active and conserved sites of the enzyme. These data were obtained by genetic characterization of about 70 ACV-resistant HSV isolates and 30 ACV-sensitive HSV isolates.[24,25] These studies revealed a large degree of polymorphism in the HSV TK gene. The mutations unrelated to resistance are located throughout the gene but

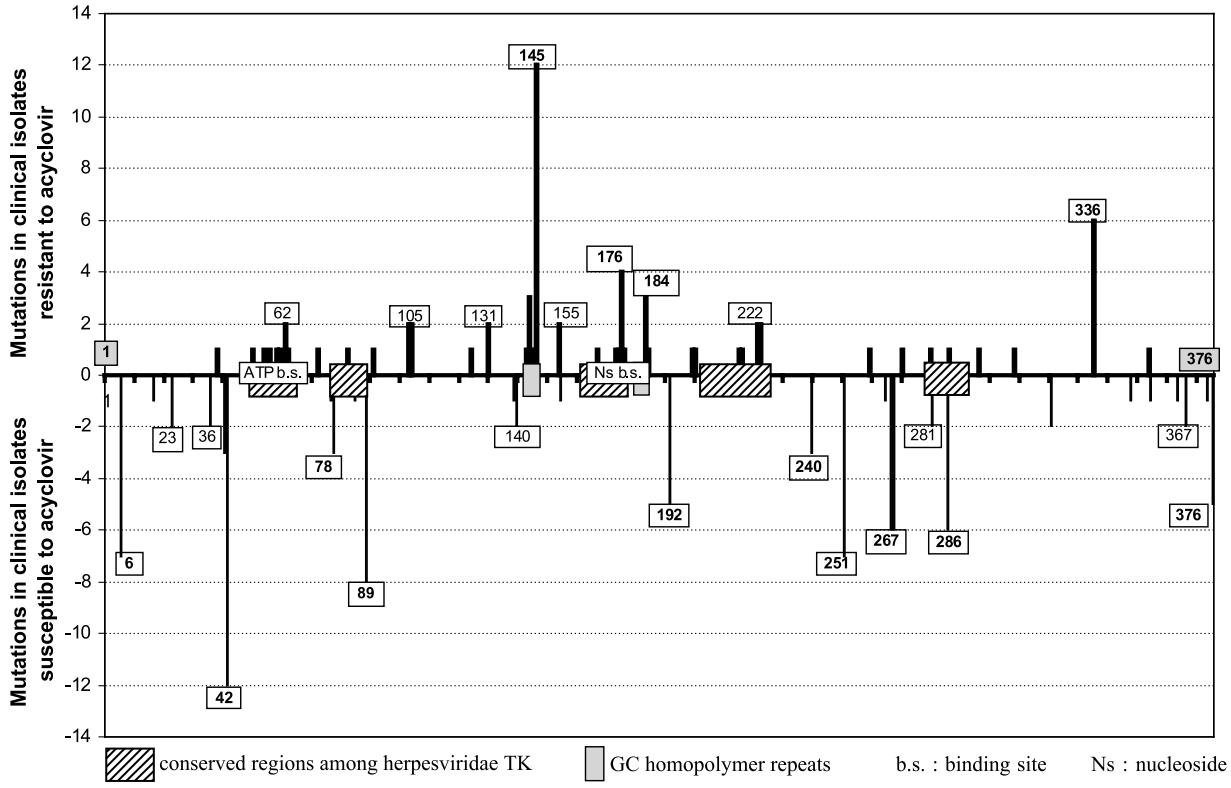

Fig. 1 Thymidine kinase mutations reported in ACV-sensitive and ACV-resistant HSV clinical isolates.

they are almost all located outside TK active and conserved sites. Gene polymorphism is four times higher for HSV 1 than for HSV 2.[26] Among mutations that could be associated with resistance, half were nucleotide insertion or deletion, and half were nucleotide substitution.[27–29] Nucleotide insertions or deletions are responsible for a frameshift and the synthesis of a truncated, nonfunctional TK. They are often located in homopolymer repeats of guanines or cytosines that are mutational hot spots. Mutations were found several times in repeats located at codon 92 (five guanines) and at codon 146 (seven guanines). The frameshift mutation in the 7Gs homopolymer of codon 146 is the most frequent mutation reported in ACV-resistant HSV isolates.[21,27,28,30] Numerous nucleotide substitutions, most often recovered in conserved regions of the TK gene, have been described in ACV-resistant clinical HSV strains, but some are reported more frequently as the substitution of arginine 176 of HSV 1 and 177 of HSV 2, and the mutation of amino acid 336.[24]

Herpes simplex virus DNA polymerase is a 1235-amino acid protein encoded by a 3705-bp gene (UL 30). It includes eight conserved regions in comparison with cellular or viral DNA polymerases; these regions are labeled I to VII according to their degree of conservation (region I being the most conserved), plus one region called A. DNA polymerase mutations associated with resistance are related to genetic characterization of about 15 resistant strains either isolated from patients[21,31] or selected in vitro.[23,32,33] As for TK, these mutations are mainly located in the conserved regions, especially in regions II and III which include more than 40% of the mutations reported so far in the DNA polymerase gene.

MOLECULAR GENETIC TESTING

Phenotypic methods require previous isolation of viral strains on cell cultures, which is time consuming and delays the change of antiviral treatment according to in vitro susceptibility. Genotypic tests are now being developed to detect resistant virus within a shorter time. The viral genes encoding the two targets of antiviral drugs (TK and DNA polymerase) are amplified by PCR and PCR products are then sequenced. The difficulty lies in the fact that numerous nucleotide substitutions may be found and these must then be identified as mutations responsible for resistance or not. This interpretation will be made easier as more and more results on mutations detected in resistant isolates are collected. In addition, site-directed mutagenesis studies are in progress to formally assess the role of the mutations in the development of antiviral drug resistance. Results already obtained by genetic characterization of clinical isolates resistant to ACV indicate that sequencing of limited areas in the TK and DNA polymerase genes is sufficient to detect most of the mutations associated with resistance. For TK, which is only 1128 nucleotides long, it is preferable to sequence all the gene, but sequencing of nucleotides 150 to 600, combined with a specific detection of mutations at codon 336, should allow the detection of 80% of the mutations associated with resistance. For DNA polymerase, we focused on the region between nucleotides 1500 and 3000, which includes 90% of the mutations reported in resistant strains.

At the present time, genetic detection of ACV-resistant HSV is based on PCR amplification and sequencing of

Table 1 Primers for TK and DNA polymerase genetic characterization

Target gene	Primers for PCR	PCR product length and localization	Primers for sequencing
Thymidine kinase (1128 bp)	**TK1**: GGGGATCCTCCCG-CACCTCTTTGGC (forward)	635 bp	• Coding strand with TK1
	TK3: CGCAAGCACCGGG-AGTACCTAGGGG (reverse)	38 nt before ATG to nt 581	• Noncoding strand with TK3
	TK2: GGGGATCCGATA-CCTTATGGGCAGC (forward)	638 bp	• Coding strand with TK2
	TK4: TGTGCCTTCCTCT-GTTACCTAGGGG (reverse)	Nt 526 to 19 after stop codon	• Noncoding strand with TK4
DNA polymerase (3705 bp)	**LP6**: GAGCACGTCCTCC-TGTTTTC (forward)	1313 bp	• Coding strand with LP6 and LP7
	RP6: CCGAGTTACACACGACCTTG (reverse)	Nt 1219 to 2449	• Noncoding strand with RP6 and RP8: ATGCAGTACTCGCCGATCAC (reverse)
	LP7: GTATTAACATCACCCGCACC (forward)	1135 bp	• Coding strand with LP7 and LP8: TGTAACTCGGTGTACGGGTTC (forward)
	RP7: CGGAGACGGTATCGTCGTAA (reverse)	Nt 1817 to 2953	• Noncoding strand with RP6 and RP7

target sequences within the TK gene and/or the viral gene encoding DNA polymerase. Primers that may be used are summarized in Table 1. These techniques have been applied to the genetic characterization of resistant isolates detected with phenotypic tests.[28] The first step of the development of genotypic tests could be the optimization of PCR amplification to increase its sensitivity so that it could be performed directly on clinical samples. A specific PCR amplification of HSV TK gene, involved in 95% of resistance cases, followed, if positive, by PCR product sequencing could allow the detection of mutations associated with resistance to ACV. A complete database of the HSV mutations associated with ACV resistance or TK polymorphism is needed to interpret sequencing results. In this way, results could be available in 24 to 48 hr, allowing early change of antiviral treatment according to virus susceptibility.

As more and more data are available on TK mutations associated with resistance, the GeneChip technology, developed by Affimetrix (Santa Clara, CA) may be considered as a promising method for the detection of resistance. This method of genetic diversity analysis relies upon the hybridization of the nucleic acid target to a large set of oligonucleotides. This technology has already been applied to detect mutations into HIV and *Mycobacterium* species genome,[34,35] giving results in a few hours. Herpes simplex virus arrays could thus represent a rapid and automatable method to detect mutations into TK and DNA polymerase genes associated with resistance to antiviral drugs.

CONCLUSION

The risk of emergence of HSV strains resistant to ACV remains a major concern because of the increase in the number of immunocompromised patients and in the use of antiviral drugs, both for prophylactic and therapeutic treatments of herpesvirus infections. So far, even if phenotypic tests are relevant to detect resistant strains, they are time consuming and delay the change of antiviral treatment according to in vitro susceptibility. Therefore it is important to develop new tests based on molecular biology techniques to allow the detection of resistant viruses more rapidly. Because of the numerous mutations reported in resistant strains, both in TK and DNA polymerase genes, and because of the high degree of polymorphism, specially in the TK gene, a complete database of the mutations associated or not with resistance to antiviral drugs has to be established for the interpretation of TK and DNA polymerase sequencing results in new isolates. Genotypic tests based on PCR associated with sequencing would be performed directly on clinical samples preventing the delay of virus isolation by culture. The development of microarrays applied to HSV could constitute a further improvement in the detection of HSV resistance. These new assays may help clinicians in the management of antiviral treatment of HSV infections.

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Human Herpesvirus 6 and 7

David H. Dockrell
University of Sheffield Medical School, Sheffield, U.K.

INTRODUCTION

Human herpesvirus-6 (HHV-6) was discovered by Salahuddin et al. and HHV-7 by Frenkel et al. Both viruses cause the childhood exanthem roseola and may reactivate in immunocompromised hosts. Advances in genomics and proteomics are providing novel insights concerning these novel immunomodulating lymphotropic herpesviruses.

TYPE OF PATHOGEN

HHV-6, HHV-7, and human cytomegalovirus (HCMV) are the three known human members of the Betaherpesviridae subgroup of herpesviruses and HHV-6 and HHV-7 form the *Roseolavirus* genus.[1,2] Genetic, immunologic, and biological differences support the existence of distinct strains of HHV-6: HHV-6A and HHV-6B. The viruses contain an envelope, icosahedral capsid, and linear double-stranded DNA.

HHV-6A, HHV-6B, and HHV-7 have a similar genomic structure (Fig. 1). The central portion contains the unique (U) segment flanked at either end by duplicated sequences (DR). The number of kilobase pairs (kbp) encoded within the left end of each DR segments termed the *het* or heterogeneous region is variable and accounts for much of the variability in size between strains. The DR termini contain an analog of human telomeric repeats, a repetitive GGGTTA sequence. At either end of DR are homologs of the herpesvirus cleavage and packing signals, *pac-1* and *pac-2*. HHV-6A strain U1102 is 159 kbp, HHV-6B strain Z29 or HST is 162 kbp, and HHV-7 strains JI and RK are 145 and 153 kbp, respectively.[3–7] The majority of genes are common to all Betaherpesviridae. HHV-6B contains 115–119 potential open reading frames (ORF) (97 unique genes), HHV-6A contains 113 ORF (85 unique genes), and HHV-7 contains 101 ORF (84 unique genes). All HHV-7 genes except one have direct HHV-6A counterparts and all genes except two in HHV-6A have HHV-7 counterparts. Of the 97 unique genes in HHV-6B, it is estimated that 88 have HHV-6A counterparts and 82 have HHV-7 counterparts. Genomic differences are encoded mainly in DR and U86-100. Overall nucleotide sequence identity between HHV-6A and HHV-6B is 90%. HHV-6A strains show greatest variability and HHV-7 strains show least with differences mainly involving the telomeric reiterations.

Clinical Description of Infection

HHV-6B is the principle cause of the exanthem roseola infantum (exanthem subitum), an illness characterized by high fever and development of a rash after fever resolves.[8] HHV-7 can also cause roseola. However, the majority of children with HHV-6B or HHV-7 infection develop an undifferentiated fever, but this may be complicated in some by febrile convulsions, encephalopathy, and hepatitis. Primary infection is rare in adults but can occur including an infectious mononucleosis-type illness.

These viruses are ubiquitous and frequently reactivate. Proof that viral replication is causal in specific disease associations is usually lacking. The greatest attention has focused on links between HHV-6A and multiple sclerosis and HHV-7 and the skin rash pityriasis rosea. Links between HHV-6/HHV-7 and chronic fatigue syndrome have not been substantiated by molecular techniques.

Interactions between HIV-1 and HHV-6A, HHV-6B, or HHV-7 replication occur. Each of the herpesviruses can up-regulate or down-regulate HIV-1 replication under specific conditions, but the significance is uncertain. HHV-6 infection in the first year of life has been associated with more rapid HIV-1 disease progression in vertically infected infants. HHV-6A may contribute to the switch between CCR5 (M-tropic) and CXCR4 (T-tropic) virus late in HIV-1 infection. Reactivation of HHV-6A, HHV-6B, or HHV-7 in immunosuppressed transplant recipients can induce clinical syndromes including undifferentiated febrile illness, cytopenias, hepatitis, encephalopathy, rash, or pneumonitis. Indirect effects of reactivation of these viruses, posttransplantation may be more frequent and include increased HCMV replication, increased severity of HCMV disease, enhanced immunosuppression leading to fungal superinfection, and in association with HCMV graft dysfunction or rejection.

Prevalence

Infection with HHV-6 is most frequent at 6–12 months of age and HHV-7 is a little later at 6–24 months. More than

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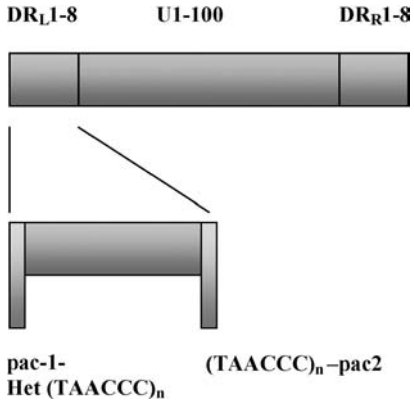

Fig. 1 Organization of viral genome. The viral genomes consist of linear double-stranded DNA with a central unique segment (U) and directly repeated sequences (DR) at each end. The DR encodes homologs of the herpesvirus cleavage and packing sequences *pac-1* and *pac-2*. The terminal and junctional segments of the DRs contain human telomere repeats $(TAACCC)_n$ and variation in length maps to the left side of each DR regions (heterogeneous or Het region). (*View this art in color at www.dekker.com.*)

90% of adults are seropositive for HHV-6 and up to 90% for HHV-7 worldwide. The majority of pediatric infections in western populations are due to HHV-6B, but HHV-6A causes pediatric infections in nonwestern populations. Neurological diseases or infections in immunocompromised individuals are more often HHV-6A-related. Saliva is likely to be an important route of transmission especially for HHV-6B and HHV-7 with mother to child transmission.

Management

Most HHV-6 or HHV-7 infections in childhood require no specific antiviral therapy, but therapy is indicated for complicated infections especially in immunocompromised patients. HHV-6 and HHV-7 lack thymidine kinase and are resistant to acyclovir. The guanine analog Ganciclovir (GCV) is, however, active: 50% effective inhibitory concentration (EC_{50}) for HHV-6A (GCV EC_{50} is 0.65 μg/mL), for HHV-6B (GCV EC_{50} is 1.33 μg/mL), and for HHV-7 (GCV EC_{50} >7 μg/mL).[9] The HHV-7 GCV EC_{50} is greater than plasma concentrations of GCV routinely achieved with administration of GCV 5 mg/kg intravenously. The U69 gene product of HHV-6 and HHV-7 is a phosphotransferase analog of HCMV UL97, a GCV kinase. It is required to phosphorylate GCV so that it inhibits the viral DNA polymerase (the U38 gene product) (Fig. 2). The HHV-7 phosphotransferase is least efficient at phosphorylating GCV. Point mutations in the U69 and U38 are associated with GCV resistance. HHV-6 and HHV-7 can respond to GCV treatment, but a report exists of prophylactic GCV, administered to transplant recipients, failing to prevent HHV-7 replication despite adequate suppression of HCMV.

The pyrophosphate analog, phosphonoformic acid (Foscarnet, FSC), inhibits the DNA polymerase of all three viruses. It may be of particular use, as in HCMV, in the treatment of infections failing to respond to or resistant to GCV. The acyclic nucleoside phosphonate, cidofovir (CDF), also inhibits DNA polymerase; CDF EC_{50} is 0.3 μg/mL for HHV-6A, CDF EC_{50} is 1.2 μg/mL for HHV-6B, and CDF EC_{50} is 3 μg/mL for HHV-7. U38 mutations may also decrease susceptibility to this agent. Because of

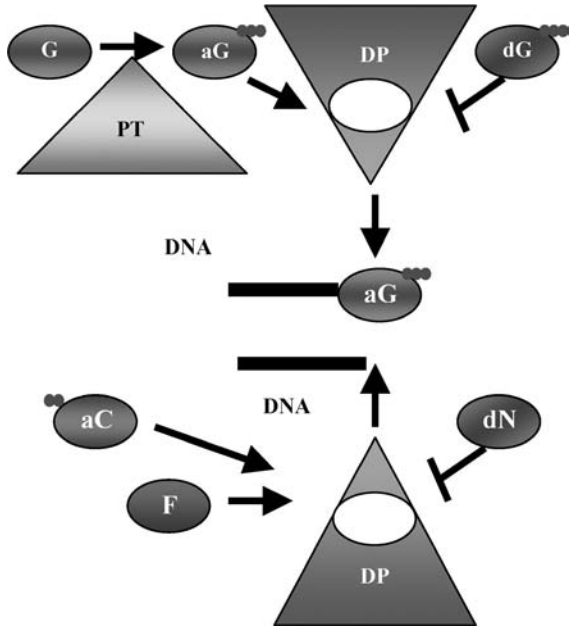

Fig. 2 Mechanism of action of antiviral compounds against HHV-6 and HHV-7. Ganciclovir (G) is phosphorylated by a phosphotransferase (PT) to become the active triphosphorylated compound (aG). aG binds to the viral DNA polymerase (DP) and competitively inhibits the binding of deoxyguanosine triphosphate (dG) to the polymerase. aG is incorporated into DNA resulting in retardation and cessation of chain elongation. Foscarnet (F) inhibits the binding of a range of deoxynucleotide triphosphates (dN) to the pyrophosphate-binding site of DP and blocks pyrophosphate cleavage. Foscarnet does not require intracellular phosphorylation. Cidofovir requires virus-independent intracellular diphosphorylation (aC). aC competitively inhibits the binding of deoxycytosine triphosphate to DP. aC is incorporated into DNA slowing elongation and, if two successive aC bind, terminating chain elongation. (*View this art in color at www.dekker.com.*)

nephrotoxicity, there is less experience with this agent in transplant recipients.

MOLECULAR CHARACTERIZATION OF PATHOGEN

Proteomic Analysis

Analysis of the sequenced genomes and by specialized protein expression systems has provided insights into protein functions. As is typical of herpesviruses, proteins are transcribed as immediate-early (IE, synthesized within minutes of infection and independently of de novo protein synthesis), early, or late proteins. Table 1 summarizes some of the principle genes and the proteins encoded.

Table 1 Human herpesvirus-6 and herpesvirus-7 genes and gene products

Gene	Product
Gene regulation	
U86–89	IE-A gene block. U89 encodes IE1
U95	IE protein (IE2)
U16–19	IE-B gene block
DNA synthesis and viral replication	
U27	Polymerase processivity factor
U38	DNA polymerase
U41	Major DNA-binding protein
U43/74/77	Helicase/primase complex
U73	Origin-binding protein
U94	RepH6
Nucleic acid metabolism	
U28	R1 subunit of ribonucleotide reductase
U45	dUTPase
U69	Phosphotransferase
U81	Uracil–DNA glycosylase
Structural proteins	
U11	Tegument protein
U14	Tegument protein
U39	gB
U48	gH
U72	gM
U82	gL
U100	Heparin-binding glycoprotein
Miscellaneous	
U53	Protease
U83	Viral chemokine, vCCL4
U12	Chemokine receptor
U51	Chemokine receptor
U69	Protein kinase
DR7	p53-binding protein

The genes encoding IE genes show variability between HHV-6 and HHV-7. The transcriptional regulation is incompletely characterized, but NF-κB and AP-1 binding sites have been identified. IE1 is a nuclear phosphoprotein that transactivates heterologous promoters and plays a role in latency-associated gene transcription. IE2 is more highly conserved between HHV-6 strains and HHV-7. The U16 gene product of HHV-6 and the p53 binding protein encoded by DR7 transactivate the HIV-1 long terminal repeat. The DR7 gene product has transforming activity.

Proteins involved in replication are highly conserved. The DNA polymerase binds a 40–41 kDa protein (DNA polymerase processivity factor) that localizes to the nucleus. Viral replication in vitro is enhanced by the presence of multiple copies of the origin of lytic replication that bind the origin of replication binding protein (OBP). U94 encodes a homolog of the human adeno-associated virus 2 (AAV-2) rep gene and is absent in HHV-7. It binds the human TATA-binding protein, a transcription factor and aids maintenance of latency by down-regulating gene transcription. It also inhibits cell transformation and transactivation of HIV-1 LTR.

Structural proteins include two heparin-binding glycoproteins, gB and the U100 product, that contain subtype-specific epitopes. U100 products include gp105 of HHV-6 (gp82–105) and gp65 of HHV-7 and are unique to HHV-6 and HHV-7. U100 can be differentially spliced and shows marked variation between HHV-6A and HHV-6B with possible biological implications. A U100-transcribed protein in HHV-6 has been identified as gQ, an 80 kDa protein, that complexes with gH and gL. gL plays a role in the transport and processing of gH. gH and gL form heterologous complexes both with each other and gH/gL of other β-herpesvirus that might facilitate viral interactions. gB, gH, and U100 products are targets for neutralizing antibodies. gM is a highly conserved glycoprotein. The glycoprotein encoded by U22 is not present in HHV-7. pp85 of HHV-7 is an immunodominant tegument phosphoprotein that forms a protein complex that is enriched in dense bodies. Other immunodominant tegument proteins include the p100 phosphoprotein of HHV-6 and p89 of HHV-7 that contain type-specific epitopes. A viral encoded protease contributes to viral maturation. HHV-6 and HHV-7 encode two G-protein-coupled receptors that are β-chemokine receptors. Signaling via the U51-encoded receptor can result in down-regulation of the chemokine RANTES. HHV-6A and HHV-6B (but not HHV-7) encode a functional chemokine

that contributes to viral immunomodulation. U83 is subject to differential splicing.

Host Pathogenesis

The roseola viruses induce acute lytic infection, latency, and some features of cell transformation. The cell receptor for HHV-6 is CD46, a ubiquitous type-1 glycoprotein that regulates complement activation. CD46 binds to gH via a gH–gL–gQ complex.[10] As CD46 is widely expressed, this explains the broad cell tropism noted. CD4+ T-lymphocytes are preferentially infected, but CD8+ T-lymphocytes, γδ T-lymphocytes, natural killer (NK) cells, macrophages, dendritic cells, bone marrow progenitors, fibroblasts, epithelial cells, and fetal astrocytes can also support viral replication. Some differences in infection of transformed lymphocytes have been noted between HHV-6A and HHV-6B, while HHV-6A has greater neurotropism. Other coreceptors for infection may exist. HHV-7 requires cell surface CD4 association with gB which is necessary but not sufficient for viral internalization. Cell surface heparin-like glycoproteins are also required for viral entry. HHV-7 tropism is mainly for CD4+ T-lymphocytes. However, HHV-7 can be identified in diverse tissue samples suggesting that HHV-7 cell tropism may be broader. In particular, HHV-7 antigens have been identified in salivary epithelial cells. HHV-7 replication has also been identified in macrophages and CD34+ hematopoietic progenitor cells.

Viral replication involves production of DNA-containing capsids in the nucleus, acquisition of tegument in the cytoplasm, and addition of a secondary envelope containing glycoproteins acquired from the annulate lamellae. Virions transit through the Golgi where glycosylation further modifies the envelope glycoproteins before release of mature virions. Infected host cells can die by apoptosis or necrotic lysis. Polypoid giant cells have been described with in vitro HHV-7 infection and result from cell cycle dysregulation. After primary infection, the virus is maintained in a latent state with low levels of gene transcription. Intermittent bouts of lytic replication allow persistence. Interestingly, specific T-lymphocyte activation stimuli can induce HHV-7 but not HHV-6B reactivation in vitro. HHV-7 replication produces HHV-6B transactivation suggesting that in vivo, there may be a sequence to viral reactivation.

The host response to infection involves production of neutralizing antibody and cell-mediated immunity. T-lymphocyte IFN-γ responses are detected against type-specific antigens. NK cells also contribute to host immunity via IL-15 production. HHV-6A and HHV-6B induce immunomodulation by depletion of CD4 T-lymphocytes via destruction of thymic progenitors and, as with HHV-7, via peripheral induction of apoptosis. The majority of apoptotic cells are uninfected and apoptosis can involve Fas ligand, TNF-α, or p53. CD4+ T-lymphocytes directly infected with HHV-7 up-regulate the antiapoptotic molecule bcl-2 and down-regulate the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) R1 receptor to prevent apoptosis and maintain viral replication, but up-regulate TRAIL to induce apoptosis in immune effector cells. HHV-6A also depletes CD4 T-lymphocytes by formation of syncytia. A further mechanism of immune evasion described for HHV-7 is blockade of viral antigen presentation to cytotoxic T-lymphocytes by the binding of the U21-encoded glycoprotein to major histocompatibility complex (MHC) class 1 molecules that diverts the MHC molecules to lysosomes.

T-lymphocyte proliferation is inhibited by down-regulation of IL-2 and function inhibited by down-modulation of CD3, CD4, and the chemokine receptor CXCR4. Type-1 response cytokines up-regulated in T-lymphocytes include TNF-α, IFN-γ, and IL-18. TGF-β is also up-regulated. IL-8 and adhesion molecule induction enhances inflammatory injury, as may production of reactive oxygen species and PGE_2 by monocytes. HHV-6 encodes a unique chemokine analog, vCCL4, that binds to the chemokine receptor CCR2 expressed on cells including macrophages. HHV-7 also up-regulates the lymphocyte-specific G-protein-coupled receptor EB 1 which aids recruitment of infected lymphocytes to areas in which they can propagate infection.

Molecular Testing

HHV-6 or HHV-7 can be cultured from clinical specimens using coculture with stimulated lymphoblasts. Use of cord blood lymphocytes limits the chance of measuring replication of endogenous virus from the lymphoblasts. Demonstration of cytopathic effect or of viral antigen supports the diagnosis. Viral culture is, however, time-consuming. Serologic diagnosis is of greatest use in seroprevalence studies. An immunofluorescence assay or an enzyme immunoassay is most commonly employed. Appropriate controls are essential to exclude cross-reactions between Betaherpesviridae and should include a preadsorption step with appropriate antigens. An immunoblot assay has been described for detection of HHV-7 using the p89 tegument protein. Immunohistochemistry often uses antibodies against gp82 (HHV-6A), p101 (HHV-6B), or pp85 (HHV-7). In situ hybridization using HHV-7 plasmid clone (pH7SB-268) has also been described.

The most sensitive diagnostic technique is quantitative PCR that is rapid and can be performed on samples obtained by noninvasive means. Real-time PCR using TaqMan reaction combines single-step amplification with

computer-based analysis. Detection of viral DNA in whole blood does not distinguish acute from previous infection but a high viral copy number, DNA in the absence of antibody, or DNA in plasma can suggest acute infection. Quantitative PCR is also of use in diagnosing reactivation in immunocompromised hosts, but HHV-6A DNA may not be identified in peripheral blood lymphocytes. Nested or multiplex PCR methodologies are also described. Published primers include a 240-bp amplicon of HHV-6 major antigen's structural protein, 5′-ATCTC-GATTCCGTTCAGTCT-3′ and 5′-TGAGACACCTGAA-GAAAAAG-3′, and a 186-bp amplicon corresponding to the U10 encoded structural protein of HHV-7, 5′-TATCCCAGCTGTTTTCATATAGTAAC-3′ and 5′-CAAAAAATCTAGTGCTACCGCAAGGC-3′.[11] For discrimination of HHV-6A and HHV-6B, primers that amplify an IE target have been described; a 278-bp amplicon of HHV-6A is generated with primers H6fA 5′-CATGAAGATGATGACAA-TAAAATG-3′, H6bA 5′-TGGAACCATCTTGTTCTGTCC-3′, and exonuclease probe H6tmA 5′-FAM-CCGCCCAGA[TAMRA]TCTGT-CACTGAGGCTG-3′p and a 145-bp amplicon of HHV-6B with primers H6fB 5′-GAGACCGGGTCTGGACAACA-3′, H6bB 5′-GAGTTGCTGAGTTGGTAAAGG-3′, and exonuclease probe H6tmB 5′-FAM-CTCCAAGTG-TACCGAAACGC[TAMRA]TTCCTGG-3′p.[12]

CONCLUSION

HHV-6A, HHV-6B, and HHV-7 are of interest as novel lymphotropic viruses. Because of the ubiquitous nature of these viruses and the subtle ways they interact with the immune system, further studies investigating the role they play in human diseases will be of great interest.

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Molecular Diagnosis of HIV-1 and HIV-2

M

John R. Clarke
Imperial College of Science, Technology and Medicine, Paddington, London, U.K.

INTRODUCTION

More than 40 million people worldwide have been infected with the human immunodeficiency virus (HIV). There are 14,000 new infections every day and approximately 10,000 people die of AIDS every day. HIV-1 and HIV-2 are retroviruses and HIV-1 is responsible for the vast majority of HIV infections worldwide but HIV-2 predominates in parts of West Africa and has been detected in Europe, Asia, and North America.

The molecular diagnosis of HIV involves two major strategies. Firstly, the monitoring of plasma HIV RNA viral load in conjunction with CD4 T-lymphocyte cell numbers as surrogate markers for disease progression and, secondly, the detection of specific point mutations in the HIV-1 *pol* gene that confer resistance to specific antiretroviral drugs. More recently, mutations in *gag* which alter the p6/p1 splice site, in *env* which confer resistance to the fusion inhibitors, and in *integrase* which confer resistance to integrase inhibitors have also become important. HIV-1 RNA viral load assays have revolutionized patient management. Quantitative measures of HIV-1 RNA have been used in the study of HIV-1 viral dynamics and as a marker for clinical endpoints in studies of new treatment regimens.[1] The dynamics of HIV-1 replication using HIV-1 show that the virus is very active during the long clinically asymptomatic stage of the disease.[2] There is a significant relationship between HIV RNA viral load in plasma and progression to AIDS. Patients with low viral load progress to AIDS at a slower rate than those with a high viral load.[3] It has become standard practice to monitor HIV-1 RNA viral load in order to assess the effectiveness of treatment and the likelihood of clinical progression.

HIV VIRAL LOAD ASSAYS

There are four commercially available HIV RNA viral load assays in common use:

Nucleic acid sequence-based amplification (NASBA), branched chain DNA amplification (bDNA), reverse transcription PCR (RT-PCR), and the ligase chain reaction (LCx). The NASBA assay (Organon Technica) utilizes the Boom method for the extraction of total RNA from a plasma sample and this method produces sufficient high-quality RNA that can be used for other investigations such as HIV genotyping. The major difficulty with NASBA is the cumbersome nature of the RNA extraction procedure and the low throughput of the method. Automation of the extraction procedure has in part addressed these difficulties but not eliminated the problem altogether. The NASBA assay utilizes three enzymes which operate at low annealing temperatures and this could result in nonspecific interactions of the primers. The absence of temperature-dependent cycling steps in NASBA reduces the extent to which the amplification process can be externally controlled and may lead to variation in amplification efficiency.

RT-PCR (Roche Amplicor) remains the most sensitive HIV viral load detection system. The new Ampliprep system provides the first fully automated system for the determination of HIV-1 viral load and this will eliminate potential variation in assay results introduced by human error. The Ampliprep system is also barcoded and is optimized for high throughput of samples. Polymerase chain reaction-based approaches increase the possibility of introduction of contaminating RNA which may then be amplified resulting in false positive results. The reliance on one set of primers derived from the *gag* gene of HIV-1 may mean that quasi-species of HIV with multiple mutations in the 3′ primer attachment sequences are inefficiently amplified leading to under-detection or not amplified at all leading to false negative results.

The bDNA method (Versant Diagnostics) amplifies signal and therefore it does not alter the number of target molecules present which reduces the effect that introduction of contamination into a sample may have .The assay is well suited to high throughput of plasma specimens with as many as 168 samples being processed per assay run in the semiautomated 340 machine. The assay has no internal control within the sample and therefore there is no way of discerning if amplification was successful or whether the test was inhibited.

More recently, Abbott Diagnostics has launched the HIV-1 LCx ligase chain reaction assay which uses similar technology to the Roche Amplicor system.

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Abbott has developed a fully automated robotic system for their assay which will reduce technical ''hands-on'' time and operator error.

COMPARISON OF VIRAL LOAD ASSAYS

A number of studies have compared the performance of these assays in clinical situations. Schuurman et al.[4] showed that interlaboratory reproducibility of the Roche, Organon, and Versant assays was very good. Others have shown that each of the assays has relatively low intra- and interassay variability.[5] The biological variation of HIV-1 RNA in plasma in clinically stable individuals is approximately threefold (0.5 log) and this variation was not associated with diurnal fluctuations.[6] The anticoagulant used is important for optimal results and the preferred anticoagulant for bDNA is EDTA while citrate or EDTA may be used for NASBA and RT-PCR. Heparin interferes with reagents used in the assays and should be avoided and there is also some question over its effectiveness as a preservative for RNA.

Some samples give discrepant results between assay systems and therefore the assays should not be used interchangeably.[7] Of concern is that all commercially available kit-based assays have been optimized against HIV-1 clade B viruses which predominate in North America and Europe but represent only 3% of infections worldwide.[8,9] The challenge for commercial nucleic acid-based tests is to detect and quantify accurately very divergent strains of HIV-1 and HIV-2. Under detection or complete lack of detection of some strains of HIV remains a major concern.[8,10] Recombination between distantly related strains may further contribute to the emergence of HIV-1 viral variants that escape detection or are underdetected by current molecular tests and there are several circulating recombinant forms or CRFs which may contain sequences from more than one strain of HIV-1. Comparison of the performance of bDNA and RT-PCR assays for the quantification of viral load from patients infected with non-clade B strains of HIV-1 has shown greater than 1.0 log variation in the detection of some strains.[10] The choice of method for quantification is dictated by throughput of samples and the genetic makeup of the patient cohort. There is an urgent requirement for international standards which should include non-clade B strains to enable quality control of viral load measurements.

VIRAL LOAD ASSAYS UNDER DEVELOPMENT

Real-time PCR methods for the determination of HIV-1 viral load uses light cycler instrumentation, and the procedure has increased speed in detection over conventional PCR methods because of the removal of manipulations required for the detection of the PCR amplicon and the incorporation of rapid amplification coupled to fluorescence detection. However, the length of time taken to extract HIV-1 RNA from biological fluids is a hindrance. A recent method which uses lysates may have resolved this difficulty.[11] A further adaptation of real-time diagnosis are tests that are able to detect low copies of HIV-1 nucleic acid[12] as low-level replication of HIV-1 below the detection of current viral load assays may have some clinical importance. The HIV-1 Taqman 5′ nuclease PCR is also under development and may have application to the large throughput applications such as the screening of plasma from blood donors.[13] Use of an internal control in each reaction is essential to monitor sensitivity. The optimization of new quantitative assays should take into consideration the diversity of HIV-1 and where possible be able to detect outlier strains and HIV-2.

Other screening assays currently in use in the molecular diagnosis of HIV-1 include the Roche Amplicor HIV-1 test which is a qualitative assay that utilizes PCR and nucleic acid hybridization for the detection of HIV DNA from whole blood. The assay was optimized for clade B viruses but there are additional primers which facilitate the detection of non-clade B viruses and has application for the detection of HIV in recently infected individuals prior to the production of antibodies. The

Table 1 Drugs available for use in combination antiretroviral therapy

Nucleoside analogues	Nonnucleoside analogues	Nucleotide inhibitors	Protease inhibitors	Fusion inhibitors
Zidovudine	Nevirapine	Tenofovir	Saquinavir	Enfuvirtide
Didanosine	Delavudine		Indinavir	
Stavudine	Efavirenz		Ritonavir	
Lamivudine			Nelfinavir	
Abacavir			Amprenavir	
Zalcitabine			Tipranavir	
			Lopinavir	
			Atazanavir	

method may also be adapted to detect HIV DNA in patients where antibody tests may be ineffective, such as infants born to HIV seropositive mothers.

MONITORING THE DEVELOPMENT OF ANTIRETROVIRAL RESISTANCE

There are currently 18 drugs licensed for use against HIV-1 and they are shown in Table 1. The compounds are active against the viral protease (protease inhibitors) and reverse transcriptase (nucleoside, nucleotide, and nonnucleoside analogues) enzymes. Other drugs in development include compounds directed against other viral targets such as integrase inhibitors and fusion inhibitors. Point mutations that confer resistance to nucleoside analogues and protease inhibitors appear to be similar for both HIV-1 and HIV-2.[14] However, HIV-2 isolates appear to be resistant to nonnucleoside reverse transcriptase inhibitors (NNRTIs) because of preexisting polymorphisms.[15] Mutations that

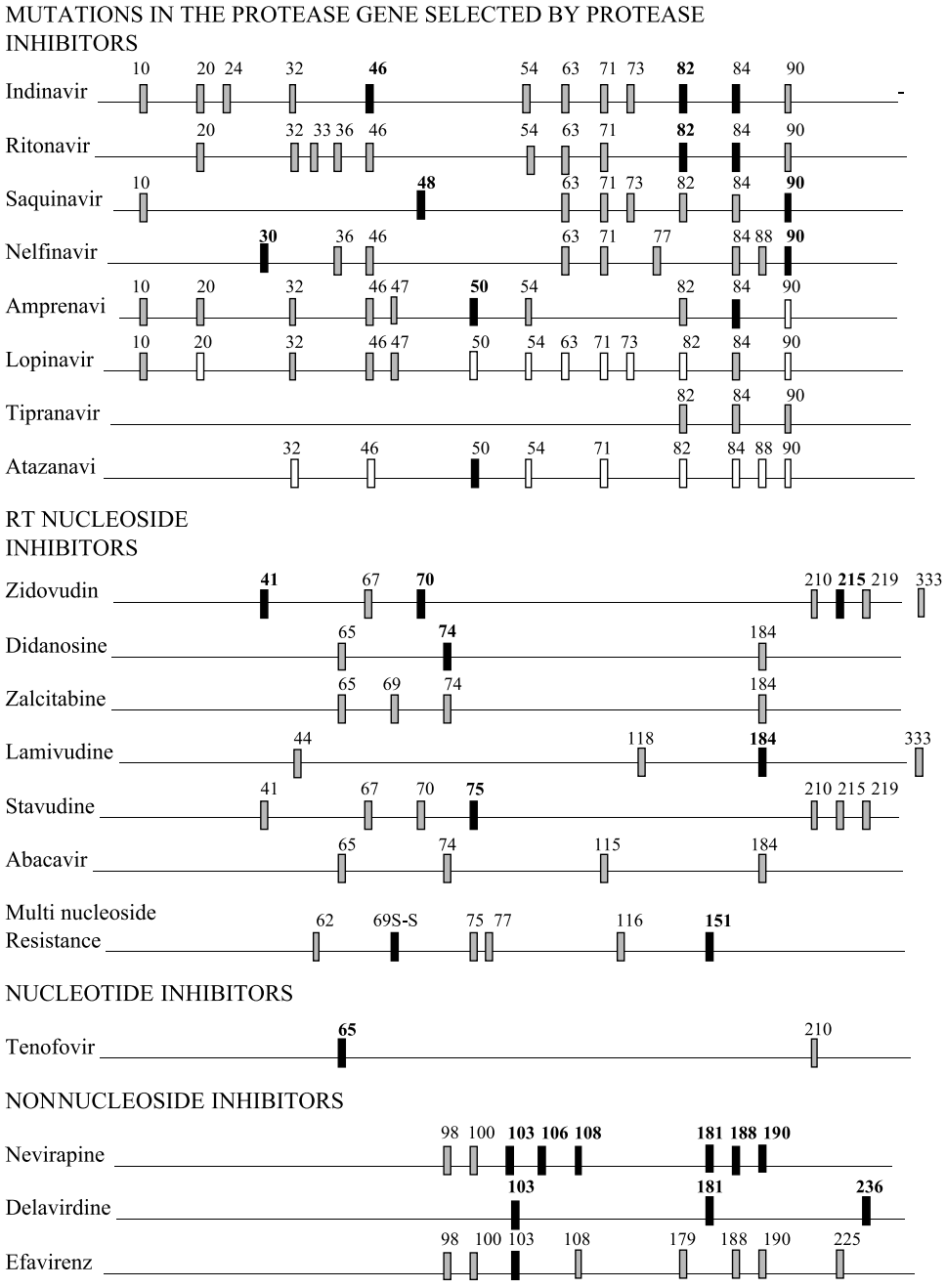

Fig. 1 Mutations in the HIV-1 *pol* gene known to reduce sensitivity of antiretroviral drugs. (*View this art in color at www.dekker.com.*)

confer resistance to antiretroviral drugs are well characterized (Fig. 1) and there are a number of PCR-based assays for the specific detection of known point mutations.

Initially, allele-specific PCR assays were developed by incorporating the specific point mutation conferring resistance into the final codon of the PCR oligonucleotide primer, and using stringent annealing temperatures to inhibit PCR amplification unless a perfect match was obtained.[16] The disadvantages of this approach were that low levels of nonspecific amplification could not be ruled out and that each mutation required a specific primer pair. An alternative approach for characterizing mutations was to use point mutation assays (PMA) which captured PCR products containing either wild-type or mutant codons using oligonucleotides containing the codon of interest.[17] The Inno-LiPA HIV-1 resistance assay is based on reverse hybridization technology.[18] The Inno-LiPA strips allow the detection of limited mutations in RT and protease. The limitation of LiPA and other hybridization assays is that HIV-1 is extremely heterogeneous and as many as 40% of samples may fail to yield results which reduces the clinical benefit of the assay in the routine detection of resistance.

The gold standard approach to the detection of mutations that confer resistance to antiretroviral drugs is DNA sequencing. Sequencing has two limitations, firstly, it is time consuming and requires well-trained personnel, and, secondly, it is relatively insensitive, the mutant strain must constitute at least 25% of the sample. Not only are known mutations detected this way, but new mutations arising with novel combinations of therapy will also be detected. Of particular concern is the emergence of new mutations that may confer cross resistance to all compounds in a class, e.g., the Q151 M mutation affecting the reverse transcriptase gene confers cross resistance to all six nucleoside analogues in clinical use.[19] Current sequencing protocols require at least 1000 copies of HIV RNA per milliliter of plasma. However, nested PCR methods increase the sensitivity of the assay and sequences may be obtained from samples containing a plasma viral load of <50 copies/mL,[20] which enables earlier detection of treatment failure. Reproducibility between kits is good with Visible Genetics and Applied Biosystems (ABI) detecting the same mutations in 91% of samples tested although some reduction in the detection of mixtures of wild type/mutant was observed in the ABI system because of the limitations of the software.[21] Viroseq (ABI) was able to sequence 97% of samples from non-clade B HIV-1 strains from Uganda.[22]

Interpretation of sequencing should take into consideration the clinical history of the patient which may be complex in the case of transmission of resistant virus either from a partner who has been extensively treated with antiretroviral drugs or as the result of the casual transmission of resistant virus from an unnamed source. A useful tool in interpreting mutations in HIV sequences and for subtyping HIV clade is the University of Stanford database which may be accessed through http://www.hivdb.stanford.edu. HIV subtype may also be determined via the National Centre for Biotechnology Information web site at http://www.ncbi.nlm.nih.gov/retroviruses/subtype/subtype.html. Usefulness of individual interpretation systems depends upon the accuracy of the algorithms used to set up the system which differ from system to system and this affects the interpretation of the influence some mutations have on the sensitivity of individual drugs.

CONCLUSION

In the next 5 years major efforts will be made to lessen the impact of HIV-1 in the developing world. The reduced costs of antiretroviral to these countries should be extended to cut-price viral load assays and sequencing machinery and kits. This would facilitate the monitoring of HIV-1 and HIV-2 infections in the growing number of infected individuals. New antiretrovirals will become available from new classes of drugs such as fusion inhibitors and integrase inhibitors which will increase the complexity of detection of resistant mutants. Improvements in the current viral load assays will allow more uniform detection of all clades of HIV-1. International standards will reduce the discrepancies observed between the current commercial viral load assays. Real-time PCR technology will further reduce the cost of carrying out viral load detection and improve the speed and sensitivity of current detection systems. The goal of molecular tests should be to detect one copy of HIV-1 RNA/mL of plasma to sequence low copies of HIV-1 RNA for residual resistance and for treatment to reduce levels of virus in plasma to absolute zero.

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HyBeacon™ Probes

David John French
LGC (Teddington) Ltd., Teddington, U.K.

INTRODUCTION

Real-time PCR methods are of great importance to a multitude of diagnostic sectors, such as health care, forensics, food analysis, and defense, which require rapid genetic analysis. Traditional methods of sequence analysis are time consuming and need to be performed in specialized facilities, requiring multiple steps and techniques to achieve target amplification and detection. A range of fluorescent probe technologies, such as TaqMan, Scorpions, molecular beacons, and hybridization probes, has been developed to combine the target amplification, detection, and analysis processes required to type samples. The combination of amplification and detection in homogeneous real-time PCR reduces the duration of tests, often to less than 30 min, and decreases the possibility of cross-contamination. Such rapid analyses may be performed at the point-of-care (e.g., at hospitals and doctors surgeries) and in-the-field (e.g., on farms and battlefields) by individuals provided with minimal training.

HyBeacon™ probes provide a homogeneous method of ultrarapid sequence analysis that allows samples to be genotyped in less than 20 min. These fluorescent probes are capable of reliably detecting specific DNA targets and discriminating closely related sequences, including those containing single nucleotide polymorphisms (SNPs). As such, HyBeacon probes may be employed in a large number of applications including genetic diagnostics, disease predisposition, pharmacogenetics, and pathogen detection. HyBeacons exhibit a simplistic mode of action, possess few design constraints, yield easily interpreted melt peak data, and can simultaneously analyze multiple target sequences with a single probe. HyBeacon assays have been demonstrated to function efficiently directly from samples, such as saliva, without prior purification of nucleic acids, making the probe technology suitable for point-of-care diagnostics.

BODY OF TEXT

HyBeacons™

HyBeacon probes are target-specific linear oligonucleotides with fluorescent dyes attached to internal nucleotides.[1–3] Probes emit significantly greater amounts of fluorescence when hybridized to complementary target sequences than were single-stranded. HyBeacon probes do not possess quencher moieties attached to the oligonucleotide and the mechanism of target detection does not rely on probe secondary structures or enzymatic digestion.[1] The inherent fluorescence-quenching properties of DNA are responsible for the lower levels of probe emission in the single-stranded state, and hybridization to complementary targets directly relieves a large proportion of that quenching. This phenomenon enables specific target detection and reliable discrimination of DNA sequences. HyBeacon functionality does not appear to be dependent on nucleotide sequence or the location of guanosine residues within probe and target as reported for other single-labeled technologies such as Simple probes, LUX primers, and Smart probes.[4–6]

Melt Peak Analysis

Because the amount of fluorescence emitted from HyBeacons is determined by the hybridization state of the probe, the nature of target sequences may be determined by melting curve analysis (Fig. 1). At low temperatures, probes are hybridized to target sequences and emit elevated levels of fluorescence. As temperature increases, the level of fluorescence emission reduces as probes dissociate (melt) from their target sequences. The melting temperature (Tm) of the probe occurs when 50% is hybridized to target sequence and 50% is single-stranded. Probes hybridized to fully complementary target sequences are considerably more stable than those bound to targets containing regions of mismatch (Fig. 1). Therefore, closely related target sequences, differing by as little as a single nucleotide may be reliably differentiated on the basis of Tm. Melt peaks are highly specific and reproducible such that probe Tms may be employed directly to determine the nature of target sequences.

Single Probe Multiplex Analysis

Real-time PCR probe technologies, such as TaqMan and Scorpions,[7–10] achieve target detection through

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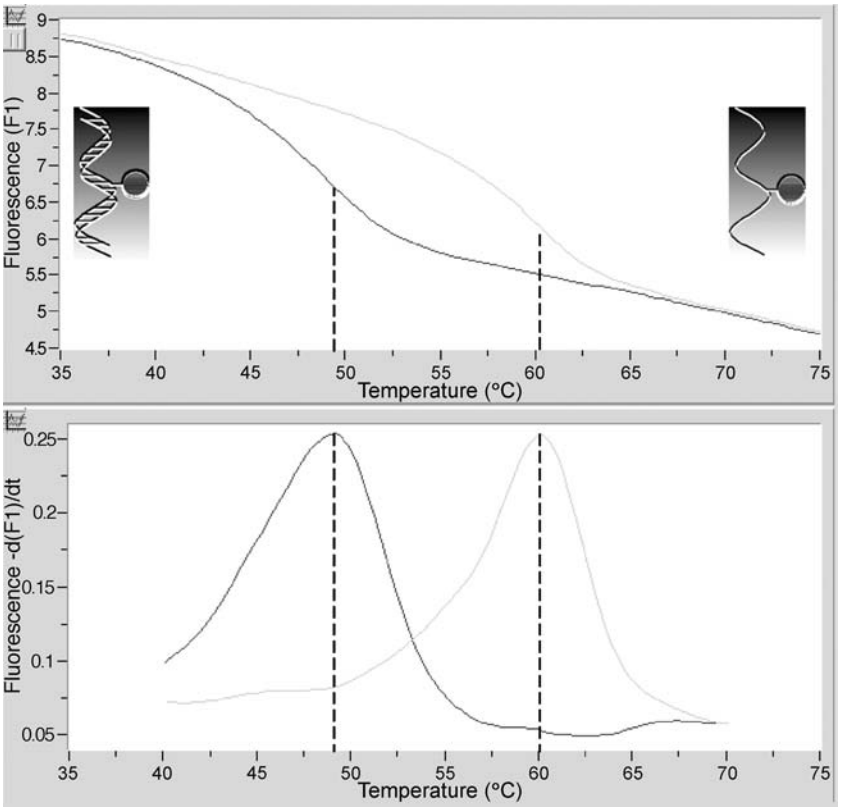

Fig. 1 Sequence analysis by Tm determination. HyBeacon fluorescence emission decreases as the temperature increases and probe/target duplexes melt to become single stranded. The upper graph displays melting curves from a probe hybridized to a fully complementary target and a sequence containing a single nucleotide mismatch. Fully complementary probe/target duplexes are more stable than duplexes containing positions of mismatch and exhibit considerably higher Tms. The lower graph displays the melt data in peaks to clearly measure probe Tm and determine the nature of the target sequence. (*View this art in color at www.dekker.com.*)

monitoring fluorescence emission during amplification. Increased levels of fluorescence are indicative of target amplification and may be employed to assess the presence or absence of particular sequences in test samples. However, numerous probes, labeled with spectrally distinct fluorophores, are typically required to simultaneously detect and differentiate multiple target sequences in a single reaction vessel. For example, different fluorescent probes are required for each allele of polymorphic genes to ensure correct identification of homozygous and heterozygous samples.

HyBeacon fluorescence typically only acquired post-amplification during melting curve analysis. Determination of melt peak Tms allows multiple target sequences to be simultaneously detected and identified using a single probe and fluorophore (Fig. 2).[1,2,4] Target sequences differing by as little as a single nucleotide may be simultaneously detected and reliably differentiated through the generation of multiple melt peaks such that samples homozygous and heterozygous for specific SNPs may be reliably genotyped with a single probe. This single-fluorophore method of multiplex sequence analysis may also be employed for applications, such as pathogen detection, that require an endogenous amplification control. Furthermore, unrelated target sequences may be simultaneously detected using multiple probes labeled with the same fluorophore. The number of target sequences that may be simultaneously analyzed depends on the Tms of each probe/target duplex, where a difference of at least 5°C is required to separate melt peaks and ensure correct identification of sequences.[1] The example presented below demonstrates simultaneous analysis of two adjacent polymorphisms in the human β globin gene, where combinations of three different melt peaks allow samples to be reliably genotyped.

Ultrarapid Diagnostics

HyBeacon sequence analysis may be performed in less than 20 min using rapid thermocyclers such as the

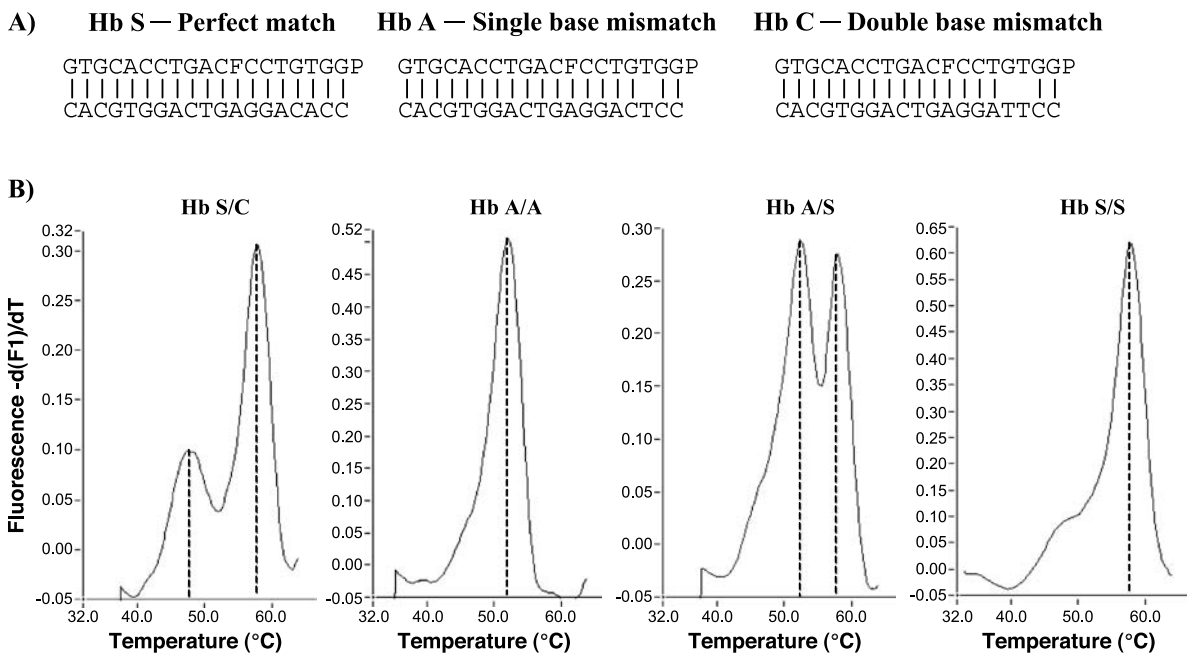

Fig. 2 Rapid analysis of sickle cell anemia polymorphisms. (A) The HyBeacon probe employed to genotype samples with respect to sickle cell disease was fully complementary to the sickle cell (HbS) allele, exhibiting single and double nucleotide mismatches when hybridized to normal β globin gene (HbA) and hemoglobin C (HbC), respectively. (B) HyBeacon probe hybridized to the fully complementary HbS allele possesses a Tm of approximately 58°C. Hybridization to the mismatched HbA and HbC alleles drops the probe Tm to 52°C and 48°C, respectively. Combinations of probe melt peaks allow determination of HbA/A, HbS/S, HbA/S, and HbA/C genotypes.

LightCycler. HyBeacon probes are capable of detecting both DNA and RNA targets, being compatible with single-tube RT-PCR, and PCR assays may comprise both three- and two-stage cycling protocols. Probes and assays may be designed such that HyBeacons are not hybridized to target sequences during PCR, thereby avoiding obstruction of DNA polymerase (cf. TaqMan) and enabling the use of extremely rapid thermal cycling conditions. Forty-five cycles of amplification have been achieved in less than 10 min using two-stage LightCycler thermal protocols and short incubation times. Following amplification, melt analysis may be completed in as little as 2 min. The length and composition of target sequences will affect amplification efficiency and determine PCR cycling conditions and assay duration. Although target sequences of 80–150 bp are typically employed in HyBeacon assays, it is sometimes necessary to analyze larger amplicons to ensure specificity in the presence of closely related sequences such as pseudogenes. The type and purity of samples to be analyzed will also affect the optimal PCR conditions and test duration.

Saliva samples may be employed directly in HyBeacon assays without the requirement for DNA purification.[2] Direct saliva analysis not only reduces the overall duration of assays, but also makes the tests noninvasive, thereby avoiding the safety issues associated with diagnostics using blood samples. Furthermore, providing saliva samples is easier, more acceptable, and less stressful for patients. Ultrarapid analyses will permit HyBeacons to be employed in point-of-care diagnostic scenarios and in-the-field applications where results are required quickly.

Model System

Sickle cell anemia is a disorder that affects red blood cells through altering the structure and function of the hemoglobin (Hb) protein that is required to carry oxygen around the body. Individuals with sickle cell anemia possess hemoglobin (HbS) which is different from the normal protein (HbA). Whereas normal red blood cells can bend and squeeze through small blood vessels easily, HbS can result in cells becoming sickle-shaped and rigid causing small blood vessels to get blocked. Such blockages can prevent sufficient passage of oxygen and may result in severe pain and damage to organs.

Sickle cell anemia was one of the first diseases found to be caused by a molecular defect, arising from a single nucleotide substitution (Fig. 2) that causes a glutamic acid

(GAG) in HbA to be changed to a valine (GTG) in the HbS protein.[11] This base difference has a major effect on the folding and functionality of the hemoglobin protein. Other types of hemoglobin, such as HbC and β thalassemia, can exacerbate the sickle cell condition when an individual possesses a copy of the HbS gene. The HbC allele arises from a base substitution adjacent to HbS that converts the glutamic acid to a lysine (AAG). A single HyBeacon probe enables multiplex analysis of the β globin variants to simultaneously genotype individuals with respect to both HbS and HbC alleles.

Rapid Analysis of Sickle Cell Samples

A HyBeacon probe was designed to simultaneously detect and differentiate HbA, HbS, and HbC target sequences. The probe was designed to be fully complementary to the HbS sickle cell allele, possessing single and double nucleotide mismatches when hybridized to HbA and HbC sequences, respectively (Fig. 2). The sickle cell HyBeacon was synthesized using methods described previously.[1,12] The probe sequence was 5′(GTGCACCTGACFCCTGTGGP)3′, where F and P were the FAM (6-carboxyfluorescein) labeled uracil and 3′ phosphate, respectively. The FAM fluorophore was included at an internal position of the oligonucleotide, and the 3′ phosphate was attached to prevent extension from probes during real-time PCR assays.

The HyBeacon probe was employed to detect a 139-bp amplicon containing the HbS and HbC loci. The sequences of the primers employed to amplify the probe target were 5′(AGGGCAGAGCCATCTATTGCT)3′ and 5′(CATCCACGTTCACCTTGCC)3′. Target sequences were amplified from genomic DNAs extracted from 12 clinical samples supplied by the National Haemoglobinopathy Reference Service (John Radcliffe Hospital, Oxford, U.K.). Samples were analyzed with a LightCycler™ instrument using materials and methods described previously.[1,2]

Melting peak analysis was performed following PCR to determine the nature of amplified targets by means of probe Tm. HyBeacon probe hybridized to the fully complementary HbS allele generated melt peaks possessing Tms of approximately 58°C (Fig. 2). Hybridization to the HbA allele resulted in a T/T nucleotide mismatch between probe and target sequence, reducing the melting temperature to 52°C. Probe hybridization to the HbC allele resulted in T/T and G/T mismatches causing the Tm to drop to 48°C. Combinations of melt peaks enabled samples to be genotyped rapidly and reliably. Melt traces possessing only 58°C or 52°C peaks were identified as being homozygous HbS/S and HbA/A, respectively. Samples that generated both 58°C and 52°C peaks were similarly typed as heterozygous for HbS and HbC alleles, whereas those producing both 58°C and 48°C peaks were typed as compound heterozygotes possessing both HbS and HbC (Fig. 2). The 12 clinical samples, analyzed blind, were found to comprise triplicates of HbS/C, HbA/A, HbA/S, and HbS/S genotypes (Table 1). Genotypes were confirmed by the National Haemoglobinopathy Reference Service.

Applications

The ultrarapid detection mechanism of HyBeacon probes will enable their use outside of specialized research facilities to analyze samples within 20 min. Utilized in hospitals and doctors' surgeries, HyBeacon assays may be employed to detect and identify nucleic acid target sequences for applications such as genetic diagnosis, disease predisposition, pathogen detection, pharmacogenetics,

Table 1 Analysis of sickle cell samples

Sample number	Hb C (46–50°C)	Hb A (50–54°C)	Hb S (56–60°C)	Genotype
1	48.04		57.78	Hb S/C
2		52.39		Hb A/A
3		52.32	58.2	Hb A/S
4			57.75	Hb S/S
5	47.29		57.68	Hb S/C
6		52.15		Hb A/A
7		52.41	58.25	Hb A/S
8			57.69	Hb S/S
9	47.3		57.86	Hb S/C
10		51.92		Hb A/A
11		52.2	58.15	Hb A/S
12			57.58	Hb S/S

Twelve genomic DNA samples were analyzed using a sickle cell-specific HyBeacon probe. Tm ranges were established to reliably detect and identify hemoglobin alleles, where combinations of peaks may be employed to determine sample genotype.

and lifestyle management. HyBeacon assays may also be utilized for in-the-field applications such as screening cattle for infectious diseases, testing water for pathogens at source, and detecting the presence of biological warfare agents for buildings, protection or on the battlefield. Because the melt peak Tms generated during analysis are characteristic of specific probe targets, samples could be typed automatically using specialized software. This would permit individuals with minimal training to interpret test data and enable HyBeacon assays to be employed in applications requiring high throughput.

CONCLUSION

HyBeacon probes are fluorescent oligonucleotides that may be employed in real-time PCR assays to rapidly detect and discriminate nucleic acid sequences for a multitude of diagnostic applications. Probes emit higher levels of fluorescence when hybridized to target sequences than when single-stranded and permit target identification through melt peak analysis and Tm determination. The lack of probe secondary structure, quencher attachment, and sequence dependence renders HyBeacons simple and relatively inexpensive to design and synthesize. Multiple target sequences may be simultaneously analyzed using HyBeacon probes and assays typically take less than 20 min to complete. Saliva samples have been utilized directly in HyBeacon assays to further decrease analytical time and increase utility for point-of-care applications. HyBeacon assays are currently in development to detect pathogens in samples of urine and blood without sample purification.

ACKNOWLEDGMENTS

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Immunoisolated Cells

Dwaine F. Emerich
LCT BioPharma, Inc., Cranston, Rhode Island, U.S.A.

Christopher G. Thanos
Brown University, Providence, Rhode Island, U.S.A.

INTRODUCTION

Transplanting a failing organ or specific cell type is a logical means of restoring function for secretory diseases. While inherently appealing, the lack of suitable donor organs or tissues makes the widespread use of cell transplantation impractical. To overcome this limitation, xenogeneic cells can be encased within a selectively permeable polymeric membrane, known as immunoisolation. Immunoisolation owes much of its foundation to investigators focused on diabetes and Parkinson's disease, showing that islets and dopamine-secreting cells are protected from host rejection by encapsulating them within a semipermeable membrane. Single cells or clusters of cells can be enclosed within a selective, semipermeable membrane barrier that admits oxygen and required nutrients and releases bioactive cell secretions, but restricts passage of larger cytotoxic agents from the host immune defense system. Immunoisolation eliminates the need for chronic immunosuppression and allows the implanted cells to be obtained from human or nonhuman sources, thus avoiding the constraints associated with cell sourcing, which limit the clinical application of unencapsulated cell transplantation.

IMMUNOISOLATORY MEMBRANES

True immunoisolation requires restricting the membrane transport of all relevant molecules belonging to the host and the encapsulated cells. Whereas a molecular weight cutoff of 50–100 kDa likely prevents infiltration of IgG, greater size restrictions must be employed to achieve a complete barrier. Growth factors, cytokines, complement, and smaller metabolites several logs lower in molecular weight exist within immunoisolated systems that are immunogenic and can lead to graft rejection. Limiting passage of these smaller molecules would retain the cell-secreted molecule of interest, and thus such a system would not be practical. However, this level of protection is not always necessary, particularly in allogeneic or syngeneic transplantation, and immunosuppressants can be administered to overcome this effect.

The polymer membrane of an immunoisolatory device is a thin barrier that restricts transport between adjacent phases.[1–4] Most membranes are produced from thermoplastics such as polysulfone or acrylic copolymers that are cast into hollow fiber membranes less than 1 mm in diameter with a wall thickness of 10–15% of the total fiber diameter.[5–7] The majority of thermoplastic ultrafiltration and microfiltration membranes used to encapsulate cells are manufactured from homogenous polymer solutions by phase inversion. Ultrafiltration membranes have pore sizes ranging from 5 nm to 0.1 μm, while microfiltration (or microporous) membranes have pores ranging from 0.1 to 3 μm. Phase inversion forms membranes with a wide variety of nominal molecular weight cutoffs, permeabilities, and morphologies. The polymer is first dissolved in an appropriate solvent and then cast as a flat sheet or extruded as a hollow fiber. As part of the casting or extrusion procedure, the polymer solution is precipitated by a phase transition brought about by temperature change or solution composition. This process involves the transfer of a single-phase liquid polymer solution into a two-phase system consisting of a polymer-rich phase that forms the membrane structure and a second liquid polymer-poor phase that forms the membrane pores. Any polymer that forms a homogenous solution that will separate into two phases can be used. Thermodynamic and kinetic parameters, such as the chemical potential of the components and the free energy of mixing of the components, determine the manner of the phase separation.

CELL IMMUNOISOLATION

There are generally two categories of encapsulation, microencapsulation and macroencapsulation, each with some benefits and limitations. Several detailed reviews are available.[3–9]

Microencapsulation

Microencapsulation involves surrounding cells with a thin, spherical, semipermeable polymer film.[8] The small size, thin wall, and spherical shape of microcapsules are

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structurally optimal for diffusion, cell viability, and release kinetics. The material components of microcapsules vary from polysaccharides to thermoplastics. There are potential benefits associated with each choice; however, during the manufacture of certain thermoplastic cell-encapsulated systems, exposure to organic solvents is not easily avoided and is a potential viability risk. Thus, work has primarily focused on the polyelectrolytes, which are nontoxic during processing but are generally mechanically fragile and chemically unstable, as well as difficult to retrieve. For the polyelectrolytes, microcapsule membranes are formed by ionic or hydrogen bonds between two weak polyelectrolytes with opposite charges. Microcapsules can be prepared by gelling droplets of a polyanion/cellular suspension (e.g., alginate) in a mixing divalent cationic bath, which immobilizes the cells in a negatively charged matrix, and then coating the immobilized cells with a thin film of a polycation, such as poly (L-lysine). The poly(L-lysine)-alginate bond creates a permselective membrane whose molecular mass cutoff is on the order of 30,000–70,000 Da. Because poly(L-lysine) is not biocompatible, a second layer of alginate is generally added to the capsule surface. Alternatively, polyanions such as polyornithine have greater reactivity during capsule formation and are biocompatible. Polyelectrolyte-based techniques avoid organic solvents, and, with proper permeability control, microcapsules represent a very effective configuration for cell viability and neurochemical diffusion.

Macroencapsulation

Macroencapsulation involves filling a hollow, usually cylindrical, selectively permeable membrane with cells, generally suspended in a matrix, and then sealing the ends to form a capsule.[4] Polymers used for macroencapsulation are biodurable, with a thicker wall than that found in microencapsulation. While thicker wall and larger implant diameters can enhance long-term implant stability, these features may also impair diffusion, compromise the viability of the tissue, and slow the release kinetics of desired factors. In theory, macrocapsules can be retrieved from the recipient and replaced if necessary.

Macroencapsulation is generally achieved by filling preformed thermoplastic hollow fibers with a cell suspension. The hollow fiber is formed by pumping a solution of polymer in a water-miscible solvent through a nozzle concurrently with an aqueous solution. The polymer solution is pumped through an outer annular region of the nozzle, while the aqueous solution is pumped through a central bore. Upon contact with the water, the polymer precipitates and forms a cylindrical hollow fiber with a permselective inner membrane. Further precipitation of the polymer occurs as the water moves through the polymer wall, forcing the organic solvent out and forming a trabecular wall structure. The hollow fiber is typically collected in a water bath, allowing complete precipitation of the polymer and dissolution of the organic solvent. The ends of the hollow fiber are sealed using a biocompatible fast-curing adhesive manually applied to the ends of the device. A second method of macroencapsulation, called coextrusion, avoids the sealing problem by entrapping cells within the lumen of a hollow fiber during the fabrication process. Pinching the fiber before complete precipitation of the polymer causes fusion of the walls, providing closure of the extremities while the cells are inside.

CELLS AND EXTRACELLULAR MATRICES USED IN ENCAPSULATION

Successful cell encapsulation involves the choice of the cells to be encapsulated, the type of intracapsular matrix used, and the ability to control membrane geometry, morphology, and transport.[3–9] Cells placed within encapsulation devices generally fall into one of three categories (Table 1) including primary postmitotic cells, immortalized (or dividing) cells, and cell lines that are genetically engineered to produce specific factors. Dividing tissue has advantages over postmitotic tissue; it can be expanded, banked, and thus more easily tested for sterility and contaminants. However, dividing tissue is also constrained by the potential for overgrowth within the capsule environment, resulting in an accumulation of necrotic tissue that could diminish the membrane's

Table 1 Essential components of an immunoisolatory device

Cells	
Primary	Islets (diabetes), hepatocytes (liver failure), chromaffin cells (pain and Parkinson's disease)
Dividing	PC12 cells (Parkinson's disease)
Genetically engineered	Fibroblasts producing neurotrophic factors
Extracellular matrix	
Hydrogels	Alginate and collagen
Scaffolds	Foamy materials including urethane
Membranes	
Dialysis membranes	PAN–PVC, polysulfone, polyethersulfone
Polyelectrolytes	Alginate, polylysine, polyornithine
Combinations	Conformal coating on surface Combined with reinforcing structures

permeability characteristics, further reducing cell viability and neurochemical output.

In vivo, extracellular matrices (ECMs) control cell function through the regulation of morphology, proliferation, differentiation, migration, and metastasis. Within a capsule, ECMs are used to prevent aggregation of cells (immobilization) and resultant central necrosis, but are also beneficial to the viability and function of cells that require immobilization. For example, adrenal chromaffin cells have been immobilized in alginate to prevent aggregation and the formation of central necrotic cores. The chromaffin cells thrive in alginate whereas mitotically active fibroblasts do not. In this case, the use of alginate is essential to the optimal functioning of this device because some anchorage-dependent cells such as fibroblasts or endothelial cells are present with the adrenal chromaffin cells. In the absence of alginate or similar immobilizing matrices, the fibroblasts expand and overgrow the encapsulated milieu, resulting in a device deficient of bioactive factors produced from the chromaffin cells. In contrast, BHK cells, a fibroblastic cell line, prefer collagen, whereas PC12 cells exhibit a preference for distribution within precipitated chitosan, which provides a scaffolding structure on which the cells anchor.

Polymeric matrices can also be used as cell scaffold material. Poly(ethylene oxide) (PEO)–star copolymers have been fabricated as a potential synthetic extracellular matrix. The star copolymers provide many hydroxyl groups where various synthetic oligopeptides can be attached to desired specifications. Other more resilient polymers such as poly(ethylene terephthalate) (PET) polymers can be extruded into unique geometries which facilitate a three-dimensional arrangement of cells within the device, such as cylinders, braids, or other geometries. Polyesters such as this can be modified to have a surface microtexture for improved cell anchoring or attachment of peptides or other components desirable for cell attachment.

ONGOING CLINICAL TRIALS

As shown in Table 2, there are extensive ongoing efforts to develop cell encapsulation across a wide range of preclinical and clinical applications. Here we highlight the ongoing clinical efforts using encapsulated cells for treating CNS diseases.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive degeneration of spinal motor neurons. Systemic delivery of ciliary neurotrophic factor (CNTF) has therapeutic potential but is limited clinically because of systemic side effects, short half-life, and the inability to cross the BBB.[10] Continuous intrathecal delivery of CNTF proximal to the nerve roots in the spinal cord could result in less side effects and better efficacy of CNTF in ALS patients.[11] After safety, toxicology, and preclinical evaluation, a clinical trial was performed to establish the safety of encapsulated CNTF-producing cells in ALS patients. Six ALS patients with early-stage disease received BHK cells that were encapsulated into 5 cm long $\times$ 0.6-mm diameter hollow membranes and implanted into the lumbar intrathecal space. CNTF from the cells was found in all six patients at 3–4 months post-implantation. All six explanted devices had viable cells and detectable CNTF secretion. More recently, a phase I/II clinical trial was initiated in 12 ALS patients using the same approach. CNTF was detectable for several weeks in the CSF of 9 out of 12 patients, and only a very weak antigenic immune response was detected with bovine fetuin as the main antigenic component.[12]

Huntington's Disease

Huntington's disease (HD) is an inherited neurological disorder characterized by an intractable course of mental deterioration and progressive motor abnormalities with death usually occurring within 15–17 years. There are no treatments for HD. Recent studies have suggested that CNTF might slow the neural degeneration in HD.[13–15] Clinical trials are underway to determine the safety and tolerability of CNTF-producing cells implanted into the lateral ventricle of HD patients.[16] Although the results of this trial are not yet available, the trial is designed using a device containing as many as 10^6 CNTF-producing BHK cells producing 0.15–0.50 μg CNTF/day implanted into the right lateral ventricle of six patients. In this phase I study, the principal goal is to evaluate the safety and tolerability of the procedure. HD symptoms will also be analyzed using neuropsychological, motor, neurological, and neurophysiological tests and the striatal pathology monitored using MRI and PET-scan imaging.

Retinitis Pigmentosa

Retinitis pigmentosa (RP) is an inherited disorder of the retina and is the sixth leading cause of blindness. Whereas current clinical strategies are primarily palliative in nature, research in animal models demonstrates that neurotrophic factors inhibit retinal degeneration caused by RP. Studies in the *rd* model of retinal degeneration demonstrated that CNTF gene transfer maintains retinal morphology,[17] and later studies in the *rcd1* dog model[18] using intravitreal implants of encapsulated CNTF-secreting cells demonstrated a dose-dependent preservation of rods. In the dog studies, untreated eyes showed, on

Table 2 Diseases for which cell encapsulation is being applied

Disease/model	Encapsulated cell/experimental paradigm	Results
Hormonal and whole-organ diseases		
Diabetes	Islets in rodents and dogs	Normoglycemia for 2 years
Hypoparathyroidism	Parathyroid tissue in rats	Normocalcemia for 30 weeks
Kidney failure	Orally delivered *Escherichia coli* bacteria to rats	Normalized urea metabolism
Growth hormone deficiency	Growth hormone-producing cells in dogs	Hormone secretion for 1 year
Single-gene diseases		
Hemophilia	Factor 9 cells in rats	Cell survival and secretion
Lysosomal storage disease	Beta-glucuronidase cells in mice	Behavioral normalization
Age-related/Neurodegenerative-diseases		
Age-related motor decline	Catecholamine and GDNF cells in rats	Improvement in motor function
Amyotrophic lateral sclerosis	CNTF-producing cells in mice	Protection of motor neurons
Alzheimer's disease	NGF cells in rat and primates	Protection of cholinergic neurons, improved memory
Huntington's disease	NGF and CNTF cells in rats and primates	Protection of neurons, improved behavior
Parkinson's disease	Catecholamine and GDNF cells in rat and primate brain	Improved behavior, protection of dopaminergic neurons
Retinitis pigmentosa	Human cells secreting hCNTF into the vitreous	Rod preservation
Spinal cord damage	BDNF cells in rats	Outgrowth of neurites
Oncology		
Colon cancer	iNOS cells (tet-regulated system) in mice	Enhanced survival
Glioblastoma	Endostatin cells in mice and rats	Reduced tumor growth, enhanced survival
HER-2/neu positive tumors	IL-2 fused with anti-HER-2/neu antibody in mice	Modest survival benefit
Leukemia	Hybridoma producing antibodies to p15E in mice	Enhanced survival
Ovarian cancer	iNOS cells (tet-regulated system) in mice	Enhanced survival, cures
Other		
Acute and chronic pain	Chromaffin cells in rats	Reduced pain
Clinical trials		
Amyotrophic lateral sclerosis	CNTF cells intrathecally	Sustained delivery, no toxicity
Chronic pain	Chromaffin cells in subarachnoid space	Prolonged cell survival, no pain reduction in phase II trials
Diabetes	Human islets intraperitoneally	Insulin independence for 9 months
Huntington's disease	CNTF cells into ventricles	Delivery for 6 months
Hypoparathyroidism	Parathyroid tissue	Successful in two patients
Pancreatic cancer	CYP2B1 cells in tumor vessel	Local tumor growth controlled, well tolerated
Retinitis pigmentosa	CNTF cells in eye	Ongoing

average, three layers of the outer nuclear layer, while treated eyes showed between three and six layers. The distribution of retinal preservation was homogenous throughout the retina. These experiments paved the way for the recently initiated clinical trials by Neurotech USA. Patients diagnosed with RP have received intravitreal implants of an immunoisolatory membrane containing a human epithelial cell line engineered to secrete CNTF. Results from this phase I safety study are anticipated in 2004–2005.

CONCLUSION

There is an abundance of work in the field of immunoisolation to highlight the importance of creating a synergistic system that is capable of maintaining the production and delivery of secreted factors while promoting device viability and rejecting cells and molecules of the immune system. An effective device relies on the ability of the cells to withstand transplant conditions, and likewise, the encapsulated cells must be in a favorable

immunoisolatory environment to optimally perform their function. Immunoisolation can be achieved using a variety of materials and techniques described, but the secreted component must be able to diffuse outward while inward transit of immune components is halted. Ongoing research and the products being investigated in clinical trials will provide the field of cell transplantation with a tangible picture of the true importance of immunoisolation.

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Introduction of Molecular Genetics and Genomics Into Clinical Practice

Ronald J. A. Trent
Bing Yu
Melody Caramins
Royal Prince Alfred Hospital, Camperdown, Australia

INTRODUCTION

The Human Genome Project (HGP), a multibillion, multinational endeavor to sequence the entire human genome, as well as the genomes of model organisms, was started in 1990, and the completion was announced in 2000. In February 2001, the first draft of the human genome sequence was published. In 2003, the 50th anniversary of the discovery of the DNA double helix, a high-quality version of the human genome sequence became available. The HGP-driven research is leading to a rethinking of genetics and the impact of genes on human health. This will have a profound influence on clinical practice.

Molecular genetics is a relatively new entry into pathology. It was only in 1978 that the first prenatal diagnosis was made with this approach. Today, the DNA-based test has become an integral component of clinical genetics. As knowledge gained from the HGP expands our understanding of human genetic disorders and genetic components of common complex diseases, the scope for DNA testing increases. Current interest in DNA diagnostics predominantly focuses on single-gene disorders. The challenging developments ahead are in the fields of DNA diagnostics involving somatic cell (acquired) disorders for which there is a genetic predisposition, and multifactorial disorders that involve genetic susceptibility interacting with the environment.

The HGP has brought about another change, with the traditional concept of genetics expanding into genomics. With the term ''genetics,'' the focus was on a single disorder. In this situation, a mutation in the DNA allowed a diagnosis to be made, and perhaps some understanding of pathogenesis was gained. In contrast, genomics has wider ramifications, and it has been used to refer to all genes in the genome rather than individual ones, including their function, sequence, and interactions with the environment.

DNA TECHNOLOGY

As will be illustrated in this encyclopedia, technological developments in molecular genetics and genomics are impressive. Particularly relevant will be microarrays (discussed further in ''Classes of DNA Tests'') and nanotechnology. The latter will allow miniaturization of all steps in DNA testing. Small, compact analytical platforms will ensure mobility, thereby allowing DNA testing to move to the bedside or the consultant's office. This will have direct relevance to the emerging field of pharmacogenetics and pharmacogenomics because it will allow health professionals to test individuals to determine their susceptibility to drugs, and, from this, to personalize various treatment regimens.[1,2] This will be discussed further under ''Classes of DNA tests.''

When the controversy about the patenting of human genetic sequences is settled, there is likelihood that commercial companies will have significant control over the range of DNA tests provided. This will be accomplished through licensing. Control will also be exerted through the development of kits. For these kits to be commercially viable, they will need to be user-friendly—and this, coupled with nanotechnology, will greatly expand the availability of DNA testing to health professionals.

CLASSES OF DNA TESTS

The unique feature of DNA in molecular genetics/genomics is its ability to use this approach to investigate many clinical scenarios (Table 1). The range is extensive, starting with the straightforward DNA diagnostic test confirming that an individual with a clinical problem has a genetic disease. The next level of complexity involves population screening, or DNA predictive testing for late-onset genetic disorders. Many of these DNA tests require

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Table 1 Classes of DNA genetic tests

DNA test and description	Examples
Diagnostic—A DNA test used to confirm a clinical suspicion that the patient has an established disorder. The DNA test in this circumstance is comparable to a blood count or measurement of a biochemical analyte, although a positive result has implications for family members.	A suspected case of hemochromatosis can be confirmed by a liver biopsy, a procedure that has associated morbidity and mortality. An alternative is a DNA test, used to look for the common hemochromatosis mutation (C282Y) to confirm the clinical suspicion.
Prenatal—DNA test to detect a genetic disorder in the fetus or embryo.	1) *Fetal prenatal diagnosis*. Straightforward DNA test in an at-risk fetus. Sources of fetal DNA include desquamated cells in the amniotic fluid (obtained during amniocentesis) or chorionic villi (chorion villus sampling, or CVS). 2) *Preimplantation genetic diagnosis* (PGD). A few cells obtained from the developing preembryo allow genetic diagnosis in vitro (i.e., in vitro fertilization, or IVF). This allows early detection of an abnormality and avoids termination of pregnancy. PGD is still not robust enough to be routinely available.
Screening—A DNA test used to look at asymptomatic individuals (or populations) to determine who are carriers or who have a genetic predisposition.[3,4] Various types of screening options are available.	1) *Reproduction screening*. DNA tests are undertaken to identify couples who are at risk for a genetic defect in the fetus. Presently, most reproduction screenings are undertaken because there is a family history, or because the couple demonstrates a risk factor (e.g., advanced maternal age). 2) *Newborn screening*. Newborns are screened for a number of genetic disorders that are treatable or preventable, provided they are diagnosed early. 3) *Community screening*. This involves the DNA testing of populations who have *no prior risk*. The populations can be whole communities (comparable to mammography screening for breast cancer) or selected communities. An example of whole community testing would be DNA-testing all pregnant women for cystic fibrosis. A selected community screening program is exemplified by testing for Tay Sachs disease in Ashkenazi Jews.
Predictive or presymptomatic—A DNA test used to predict the development of a genetic disorder in advance of any signs or symptoms (for convenience, a distinction is not made between predictive and presymptomatic).[5]	1) *Neurological adult-onset disorders*. Huntington disease (HD) develops around the fourth decade of life. The DNA predictive test is used to identify whether an at-risk individual has inherited the mutant or normal gene from an affected parent. Although no treatment is available, the HD predictive test is useful because penetrance is very high in HD. 2) *Cancers*. Individuals with a high risk of familial breast cancer can have DNA testing of *BRCA1* and *BRCA2* genes to look for a mutation that might guide them in future treatment decisions, as well as identify risks in other family members. However, unlike genetic forms of colon cancer, such as familial adenomatous polyposis, the penetrance with *BRCA1* and *BRCA2* is quite variable (36–85% lifetime risk) and so understanding the significance of breast cancer gene mutations is not easy.

(*Continued*)

Table 1 Classes of DNA genetic tests (*Continued*)

DNA test and description	Examples
Individualizing—A form of DNA screening test involving drug metabolism pathways to predict a patient's response to treatment.[6] Unlike screening mentioned above, this type of test is directed to the individual.	1) *Malignant hyperthermia*. Individuals exposed to anesthetic agents can develop life-threatening complications if they have a genetic abnormality involving the ryanodine receptor genes (*RYR1*).[4] 2) *Oral anticoagulation with warfarin*. Side effects of anticoagulation treatment include life-threatening bleeding episodes. Two genetic variants of the important warfarin-metabolizing enzyme (hepatic microsomal enzyme CYP2C9) are associated with decreased enzymatic activity.[2]

a more sophisticated approach to interpretation and greater resources to ensure that they are undertaken optimally. A final class that is not yet a component of routine practice is the potential to individualize treatment options to avoid drug-induced morbidity and mortality.[7]

DNA screening is worth considering in more detail because it will continue to remain topical for some time. Newborn screening is well accepted as an approach to detecting genetic or metabolic disorders for which intervention is possible, thereby preventing the disease or its consequences from developing. A good example is phenylketonuria. By identifying this early in the newborn, the development of mental retardation can be prevented by a phenylalanine-restricted diet. More controversial is DNA newborn screening for cystic fibrosis. Because there are no specific preventative measures, the value of this test has been questioned. However, it has been justified on the basis that the earlier detection of cystic fibrosis avoids anxiety and uncertainty in the parents if their child is ill and a diagnosis is not made. There is also some evidence that the long-term outlook is improved.[8]

Another type of DNA screening, which involves the testing of *asymptomatic* individuals in the community for recessive conditions, has provoked a lot of discussion. The article by Grody[4] notes that cystic fibrosis has the ''honor of being the first disease subject to nationwide, pan-ethnic molecular genetic screening.'' However, the National Institutes of Health (NIH) reference quoted specifically advises against cystic fibrosis genetic testing in the general population or newborns. Apart from DNA testing in those with a positive family history, the NIH consensus statement recommends cystic fibrosis DNA testing in couples planning a pregnancy or seeking prenatal care.[9] Despite this, the routine DNA testing of couples in relation to reproduction has not been adopted universally. Problems that need to be addressed include the logistics of pretest and posttest counseling. In multicultural societies, there is the additional consideration of risk variability and the optimal mutation profile is required. The latter is relevant for cystic fibrosis DNA testing, which has low sensitivity because of the large number of mutations present.

The potential utility for genomics on clinical decision making is illustrated by a microarray example, which allowed 295 patients with primary breast cancer to be studied by measuring the expression of 70 genes likely to influence the natural history.[10] These 70 genes involving cell cycle regulation, invasion, metastasis, and angiogenesis were initially selected from a 25,000-gene array. Based on gene expression patterns, patients were stratified into high-risk and low-risk groups. It was claimed that this was a more powerful predictor of clinical outcomes in breast cancer than conventional clinical or histopathological criteria. Decisions about benefits patients would derive from adjuvant treatment (hormonal or chemotherapy) could then be based on more objective data.[10]

UNDERSTANDING DISEASE PATHOGENESIS

Cystic fibrosis illustrates how our understanding of this disorder has changed through molecular genetics. What was previously a ''disease'' is now a syndrome because DNA-based knowledge has shown that a number of sporadic conditions are part of the cystic fibrosis spectrum. These include meconium ileus in the fetus, male infertility related to congenital bilateral absence of vas deferens (CBAVD), some forms of disseminated bronchiectasis,

and chronic idiopathic pancreatitis. Evidence that the aforementioned are mild forms of cystic fibrosis has come from population studies in which it was shown that mutations in the cystic fibrosis gene known as *CFTR* (cystic fibrosis transmembrane conductance regulator) were more commonly found in the patient group compared with controls. For example, 13% of 134 patients with chronic pancreatitis had one *CFTR* mutation compared with the population frequency of 5%. In addition, 10% of the patients (twice the expected frequency) had 5T splicing abnormality, which is associated with impaired function of *CFTR*.[11] A similar story is seen in disseminated bronchiectasis, with increased finding of 5T defect and a greater number of *CFTR* mutations compared with what would be expected in the normal population. In CBAVD, the 5T allele and a *CFTR* mutation (R117H) are often detected. In up to 50% of patients with CBAVD, it is possible to detect two *CFTR* mutations.[12]

RESOURCES FOR EDUCATION

The rapid changes occurring in molecular genetics mean that Internet-based resources play an important role in educating health professionals. Patients and families can be well informed about genetic disorders, and this places additional pressure on health professionals to have access to the most recent developments. OMIM (Online Mendelian Inheritance in Man™: http://www.ncbi.nlm.nih.gov/Omim/)[13] is a reputable catalogue of human genetic disorders. Two other sites providing teaching and learning resources include http://www.nchpeg.org[14] and http://genes-r-us.uthscsa.edu/resources/genetics/primary_care.htm.[15] Computer-based decision making is another option to consider in the rapidly progressing field of molecular genetics and genomics. An example of this is shown in a pilot study utilizing 36 UK general practitioners. Patients with a positive family history of breast cancer were assessed using the traditional pen-and-paper approach vs. a computerized pedigree drawing package or a computerized decision support package. Perhaps not surprisingly, decision making was superior with the latter option.[16]

ETHICAL, SOCIAL, AND LEGAL ISSUES

There are a number of potential ethical and social issues emerging in molecular genetics/genomics.[17] The main areas of concern include access, equity, and an enlarging gap between what is being tested for and what can be done in terms of therapy and prevention. There are also issues related to privacy, discrimination, and stigmatization. The first three require societally determined standards to ensure that DNA tests are used appropriately, particularly when resources are limited. To complicate this further, there are likely to be tensions between industry and the publicly funded health and research organizations. This union is of mutual benefit and is needed to provide expensive resources for many of the large gene discovery and genomics enterprises (an example of this is the microarray study described earlier). However, as industry has greater involvement in clinical genetics, there is the potential for more extensive (but not necessarily relevant) DNA testing. In this environment, health professionals can also be bypassed by direct company-to-consumer advertising and DNA testing.[18]

Issues with more implicit legal implications arise when there are threats to privacy/confidentiality raised by the specter of DNA tests identifying information on individuals for which consent has not been given. Related to this is the potential for DNA information to lead to discrimination or stigmatization because an individual has a particular DNA change, which either has the propensity for developing into disease at some time in the future, or, even more complex, is associated with a behavioral trait. Because of the community's fear that these may occur, there is often pressure for new laws to be passed. In some circumstances, a law may be necessary, but rapid changes occurring in genetics/genomics make legal options less than ideal because laws can be inflexible. Hence, alternative approaches such as education should be tried, as many of the problems that arise do so because of ignorance.

From the clinical perspective, the ethical, social, and legal issues add another layer of complexity when dealing with molecular genetics/genomics. The concepts of informed consent and duty of care are well established and provide health professionals with broad guidelines for clinical consultation. However, molecular genetics (and particularly genomics) adds new dimensions: 1) knowledge is often changing; and 2) effects can be far-reaching (i.e., results for a patient will have implications for other family members). In this environment, continuity of care and long-term follow-up become particularly important.

CONCLUSION

Molecular genetics—and now the genomics era—has brought clinical genetics to the cutting edge of medical practice. Not only is clinical genetics impacting on internal medicine and family practice, but it is relevant to many other disciplines. Specialized clinical genetics

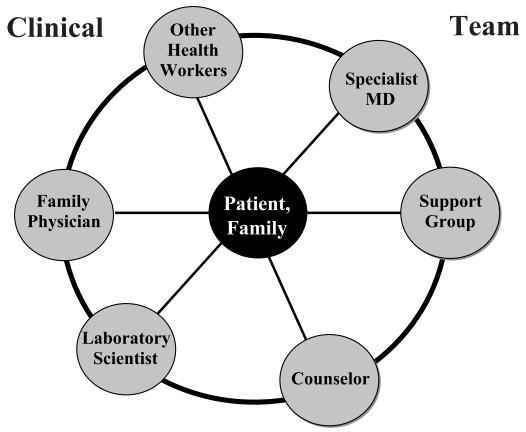

Fig. 1 Central to genetic issues are the patient and the family. Various interactions involving a range of health professionals and support groups provide the optimal approach in dealing with molecular genetics (genomics) issues. What particular combination is needed will depend on the underlying problem and the ability of the patient and the family to deal with associated issues.

units have formed in many medical centers, and the work undertaken extends from traditional fetal medicine and pediatrics problems to adult genetics. The distinction is made because training in genetics has often come through pediatrics, but this is now changing because molecular genetics has relevance to any age group. For example, the geriatrician of the future will need to understand molecular genetics as knowledge is gained about the molecular basis for aging, including common disorders at this time of life such as dementia, heart disease, and osteoporosis, to name a few.

Genetics clinics, comprising the specialist physician and the genetic counselor, ensure that DNA testing is appropriate. The clinics provide relevant counseling and support, particularly in circumstances of predictive DNA testing where an adverse result has implications for the patient and the family members. Because of the nature of molecular genetics, these clinics are often broadly based and deal with issues ranging from dysmorphology to pediatric genetic problems, to reproductive genetics, and to an increasing range of adult-based genetic issues and community ''public health'' screening genetics. This arrangement has allowed molecular genetics to develop rapidly. However, it is now time to rethink and consider the future model for clinical genetics, particularly in the light of the vast amount of data that will emerge in the next few years.

The clinical practice of the future will need to contend with an expanding database of knowledge gained from genomics that will overlap many clinical disciplines. The physician of first contact, the family physician, is asked by patients about genetic tests that have been featured in the media. Patients and families are becoming increasingly aware and knowledgeable about genetic disorders and DNA testing because of the media and access to the Internet. The complexities and sheer volume of knowledge about the genome require novel and computer-based learning activities. Greater specialization is inevitable, and general genetics clinics will increasingly need to move into multidisciplinary clinics involving a number of health professionals including family support groups. This *molecular medicine team* will ensure that the level of knowledge is contemporary, and that the patients and their families can have access to advice from a range of experts, as well as access to support from other parents or families with experience in genetics problems (Fig. 1). Without this division of work and close interactions between various disciplines, resources and expertise will be severely limited when our understanding of multifactorial genetic disorders (common conditions such as dementia, heart disease, stroke, and sporadic cancers) adds a DNA dimension to their prevention and treatment.

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Invader® Assay

Stephen Day
Andrea Mast
Third Wave Technologies, Madison, Wisconsin, U.S.A.

INTRODUCTION

The Invader® platform (Third Wave Technologies Inc., Madison, WI) is a homogeneous, isothermal, DNA probe-based system for highly sensitive, quantitative detection of specific nucleic acid sequences. It is an accurate and specific detection method for single-base changes, insertions, deletions, gene copy number, infectious agents, and gene expression. Invader reactions can be performed directly on genomic DNA- or on RNA-, PCR-, or RT-PCR-amplified products. A target-specific signal is amplified but not the target itself.

BACKGROUND

The Invader technology is based on the ability of a Cleavase® enzyme to recognize and cleave a specific nucleic acid structure generated by an overlap of two oligonucleotides (oligos)—the Invader oligo and the primary probe—on the nucleic acid target. Cleavase enzymes are a family of naturally occurring as well as engineered thermophilic structure-specific 5′ endonucleases.[1] Cleavase enzymes for use in DNA assays are derived from members of the flap endonuclease (FEN-1) family, typically found in thermophilic archaebacteria.[1] Invader RNA assays use Cleavase enzymes that are engineered from the 5′-exonuclease domain of DNA polymerase I of thermophilic eubacteria.[2] The optimal substrate for these nucleases is composed of distinct upstream, downstream, and template strands, which mimic the replication fork formed during displacement synthesis. The enzymes cleave at the 5′ end of the downstream strand between the first 2 base pairs and remove the single-stranded 5′ arm or flap.[1]

STRUCTURE FORMATION

The primary probe consists of two functionally different regions: a 5′-flap sequence and a 3′ target-specific region (TSR). The sequence of the 5′-flap varies in length (typically 1–15 nucleotides) and, because it is independent of the target, can consist of any sequence. The 3′ base of the Invader oligo overlaps with the TSR of the primary probe at a base referred to as ''position 1,'' creating a substrate for the Cleavase enzyme. The specificity of the Cleavase enzymes requires that position 1 of the primary probe be complementary to the target for cleavage to occur, providing the ability to discriminate single-base changes. The generation of the proper enzyme substrate is dependent on association between the primary probe, Invader oligo, and target. A noncomplementary base in the target at position 1 results in the formation of a nicked structure, rather than an invasive structure, because position 1 of the primary probe effectively becomes part of the flap. The nicked structure is not a substrate for the Cleavase enzyme, and thus the noncomplementary primary probe does not get cleaved. Discrimination relies on enzymatic recognition of the properly assembled structure in addition to the sequence specificity of oligonucleotides to the target sequence.

DNA DETECTION

The target-specific region of the primary probe is designed to have a melting temperature close to the reaction temperature, typically 63°C. Its inherent instability allows for the exchange of cleaved and uncleaved probes onto the target. When the specific sequence is present, the Invader oligo and TSR of the primary probe form an invasive structure on the target. The Cleavase enzyme removes the 5′-flap plus position 1 of the target-specific region. Following cleavage, the primary probe dissociates from the target and is replaced with another uncleaved primary probe, which is present in excess. Thus numerous 5′-flaps are released for each target molecule present, resulting in a linear amplification of signal, but not target.

The cleaved 5′-flap then acts as an Invader oligo to generate another invasive structure with a synthetic molecule, the fluorescence resonance energy transfer (FRET) oligo, which contains a donor fluorophore on the overlapping base (position 1) and a quencher fluorophore on the other side of the cleavage site.

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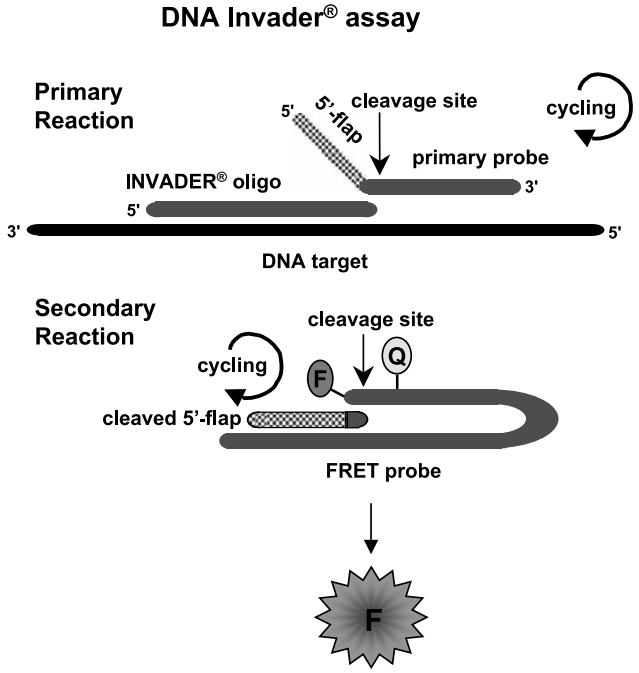

Fig. 1 Schematic of the Invader® DNA assay. (*View this art in color at www.dekker.com.*)

Cleavage of the FRET oligo separates the donor fluorophore (F) from the quencher fluorophore (Q) to generate a fluorescent signal. Using two different 5′-flap sequences and their complementary FRET oligos, each labeled with different fluorophores, two different target sequences may be detected in a single well. In addition to producing signal, the FRET oligo provides a second level of signal amplification. In the DNA assay, the 5′-flap forms an unstable, invasive duplex with a FRET oligo. After the fluorophore has been released, the 5′-flap dissociates and is able to form an invasive structure with a new, uncleaved FRET oligo. Signal amplification results from the combined effect of having multiple flaps cleaved for each target molecule and from each of those released flaps driving the cleavage of multiple FRET oligos. Both reactions—cleavage of the primary probes and of the FRET probes—occur simultaneously at a single temperature near the melting temperature of the primary probe (Fig. 1).[3–5]

RNA DETECTION

The FEN-type Cleavase enzymes that are used in Invader DNA assays are not able to recognize RNA targets; instead, pol-type Cleavase enzymes that recognize both DNA and RNA targets are used. The altered substrate specificity of the pol-type Cleavase enzymes requires an adaptation to the reaction used to cleave the FRET oligo. RNA assays occur in two sequential steps: the primary reaction and the secondary reaction. During the primary reaction, the Invader oligo and primary probe form an invasive structure on the RNA target. An additional oligo, the stacker oligo, is designed to coaxially stack with the 3′ end of the primary probe. The stacker oligo allows the primary probe to cycle on and off the target at a higher temperature and increases the sensitivity of the assay. In the secondary reaction, the cleavage product of the primary reaction (the cleaved 5′-flap plus one base of the TSR) forms a one-base overlap structure with a secondary reaction template (SRT) and a FRET oligo. The enzymatic cleavage of the FRET oligo separates a fluorophore from a quencher molecule, as described for DNA assays, generating fluorescent signal. In contrast to the DNA format, the released 5′-flap remains bound to the SRT while the FRET oligos cycle on and off the SRT. Consequently, multiple FRET oligos are cleaved per 5′-flap, resulting in a linear accumulation of fluorescence signal. An arrestor oligo, which hybridizes to the TSR plus a portion of the 5′-flap of uncleaved primary probes, is also included in the secondary reaction. The arrestor oligo allows for greater signal generation by sequestering uncleaved primary probes and preventing competitive inhibition of 5′-flap hybridization with the SRT during the secondary reaction (Fig. 2).[3,4,6]

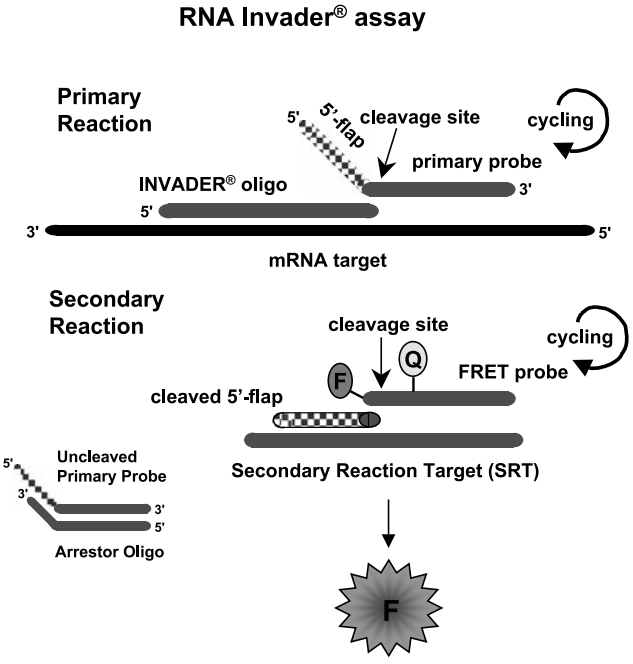

Fig. 2 Schematic of the Invader® RNA assay. (*View this art in color at www.dekker.com.*)

DETECTION FORMATS

Because its composition is independent of the target being detected, the 5′-flap can be detected in a variety of ways, e.g., by size, sequence, charge, or fluorescence. Based on these properties, the Invader technology can be applied to numerous alternate detection formats including mass spectrometry, capillary electrophoresis, microfluidics, universal array chips, capture, eSensor™, fluorescence polarization, and time-release fluorescence.

ACLARA Biosciences (Mountain View, CA) produces small, electrophoretically distinct, fluorescent molecules called eTag reporter molecules that can be incorporated into primary probes as 5′-flaps. The released eTag flaps are resolved and quantitated using universal separation conditions on standard capillary DNA-sequencing instrumentation. High-level multiplex capabilities are possible with the development of hundreds of eTag reporters. ACLARA has demonstrated the ability to detect 26 Invader RNA assays from a single tube.

SPECIFICITY

Superior specificity has been achieved with the Invader technology by combining sequence identification with enzyme structure recognition. The key to the assay's specificity is the strict requirement for an invasive structure. If the sequence at the cleavage site is not complementary to the intended target, an invasive structure does not form, and the 5′-flap is not released for detection in the secondary reaction. For mutation detection applications, such as cystic fibrosis transmembrane conductance regulator (CFTR) gene screening, this discrimination capability provides high accuracy, as demonstrated by its ability to classify the three possible CFTR ΔF508 genotypes and discriminate between the ΔI507 and ΔF508 mutations (Fig. 3). In a multisite study to determine the accuracy of genotyping five polymorphisms associated with venous thrombosis, the Invader assays correctly genotyped 99.9% of samples compared with 98.5% correct genotypes called by PCR-based methods.[7] Such ability to discriminate single-base changes also makes the Invader platform ideally suited for genotyping highly homologous strains of infectious agents.

The Invader platform is capable of specifically genotyping highly polymorphic regions such as the cytochrome P450 gene family, which contain multiple pseudogenes, gene deletions, and gene duplications. To prevent false positive results or inflated wild-type signals caused by association of Invader oligos to the pseudogenes, the region of interest can be amplified using PCR primers specific to the region of interest. The resulting amplicons can be used as templates for Invader assay-based detection. Using this approach, results from 11 Invader CYP2D6 assays provided a clear genotype for 100% of the 171 anonymous donor samples that had a visible PCR product on an agarose gel.[5]

The quantitative aspect of the Invader platform allows for the determination of chromosome and gene copy number directly from genomic DNA. This analysis may be accomplished by comparing the specific signal generated from the gene or chromosome of interest with that of a reference gene such as α-actin, which is not known to be polymorphic for either duplication or deletion.[5] Careful design of the Invader oligos to regions with minimal homology to other regions within the genome enables accurate quantitation of the desired target sequence.

Invader RNA assays have the same ability as the DNA assays to discriminate single-base changes. By positioning the cleavage site of the primary probe at a nonconserved site within the mRNA target, Invader assays are able to differentiate highly homologous RNA sequences. Cross-reactivity is undetectable in the presence of 10,000-fold excess of highly related mRNA.[4] In addition, assays with cleavage sites located at particular exons or splice junctions can distinguish alternatively spliced mRNA variants.[6]

SENSITIVITY

Because of its high level of precision, Invader assays can determine changes in gene expression as low as 1.2-fold.[6] Intraassay coefficients of variation (CVs) are typically less than 10% and range, on average, from 2% to 3%.[4,6] Reading the assays in real time or at multiple time points can extend the dynamic range of detection, which covers three orders of magnitude with a single endpoint read.[4]

Signal amplification eliminates the need for target amplification prior to analysis, which, in turn, simplifies sample processing. Genomic DNA obtained from small blood volumes can be used directly in genotyping assays. Invader DNA assays are able to statistically detect as little as 10 ng of genomic DNA (approximately 1500 heterozygous genomic copies);[3] however, a minimum of 100 ng is generally recommended. For RNA assays, sample preparation can be simplified by using unpurified cell lysates directly in the assay. The Invader RNA assays can routinely detect as little as 0.005–0.01 amol RNA target (approximately 3000–6000 copies of RNA transcript or messenger RNA).[6]

Invader assays have the unique ability to tolerate the presence of secondary polymorphisms. Whereas the formation of the proper enzyme substrate is dependent on base pairing at critical positions within the primary probe, other regions may be mismatched. This provides the ability to detect or disregard polymorphisms, which

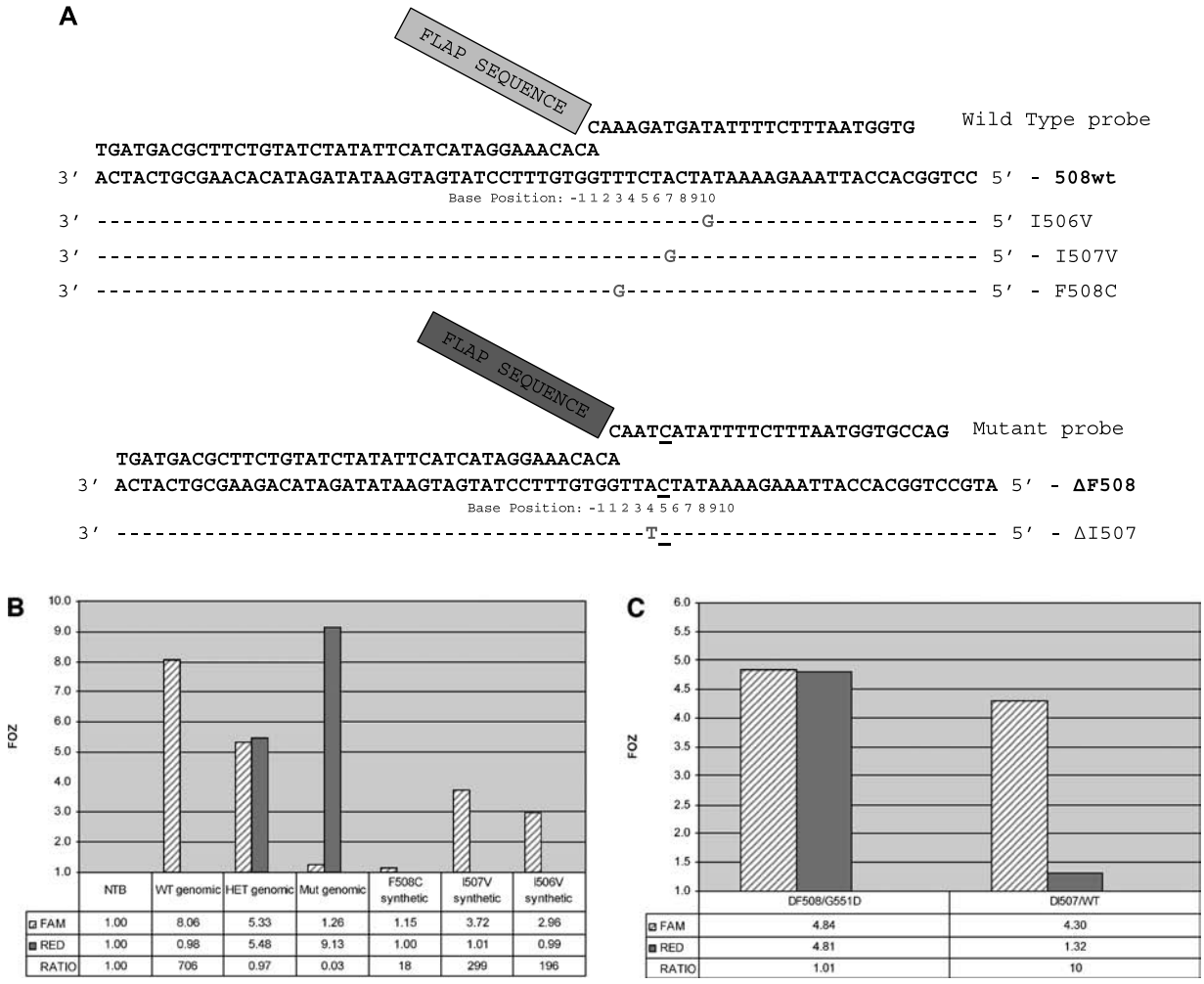

Fig. 3 Discrimination and detection of ΔF508 DNA. (A) ΔF508 assay design and alignment of WT, I506V, I507V, F508C, ΔF508, and ΔI507 DNA sequences. The wild-type (WT) probe is completely homologous to the WT target but mismatches at position 3, 7, and 10 on the respective targets F508C, I507V, and I506V. The Mutant (Mut) probe has a single mismatch at position 5 (underlined) on the ΔF508 target and two mismatches (positions 4 and 5) on the ΔI507 target. The position 5 mismatch was designed to reduce cross-reactive signal on the ΔI507 target, which differs from ΔF508 only at position 4. (B) The specific signal generated on WT, HET (WT/ΔF508), and Mut (ΔF508/ΔF508) genomic samples. The WT probe generates sufficient signal with the I507V and I506V targets (mismatches at positions 7 and 10, respectively), but not with the F508C target (mismatch at position 3). An additional test is required to determine F508C genotype. (C) The specificity of the Mut probe: the mismatch at position 5 does not hinder the signal generated from a ΔF508 HET genomic sample and creates additional discrimination of the ΔI507 genomic sample. (*View this art in color at www.dekker.com.*)

increases the assays' detection sensitivity in highly polymorphic genes or strains of infectious agents.

ROBUSTNESS

Ease of use, flexibility, and scalability are some of the advantages that make the Invader platform ideally suited for automation. Assays are readily adaptable from manual to semiautomated to fully automated ultra-high-throughput settings using a variety of standard plate formats. Most automated liquid handling systems can handle the assay setup, which requires only a few pipetting steps. Regardless of format, e.g., 96 or 384 wells, or setup, e.g., manual or automated, the robustness of the platform remains constant.[3]

The Invader platform—a highly accurate, cost-effective, and specific method for the detection of specific nucleic acid sequences—has been applied to the detection of thousands of SNPs in genome-wide association studies.[8,9] Simple, fast, reliable, and inexpensive diagnostic tools are essential not only for research purposes, but

for clinical settings as well. Several analyte-specific reagents (ASRs) have been developed for use in clinical diagnostics, including Factor V Leiden (FVL), apolipoprotein E (ApoE), and cystic fibrosis (CFTR) mutation detection. The current clinical applications of the Invader platform include detection of coagulopathies and inherited diseases.

CONCLUSION

The Invader platform is a homogeneous, isothermal DNA probe-based system for the quantitative detection of specific nucleic acid sequences. Invader assays can be performed directly on DNA-, RNA-, PCR-, or RT-PCR-amplified products. Based on the ability of the Cleavase enzymes to recognize and cleave-specific nucleic acid structures, the Invader platform is a highly specific method for detecting single nucleotide polymorphisms, insertions, deletions, and gene copy number. Its ability to amplify signal and tolerate secondary polymorphisms makes the Invader platform extremely sensitive as well. Because of the flexibility, ease of use, and robustness of the technology, it can be used in both small and high-throughput laboratories.

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Inverse PCR

I

Philip Lewis White
Sam Hibbitts
University of Wales College of Medicine, Cardiff, U.K.

INTRODUCTION

Polymerase chain reaction (PCR) is widely accepted as one of the most powerful tools in molecular biology capable of amplifying over 10^6 identical copies of small (few kb), specific DNA regions. It utilizes the thermostability of the enzyme *Taq* DNA polymerase I, and allows quick and efficient exponential amplification of a target region between known DNA sequences. This removes the need for time-consuming cloning, although it does have two key limitations. It is only efficient in amplifying relatively small DNA fragments and secondly, but more importantly, PCR can only be used to amplify regions of known DNA sequence. As such, PCR alone cannot be used to amplify DNA sequences adjacent to an unknown sequence, thus making chromosomal walking unfeasible.

In the late 1980s and early 1990s, research was performed to overcome this restriction, and inverse PCR (IPCR) was one of the developments.[1–9] Inverse PCR is a novel molecular method utilizing the basic principles of PCR, but in a reverse manner so regions of unknown sequence can be amplified. This procedure is one of numerous tools utilized for furthering our understanding of the complexities of DNA, and facilitates cloning and sequencing of otherwise unknown elements of a genome.

TECHNICAL OVERVIEW

The basic theory behind inverse PCR requires a core DNA region of known sequence to be adjacent to an unknown target. This region is digested to produce a fragment of suitable size for PCR that is ligated together to produce a circular construct. The core region provides the sequence information required for synthesis of primers, designed to hybridize to the ends of this core region, which, because of the nature of the circular construct, flank the unknown sequence. Extension occurs divergently toward the unknown sequence with PCR amplification of the uncharacterized region.

BASIC PROTOCOL FOR PCR OF AN UNKNOWN DNA TARGET

IPCR within a Circular Construct

A quality target genomic DNA extract should be obtained, and 500 ng digested with an appropriate restriction enzyme in a 30 μL volume and adequate digestion should be confirmed by agarose gel electrophoresis. The resulting fragment should contain a region of known sequence flanked by the unknown target. The enzyme is then inactivated, DNA-precipitated, and diluted (approximately 1:5) prior to ligation in a media of ligase buffer, water, and ligase to optimize conditions for intramolecular ligation. Following incubation according to the manufacturer's instructions (approx. 1–12 hr at room temperature), complete ligation with production of a circular construct can be verified by Southern blotting using the known sequence as a probe.

A titration series (5–10 μL) of the circular construct should be used for the PCR reaction in a final volume of 50 μl to optimize the chances of target DNA amplification. Primers should be designed based on the known sequence and PCR conditions to include a high annealing temperature to prevent any nonspecific priming, a long extension time (2 min), and up to 40 cycles to maximize the chances of amplification of rare templates.

A nested PCR maybe performed to increase the specificity of the reaction (Fig. 1). In this case, following the first-round PCR and analysis of the product by agarose gel electrophoresis, another titration series of the first-round product (up to 15 μL) should be added to a second-round PCR reaction containing primers located inside the region previously amplified. Annealing temperatures should be increased with an extension time of 1 min (approx.). Final products should be analyzed by electrophoresis.

In general, the objective of inverse PCR is to elucidate the sequence of the unknown region, and so the inclusion of a proofreading enzyme (e.g., *PfU*) in the PCR reactions will reduce errors that may be introduced during amplification. Products obtained after PCR are then cloned into a vector for subsequent DNA sequencing.

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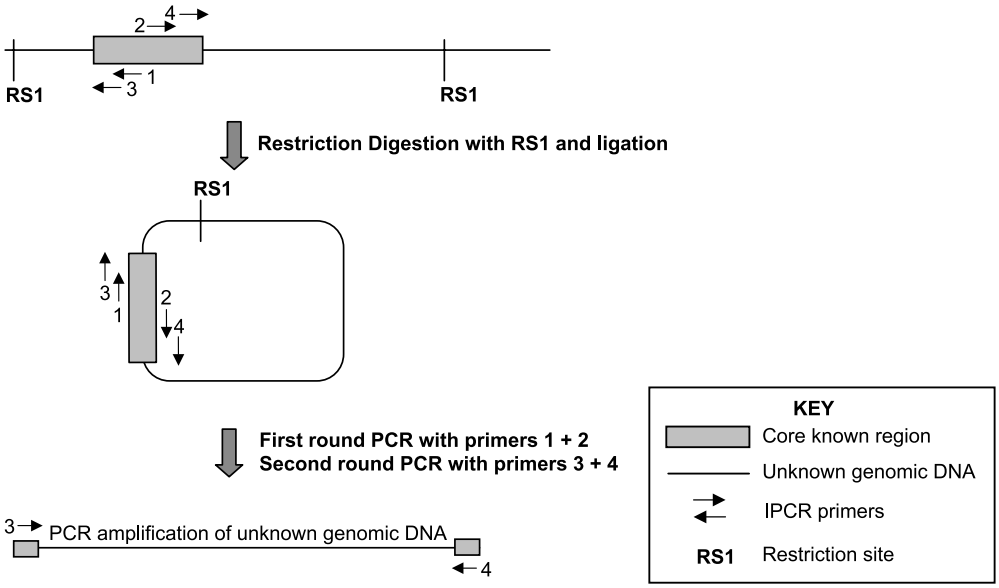

Fig. 1 Inverse PCR using a circular construct. Schematic for the PCR amplification of an unknown region from a circular construct.

IPCR of a Linearized Construct

An alternative procedure for PCR amplification of an unknown target requires that the circular construct produced as described in ''IPCR within a Circular Construct'' should be linearized by using a restriction enzyme located in the known sequence between the 5′ regions of the PCR primers. This additional step has been shown to increase the efficiency of the PCR amplification reaction up to 100-fold.[4] In theory, covalently closed circular double-stranded DNA is a poor substrate for PCR with reannealing of the DNA strands during the extension step of PCR. However, linearization or nicking of such a construct will reduce this and make it a more ideal template for amplification (Fig. 2).

INVERSE PCR MUTAGENESIS

This procedure utilizes the basic principle of IPCR to introduce a genetic change within a plasmid. As in traditional IPCR, the primers are oriented ''back-to-back'' but anneal in opposite directions around the plasmid, and one of the primers contain the required mutation. PCR of the plasmid with a proofreading enzyme will introduce the genetic change present in one primer and yield a blunt-ended product. Self-ligation of the final product will reproduce the original plasmid plus the introduced mutation (Fig. 3). This procedure can be utilized for point mutations or insertions. If a region of the plasmid is to be deleted, primers can be designed on either side of this area, and the subsequent PCR product when religated will be complete except for the region that the primers flanked (Fig. 4).

Transformation of ligated mutated plasmids into *Escherichia coli* gives a high efficiency of mutant plasmid isolation (approximately 80%).

POINTS TO CONSIDER/LIMITATIONS

Irrespective of the application of inverse PCR, the basic principle remains the same with an optimized protocol designed according to: 1) DNA origin; 2) restriction enzymes utilized; and 3) PCR conditions.

The origin of the DNA will determine the extraction procedure used to obtain a quality DNA sample. Various commercial kits exist for DNA extraction, and optimal procedures vary depending on the starting material. For example, commercial kits are available for both manual and automated genomic DNA extraction from whole blood and include Qiamp DNA Blood Kits (Qiagen, Crawley, UK), Genelute Blood Genomic DNA Kit (Sigma, Poole, UK), Generation Capture Column Kit (Gentra, Minneapolis, USA), and Magna Pure LC DNA isolation Kit I (Roche, Lewes, UK). Meanwhile, genomic DNA extraction from human/animal/plant tissue tend to utilize alternative extraction kits or procedures [DNeasy Plant and Tissue System (Qiagen); Magna Pure LC DNA Isolation Kit II (Roche); Genelute plant or mammalian genomic DNA kit (Sigma) and NuCleon® phytopure genomic DNA extraction kit (Amersham Pharmacia

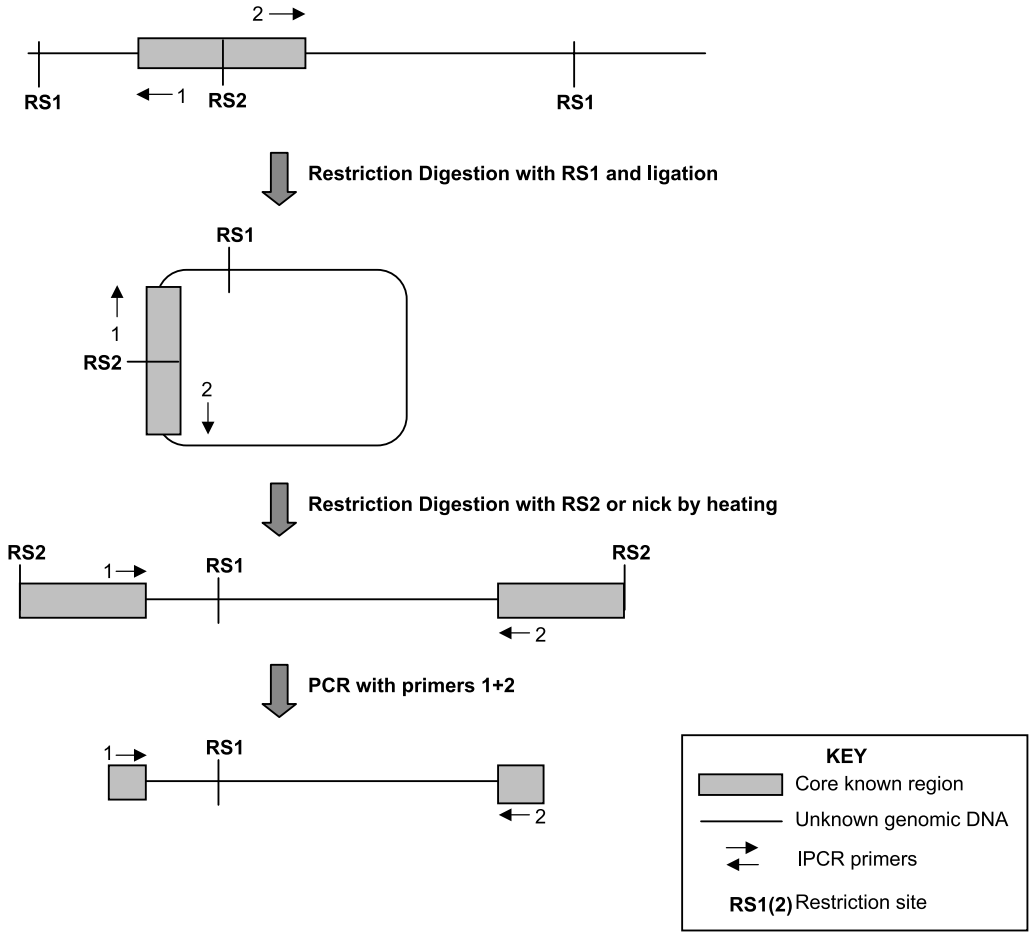

Fig. 2 Inverse PCR of a linear construct. Schematic for the PCR amplification of an unknown region from a linear construct.

Biotech, Little Chalfont, UK)]. Because a quality DNA starting material is important for efficient IPCR, this should be the first issue that needs to be addressed.

The restriction enzyme required to cleave the target DNA region is crucial in that it must produce a fragment of appropriate length for the subsequent ligation and PCR. Small fragments (<200 bp) prove difficult to circularize, whereas larger fragments (>3 kb) may be problematic for PCR amplification. However, Benkel and Fong[10] have published a hybrid method allowing amplification of larger fragments by combining long-range (distance) PCR with IPCR, and accordingly labeled it long-range inverse PCR (LR-IPCR). In addition to the fragment size, the positions of the restriction sites are important when deciding what is required from the amplification. If amplification of both flanking regions is sought, then it is essential that the restriction enzyme of choice does not cleave within the known core region. Alternatively, if only amplification of one side of the core region is necessary, the enzyme must cleave inside the core region, but leave enough core sequence to allow the design of a primer pair for PCR.[3,11]

The element of IPCR becomes more difficult when there is no restriction map available of the unknown region. It has been suggested that by using a restriction enzyme with a four-base recognition sequence, enough IPCR suitable fragments would be generated.[11] However, this method could be very random and the use of Southern blots to identify restriction sites is a more controlled option. In all cases, numerous restriction digests should be evaluated to elucidate the most appropriate and efficient for PCR of the unknown region.

If IPCR on a linear construct is to be undertaken, care should be taken regarding the choice of restriction enzyme used to linearize the circular construct. The restriction site has to be within the known region between the 5′ ends of the primers, not downstream of this or in the unknown sequence, or the template for subsequent PCR will be destroyed. An alternative to restriction digestion of the circular construct, yet still enhancing it as a PCR template,

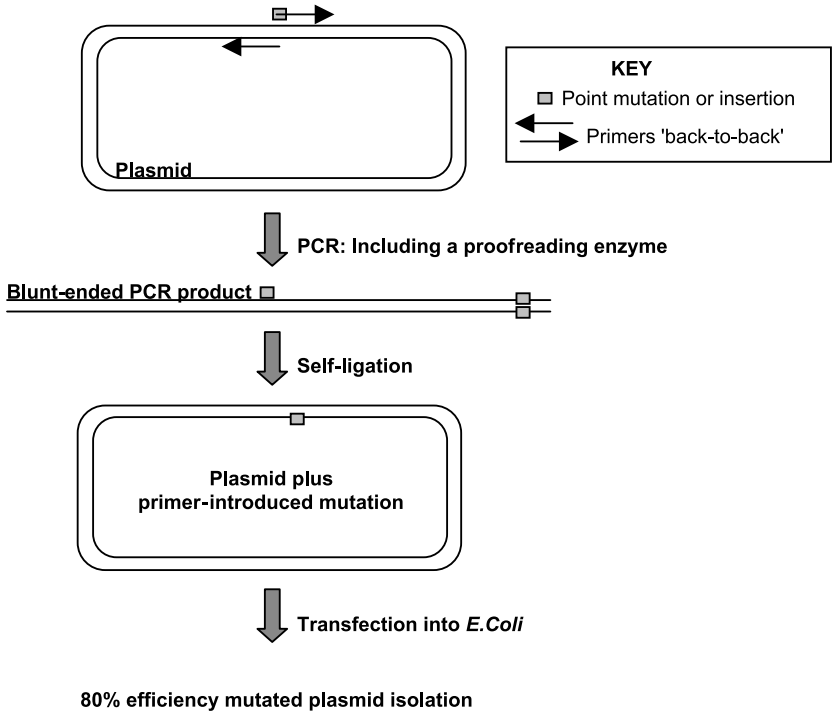

Fig. 3 Inverse PCR mutagenesis 1. Schematic for the introduction of a point mutation or insertion into a plasmid.

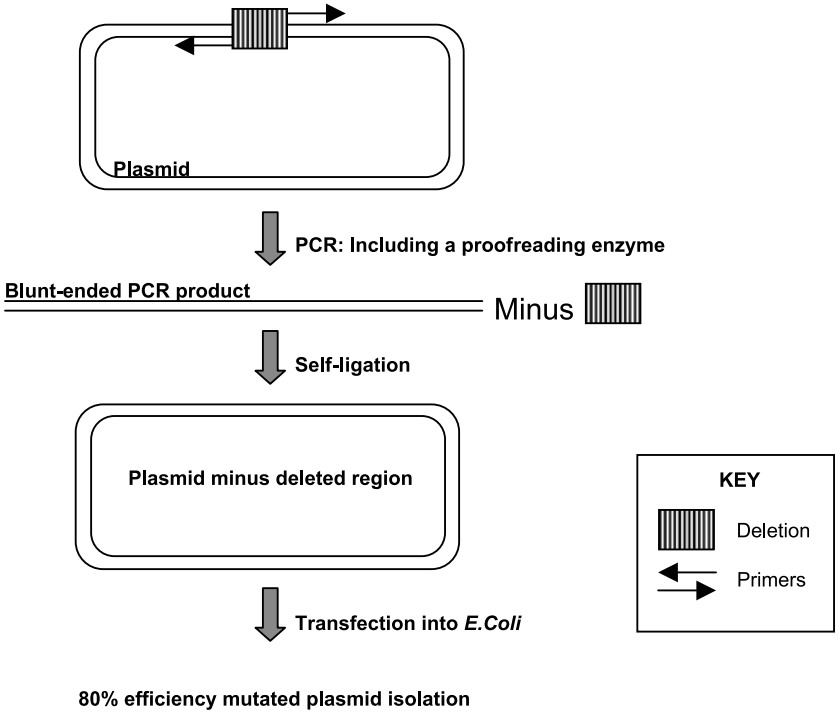

Fig. 4 Inverse PCR mutagenesis 2. Schematic for the deletion of a region within a plasmid.

would involve introducing random nicks that can be caused by heating[1] or limited DNase treatment. However, a major drawback to this approach is the difficulty in controlling the degree of nicking to the construct.

The PCR component of the IPCR reaction needs to be optimized both in relation to the quantity of template, primers, and *Taq* polymerase, and in terms of the cycling conditions. As discussed, titration of the starting material in the PCR reactions will help to overcome some of these problems, and it is essential that appropriate controls are included in the PCR reaction. Analysis of products by electrophoresis will also elucidate any technical difficulties. A common problem is the formation of primer dimers or multimers, as is smearing recognized after the first-round PCR, which can be attributed to nonspecific amplification that maybe caused by an excess of template or inefficient digestion and/or ligation. Increased annealing temperatures as well as decreased primer and *Taq* polymerase concentrations may alleviate nonspecific primer binding and also minimize the formation of primer dimers, although it is recommended that when designing primers, the appropriate software packages (e.g., Netprimer: www.premierbiosoft.com/netprimer) are used to reduce the opportunity of primer dimer/polymer formation.

CLINICAL AND GENETIC APPLICATIONS

The genetic applications of inverse PCR have been widespread and diverse. They include:

1. characterization of long stretches of unknown DNA sequence adjacent to specific genes, e.g., chromosome mapping/walking[5] and identification of upstream and downstream regulatory elements;
2. establishing the insertion sites of mobile genetic elements,[12] including the integration of viral genomes into the host;[13–15]
3. aiding in the understanding of mutational mechanism involved in deletions and translocations;[16]
4. the construction of knockout vectors to disrupt genes;[17]
5. fingerprinting of microorganisms;[18,19]
6. evolution of organisms;[20]
7. generation of end-specific probes.[3]
8. defining new pathways of disease;[21]
9. determining the differential expression of genes.[22]

CONCLUSION

Despite the numerous benefits of PCR, it was limited by its inability to amplify unknown genomic regions. The development of IPCR overcame this limitation and has helped to further our understanding of unidentified genes without the need for more complex chromosomal walking techniques.

Note that a new edition of PCR Technology: Principles and Applications for DNA amplification was published by Oxford University Press in 2002.

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Keratin Disorders

Eli Sprecher
Rambam Medical Center, Haifa, Israel

INTRODUCTION

Keratins are part of a large group of proteins known as intermediate filaments, which, together with actin filaments and microtubules, form the cell cytoskeleton. Keratins are widely expressed in the cytoplasm of epithelial cells, to which they confer mechanical resilience. Keratins are expressed in a tissue-specific manner and, accordingly, often demarcate pathways of embryonic development and cell differentiation. For example, keratins 8 and 18 are the only keratins detectable in preimplantation blastocyst; in the epidermis, keratins 5 and 14 are expressed in proliferating basal cells, whereas keratins 1 and 10 are the major keratins produced in more differentiated suprabasal cells.

OVERVIEW

Keratin intermediate filament (KIF) assembly initiates with the formation of a heterodimer composed of a basic keratin molecule and an acidic keratin molecule intertwining in a coiled coil parallel fashion. Keratin heterodimers then interact in a staggered antiparallel manner to form stable tetramers, which associate longitudinally and laterally to generate 2-nm protofilaments. Protofilaments are then assembled into protofibrils, which eventually interact to yield 10-nm KIF.

Types I and II keratin-encoding genes are clustered on 17q and 12q, respectively. Approximately 50 distinct keratin or keratin-like genes, and more than 150 keratin pseudogenes have been identified in silico in the human genome. Some of those pseudogenes are readily expressed as mRNA, thus significantly complicating the molecular diagnostics of inherited keratin disorders.[1] A number of techniques are now available to avoid keratin pseudogene amplification.[2]

The following article presents a succinct description of keratinopathies and relevant diagnostic strategies. More detailed information can be found at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM using the OMIM numbers given in Table 1. References including specific molecular diagnostic strategies, primer lists, and polymerase chain reaction (PCR) conditions for amplification and sequencing of various keratin genes are given in Table 1. An exhaustive list of keratin mutations can be obtained at http://www.interfil.org.

Keratins can be classified according to their isoelectric point and amino acid sequence (e.g., basic or type I, and acidic or type II), their major site of expression (e.g., epithelial keratins and hair keratins), or, their cysteine content (e.g., hard and soft keratins).

As for all other intermediate filaments, keratins are organized into two nonhelical end domains of varying sequence, flanking a central α-helical rod segment (Fig. 1). The sequences found at both ends of the rod segment are remarkably conserved across species and keratin types. They are known as the helix initiation motif (HIM) and the helix termination motif (HTM).[1]

EPIDERMAL DISORDERS

Epidermolysis Bullosa Simplex

Inheritance: Autosomal dominant (rarely autosomal recessive).

Clinical description: Epidermolysis bullosa simplex (EBS) is part of a heterogeneous group of inherited blistering diseases collectively known as epidermolysis bullosa (EB). All EB subtypes are characterized by the appearance of cutaneous blisters upon exposure of the skin to mechanical trauma (Fig. 2a). The major EB subtypes are defined by the location of blister formation within the skin.[19] In EBS, blisters form within the epidermal basal cells. The age of onset of EBS is variable. Progressive improvement with age is common. Three clinical forms of EBS have been delineated. Blisters occurring exclusively over the feet and hands distinguish the Weber–Cockayne type of EBS. In EBS Koebner type, blistering is generalized. The most severe, fortunately rare, and often life-threatening form of EBS is known as Dowling–Meara EBS and is characterized by skin and mucosal hemorrhagic blisters arranged in small clusters. EBS Dowling–Meara type is occasionally associated with milia formation, nail dystrophy, and severe palmoplantar keratoderma (PPK).

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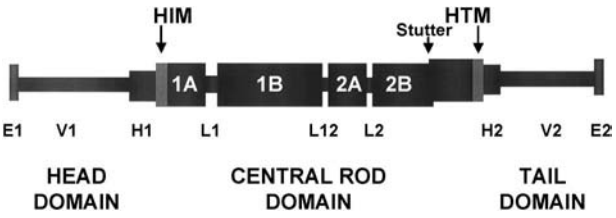

Fig. 1 Schematic structure of a prototypic keratin molecule. The central rod domain is divided into four regions, which are separated by three nonhelical short linker sequences and the stutter sequence, where helix polarity is reversed. The rod domain is flanked by the highly conserved HIM and HTM. The head and tail domains consist of extreme end domains (E1, E2), variable domains (V1, V2), and homology domains (H1, H2). In contrast with the central rod domain, head and tail domains greatly vary in structure between different keratin types.

Skin pathology: Blister formation and typical vacuolar changes are seen within the epidermal basal cell layer. Keratin filament clumping is typical of Dowling–Meara cases.

Molecular genetics: Mutations in two keratins specifically expressed in epidermal basal cells, K5 and K14, underlie most cases of EBS.[2,20] The majority of EBS-causing mutations are missense mutations, affecting amino acids located within the rod segment or at its boundaries. The same regions have been shown in many other keratin disorders to carry deleterious mutations,[2] leading to the implementation of a diagnostic strategy based on initial pathological examination of skin tissues, followed by mutation analysis of rod boundaries-encoding regions, and ending with examination of the rest of the keratin gene (Fig. 3). Mutations affecting the end domains of the rod segment have been shown to exert a dominant negative effect, resulting in the synthesis of aberrant keratin proteins with altered three-dimensional structure, which interferes with normal protein functions during KIF assembly. Abnormal KIF affects cell resilience so that on exposure of the skin to friction, the cytoskeleton collapses, leading to cell cytolysis and intraepidermal blistering. Recessive K14 nonsense mutations are considered to be rare except in populations characterized by a high inbreeding coefficient.[21] As a rule, recessive mutations as well as dominant mutations located within the conserved helix end motifs are associated with severe phenotypes; missense mutations located outside these regions generally cause milder forms of EBS. The nature of an amino acid substitution, and not only its location, is also a major determinant of phenotype severity.[20]

Mutations in K5 end domains have also been reported. A recurrent mutation, P25L, located in the V1 head domain of K5 has been found to cause a rare subtype of EBS associated with mottled pigmentation. P25L has been suggested to impair melanin granule aggregation and keratin filament function by interfering with posttranslational processing.[20] Mutations in K5 tail domain have been shown to cause dominant EBS, possibly because of abnormal protein folding.[22,23]

Those major advances in our understanding of the molecular basis of EBS and other keratin disorders have led to the development of DNA-based prenatal testing, usually carried out during the first trimester (Fig. 4). Prenatal diagnosis is provided based on previous knowledge of mutation(s) in the family, sequencing of fetal and parental DNA, and confirmation of mutation using ancillary tests such as polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) or allele-specific PCR. A particular problem arises when prenatal diagnosis is requested with no prior knowledge of the mutation. Microscopical examination of fetal skin biopsy, which entails significant risks and is carried out late in pregnancy, has rarely been performed in such cases.

Differential diagnosis: The differential diagnosis of EBS encompasses a large spectrum of inherited blistering conditions, including other EB types[19]; epidermolytic hyperkeratosis (EHK); Kindler syndrome (MIM173650), caused by mutations in kindlin, typically accompanied by pigmentary and atrophic changes affecting sun-exposed skin; ectodermal dysplasia with skin fragility (MIM604536), typified by suprabasal separation and due to plakophilin 1 deficiency; EB with muscular dystrophy (MIM226670), due to mutations in plectin gene; and incontinentia pigmenti (MIM318310), characterized by congenital blisters distributed along the lines of Blashko. In addition, EBS can result from mutations in genes coding for integrinβ4, plectin, and collagen XVII, known to be usually involved in the pathogenesis of non-EBS phenotypes.[24–26] EBS should also be distinguished from congenital blistering resulting from infectious, autoimmune, and neoplastic causes.

Management: Patient care involves wound care, including sterile dressings and lancing of blisters to prevent spread; infection control; and nutritional support. Novel therapeutic approaches include the use of biological dressings or skin equivalents, and burgeoning attempts to cure EB through gene therapy.

EHK and Other Epidermal Disorders

Inheritance: Autosomal dominant.

Clinical description: EHK, also known as bullous congenital ichthyosiform erythroderma, manifests at birth with skin erythema and blistering.[2] Over time, blisters become less prominent and patients develop generalized

Table 1 Spectrum of keratin disorders

Keratin	Disease	OMIM number	References for primer lists and PCR conditions
K1	EHK	113800	[3]
	Nonepidermolytic PPK	600962	
	Ichthyosis hystrix	146590	
	Striate PPK III	607654	
K2e	IBS	146800	[4]
K3	Meesman's corneal dystrophy	122100	[5]
K4	White sponge nevus	193900	[6]
K5	EBS Weber–Cockayne	131900	[3]
	EBS Koebner	131800	
	EBS Dowling–Meara	131760	
	EBS with mottled pigmentation	131960	
K6a	PC-1	167200	[7]
K6b	PC-2	167210	[8]
K8	Cryptogenic cirrhosis	215600	[9]
K9	Epidermolytic PPK	144200	[10]
K10	EHK	113800	[11]
	Annular epidermolytic ichthyosis	607602	
K12	Meesman's corneal dystrophy	122100	[12]
K13	White sponge nevus	193900	[13]
K14	EBS Weber–Cockayne	131900	[14]
	EBS Koebner	131800	
	EBS Dowling–Meara	131760	
	Autosomal recessive EBS	601001	
K16	PC-1	167200	[15]
	Nonepidermolytic PPK	600692	
K17	PC-2	167210	[16]
	Steatocystoma multiplex	184500	
hHb6	Monilethrix	158000	[17]
hHb1	Monilethrix	158000	[31]
K6hf	Loose anagen syndrome	600628	[18]

ichthyosis of variable severity, often accompanied by PPK (Fig. 1B).

Skin pathology: In contrast with most ichthyoses, histopathological features of EHK are distinctive and include orthohyperkeratosis, hypergranulosis, and vacuolar degeneration confined to the upper epidermal layers. Electron microscopical examination demonstrates an increased number of clumped tonofilaments located at the periphery of suprabasal cells and poorly attached to plasma membrane desmosomes.

Molecular genetics: EHK results from mutations in K1 or K10, which are expressed in the suprabasal layers of the epidermis. As for other keratin disorders, most EHK-causing mutations affect conserved amino acid residues located at both ends of the central K1/K10 rod segment and result in aberrant KIF formation, leading to perinatal skin blistering.[2,20] Mutations in *K1* or *K10* genes also interfere with normal epidermal outer barrier formation, causing subsequent hyperkeratosis. Epidermolytic PPK results from mutations in K9, a type I keratin that is specifically expressed in palmoplantar skin.[2] K9 may compensate for K10 abnormal function, explaining the fact that PPK in EHK is strongly associated with K1, but not with K10 mutations.[27] In addition, marked interfamilial and intrafamilial variations in phenotypic expression of K1/K10 mutations are typical.[27] Somatic mutations in K10 have been identified in individuals with epidermolytic epidermal nevi, manifesting with a mosaic distribution of hyperkeratotic lesions. Affected individuals are at risk for children affected with EHK; therefore, they qualify for prenatal diagnosis.[2] Finally, mutations affecting the nonhelical end domains of K1 have been shown to underlie a number of phenotypically distinct clinical entities. The pathophysiology of this group of disorders is likely to involve aberrant interactions between KIF and desmosomes.[20,22]

Differential diagnosis: Congenital ichthyosiform erythroderma (MIM242100) is characterized by erythroderma

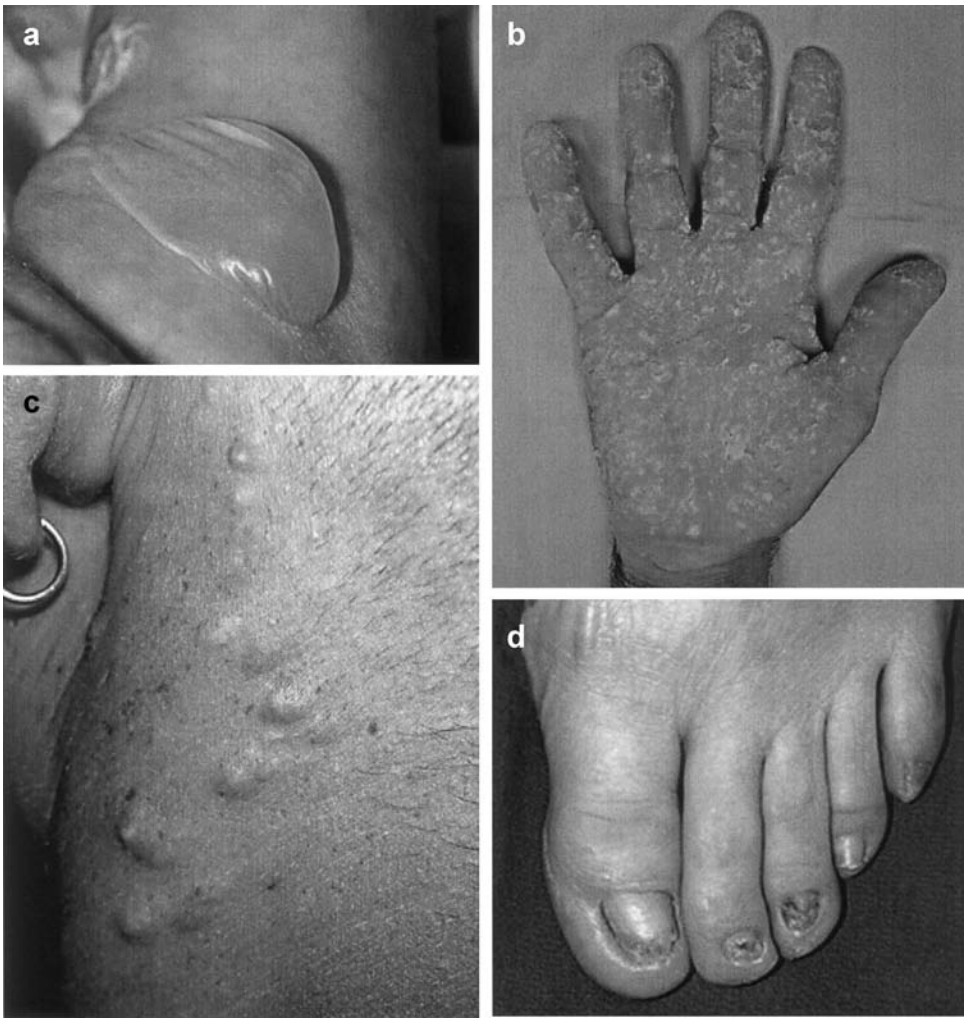

Fig. 2 Clinical spectrum of keratin disorders. (a) Skin blister in a 1-month-old girl with EBS Dowling–Meara type. (b) Palmar hyperkeratosis in a 26-year-old individual with EHK. (c) Multiple steatocystomas and (d) nail dystrophy in a 25-year-old patient with PC-2.

and scaling at birth; however, blistering is absent and inheritance is recessive. In contrast, EBS manifests with blistering, but hyperkeratosis is usually absent. Ichthyosis bullosa of Siemens (IBS) is caused by mutations in K2e.[2] Affected individuals display mild scaling in the flexural areas, over the shins and elbows, as well as a superficial form of skin peeling, known as mauserung phenomenon. Intraepidermal separation and tonofilament clumping occur in a layer higher than that in EHK—within the granular layer.

Management: Conservative treatment measures such as lubrication, wound dressing, management of skin infections, and nutritional support are essential, especially during the first years. Satisfactory response to retinoids has been reported more often in carriers of K10 mutations than in carriers of K1 mutations.[28]

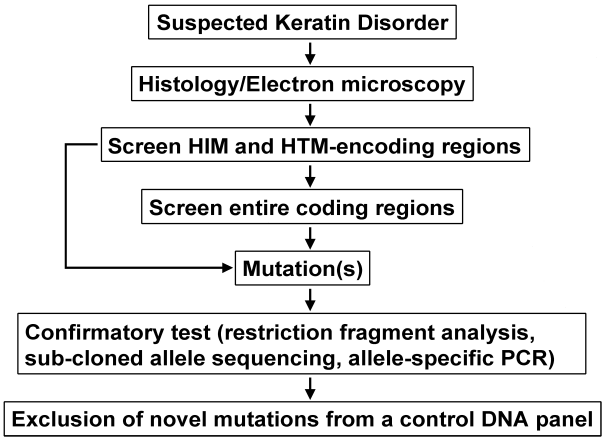

Fig. 3 Diagnostic strategy for identification of mutations in keratin genes.

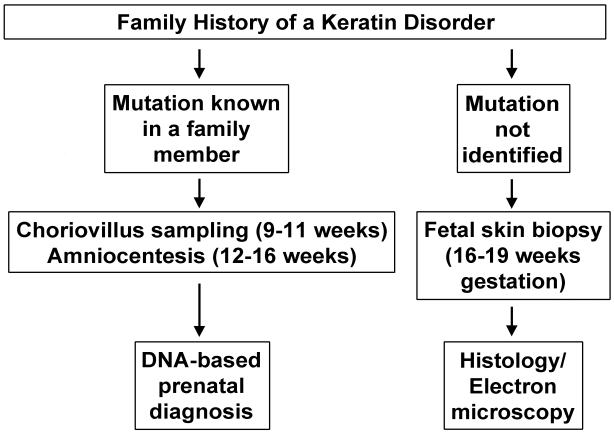

Fig. 4 Methods in use for prenatal testing of pregnancies at risk for keratin disorders.

NAIL, HAIR, AND MUCOSAL DISORDERS

Pachyonychia Congenita

Inheritance: Autosomal dominant.

Clinical description: Pachyonychia congenita (PC) type I or Jadassohn–Levandowsky syndrome (PC-1) is characterized by subungual hyperkeratosis, resulting in nail thickening and yellowish discoloration, accompanied by focal PPK, follicular hyperkeratosis, and oral leukeratosis. In contrast, PC-2, also known as Jackson–Lawler syndrome, is accompanied by less severe nail dystrophy and milder keratoderma, and is distinguished by the presence of multiple steatocystomas, a history of neonatal teeth, and the occasional occurrence of pili torti and/or alopecia (Fig. 2c–d). Additional variants with corneal leukokeratosis (PC-3) and laryngeal lesions with mental retardation (PC-4) have been described.[2]

Molecular genetics: PC-1 is caused by mutations in *K6a* or *K16* keratin genes, whereas PC-2 results from mutations in *K6b* or *K17* keratin genes.[2] The pattern of expression of these two pairs of keratin underlies the specific clinical manifestations of the two PC subtypes: K6a/K16 are expressed in the nail bed and nail fold as well as in palmoplantar skin and oral mucosa; K6b/K17 are found in the nail bed, hair follicle, and selected areas of palmoplantar skin. Although most PC-causing mutations are located at both ends of the keratin central rod segment, a somatic mutation in the head domain of K16 was recently shown to cause a mosaic form of PPK.[29]

Phenotypic heterogeneity is typical of PC. Mutations in K16 have been shown to underlie keratoderma in the absence of nail changes, and mutations in K17 have been shown to cause steatocystoma multiplex with no other PC-2 manifestations.[2] To complicate the picture even further, combined features of PC-1 and PC-2 have recently been shown to result from a deleterious mutation in K6a.[30]

Differential diagnosis: PC differential diagnosis includes toenail dermatophytic infection; psoriasis; lichen planus; dyskeratosis congenita (MIM305000), characterized by hypoplasia of the nails, oral leukokeratosis, hyperpigmentation, and hematological abnormalities; and hidrotic ectodermal dysplasia (MIM129500), manifesting with congenital nail dysplasia and focal PPK.

Management: Treatment is mainly symptomatic and involves the use of emollients or keratolytics to treat the PPK. Retinoids have been found useful in some patients. When hand or foot function is compromised by nail disease, nail matrix ablation can be considered.

Monilethrix

Inheritance: Autosomal dominant.

Clinical description: Monilethrix is characterized by the beaded appearance of hair shafts due to alternating elliptical wide (nodes) and narrow (internodes) hair segments. Hairs tend to break at the constricted sites, resulting in varying degrees of alopecia. Abnormal hair may be visible at birth or, more often, appears during the first months of life. Marked improvement is usual after puberty. Interfamilial and intrafamilial variations in disease severity have been observed.[31] In addition, patients may display additional cutaneous anomalies including follicular hyperkeratosis, and nail and tooth abnormalities.[2]

Molecular genetics: Pathogenic mutations have been identified in hair cortex keratin genes, *hHb6* and *hHb1*.[2] As for most other keratin disorders, mutations are clustered at the ends of the central α-helical rod.

Differential diagnosis: Monilethrix hair has been observed in Menkes syndrome (MIM309400), an X-linked disorder characterized by neurological and hair manifestations due to deleterious mutations in the *ATPA7A* gene, encoding a copper-binding protein.[32] Abnormal posttranslational processing of keratin molecules due to intracellular copper deficiency may explain the occurrence of beaded hair in Menkes syndrome. Pseudo-monilethrix (MIM177750) is characterized, in contrast with monilethrix, by irregularly spaced nodes with narrow internodes representing normal hair shafts.

Management: Spontaneous improvement is frequent.

White Sponge Nevus

Inheritance: Autosomal dominant.

Clinical description: Buccal involvement is most common, although the disease also manifests in other noncornified stratified squamous epithelia, including the

esophagus, nose, vagina, and rectum. Clinically, white, spongy plaques cover involved areas as a result of massive mucosal overgrowth.

Molecular genetics: The disorder is caused by mutations in two keratin genes, *K4* and *K13*, specifically expressed in mucosal tissues.[2]

Differential diagnosis: White sponge nevus may be confused with PC-1, Darier disease (MIM124200), dyskeratosis congenita, oral lichen planus, lupus erythematosus, candidiasis, and precancerous leukoplakia and oral florid papillomatosis.

Management: Antibiotics, including topical tetracyclines, have been shown to provide significant improvement. In most cases, no treatment is necessary.

OTHER KERATIN DISORDERS

The spectrum of keratin disorders also encompasses a number of extracutaneous diseases. Meesmann corneal dystrophy (MSD) is inherited in an autosomal-dominant fashion with incomplete penetrance. Slit lamp identification of typical fine, round cysts in the corneal epithelium aids in differentiating this disorder from other inherited corneal dystrophies.[33] Although vision is only rarely impaired to a serious degree in MSD, cyst rupture can cause corneal erosions and intermittent decrease in visual acuity during adulthood.[33] The disorder results from dominant mutations in *K3* and *K12* genes that are specifically expressed in the corneal epithelium.[2]

Recent studies have revealed that mutations in keratins expressed in simple epithelia may be involved in the pathogenesis of a number of gastrointestinal diseases.[20] Cryptogenic cirrhosis is a diagnosis of exclusion applicable to an individual with cirrhosis who does not carry a hepatitis B or C virus; who does not test positive for serological markers associated with autoimmune hepatitis or primary biliary cirrhosis; who has normal iron, ceruloplasmin, and α_1-antitrypsin levels; and who has no history of alcohol or toxin ingestion. Recurrent mutations in human *K8*/*K18* genes have been shown to predispose individuals to cryptogenic cirrhosis, chronic pancreatitis, and inflammatory bowel disease.[20,34,35] How K8/K18 mutations cause liver disease is still a matter of debate. Animals deficient in K8/K18 are highly susceptible to proapoptotic signals, suggesting that keratins may play a cytoprotective role in the gastrointestinal tract.[20]

CONCLUSION

The past recent years have witnessed tremendous progress in our understanding of keratinopathies, which in turn has led to dramatic advances in the management of this group of disorders. Prenatal diagnosis of EBS or EHK has become widely available, and novel gene therapy approaches for dominant—and not only recessive—keratin disorders are surfacing.[36] Beyond these immediate benefits, keratin disease research is radically transforming our traditional classification schemes of inherited skin disorders, establishing keratin disorders as a separate and legitimate clinical entity.[2] With the completion of the Human Genome Project and the ensuing identification of numerous novel keratin and keratin-associated genes,[20] one can logically anticipate seeing the spectrum of keratin disorders to further enfold and rechallenge our current conceptions.

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Lamivudine Resistance-Associated Gene Mutations

Harald H. Kessler
Evelyn Stelzl
Medical University Graz, Graz, Austria

Alexander C. Haushofer
General Hospital St. Pölten, St. Pölten, Austria

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health problem in many regions of the world. Antiviral treatment of chronic hepatitis B aims to reduce viral replication and/or to affect the immune response to the virus and virus-infected cells. Development of reverse transcriptase inhibitors such as lamivudine, which has been shown to be a safe and potent inhibitor of HBV replication, has facilitated major advances in the antiviral treatment of chronic hepatitis B.[1,2] Today, lamivudine is a first-line therapy for prophylaxis of HBV recurrence in decompensated cirrhotic patients and liver transplant recipients. In most patients, treatment with lamivudine results in a substantial reduction of the serum HBV DNA level and normalization of serum alanine aminotransferase.[2,3] Moreover, therapy with lamivudine has been shown to improve liver function and histology, to produce hepatitis B e antigen/antibody seroconversion in a substantial proportion of patients, and slow disease progression in patients with cirrhotic liver disease.[2,3] In combination with hepatitis B immunoglobulin, lamivudine has been demonstrated to be effective in preventing recurrence of infection after liver transplantation.[4] Lamivudine is also effective in patients infected with precore mutants of HBV.[5] Relapse following the cessation of lamivudine therapy, however, is common and seroreversion from hepatitis B e antibodies to hepatitis B e antigen may occur.[6]

LAMIVUDINE-ASSOCIATED MUTATIONS IN THE HBV POLYMERASE GENE

A major problem with lamivudine therapy is the emergence of drug resistance, which increases with extended duration of therapy. The selection of drug-resistant HBV mutants is associated with viral breakthrough.[2,3,7] In 57% of patients treated with lamivudine continuously for 3 years, lamivudine-resistant HBV mutants developed.[8] Resistant variants have been localized in the reverse transcriptase (rt) region of the HBV polymerase (P) gene. Lamivudine-resistant amino acids have been described at position rt204 (rtM204V, rtM204I, or rtM204S) in the YMDD motif that encodes the catalytic center of the enzyme.[9,10] Another lamivudine-related mutation has been localized at position rt180 (rtL180M) and linked to the rtM204V or rtM204I changes.[11,12] More recently, mutations at positions rt173 (rtV173L) and rt207 (rtV207I), which had earlier been described as associated with famciclovir treatment, have been found in patients with lamivudine monotherapy and significant increase of serum HBV DNA load.[13]

Steric blockade between mutant amino acid side chains and lamivudine is the basis for the lamivudine-resistance phenotype as shown by molecular modeling studies using an HBV polymerase homology model.[14] At position rt204, a steric conflict between the methyl group of isoleucine or valine and the sulfur atom in the oxathiolane ring of lamivudine accounts for the resistance observed on rtM204V/I mutations.[14]

Lamivudine-resistant mutants have impaired replication capacity compared with the wild type.[15] Nevertheless, the selection of lamivudine-resistant mutants is associated with reappearance or significant increase of serum HBV DNA and often leads to deterioration of liver function, which occasionally may be severe or even fatal.[3]

LAMIVUDINE-ASSOCIATED MUTATIONS IN THE HBV S GENE

Four open reading frames, the S, C, X, and P genes, have been identified within the HBV genome. Because of the overlap of the P and S genes, lamivudine-associated mutations produce changes in the S gene, too. Consequently, lamivudine-resistant HBV isolates show alterations in the ''a'' determinant of the HBsAg protein.[16] Lamivudine-selected HBsAg protein changes may have important virological and clinical implications: They may escape neutralization by vaccine-induced anti-HBs antibodies and thus may have the potential to become

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vaccine escape mutants. Widespread use of lamivudine may lead to infection of vaccinated individuals and potentially reduce the efficacy of current HBV vaccines.

LABORATORY DIAGNOSIS OF LAMIVUDINE-ASSOCIATED GENE MUTATIONS

Methods for the identification of mutations in the HBV P gene include DNA sequencing, restriction fragment length polymorphism analysis, and reverse hybridization. Conventional direct DNA sequencing has been the gold standard method. Both conventional direct DNA sequencing and restriction fragment length polymorphism analysis, however, are labor-intensive and time-consuming methods and thus not useful for the routine diagnostic laboratory. Presently, two assays based on the reverse hybridization principle on DNA sequencing are commercially available.

The INNO-LiPA HBV DR assay (Innogenetics, Ghent, Belgium) is based on the reverse hybridization principle. After extraction, HBV DNA is amplified with a two-round PCR. Both PCR products are visualized on an agarose gel. The second-round PCR product is used for hybridization to the LiPA strip. The probes on the INNO-LiPA HBV DR strip cover codon 180 (wild-type L and mutant M), codon 204 (wild-type M and mutants V and I), and codon 207 (wild-types V, L, and M and mutant I). This assay cannot detect any mutations other than those on the strip but it is highly sensitive and easily applicable.[17]

The Trugene™ HBV Genotyping Kit (Bayer HealthCare, Toronto, Ontario, Canada) is a standardized and largely automated sequencing assay. After HBV DNA extraction, a 1.2-kb sequence of the HBV P gene, representing the central portion of the rt domain, is amplified by PCR, and sequencing reactions are then performed on this amplification product with the CLIP™ sequencing (Bayer HealthCare) technology. CLIP sequencing allows both directions of the amplification products to be sequenced simultaneously in the same tube with use of two different dye-labeled primers for each of the four sequencing reactions. Electrophoresis and subsequent data analysis are performed automatically with the automated OpenGene™ and GeneObjects™ DNA sequence analysis system (Bayer HealthCare). Data are acquired with the GeneLibrarian module of GeneObjects software by combination of the forward and reverse sequences. The query sequence is compared with the consensus sequences of HBV genotypes A to G in the Trugene HBV Module of the OpenGene software to determine the HBV genotype of the sample. Mutations in the rt region of the HBV P gene as well as in the overlapping S gene are also automatically detected and reported. The detection limit of this assay is $\sim 2.0 \times 10^3$ HBV DNA copies/mL, and all viral variants present at concentrations $\geq 20\%$ of the total can be detected. This assay is able to provide HBV genotype and resistance information from the same data and is useful for the molecular diagnostic laboratory.[13]

THERAPY FOR LAMIVUDINE-RESISTANT HEPATITIS B

The benefit of long-term lamivudine therapy must be balanced against concern about any possible risk produced by lamivudine-associated mutations. Alternative treatment includes adefovir dipivoxil and tenofovir.

Adefovir dipivoxil has been shown to significantly reduce serum HBV DNA levels in patients with lamivudine-associated mutations and to produce improvement in liver histology and decrease of serum alanine aminotransferase (ALT) levels.[18] Molecular modeling studies have shown that adefovir dipivoxil can be accommodated more effectively in the more constrained and ''crowded'' deoxynucleoside triphosphate-binding pocket that carries the rtM204V/I mutations, which may explain the low level of resistance that has so far been observed.[19] Recently, adefovir dipivoxil resistance has been associated with a mutation at position 236 (rtN236T).[20,21] Development of adefovir dipivoxil resistance, however, seems to be uncommon within the first 12 months of treatment.

Tenofovir has shown excellent short-term results in management of chronic hepatitis B in HIV-positive patients.[22] No tenofovir-associated mutation in the HBV P gene has been observed so far.

CONCLUSION

For adequate clinical management of HBV infection, detailed molecular information on the type or virus is clearly important. The most accurate way to provide detailed molecular information is by rapid accumulation of full genomic sequence information. Recent developments in automated sequencing technologies permit reproducible and sensitive sequence analysis to be used in the routine diagnostic laboratory. For patients with lamivudine-resistant hepatitis B, alternative drugs have recently been introduced.

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Laser Capture Microdissection

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Falko Fend
Technical University Munich, Munich, Germany

INTRODUCTION

The molecular analysis of diagnostic tissue specimens has given significant insight into the pathogenesis of human disease and led to the identification of a broad range of diagnostic and prognostic markers, especially for neoplastic disorders. High-throughput approaches for the examination of DNA, mRNA, and proteins will have a profound impact on tissue-based diagnostics and will complement conventional histological examination. However, the reliability of many tests based on tissue or cell extracts critically depends on the abundance of the cell population in question. Primary tissues contain a variety of cellular elements including specialized, organotypic parenchyma and a large range of stromal and inflammatory cells. The relative percentages of these different cell types vary widely, especially in pathologically altered tissues. This inherent complexity of primary tissues can lead to false negative results in diagnostic molecular tests. Whereas the PCR detection threshold for some genetic alterations such as tumor-specific chromosomal translocations are in the range of 1 in 10^4 cells or less carrying the marker, other tests such as the detection of clonal immunoglobulin or T-cell receptor gene rearrangements with consensus primers or loss of heterozygosity at tumor suppressor gene loci require higher percentages of target cells. In addition, the examination of early or precursor lesions such as carcinoma in situ and epithelial dysplasias, which can shed light on the first seminal steps of carcinogenesis, is virtually impossible from bulk tissue extracts.

These problems are magnified at the mRNA and protein level, as, frequently, the relative abundance of the target, rather than its presence or absence, is of diagnostic relevance. In situ studies such as immunohistochemistry and in situ hybridization provide valuable alternatives, but are limited in their application range. For these reasons, microdissection strategies, ranging from relatively crude, manual microdissection to micromanipulation of single cells, have been used increasingly for the isolation of pure cell populations from primary tissues.

PRINCIPLES OF LASER CAPTURE MICRODISSECTION

Recently, a variety of laser-assisted microdissection devices have been developed and are available on the market. Two different technical principles can be discerned. One of them, initially termed laser microbeam microdissection (LMM), uses a pulsed UV laser with a small beam focus to cut out areas or cells of interest by photoablation of adjacent tissue.[1] The second technique is laser capture microdissection (LCM), which is described in more detail below.[2–4]

Laser capture microdissection is based on the selective adherence of visually targeted cells and tissue fragments to a thermoplastic membrane activated by a low-energy infrared laser pulse (Fig. 1). The basic system (marketed by Arcturus, Mountain View, CA) consists of an inverted microscope, a solid-state near-infrared laser diode, a joystick-controlled microscope stage, and hardware and software for laser control and image archiving. A new system for automated microdissection with expanded technical capabilities has recently been introduced by the same company. The thermoplastic membrane used for the transfer of selected cells is mounted on an optically clear cap which fits on standard 0.5-mL microcentrifuge tubes for further processing.

After visual selection of the targeted cells, laser activation leads to focal melting of the ethylene vinyl acetate (EVA) membrane, which has its absorption maximum near the wavelength of the laser. The melted polymer expands into the section, resolidifies within milliseconds, and forms a composite with the tissue. This allows selective removal of the cells attached to the activated membrane. Laser ''shots'' can be repeated multiple times across the whole cap surface to collect large numbers of cells.[2] Depending on the chosen laser spot size, the architectural features of the tissue, and the desired precision of the microdissection, even thousands of cells can be collected within a few minutes. After dissection, the cap is transferred to a tube containing the buffer solutions required for isolation of the molecules of interest. As most of the energy is absorbed by the

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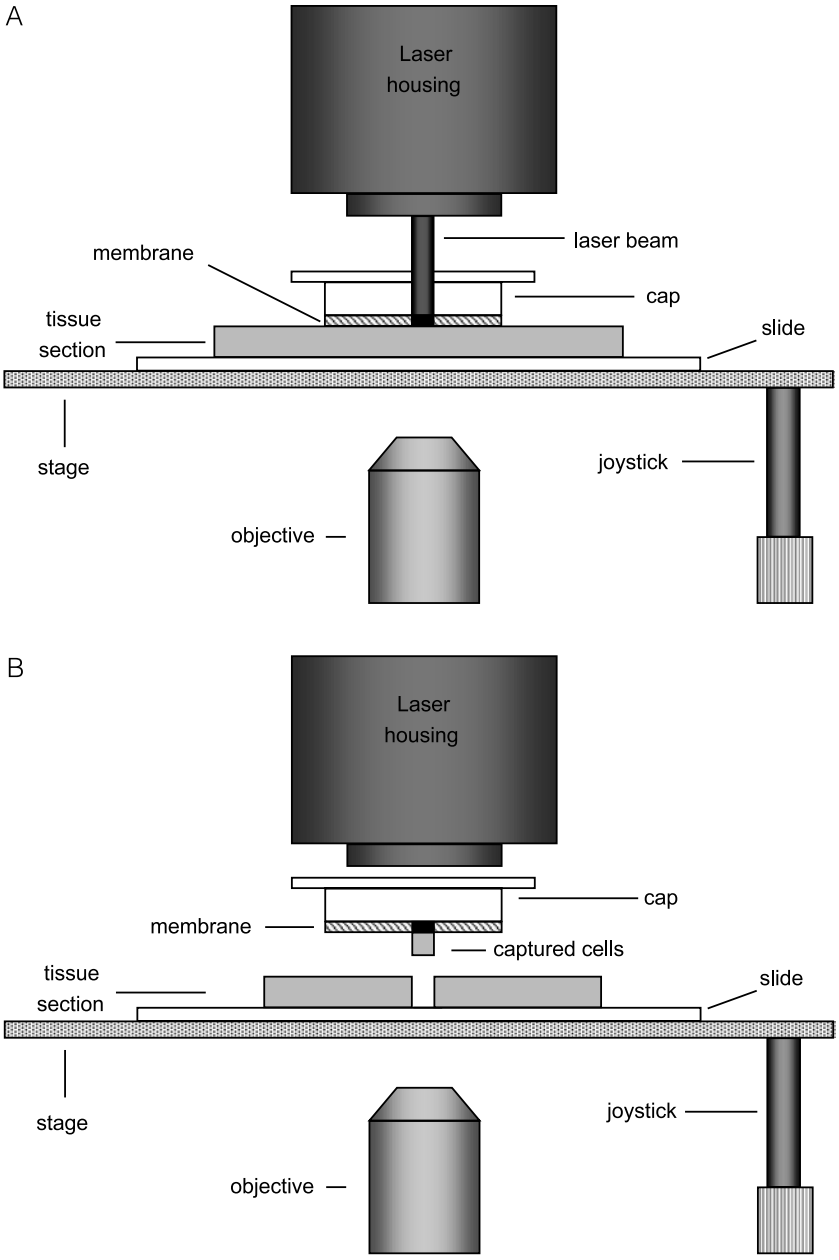

Fig. 1 Schematic representation of laser capture microdissection. A) Activation of the laser leads to focal melting of the thermoplastic membrane attached to the lower portion of the optically transparent cap. B) Lifting of the cap leads top selective detachment of cells adherent to the molten (activated) parts of the membrane. (From Ref. [3].) (*View this art in color at www.dekker.com.*)

membrane, the maximum temperature reached by the tissue upon laser activation is in the range of 90°C for several milliseconds, thus leaving intact biological macromolecules of interest. The precision of LCM mainly depends on the ability to visually identify the desired cell population on the noncoverslipped slide, and to a lesser degree on the size and configuration of target cells. The current smallest spot size achievable is approximately 7.5 μm. Special caps hovering slightly above the section surface rather than being in direct physical contact with it have been designed for single-cell applications.

APPLICATIONS OF LCM

Laser capture microdissection can be applied to a wide range of different cell and tissue preparations, including

frozen sections, sections from fixed, paraffin-embedded archival material, and certain cytological preparations.[3,4] The most important parameters to consider for LCM are the type and fixation of the tissue to be examined, the morphologic features suitable for target identification, the desired target biomolecule—DNA, mRNA, or protein—and the type of downstream analysis.

DNA is relatively resistant to fixation and staining procedures and provides an ideal target for PCR-based analysis. Detection of point mutations or loss of heterozygosity (LOH), or identification of lymphocyte clonality in lymphomas can be greatly facilitated by collecting highly enriched tumor cell populations with LCM, thus increasing the diagnostic yield and obviating the need for more labor-intensive analytical strategies. Microdissection is crucial for the molecular analysis of discrete functional anatomic units such as glomeruli, or small lesions such as preneoplastic changes or areas of tumor heterogeneity which are not amenable to conventional examination of bulk tissue extracts. The investigation of precursor lesions allows the correlation genetic alterations with the morphological stages of tumor development, thus testing models of the stepwise acquisition of progressive genetic changes in vivo. In combination with techniques for the random amplification of the whole genome, comparative genomic hybridization (CGH), whole genome allelotyping, or other applications can be performed on a small number or even single cells.[4] However, the smaller the amount of template available, the higher is the risk for artefacts such as allelic dropout, biased amplification of certain sequences, or introduction of Taq polymerase errors. Critical evaluation of results and introduction of multiple controls, such as repeat analyses and continuous parallel investigation of adjacent normal tissue, are mandatory.

For most purposes, detachment of captured cells from the membrane by proteinase digestion without further purification steps renders DNA template of sufficient quality. Although frozen tissues usually show superior DNA quality, paraffin-embedded archival tissues can be used for most DNA-based analyses.[3]

Gene Expression Analysis

The analysis of tissue- or cell-specific gene expression is of paramount importance for many fields of biological and biomedical research. However, tissue heterogeneity can make it very difficult to assign gene expression profiles or specific messages to defined cell populations if gross tissue extracts serve as a source for mRNA, and significant efforts have been put into developing microdissection strategies which allow cell- or tissue-specific gene expression analysis. In contrast to DNA, mRNA is more sensitive to fixation, is quickly degraded by ubiquitous RNases, and requires stringent RNase-free conditions during specimen handling. Keeping these requirements in mind, mRNA of good quality can be recovered from microdissected frozen tissue samples down to the single-cell level, suitable for RT-PCR or quantitative real-time RT-PCR.[5] Rapid, optimized immunohistochemical or immunofluorescence staining protocols allow the combination of phenotypical identification of target cells with mRNA analysis.[6,7] The superior speed of LCM and the fact that capturing is performed from dried slides allow sampling of large numbers of cells without significant RNA degradation during the procurement procedure. If very small numbers of cells serve as template or if large-scale expression profiling using cDNA or oligonucleotide arrays is used, random mRNA amplification protocols can be employed.[8] In most studies, T7 RNA polymerase-based strategies have been the preferred technique in order to achieve an unbiased mRNA amplification from small amounts of tissue. Array-based expression screening of LCM-captured tissues has been used successfully for delineating expression differences of microdissected small and large neurons from dorsal root ganglia[9] or expression changes during breast cancer progression,[10] to name only a few examples.

Another application of LCM is the generation of expression libraries from purified cell populations, which has been performed at a large scale for the cancer genome anatomy project (CGAP; http://www.ncbi.nlm.nih.gov/ncicgap) sponsored by the National Cancer Institute.[11] This approach is intended to give a more faithful representation of gene expression patterns in epithelial neoplasms at all stages of development, in contrast to established cell lines which are usually derived from terminal-stage cancer patients, and in addition may show the artefacts of prolonged culture and the lack of an accompanying tissue microenvironment.

Although frozen tissue is the preferred source for mRNA analysis, the vast amounts of available archival specimens make paraffin-embedded tissues a valuable resource, and several groups have demonstrated that RNA derived from formalin-fixed paraffin blocks is suitable for certain types of analysis, such as conventional and real-time RT-PCR. A few hundred cells from paraffin sections render enough RNA for several RT-PCR assays without the need for preamplification steps, provided that the amplified fragment is below 100–120 bp.[12] Using the TaqMan technology with fluorescence-labeled probes, highly reproducible expression values can be obtained from microdissected archival material over a broad range of starting material and template concentrations.[13] The precision achievable by combining target cell purification by LCM with quantitative

RT-PCR and the ease with which these two techniques can be applied to paraffin sections make this approach a valuable tool for advanced molecular diagnostics.

Protein Analysis

Proteins are the effector molecules of the cell and can be modified through a variety of posttranscriptional mechanisms. Therefore a cell-specific analysis of protein patterns may ultimately prove to be more informative than expression profiling in primary tissues. For these reasons, a variety of proteomic techniques have recently been applied to microdissected tissues, although the inherent limitation of starting material can present a major obstacle for protein analyses.[14–17] Nevertheless, standard techniques such as the two-dimensional polyacrylamide electrophoresis for the detection of differential protein expression have been tested successfully on microdissected samples, and protocols for optimal fixation and protein recovery have been published.[18,19] Microdissection may also prove valuable to reduce sample complexity for mass spectrometric techniques such as surface-enhanced laser desorption/ionization time-of-flight (SELDI-ToF),[14] which need significantly less starting material than most gel-based techniques. Novel approaches such as the reverse-phase protein microarray[20] may prove useful to study multiple proteins, such as whole signal transduction pathways, in microdissected samples.

CONCLUSION

Laser capture microdissection and related laser-assisted microdissection techniques have developed into a key technology for the in-depth molecular analysis of primary tissues for both diagnostic and research purposes. The ease of use and versatility of LCM help to integrate microdissection into the workflow of any molecular pathology laboratory without much additional effort. Combined with downstream high-throughput techniques, microdissection is a big step toward ''molecular morphology.''

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Legionella spp.

Nele Wellinghausen
University of Ulm, Ulm, Germany

Michael Steinert
Universität Würzburg, Würzburg, Germany

INTRODUCTION

Legionella species are aquatic microbes and pathogens of protozoa. Only a few of the more than 40 species cause infections in humans. The disease spectrum ranges from mild flulike symptoms to a highly lethal, severe form of pneumonia, called Legionnaires' disease. The pathogenic potential of *Legionella* was first recognized during an outbreak at the 56th annual American Legion convention in Philadelphia, PA, USA, in 1976, which led to the description of the pathogen. Today, legionellae are considered as one of the most important causative agents of community- and nosocomial-acquired pneumonia. Intensive research in the areas of molecular biology and genomics has greatly improved our understanding of the environmental fitness and pathogenicity of *Legionella* spp. Recent advances in molecular diagnostics offer a fast and specific diagnosis of the disease caused by *Legionella* species.

TAXONOMY AND DESCRIPTION OF THE PATHOGEN

Legionellae belong to the Gammaproteobacteria and are non-spore-forming gram-negative rods. To date, 42 species and 65 serogroups of *Legionella* have been described. *Legionella pneumophila* with its 15 serogroups is the main pathogen and accounts for more than 90% of infections, with serogroup 1 being the most common one. Several other legionellae, such as *Legionella micdadei*, *Legionella bozemanii*, and *Legionella dumoffii*, also cause disease.[1]

Legionellae are intracellularly replicating bacteria. They live primarily in environmental aquatic habitats and replicate within protozoa, such as amoeba and flagellates. Upon inhalation by humans of aerosolized water from contaminated tap waters, air-conditioning systems, or cooling towers, *Legionella* replicates in monocytes, alveolar macrophages, and epithelial cells.[1] In vitro, legionellae show fastidious growth characteristics.

Highest concentrations of legionellae are found in man-made hot-water systems because legionellae grow best at temperatures between 37°C and 50°C and can even tolerate temperatures above 60°C.[2]

INTRACELLULAR LIFE CYCLE

The broad protozoal host spectrum of *Legionella* and the exploitation of basic cellular mechanisms of eukaryotes enables *Legionella* to replicate within human cells. Striking similarities in both host systems include the uptake by phagocytosis, the formation of a *Legionella*-specific vacuole that does not follow the endosomal pathway, the association of this vacuole with ribosome-studded membranes that originate from the endoplasmic reticulum, and finally, host cell lysis. In this life cycle, *Legionella* switches from a nonreplicating, flagellated, stress-resistant, cytotoxic phase (transmission form) to a replicating, nonflagellated phase (growth form). The conversion is triggered by a stringent response-like mechanism.[2]

In recent years, much progress has been made toward the characterization of how *Legionella* infects host cells. Because the reprogramming of the endocytic pathway occurs between the initial attachment and the replicative phase of the bacteria, it is highly likely that cell surface components and secreted factors are involved in this process. Various surface structures including the major outer membrane protein (MOMP), which mediates the uptake of *Legionella* via the CR1 and CR3 receptors of macrophages, the type IV pilus biogenesis, and the macrophage infectivity potentiator (Mip) protein have been characterized. Moreover, the Dot/Icm secretion system plays a pivotal role during infection. Yet, unidentified factors that are injected into host cells by this type IV secretion system obviously prevent the *Legionella*-containing vacuole from endosomal maturation. Recently, macrophage-specific virulence genes such as *mil* (macrophage-specific infectivity loci) and protozoa specific virulence genes such as legionella infectivity gene (*ligA*), type II secretion system (*lsp*), alternative sigma factors (*rpoS*) have been identified. This suggests that besides many similarities, notable differences in the mechanisms of entering and exiting the host cell exist.[3]

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Comparison of the nucleotide sequence of different *Legionella* species revealed 23% nucleotide sequence variation within the 16S rRNA gene and 56% variation for the *mip* gene. However, knowledge of genetic diversity between different isolates of the same or of different *Legionella* species is still very limited. The current *Legionella* genome project (http://genome3.cpmc.columbia.edu/~legion/) may soon reveal the relation between the presence of specific genes and the prevalence of certain species in epidemiological investigations.[4]

In order to study the host side of infection, guinea pigs, primary cell culture systems, and cell lines derived from human leukemias such as U937, HL60, HeLa, Vero, and McCoy cells have been used. In addition, the infection of axenically grown protozoa such as *Acanthamoeba*, *Hartmannella*, and *Naegleria* have increased our knowledge about the ecology of *Legionella*.[5] Especially, the haploid soil amoeba *Dictyostelium discoideum* has proven to be a suitable model system for studying cellular mechanisms of pathogenesis. This surrogate host system is amenable to genetic manipulation and evolutionarily expresses highly conserved cellular markers. In the future, the combination of full-scale genomic sequencing and high-throughput expression analysis with *Legionella* and *Dictyostelium* should reveal important aspects of the pathogen–host cross-talk.[6,7]

CLINICAL SIGNIFICANCE OF *LEGIONELLA* SPP.

Legionella causes two different manifestations of pulmonary disease: a mild, flulike illness, called Pontiac fever, and pneumonia. Pontiac fever has an incubation period of 1–2 days and is characterized by malaise, myalgias, fever, headache, and, sometimes, nonproductive cough. Only symptomatic therapy is required, and complete recovery within 1 week can be expected. Pneumonia, on the other hand, is the predominant manifestation of the more severe form of legionellosis, Legionnaires' disease. This pneumonia can be associated with multiorgan failure. The incubation period of legionnaires' disease ranges from 2 to 10 days, and antimicrobial chemotherapy is necessary for complete recovery of the patients. Nevertheless, in elderly or pulmonary-compromised patients, Legionnaires' disease has a mortality rate of up to 50%.[5,8]

Infection of humans mainly occurs through close contact with contaminated water sources, such as droplets and aerosols in spas, hot showers, and air-conditioning systems. Legionellae are responsible for 2–15% of community-acquired pneumonias, but the estimated number of infections is much higher. In addition, legionellae are a significant cause of nosocomial pneumonias, especially in immunocompromised and ICU patients.[5,8] Nosocomial infections caused by legionellae are more often caused by *L. pneumophila* nonserogroup 1 and by legionellae other than *L. pneumophila*,[9] corresponding to the epidemiology of the different *Legionella* species in water sources.

DETECTION OF *LEGIONELLA* SPP. IN CLINICAL AND ENVIRONMENTAL SAMPLES

Culture is still regarded as the gold standard for diagnosis of *Legionella* infection, but it is hampered by the slow replication rate and the fastidious growth requirements of legionellae. *Legionella* urinary antigen detection assays have greatly improved the diagnosis of acute *Legionella* infection. However, they only detect *L. pneumophila* serogroup 1 with high sensitivity and specificity.[10]

More recently, nucleic acid amplification techniques, such as the polymerase chain reaction (PCR), have shown promise for the rapid diagnosis of *Legionella* infection.[11] Nucleic acid amplification techniques enable specific amplification of minute amounts of *Legionella* DNA, provide results within a short time frame, and have the potential to detect infections caused by any *Legionella* species and serogroup. New real-time PCR methods, such as the LightCycler® PCR technique, can even speed up the process of detection to a few hours.

Molecular Detection of *Legionella* in Water Samples

Detection of legionellae in environmental and building water samples by PCR has been centered on hospital and cooling tower water samples. Target genes included the 16S and 5S rRNA gene of *Legionella* spp. and the *mip* gene of *L. pneumophila*.[12–15] Compared to conventional culture, the published PCR protocols showed a sensitivity of 71.8% to 98.1% and 54.5% to 82.1% for the detection of legionellae and *L. pneumophila*, respectively. However, the main limitation of all PCR assays is the high prevalence of PCR inhibitors in water samples. The presence of waterborne PCR inhibitors that will be enriched during concentration of the water samples also limits the sample size and thus the sensitivity of the PCR assays. Nevertheless, legionellae were detected in 60% to 100% of culture-negative water samples in several studies.[12,14,15] Because of the labor- and time-intensive nature of *Legionella* culture and the presence of viable and infectious but nonculturable Legionellae and *Legionella*-like organisms in water samples, PCR methods are a valuable tool for the detection of legionellae in water samples. However, further studies are necessary to clarify the significance of PCR-positive but culture-negative

Table 1 Nucleic acid amplification tests for detection of legionellae in respiratory samples [bronchoalveolar lavage (BAL), sputum, bronchial washing]

Ref.	Target gene	Specificity	Type of test	Analytical sensitivity	PCR-positive per culture-positive samples	PCR-positive per culture-negative samples
Jaulhac et al.[18]	*mip*	*L. pneumophila*	PCR/Southern Blot	25 CFU/mL 50 fg DNA/PCR	8/8	7/60
Matsiota-Bernard et al.[20]	5S rRNA *mip*	*Legionella* spp. *L. pneumophila*	EnviroAmp *Legionella* PCR kit (PCR/dot blot)	100 CFU/PCR	11/12	0/17
Jonas et al.[19]	16S rRNA	*Legionella* spp.	PCR/ELISA	100 CFU/mL 200 fg DNA/PCR	8/8	6/250
Bernander et al.[23]	*mip*	*L. pneumophila*	Nested PCR	1–5 CFU/PCR	13/14	0/33
Cloud et al.[16]	16S rRNA	*Legionella* spp.	PCR	1–10 fg DNA/PCR	31/31	12/181
Hayden et al.[17]	*mip*	*L. pneumophila*	LightCycler PCR	<10 CFU/mL	7/7	0/10
	5S rRNA	*Legionella* spp.		<10 CFU/mL	9/9	0/10
Raggam et al.[21]	16S rRNA	*Legionella* spp.x	LightCycler PCR	5 CFU/PCR	3/3	2/58
Reischl et al.[22]	16S rRNA	*Legionella* spp. and *L. pneumophila*	LightCycler PCR	10 fg DNA/PCR	26/26	0/42

The table exclusively shows studies that investigate a minimum of 10 patients.

samples and to establish limits for PCR results to be used in infection control and surveillance practice.

Molecular Detection of *Legionella* in Clinical Samples

Target sequences of the hitherto developed *Legionella*-specific PCR assays were the 16S rRNA gene and the 5S rRNA gene of *Legionella* spp. and the *mip* gene of *L. pneumophila*. Due to the nature of infection, materials from the deep respiratory tract, such as bronchial alveolar lavage fluid and sputum, are the samples of choice for investigation and have been studied extensively during the last decade (Table 1). In the studies, the PCR assays consistently detected all culture-positive samples. Real-time PCR assays even allowed a diagnosis of *Legionella* infection within a few hours. Notably, in most studies PCR-positive samples that were negative by culture were also observed, ranging from 3% to 12% of the samples investigated.[16–23] Because most of these samples were obtained from patients with a serologically confirmed *Legionella* infection or from patients with otherwise undefined pneumonia, a higher sensitivity of the PCR assays compared to culture can be assumed. Although all studies suffer from a small sample size, PCR may be considered the method of choice for detection of acute legionellosis, especially in patients with a higher risk of infections caused by legionellae other than *L. pneumophila* serogroup 1 in nosocomial settings or in certain geographical regions.

However, less than half of the patients with legionellosis can produce sputum, and, thus, PCR testing of other materials has been evaluated in several studies. In accordance with antigen detection assays, legionellae can be detected in urine of affected patients; however, the sensitivity was much lower than in respiratory samples and adds little to that of antigen detection assays.[11] In addition to urine, serum was evaluated in a few studies. Whereas Lindsay et al.[25] recorded positive results by PCR and probing in five out of five patients with legionellosis, others found sensitivities for serum PCR assays of only 30% to 43% in studies comprising up to 28 patients.[11] Clearly, further evaluation of nonrespiratory samples and sample types is necessary to determine their role in the diagnosis of legionellosis.

DISINFECTION AND TREATMENT

Person-to-person transmission of legionellosis has not been published. Therefore, prevention of *Legionella* infections refers to the elimination of the pathogen from water supplies. Several methods for controlling the growth of legionellae in drinking water (superheating, ultraviolet light irradiation, ozonation, metal ionization, chlorination) have been described.[24]

With respect to human infection, prompt diagnosis and treatment appear to be critical, because delay in starting an appropriate therapy has been associated with increased mortality. Legionellae are intracellular pathogens, and thus antibiotics that achieve high intracellular concentration are more efficacious than those with poor intracellular penetration. Historically, erythromycin has been the drug of choice. Macrolides, quinolones, rifampicin, trimethorpim–sulfamethoxazole, and tetracyclines are effective agents in vitro. According to in vitro studies, the newer macrolides, especially clarithromycin and azithromycin, and quinolones are the drugs of choice for *Legionella* infections.[5,8]

CONCLUSION

Because the coevolution of the legionellae–protozoa interaction can be viewed as a ''living fossil'' of the adaptation of bacteria to professional phagocytes this pathogen has become a prominent model system in this respect. Main characteristics of the intracellular lifestyle and the regulation and function of replication- and pathogenicity-associated genes have already been elucidated. In addition, detection of legionellae by molecular methods has taken a significant role in clinical as well as in environmental diagnostics. The characterization of the genetic diversity of different *Legionella* species will provide information about the core genome of *Legionella* and genes present only in certain isolates. This may allow the identification of marker genes that can be used as tools for detection and risk assessment. In a long perspective, the molecular approach may also help to understand the infection cycle and the life cycle in the environment, which is the prerequisite for successful elimination of *Legionella* from high-risk areas.

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Ligase Chain Reaction (LCR) and Ligase Detection Reaction (LDR)

Jürgen Fuchs
Maurizio Podda
J.W. Goethe University, Frankfurt, Germany

INTRODUCTION

The polymerase chain reaction (PCR) has revolutionized molecular biology and has become an indispensable tool in molecular diagnostics. The ligase chain reaction (LCR) and the ligase detection reaction (LDR) were developed as alternative nucleic acid amplification methods for analyzing known point mutations associated with genetic diseases. In contrast to target amplification techniques such as PCR, SDA, 3 SR, and NASBA, LCR and LDR are not based on primer extension, but on amplification of ligated probes.[1,2] Excellent reviews on LCR and its diagnostic applications have been published.[3–7]

HISTORIC DEVELOPMENTS

DNA ligase was discovered in the late 1960s and the enzyme is an essential component of DNA replication, recombination, and repair systems, which catalyzes the formation of covalent phosphodiester bonds at single-stranded breaks on dsDNA. In 1972, ligation of flanking oligonucleotides while hybridized to a template was investigated,[8] and soon it became apparent that DNA ligase could be used as an analytical tool for detecting the presence of two adjacent pieces of oligonucleotides hybridized to a complementary target DNA strand.[3,9] After synthetic oligonucleotides became readily available, the practical use of this concept was further exploited and Landegren et al. developed the oligonucleotide ligation assay (OLA).[5] Wu and Wallace described a technique called ligase amplification reaction (LAR), which uses two sets of complementary oligonucleotide probes and repeated cycles of heat denaturation, hybridization, and ligation using a mesophilic DNA ligase, leading to exponential amplification.[10] Barany reported a technique called ligase detection reaction (LDR) and ligase chain reaction (LCR) that uses a thermostable ligase which dramatically improved the sensitivity of the reaction and exquisitely discriminated between single-base mismatches at the junction of the two oligonucleotide probes.[3]

LIGASE CHAIN REACTION AND LIGASE DETECTION REACTION

Ligase chain reaction utilizes two complementary oligonucleotide probe pairs, which are specific for a DNA target sequence. In the presence of the target sequence, each probe of a pair hybridizes to adjacent positions on the template such that the 5′-phosphate of one oligonucleotide abuts the 3′-hydroxyl of the other. The resulting nick is subsequently sealed by a DNA ligase. By temperature cycling, bound ligated units dissociate from the target and the released ligated unit becomes the target for the next round of ligation which is initiated by aligning the opposite sense adjacent probes. After repeated cycles of denaturation, annealing, and ligation the target sequence is amplified exponentially (Fig. 1). Ligase detection reaction employs only one set of oligonucleotide probes in a thermal cycling reaction similar to the one described above, resulting in linear product increase. Attaching biotin on the first probe and a suitable reporter group on the second probe allows for product capture and detection in a manner amenable to automation.[3,4] Ligase chain reaction and ligase detection reaction are very sensitive assays for discriminating DNA sequences that differ by only one single base pair. A mismatch in the ligation junction inhibits the ligation reaction thus conferring an excellent resolution power to hybridization, decreasing the need for high stringency conditions and thus providing excellent conditions for multiplexing reactions.[3,5] A great potential of LCR is its compatibility with other nucleic acid amplification techniques such as PCR, 3 SR, and the Qβ replicase assay. Real-time applications,[11,12] multiplexing assays,[13,14] competitive LCR and multiplex-competitive LCR,[15] quantitative LCR,[16] and nested LCR[4] have been described. The sensitivity of LCR is dependent on the fidelity of the ligase. Four thermostable ligases are commercially available: 1) Taq ligase (New England Biolabs, Beverly, MA); 2) Tth (Abbott Diagnostics, Abbott Park, IL); 3) Pfu (Stratagene, La Jolla, CA); and 4) Ampligase (Epicenter Technologies, Madison, WI). Pfu was advertised to have higher ligation specificity and lower background than Tth.[7] Although

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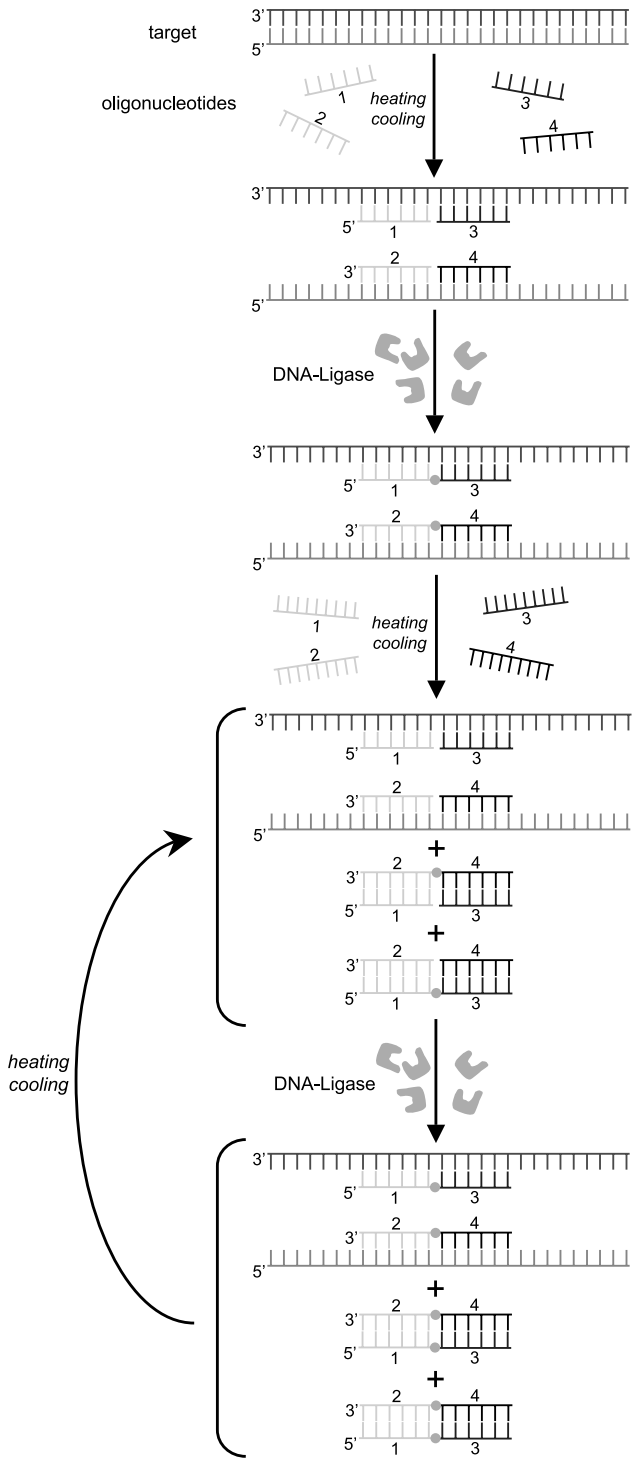

Fig. 1 Ligase chain reaction. The probes are annealed to template molecules in a head-to-tail fashion with the 3′ end of one probe abutting the 5′ end of the second probe. DNA ligase then joins the adjacent 3′ and 5′ ends to form a copy of one strand of the target. The copy is subsequently separated from the target by heat denaturation. Released ssDNA forms new templates for further probe binding. A second set of probes, complementary to the first, uses the duplicated strand, as well as the original target, as a template for ligation. (*View this art in color at www.dekker.com.*)

LCR was reported to be less affected by inhibitors in some specimens than PCR, LCR is subject to contamination that is at least as much of a problem as for PCR.[7] In particular, the inhibitory effect of phosphate on LCR can pose a problem,[17] and duplicate dilution analysis of negative specimens was recommended.[18]

PCR/LDR AND Gap-LCR

A major problem of the LCR reaction is joining of blunt end duplexes to each other and to themselves, all of which can serve as templates for further amplification, although no real target DNA is present in the original sample[7] and this poses significant limitations on the sensitivity of LCR.[6] To overcome this problem, several modifications have been developed, including a combined PCR/LDR method and Gap-LCR (G-LCR). In PCR/LDR, the amplification step for LDR is PCR utilizing outside primers and employing only one pair of adjacent probes to detect the proper sequence in the PCR produced. This procedure does not produce double-stranded oligonucleotides to blunt end ligate.[4] A recent study showed that PCR/LDR was more accurate than FISH in the quantification of HER2/neu gene copy numbers.[19] Gap-LCR is a version of LCR where the probes are not adjacent but are separated by one to three bases on both the sense and antisense strand. A nonstrand displacing thermostable DNA polymerase, supplied with the necessary dNTPs, adds the missing bases to the 3′ internal end of one of the two probes, thus initiating a template-dependent extension. Once the gap between the two probes is filled, the probes are once again adjacent and can be ligated. The same process occurs on the opposite strand (Fig. 2). By this modification, G-LCR prevents target-independent ligation very efficiently[20,21] and has the advantage of having the additive effect of polymerase and ligase on specificity.[22] Excellent discrimination of mutations was achieved using multiplex G-LCR,[13,14] and a real-time G-LCR for rapid detection of p53 mutations in clinical specimens was recently described.[23] Gap-LCR assays have been developed for the detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Mycobacterium tuberculosis* (Abbott, IL, USA). Several studies have evaluated the usefulness of commercially available nucleic acid amplification assays, comparing G-LCR with PCR and SDA for detection of *C. trachomatis* and *N. gonorrhoeae*. In these comparative studies G-LCR proved to be a robust, specific, and sensitive technique.[24–29]

AMPLIFICATION OF RNA TARGETS

The natural target of DNA ligase is dsDNA. DNA ligase has been shown to join DNA oligonucleotides hybridized

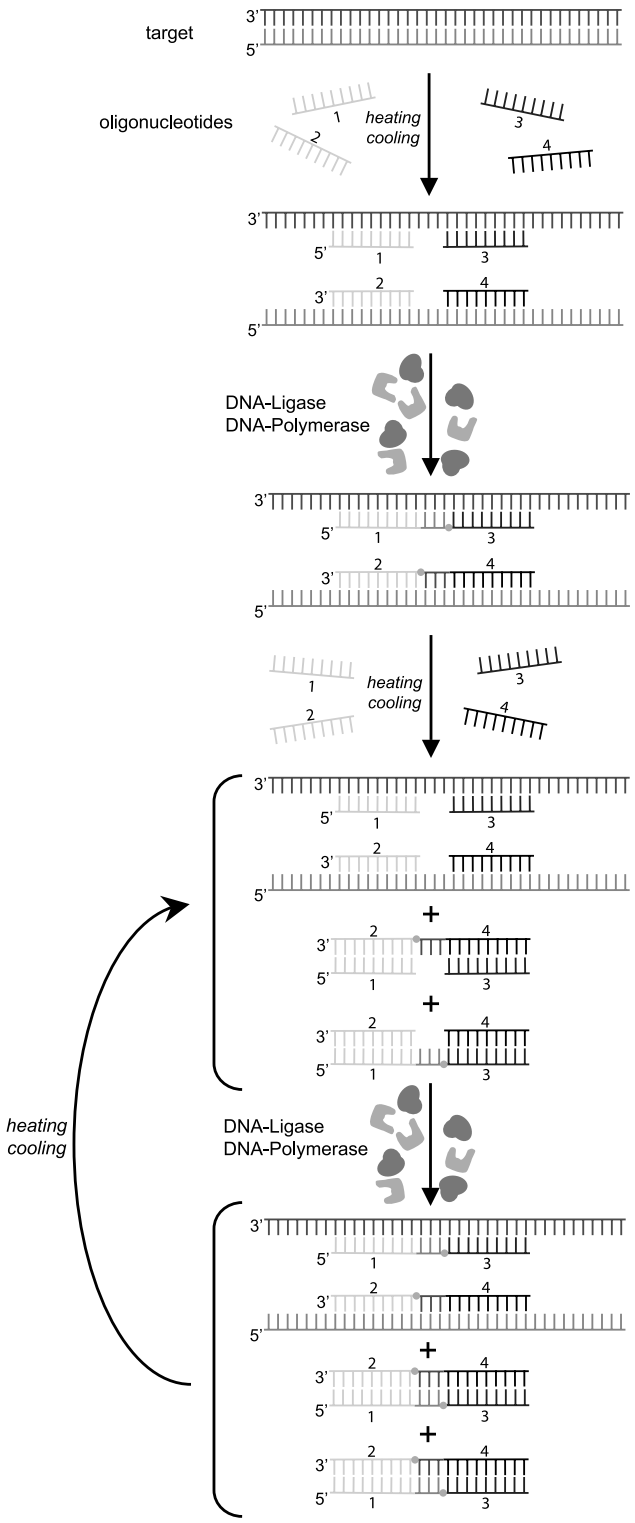

Fig. 2 Gap-ligase chain reaction. Gap-LCR differs from LCR in that a short gap is formed after annealing of the four oligonucleotide probes to the template. The gap is filled in by a non-strand-displacing thermostable DNA polymerase and the resulting nick is ligated. (*View this art in color at www.dekker.com.*)

to RNA templates, although with a significantly lower efficiency than DNA-templated reactions.[30] Consequently, LCR and G-LCR cannot amplify RNA efficiently. To overcome this problem, an asymmetric G-LCR was developed that allows for the detection of RNA via a cDNA step.[21] Another approach allows direct analysis of RNA sequences without a preceding cDNA synthesis by defining specific reaction conditions to increase the efficiency of the enzyme on a DNA/RNA hybrid.[31,32]

CONCLUSION

In the mid-1990s commercial G-LCR kits were developed for the detection of *C. trachomatis*, *N. gonorrhoeae*, and *M. tuberculosis* (Abbott), and these assays played a significant role in clinical STD (sexually transmitted disease) diagnostics. Due to modernized regulations in quality assurance, modifications performed in the manufacturing process and component changes made these assays not as robust as they used to be, resulting in high failure rates in internal testing since 2001 (personal communication, Judy H. Yu, Abbott, IL). In 2003, Abbott discontinued the distribution of its LCR-based diagnostic products and is now using PCR-based technology for its commercial diagnostic kits (LCx HCV and LCx HIV). Currently, there are no commercial LCR-based nucleic acid amplification tests on the market, and in molecular STD diagnostics LCR has lost ground against PCR. Nonetheless, DNA ligation assays continue to play an important role in molecular diagnostics, as is exemplified by the development of ligase-detection PCR (LD-PCR)[31] and the use of LDR in combination with DNA array technology.[33] DNA ligase was used in combination with PCR in a probe amplification assay named LD-PCR to increase the accuracy and sensitivity of target nucleic acid determination.[31] This method utilizes a capture probe for target isolation, two hemiprobes for target detection, and a PCR step to amplify the ligated full probe. Ligase-detection PCR was more sensitive than conventional RT-PCR for the detection of HCV DNA in formalin-fixed paraffin-embedded liver specimens.[34] Ligation assays are useful methods for mutation analysis on microarray platforms and are gaining wide attention due to their excellent discriminatory power of allele variants.[35] Ligase detection reaction was successfully used on microarrays for discriminating between different bacterial groups,[36] for rapid and sensitive identification of drug-resistant *M. tuberculosis* strains,[37] and, in combination with PCR, for detecting and genotyping human papillomavirus[38] or for characterizing p53 mutations in a multiplex PCR/LDR assay.[39–41]

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LightUp® Probes

Mikael Leijon
LightUp Technologies AB, Huddinge, Sweden

INTRODUCTION

Real-time monitoring of the polymerase chain reaction (PCR) product by fluorescent probes has recently enabled the homogeneous detection of pathogen deoxyribonucleic acid (DNA) or ribonucleic acid (RNA0) in a closed-tube format. The reduced risk of carry-over contamination and the superior sensitivity of PCR-based assays have made real-time PCR the method of choice in many clinical settings. The LightUp® Probe is a novel of fluorescent probe where the fluorescent reporter dye is coupled to an oligomer of the uncharged DNA analog peptide nucleic acid (PNA). Peptide nucleic acid provides strong, but still specific, binding and serves as the sequence-recognizing element of the probe. The fluorophore is an asymmetric cyanine dye that fluoresces when the probe binds its target DNA, but is virtually nonfluorescent when the probe is free in solution.

RESULTS

The extraordinary sensitivity of PCR-based assays is an important factor behind the recent tour de force of real-time PCR in molecular biology in general and viral diagnostics in particular.[1,2] Besides the inherent sensitivity of the PCR technique, the real-time fluorescence detection used in modern PCR instrumentation has the added advantage of providing accurate quantification of the initial amount of the amplified nucleic acid. This is achieved by comparing C_t values, i.e., the PCR cycle where the fluorescence signal reaches a certain level, for unknown clinical samples with the C_t values of a dilution series of a reference material with known concentration. This is generally carried out via a standard curve:

$$C_t = \frac{\log(c)}{\log(1+E)^{-1}} + C_t(1)$$

where the parameter E is the PCR efficiency, ideally equal to unity, and $C_t(1)$ is the threshold cycle value where one copy of the standard would appear. Assuming that the standard curve parameters also apply to the clinical samples, the concentration of the clinical samples can be determined from the measured C_t values.

DNA-based sequence recognizing fluorescent probes commonly utilize a fluorophor/quencher pair that is, in some manner, separated in the course of the amplification reaction. TaqMan® probes, for example, are degraded by the 5′-nuclease activity of the DNA polymerase, which releases the fluorophore from its sequence-recognizing element, and thereby separates it from the quencher, so that a light signal is emitted upon excitation.[3] Molecular beacons achieve the same goal by a rearrangement of the secondary structure upon hybridization, which causes a separation of the quencher and the fluorophore.[4] Fluorescence resonance energy transfer (FRET) probes work slightly differently. Instead of the acceptor dye functioning as a quencher, it emits the detectable fluorescence when the two probes bind adjacent to each other on the target DNA molecule.[5]

The LightUp® Probe[6] (Fig. 1) represents a significant departure from this paradigm, because the DNA has been entirely replaced by the homomorphous but uncharged and achiral DNA analog PNA (Fig. 2). Peptide nucleic acid is composed of *N*-(2-aminoethyl)glycine subunits[7] and forms very stable hybrid complexes with both DNA and RNA with a sequence specificity that is higher than that DNA has for itself.[8] An asymmetric cyanine dye, such as a thiazole orange derivative or 4-[(3-methyl-6-(benzothiazol-2-yl)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium iodide (BEBO),[9] is generally used as the fluorescent marker (Fig. 1). This type of dye releases excitation energy by internal motion when it is free in solution and is, under these conditions, virtually nonfluorescent. When bound to DNA, however, the dye becomes brightly fluorescent.[10–13] Because the dye is positively charged, a significantly higher background signal would result from internal binding of the dye if DNA is used instead of PNA.

The LightUp Probes have several advantages compared to probes with sequence recognizing elements consisting of DNA. Because it binds during the annealing phase, an optimal three-step PCR cycle can be used. The primer design is also very flexible because the amplicon does not need to be kept short, apart from what is compatible with maximum sensitivity of the PCR system. A typical amplicon length is generally in the range of 100–500 bp. Because PNA is not degraded by proteases or nucleases, kits utilizing LightUp Probe detection are very stable with

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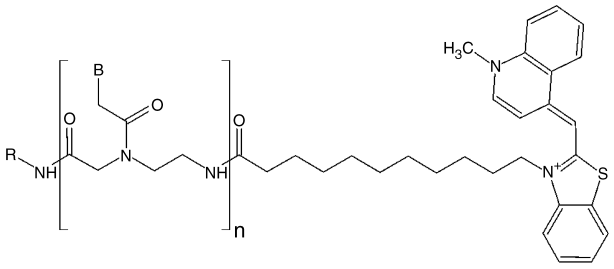

Fig. 1 A schematic view of the LightUp Probe. The dye may be different.

typical storage conditions at 4–6°C for at least 1 year. Possibly, the different chemistry by which the dye is attached to the PNA as compared to DNA contributes to this favorable stability. Recently, it has become increasingly apparent that the long (typically 20–40 bases) DNA probes are not suitable in some applications where high specificity is desirable, for instance, in SNP detections. Obviously, a one base mismatch out of 40 bases makes a smaller difference than one base out of ten. For this reason, minor groove binding (MGB) probes were designed, where minor groove binding ligands have been covalently attached to the DNA probes to increase stability upon hybridization and enable a shortening of the probe.[14] The uncharged LightUp Probe is intrinsically much more stable than a DNA probe because of the absence of electrostatic backbone–backbone repulsion. Furthermore, the positively charged dye (Fig. 1) plays a similar role as the minor groove-binding moiety of the MGB probes. Hence, the LightUp Probes are typically 8–12 bases long, about one-third of the length of a typical DNA probe and one-sixth of the length of a FRET pair. Many viral pathogens have rapidly mutating genomes, making it very challenging to find conserved target regions for DNA-based probes/quencher system. The LightUp technology provides a significant advantage in these applications.[13] Finally, the spectral region occupied by a LightUp Probe is just one-half that of a fluorophor/quencher pair or a FRET pair, because the LightUp Probe contains only a single dye. This should be advantageous in multiplex applications, but because

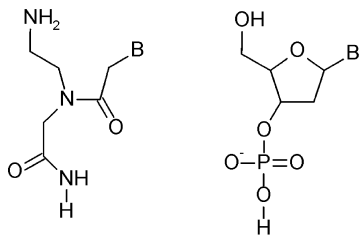

Fig. 2 The homomorphous DNA and PNA subunits.

several instrument platforms use a single wavelength excitation source (e.g., the ABI real-time PCR instruments and the Roche LightCycler), it is necessary to design asymmetric cyanine dyes with greatly varying Stokes' shift, which may prove difficult, to enable multiplex detection with LightUp Probes. However, the trend is that new real-time PCR instrumentation provides several excitation wavelengths (e.g., the Rotor-Gene™ from Corbett Research, the SmartCycler® from Cepheid Research and the iCycler from Bio-Rad).

Figure 3A shows the real-time amplification curves, in duplicates, for a successive dilution of an Epstein–Barr virus (EBV) DNA solution, obtained by using the LightUp ReSSQ EBV Assay. The initial EBV template concentration ranges from 20 to 200,000 genome copies, as indicated. Prominent Hook effects are observed at all concentrations and the end signal is independent of the initial template amount. This shows that the probe is stable throughout the PCR run. With DNA probes, the end signal commonly decreases for low template concentrations, which is likely to be an effect of probe degradation. By fitting a standard curve to the threshold cycle numbers in Fig. 3A and the logarithm of the known concentrations (Fig. 3B), it is found that the PCR efficiency is 96% and that the $C_t(1)$ value is 36.4 cycles.

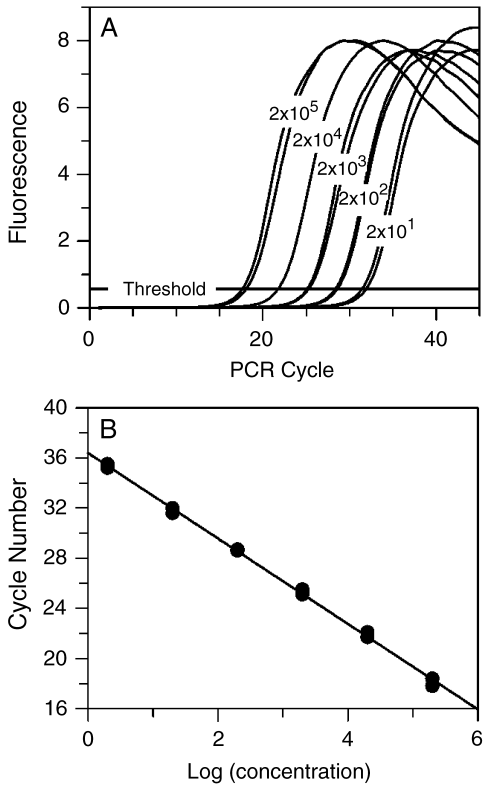

Fig. 3 The LightUp ReSSQ™ Epstein–Barr virus assay.

The viral load of an unknown clinical sample could easily be determined from its C_t value and this standard curve. As an example, a clinical evaluation study shows that quantification with the LightUp Cytomegalovirus (CMV) ReSSQ Assay is very similar to that of the Roche COBAS Amplicor CMV Monitor, but with higher sensitivity (to be published).

Applications of LightUp Probes also include bacterial DNA diagnostics. One example is Salmonella typing. It has been shown that the *Salmonella bongori* serotype could readily be distinguished from the *Salmonella entertidis* and *Salmonella anatum* serotypes from a single mismatch in the probe target region.[13] In another study, *Yersinia enterocolitica* was quantified over four log units.[15] LightUp probes have also found application in cancer diagnostics. The relative expression of the immunoglobulin κ and λ genes is a sensitive marker for non-Hodgkin B-cell lymphomas. In a healthy individual, 60% of the B-cells produce κ chains, while the rest produce λ chains. A significant perturbation of this ratio is indicative of B-cell lymphoma. In a recently published study, 28 of 32 clinical samples were correctly diagnosed B-cell lymphoma by cDNA quantification using LightUp Probes.[16]

CONCLUSION

LightUp Probes provide a useful combination of strong affinity and high specificity. The resistance against degradation is an important advantage when storing and manufacturing diagnostic kits. Freeze–thaw cycles are often deleterious to long DNA, such as viral genomes and plasmids. This can be avoided by using LightUp Probe technology, because the probe does not need to be stored frozen. Furthermore, the probes can be made short: 8–12 residues is typical. This is a very useful property in viral diagnostics, where the mutation frequency often is high and long conserved target regions may be rare. This is particularly true for RNA viruses such as HIV, influenza, and severe acute respiratory syndrome (SARS). In fact, several probes could, in principle, be targeting the same amplicon-generating independent signals, thereby making the assay even more robust and less prone to false negatives. The greatest challenge for the future success of the LightUp Probe technology will be to develop and implement fluorescent dyes suitable for multiplex applications.

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Liposomal Nonviral Delivery Vehicles

Nancy Smyth Templeton
Baylor College of Medicine, Houston, Texas, U.S.A.

INTRODUCTION

Optimization of cationic liposomal complexes for in vivo applications is complex, involving many diverse components including nucleic acid purification, plasmid design, delivery vehicle formulation, administration route and schedule, dosing, detection of gene expression, and others. Optimizing all components of the delivery system is pivotal and will allow broad use of liposomal complexes to treat human diseases or disorders. This article will highlight the features of liposomes that contribute to successful delivery, gene expression, and efficacy.

Delivery of nucleic acids using liposomes is promising as a safe and nonimmunogenic approach to gene therapy and would overcome the numerous disadvantages of viral vectors. Furthermore, gene therapeutics composed of artificial reagents can be standardized and regulated as drugs rather than as biologics. Cationic lipids have been used for efficient delivery of nucleic acids to cells in tissue culture for several years.[1] Much effort has also been directed toward developing cationic liposomes for efficient delivery of nucleic acids in animals and in humans. Most frequently, the formulations that are best to use for transfection of a broad range of cell types in culture are not optimal for achieving efficacy in animals or people. Functional properties defined in vitro do not assess the stability of the complexes in plasma, pharmacokinetics, biodistribution, and colloidal properties that are essential for optimal activity in vivo. Nucleic acid–liposome complexes must also traverse tight barriers in vivo and penetrate throughout the target tissue to produce efficacy for the treatment of many diseases. These are not issues for achieving efficient transfection of cells in culture with the exception of polarized tissue culture cells. Therefore, optimized in vivo liposomes may differ from those used for efficient tissue culture transfection.

Different types of nucleic acids of unlimited size can be delivered using liposomes. Recent advances have dramatically improved transfection efficiencies and efficacy of liposomal vectors.[2–6] We demonstrated broad efficacy of a robust liposomal delivery system in small and large animal models for lung,[3] breast,[5] head and neck (Hung and Templeton, unpublished data), and pancreatic cancers,[4] and for hepatitis B and C (Clawson and Templeton, unpublished data). This liposomal delivery system is currently used in a clinical trial to treat non-small-cell lung cancer and will be used in upcoming clinical trials to treat other cancers and cardiovascular diseases. Reviews of other in vivo delivery systems and improvements using cationic liposomes have been published recently.[7,8]

OPTIMIZATION OF LIPOSOMAL DELIVERY

Efficient in vivo nucleic acid–liposome complexes have unique features, including their morphology, mechanisms for cell and nuclear entry, targeted delivery, and ability to penetrate across tight barriers and throughout target tissues. Liposomes have different morphologies based on their composition and the formulation method that contribute to their ability to deliver nucleic acids in vivo. Formulations frequently used for the delivery of nucleic acids are lamellar structures, including small unilamellar vesicles (SUVs), multilamellar vesicles (MLVs), or bilamellar invaginated vesicles (BIVs) recently developed in our laboratory (Fig. 1). Hexagonal structures have demonstrated efficiency primarily for the transfection of some cell types in culture and not for in vivo delivery. SUVs condense nucleic acids on the surface and form ''spaghetti and meatballs'' structures.[9] SUV complexes produce little or no gene expression upon systemic delivery, although these complexes transfect numerous cell types efficiently in vitro.[1] Furthermore, SUV liposome–DNA complexes cannot be targeted efficiently.

SUV complexes also have a short half-life in circulation, about 5 to 10 min. Polyethylene glycol (PEG) has been added to liposome formulations to extend their half-life;[10–12] however, PEGylation created other unresolved problems. PEG also hinders delivery of cationic complexes into cells because of its sterically hindering ionic interactions and interferes with optimal condensation of nucleic acids into complexes. Furthermore, extremely long half-life in the circulation, e.g., several days, caused problems for patients because the bulk of the PEGylated liposomal formulation, doxil, which encapsulates the cytotoxic agent, doxorubicin, accumulates in the skin, hands, and feet. Patients contract mucositis and hand and foot syndrome[13,14] that cause

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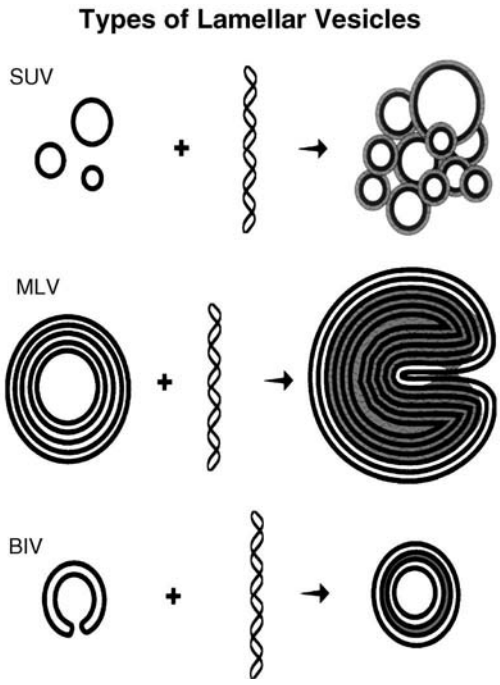

Fig. 1 Diagrams drawn from cryoelectron micrographs of lamellar liposomes and complexes. SUVs, ''spaghetti and meatballs'' structures, condense nucleic acids on the surface. MLV complexes appear as ''Swiss rolls.'' BIVs efficiently encapsulate nucleic acids between two bilamellar invaginated structures. (From Ref. [2].)

extreme discomfort to the patient. Adding ligands to doxil for delivery to specific cell surface receptors did not result in much cell-specific delivery, and most of the injected targeted formulation still accumulated in the skin, hands, and feet. Addition of PEG in BIVs also caused steric hindrance that prevented efficient encapsulation of nucleic acids, and gene expression was substantially diminished. MLV complexes appear as ''Swiss rolls'' when viewing cross sections by cryoelectron microscopy.[15] These complexes can become too large for systemic administration or deliver nucleic acids inefficiently into cells because of inability to ''unravel'' at the cell surface. Addition of ligands onto MLV liposome–DNA complexes further aggravates these problems.

BIVs efficiently encapsulate large amounts of nucleic acids of any size between two bilamellar invaginated vesicles.[2] We created these unique structures using 1,2-*bis*(oleoyloxy)-3-(trimethylammino)propane (DOTAP) and synthetic cholesterol (Chol) and a novel formulation procedure. Addition of other DNA condensing agents including polymers is not necessary, and encapsulation of nucleic acids by BIVs alone is spontaneous and immediate. The BIV complexes are also large enough so that they are not cleared rapidly by Kupffer cells in the liver, and yet extravasate and penetrate across tight barriers. BIVs penetrated barriers including the endothelial cell barrier in normal mice and the posterior blood retinal barrier in adult mouse eyes, and diffused throughout large tumors[3] and several layers of smooth muscle cells in pig arteries. We demonstrated efficacy for treatment of non-small-cell lung cancer[3] using BIV DOTAP:Chol-p53 DNA–liposome complexes and not by using SUV DOTAP:Chol-p53 DNA–liposome complexes. Therefore, morphology of the complexes is essential.

A common belief is that artificial vehicles must be 100 nm or smaller to be effective for systemic delivery. However, this belief is most likely true only for large, inflexible delivery vehicles. Blood cells are several micrometers (up to 7000 nm) in size, and yet have no difficulty circulating in the blood including through the smallest capillaries. However, sickle cell blood cells, which are rigid, do have problems in the circulation. Therefore, we believe that flexibility is a more important issue than size. In fact, BIV DNA–liposome complexes in the size range of 200 to 450 nm produced the highest levels of gene expression in all tissues after intravenous injection.[2] Kupffer cells in the liver quickly clear delivery vehicles (including nonviral vectors and viruses) that are not PEGylated and are smaller than 200 nm. Therefore, increased size of liposomal complexes could extend their circulation time particularly when combined with injection of high colloidal suspensions. BIVs encapsulate nucleic acids and viruses apparently because of the presence of cholesterol in the bilayer, whereas formulations including L-alpha dioleoyl phosphatidylethandamine (DOPE) instead of cholesterol could not assemble nucleic acids by a ''wrapping type'' of mechanism, and produced little gene expression in the lungs and no expression in other tissues after intravenous injections. The DOTAP:Chol BIV complexes are flexible and not rigid, are stable in high concentrations of serum, have extended half-life, and circulate efficiently in the bloodstream.

Colloidal properties of nucleic acid–liposome complexes also determine the levels of gene expression produced after in vivo delivery.[2,16] These properties include the DNA:lipid ratio, the overall charge density of the complexes, complex size and shape, lipid composition, formulation, and encapsulation efficiency and the colloidal suspension monitored by turbidity measurements at OD400. Our data showed that transfection efficiency in all tissues corresponded to the OD400 of complexes measured prior to intravenous injection. Colloidal properties affect serum stability, protection from nuclease degradation, blood circulation time, and biodistribution of the complexes.

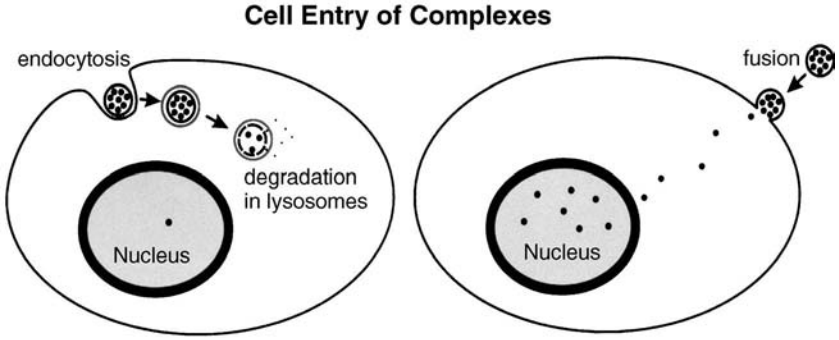

Fig. 2 Mechanisms for cell entry of complexes by endocytosis or by direct fusion with the cell membrane. Cell fusion allows delivery of more nucleic acids to the nucleus because the bulk of the nucleic acids do not enter endosomes.

The BIV complexes efficiently deliver DNA into cells by fusion with the cell membrane and avoid the endocytic pathway (Fig. 2). Cells are negatively charged on the surface, and cationic complexes have nonspecific ionic charge interactions with cell surfaces that, in part, contribute to efficient transfection. Therefore, we create targeted delivery of our complexes in vivo without the use of PEG to retain predominant entry into cells by direct fusion. These ligand-coated complexes reexpose the overall positive charge by shedding the ''mask'' as they approach the target cells, using a reversible masking technology to bypass nontarget organs and tissues. Through ionic interactions or covalent attachments, we have added monoclonal antibodies, Fab fragments, proteins, partial proteins, peptides, peptide mimetics, small molecules, and drugs to the surface of BIV complexes after mixing. Using novel methods for addition of these ligands to the complexes results in further increased gene expression in the target cells after transfection. Figure 3 shows our optimized strategy to achieve targeted delivery, deshielding, cell fusion, cell and nuclear entry of nucleic acids, and production of gene expression.

Stability of BIV complexes was studied at 37°C out to 24 hr at concentrations of serum ranging from 0% to 100%. The results showed serum stability at the highest

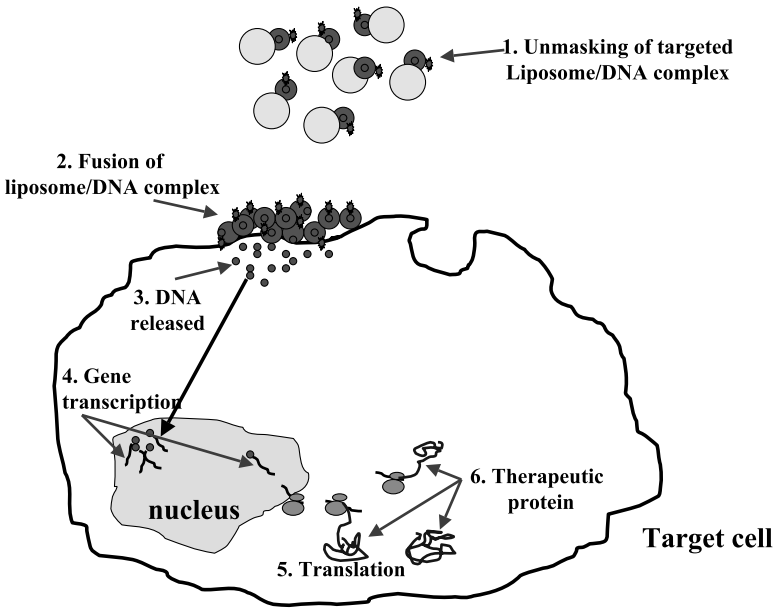

Fig. 3 Optimized strategy for delivery and gene expression in the target cell including targeted delivery, deshielding, fusion with the cell membrane, entry of nucleic acids into the cell and to the nucleus, and production of gene expression of a cDNA cloned in a plasmid. (*View this art in color at www.dekker.com.*)

concentrations of serum, about 70% to 100%, which are physiological concentrations of serum found in the bloodstream, and at no or low concentrations of serum. The complexes were unstable at 10% to 50% serum, perhaps because of salt bridging. Therefore, in vitro optimization of serum stability for formulations of cationic complexes must be performed over a broad range of serum concentration to be useful for applications in vivo.

Delivery of DNA to the nucleus and subsequent gene expression may be poorly correlated.[6,17] The following issues should be considered independent of the delivery formulation, including suboptimal promoter-enhancers in the plasmid, poor preparation of plasmid DNA, and insensitive detection of gene expression. Plasmid expression cassettes typically have not been optimized for animal studies. For example, many plasmids lack a full-length CMV promoter–enhancer, and some variations produce greatly reduced or no gene expression in certain cell types.[6] Ideally, investigators design custom promoter–enhancer chimeras that produce the highest levels of gene expression in their target cells. For example, we designed a systematic approach for customizing plasmids used for breast cancer gene therapy by using expression profiling.[6] To increase long-term expression, use of replication-competent plasmids increase gene expression over time posttransfection. Plasmids can also be engineered to provide for specific or long-term gene expression, replication, or integration.

The transfection quality of plasmid DNA is dependent on the preparation protocol and training of the person preparing the DNA. We have also identified large amounts of contaminants in laboratory- and clinical-grade preparations of plasmid DNA and developed three proprietary methods for their detection. These contaminants copurify with DNA by anion exchange chromatography and by cesium chloride density gradient centrifugation. Endotoxin removal does not remove these contaminants, and HPLC cannot detect them. To provide the greatest efficacy, safety, and gene expression, these contaminants must be assessed and removed from plasmid DNA preparations. These contaminants belong to a class of molecules known to inhibit both DNA and RNA polymerase activities. Our group and other investigators have shown that intravenous injections of high doses of improved liposomes alone cause no adverse effects. Plasmids with most of CpG sequences removed apparently reduced toxicity after intravenous injections of cationic complexes.[18] However, only low doses containing up to 16.5 μg of DNA per injection into each mouse reduced toxicity, and no significant dose response to CpG motifs in plasmid DNA was demonstrated. Therefore, we believe that removal of the other contaminants in current DNA preparations is the major block to safe intravenous injection of high doses of DNA–liposome complexes.

Choosing the most sensitive detection method for gene expression is also essential. For example, detection of β-galactosidase (β-gal) expression is far more sensitive than that for the green fluorescent protein (GFP). Specifically, 500 molecules of β-gal per cell are required for detection using X-gal staining, whereas about 1 million molecules of GFP per cell are required for direct detection, and its detection may be impossible if the fluorescence background of the target cell or tissue is too high. Detection of chloramphenicol acetyltransferase (CAT) is extremely sensitive with little or no background detected in untransfected cells. Few molecules of luciferase in a cell can be detected by luminescence assays of cell or tissue extracts posttransfection, and the sensitivity is highly dependent on the type of instrument used to measure luminescence. Luciferase data may not predict the therapeutic potential of a nonviral delivery system if several hundred or thousand molecules per cell of a therapeutic protein are required to produce efficacy for a certain disease. Furthermore, noninvasive detection of luciferase expression in vivo is not as sensitive as luminescence assays of cell or tissue extracts posttransfection. My colleagues detected luciferase expression in mice by cooled charge-coupled device (CCD) imaging after intravenous injections of BIV DOTAP:Chol-luciferase DNA–liposome complexes and detection of HSV-TK gene expression using microPET imaging.[19]

To establish the maximal efficacy for the treatment of certain diseases or for vaccine production, administration of the nonviral gene therapeutic, etc. via different routes may be required. The optimal dose and administration schedule should be determined because administering the highest tolerable dose most frequently may not necessarily produce maximal efficacy. Loss of the therapeutic gene product will vary with the half-life of the protein produced. Therefore, if a therapeutic protein has a longer half-life, then the gene therapy could perhaps be administered less frequently.

CONCLUSION

Some hurdles remain in the broad application of nonviral delivery; however, we are confident that we will successfully overcome these challenges. We predict that eventually the majority of gene therapies will utilize artificial reagents that can be standardized and regulated as drugs rather than biologics. We will also continue to incorporate the molecular mechanisms of viral delivery that produce efficient delivery to cells into artificial systems. Therefore, the artificial systems, including liposomal delivery vehicles, will be further engineered to mimic the most beneficial parts of the viral delivery systems while circumventing their limitations. We will

also maintain the numerous benefits of the liposomal delivery systems discussed in this article.

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Listeria spp.: DNA Probes and Conventional PCR Assays

Philippe Gilot
Institut National de la Recherche Agronomique, Nouzilly, France

INTRODUCTION

Listeria spp. are facultatively anaerobic, nonspore-forming rod-shaped bacteria which belong to the gram-positive bacteria with a low G+C DNA content. The genus *Listeria* comprises six species: *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri*, and *Listeria grayi*. All of these species are widespread in the environment, but only *L. monocytogenes* is considered as a significant human and animal pathogen. Besides *L. monocytogenes*, however, occasional human infections because of *L. ivanovii* and *L. seeligeri* have also been reported. *L. ivanovii* is nevertheless mainly responsible for abortion in sheep. The identification of *Listeria* species has long been hampered by small number of tests allowing the differentiation between these closely related species. Hemolysis, a major characteristic for *Listeria* species identification, may, in some cases (and especially for environmental and food isolates), be difficult to read on blood agar and tests monitoring the acid production from carbohydrates are time-consuming. In last years, the necessity to rapidly identify *Listeria* spp. has nevertheless led to the development of some new methods, including commercial methods. All rapid commercial methods need a selective enrichment step before the application of the assay. Some of them use an immunocapture step with antibodies specific to *Listeria* genus or *L. monocytogenes* bound to solid matrices; others are based on the unique characteristics of the DNA molecules. Herein and in the next entry, we will focus on molecular techniques based on the DNA molecule, which, unlike identification techniques based on phenotypic characters, are uninfluenced by environmental growth conditions and protein expression mechanisms.

HUMAN LISTERIOSIS

Human disease because of *L. monocytogenes* usually occurs in pregnant women, the elderly, and immunocompromised patients. Clinical manifestations range from mild flulike symptoms and gastroenteritis to septicemia, central nervous infections, and feto-maternal infections with abortion, premature labor, or birth of an infected child. Despite the low incidence of listeriosis (2–15 people per 100,000 people per year), the disease is associated with high mortality rates (20–30%).[1–3] At first sight, this is difficult to understand because *Listeria* strains are, in general, susceptible to a wide range of antibiotics. Unfortunately, most antibiotics are not bactericidal for *Listeria*. Furthermore, the efficacy of therapy is limited by the fact of intracellular habitat of pathogenic *Listeria*. The clinical experience shows that the combination of amoxicillin and gentamicin is the best therapeutic option for listeriosis (for a review, see Ref. [4]).

VIRULENCE-ASSOCIATED GENES

The pathogenesis and molecular mechanisms underlying *Listeria* virulence have been particularly well studied. Many of the genes encoding proteins involved in *Listeria* pathogenesis are clustered in a 9-kb chromosomal fragment that is involved in functions essential to intracellular survival. This locus comprises genes coding for the hemolysin (*hly*A), the phospholipases C (*plc*A and *plc*B), the actin-polymerizing protein (*act*A), the metaloprotease (*mpl*), and the virulence factor regulator (*prf*A). Some other genetic determinants also implicated in the virulence of the bacteria, such as, for example, the internalin locus (*inlAB*), the invasion-associated protein gene (*iap*), or the *clp* stress response mediator locus, were also identified outside of the virulence cluster (for a review, see Ref. [5]). Heterogeneity in the virulence of *L. monocytogenes* strains has been observed. Whereas in most cases this heterogeneity is unexplained, attenuated or nonvirulent strains could be associated with truncated forms of the virulence factors InlA and ActA.[6–9]

ECOLOGY

All *Listeria* species have been isolated from soil, decaying vegetable matter, silage, sewage, water, animal feed, fresh and processed meats, raw milk, cheese, slaughterhouse waste, and asymptomatic human and animal carriers. Because of their widespread occurrence, *Listeria* species have many opportunities to enter food production and processing environments. Because of their psychrotrophic

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nature, they are then able to grow in food, even at temperatures such as those of refrigerators.[1,2] Consequently, outbreaks and sporadic cases of listeriosis have been traced to different foodstuffs such as pasteurized milk, cheese, coleslaw, and meat products.[2,3,10] The coexistence of several *Listeria* species on the same food is not unusual, and often, the incidence of *Listeria* species other than *L. monocytogenes* is higher than the incidence of *L. monocytogenes* itself. Because all *Listeria* species are potential food contaminants, the presence on foodstuffs of any of these species can be considered as an indicator of their contamination and of the potential presence of *L. monocytogenes*.[11] However, as the threat to public health posed by contamination of foods by each *Listeria* species is not similar, it is very important that all of them could be rapidly and reliably detected and identified.

DNA-BASED IDENTIFICATION

DNA Probes

Several probes targeting genes such as those encoding the hemolysin (LLO), the invasion-associated protein, the DTH-18 gene, the PrfA regulator, the 16S rRNA gene, or the 16S/23S rRNA spacer have been described to differentiate *Listeria* genus and mainly the species *L. monocytogenes*.[12–18] Commercial DNA probe-based assays are now available for the rapid confirmation of *L. monocytogenes* from colonies (AccuProbe®, Gene-Probe, San Diego, CA; GENE-TRAK® *L. monocytogenes* Assay, Gene-trak system, Framingham, MA). Both assays are nonradioactive hybridization methods (chemiluminescently labeled probe or fluorescein-labeled probe for AccuProbe and Gene-trak, respectively) based on the detection of unique sequence in the 16S rRNA region. The sensitivity of both methods is achieved by the high copy number of 16S rRNA in the bacterium cell. The Gene-trak system is also usable for the rapid detection of *Listeria* spp. or *L. monocytogenes* from samples previously grown in selective enrichment broths.[19] The usefulness of these probes is restricted to the species containing nucleotide sequence that hybridize with the specific probe. In contrast, the analysis of the rRNA gene restriction patterns, after restriction hydrolysis of whole chromosomal DNA and hybridization with a 16S rRNA gene, allowed the identification of each *Listeria* species.[20] More recently, a fluorescence in situ hybridization (FISH) technique with 16S rRNA-targeted oligonucleotide has been developed for the identification of *Listeria* genus (but not *L. grayi*) by epifluorescence microscopy in a mixture with other bacteria. In addition, a technique for in situ detection of *iap* mRNA within intact cells of *L. monocytogenes* has also been devised.[21,22] These latter two methods will help in the analysis of the structure and dynamics of complex microbial communities and of the spatial distribution of probe–target populations in microbial biofilms and tissue samples.

Conventional Polymerase Chain Reaction Assays

As an alternative to DNA hybridization methods, PCR procedures were proved to be rapid and highly specific for the detection of a number of pathogens. Polymerase chain reaction assays based on the flagellin A encoding gene (*fla*A), the 16S/23S intergenic spacer region of the rRNA operon, and the *iap* gene have been developed for the identification of the *Listeria* genus.[23–25] Continuous efforts for the specific identification of *L. monocytogenes* using polymerase chain reaction assays were made. Apart from PCR identification procedures targeting the 16S rRNA gene, the 16S/23S spacer region, and the aminopeptidase C gene (*pep*C), these assays amplified DNA sequences derived from genes proved or suspected to be implicated in the virulence mechanism of this species, such as *hly*A, *plc*B, *act*A, *prf*A, *inl*A, *inl*B, *iap*, the gene coding for LmA antigen (*lma*A or Dth18 gene), or the fibronectin-binding protein (*fbp*) gene.[17,23,24,26–36] The most frequently chosen target has been the *hly*A gene from which amplification fragment of different sizes has been generated with specific primers designed by several authors.[37] As *L. monocytogenes* strains with mutations or deletions in one or more virulence determinants can be encountered, a multiplex PCR for the simultaneous detection of multiple genes in a single step has also been developed.[6–9,26] Whereas vast number of protocols for the PCR identification of *L. monocytogenes* have been published, only few studies have considered the identification of the other members of the genus *Listeria*.[37] Nevertheless, the identification of non-*L. monocytogenes* species can also be achieved by the specific amplification of part of the 16S rRNA gene of *L. ivanovii*, internal *iap* sequences of *L. grayi*, *L. seeligeri*, *L. welshimeri*, and *L. innocua*, part of the *L. welshimeri fbp* gene, and part of the *L. innocua* gene encoding the *lin046* putative transcriptional regulator.[16,27,38,39] One of the above PCR assay is a multiplex PCR that allows the simultaneous detection of all *Listeria* species and the differentiation between some (but not all) of them, in a single step.[38] The comparative analysis of genome sequences of *L. monocytogenes* and *L. innocua* revealed also the presence of 270 *L. monocytogenes* and 149 *L. innocua* strain-specific genes.[40] These genes are putative targets for the further development of PCR assays for the specific identification of both species.

The introduction of commercial PCR kits such as the Probelia *L. monocytogenes* system which targets the *hly*A gene (Bio-Rad Lab., Richmond, CA) and the Bax system for *L. monocytogenes* and also for *Listeria* genus (Qualicon-Dupont, Willington, DE) has made easier the routine use of the PCR method for the identification of *Listeria*. PCR products are detected by agarose gel electrophoresis for the Bax system or in a microplate postamplification capture hybridization assay for the Probelia system. These automatized methods, developed to screen food and environmental samples, need a pre-enrichment step of the samples before the PCR assays to increase the number of target bacteria and to avoid the detection of dead cells by diluting out any nonviable cells that may be present.

CONCLUSION

DNA probes and conventional PCR assays were developed to facilitate the identification of this bacterium from both clinical samples as well as in contaminated food. In the next entry, less conventional DNA-based identification procedures and DNA-based typing methods will be reviewed. It can be expected that the application of all of these methodologies will allow an early and efficient identification of *Listeria* spp. and will lower the risk associated with listeriosis.

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Listeria spp.: Other DNA-Based Identification Procedures and DNA-Based Typing Methods

Philippe Gilot
Institut National de la Recherche Agronomique, Nouzilly, France

INTRODUCTION

In the previous *Listeria* spp. entry, the clinical spectrum and management of human listeriosis, the ecology of *Listeria* species, and the identification methods based on DNA probes and standard polymerase chain reaction (PCR) assays were described. In this entry, less conventional DNA-based identification procedures and DNA-based typing methods will be reviewed.

DNA-BASED IDENTIFICATION

Ligase Chain Reaction

A ligase chain reaction (LCR) assay with the thermostable *Thermus aquaticus* DNA ligase has been devised for the specific detection of *Listeria monocytogenes*. This assay is based on a single base-pair difference in the V9 region of the 16S sequence of the genes coding for rRNA, which distinguishes *L. monocytogenes* from other closely related *Listeria* spp. To achieve a higher sensitivity, the 16S rRNA gene was initially amplified by PCR prior to LCR. The LCR was originally performed with radioactive-labeled primers, and ligation products were detected by autoradiography after denaturing gel electrophoresis (DGGE). The method was further adapted for the detection of LCR products with a nonisotopic readout using chemiluminescent or colorimetric substrates. The latter method gave a sensitivity of 10 CFU of *L. monocytogenes* and is amenable to automation.[1,2]

Microarray-Based Assays

A rapid oligonucleotide microarray hybridization assay for the discrimination of the six species of the *Listeria* genus has been developed. The approach involves one-tube multiplex PCR of six target virulence factor genes (*iap*, *hly*A, *inl*B, *plc*A, *plc*B, and *clp*E), synthesis of fluorescently labeled single-stranded DNA with primer extension of DNA synthesized in the first round of multiplex PCR, and hybridization to the multiple individual oligonucleotide probes specific for each *Listeria* species and immobilized on a glass surface. This microarray technique was proven to correctly identify all *Listeria* species.[3]

Quantitative PCR

Recently, a number of investigators have described semiquantitative PCR methods that allow to identify and to know the number of bacteria present in the sample, and that simplify the need to perform postamplification techniques. An enzyme-linked immunosorbent assay-mediated PCR technique was described to detect and quantify *L. monocytogenes* in food products. Using this method, a fragment of the *iap* gene and of an internal standard are amplified in the presence of fluorescein dUTP. PCR products were then hybridized in streptavidin-coated microtiter plate prepared with biotinylated specific DNA probes. After the addition of an alkaline phosphatase-conjugated antibody to fluorescein, the PCR products are quantitated based on an optical density reading.[4] Bassler et al.[5] described a 5′ nuclease PCR-based assay for *L. monocytogenes* that uses the hydrolysis of a dually labeled internal fluorogenic probe (TaqMan® PCR detection, Applied Biosystems, Foster City, CA) to monitor the amplification of the target (a region of the *hly*A gene). Quantification was found to be linear over a range of 5×10 to 5×10^5 CFU. A variation of the above method was described by Koo and Jaykus[6] for the detection of *L. monocytogenes* using *hly*A or *iap* as a target, and an inexpensive asymmetric probe set in place of the TaqMan probe. The above methods are endpoint detection assays and the PCR products have to be detected by fluorescence or optical density reading immediately following termination of the PCR.

Strategies based on the detection of PCR products simultaneously as the PCR is progressing (real-time PCR) and avoiding further postprocessing were also developed for the detection and quantification of *L. monocytogenes* and *Listeria innocua*. Those methods exploit also the 5′ nuclease activity of the *Taq* DNA polymerase and a dually labeled fluorogenic TaqMan hybridization probe, but use the ABI Prism® 7700 sequence detection system of Perkin-Elmer (Foster City,

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CA) and either the *hly*A or *iap* genes as target.[7–9] A protocol enabling the simultaneous detection and quantification of *Escherichia coli* O157:H7, *L. monocytogenes*, and *Salmonella* strains was also formatted using commercial Bax PCR kits (for Bax, see *Listeria* spp. entry: DNA probes and standard PCR assays) and by monitoring the PCR in real time with the iCycler of Bio-Rad Laboratories (Hearles, CA) after the addition of the nonspecific fluorescent dye SYBR Green I. A melting curve analysis of PCR amplicons serves as a confirmatory test, with the T_m of each amplicon being dependent on the length as well as the G+C content of the sequence.[10]

Other PCR-Based Assays

The PCR single-strand conformation polymorphism (PCR-SSCP) technique was also tested for the identification of *Listeria* genus and individual *Listeria* species. This approach employs the PCR amplification of a variable region of the 16S rRNA, and the analysis of the denatured amplified product by electrophoresis in a nondenaturating polyacrylamide gel. The method was found to be able to identify almost all species of the *Listeria* genus.[11,12] A variation of this method was also used by Manzano et al.,[13] who analyzed a short amplified fragment of the 16S rRNA gene by PCR temperature gradient gel electrophoresis (PCR-TGGE) in a polyacrylamide gel. By the use of PCR-TGGE, the species *L. monocytogenes*, *Listeria seeligeri*, and *Listeria ivanovii* were distinguished, whereas the *L. innocua* and *Listeria welshimeri* PCR products migrated to the same position in the gel. More recently, an identification approach based on the PCR of a fragment of the *iap* gene and on the analysis of the PCR product obtained by DGGE (PCR-DGGE) was also developed for the direct identification of *Listeria* spp. in food samples. Species-specific DGGE migration that allows the identification of individual *Listeria* species was obtained. Nevertheless, it should be noted that the specificity of the PCR-TGGE and PCR-DGGE methods was not tested toward *Listeria grayi*.[14] Other PCR-based identification approaches, such as repetitive sequence base PCR (rep-PCR) with primer sets targeting the enterobacterial repetitive intergenic consensus sequence (ERIC), 16S-amplified rDNA-RFLP analysis (ARDA), randomly amplified polymorphic DNA (RAPD) technique, and rDNA spacer PCR analysis, have also been described.[11,15–18]

Reverse Transcription PCR

Detection of pathogens in contaminated food products by PCR can result in false positive data due to amplification of DNA from nonviable cells. Most bacterial mRNA have very short half-life due to rapid degradation by endogenous RNases. As a consequence, nonviable cells that are disrupted in cellular transcription will rapidly lose their cellular mRNA. Studies were undertaken to develop a sensitive method for the detection of viable *L. monocytogenes* based on amplification of mRNA by reverse transcription PCR (RT-PCR). In a first attempt, Klein and Junega[19] isolated total RNA from *L. monocytogenes* samples and, following DNase treatment, amplified the RNA by RT-PCR with recombinant *Thermus thermophilus* DNA polymerase and primers specific for the *iap* gene. Amplicon detection was then accomplished by Southern hybridization to a digoxigenin-labeled gene probe. Viable *L. monocytogenes* in a cooked meat sample that was originally inoculated with ca. 3 CFU/g could be detected. A modification of the fluorogenic 5′ nuclease PCR assay of Bassler et al.[5] to detect mRNA as a monitor of viable *L. monocytogenes* has also been developed.[20] This assay, after a DNase treatment of the sample, amplifies *hly*A transcript by using the *Tth* DNA polymerase. The test, with primers that included the 3′-end of the transcript, was proven to be an accurate indicator of viability as measured by colony-forming unit determination.[20] Koo and Jaykus have also adapted their quantitative PCR method with fluorogenic asymmetric probes for the amplification of *hly*A or *iap* transcripts. Because of a special design of the RT-PCR primer set, they used no DNase treatment of the RNA extract prior to RT. The method utilized the AMV reverse transcriptase and *Taq* DNA polymerase in place of the *Tth* DNA polymerase for cDNA synthesis and both cDNA amplification and 5′ hydrolysis of the fluorogenic probe, respectively.[6] Based on a different concept, an isothermal nucleic acid sequence-based amplification assay (NASBA) was devised to detect *L. monocytogenes* by targeting *hly*A mRNA. The generated amplicons, which incorporated biotin-labeled UTP, were then hybridized with an immobilized capture probe and detected by colorimetry.[21] Other investigators reported the development of a NASBA system amplifying *Listeria* 16S rRNA sequences. The latter system utilizes a hybridization method to specifically detect *L. monocytogenes* amplimers.[21] The specificity of the method was not tested toward *L. grayi*, and is not amenable to the detection of viable bacteria as, contrary to mRNA, rRNA is an extremely stable molecule.

Other DNA-Based Techniques

Genomic fingerprinting of *Listeria* DNA via pulsed-field gel electrophoresis (PFGE) was also found to discriminate between *Listeria* species.[22] Finally, the A511 phage, a

broad host range bacteriophage specific for the genus *Listeria*, has been genetically engineered by the incorporation of the *lux* operon of *Vibrio fischeri* in its chromosome. The A511*luxAB* phage transduces bacterial bioluminescence into infected cells, allowing a rapid and easy testing of contaminated food and environmental sample for the presence of viable *Listeria* cells.[23]

DNA-BASED TYPING

Serotyping and bacteriophage typing have traditionally been used for intraspecies differentiation of *L. monocytogenes* isolates and epidemiological survey of listeriosis. An improved discrimination between *L. monocytogenes* isolates has been obtained following the development of multilocus enzyme electrophoresis, which differentiates isolates according to the mobility of a great number of housekeeping enzymes.[24] Several DNA-based typing methods such as restriction enzyme analysis (REA) of chromosomal DNA, plasmid profiling, restriction fragment length polymorphism (RFLP), and PCR-based fingerprinting techniques have also been developed for this purpose.[25] Some standardized protocols have been defined to allow comparisons between laboratories and to favor the recognition of potential outbreaks as quickly as possible. For that aim, ribotyping, an RFLP technique that allows the analysis of the rRNA gene restriction patterns after hybridization with a rRNA gene, has been automatized (Riboprinter Qualicon, Willmingtonn, DE).[26] A standardized protocol of PFGE has also been developed by a national network of public health and food regulatory laboratories in the United States (PulseNet). PFGE patterns identified by PulseNet are stored in a national electronic database maintained at the Centers for Disease Control and Prevention (Atlanta, GA). Fingerprinting techniques based on PCR amplification of DNA segments flanking either undetermined sequences [RAPD and arbitrarily primed PCR (AP-PCR)] or defined and conserved sequences [rRNA genes (PCR ribotyping), the repetitive elements REP and ERIC (rep-PCR) and infrequent DNA restriction fragments (IRS-PCR and AFLP)] have also been used to study the genetic structure and epidemiology of *L. monocytogenes*.[17,27–29] Restriction endonuclease analysis of PCR-amplified virulence-associated genes (RE-PCR) has also been devised for that aim.[30,31] More recently, DNA sequence-based subtyping strategies were developed to reduce interpretation ambiguity and to make easier the exchange and comparison of subtype data among laboratories. These methods to determine allelic variations include pyrosequencing of a short sequence of the *inl*B gene, multilocus sequence typing (MLST) of several housekeeping genes, and sequencing of multiple genes with various evolving speeds (virulence genes, stress response genes, intergenic region, and housekeeping genes).[32–34] Finally, DNA microarrays were found to be promising tools for differentiating among closely related *L. monocytogenes* isolates.[35,36]

Studies of the genetic diversity and population structure of *L. monocytogenes* by using many of the above methods revealed that this species is composed of two distant phylogenetic divisions of strains, which are correlated with the flagellar antigen groups, division (division I, which is composed of strains of serotypes 1/2a and 1/2c, and division II, which is composed of strains of serotypes 1/2b and 4b). An additional division has also been proposed based on the sequence of several genes involved in virulence. This latter division, consisting of serotypes 4a and 4c strains, has recently been shown to be a branch of the first division.[37] A mismatch amplification mutation (MAMA-PCR) assay targeting the hemolysin gene has been developed to characterize *L. monocytogenes* isolates with regard to these lineage types.[38]

CONCLUSION

As the detection and identification of *L. monocytogenes* by conventional methods are not easy, new procedures were developed to facilitate the recovery and characterization of this bacterium from both clinical samples as well as in contaminated food. Among these methods, a great variety of nucleic acid-based techniques were devised by many researchers. Some of these methods are now available as commercial assays. The sequencing of the complete genome of *L. monocytogenes* and *L. innocua* has opened the way for the development of microarrays, sequence-based standardized strategies, and semiautomated platforms. By simplifying application methods and by allowing an early and efficient identification of *Listeria* spp., these procedures will put molecular-based methods within the reach of most routine laboratories and will certainly lower the risk associated with listeriosis.

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Long-Distance PCR

Philip Lewis White
Sam Hibbitts
University of Wales College of Medicine, Cardiff, U.K.

INTRODUCTION

The polymerase chain reaction (PCR) is a common tool of molecular biology and is utilized in the field of medical diagnostics to identify genetic disorders and the causative agents of human infections. PCR is also used for typing microorganisms, thus playing an important role in both epidemiology and phylogenetics. The PCR method is a starting point that when used together with cloning is influential in the sequencing of novel genes and genomes.

When compared to cloning techniques, PCR is both more economical and straightforward to perform; however, it is limited by the fidelity and size (<5 kb) of the amplicon generated. Despite various publications[1–3] reporting longer PCR amplifications (>10 kb), only low yields of amplicon are produced, thus limiting further applications.[4]

Long-distance/range PCR (LD-PCR) has been developed to overcome these restrictions and allows amplification of up to 40 kb.[4–7] This methodology has extensive applications in the field of molecular biology and these will be discussed later.

TECHNICAL OVERVIEW

The principle of LD-PCR is essentially the same as for any standard PCR technique, with primers specific for two known regions flanking a target DNA sequence, enabling quick and efficient exponential amplification of the target amplicon. Standard PCR procedures tend to use only *Taq* polymerase, whereas in LD-PCR a proofreading enzyme (*Pfu* polymerase; from *Pyrococcus fuirosus*) must be used to correct possible nucleotide mismatches and prevent stalling of the extension process or introduction of a mutation into the sequence. Furthermore, it is preferential to replace the *Taq* polymerase with a more thermostable enzyme such as r*Tth* DNA polymerase (the recombinant form of the DNA polymerase from *Thermus thermophilus*). As with all PCR procedures, the reaction conditions and reagents must be optimized, but for LD-PCR it is also beneficial to obtain a high-quality (purity and length) DNA template, design primers optimally, and use a buffer composition optimal for the polymerase activity.

BASIC PROTOCOL FOR LONG-DISTANCE PCR

A quality target genomic DNA extract is essential for LD-PCR, and time should be taken to develop an extraction procedure. Standard concentrations of PCR reagents do not guarantee a successful amplification, and it is necessary to optimize all components for each LD-PCR reaction. In addition, the thermal cycling parameters and DNA template concentrations have to be evaluated, with too much DNA leading to nonspecific binding or even PCR inhibition and too little DNA leading to non-detectable amplicons, especially for low copy number targets. LD-PCR amplifications have been performed in as little as 10 μL[8] and up to 100 μL[5] reaction volume, although 50 μL is now more regularly used because of enhanced heat transfer over 100 μL. Individual reagent concentrations may or may not vary from a normal 50-μL PCR reaction; listed below are concentrations that have been used:

1. Mg^{2+} [$MgCl_2$, $Mg(OAc)_2$] concentration: Range 1.1[4] to 3.5 mM.[5] Typical concentration 2.5 mM.
2. dNTP concentration: Range 0.2[4] to 1.0 mM (non-limiting).[9] Typical concentration 0.2–0.25 mM, although 0.5 mM is regularly used.
3. Primer concentration: Range 0.12[10] to 2.0 μM.[11] Typical concentration 0.4 to 1.0 μM. Usual length 21–34 bp, with a balanced T_m of >58°C (usually 65–70°C).[7]
4. Typical PCR buffer: Tris–HCl 20–50 mM (pH 8.3–9.2) or 20–25 mM Tricine (pH 8.5–8.7), KOAc 85 mM, >10% glycerol, 2% DMSO.[4] Other reagents used in LD-PCR buffers include Tween 20 (0.1%), BSA (150–200 μg/mL), and $(NH_4)_2SO_4$ (16 mM), which is used instead of K^+ salts for certain polymerases.
5. Polymerase concentration: 2.6 U for a mix *Taq* and *Pwo* polymerases;[12] 1.5–2.5 U for *Tth* polymerase;[9,13,14] 0.9/0.02–1.75/0.02 U for a mix of r*Tth* and Vent polymerases, respectively;[4] 3.3 U of Expand Long-Template DNA polymerases.[10]
6. DNA template: Genomic template DNA Range 50[9]–625 ng (125 ng/10 μL reaction).[8] Typical

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Table 1 Commercially available LD-PCR polymerases and kits

Kit name	Manufacturer	Enzyme	PCR buffer	Maximum amplification
KlenTaq LA Core Kit	Sigma	KlenTaq-1 DNA polymerase[a] and a proofreading enzyme	10X optimized buffer supplied	Up to 10 kb
Accutaq LA DNA Core Kit	Sigma	*Taq* DNA polymerase and a proofreading enzyme	10X optimized buffer and a vial of DMSO supplied	>20 kb
Expand Long-Template PCR system	Roche	*Taq* and *Pwo* polymerases	Three types of 10X optimized buffers supplied	0.5–12 kb 12–15 kb >15 kb[b]
Expand 20-kb^{plus} PCR system	Roche	*Taq* and *Pwo* polymerases	10X buffer (27.5 mM $MgCl_2$) with an extra vial of $MgCl_2$ for optimization	>20 kb
Arrow *Taq* DNA polymerase	Qbiogene	*Taq* and *Tfu* polymerases	10X buffer supplied	Up to 21 kb
KOD XL DNA polymerase	Novagen	KOD HiFi DNA polymerase[c] and a 3′–5′ exonuclease-deficient KOD HiFi mutant	10X buffer and dNTP mix supplied	Up to 30 kb
Extensor Hi-Fidelity PCR Master Mix	Abgene	Thermoprime plus DNA polymerase and a thermostable proofreading enzyme	Two types of 10X buffer (22.5 mM $MgCl_2$) are supplied with an extra vial of $MgCl_2$ for optimization. dNTPs are included	Up to 12 kb >12 kb[b]
Advantage™ 2 PCR Kit	Clontech	Advantaq™ DNA polymerase, proofreading enzyme and Taqstart antibody	*Advantage* 2 PCR buffer, dNTP mix, control DNA, and primer mixes. PCR-grade water	Up to 18.5 kb
Advantage Genomic and GC Genomic Kit	Clontech	*Tth* DNA polymerase and a proofreading enzyme	Buffer, dNTPs, and controls (as above), magnesium acetate. In GC kit GC-melt reagent to aid in amplification of GC-rich templates	Up to 10 kb (complex templates) Up to 40 kb
Hot Tub DNA polymerase	Amersham Biosciences	DNA polymerase from *Thermus* ''ubiquitous''	10X buffer supplied[d]	>10 kb
Elongase® Amplification system	Invitrogen	*Taq* DNA polymerase and *Pyrococcus* species *GB-D* polymerase	Two types of 10X buffer (either 5 or 10 mM $MgSO_4$), dNTPs, control primers, and DNA. Water and oil	Up to 30 kb
TaqPlus® *Long PCR system*	Stratagene	*Taq2000*™ and *PFU* DNA polymerases	Two types of 10X buffer (low salt and high salt)	Up to 35 kb

[a]A 5′ exo-minus, N-terminal deletion of *Taq* DNA polymerase.
[b]Range of fragments amplified is dependent on what buffer is used.
[c]KOD HiFi DNA polymerase is a recombinant form of *Thermococcus kodakaraensis* KOD1 DNA polymerase.
[d]Amersham now supplies premixed nucleotide blends (Nucleix Plus PCR) that are functionally tested in long PCR.

concentration 50–250 ng. Phage template DNA range 0.1–100 ng.[5] Typical concentration 0.1–10 ng.

There is a broad diversity in LD-PCR master-mix compositions and this highlights the necessity to optimize each component in the master mix when developing an in-house method. Foord and Rose[9] reported initial parameters to be used as a starting point for developing LD-PCR protocols. Since then, an extensive range of LD-PCR commercial kits and DNA polymerase mixes have been developed, providing the user with a choice of starting reagents complete with DNA polymerases with their respective buffers, and occasionally dNTP mixes and controls (Table 1). This removes the need to optimize polymerase/proofreader mixes and the buffer composition (apart from Mg^{2+} concentration), thus saving both time and expense.

Like every other aspect of LD-PCR the thermal cycling parameters must also have extensive optimization, but the most widely used conditions are as follows:

Initial denaturation: 92–95°C for up to 2 min.

10–30 cycles of:

Denature: 92–95°C for 10–30 sec.
Anneal: >58°C (dependent on T_m of primers) for 30–60 sec.
Extension: 68°C (with DMSO/glycerol)/72°C (without DMSO/glycerol) for 3–15 min (dependent on size of fragment being amplified).

20 cycles of:

Denature: 92–95°C for 10–30 sec.
Anneal: >58°C for 30–60 sec.
Extension: 68°C/72°C for 3–15 min (with a 15- to 30-sec extension every cycle).
Final extension/polish: 68°C/72°C for 5–6 min.

Another method, based on the above parameters, utilizes primers that bind at 68°C, and so the anneal and extension steps are combined into one. Alternatively, other methods exclude the cyclic addition of 15–30 sec to the extension time and simply have a longer extension time throughout the procedure. All appear to work and it is recommended that different thermal cycling protocols be tried to achieve maximum efficiency.

To separate LD-PCR amplicons, standard gel electrophoresis is generally the method of choice for fragments below 20 kb. However, when differentiating larger amplicons (20–50 kb), gels must be of a lower agarose percentage and be longer to acquire a good resolution. For accurate separation and sizing of very large products (>50 kb), pulsed-field gel electrophoresis is recommended. Staining with ethidium bromide in conjunction with UV illumination is commonly used to visualize high molecular weight amplicons, although a large (entire) volume of the reaction mix is usually needed to achieve the required sensitivity.[9]

To avoid the hazards associated with ethidium bromide it is worth considering the use of the fluorescent stain sybr gold. Like ethidium bromide it binds to the minor groove in double-stranded DNA, and although it can be visualized using UV light it is optimal to use a blue light transilluminator (e.g., a Dark reader, Clare Chemical Research, Denver, USA). One drawback with sybr gold staining is that it can retard movement through a gel, and so it is essential to stain gels after they have been completed. To check amplicon specificity Southern hybridization can be performed, and although radio-labeled probes have been used, it is preferential to use fluorescent-labeled probes or biotinylated probes, which avoid the hazards of working with radioactive material (e.g., ^{32}P). The use of fluorescent-labeled uracil nucleotides has also been successfully performed[9] and is another alternative to be considered.

POINTS TO CONSIDER/LIMITATIONS

As with all molecular methods, the quality of the DNA/RNA is of particular importance, and this is highlighted in LD-PCR where obtaining high-quality DNA template is paramount. If the DNA is nicked or fragmented, then the size of amplicon that can be produced will obviously be limited, and if the DNA is impure, then the PCR amplification is going to be inhibited.

Many commercial kits that extract genomic DNA from a variety of starting materials are now available (as discussed in Inverse PCR). They provide DNA of high purity and length (average length 50–100 kb Genomic-tip 100/G columns, Qiagen, Crawley, UK)[13] and remove the need for time-consuming purification procedures e.g., pulsed-field, low-melting point agarose gel electrophoresis.

As for all PCR primer pairs, the quality of their design can only be conclusively demonstrated by experimentation, although if certain strategies are adhered to optimal theoretical design can be achieved. For LD-PCR, it is essential to minimize secondary structures, especially at the 3′ ends, as the formation of dimers/polymers will create a competition for reagents and reduce the concentration of the amplicon. Thus, it is important to ensure that the primers are of the highest specificity to minimize nonspecific amplification and prevent the design of a competitive PCR. This can be achieved by design of longer primers, although there is a balance between specificity and optimal amplification, and reports vary as to the optimal length for LD-PCR. In general, optimal primer

length is similar to that for normal PCR (18–25 bp),[4,9] although longer primers (27–33 bp) have been shown to reduce the frequency of failed LD-PCR reactions.[5] The concentrations of LD-PCR primers are similar to a normal PCR (0.1–1.0 μM), although increased concentrations (4 mM) have been used, in an attempt to reduce the annealing time required.[9] However, at higher concentrations this benefit may be negated by the formation of secondary structures and the possibility of mispriming. For further information on primer design, see Foord and Rose.[9]

When LD-PCR was initially developed, the use of many individual polymerases was evaluated and r*Tth* DNA polymerase provided a consistent and reproducible amplification.[4,7,9] As the LD-PCR technique progressed, it was discovered that the use of a polymerase/exonuclease mix could benefit the LD-PCR amplification,[4,5,7,12] but had the drawback of determining optimal enzyme concentrations and buffer solutions. Since then, a variety of commercial LD-PCR kits and enzyme mixes are available (Table 1), providing optimal relative enzyme concentrations and buffers. Recently, the use of *Escherichia coli* exonuclease III was found beneficial when trying to perform LD-PCR. It permitted amplification of moderately damaged DNA templates when added to the polymerase mix, although it had no benefit where extensive damage had occurred.[15]

Typical buffer compositions have been stated above and generally are similar to normal PCR buffers except concentrations are nonlimiting. Certain protocols report the use of Tricine instead of Tris–HCl because its pK_a is less temperature dependent, and so provides DNA with a greater protection against nicking and depurination.[7] By adding certain cosolvents, i.e., glycerol (up to 8%), DMSO (1–3%) or a mix of the two (5% of each), the melting and strand-separation temperature can be lowered. This can increase the length of amplification by 10 kb by facilitating denaturation[4] and minimizing the exposure of any heat-labile components (DNA template) to extended high temperatures.[5]

CLINICAL AND GENETIC APPLICATIONS

LD-PCR has extensive uses as a supplement to cloning, as a time and cost-saving alternative, or where the DNA sequence is unclonable. They include the following:

1. Detection of DNA mutations such as:
   a) deletions within human mitochondrial DNA (mtDNA)[12] and viral genomes.[16]
   b) insertions, e.g., duplications, in human mtDNA.[17]
   c) rearrangements, e.g., inversions in disorders such as hemophilia.[8,18]
2. Chromosomal and genetic analysis of single cells for possible medical, forensic, and archaeological purposes.[11]
3. Typing of genetic markers for genome diversity studies.[14]
4. It has now been adapted to perform long-distance RT-PCR[19] and long-distance vectorette PCR.[20]
5. Fingerprinting of organisms by amplification of specific interspersed repetitive elements.[9]
6. Characterization of unknown, unclonable, long DNA sequences.[4]
7. Analysis of viral heterogeneity to aid in drug design and viral association in certain disorders.[7]

CONCLUSION

PCR is an important tool in many aspects of molecular biology, and in association with cloning is imperative in identifying and sequencing novel genes and genomes. However, it is limited by the size and fidelity of the amplicon generated and because of this, LD-PCR was developed permitting the accurate amplification of long DNA sequences (>40 kb).

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Long QT Syndrome

Carlo Napolitano
IRCCS, Fondazione S. Maugeri, Pavia, Italy

Silvia G. Priori
IRCCS, Foundazione S. Maugeri, and University of Pavia, Pavia, Italy

INTRODUCTION

The long QT syndrome (LQTS) is an inherited arrhythmogenic disease occurring in the structurally normal heart that may cause sudden death and that usually manifests in children and teenagers. The estimated prevalence of this disorder is between 1:10,000 and 1:5000. Two major phenotypic variants have been originally described in early 1960s one autosomal dominant, the Romano–Ward syndrome[1] and one autosomal recessive, the Jervell and Lange-Nielsen syndrome. Both variants share a common cardiac phenotype but Jervell and Lange-Nielsen syndrome is also associated with neurosensorial deafness. The Romano–Ward syndrome accounts for the vast majority of LQTS cases.[2] In early 1990s, the discovery of the genetic bases of LQTS provided novel tools for the understanding of the pathophysiology of this disease. The most relevant genetic and phenotypic features will be reviewed in this article.

CLINICAL MANIFESTATIONS

The LQTS is caused by a genetically determined abnormality of cardiac excitability. As a result, the affected patients have prolonged repolarization (QT interval at the surface electrocardiogram), abnormal T-wave morphology, and life-threatening cardiac arrhythmias, often with the typical pattern of ''torsades des pointes''[2] (Fig. 1). Syncope and a high risk of sudden death are the main features. The mean age of onset of symptoms is 12 years and earlier onset of symptoms is usually associated with a more severe form of the disease. Cardiac events are often precipitated by physical or emotional stress even if in a smaller subset of individuals, cardiac events occur at rest.[2]

CLINICAL MANAGEMENT

The link between the onset of cardiac events and increased adrenergic activity suggested the use of beta-blockers as a therapy for LQTS.[2] The clinical efficacy of this approach was confirmed in an international collaborative project.[3] Left cardiac sympathetic denervation (LCSD) might be an option when beta-blockers fail or are not tolerated.[4] Cardiac arrest survivors are always to be considered a high-risk subgroup. Therefore, the implant of an implantable cardioverter defibrillator (ICD) is recommended in these patients.

GENETIC BASES OF LONG QT SYNDROME

The discovery of the genetic basis of LQTS started in the early 1990s with the mapping of four LQTS loci on chromosomes 11, 3, 7, and 4[5] (OMIM IDs: 192500, 152427, 603830, 600919) (Table 1). Subsequently, the gene on chromosome 11 (LQT1) was identified as *KCNQ1* by Wang et al.[6] using positional cloning whereas the candidate gene approach led to the identification of *KCNH2* and *SCN5A* as the genes on chromosomes 7 (LQT2) and 3 (LQT3).[7,8] More recently, mutations in two additional genes on chromosome 21, *KCNE1* (LQT5; OMIM id: 176261) and *KCNE2* (LQT6; OMIM id: 603796), were reported. All the LQT1-3 and LQT5-6 genes encode for cardiac ion channel subunits. Based on these evidences, LQTS was initially considered a disorder specifically affecting plasmalemmal ion channel controlling the cardiac excitability. However, recent data show that the gene of LQT4 is *ANK2*: an intracellular protein called ankyrin involved in ion channels anchoring to the cellular membrane (see below).[9]

MOLECULAR EPIDEMIOLOGY

KCNQ1 (LQT1) and *KCNE1* (LQT5)

KCNQ1 and *KCNE1* encode, respectively, for the alpha (KVLQT1) and the beta (MinK) subunit of the potassium channel conducting the I_{Ks} current, the slow component of the delayed rectifier current (I_K), and the major repolarizing current during phase 3 of the cardiac action potential.

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Table 1 Clinical and genetic variants of LQTS

	Symbol	OMIM ID	Gene	Protein	Function
Romano–Ward	LQT1	192500	*KCNQ1*	KvLQT1	I_{Ks} alpha subunit
	LQT2	152427	*KCNH2*	HERG	I_{Kr} alpha subunit
	LQT3	603830	*SCN5A*	NaV1.5	I_{Na} alpha subunit
	LQT4	600919	*ANK2*	Ankyrin B	Anchoring protein
	LQT5	176261	*KCNE1*	MinK	I_{Ks} beta subunit
	LQT6	603796	*KCNE2*	MiRP	I_{Kr} beta subunit
Jervell and Lange-Nielsen	JLN1	220400[a]	*KCNQ1*	KvLQT1	I_{Ks} alpha subunit
	JLN2	–	*KCNE1*	MinK	I_{Ks} beta subunit

[a]OMIM database has only one entry for JLN1 and JLN2.

LQT1 is the most prevalent genetic form of LQTS accounting for approximately 50% of genotyped patients. Several different mutations have been reported (http://pc4.fsm.it:81/cardmoc) and in vitro expression studies of mutated proteins suggested multiple biophysical consequences, all of them ultimately inducing a loss of function, with or without a dominant negative effect on the wild-type protein.[10] Homozygous or compound heterozygous mutations of *KCNQ1* also cause Jervell and Lange-Nielsen form of LQTS (JLN1).[11]

KCNE1 (LQT5) mutations are rather infrequent accounting for approximately 2–3% of genotyped LQTS patients and they may cause both Romano–Ward (LQT5) and Jervell and Lange-Nielsen (JLN2) syndromes.[12,13]

KCNH2 (LQT2) and *KCNE2* (LQT6)

KCNH2 and *KCNE2* gene encode, respectively, for the alpha (HERG) and the beta (MiRP) subunit of the potassium channel conducting the I_{Kr} current, the rapid component of the cardiac delayed rectifier (Fig. 1). LQT2 is the second most common variant of LQTS accounting for 35–40% of mutations (http://pc4.fsm.it:81/cardmoc). Functional expression studies have demonstrated that *KCNH2* mutations cause a reduction of I_{Kr} current,[14] but, similarly to LQT1 mutations, this effect is realized through different biophysical mechanisms.[10] Mutations in the *KCNE2* gene[15] cause the LQT6 variant of LQTS, which is the most uncommon subtype of the disease (<1%).

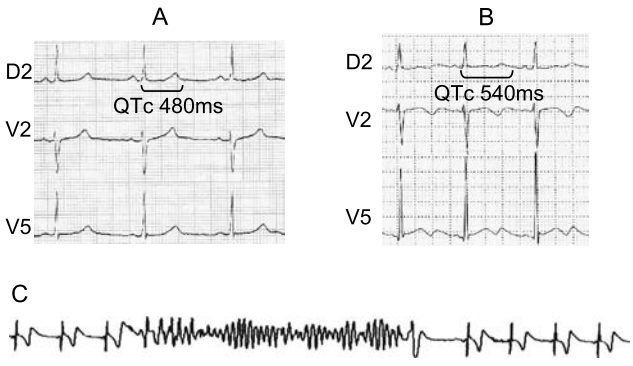

Fig. 1 Electrocardiogram (ECG) in the long QT syndrome. Examples of resting ECG in patients with a KCNQ1 mutation (A) and a KCNH2 mutation (B). Panel C shows a typical self-terminating polymorphic ventricular tachycardia in a LQTS patient. The arrhythmia was recorded by means of an insertable loop recorder (Reveal™).

SCN5A (LQT3)

SCN5A encodes for cardiac sodium channel conducting the sodium inward current (I_{Na}). The first reported *SCN5A* mutations[8] were clustered in the regions functionally associated with channel inactivation. Subsequently, several allelic variants have been reported (http://pc4.fsm.it:81/cardmoc) and functional expression studies showed that, at variance with LQT1- and LQT2-asociated mutations, LQT3 defects cause a gain of function with an increased I_{Na}.[16] Interestingly, *SCN5A* mutations are also found in other clinical syndromes allelic to LQT3: Brugada syndrome, cardiac conduction defect, and sinus node disease (for a review see Ref. [17]). The prevalence of LQT3 among LQTS patient is estimated to be 10–15%.

ANK2 (LQT4)

As of today, only one family linked to this locus (4q25–q27) has been reported.[5] Of note, the phenotype of the LQT4 patients differs from the typical LQTS. Most of the affected individuals, besides QT interval prolongation, also present with severe sinus bradycardia, paroxysmal atrial fibrillation (detected in >50% of the patients), and polyphasic T waves. Recently, a missense mutation in the *ANK2* gene was identified in this family.[9] *ANK2* encodes for an intracellular protein (ankyrin) that regulates the proper intracellular localization of plasmalemmal ion channels (calcium channel, sodium channel, sodium/calcium exchanger), sarcoplasmic reticulum channels

(ryanodine receptor, inositol triphosphate receptor), and other adhesion molecules.[18]

MOLECULAR SCREENING OF THE LQTS GENES

The primary consequence of the large heterogeneity of LQTS is that the vast majority of probands have their own ''private mutation'' with no or few hot spots so far identified. Therefore, the molecular screening of the entire ORF on the known gene is the only available option. In order to complete the analysis, 16 exons of *KCNQ1*, 15 exons of *KCNH2*, 28 exons of *SCN5A*, and 2 exons of *KCNE1* and *KCNE2* must be completely screened. *ANK2* encompasses 46 exons, but a routine analysis did not indicate that the only LQT4 family so far reported has a very peculiar phenotype (see above).

A fast and standardized screening methodology is still not available. Denaturating high-pressure liquid chromatography (DHPLC) and DNA sequencing are the most widely used techniques among laboratories offering LQTS genotyping.

Nonetheless, the number of cardiologist and geneticists requesting the screening is increasing and it is therefore important to make few relevant statements concerning implications and value of genetic screening. The major implication of finding the molecular alteration in LQTS lies in the possibility to obtain information that adds to the phenotypic assessment. This is true especially for LQT1, LQT2, and LQT3 genetic variants where a genotype-based clinical management is progressively entering the clinical practice.

When the genetic defect is identified in a proband, genetic screening could be made available to family members. In this step, genetic testing moves from being a method that confirms and refines a known diagnosis to being a diagnostic technique may be applied to healthy individuals (nonpenetrant carriers). Learning to be a ''gene carrier'' may expose asymptomatic individuals without clinical phenotype of the disease to the evidence that they carry and may transmit a potentially fatal disease. Counseling these patients is very difficult. It is recommended that before undergoing genetic testing these individuals are informed about the implication of a positive result.

GENOTYPE–PHENOTYPE CORRELATION

Electrocardiogram and Cardiac Events

In the last few years, several studies have outlined the distinguishing features of the three most common genetic variants of LQTS (LQT1, LQT2, LQT3), while the low prevalence of the other genetic variants so far prevented a precise characterization of the associated phenotypes.

Gene-specific EGC features have been initially described by Moss et al.[19] and subsequently refined by Zhang et al.[20] Schwartz et al.[21] have provided evidence that LQT1 patients present 97% of cardiac events during physical activity as opposed to LQT3 who present the majority of cardiac events at rest. Furthermore, it has been proposed that auditory stimuli and arousal are a relatively specific trigger for LQT2 patients, whereas swimming is a predisposing setting for cardiac events in LQT1 patients.[22,23]

Natural History and Risk Stratification

The increased availability of data collected among genotyped LQTS patients has allowed developing risk-stratification models based on the genetic substrate. Zareba et al. in 1998 provided the first gene-specific assessment of the natural history of the disease.[24] Such observations were further refined by Priori et al.[25] who also provided the first risk-stratification scheme based on the genotype. This study showed that the QT interval duration, the genotype, and the gender are significantly associated with the outcome. A QTc interval >500 msec determines a worse prognosis. However, LQT1 patients have a better prognosis vs. LQT2 and LQT3 with similar QT. Gender has no influence among LQT1 patients, whereas a higher risk was identified for LQT2 females and LQT3 males.[25]

Recent, still preliminary evidences suggested that risk stratification may be further refined when the position of a mutation on the predicted protein topology is taken into consideration. Among others, epidemiological findings from the International LQTS Registry[26] showed that LQT2 patients with mutation in the pore region are at greater risk of cardiac events (syncope and cardiac arrest) than patients with nonpore mutations.

LOW PENETRANCE AND VARIABLE EXPRESSIVITY IN LQTS

Likewise, as in other mendelian diseases, LQTS is characterized by a high degree of genetic heterogeneity and variable penetrance that translates into a range of possible phenotypes including subclinical forms with borderline of QT interval and no arrhythmias or syncope.[27,28] The demonstration of incomplete penetrance in LQTS leads to the speculation that carriers' subclinical mutations, although not manifesting any phenotype, may be more susceptible to develop arrhythmias when exposed to precipitating factors. The prevalence of such variants in the population is not known, but

it is appealing to speculate, and preliminary evidences exist,[29] that these defects may have a role in modulating the phenotype and may create a vulnerable substrate.[15,30]

GENETIC DIAGNOSIS AND COUNSELING

Long QT syndrome genotyping is progressively entering in the clinical practice. The availability of the genetic diagnosis may allow a more thoughtful clinical management and risk stratification. Genetic counseling should be regarded as a fundamental component of the clinical management, and the availability of effective therapeutic options is the main reason to encourage an early identification of all gene carriers in a family.

Unfortunately, gaps of knowledge and availability still exist and limit a wide spread applicability of genetic testing.

The ''knowledge'' issue is represented by the fact that the molecular screening of the entire open reading frame of the known genes allows identifying a pathogenetic mutation in 60–65% of probands. This implies that there is one or more LQTS gene(s) still to be identified, but it also leads to the practical consequence that a negative result from a molecular genetic lab has no capability of excluding the diagnosis.

On the other hand, the limited availability of laboratories offering clinical genetic testing for LQTS and the lack of fast and low-cost screening methodologies constitute additional limitations.

CONCLUSION

In the last decade, major achievements have been made in the understanding of the genetic basis of LQTS, the first inherited arrhythmogenic disease to enter the ''molecular era.'' Thanks to the contribution of several research laboratories pioneered by the seminal work of Mark Keating, it is now clear that the LQTS phenotype is the common pathway of mutations in several different genes.

Genotype–phenotype correlation analysis showed gene-specific distinguishing clinical features and risk of life-threatening cardiac. However, the power of such studies is partially limited by the variable penetrance identified in several families.

Looking to the future and trying to foresee directions of research, one would certainly anticipate that studies should be directed at the identification of factors (genetic or epigenetic) that may modify the penetrance and the clinical manifestations of the disease in patients with the same primary genetic defect. A natural extension to this approach would be the definition on the prevalence of these modifiers in the general population to define their role in arrhythmogenesis.

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Loop-Mediated Isothermal Amplification (LAMP)

Kentaro Nagamine
Tsugunori Notomi
Eiken Chemical Co., Ltd., Tochigi Pref., Japan

INTRODUCTION

To perform molecular biology studies based on the analysis of nucleic acids such as DNA and RNA, nucleic acid amplification technology is necessary because the gene content of almost all specimen is extremely low. Because these methods can be useful in the diagnosis of infections and genetic diseases, including predictive genetic and pharmacogenetic testing, diagnostic laboratories including hospital laboratories frequently use such technology as molecular diagnostic tools. In the medical field, amplification technology will play an important role in facilitating point-of-care testing to improve patient care. To widely apply such technology for practical use in the medical field, low cost and rapid performance are important. To achieve a cost-effective method, the apparatus being used must be simple. Moreover, the time from the start of a reaction until the result is obtained should be no more than 30–60 min. For example, methods amplifying genes under isothermal conditions have been developed (see other sections), but they require more than one enzyme and present difficulty in the selection of suitable reaction conditions.

To solve such problems, we have developed a new method of gene amplification. This method is named loop-mediated isothermal amplification (LAMP), and it involves gene amplification under isothermal conditions using only one enzyme with a high specificity, which is of particular importance for diagnostic techniques. Indeed, LAMP can be used for a direct detection of *Mycobacterium tuberculosis* in clinical diagnostic applications. However, compared to other methods [including polymerase chain reaction (PCR)], LAMP seems to be much less frequently used in laboratory diagnostics, because it was only developed recently or the principle and the primer design are a little complex (although the experimental procedure is simple).

In this section, we introduce the features and principles of LAMP, including the rapid method using a loop primer and single nucleotide polymorphism (SNP) typing.

FEATURES OF THE LAMP METHOD

Amplification Under Isothermal Conditions

The LAMP method, which differs from conventional methods of amplification, has several advantages.[1] Although there are other methods that can amplify genes under isothermal conditions, only the LAMP method does so with the aid of a single enzyme. Because no special reagents are required, suitable reaction conditions can be easily established and the procedure is inexpensive. For the LAMP method, the reaction, which used betaine at a temperature of around 60°C, destabilizes the double-stranded structure of nucleic acids, so this reaction can be initiated without previously denaturing double-stranded DNA.[2]

High Specificity

Conventional methods of gene amplification determine specificity on the basis of two regions, whereas the LAMP method requires six regions and four primers for gene amplification. A very high specificity of gene amplification ensures highly reproducible results, so that the presence of the target nucleic acid in the specimen is judged just by the amplification reaction.

Direct Gene Amplification From RNA

When the target is ribonucleic acid (RNA), complementary DNA (cDNA) is usually synthesized by using reverse transcriptase before amplification reaction is performed.[1] Because reverse transcriptase also substitutes the synthesized strands, LAMP is capable of amplifying a gene from RNA in exactly the same way as from DNA when reverse transcriptase is added to the reaction system.

Rapidity and High Sensitivity

Because the LAMP method has a high specificity, the target gene alone can be amplified with high efficiency. In

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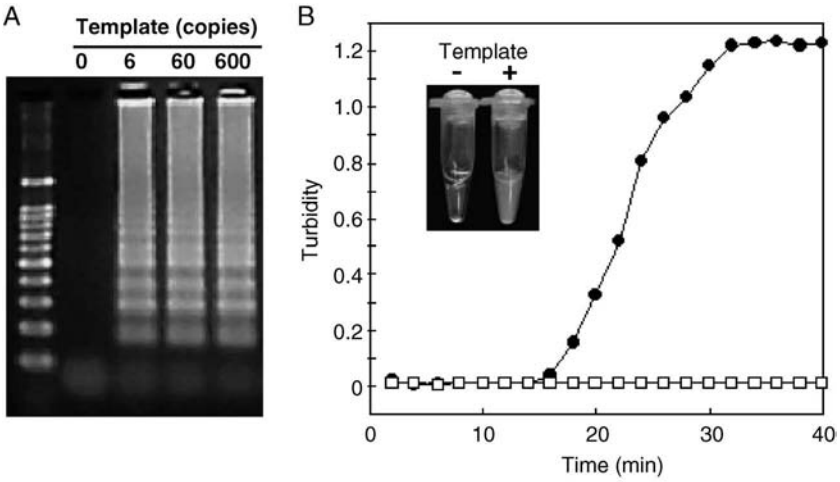

Fig. 1 Detection of LAMP products. (A) Electrophoresis of the LAMP products. Various copy numbers of HBV DNA were amplified at 60°C for 60 min. (B) Real-time detection using turbidity. The LAMP reaction was conducted with 6000 copies DNA at 65°C. The inset shows the tube after reaction. (*View this art in color at www.dekker.com.*)

fact, it is possible to detect a target gene within 1hr. We have also developed a method of rapid gene amplification by adding new primers called loop primers to amplify the loop region of products with which the inner primers do not anneal.[3] The LAMP method, like PCR, has a sensitivity of a few copies (Fig. 1A). Because a strand substitution reaction is used for the synthesis, no amplicon leads to product inhibition during the amplification, so that the yield of the products reaches as high as 0.5 mg/mL.

Easy Detection

The reliability of results is of critical importance when gene amplification is performed for testing and diagnosis. With conventional methods, a separate detection reaction is required after amplification to confirm the gene that has been amplified. This adds to the complexity of genetic testing. On the other hand, the LAMP method accomplishes very specific gene amplification and the results can be judged directly. Furthermore, because it produces amplification products in far larger quantities, a simple method of detection can be selected. For example, if gene amplification is performed in the presence of a fluorescent intercalating dye such as ethidium bromide (EtBr), which binds to double-stranded nucleic acids, amplification products can be detected with the aid of an ultraviolet lamp. The efficiency of amplification is so high that pyrophosphoric acid is formed in large quantities as a product of DNA synthesis. When it combines with magnesium, turbidity is produced. Gene amplification can therefore be confirmed by visual detection of turbidity or a white precipitate.[4] Turbidity can also be measured so that gene amplification can be detected in real-time (Fig. 1B).

PRINCIPLE OF THE LAMP METHOD

Original LAMP Method

The LAMP method is performed with two inner primers, forward inner primer (FIP) and backward inner primer (BIP), and two outer primers, F3 and B3 primers. Forward inner primer has the same sequences as the F2 region and the F1C region of the target DNA, while BIP has the same sequences as the B2 region and the B1C region. The mechanism of the method and the expected reaction steps are illustrated in Fig. 2. The reaction involves three steps—''stating material producing step,'' ''cycling amplification step,'' and ''elongation step.''

First, for stating material producing step, the inner primer BIP hybridizes to B2c in the target DNA and initiates complementary strand synthesis (structure 1). The outer primer B3, which is a few bases shorter and is present at a low concentration, slowly hybridizes to B3c in the target DNA and initiates strand displacement DNA synthesis that releases a BIP-linked complementary strand (structure 2). Next, the inner primer FIP hybridizes to F2c in the released single-stranded DNA, and outer primer F3 hybridizes to F3c, releasing DNA as a dumbbell-like structure (structures 3 and 4).

The dumbbell-like DNA (structure 4) is quickly converted to stem–loop DNA (structure 5) by self-primed DNA synthesis. This stem–loop DNA then serves as the starting material for LAMP cycling, which is the second stage of the reaction (cycling amplification step; structures 4, 5, 6, and 7).

The DNA derived from structure 5 is also involved in ''elongation step'' of the reaction. Thus the 3′ region of structure 5 is released by BIP-primed DNA synthesis,

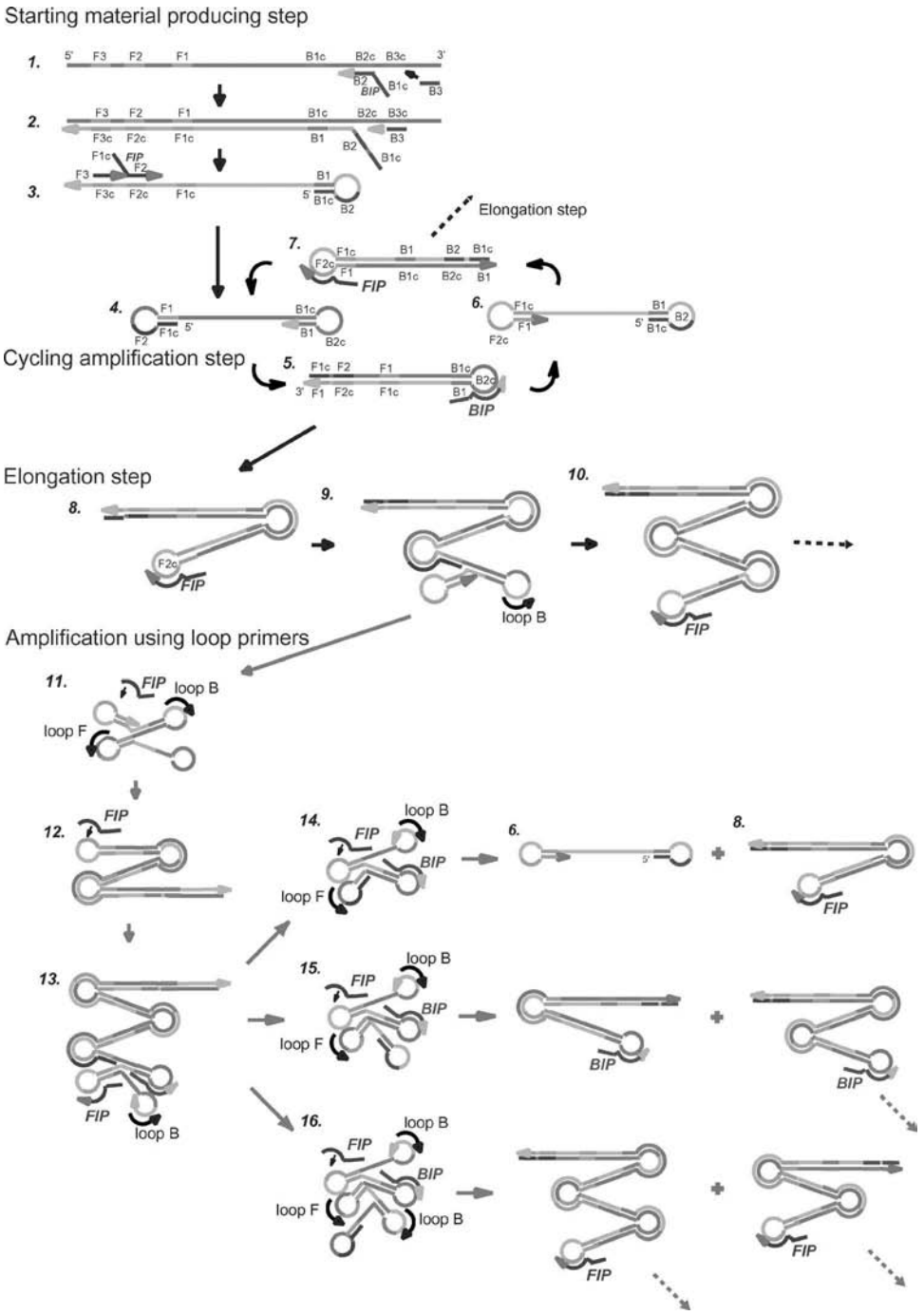

Fig. 2 Schematic representation of the LAMP mechanism. In the first step, the starting structure is generated with dumbbell-like DNA (structure 4) being formed. Then, the starting structures are cyclically produced in cycling amplification step. The elongation reaction starts from the subproducts (structures 5 and 7) of the cycling amplification step, generating products of various sizes. F2c and B2c regions on the target gene are hybridized by the inner primers. Structure 11 is generated by the reaction starting from the loop primers. The region between F1 and F2 (or B1c and B2c) is hybridized by the loop primer. Dotted arrows represent continuous reactions. (*View this art in color at www.dekker.com.*)

and then self-primed DNA synthesis generates stem–loop DNA with a stem that is elongated to twice as long (structure 8). This reaction also occurs from structure 7 (following reactions not shown). Because the stem loop—including the single-stranded region containing the target sequence of the primer—is present in each structure, this reaction continues until the decay of the substrates or primers. The final products are a mixture of

stem–loop DNAs of various stem lengths and cauliflower-like structures, with multiple loops induced by annealing between alternately inverted repeats of the target sequence on the same strand. When the products are electrophoresed on agarose gel, followed by staining with an intercalating dye such as EtBr, many bands of different sizes are detected (Fig. 1A).

Rapid LAMP Method

To accelerate the LAMP reaction, loop primers act from the elongation steps of the original LAMP reaction (Fig. 2, lower). The loop primer called loop B hybridizes to single-stranded loop region of the stem–loop DNA in structure 9 before initiating strand displacement DNA synthesis. Then, DNA synthesized from this loop primer is displaced by extension from the 3′ end of the target DNA, generating structure 11, which is not created in the original LAMP reaction. Structures 6 and 8 generated by loop primers are also obtained in the original method and are reused in the LAMP reaction. All of the stem–loop DNAs are used by either the inner or loop primers, e.g., structures 14, 15, and 16. The loop primers hybridize to the stem–loop region in structure 9, as well as in the other structures that are generated by the LAMP reaction, resulting in dramatic amplification.

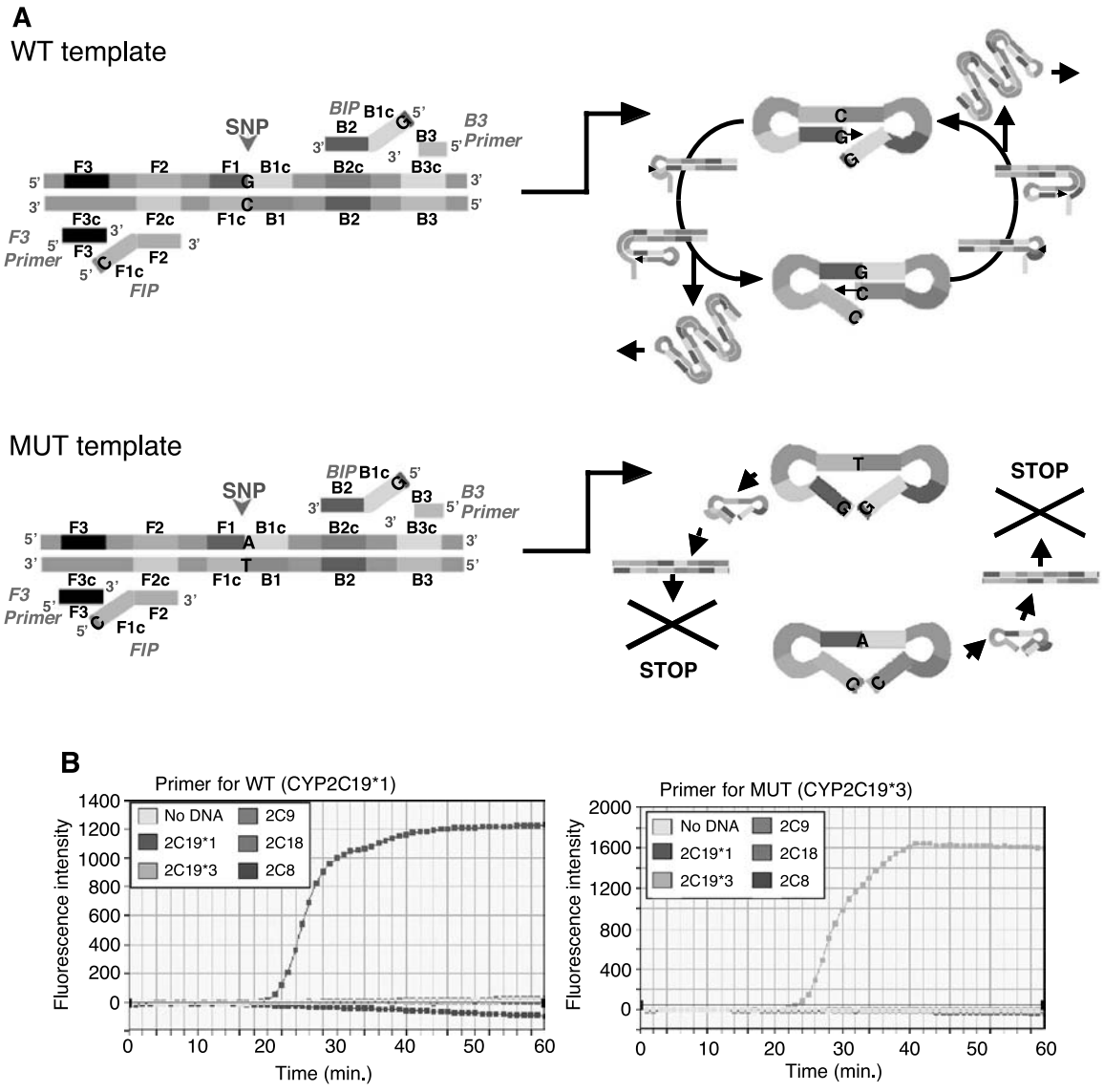

Fig. 3 The principle of SNP testing using the LAMP method, and SNP typing of CYP2C19. (A) Schematic representation of the SNP typing mechanism. Upper and lower panels show the reactions using wild-type and mutant templates, respectively. B: Real-time detection of SNP in CYP2C19. The CYP2C19*1 (636G) primer (left) and CYP2C19*3 (636A) primer (right) were used for the LAMP reaction. (*View this art in color at www.dekker.com.*)

SNP TYPING USING THE LAMP METHOD

To detect a single nucleotide difference by LAMP-based SNP typing, both FIP and BIP were designed to contain SNP nucleotides on the target sequence at each 5′ end. Figure 3A shows the basic principles of the LAMP cycling reaction with the WT primer set, using WT DNA (Fig. 3A, upper) and MUT DNA (Fig. 3A, lower) as templates.

When LAMP reaction using the WT primer set is carried out with WT DNA as the template, a dumbbell-like structure is generated with both ends being overlapped by self-annealing (Fig. 3A, upper). This dumbbell-like structure served as the starting point for cycling amplification step (Fig. 2, structure 4 or 6). DNA synthesis is initiated from the 3′ end, using the structure itself as the template (similar to the original LAMP method). As a result, DNA strands consisting of inverted repeats of the target sequence are formed. When MUT DNA is used as the template, the 3′ end of the dumbbell-like structure cannot self-anneal completely at its end (Fig. 3A, lower). Thus DNA synthesis is only primed from the 3′ end of FIP or BIP that anneals to the loop region of the structure, resulting in the formation of double-stranded DNA and the halt of the LAMP cycling process. When DNA synthesis proceeded as a result of a miscopy and the dumbbell-like structure was produced, further DNA synthesis from the turnover structure is halted through the steps mentioned above. Because two primers are designed to recognize a single nucleotide difference of the dumbbell-like structure or its turnover structure, LAMP-based SNP typing could precisely discriminate single nucleotide differences.

To demonstrate the usefulness of LAMP-based SNP typing, we assessed the typing of CYP2C19, a gene belonging to the cytochrome P450 family and showing various polymorphisms. Although homology between the CYP2C subfamilies is very high, the LAMP method was capable of specifically amplifying CYP2C19 and detecting a single nucleotide difference during single-step amplification, because the method uses four primers that recognize six regions and detects a single-base difference after each cycle of amplification. To demonstrate the value of LAMP, CYP2C19*1 (WT), 2C19*3 (G636A), 2C9, 2C18, and 2C8 genes were used as templates and the reaction was carried out in the presence of an intercalater for real-time detection (Fig. 3B). Only 2C19*1 alone was amplified when the 2C19*1 primer was used, while the 2C19*3 gene with a single nucleotide difference and genes of other 2C subfamilies were not amplified (Fig. 3B, left). Similarly, only the 2C19*3 gene was amplified and genes of the other 2C subfamilies were not amplified when the 2C19*3 primer was used (Fig. 3B, right). These results indicate that the LAMP reaction can detect a single nucleotide difference on a target gene when LAMP-based SNP typing is performed with human genomic DNA in the presence of many homologous family genes.

CONCLUSION

The LAMP method bears the following features: 1) it can be performed under isothermal conditions; 2) the reaction can be accelerated by using a loop primer; 3) it generates large amounts of the amplified products; and 4) it can also amplify RNA by addition of reverse transcriptase with a single step.

Furthermore, the LAMP method can be used for SNP typing with a distinctive primer. Considering that SNPs are associated with constitutional factors, such as disease susceptibility, drug responsiveness, and adverse drug reactions, the LAMP method holds the promise of application to tailored medical care. The LAMP method can be used for tests in various fields such as the diagnosis of hereditary disease, identification of viral infections as point-of-care testing, sex determination (humans and other animals), and testing for contamination of food or water by bacteria and other agents.[5,6]

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MALDI-MS Coupled with Capillary LC and CE

Robert T. Kennedy
Kerstin Nolkrantz
University of Michigan, Ann Arbor, Michigan, U.S.A.

INTRODUCTION

Proteomics is made possible by the high-throughput detection, identification, and characterization of proteins in complex mixtures. High throughput refers to both the ability to detect many proteins per sample and to analyze many samples. While no method presently allows the rapid identification and quantification of all proteins in a mixture, intense research over the past decade has considerably accelerated the pace of protein measurements. Such measurements open the door to many applications in clinical and basic research.

Proteomics measurements rely on a combination of chemical separations, such as chromatography or electrophoresis and mass spectrometry (MS). For top–down proteomics, the proteins themselves are separated and then analyzed, whereas for bottom–up proteomics the proteins are first digested with enzymes; the resulting peptides are then separated and analyzed by MS. Peptides detected are used to identify proteins by bioinformatics. The classical proteomic analysis tool is two-dimensional gel electrophoresis (2-D GE). This technique is powerful, allowing as many as 10,000 proteins to be detected in a single analysis. Spots detected on gels can be identified by extraction from the gel followed by enzyme digestion and MS analysis. 2-D GE is hindered by two disadvantages: 1) it requires relatively large samples (10^{-13} to 10^{-12} mol); 2) it is slow and laborious, often requiring days for separation and much longer to extract and perform MS analysis of the spots.

Novel, automatable techniques that are faster and utilize miniscule samples are presently being developed to replace 2-D GE. One technique under investigation is microscale separation, such as capillary liquid chromatography (cLC) or capillary electrophoresis (CE), coupled with matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). In this approach, protein or peptide samples are resolved by cLC or CE and the fractions stored on the surface of a plate that is inserted into the mass spectrometer. Samples are examined by MALDI-MS to determine molecular weights and ultimately identify the protein or peptide. The method has been demonstrated to be able to detect as little as 10^{-18} to 10^{-15} mol of analyte in an automated, parallel format for high-throughput analysis. This potentially important proteomic technique is not presently commercially available, but is rapidly being developed. This article will discuss the state-of-the-art in coupling CE and cLC to MALDI-MS.

IONIZATION TECHNIQUES: MALDI AND ESI

MS provides highly accurate measurements of the mass-to-charge ratio (m/z) of gaseous ions in vacuum. The m/z can be used to calculate the mass of the ion and, from this, the identification of the species can often be deduced. Peptides and proteins are not volatile enough to form gaseous ions using traditional approaches; however, the advent of electrospray ionization (ESI) and MALDI has rendered these important analytes amenable to MS analysis and, along with several other innovations, led to the ongoing revolution in proteomics. In ESI, sample flows out of a capillary tube that has voltage applied at the outlet, resulting in the formation of a spray of sample droplets. As the droplets evaporate, ionized sample molecules are formed and introduced to the mass spectrometer. In MALDI, samples are cocrystallized with a radiation-absorbing molecule (matrix) of low molecular weight, such as α-cyano-4-hydroxycinnamic acid (CHCA) or 2,5-dihydroxybenzoic acid (DHB). Ionization is performed by exposing the sample to short bursts of radiation from a laser. Either ultraviolet (UV)-absorbing matrix with a UV-emitting laser (UV-MALDI) or infrared (IR)-absorbing matrix with an IR-emitting laser (IR-MALDI) can be utilized. The rapid heating caused by radiation absorption results in explosive volatilization of the matrix and formation of charged analytes in the gas state that can be transferred to the mass spectrometer. Usually, a tiny fraction of the sample is vaporized by a single laser shot, so signal from multiple shots are averaged to obtain good-quality spectra. MALDI-MS has a high mass range (routinely up to 30 kDa), good detection limits (attomol range), and high mass accuracy (0.01% at high mass).

MALDI is tolerant of additives (such as salts or surfactants) that might be contained in a sample, and is

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compatible with parallel analysis as discussed below. Because the sample is deposited on a surface in MALDI, the sample can be stored and reanalyzed. The latter feature enables data-dependent workflows, wherein results from an analysis can be used to determine the next type of experiment to perform on the sample. Experiments may include multidimensional MS (MS^n), wherein a selected ion is degraded in the gas state by irradiation with an infrared laser or collision with neutral molecules (collision-induced dissociation), resulting in dissociation of the ion into characteristic fragments, called product ions or daughter ions, which are then analyzed. The resulting spectra can be used like puzzle pieces to identify the parent ion. Alternatively, a sample may be chemically degraded or modified, such as digestion with an enzyme, and reanalyzed providing further information.

A significant weakness of MALDI is the difficulty of quantification. As the laser spot is focused on different regions of sample, the relative amounts of analyte detected will vary. In many cases, this effect can be quite extreme so that in some regions, no signal is detected and in others, the so-called ''sweet spots,'' strong signals are observed.

ESI and MALDI have proven to be complementary in many ways.[1] Perhaps, the most important reason for both MALDI and ESI to be developed is that they appear to be effective in ionizing different classes of peptides. Thus, in analysis of complex mixtures, detected peptides will vary significantly depending on the ionization method used.[1] Therefore, application of both methods may prove effective.

CAPILLARY SEPARATIONS COUPLED TO MS

While ESI and MALDI have enabled proteins and peptides to be analyzed by MS, the complex mixtures represented by clinical and other biological samples require fractionation prior to analysis. Without fractionation, competitive ionization induces only the species that most avidly take up charge to be detected in a sample, resulting in poor sensitivity for many ions; this effect is known as ion suppression. In addition, the mass spectra of unfractionated mixtures can be so complex as to defy interpretation even if extremely high resolution mass spectrometers are used. Resolution of mixtures by a separation technique prior to MS ameliorates these problems. Of the separation techniques available, cLC and CE are two that have received considerable attention for coupling to MALDI-MS.

cLC is comparable to high-performance liquid chromatography (HPLC), with the main difference being a much reduced internal bore of the column for cLC. Whereas an HPLC column may have an inner bore of 2–5 mm, a cLC column may have an inner bore of 25–300 μm. As mobile phase flow and sample volume requirements scale with the square of inner diameter (i.d.), this miniaturization results in much reduced sample consumption, increased sensitivity (for same amount injected), reduced operating costs (less mobile phase and disposal), and feasibility of parallel operation. Most of these advantages have been demonstrated in a research setting; however, commercial cLC systems are just now becoming available.

In CE, samples are separated by applying an electric field along a capillary tube, typically 25–75-μm inner diameter and 10–100 cm long, filled with buffer. The extremely narrow bore of the capillaries allows rapid heat dissipation, because of the high surface area/volume ratio. Because the limit to the electric field applied is typically set by heat buildup from current flow, the capillary arrangement allows electric fields (100–1000 V/cm) that are much higher than those observed in conventional gel electrophoresis (10–30 V/cm). As the resolving power and speed of an electrophoretic separation are directly dependent upon the field applied, CE allows for much faster and higher resolution separations than gel electrophoresis.[2,3] In addition, the extremely small inner diameter capillaries require only nanoliter volume samples, thus reducing sample consumption. In CE, samples are typically injected onto one end of the capillary and then detected near the opposite end. Electroosmotic flow (a type of flow generated in tubes with voltage applied) causes most analytes, regardless of charge, to migrate in the same direction—allowing detection at a single point. Analyte molecules are separated according to their electrophoretic mobility, which is a function of their charge and hydrodynamic radius in solution.

Coupling of liquid separation with MS is relatively straightforward when using continuous flow ionization methods such as ESI. Therefore, ESI has been the method of choice for coupling to modern separation methods, and many proteomic methods have been developed around this combination. MALDI requires samples to be crystallized with matrix, and it is conventionally a vacuum ionization technique. Therefore the coupling of liquid separation to MALDI-MS is more difficult, resulting in a longer development time. Most systems utilize off-line coupling, meaning that the sample is stored on a surface (target plate) that is later introduced to the MS. While off-line methods are seemingly cumbersome, they can present significant advantages such as: 1) independent optimization of separation and MS conditions, and 2) saving the sample after analysis and allowing reanalysis or other experiments such as protein digest to be performed later.

cLC–MALDI–MS

While several combinations of cLC–MALDI-MS have been reported, a system recently developed at the Genomics Institute of the Novartis Research Foundation is indicative of the state-of-the-art.[4] As illustrated in Fig. 1, the outlet of the cLC column is fixed over the target plate mounted on a motor–driven translation stage, allowing fractions to be collected at discrete locations on the plate. To control the deposition of a fraction, droplets formed on the column outlet are pulled down to the plate by application of a voltage pulse. Novel use of voltage to dislodge droplets allows complete and automated control of sample deposition and, because the column is not in contact with the plate surface, opportunities for cross-contamination and damage to the column or plate are minimized. The target plate consists of hydrophobic surface punctuated with an array of 400-μm-diameter wells containing a hydrophilic surface coating.[5] The use of a prestructured surface prevents a deposited fraction from spreading on the surface and confines it to the predetermined area. In addition, it allows for fairly large samples to be concentrated onto a small area because large droplets adhere to the hydrophilic spot and are confined to the spot as they evaporate. The small sample area allows the laser to irradiate the entire spot, eliminating the ''sweet spot'' effect. Matrix may either be added postcolumn via a Y-connector or precoated onto the target plate in a multistep deposition, depending on the properties of the matrix. This prototype instrument utilizes up to four cLC columns (300-μm i.d., 15 cm long) in an array. Because chromatography separations can take 10–100 min, the use of the array can substantially increase the throughput of analysis.

The performance of this instrument was demonstrated by analysis of a tryptic digest of bovine serum albumin using a Fourier-transform ion cyclotron resonance (FT-ICR) instrument as the MS. A sample of the three-dimensional data output with separation time, *m*/*z*, and signal intensity as the dimensions is illustrated in Fig. 2.

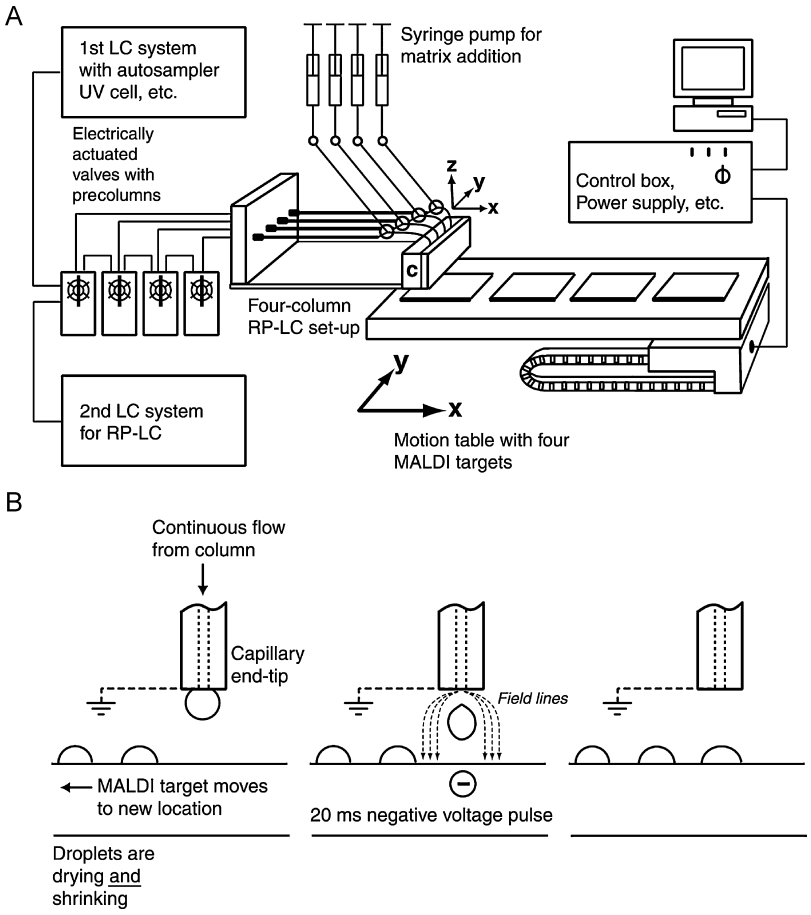

Fig. 1 Automatic deposition of effluent from cLC directly down to a MALDI target plate. A) The automated off-line system for cLC-MALDI. B) Deposition of the effluent from the LC column down to the target plate by application of a negative voltage. (From Ref. [4].)

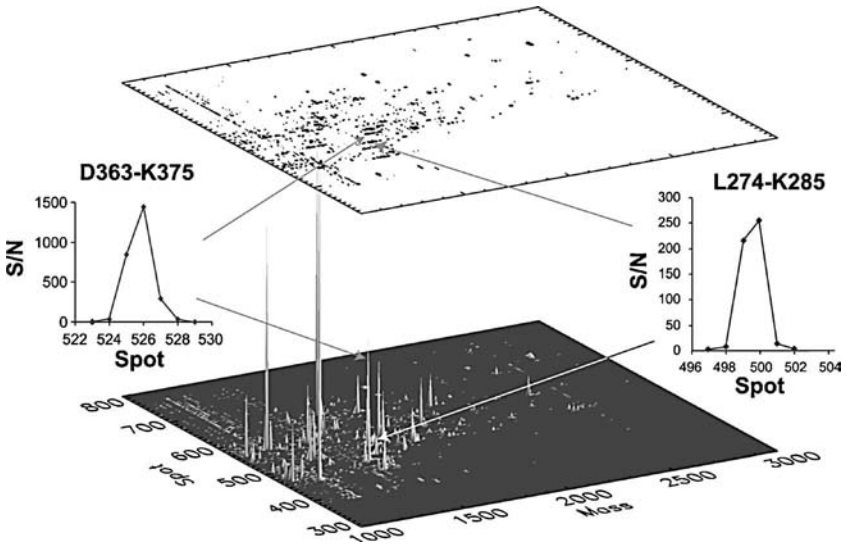

Fig. 2 3-D plot of LC/MALDI-MS analysis of 200 fmol BSA tryptic digest with elution profiles for two peptides (D363–K375 and L274–K285). 20 μL of a 10 fmol/mL digest solution was injected each onto four parallel columns. The effluent was deposited every 10 sec onto the target plate. A sequence coverage for BSA of 78% was obtained. (From Ref. [4].) (*View this art in color at www.dekker.com.*)

The detection limit for the instrument, when coupled to a time-of-flight mass spectrometer (TOF-MS), was better than 50 attomol. The instrument could collect up to 384 fractions from 4 columns, or 1536 fractions from a single column. This large number of fractions would make it possible for operators to obtain multiple fractions for all resolved analytes, because the peak capacity of an LC column, i.e., the maximum number of resolvable components, is typically $\sim$200.

Off-line coupling of cLC–MALDI-MS may be achieved in other ways as well. For example, the effluent can also be deposited as a continuous trace on the target instead of discrete spots.[6] In this case, the column effluent passes through a heated nebulizer that sprays the effluent directly onto the target plate. The continuous trace deposition method allows the full chromatographic peak capacity to be retained.

CE-MALDI

As with cLC–MALDI-MS, CE–MALDI-MS is still under development and several designs have been reported. Off-line deposition onto a continuously moving target was one of the earliest designs used.[7] In another type of off-line coupling, a piezoelectric flow-through microdispenser was connected to a CE system.[8] Pressure pulses cause the microdispenser to eject droplets with volumes of 60–100 pL collected at the target plate. This decreased the spot size to $\sim$400 μm in diameter, compared to a diameter of 1–4 mm for the dried-droplet method. The sensitivity increased as well as the detection limits (100 attomol for peptides).

More recently, on-line interfacing has been performed by depositing the capillary effluent onto a moving surface under vacuum inside the MS. Moving surfaces used include a rotating ball,[9] a wheel,[10] and a moving tape.[11] In the moving-tape design (Fig. 3), the CE capillary outlet is juxtaposed with an infusion capillary in a liquid junction. The liquid junction allows the circuit for the electrophoresis to be separated from the MS. In addition, the gap in the capillaries is used to mix matrix with capillary effluent. Flow from the separation capillary across the junction is maintained by electroosmosis (100–400 nL/min). The infusion capillary transfers sample and matrix mixture into the vacuum of the mass spectrometer and is in contact with the tape. Small volumes of deposited solvent are rapidly evaporated within the vacuum region, leaving fractionated sample crystallized with matrix deposited as a continuous trace 40 μm wide on the tape. The tape transfers the sample to the laser irradiation region for analysis by MALDI-TOF-MS. The laser spot is wider (100×100 μm) than the deposited trace; therefore, a full segment of the trace is irradiated with each shot. This arrangement yields extremely high sensitivity; detection limits of 10^{-18} mol for peptide standards demonstrated. In addition, the reproducibility of 15% was very good when compared to 20–90% for conventional MALDI experiments. The instrument is robust enough to operate for 24 hr without interruption. In principle, this

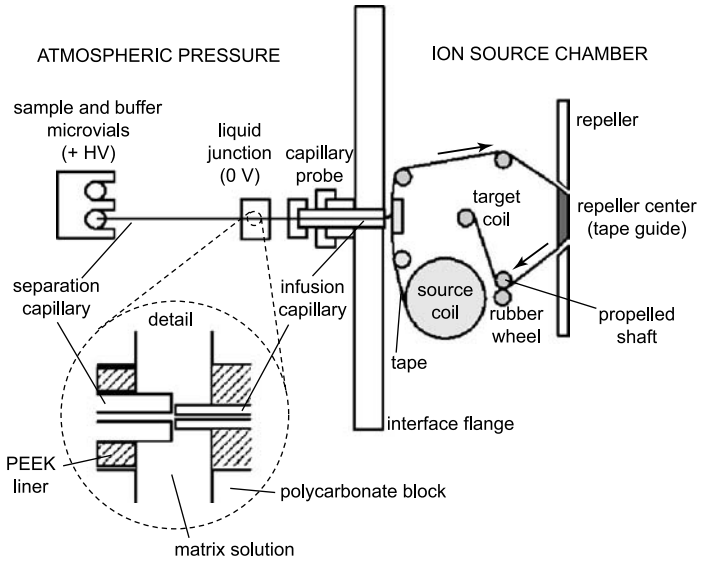

Fig. 3 Schematic of the on-line coupling of CE to MALDI. (From Ref. [11].)

system could also be operated in parallel with multiple capillaries and the laser directed toward different traces on the same tape.

CONCLUSION

It is likely that cLC and CE coupled to MALDI will continue to evolve, ultimately leading to commercial instruments and a plethora of new proteomic applications. cLC–MALDI-MS or CE–MALDI-MS is inherently compatible with other proteomics innovations, such as the isotope-coded affinity tag (ICAT) reagents for quantification and database searching for protein identification. It is anticipated that parallel operation will become the standard for high-throughput analysis. The advent of MALDI performed under atmospheric pressure[12] may facilitate simpler interfaces between the separation and the mass spectrometer. While MALDI is inherently a pulsed source lending itself to mass spectrometers acquiring rapid spectra, such as the TOF-MS, innovative ion optics have enabled the MALDI to be coupled to other spectrometers such as the FT-ICR (see above), quadrupole time-of-flight, and quadrupole ion trap. These instruments, along with the TOF–TOF, enable operators to perform MS^n, which provides much more powerful chemical identification capability than single-dimensional MS. In this regard, the off-line coupling schemes have a significant advantage over on-line methods in that time is available to acquire multiple spectra from a single fraction, whereas the on-line coupling schemes require on-the-fly spectra acquisition. A possible scenario for future development is off-line systems for detailed, complex analyses and on-line systems for high-throughput, routine proteomics applications.

The advent of such techniques would enable a variety of proteomics measurements, including top–down or bottom–up approaches, to be performed in a rapid, sensitive, and automated format. The methods described in this article are clearly more sensitive, automated, and faster than 2-D GE. The individual separation techniques such as CE and cLC have a peak capacity of $\sim$200, and cannot match the resolving power for 2-D GE. More extensive separation prior to MS analysis will likely be necessary for complete proteome analysis.

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ARTICLE OF FURTHER INTEREST

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MALDI-TOF Mass Spectrometry

Christian Jurinke
SEQUENOM, Inc., San Diego, California, U.S.A.

INTRODUCTION

Mass spectrometry (MS), in general, is concerned with generating, separating, and detecting gas-phase ions. The underlying concept goes back to 1913 and was demonstrated by Thomson[1] in a series of cathode ray tube experiments. One of the inherent limitations of MS is the need to convert the analyte molecules or atoms into gas-phase ions because electromagnetic forces can easily manipulate those and they can readily be detected. This necessity has hampered the widespread use of mass spectrometric techniques in the biological sciences because it usually results in severe decomposition of biological macromolecules. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS, as practiced today, goes back to a concept introduced in 1989 by Karas and Hillenkamp.[2] With the introduction of this technique, limitations resulting from desorption efficiencies for large biomolecules have been overcome. Consequently, the use of MS techniques is exploding and is becoming more and more a routine.

THE MALDI PROCESS

The main principle of MALDI goes back to the observation that in a laser-irradiated mixture of substances that differ in their absorption efficiency, the less efficient absorbing substance is already desorbed into the gas phase at less laser energy than would be required for this substance alone, if the more efficiently absorbing substance is provided in excess. This enables desorption of substances (e.g., large biomolecules) that would undergo fragmentation and decomposition if desorption is attempted without matrix assistance. In a typical MALDI experiment, the matrix is present in a 10^3-fold to 10^4-fold excess.

To conduct the MALDI process, the sample is deposited on a conductive sample support and cocrystallized with matrix. Typical matrix substances are small organic molecules (mainly acids) that have an absorption maximum at the laser wavelength and are able to efficiently cocrystallize with the analyte and to induce its ionization in the gas phase. The choice of the appropriate matrix is key to a successful MALDI-TOF experiment. Some common matrix compounds, their solvents, absorption wavelength, and the compatible analytes are listed in Table 1.

Several techniques have been reported to efficiently cocrystallize the matrix and the sample. The most commonly used is the so-called dried droplet method. Here, the sample is premixed with the matrix and the mixture is allowed to evaporate slowly at ambient temperature. Other techniques include the fast evaporation and sandwich matrix technique. For the fast evaporation method, a small amount of matrix (usually in a water–acetone mixture) is deposited on the sample. The acetone (or a comparable solvent) rapidly evaporates and leaves a homogeneous crystal surface. The sandwich matrix technique starts with a thin layer of matrix that is applied first, followed by the sample solution and an additional layer of matrix. There is a vast variety of variations of these techniques and usually the user will have to determine empirically which one is suited best for a particular application.

To induce desorption, the matrix sample cocrystals are irradiated with a nanosecond laser beam [e.g., from an ultraviolet (UV) laser with a wavelength of 266 or 337 nm]. Lasers used for MALDI-TOF purposes are mainly UV lasers [e.g., frequency-quadrupled neodymium/yttrium aluminum garnet (Nd/YAG) lasers], but nitrogen and CO_2 lasers are also in use. The use of infrared (IR) lasers is suitable for several applications and has been reported to successfully analyze large DNA fragments.[3] The energies introduced by the laser irradiation are in the range of 1×10^7 to 5×10^7 W/cm^2. This energy introduction causes a structural decomposition of the crystal and generates a particle cloud (the plume). The process of desorption can be described as a conversion of laser energy to vibrational oscillation of the crystal molecules. This results in the disintegration of the crystal. The plume contains ions that follow a Boltzmann distribution of their initial velocities. Out of the plume, ions are extracted by an electric field.

TOF-BASED SEPARATION AND DETECTION

Following acceleration, the extracted ions drift through a field-free path and finally reach a detector (e.g., a

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Table 1 Common matrices in use for MALDI-TOF MS

Matrix compound	Laser λ (nm)	Solvent	Analyte
2,5-Dihydroxybenzoic acid (DHB)	266, 337, 355	ACN[a], water	Peptides, oligosaccharides, oligonucleotides
Cinnamic acid	337	ACN, water	General purpose
α-Cyano-4-hydroxycinamic acid	337, 355	ACN, water, EtOH[b], acetone	Peptides, lipids, nucleotides
Sinapinic acid	266, 337, 355	ACN, water, chloroform, acetone	Lipids, peptides, proteins
3-Hydroxypicolinic acid (3-HPA)	337, 355	ACN, water, EtOH	Oligonucleotides
6-Aza-2-thiothymine acid (ATT)	266, 337, 355	ACN, water, MetOH[c]	Oligonucleotides, lipids
2,6-Hydroxyacetophenone	337, 355	ACN, water	Proteins, oligonucleotides
Nicotinic acid	266, 337, 355	Water	Proteins, oligonucleotides
Succinic acid	2940	ACN, water	Oligonucleotides, peptides

[a]Acetonitrile.
[b]Ethanol.
[c]Methanol.

secondary electron multiplier, or SEM). Ion masses (mass-to-charge ratios, m/z) are typically calculated by measuring their TOF, which is longer for larger molecules than for smaller ones (provided their initial energies are identical). In mathematical terms, the velocity (v) of desorbed ions that are accelerated through a constant electric field is inversely proportional to the square root of their mass-to-charge ratio.

The most prominent detectors that are used to analyze the ion beam resulting from a MALDI experiment are SEMs. The basic concept relies on the emission of so-called secondary electrons from a coated surface resulting from a strike of fast-moving ions. In these detection devices, a series of dynodes is connected together via a resistor chain. A high voltage is applied across the first dynode (conversion dynode) and the last dynode (anode). The impact of ions on the conversion dynode results in the generation of secondary electrons that are accelerated toward the second dynode where more secondary ions are emitted. This process is repeated multiple times and leads to an amplification of secondary ions at each successive step. The achieved gain in signal amplification is in the range of 10^7. It has to be noted that the sensitivity of these devices decreases with increasing analyte mass because the electron yield depends on the momentum of the incoming particle, not its energy. A commonly used version of the electron multiplier is the channel electron multiplier (CEM). The CEM provides a higher amplification gain and is more compact and less expensive. Another detector device is the channel plate that essentially is a multichannel version of the CEM. It consists of a multitude of fiber optic cables that provides an increased length-to-diameter ratio. In addition, photomultipliers that convert an incoming beam of ions into photons that are amplified by a conventional photomultiplier are used.

Because the MALDI process predominantly generates single-charged, nonfragmented ions, parent ion masses can easily be determined from the resulting spectrum without the need for complex data processing. The masses are accessible as numerical data for direct processing and subsequent analysis. TOFs measured during a typical MALDI experiment are in the range of microseconds. The MALDI process and instrumentation as described in the above paragraphs are illustrated in Fig. 1.

PERFORMANCE CHARACTERISTICS OF MASS SPECTRA

The most important performance characteristics for a MALDI-TOF spectrum are resolution and signal-to-noise (S/N) ratio. The resolving power of a mass spectrometer is its ability to distinguish between ions with slightly different m/z ratios. Usually, the resolution of a particular peak in the mass spectrum is expressed as the peak width measured at half of its maximum height (full width at half maximum, or FWHM). Numerically, resolution is expressed as $R = m/\Delta m$, or the ratio of the mass of one ion to the difference of a closely neighbored second ion. The intensity is given in arbitrary units or in relative units, where the most abundant signal is assigned the 100% value. The S/N ratio is an additional factor to characterize the quality of a mass spectrum. It is expressed as the ratio of a particular signal intensity to the average noise intensity. To increase sensitivity and/or S/N ratios, spectra can be summed up, which simply means that spectra from multiple laser shots are averaged. Typically, sensitivities are in the low femtomole to attomole range.

The quantitative abilities of MALDI-TOF MS are limited. Absolute quantitation of isolated signals cannot

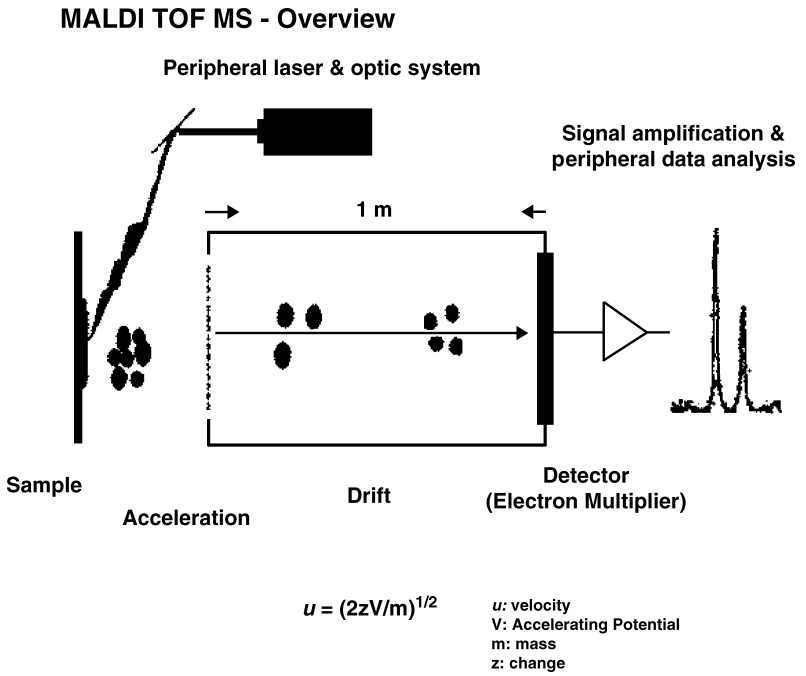

Fig. 1 Depicted is a schematic overview of the MALDI-TOF process. The text provides a detailed description.

be achieved. However, relative quantitation employing a reference standard with a known concentration is possible. Relative quantitation with MALDI-TOF is, for example, routinely used to analyze the allele frequency of single nucleotide polymorphisms (SNPs) in equimolar mixtures of genomic DNA (DNA pools). For more details, the reader is referred to the respective chapter in this encyclopedia.

ADVANCED TECHNIQUES AND INSTRUMENTATION

Important enhancements to MALDI-TOF experiments are provided by additional instrumentation. Examples for improving instrumentation are the use of delayed extraction methods and reflectron devices. The principle of delayed extraction is to have a small time window between the ionization pulse and the extraction pulse. During this time window, ions are allowed to disperse according to their initial velocities, and neutral (non-ionized) molecules that are generated during desorption are pumped away. Therefore ion–molecule collisions are minimized, which otherwise would contribute to a spread of translational energies. The application of delayed extraction[4] leads to significant increases in mass resolution (e.g., at 3000 Da from about 5000 without DE to over 10,000 with DE) and accuracy[5] (from about 100 ppm to about 10 ppm). Another important performance enhancement comes with the application of reflectron devices. A reflectron essentially is an electrostatic ion mirror. It consists of a series of electrical lenses with increasing repelling potential. The reflectron corrects for initial energy and positional dispersions of isomass ions. Faster-moving ions spend less time in the drift region but enter to a greater depth into the reflecting field. The extra time spent in the field compensates for their shorter flight time in the drift regions, with all isomass ions arriving simultaneously at the detector. The performance enhancement compared to nonreflectron instruments (called linear TOF instruments) is significant, especially for molecules with masses greater than 10 kDa. An excellent overview on the underlying principles of MALDI-TOF and the effect of reflectron devices is provided by Guilhaus.[6]

An important technique used to conduct structural studies with MALDI-TOF MS is the so-called postsource decay (PSD). The MALDI process results in different classes of desorbed particles, ions, neutral particles, and metastable particles that undergo decay during their drift toward the detector. The decay is initiated by low-energy collisions of neutral matrix molecules with analyte ions. To analyze the PSD products, precursor ions are preselected (based on mass) via an ion gating device. Products and precursors are resolved through the use of single-staged or two-staged reflectrons. Some applications for the PSD approach are described in ''Applications in Proteomics.''

APPLICATIONS IN PROTEOMICS

Peptide and protein analyses are the most widespread applications of MALDI-TOF MS in the biological sciences today. The most important applications are structural analysis of peptides and proteins (such as sequencing and investigation of bisulfide bonds), modification analysis (investigation of phosphorylation and glycosylation sites), investigation of noncovalent interactions, and protein identification in mixtures or from two-dimensional gels. Because of the number of applications, their complexity, and space constraints, this section can only provide a brief outline and the reader needs to be referred to the respective scientific publications for more details.[7]

Structural Peptide and Protein Analysis

A generic outline to the structural analysis includes the following steps:

1. Purification of the targeted protein or peptide out of the sample (tissue, cell culture, etc.) via gel electrophoretic (e.g., two-dimensional), affinity-based, or gel permeation-based techniques.
2. Mass measurement of the intact protein via MALDI-TOF. At this point of time, databases (such as SWISSPROT, PIR, EMBL, and others) can be used to investigate the identity of the targeted protein.
3. Analysis of disulfide bonds and free suflhydryl groups via chemical reduction and carboxymethylation. The protein is measured prior to and after reduction of disulfide bonds (e.g., using mercaptoethanol) and the subsequent carboxymethylation (e.g., via iodacetamide). This treatment provides information on the number of disulfide bonds and free sulfhydryl groups, respectively. In addition, this procedure facilitates the subsequent cleavage step into peptide fragments by eliminating secondary and tertiary structures, thus providing additional cleavage sites.
4. Chemical and or enzymatic cleavage into smaller peptide fragments is accomplished through amino acid-specific cleavage reagents. Common reagents to perform specific cleavage reactions are specific proteases (e.g., trypsin and certain endoproteinases), nonspecific enzymes (e.g., chymotrypsin), and chemical agents (e.g., chlorosuccinimide or cyanogen bromide). The cleavage products are usually fractionated by high-performance liquid chromatography (HPLC)-based techniques prior to MALDI-TOF MS-based analysis. The obtained masses provide a specific signature (fingerprint) that can be used in databases searches. If the information generated in this step is not sufficient to accomplish the identification of the analyte (e.g., for de novo protein and peptide analysis), an additional step has to be performed to obtain the sequence of the purified cleavage products.
5. Peptide ladder sequencing is performed through the generation of C-terminal and N-terminal peptide ladders. For this purpose, carboxypeptidases (e.g., carboxypeptidase Y) are used. Usually, several different carboxypeptidases have to be applied to cover the whole sequence because most of them exhibit preferences to release certain residues out of the chain. The N-terminal information is obtained similarly through treatment with aminopeptidases or controlled Edman degradation. Analysis of the products is performed on reflectron TOF instruments or with electrospray ionization (ESI) MS. PSD MALDI provides a very sensitive alternative analysis format.

Modification Analysis

The analysis of posttranslational modifications (PTMs) of peptides and proteins, such as phosphorylation and glycosylation, is important to elucidate the functions and interactions of peptides and proteins. The initial steps to analyze PTMs are similar to those performed for structural analysis. In fact, following the initial purification step, the material will usually be fractionated for structural and modification analyses.

A straightforward method for the analysis of peptide and protein phosphorylation is to generate short peptide fragments and to compare mass spectra recorded before and after treatment with phosphatases. Residues that are potential phosphorylation sites are: serine, threonine, and thyrosine. Antibodies, specific to the phosphorylated form of the protein, provide a convenient tool for purification.

The analysis of glycosylation can be performed through treatment with residue-specific glycosidases. Similar to phosphoproteins, glycoproteins can be specifically purified via affinity chromatography. The residues that are usually potential glycosylation sites are serine, asparagine, and threonine. To release *N*-linked carbohydrate chains from glycopeptides, endoglycosidases (mainly peptide *N*-glycosidase F, PNGase F) are used. In addition, chemical reagents such as trifluoromethanesulfonic acid can be used. *O*-linked carbohydrate chains are released via reductive β-elimination (through $NaBH_4$ treatment). Comparative analysis of tryptic digests before and after treatment is used to identify the extent of the glycosilation and to identify the glycosilation sites. The structural characterization of carbohydrate site chains is a complex undertaking. Partially, it can be achieved through mass measurement of the isolated carbohydrate

Table 2 Overview on common covalent posttranslational modifications

Modification	Amino acid residue	Function	Δm
Phosphorylation	Tyr, Ser, Thr	Reversible activity regulation	+80
Glycosylation	Asn, Ser, Thr	Cell–cell recognition, molecular targeting	*N*-linked: >800; *O*-linked: +203, >800
Acylation	Cys, N-terminal Gly	Cellular localization, membrane anchoring	Farnesyl: +204, myristoyl: +210, palmitoyl: +238
Acetylation	Lys, N-terminus	Modulation of protein–DNA interaction and protein stability	+42
Hydroxylation	Pro, Lys	Modulation of protein stability and interactions (collagen triple helix formation)	+16
Carboxylation	Glu, Asp	Ca^{2+} storage, prothrombin clotting	+44
Sulfation	Tyr	Modulation of protein–protein interactions	+80
Proteolytic cleavage	Various	Activation/deactivation	—
Ubiquitination		Degradation signal	>1000
GPI anchor	C-terminus	Membrane anchoring	>1000
C-terminal amidation	C-terminus	Activity modulation	−1

Amino acid abbreviations are standard symbols. Δm is mass difference associated with the modification (in daltons).

component and subsequent database searches (e.g., http://us.expasy.org/tools/glycomod/). In addition, sequential exoglycosidase treatments can be used to release individual residues from complex carbohydrate chains to identify the structure. A detailed discussion on all techniques used to investigate the structural composition of complex carbohydrates is beyond the scope of this contribution.

Other posttranslational modifications occurring (such as acetylation, acylation, carboxylation, methylation, etc.) cannot be discussed in sufficient detail in the scope of this contribution. Table 2 provides an overview of common PTMs and their function, as well as associated changes in mass. For a review, the reader is referred to the scientific literature.[8]

APPLICATIONS IN GENOMICS

The main application of MALDI-TOF MS for DNA analysis today is in the field of SNP analysis and the quality control of oligonucleotide synthesis. Applications for resequencing, gene expression analysis, and haplotyping have been reported and are of increasing importance. In addition, the analysis of methylation patterns (imprinting) is a growing field of interest. In general, the analysis of nucleic acids with MALDI-TOF is more cumbersome than that of proteins and consequently less widespread. For a more detailed information on SNP and sequence analysis with MALDI-TOF MS, the reader is referred to the respective chapters in this encyclopedia and the scientific literature.[9]

CONCLUSION

MALDI-TOF MS provides a superior detection format for qualitative and quantitative analyses of all main classes of biomolecules and their modifications.

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Marfan Syndrome and Related Fibrillinopathies

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Gwenaëlle Collod-Béroud
Catherine Boileau
Université Paris V, Hôpital Necker-Enfants Malades, Paris, France

INTRODUCTION

Marfan syndrome (MFS; OMIM no. 154700) is the founding member of connective tissue disorders. It is autosomal dominant and has an estimated incidence of 1 in 5000 with probably over 25% of sporadic cases. The syndrome involves many systems (skeletal, ocular, cardiovascular, pulmonary, skin and integumentary, and dural), but its more prominent manifestations are skeletal, ocular, and cardiovascular. It is characterized by extreme variability in phenotype severity between different affected members of a given family. At least two genes are implicated in MFS: in the majority of cases, mutations in *FBN1*, the gene encoding fibrillin-1 (the major component of microfibrils), are implicated. Linkage analyses have localized a second gene in 3p24.2–p25. Because no specific clinical anomaly is associated with mutations in each gene, both must be tested for molecular diagnosis.

CLINICAL DEFINITION

In 1986, an international group of experts agreed on diagnostic criteria to distinguish classical MFS from many related disorders. These criteria constitute what is currently referred to as the ''Berlin nosology.''[1] Patients are diagnosed based on the involvement of the skeletal system and two other systems with at least one major manifestation (ectopia lentis, aortic dilation/dissection, or dural ectasia). Patients with an affected first-degree relative are required to have involvement of at least two other systems, with one major manifestation preferred but not required. This nosology has been revised in 1996 and referred to as the ''Ghent nosology.''[2] This new formulation requires involvement of three systems with two major diagnostic manifestations. It provides for major skeletal manifestations and considers affected first-degree relatives or molecular data as major diagnostic criteria.

MFS AND *FBN1*

In 1986, Sakai et al.[3] identified a new extracellular matrix protein, which they named ''fibrillin'' (OMIM no. 134797). This protein is the major component of microfibrils, which are structures found in the extracellular matrix either as isolated aggregates or closely associated with elastin fibers. Ultrastructurally, microfibrils display a typical ''beads-on-a-string'' appearance consisting of a long series of globules connected by multiple filaments. In 1990, Hollister et al.,[4] using a monoclonal antibody against fibrillin, reported abnormalities of the microfibrillar system in MFS. Kainulainen et al.[5] demonstrated through linkage analysis that the gene involved in classical complete forms of the MFS was located on human chromosome 15. The gene encoding fibrillin-1 (*FBN1*) was cloned and located in 15q15–q21.1,[6,7] and the first mutations in the gene were identified in MFS patients.[8]

FBN1 GENE

The gene encoding type 1 fibrillin (*FBN1*) is very large (over 230 kb) and is highly fragmented into 65 exons, transcribed in a 10-kb mRNA that encodes a 2871-amino acid protein.[7,9] Three additional alternatively spliced exons, most likely untranslated and well conserved between humans, mice, and porcine, were found upstream of exon 1. This region is GC-rich, contains a CpG island, and lacks conventional TATA or CCAAT boxes.[10]

The deduced primary structure reveals a highly repetitive protein that contains essentially three repeated modules:

- *EGF-like module*, which is homologous to the one found in the epidermal growth factor. These modules contain six cysteine residues that form three intradomain disulfide bonds. There are 47 of these throughout the fibrillin-1 protein. Among these, 43 contain a conserved consensus sequence for calcium binding and are called cb EGF-like modules. The presence of calcium ions significantly protects full-length or recombinant fragments of fibrillin-1 from proteolysis by trypsin, elastase, endoproteinase Glu-C, plasmin, and matrix metalloproteinases.[11]
- *Transforming growth factor (TGF) β1-binding protein-like module* (TGF β1-BP-like module), which is found seven times interspaced with cb EGF-like in the

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protein and is homologous to modules found in the TGF-β1 binding protein. This domain appears to be limited to proteins that localize to matrix fibrils [fibrillins and latent TGF β-binding proteins (LTBPs)]. These modules contain eight cysteine residues. No specific function has yet been ascribed to these modules. However, some evidence suggest that these domains mediate specific protein–protein interactions.[12]

- *Hybrid module*, which combines features of the former two. This consists of approximately 65 amino acids, and is found twice in the protein. This module is also found in LTBPs, which have a single hybrid domain.

Finally, the protein contains three unique regions: a *proline-rich region* that may act as a ''hingelike'' region[13] and the *amino* and *carboxy terminal domains*. The N-terminal and C-terminal domains of fibrillin display two prominent features: the presence of an even number of cysteine residues (four in the N-terminal and two in the C-terminal domains) and the presence of the basic consensus sequence for processing by furin-type enzymes BXBB (B=basic amino acid residue, K or R) in each domain. The four-cysteine domain in the N-terminus of fibrillin is homologous to similar four-cysteine domains in the N-terminal extended forms of the LTBPs. The C-terminal domain of fibrillin is homologous to the C-terminal domain of all four members of the fibulin family; thus a new type of extracellular module of approximately 120 amino acid residues in length has been proposed.[14] This type of homology is not shared by LTBPs.

Other members of the fibrillin family were identified: *FBN2* gene in 5q23–q31 (fibrillin-2 protein) carrying mutations in congenital contractural arachnodactyly (CCA) (OMIM no. 120150); and *FBNL* gene (or *EFEMP1* gene) in 2p16 (fibrillin-like protein) carrying mutations in Doyne honeycomb retinal dystrophy (DHRD; OMIM no. 126600) or malattia Leventinese (MLVT).

FIBRILLIN PROTEINS

Fibrillins are extracellular matrix glycoproteins that show a wide distribution in both elastic and nonelastic tissues and are integral components of 10-nm-diameter microfibrils. Fibrillin-1 is synthesized as profibrillin and is proteolytically processed to fibrillin. Wild-type profibrillin is not incorporated into extracellular matrix until it is converted to fibrillin. The N-terminal region of each protein directs the formation of homodimers within a few hours after secretion, and disulphide bonds stabilize the interaction.[15] Dimer formation occurs intracellularly, suggesting that the process of fibrillin aggregation is initiated early after biosynthesis of the molecules. Fibrillin is posttranslationally modified by β-hydroxylation and N-linked and O-linked carbohydrate formation.[16] Baldock et al.[17] predicted fibrillin maturation from a parallel head-to-tail alignment. This model accounts for all microfibril structural features, suggests that intermolecular and intramolecular interactions drive conformation changes to form extensible microfibrils, and defines the number of molecules in cross section.

Fibrillin-1 and fibrillin-2 codistribute in elastic and nonelastic connective tissues of the developing embryo, with a preferential accumulation of the *FBN2* gene product in elastic fiber-rich matrices. Mouse study of the developmental expression of fibrillin genes has revealed different patterns. Except for the cardiovascular system, in which *Fbn1* gene activity is early and always higher than *Fbn2*, *Fbn2* transcripts appear earlier than *Fbn1* transcripts and accumulate for a short period of time just before overt tissue differentiation (i.e., a window of time immediately preceding elastogenesis). In contrast, the amount of *Fbn1* transcripts increases at an apparently gradual rate throughout morphogenesis and is mainly expressed during late morphogenesis and well-defined organ structures. Furthermore, *Fbn1* transcripts are predominantly represented in stress-bearing and load-bearing structures such as aortic adventitia, suspensory ligament of the lens, and skin. Thus spatio-temporal patterns of gene expression suggest distinct but related roles in microfibril physiology. Fibrillin-1 would provide mostly force-bearing structural support, whereas fibrillin-2 would predominantly regulate the early process of elastic fiber assembly.[18] Fibrillins could contribute to the structural and functional heterogeneities of microfibrils.

FIBRILLINOPATHIES

To date, over 600 mutations have been identified in the *FBN1* gene in MFS patients and related diseases[19] (http://www.umd.be). *FBN1* gene mutations have been identified in complete and incomplete forms of MFS, but also in various overlapping disorders: severe neonatal MFS, dominantly inherited ectopia lentis, isolated skeletal features of MFS, Shprintzen–Goldberg syndrome, Weil–Marchesani syndrome, and familial or isolated forms of aortic aneurysms. These results define the new molecular group of ''type 1 fibrillinopathies.''

PATHOGENIC MECHANISMS

Microfibrils are suggested to determine the form and the orientation of elastic fibers, thus directing fiber assembly as a scaffold on which elastin is deposited.[18] This model explains the typical fragmentation and disarray of elastic fibers observed in the media of Marfan patients. However,

unlike elastin, fibrillin-1 is also highly expressed in the vascular adventitia. Therefore reduction of this protein in the adventia is very likely also involved in the mechanism for dilatation and for increased risk of aneurysm because the role of the adventia is to maintain the vascular diameter. The pleiotropic manifestations of the disease can be explained by the observation that numerous microfibrillar aggregates devoid of elastin are found in the zonule, as well as cartilage and the extracellular matrix of many organs. However, the actual pathogenic mechanisms in these tissues still remain speculative.

At the molecular level, two different groups of mutations are distinguishable: mutations leading to a shortened protein and missense mutations. The first group corresponds to one third of the mutations. They can be responsible for: 1) the appearance of a premature stop codon that reduces the stability of the mutant transcript and, consequently, greatly reduces protein production from the mutated copy of the gene (in the affected subjects, the amount of fibrillin-1 protein produced is 50% that of normal and is produced only from the normal gene copy), or, 2) the production from the mutated copy of an abnormal monomer that considerably interferes with the assembly (polymerization) of fibrillin molecules (the amount of fibrillin is greatly reduced, <35%). The second group represent two thirds of mutations and corresponds to missense mutation. Among them, three quarters are located in calcium-binding modules. They are implicated either in creating or substituting cysteine residues potentially implicated in disulfide bonding and, consequently, in the correct folding of the monomer. The majority of remaining mutations of this type of module affect residues of the calcium consensus sequence that playa major role in defining interdomain linkage. An increased protease susceptibility is a mechanism also suggested for missense mutations. Other modules are carriers of one quarter of missense mutations and pathological mechanisms have yet to be clearly demonstrated.

What is still unknown are the multiple consequences triggered by the various mutations and the effects of unknown modifier (enhancing or protecting) genes on clinical expression. These mechanisms and the great number of mutations identified in the *FBN1* gene explain the great variability of the disease observed not only between families but also among affected individuals in a single family.

GENETIC HETEROGENEITY

The clinical variability of MFS is only partly explained by the great number of mutations identified in the *FBN1* gene. In effect, we have demonstrated the existence of genetic heterogeneity (i.e., the involvement) in certain cases of MFS of mutations located in another gene named *MFS2* (for MFS type 2) through the study of a very large French family in which affected individuals display an incomplete form of the syndrome: typical skeletal and cardiovascular features as well as involvement of the skin and integument. No ocular manifestations were observed until one of the children in the fourth generation developed ectopia lentis. We showed that fibrillin-1 was normal in several affected family members and excluded linkage between the *FBN1* gene and the disease in the family.[20] By exclusion mapping, we located the *MFS2* gene on the short arm of chromosome 3.[21] We are now identifying *MFS2* through positional cloning. Several families comparable to the French family in that they are not linked to, or do not carry, a mutation in the *FBN1* gene have been identified. Other teams, through protein studies, have identified between 7% and 16% of MFS patients with normal fibrillin metabolism.[22,23] The precise determination of this percentage is important for laboratories involved in the diagnosis of MFS because it will give the risk associated with investigation of only the *FBN1* gene.

DIAGNOSTICS

The discovery of the involvement of fibrillin-1 has raised high hopes for a protein or DNA test applicable to MFS patients. Immunofluorescence studies of cultured fibroblasts and skin sections of patients using monoclonal antibodies against fibrillin have revealed that the amount of fibrillin deposition or fibrillin microfibrils is greatly reduced.[4] Therefore immunofluorescence analysis could be helpful in diagnosis. However, the method has proven to be insufficiently sensitive and specific because of the existence of non-MFS type 1 fibrillinopathies and genetic heterogeneity. Therefore an abnormal test result does not diagnose MFS, and a normal test result does not rule out MFS.

The identification of the *FBN1* gene has allowed the development of two types of diagnostic tests: genetic family studies or mutation identification. Family studies can be performed with specific *FBN1* polymorphic markers to identify the mutation-bearing haplotype.[24] These studies are only reliable in families in which several affected individuals are available because the involvement of an *FBN1* mutation (and not that of another gene) must be clearly demonstrated. However, most family structures do not comply with this requirement. Furthermore, the method is inappropriate in sporadic cases. In practice, these instances represent over 40% of the cases referred for biological diagnosis. The second molecular test is mutation identification. Mutation identification is very costly and long. In effect, there is no quick and 100% reliable method to investigate a large ($\sim$230 kb) and highly fragmented (10 kb of coding sequence fragmented

in 65 exons) gene, knowing that almost each family has its own specific defects and that the mutations are essentially point mutations. Finally, this very costly analysis may fail to identify a mutation because only the coding sequence and closely surrounding regions are investigated. However, in the case of neonatal MFS, where a clustering of mutations is found in exons 24–32, molecular diagnosis can be performed. In all other instances and until better molecular tools are available, mutation identification cannot be performed on a systematic basis. However, in a few cases where family mutation had been identified, it was possible to perform prenatal diagnosis on chorionic villus samples, or to offer presymptomatic diagnosis in children of affected subjects who are at risk.

CONCLUSION

Although no specific therapy exists for MFS, it is of great importance to confirm or firmly exclude the diagnosis in family members at risk as early as possible because of the potential fatal complications of the disease. Development of preventive measures, and surgery for aortic aneurysms and dissection have led to treatments of life-threatening cardiovascular complications associated with MFS and have considerably altered life expectancy for patients. At present, diagnosis is still based on thorough clinical examination, including measurements of body proportions, echocardiography of the aorta, slit-lamp ophthalmological evaluation, and radiographs. A complete family history is also an essential part of the diagnosis. However, in some cases, the manifestations are not evident until adolescence and the clinical expression of the disease varies greatly between affected members of a single family. Therefore there is an absolute need for an accurate diagnostic test.

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Mass Spectrometric Genotyping of Single Nucleotide Polymorphisms

Christian Jurinke
Dirk van den Boom
SEQUENOM, Inc., San Diego, California, U.S.A.

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are biallelic single-base changes with a minor-allele frequency of at least 1%. Genotyping of SNPs has become of increasing importance to elucidate the interindividual differences in populations and to investigate the genetic foundations of major common diseases.

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) has become a powerful tool for the analysis of biomolecules in the past decades. Besides its earlier focus nearly entirely on peptide and protein analysis, MALDI-TOF MS is more widely used today for the investigation of queries related to the structure and function of genes and genomes.

SNP GENOTYPING

Single nucleotide polymorphisms provide powerful markers for genetic analysis. Their high-frequent abundance within genomes, especially their presence within coding regions of genes, discriminates them from markers used earlier (e.g., microsatellites). Single nucleotide polymorphisms are not merely markers but can also be the causative agent for diseases on a genetic level. In addition, the biallelic nature of SNPs makes them easier to analyze and the corresponding analytical procedures more amenable to automation, enabling large-scale whole-genome studies. These properties contributed to the unprecedented success of SNP markers in many genetic studies. For more details on the nature of SNPs and typing methods the reader is referred to the respective chapters in this encyclopedia.

MALDI-TOF MASS SPECTROMETRY

MALDI-TOF MS basically relies on the laser-induced desorption of large molecules that are cocrystallized with an excess of matrix substance. The so-called matrix absorbs laser energy and evaporates into the vacuum of the mass spectrometer. The analyte molecules are co-desorbed with the matrix and ionized by proton transfer. Molecules are accelerated in an electric field and their flight time is measured following their impact on a secondary electron multiplier. The advantages for using MALDI-TOF for the analysis of DNA molecules are that no surrogates (e.g., labels) are detected but an intrinsic property of the analyte molecule (the molecular mass) is used for the identification. The analysis time is in the range of seconds for any given assay. The data analysis is completely automated and straightforward.

For more details on the principles and applications of MALDI-TOF MS to proteomics the reader is referred to the respective chapters in this encyclopedia.

MALDI-TOF MS FOR SNP GENOTYPING

Nucleic acids are more difficult to analyze under MALDI conditions than peptides, which had limited its application in this field. Because of their negatively charged phosphate backbone nucleic acids are susceptible to adduct formation. They form salt adducts with cations present in the surrounding medium (e.g., buffers). Adduct formation results in a broader distribution of the signal. For example, sodium ions attached to the analyte will cause an additional signal with a mass of plus 22 Da (23 for sodium minus 1 for an exchanged hydrogen). Consequently, adduct formation lowers sensitivity and analytical accuracy. In addition to strong adduct formation, nucleic acids also tend to fragment under MALDI conditions.

The main reaction contributing to DNA fragmentation under MALDI conditions is depurination. Depurination occurs through protonation of the nucleobases A or G (at position N7). The protonation induces polarization of the *N*-glycosidic bond between sugar and nucleobase. The result is nucleobase elimination. Subsequent to depurination reactions, further fragmentation occurs via backbone cleavage.

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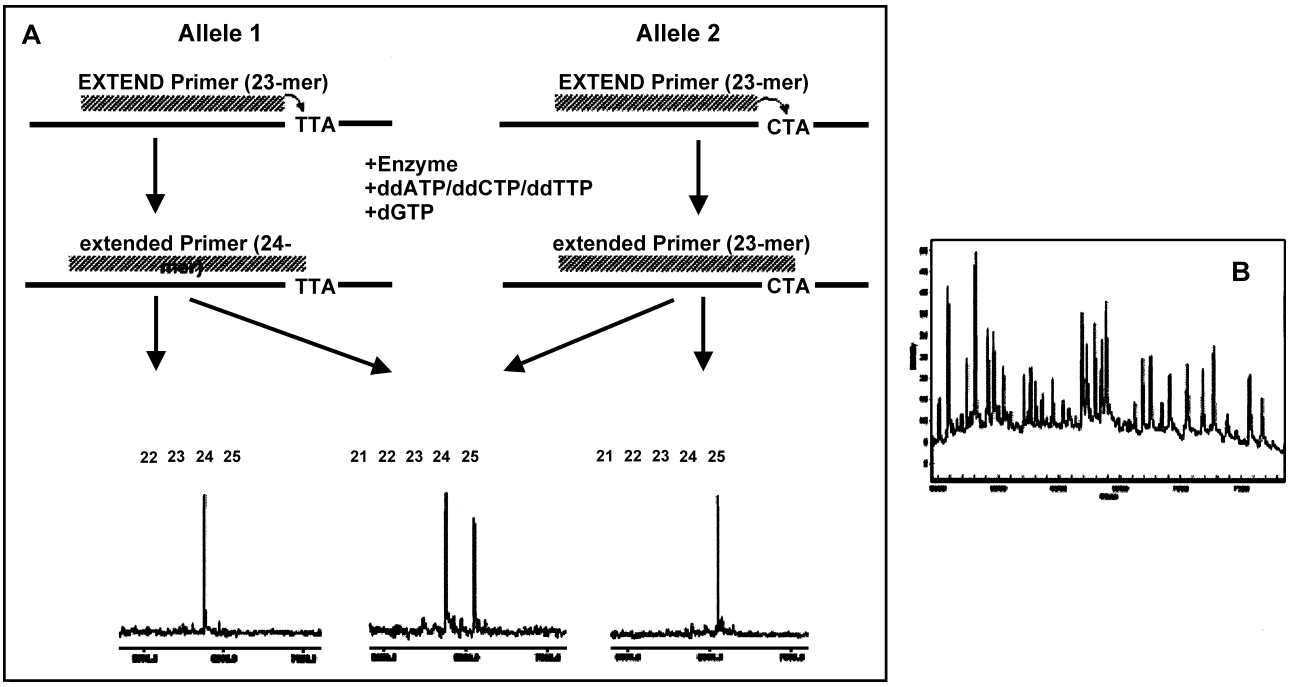

Fig. 1 Box A illustrates the MassEXTEND® primer extension concept for SNP genotyping. Spectra derived from a uniplex reaction are displayed for both homozygote cases and the heterozygote condition. Box B provides raw data for a highly multiplexed reaction for SNP genotyping following the same schema as outlined in A but employing multiple PCR and MassEXTEND® primer in one reaction vessel.

The availability of favorable matrices for DNA analysis is an important factor for the success of MALDI-TOF MS in the field of DNA analysis. Especially, the introduction of 3-hydroxypicolinic acid (3-HPA) as matrix[1] is a cornerstone in this respect.

The most predominant method used for MALDI-TOF MS-based SNP genotyping relies on primer extension concepts—the so-called MassEXTEND® assay.[2] MassEXTEND® assays combine primer extension with MALDI-TOF MS. The assay consists of a post-PCR primer extension reaction that is carried out in the presence of one or more dideoxynucleotides (ddNTPs) generating allele-specific terminated extension products. Primers are designed to anneal adjacent to the SNPs of interest. Depending on the SNP identity, extension products of different lengths and masses are generated. In the case of heterozygosity, two products with distinguishable mass are generated. Following PCR amplification, shrimp alkaline phosphatase (SAP) is added to dephosphorylate remaining dNTPs from the PCR cocktail. The samples are diluted with water and cation exchange beads (NH_4^+ form) are added and the supernatant is used for MALDI-TOF MS analysis. A schematic representation of this reaction is presented in Fig. 1. Other approaches for MALDI-TOF-based genotyping include the so-called invader[3] and GOOD[4] assays.

Multiplexing

Performing multiple PCR and extension reactions in a single well (i.e., multiplexing) is a way to increase the throughput and to reduce the effective cost per genotype. In other words, assays for different SNPs can be combined in a single reaction to save time, reagents, and to preserve DNA samples. MALDI-TOF MS provides the high resolution, high accuracy, and wide mass range needed to perform highly multiplexed genotyping assays.

To design SNP assays for multiplexing, several considerations must be taken. Among those:

1. Primers for the PCR amplification reactions must be designed in a way to avoid cross-loci amplification. In addition, PCR primer designs should have the following properties: 1) optimal primer length (20 mer); 2) optimal T_m (60°C); 3) optimal G-C content (50%); and 4) optimal amplicon length ($\sim$100 bp). Potential primers should be blasted against public databases to avoid individual assay failures due to competing kinetics or cross-hybridization.

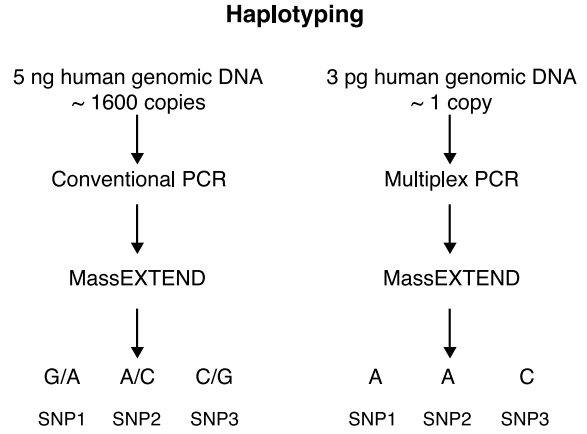

Fig. 2 The differences between molecular haplotyping and genotyping methods are exemplified and illustrated in this graph. The main difference is the use of dilutions of genomic DNA down to a level where statistically only one molecule is present in the sample.

2. Primers for the MassEXTEND® reaction must be designed so the combination of expected peak masses are resolvable for unambiguous genotyping. For the MassEXTEND® primer design there are two choices available as either site adjacent to the SNP can be used. The following should be considered: 1) optimal length (17–24 mer); 2) primers may not contain uncertain bases in the target sequence; 3) primers with low T_m should be disregarded because of hairpin formation, false priming, and primer–dimer potential; 4) problematic sequence repeats such as GGGG should be avoided; and 5) potential mass conflicts with by-products (e.g., depurination products and possible extend-pausing products) need to be avoided. For example, a MassEXTEND® primer prematurely terminated with dA would have exactly the same mass as if normally terminated with ddG.

A 12- to 15-fold multiplex assay can be routinely carried out and 20–30-fold plexing levels may be achievable with assay optimization. The 15-fold multiplexing with ~1 hr acquisition/real-time analysis of 384 elements translates into an analytical speed of ~100 genotypes per minute (or 5760 genotypes/hr). This throughput coupled with a cost/genotype of currently below 10¢ renders whole-genome scans studies feasible and affordable.

Haplotyping

Haplotypes are combinations of phase-determined genetic markers. Their unambiguous identification provides additional statistical power for detecting genes involved in common diseases. One approach that can be directly translated to the above-described MALDI-TOF platforms employs multiple short PCR reactions spanning several kilobases which are amplified from a genomic DNA template that is diluted down to the single molecule level. A subsequent multiplex primer extension reaction delivers the genotypes for the targeted SNPs. This approach has successfully been used to haplotype markers that are separated over 24 kb.[5] The process is outlined in Fig. 2.

QUANTITATIVE ANALYSIS OF SNPs IN DNA POOLS

Genotyping thousands of SNPs over hundreds to thousands of individuals still remains cost intensive. Pooling DNA samples from case and control populations has therefore been proposed as an alternative to individually genotyping populations for association studies.[6] The analysis of allele frequency distributions in pools of individual DNA samples is a tool to study the abundance of particular alleles in populations and to compare these between different populations. This information can be used to identify causative genetic loci associated with complex diseases via linkage disequilibrium between two or more loci.

Relative quantitation of allele frequencies with MALDI-TOF MS is achieved by calculating the areas of peaks associated with specific primer extension products. Peak characteristics (such as intensity, etc.) are generally stored when spectra are acquired on the MALDI-TOF MS. The area calculations can be conducted by a mathematical

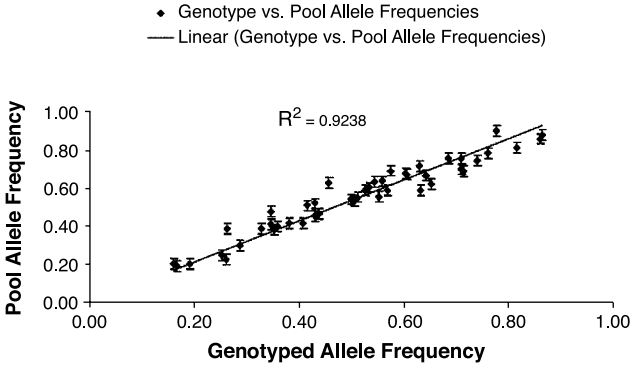

Fig. 3 The scatter plot represents quantitative results derived from a comparison of data for multiple SNPs resulting from genotyping of individual DNA samples and ''allelotyping'' of a pool of the respective individual DNAs.

method of choice. Today, software is available to automatically perform the peak area calculations in real time, during the measurement. The allele frequency of a particular SNP is calculated as the ratio of the area of each allele to the total summed area of both alleles. The sum of the two alleles is always 1. Allele frequencies down to 5% can be accurately discerned using MALDI-TOF MS in DNA pools. Frequencies below 5% are routinely detected but their accuracy must be approached with caution because of the small peak area associated with a minor allele at a 50:1 ratio relative to the major allele.

MALDI-TOF MS measurement of primer extension reactions has been found to be at least as sensitive and reproducible as other available technologies based on several studies. Figure 3 is a scatter plot with allele frequencies determined for 48 assays in a DNA pool of 96 individuals vs. the observed frequency based on the genotype for all 96 individual samples for each of the 48 assays. As can be seen from the coefficient of determination (R^2) there is not a perfect 1:1 correlation between the allele frequencies calculated from a pool of individuals and the actual frequency determined by genotyping. Several factors contribute to this difference independent of the technology used. However, a ''correction'' factor can be applied for each individual assay based on the peak areas observed for individual heterozygous samples from the population under investigation. Individual heterozygotes have two alleles at a 1:1 ratio for any given biallelic SNP. Based on this, the peak areas observed for each allele on the MALDI-TOF MS should be equal. If they are not, then a ''skewing'' of one allele over the other has occurred at some point in the process (whether it be at the level of PCR, EXTEND, or analyte ionization during MALDI-TOF or at some other point). This bias can be quantified on the MALDI-TOF MS and corrected for. This correction factor has already been applied to generate the plot in Fig. 3. The precision of this method is sufficient for many semiquantitative applications such as the comparison of SNP allele frequency in nucleic acid pools or the differential protein–DNA binding associated with allelic variants of a gene.

CONCLUSION

MALDI-TOF MS as a detection format provides accuracy, throughput, and semiquantitative capabilities for the analysis of SNPs in individual and pooled DNA samples. Furthermore, it provides the means for generating haplotype information. The application of MALDI-TOF MS to the analysis of genes and genomes will be an important tool for future research projects.

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Mastocytosis—c-Kit Mutations

M. Mayerhofer
C. Sillaber
M. Födinger
Medical University of Vienna, Vienna, Austria

K. Sotlar
University of Tübingen, Tübingen, Germany

W. R. Sperr
Medical University of Vienna, Vienna, Austria

M. Arock
Faculty of Pharmacy, Paris, France

P. Valent
Medical University of Vienna, Vienna, Austria

INTRODUCTION

Mast cells (MCs) are tissue-fixed hematopoietic cells that store numerous vasoactive and immunoregulatory mediators.[1] A complex network of cytokines is involved in the regulation of growth and differentiation of MCs.[1] A most important growth factor for MCs appears to be stem cell factor (SCF). Notably, SCF induces the development of MCs from uncommitted $CD34^+$ progenitors.[2] The effects of SCF on MCs and MC progenitors are mediated through c-kit, a tyrosine kinase receptor for SCF encoded by the c-*kit* proto-oncogene.

Systemic mastocytosis (SM) is a clonal disease leading to pathological accumulation of MCs in the bone marrow (BM) and other internal organs.[3] In a high proportion of cases, point mutations in the c-*kit* gene are detectable.[3–5] The most frequent mutation leads to substitution of valine by aspartate at codon 816 (D816V). This mutation is associated with constitutive activation of c-kit and is considered to play a critical role in the pathogenesis of SM.[5,6] However, the clinical picture in SM is variable, ranging from asymptomatic to highly aggressive courses.[3] Therefore, apart from this mutation, several other factors may influence the clinical course in these patients. The current article provides a short overview on c-kit mutation D816V in SM, with special reference to the application of this molecular defect in the diagnostic workup and staging of SM patients.

CLINICAL ASPECTS OF MASTOCYTOSIS

Based on clinical findings, four major types of SM have been defined by the World Health Organization (WHO): indolent systemic mastocytosis (ISM), SM with an associated clonal hematological non-MC lineage disease (SM-AHNMD), aggressive systemic mastocytosis (ASM), and mast cell leukemia (MCL).[3] In patients with ASM and MCL, significant organopathy caused by MC infiltration is found.[3] The organ systems most frequently affected are the BM, skeletal system, liver, and gastrointestinal tract. In some cases, eosinophilia and lymphadenopathy are prominent clinical features.[7] Mast cell leukemia is a rare form of SM characterized by progressive organopathy, circulating MCs, and short survival.[3,8]

So far, no curative therapy for ASM or MCL has become available. Some patients may benefit from interferon-α (IFN-α), glucocorticoids, or cladribine.[8] However, in most cases, treatment responses are only short-lived. Therefore, a number of attempts are currently made to identify molecular targets and novel effective

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drugs that interfere with neoplastic cell growth in ASM and MCL.

PATHOLOGY OF SM AND PHENOTYPE OF NEOPLASTIC MCs

The pathological hallmark in SM is the multifocal dense infiltrate of MCs in the BM (or/and other visceral organs).[9] Other typical features of SM include BM fibrosis,[9] increased BM angiogenesis,[10] and tissue and blood eosinophilia.[7,11] Recent data suggest that neoplastic MCs express several angiogenic and fibrogenic mediators such as vascular endothelial growth factor (VEGF), tryptase, or bFGF.[10] Other data suggest that several surface molecules such as CD2 or CD25 are overexpressed in MCs in patients with SM compared to normal MCs.[12,13] However, little is known about the regulation of expression of these molecules in neoplastic MCs. An attractive hypothesis is that the c-kit mutation D816V is involved in the regulation of expression of such genes.

SIGNAL TRANSDUCTION PATHWAYS ACTIVATED BY THE c-Kit RECEPTOR

The c-kit receptor is a glycoprotein of 145 kDa and belongs to the type III class of the receptor tyrosine kinase family.[14] c-Kit contains five immunoglobulin-like domains in its extracellular portion and an inter-kinase sequence that splits the intracytoplasmic domain into the ATP-binding domain and the phosphotransferase domain (Fig. 1).[15] Binding of SCF to c-kit leads to receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic domain. As a consequence, c-kit activates a number of signal transduction pathways.[16] Especially the activation of PI3 kinase has been shown to contribute to important c-kit-dependent cellular functions such as proliferation, survival, or adhesion.[17,18] Apart from PI3 kinase, c-kit also leads to activation of Ras and MAP kinases.[16,19] Other signaling pathways reportedly activated by c-kit include JAK2, STAT family members,[20] as well as members of the Src family.[16]

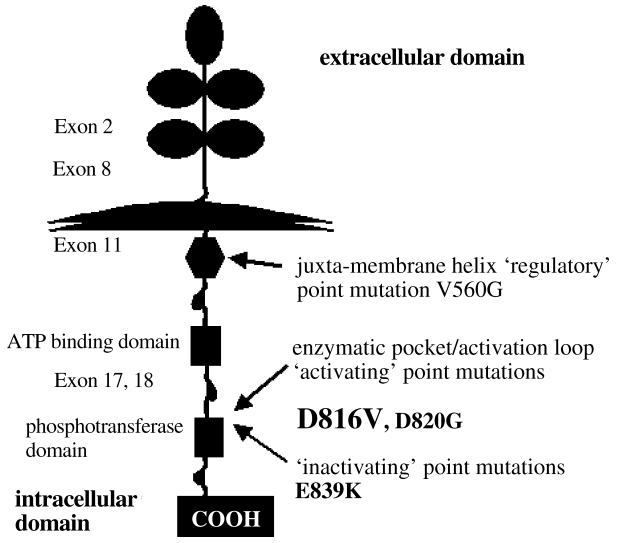

Fig. 1 Structure of c-kit receptor and localization of mutations. The c-kit receptor is composed of extracellular and intracellular domains. The most common mutation in c-kit, D816V, is located in the activation loop at the entrance to the enzymatic pocket of the c-kit kinase.

THE c-Kit MUTATION D816V AND ITS ROLE IN THE PATHOGENESIS OF SM

D816V is a point mutation in the c-kit codon 816 that leads to substitution of valine by aspartate and affects the activation loop at the entrance to the enzymatic pocket of the c-kit kinase (Fig. 1).[5] c-Kit D816V exhibits constitutive tyrosine kinase activity and leads to cytokine-independent growth of MCs.[21] Moreover, c-kit D814V (the murine homologue of D816V) transforms factor-dependent cell lines to growth factor independence and acts oncogenically in mice.[21] However, the exact mechanisms by which D816V leads to constitutive activation of c-kit are unknown. A number of previous and more recent studies support the notion that D816V alters several features of the c-kit receptor including dimerization and signaling.[21,22] One important mechanism appears to be an alteration in the activation of signal transduction pathways. Thus, recent data suggest that the signaling pathways activated by wild-type (WT) c-kit (in the presence of the c-kit ligand SCF) differ from the signaling pathways (constitutively) activated by c-kit D816V.[23] As a consequence, c-kit D816V may modulate the expression of molecules that are critical for transformation, although the identity of these genes remains largely unknown.

METHODS FOR DETECTION OF THE c-Kit MUTATION D816V

Several methods for the detection of c-kit mutations have been reported. The standard procedure for detection of c-kit D816V is restriction fragment length polymorphism (RFLP; Fig. 2).[24] In addition, direct sequencing of the c-*kit* gene can be performed[6] and may reveal additional c-kit mutations (apart from D816V). Recently, a novel

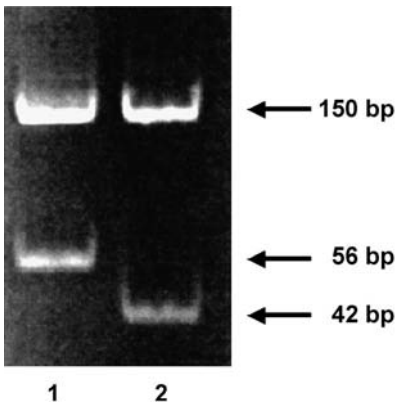

Fig. 2 RFLP analysis of c-kit. After amplification of the mutation-containing region of the c-kit cDNA, PCR products were digested with *Hin*fI and analyzed by electrophoresis on a polyacrylamide gel. Compared to WT c-kit (1), the c-kit D816V mutation (2) generates a second restriction site for the endonuclease *Hin*fI within the amplified region. Predicted sizes for the WT sequence are 150 and 56 bp, and are 150, 42, and 14 bp for the mutated sequence.

approach combining peptide nucleic acid-mediated polymerase chain reaction (PCR) clamping and detection of c-kit mutations with hybridization probes has been described.[25] The major advantage of this method is that the mutant allele is amplified selectively and can be detected in case of a more than 1000-fold excess of WT over mutated c-kit. In addition, sequence variations other than the D816V c-kit mutation can be detected within the region covered by the probes. However, all these techniques are not sufficient to identify the cell type (MCs vs. other cells in tissues or blood) in which the mutation is present. To address this subject, isolation of the cell type(s) of interest is required. One possible approach is to collect immunohistochemically defined subpopulations of cells from stained tissue sections by microdissection.[24] An alternative strategy is sorting of cell subpopulation from BM or peripheral blood (PB) by flow cytometry.[26] Using these techniques, even single cells can be isolated and analyzed for c-kit D816V.[24,26] However, these techniques are not used in the routine workup of patients with (suspected) SM. Rather, RFLP analysis of D816V is considered to be appropriate and sufficient for routine diagnostic purposes.

DETECTION OF c-Kit D816V IN VARIOUS SUBTYPES OF MASTOCYTOSIS

Detection of the D816V mutation in an extracutaneous organ is a minor criterion for the diagnosis of SM (SM criterion).[3] In the presence of other (i.e., one major or two minor) SM criteria, the final diagnosis SM can be established[3] (Table 1). Patients with cutaneous mastocytosis (CM) may also exhibit D816V (in their skin or even in BM), but other criteria for SM are not fulfilled.

In the diagnostic workup of suspected SM, analysis for the D816V mutation is routinely performed on BM and PB samples.[3–5,27] However, because SM can involve virtually all organs, the D816V mutation may be detected not only in BM and PB cells but also in other tissues such as the skin, liver, or gastrointestinal tract.[25] For most patients, it is recommended to isolate mononuclear cells (MNCs) from the BM and PB to seek for the c-kit mutation D816V.[3,27] Although c-kit D816V is detectable in BM MNC in the majority of all patients with ISM by RFLP, the organs affected and the distribution of the mutation in various hematopoietic cell lineages vary among patients.[3] In typical ISM, D816V is usually detectable in the BM, but not in unfractionated PB MNCs. By contrast, in smoldering SM, a novel subtype of (I)SM characterized by a high burden of MCs and multilineage involvement, the mutation is detectable in BM MNCs as well in unfractionated PB MNCs.[28–30] In these patients, the mutation appears to be present not only in MCs but also in monocytes and even in B lymphocytes.[26] By contrast, in typical ISM, the D816V mutation is only detectable in samples containing MCs.

In a group of patients with SM, an AHNMD is detectable.[3,9] In most cases, a myeloid neoplasm such as a myeloid leukemia or myeloproliferative disorder is found.[3,9] Similar to patients with smoldering SM (which can transform to SM-AHNMD), the c-kit mutation D816V is often detectable in unfractionated PB MNCs in these patients. Especially in patients with an associated chronic myelomonocytic leukemia (CMML), the D816V mutation was found to be expressed in leukemic cells.[24] In patients with an associated AML, leukemic blasts may also

Table 1 WHO classification of mastocytosis and typical patterns of expression of D816V in various organs

	c-Kit D816V		
Disease variant	**BM**	**Blood MNCs**	**Skin**
CM	−	−	±
ISM	+	−	±
Smoldering systemic mastocytosis (SSM)	+	+	±
Isolated bone marrow mastocytosis (BMM)	+	−	−
SM-AHNMD	+	±	∓
ASM	±	±	−
MCL	±	±	−

express the mutation. However, not all patients with SM and an associated AML exhibit c-kit D816V.

In patients with ASM and MCL, the D816V mutation has also been reported.[3] However, again, not all of these patients display this mutation.

MUTATED c-Kit AS A NOVEL TARGET OF THERAPY

Because the c-kit mutation D816V is a recurrent defect considered to be of pathogenetic significance, recent attempts have focused on agents that may be capable of inhibiting the kinase activity of D816V c-kit. A first promising observation has been that the tyrosine kinase inhibitor STI571 that is successfully applied in chronic myeloid leukemia (CML) is also capable of inhibiting the c-kit tyrosine kinase.[31,32] Moreover, it has been shown that STI571 downregulates (SCF-dependent) in vitro growth of human MCs at pharmacological concentrations.[33] However, unfortunately, the D816V-mutated form of c-kit is far less susceptible to STI571 compared to the WT receptor.[32,34] Therefore current research is focusing on novel inhibitory drugs that may counteract c-kit D816V.[35]

CONCLUSION

The c-kit D816V mutation is a pathogenetically important defect that is highly associated with the clinical picture of SM and has been proposed as a diagnostic criterion. Therefore much effort has been performed to standardize diagnostic procedures for the detection and analysis of the mutation. In addition, the mutated c-kit may be a novel promising target of therapy in SM.

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Medullary Thyroid Carcinoma (MTC) and Multiple Endocrine Neoplasia (MEN 1, MEN 2)

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Nader Hanna
University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.

INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN 1) and type 2 (MEN 2) are genetically and clinically distinct hereditary endocrinopathy syndromes with classical characteristics of early age of cancer onset, which is frequently bilateral or multifocal, multiple primary tumors, vertical transmission through either parent, frequent association with distinguished phenotypic stigmata, and occasional special pathological features. MEN 1-associated tumors, affecting mainly the parathyroid glands, the pancreatic-islet cells, and the anterior pituitary gland, are due to inactivation of the MEN 1 tumor suppressor gene located in 11q13, with no genotype–phenotype correlation. MEN 2-associated tumors, affecting mainly the thyroid parafollicular C cells, the parathyroid glands, and the adrenal medulla, are due to oncogenic point mutations of the *RET* proto-oncogene located in 10q11.2. There is a significant genotype–phenotype correlation in MEN 2 phenotypic variants, which has led to development of guidelines for the timing and extent of prophylactic surgery.

OVERVIEW

Medullary thyroid cancer (MTC) originates from the parafollicular calcitonin secreting cells of the thyroid gland and represents 5% of all thyroid cancers. Seventy-five to 80% of cases are sporadic and present in the fifth or sixth decade of life. Twenty to 25% of cases are hereditary, associated with multiple endocrine neoplasia (MEN) syndrome and characterized by younger age at presentation, and bilateral or multifocal tumors.[1] There are three phenotypic presentations of MTC in the MEN syndrome: MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC) (Table 1). Other variants of MEN 2 are rare and include MEN 2A with cutaneous lichen amyloidosis often located over upper back, MEN 2A with Hirschsprung's disease, and FMTC with Hirschsprung's disease.[2,3] Medullary thyroid cancer is the first manifestation in most MEN 2 kindreds because of its higher penetrance. Average 5-year survival for MTC is 85% compared to 98% 5-year survival for papillary and follicular thyroid cancer. Survival is correlated with age, stage at diagnosis, familial vs. sporadic by biochemical screening vs. by symptoms.[4] The aggressiveness of MTC correlates with the MEN 2 variant syndrome and with the mutant *RET* codon. Most patients with MTC in MEN 2A will present with neck mass or neck pain at age 5–20 years; 30% will present with diarrhea and advanced disease. More than 50% will have cervical nodal metastasis. In MEN 2A syndrome, the biochemical manifestation of MTC with annual basal and stimulated calcitonin levels can be detected at a mean age of 5 years. In MEN 2B, MTC is very aggressive and presents early. Without prophylactic thyroidectomy, the average age of death in MEN 2B is 21 years. Parathyroid abnormalities are often found concomitantly with surgery for medullary thyroid carcinoma. MEN 2-related hyperparathyroidism (HPT) is rarely the first manifestation with a median age at presentation of 38 years. Pheochromocytomas in MEN 2 syndrome are characterized by high frequency of multiple tumors, which are often bilateral and extra-adrenal. The majority are benign (4% malignant). Other genetic disorders with heritable predisposition to pheochromocytoma include neurofibromatosis type 1, von Hippel–Lindau disease (VHL), hereditary pheochromocytoma, and the hereditary paraganglioma syndrome.

GENETIC ALTERATIONS IN MEN 2

MEN 2 is an inherited autosomal dominant disorder due to hereditary germline one amino acid point mutation in the *RET* gene (chromosomal locus 10q11.2).[5–7] It is a proto-oncogene composed of 21 exons over 55 kb of genomic material. It codes for a receptor tyrosine kinase receptor, termed ret, with extracellular, transmembrane, and intracellular domains. Recently, the glial cell line-derived neurotrophic factor (GDNF) has been identified to be a ligand for *RET*. Glial cell line-derived neurotrophic factor receptor (GDNFR)-alpha, a novel glycosylphosphatidylinositol-linked cell surface receptor, binds GDNF specifically and mediates the activation of the Ret protein-tyrosine kinase (PTK).[8] Ligand-independent homodimerization of the ret enzyme (cycteine-rich extracellular

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Table 1 Characteristics of medullary thyroid carcinoma (MTC) in phenotypic variants of MEN 2 syndrome

	Multiple endocrine neoplasia type 2A (MEN 2A) (Sipple syndrome)	**Multiple endocrine neoplasia type 2B (MEN 2B) (mucosal neuroma syndrome or Wagenmann–Froboese syndrome)**	**Familial MTC**
Proportion of hereditary MTC cases in MEN2 syndrome by phenotype	60–90%	5%	5–35%
Incidence of MTC and age of onset	95% Early adulthood	100% Early childhood, most distinctive and aggressive variant	100% Middle age, mildest variant
Risk for pheochromocytoma	50%. Highest frequency with mutations in codon 634 and 918	50% (often bilateral and multiple)	0%
Risk for parathyroid disease (multigland hyperplasia and adenoma)	20–30%. Highest frequency with codon 634 mutation	Uncommon	0%
Requirements for clinic diagnosis in suspected families	2 or more specific endocrine tumors	MTC associated with mucosal neuromas, distinctive facies with enlarge lips, Marfanoid habitus, GI ganglioneuromatosis	Multiple MTC or *RET* mutation carriers over the age of 50
	95% have affected parent and 5% will have de novo gene mutations	50% have affected parent (usually paternal) and 50% will have de novo gene mutations.[25]	No pheochromocytoma or parathyroid disease
RET mutation analysis	95% in exon 10 or 11 (codon Cys634 in approximately 85% of families. Other identified mutations include Cys 609, 611, 618, 620)	95% single point mutation in exon 16 (codon Met918). A second mutation in exon 15 (codon Ala883 has also been identified)	85%, mostly mutation of cysteine codons 618, 620, 634. Other identified mutations include Cys 609, 611, 613, 790, 791, 844, and 891, Glu 768, and Val804
Timing for prophylactic thyroidectomy	Before age 5 years	Before age 5 years	Before age 1 year

Source: Adapted from Refs. [7,24].

domain mutants in most MEN2A) or activation of its catalytic site (intracellular domain mutants in MEN2B) results in inappropriate constitutive activation of the *RET* oncogene. Only one mutated copy of the oncogene is needed for a gain of function leading to uncontrolled proliferation and carcinogenesis.

CLINICAL DIAGNOSIS AND GENETIC TESTING

The diagnosis of the MEN 2 clinical subtypes relies on a combination of clinical findings, family history, and molecular genetic testing for *RET* gene mutation, which is recommended for all patients with sporadic MTC, pheochromocytoma (particularly if bilateral and/or before age 18), Hirschsprung's disease, and patients with physical features or gastrointestinal manifestation suggestive of MEN 2B. Genetic testing is also offered to first-degree relatives (before age 5 in MEN 2A and FMTC and before age 1 in MEN 2B) of *RET* mutation carriers to identify asymptomatic at-risk individuals for early prophylactic thyroidectomy, which could be curative when the disease is microscopic and localized to the thyroid gland. Genetic testing is generally done on DNA extracted from peripheral blood leucocytes for common mutations in exons 10 (codons 609, 611, 618, or 620), 11 (codon 634), and 16.[9] Because of the established genotypical–phenotypical correlation, *RET* mutation stratification has clinical and management implications.[10–13] Hyperparathyroidism is most commonly associated with mutations in codon 634, less frequent with mutations in codons 609, 611, 618, 620, 790, and 791. Individuals with MEN 2B (mutation in codons Met918 in exon 16, Ala883 in exon 15, or 922) do not develop HPT. Pheochromocytoma has been reported in all *RET* mutations except codons 768, Val804met, and 891 and is most commonly associated with mutations in codon 634 (in exon 11) and 918. Mutations in codons 609, 768, 790, 791, and 891 are associated with the lowest risk for MTC, whereas mutations in codon 611, 618, 620, or 634 are associated with high risk for MTC. Mutations in codon 883, 918, or 922 are associated with the highest risk for aggressive MTC. Mutations in codons 609, 618, and 620 in exon 10 are associated with MEN A2, FMTC, or Hirschsprung's disease. Therefore germline mutation analysis of *RET* exon 10 containing these codons is indicated in all children with Hirschsprung's disease.

Mutation detection rate is summarized in Table 1. Various molecular methods used to detect mutations in *RET* include DNA sequencing, restriction enzymatic analyses, chemical cleavage mismatch, single-stranded conformational polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE). Most recently, a multiplex PCR assay to amplify exons 10, 11, and 13 of the *RET* proto-oncogene has been developed. The multiplex PCR product is then analyzed on a modified mutation detection enhancement (MDE) matrix for heteroduplex identification and visualized with ethidium bromide.[14] Confirmation of the specific mutation can be achieved by restriction enzymatic digestion (if feasible) or by DNA sequencing.

Genetic testing should always be done in the setting of pre- and post-test genetic counseling, as endorsed by the American Society of Clinical Oncology in its position statement on genetic testing for hereditary cancer.[15] The process of genetic counseling includes providing risk assessment, educating patients about testing options, discussing the implications for medical management, and providing supportive counseling to the individual and their family.

SURVEILLANCE

RET germline mutation testing has replaced biochemical screening with calcitonin to identify mutation carriers in MEN 2 families because calcitonin levels lack sensitivity and specificity, normal basal or stimulated calcitonin levels cannot exclude or predict development of MTC, pathological calcitonin levels cannot differentiate between chief cell hyperplasia and MTC, and stimulated calcitonin studies can occasionally produce false-positive results. Screening for pheochromocytoma and HPT is indicated in all variants of MEN 2 syndrome as some kindreds with MEN 2A syndrome manifest only MTC and thus incorrectly designated as FMTC. The presence of a functioning pheochromocytoma should always be excluded by appropriate biochemical screening before thyroidectomy in any patient with MEN 2. Annual screening for pheochromocytoma with 24-hr urinary levels of catecholamines and catecholamine metabolites should continue until age 35 in all phenotypical variants of MEN 2 syndrome. Because pheochromocytomas in MEN 2 syndrome are often multiple and extra-adrenal, MIBG (131I-metaiodobenzylguanidine) scintigraphy and MRI are needed for localization, only if the biochemical results are abnormal. Annual biochemical screening for HPT with serum parathyroid hormone (PTH) and ionized calcium is recommended for those patients who have not had parathyroidectomy and autotransplantation.

SURGICAL MANAGEMENT

Stratification of codon mutation has led to development of guidelines for the timing and extent of prophylactic total thyroidectomy, which is generally recommended before

age 5 years in MEN 2A and FMTC, and before age 1 year in MEN 2B.[12] Total thyroidectomy with central lymph node dissection and autotransplantation of parathyroid glands into the neck or nondominant forearm following positive testing for *RET* mutations is a preventive option for all subtypes of MEN 2. Postoperatively, all patients will need thyroid hormone replacement therapy and monitoring for possible hypoparathyroidism. Although thyroidectomy prior to biochemical evidence of disease may reduce the risk of recurrent disease, continued monitoring with calcitonin levels for residual or recurrent MTC is still recommended.

For HPT, surgical excision of only enlarged glands, similar to surgical management of primary sporadic HPT, is indicated.

For unilateral pheochromocytoma, adrenalectomy, preferably laparoscopic, is indicated. Cortical-sparing adrenalectomy to avoid adrenal insufficiency should be considered in patients with bilateral pheochromocytomas. Long-term follow-up, with periodic surveillance (serum or urinary catecholamine measurements), is recommended as contralateral pheochromocytoma or recurrence may develop many years after the initial operation.

MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN 1 SYNDROME)

Multiple endocrine neoplasia type 1 (MEN 1) is an autosomal dominant endocrinopathy syndrome caused by mutations (mostly truncating) in the *MEN1* gene (chromosomal locus 11q13).[16] *MEN1* gene is a tumor suppressor gene that consists of 10 exons and its product is a 610-amino acid protein called menin.[16–18] MEN 1 syndrome is characterized by various combination of endocrine tumors involving the parathyroid glands (HPT is usually the first manifestation with near 100% penetrance by age 50), entero-pancreatic islet cell tumors (gastrinoma in 40%, insulinoma in 10%, nonfunctioning in 20%), and anterior pituitary adenomas (prolactinoma is the most common and occurs in 20% of patients).[12,19] Less prevalent tumors include adrenal cortical tumors, foregut carcinoid tumors, and lipomas. Pheochromocytomas are rare in MEN 1. Neuroendocrine tumors of the pancreas are often multifocal and malignant, and are the leading cause of mortality in patients with MEN 1. Genetic testing for MEN 1 is commercially available using single-strand conformation analysis (SSCA) and sequencing. It can identify mutation in the MEN 1 gene in approximately 80–90% of probands from well-defined MEN 1 families.[20–23] It is generally offered to patients with clinical criteria of MEN 1, their at-risk relatives, and to confirm diagnosis in cases with features atypical for MEN 1. Presymptomatic MEN 1 genetic testing for family members, while informative, its usefulness is controversial because it rarely mandates therapeutic intervention because of lack of consensus on prophylactic interventions and the inability to predict the extent, spectrum, or clinical onset of future disease.

CONCLUSION

Medullary thyroid carcinoma is the first neoplastic manifestation in MEN 2, the only consistently malignant neoplasm in all phenotypical variants, and the most common cause of death in affected patients. Recent advances in genetic profiling and stratification of RET codon mutations have contributed to accurate genotype–phenotype correlation, proper genetic counseling, targeted surveillance, and timing and extent of prophylactic thyroidectomy in accordance with the natural history of this hereditary syndrome. Genetic testing in high-risk family members is strongly recommended. The available current data indicate superiority of surgical prophylaxis compared with biochemical surveillance in reducing MTC risk in carriers of RET mutations.

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Medulloblastoma—*MYC* Messenger RNA Expression

Michael A. Grotzer
University Children's Hospital, Zürich, Switzerland

INTRODUCTION

Cerebellar primitive neuroectodermal tumors/medulloblastomas (MBs) are the most common malignant brain tumors of childhood. Because of the high risk of leptomeningeal dissemination, standard postoperative treatment for MB includes craniospinal radiotherapy and chemotherapy. Such treatment causes long-term morbidity including endocrine and growth disturbances, as well as neurocognitive dysfunction, which is particularly severe in young children. Over the last years, oncogene *MYC* has been identified as one of the most important biological prognostic factors in MB. Assessment of *MYC* mRNA levels by quantitative RT-PCR is simple, rapid, and reproducible. It is therefore suitable for routine quantification of *MYC* mRNA expression in MB and will be a powerful tool in large prospective cooperative group trials. The goal of these important biological studies will be to evaluate whether *MYC* mRNA expression can be incorporated in future risk-classification systems for clinical use.

CHILDHOOD MEDULLOBLASTOMA

CNS primitive neuroectodermal tumors (PNETs), including cerebellar PNET (medulloblastoma, MB), are the most common malignant brain tumors in children and constitute 20–25% of all pediatric brain tumors. Because of the high risk of leptomeningeal dissemination, standard postoperative treatment for PNET includes not only local radiotherapy but also craniospinal radiotherapy and chemotherapy. Such treatment causes long-term morbidity, including endocrine and growth disturbances, and neurocognitive dysfunction, which is particularly severe in young children.

Clinical prognostic factors for PNET have been identified over the last two decades. These include metastatic stage, extent of tumor resection, tumor location, and age, and are currently used to distinguish a ''high-risk'' group of patients (M stage ≥ 1, residual tumor bulk ≥ 1.5 cm^2, age <3 years, supratentorial tumor location) from a ''standard-risk'' group. However, investigators have appropriately avoided the use of the term ''low risk'' or ''good outcome,'' because published survival rates in the 50–60% range do not justify such terms. At present, clinical prognostic factors cannot identify a good-outcome group of MB patients suitable for treatment with a substantially less toxic treatment strategy than the standard treatment. As it is unlikely that new clinical prognostic factors will be found, biological prognostic factors must be identified to further improve MB prognostic systems.

MYC PROTOONCOGENE

The *MYC* (*c-myc*) protooncogene, a member of a family of closely related genes that includes *MYCN* and *MYCL*, encodes a nuclear phosphoprotein involved in the transcription of genes central to regulating the cell cycle, cellular proliferation, apoptosis, embryonic development, and differentiation.[1,2] MYC proteins form heterodimers with Max, another helix–loop–helix leucine zipper protein. MYC/Max heterodimers bind to specific DNA sequences located in the transcriptional control region of target genes and alter the transcription of these target genes by transactivation or transrepression.

MYC expression is normally tightly regulated throughout the cell cycle, but may become deregulated or activated, thus contributing to malignant transformation.[3] Dysregulation of *MYC* has been implicated in the pathogenesis of a variety of human neoplasms. One important mechanism of *MYC* dysregulation is gene amplification. In childhood neuroblastoma, amplification of *MYCN*, a *MYC* family member, is found to occur in about 25% of primary tumors and is strongly associated with advanced-stage disease, rapid tumor progression, and poor prognosis.[4]

MYC GENE AMPLIFICATION IN MEDULLOBLASTOMA

MYC has been the subject of several published studies attempting to identify biological prognostic markers in human MB. Most show that *MYC* gene amplification is uncommon in MB, with an incidence of $\sim 8\%$ in primary tumors.[5,6] The incidence of *MYC* gene amplification in MB cell lines and xenografts may be as high as 67%,[7] suggesting that *MYC* gene amplification correlates with cell line establishment and tumorigenicity. *MYC* gene amplification has been suggested as an indicator of poor prognosis in some case reports and in a recently published

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study of 29 MB patients.[5] In aggregate, however, published studies suggest that the frequency of *MYC* amplification is not sufficiently high to provide prognostic information of greater value than clinical variables.

MYC MESSENGER RNA EXPRESSION IN MEDULLOBLASTOMA

PNET cell lines show a wide range of *MYC* mRNA expression with lowest expression in DAOY PNET cells and highest expression in D425 PNET cells.[8–10] In DAOY, PFSK and UW288-2 PNET cell lines that do not have *MYC* gene amplification, *MYC* mRNA expression levels show a 10-fold difference. Mechanisms other than gene amplification that activate *MYC* are well recognized in various solid tumors. These include retroviral insertional mutagenesis, chromosomal translocation, somatic mutations, or activation by transcription factors (reviewed in Ref. [11]).

Our own studies demonstrate that MB primary tumors also show a wide range of *MYC* mRNA expression levels, with a 22-fold difference between the highest and lowest value.[8] Like the PNET cell lines, *MYC* mRNA expression is highly variable in those MBs with no *MYC* gene amplification (showing an 11.6-fold difference between the highest and lowest values).

Transcriptional regulation of *MYC* in MB may involve the adenomatous polyposis coli (APC) and β-catenin (CTNNB1) pathways. A subset of MB is associated with Turcot's syndrome,[12] in which colonic cancer is associated with primary brain tumors and which is characterized by germline mutations in the APC gene. While mutations of APC have not been detected in sporadic MB,[13,14] approximately 5% of sporadic MB have been reported to contain mutations in a second Wingless/Wnt pathway member, β-catenin.[15,16] Mutations in either APC or β-catenin act to stabilize β-catenin protein. Stabilized β-catenin protein accumulates and translocates into the nucleus where it forms a complex with Tcf4 and up-regulates the transcription of *MYC* and other target genes.[17,18] In a recent study, aberrant nuclear β-catenin staining was demonstrated in 18% of sporadic MB and in one MB from a Turcot's patient.[16] Therefore, mechanisms other than gene amplification may activate *MYC* transcription in MB.

MYC MESSENGER RNA EXPRESSION AND SURVIVAL

Performing univariate Cox regression analysis, high levels of *MYC* mRNA expression were found to correlate strongly with an unfavorable survival outcome in MB patients (Table 1). *MYC* mRNA expression levels did not correlate with metastatic stage, age, or sex of PNET patients. After correcting for these factors in multivariable analysis, the hazard ratio for *MYC* mRNA expression remained significant, indicating that *MYC* mRNA is of independent prognostic significance.[8] Accordingly, *MYC* mRNA expression represents one of four individual biological prognostic factors we have identified in PNET.[8,19–21] Of these, *TrkC* mRNA and *MYC* mRNA expressions seem to be the most potent prognostic factors when hazard ratios are compared and when the results of independent research groups are considered. Kim et al.[22] determined *TrkC* mRNA expression in 42 MB patients by Northern blot analysis and found that tumors with high *TrkC* mRNA expression were less likely to progress than tumors with low *TrkC* mRNA expression. Herms et al.[6] determined *MYC* expression in 72 MBs by in situ hybridization, and using their assay found that 30 patients with *MYC* mRNA-expressing MB had a worse survival outcome than 42 patients whose tumors did not express *MYC* mRNA.

PROGNOSTIC MODEL COMBINING *MYC* AND *TrkC* MESSENGER RNA EXPRESSION

It is well known that the regulation of tumorigenesis is multifactorial. Therefore, it is unlikely that any single biological prognostic factor will be sufficiently robust to segregate all MB patients optimally into risk-based

Table 1 Univariate analysis of clinical and laboratory variables and progression-free survival in 26 patients with medulloblastoma

Variable	Hazard ratio	(95% CI)	*P* value
MYC mRNA expression	10.89	(1.37–86.38)	0.024
TrkC mRNA expression	3.78	(0.92–15.51)	0.065
Age	20.35	(0.65–8.51)	0.19
Surgery	2.41	(0.60–9.75)	0.22
Therapy	2.40	(0.59–9.83)	0.22
Gender	1.99	(0.42–9.39)	0.39
Metastatic stage	0.88	(0.19–4.18)	0.88

The variables were compared in the following ways: *MYC* mRNA expression, high vs. low; *TrkC* mRNA expression, low vs. high; age, <3 years vs. ≥ 3 years; surgery, extent of resection $<90\%$ vs. $\geq 90\%$; therapy, radiotherapy, or chemotherapy alone vs. radiotherapy + chemotherapy; gender, male vs. female; metastatic stage, M1-3 vs. M0.
Source: Ref. [8].

strata. Accordingly, multiple factors of biological relevance are more likely to provide an accurate system of defining risk groups. We have demonstrated this by combining *MYC* and *TrkC* mRNA expression (Fig. 1). The group with low *MYC* and high *TrkC* mRNA expression had a 100% 5-year progression-free survival outcome after a median follow-up time of 55 months (range, 15–91 months). Notably, three patients in this group were infants who did not receive radiotherapy.[8] Although the number of patients is too small to draw firm conclusions, it is conceivable that for this age group, where the neurotoxic consequences of radiation therapy are greatest, a good-outcome group can be identified by biological factors and then treated using chemotherapy alone. The prognostic model using *MYC* and *TrkC* mRNA expression was superior to known clinical factors in identifying a good-outcome group of patients.

MYC QUANTITATIVE REAL-TIME RT-PCR

To optimize quantification of *MYC* mRNA expression, we developed a real-time quantitative RT-PCR assay based on TaqMan fluorescence methodology using 40 primary tumor and 7 normal brain samples. Isolation of total RNA and cDNA synthesis was performed as previously described.[9,23,24] Kinetic real-time PCR quantification of hTERT mRNA was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) as described previously.[25] For each PCR run, a master mix (Applied Biosystems) was prepared containing 200 nM of each primer and 400 nM probe. Thirty nanograms cDNA per RT sample was added to a final volume of 25 μL PCR master mix. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, and 50 cycles at 95°C for 15 sec and 60°C for 1 min. Experiments were performed in triplicate for each data point. Each PCR run included the five points of the standard curve (serially diluted DAOY PNET cell line cDNAs), a no-template control, the calibrator cDNA, and patient cDNAs.

The precise amount of total RNA added to each reaction mix (based on absorbance) and its quality are difficult to assess. We therefore also quantified transcripts of the *18S* rRNA housekeeping gene as endogenous control, and each sample was normalized on the basis of its *18S* rRNA content. The relative expression of *MYC* mRNA was calculated using the comparative C_T method.[26] The amount of *MYC*, normalized to *18S* rRNA and relative to the calibrator, is then given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ (sample) $- \Delta C_T$ (calibrator), and ΔC_T is the C_T of the target gene (*MYC*) subtracted from the C_T of the housekeeping gene (*18S* rRNA). PCR efficiencies for *MYC* and *18S* rRNA were measured as previously described[27,28] and were found to be >95%. Thus, the $\Delta\Delta C_T$ method, in which the efficiency of PCR amplification of the target gene must be approximately equal to that of the housekeeping gene, is applicable.

We validated the method on a series of PNET cell lines with known *MYC* mRNA expression. *MYC* mRNA

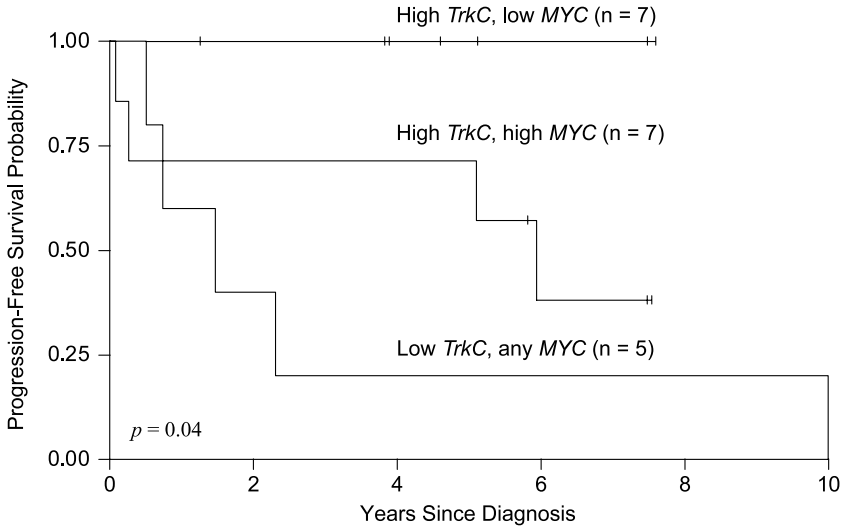

Fig. 1 Cumulative progression-free survival of patients with medulloblastoma, according to expression levels of both *MYC* mRNA and *TrkC* mRNA.

expression levels in primary PNET varied widely with >100-fold (*MYC*) differences between the highest and lowest values. This is higher than previously measured with less quantitative methods. Compared to normal cerebellum, *MYC* was overexpressed in 60% of the PNET samples evaluated.

CONCLUSION

We anticipate that biological prognostic factors, in addition to clinical factors, will define risk groups and help direct therapy decisions for children with MB. We therefore strongly recommend prospectively assessing *MYC* mRNA expression levels in future MB clinical trials in an effort to validate this model for future incorporation in risk-classification systems for clinical use.

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Melanoma Biomarkers—Molecular Profiling and Its Clinical Implications

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Pia Vihinen
Turku University Hospital, Turku, Finland

Veli-Matti Kähäri
University of Turku, Turku, Finland

INTRODUCTION

The incidence of cutaneous malignant melanoma is rising, and new clinical and histological variables that predict outcome have been identified. Cutaneous melanomas are also a tumor group, in which multiple new molecular profiling methods, such as DNA arrays, proteomics, and tissue microarrays (TMAs), have been extensively utilized to identify new prognostic factors and therapeutic targets. In this review we discuss how these new techniques may improve the diagnosis, prognosis, and therapy of malignant melanoma.

MELANOMA GENETICS

Altering the genetic message in cancer is essential for its development and can be caused by DNA mutations, chromosomal aberrations (CNAs), epigenetic modification, and protein–protein interaction.[1] Mutations in the genomic DNA can lead to alterations in gene expression and function. Multiple genetic alterations have been described during development and progression of cutaneous melanoma.[2] Investigation of primary melanomas by comparative genomic hybridization has resulted in detection of losses of chromosomes 6q, 8p, and 10 as well as increases in copy numbers of chromosomes 1q, 6p, 7, and 8.[2] DNA modification, mutation, and viral genome integration can lead to chromosomal instability, i.e., CNA, where particular regional amplifications and deletions of genes are found. Chromosomal aberrations have been found in genomes of multiple solid tumors. O'Hagan et al.[3] have been able to distinguish primary melanoma tumors with different etiology by using array-based comparative genomic hybridization analysis of genome-wide CNAs in a genetically modified mouse model.

There are also several specific gene mutations frequently found in cutaneous melanoma. Cyclin-dependent kinase gene *CDKN2A* is a tumor suppressor gene whose function is suppressed with malignant transformation.[4] *CDKN2A* encodes for p16 protein that binds and inhibits cyclin-dependent kinases, leading to cell-cycle arrest in G1 phase and continuous cell proliferation.[4] Mutations in genes, such as NRAS- and BRAF-encoding components of Ras–Raf–mitogen-activated protein kinase (MAPK) signaling pathway, have been found in nearly 90% of melanomas studied.[5] However, simultaneous mutation in both genes is rare.[5] These mutations cause uncontrolled activation of these genes, which mediate cellular responses to growth factor stimulation resulting in continuous growth signal.[5] Recent studies also show that there is a specific melanoma metastasis suppressor gene, which is located in chromosome 6.[6] Similarly, a mutation in the gene coding for tumor suppressor PTEN, a phosphatase, which opposes the action of PI-3-kinase, is found in late phases of melanoma progression.[7] In addition to alterations in individual chromosomes and genes, new array technology gives a possibility to detect multiple expression changes in thousands of specified genes simultaneously in a studied tumor model.

MELANOMA BIOMARKERS—HOW TO FIND THEM

Sample-based screening of melanoma tumors for desired markers can be performed with traditional immunohistochemistry (IHC) or with more sophisticated gene arrays, TMAs, or tumor protein lysate array.

Melanocytes and melanoma cells in vitro and in tumor samples from different stages of melanoma progression have been studied widely by immunohistochemistry to identify specific proteins to be used as biomarkers for prognosis. Markers which have shown prognostic value in restricted patient groups include S-100B, melanoma inhibitory activity protein (MIA), tyrosinase, certain matrix metalloproteinases, integrins, interleukin-8, and CD 44 (Ref. [8] and references therein). Some of these markers can also be measured in patient sera. The increased expression levels or bioactivity of these markers

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is usually associated with melanoma progression, recurrence, or poorer survival. However, the clinical use of these markers is limited to oncology departments with research activity.

In microarray studies thousands of marker genes can be simultaneously studied to diagnose melanoma, to classify patients into different prognostic subgroups, and to identify new targets for therapy.[9,10] DNA microarrays are based on high-density oligonucleotide or cDNA microarrays, which measure in parallel thousands of gene-specific mRNAs in a single RNA sample.[1] This kind of large-scale gene expression analysis has proved to be a valid strategy for developing sample-specific gene expression profiles.[1] DNA microarrays have potential application in clinical practice, but their application is limited by the requirement of fresh or frozen tissue for analysis (Fig. 1).

DNA microarrays determine the levels of target gene mRNA in studied tumors, but do not give information on gene function. Study of protein complement of the genome by functional proteomics can be used to screen for alterations in protein expression and posttranslational modifications under melanoma progression.[1]

Tissue microarrays are arrays in which hundreds of small core (0.6–2.0 mm) tissue sections are arrayed on a glass slide.[11] Tissue microarrays give a good impression of the whole tumor area and they can be used for IHC and in situ hybridization. Tissue microarrays are useful in screening of a desired marker rapidly in a large patient group.[11]

Tumor protein lysate array by proteomics is a new technique based on detection of a particular protein expression in patient samples. This array also requires frozen or fresh tissue samples.

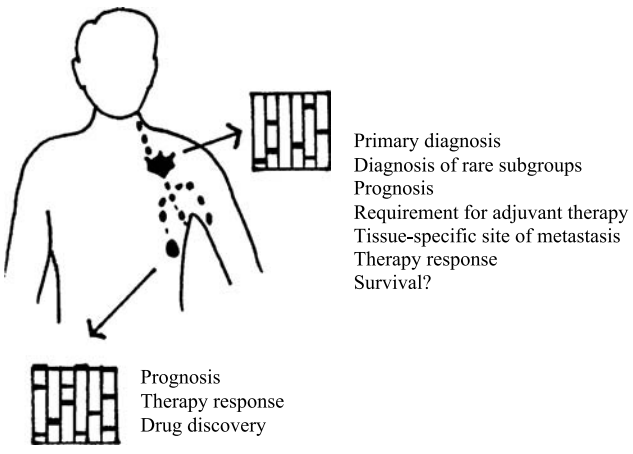

Fig. 1 Putative clinical value of DNA or tissue microarrays of primary or metastatic melanoma specimen.

Molecular Profiling of Cutaneous Melanoma

Molecular profiling of melanomas by gene expression analysis has emerged as a new possibility to predict melanoma prognosis and treatment planning. Detailed genetic profiles found by microarray analyses are available on the internet (http://www.nhgri.nih.gov). Bittner et al.[9] compared the tumor cell mRNA with a single reference probe, providing normalized measures of the expression of each gene in each sample relative to the standard. They found several genes whose expression levels were altered giving a possibility to discriminate studied genes into different clusters.[9] Interestingly, the melanoma cells studied belonging to different groups also had different biological properties, i.e., their ability to migrate, form tubular networks, and contract collagen gels in vitro was different.[9] It has become evident that there are genes whose expression levels are elevated in aggressive melanoma cell lines or tumors, as well as genes whose expression levels are downregulated. The expression of several extracellular matrix components, such as fibronectin, $\gamma 2$ chain of laminin 5, and $\alpha 2$ chain of collagen IV, is upregulated in aggressive melanoma cells,[9] suggesting that invasive tumors have increased capacity to regulate cytoskeletal organization, cell movement, and invasion by modifying the composition of the surrounding extracellular matrix. Similarly, the expression level of *Wnt5A*, a gene involved in melanoma cell motility and invasion, shows correlation with tumor grade while inversely correlating with survival.[12] In addition, several studies have shown upregulation of invasion-promoting matrix-degrading proteinases, such as cathepsin Z.[12] Melanocyte-specific markers, such as tyrosinase and melan-A, are downregulated suggesting that highly aggressive tumor cells undergo dedifferentiation into less melanocyte-like cells. Several markers of angiogenesis, such as endothelial protein receptor TIE-1, vascular endothelial growth factor-C (VEGF-C), and vascular endothelial cadherin (VE-cadherin) have been found to be overexpressed in melanomas.[10] The expression of these genes is related to a phenomenon called vascular mimicry, in which highly aggressive tumor cells are able to undergo a genetic reversion to a pluripotent, more embryonic-like phenotype, which may lead to the formation of new vessels by tumor cells themselves. Neovascularization by tumor cells may explain the aggressiveness of metastatic melanoma and provide a basis for therapy targeting angiogenic molecules and signal transduction pathways.

The possible clinical use of these arrays in diagnosis, prognosis, and treatment planning is further discussed by describing recent findings in in vitro models as well as in patient-derived sample analysis.

Molecular Profiling of Sentinel Nodes

The importance of sentinel node evaluation and detection of micrometastases in lymph nodes has been shown in a systematic evaluation of 17,600 patients with cutaneous melanoma.[13] Patients with detected micrometastases have less favorable prognosis than those whose nodes have been found to be unaffected.[13] However, this implication may change to a more detailed, molecular analysis of dissected lymph nodes.[14] Kuo et al.[14] have shown the feasibility of PCR-based molecular multimarker analysis of sentinel lymph nodes in the detection and prognostication of recurrence in patients with early-stage melanoma. The PCR-based multimarker analysis might even detect single metastatic melanoma cells in sentinel lymph nodes thought to be normal in microscopic IHC analysis.

CLINICAL IMPLICATIONS

Diagnosis

Detailed analysis of the expression patterns of thousands of genes simultaneously has made it possible to identify novel disease genes, such as *Wnt5A*, *RhoC*, and BRAF, for cutaneous melanoma.[12] De Wit et al.[15] have been able to identify new melanoma-specific antigens, such as MMA-1a and MMA-1b, by using oligonucleotide array-based analysis. In addition to diagnosis, gene expression profiling can be used in tumor classification. On the basis of melanoma-specific biomarker listing it would be possible to molecularly distinguish atypical melanomas from nonmelanoma skin cancers.

Gene array and cluster analysis might also help in the characterization of rare melanoma subgroups. Tschentscher and coworkers[16] have found new subtypes of uveal melanoma, and Segal et al.[17] have shown that clear-cell sarcoma is a distinct subtype of cutaneous melanoma.

Prognosis

Classification of tumors with similar histology into different prognostic subgroups is a clinically valuable benefit of DNA arrays. In a pronounced work by Clark et al.,[18] specific genes, such as *RhoC*, responsible for melanoma progression were identified. Alonso et al.[19] were able to identify distinct gene expression profiles distinguishing specific melanoma progression stages. Genes whose expression was reduced in advanced melanoma stages included *p16*, *p27*, and cyclin D, suggesting that losing cell-cycle control is essential in melanoma progression.[19] Previous work with primary breast cancer and its node-positive counterparts has suggested that gene expression pattern does not differ much between primary tumor and its node metastasis.[20] This may also be true in melanoma, as at least early mutation status of NRAS and BRAF is maintained from dysplastic nevi to primary melanomas and their metastases.[5] Thus detection of upregulation of genes responsible for dissemination in primary tumors would be clinically valuable as prognostic factor. Furthermore, DNA microarray analyses performed in murine melanoma models have even defined genes which are consistently elevated in pulmonary metastases,[21] suggesting that predictable, organ-specific metastasis gene expression profiles could be found and used in further patient surveillance. Similar hypothesis has been presented concerning breast cancer, where new findings suggest that primary tumors with metastatic capacity possess the signature for poor prognosis, but subpopulations of cells also display a tissue-specific expression profile predicting the site of metastasis.[22] According to these findings, the prevailing model of metastasis, where it is suggested to be a late and rare event in tumorigenesis, may change.

Direct analysis of melanocytic lesions by tissue microarrays has also helped in finding molecules which predict melanoma progression. Shen et al.[23] analyzed several benign and dysplastic nevi as well as different growth-phase melanomas to show that particular growth factor receptor tyrosine kinases are differently expressed in distinct stages of tumor development. They found that nearly 100% of dysplastic nevi and vertical growth-phase melanomas studied express c-kit and platelet-derived growth factor receptor β (PDGFR-β). Interestingly, metastases seemed to be associated with loss of c-kit and PDGFR-β expression, suggesting that patients with an earlier stage of disease might benefit from imatinib, a PTK inhibitor targeted toward these receptors.

Treatment

Different adjuvant treatment modalities, i.e., interferon-α (IFN-α) and vaccine therapy, have been extensively studied among melanoma patients. It is not known whether melanoma patients with high metastasis risk gain survival benefit from adjuvant therapy. Interferon-α is the most studied adjuvant therapy agent, although its routine use cannot be recommended in high-risk primary melanoma because of lack of phase III studies with evidence for survival benefit. Interferon-α exerts its effects through antiproliferative, apoptosis-inducing, and antiangiogenic effects in addition to immunological modulation. Certa et al.[24] have found differently regulated IFN-α responsive gene groups in IFN-α-

sensitive and -resistant melanoma cells. It is also clinically evident that an unidentified subpopulation of patients seems to benefit from IFN-α-based adjuvant therapy and thus array technology might help in identifying this population. Furthermore, microarray analysis may be used as a feasible test to identify patients who are cured by surgery alone and do not need any systemic adjuvant therapy.

Interleukin-2 (IL-2) is a cytokine which has been used in the treatment of metastatic melanoma. Panelli et al.[25] have shown that systemic IL-2 administration leads to gene expression changes in both blood mononuclear cells and melanoma metastases. The activation of genes was predominantly associated with monocyte function, but also genes responsible for secondary recruitment of immunological cells to the tumor site were upregulated.[25] Also, other types of immunotherapy have been studied in melanoma patients, especially in the form of different vaccines. Durable antitumor responses have been infrequent, which may be due to patient's specific lack of activation or trafficking of T cells. Wang et al.[26] examined gene profiles of melanoma metastasis samples and found a 30-gene cluster, which was predictive of clinical response to immunotherapy. Half of these genes are related to T-cell regulation.[26] Kershaw et al.[27] have shown in their model that melanoma cells express various chemokines, which might redirect the migration properties of activated T cells. Grolleau et al.[28] have made similar findings in their studies with dendritic cells (DCs), which can be used antigen-pulsed or -unpulsed in vaccine therapy of melanoma. They show that pulsing of DCs with tumor cell lysate leads to upregulation of genes important for DC effector function, but also induces macrophage receptor expression.[28] Differently regulated immunoresponse genes may explain some of the detected variations in the effectiveness of vaccines among individual patients. Microarray profiles might be useful when selecting between optimal therapy regimens, i.e., IFN-α, IL-2, or vaccination for a patient.

Neoangiogenesis is a pronounced phenomenon in melanoma progression and it is promoted by several factors including hypoxia. Upregulation of angiogenesis-related genes has been shown in several melanoma models, as discussed above. Specific genes involved in vascularization have been detected. In addition, progressing melanoma cells show molecular plasticity suggesting that they themselves are able to form new vessels via vascular mimicry, as discussed above. Targeting these factors might help in planning tailored therapy to each patient.

DRUG DISCOVERY

Drug discovery is an important area where gene arrays and proteomics might be used. Genomic studies that produce large databases of molecular information on cancers might be linked to patterns of drug activity. First, pharmacogenomic analyses, where genomic information is coupled with structure-based data to identify classes of compounds for which detailed experimental structure–activity studies might be used, have been performed.[29] Blower et al.[29] have identified two quinine subclasses, whose patterns of activity in tested cell lines (including melanoma) correlate strongly with expression patterns of particular genes.

Microarray studies on signaling molecules and transcriptional factors in melanocytes have identified potential therapeutic targets, such as bcl-2, which is known to rescue melanoma cells from apoptosis.[30] This antiapoptotic molecule was identified as the transcriptional target of the microphtalmia gene, *Mitf*, a transcription factor expressed in the majority of primary melanomas.[12] Bcl-2 has been used as a target of a new type of therapy, where inhibition of its expression by a specific antisense oligonucleotide has increased time to progression of metastatic melanoma patients when used together with DTIC.[31]

PERSPECTIVE

DNA and tissue microarrays have rapidly increased the molecular information on melanoma development and progression. However, there are several challenges left when different arrays are estimated in a clinical point of view. Why are the same markers measured in IHC and serum analysis assays only rarely upregulated in gene array-based analysis? There can be several explanations. The expression levels of studied ''traditional'' markers are usually estimated between different patients, thus there is always an artificial cut-off point, which is often more researcher-dependent than related to negative and positive controls. It is also evident that even if a gene is upregulated in an array analysis, it does not necessarily mean that there is an increase in the function of the protein which it codes. Comparison of results gained from distinct DNA microarray studies has brought specificity into array analyses. The function of a desired gene can be studied by RNA interference (RNAi) method, by specific gene knockdowns in mammalian cells. RNA interference analysis shows the biological effect of a specific inhibition of gene expression and thus suggests its therapeutic significance. However, there is no consensus at the moment on how to translate these microarray profiles into clinical utility.

Heterogeneity of gained gene profiles between different studies is a problem. Although several melanoma-''specific'' array results have been published there is still no melanoma-specific repeatable gene profile available. In in vitro studies with cultured cell lines this can be partly

explained by the heterogeneity of cellular subpopulations of the same pathological origin. However, clustering analyses also only rarely end up in a similar gene expression profile despite the fact that patients' clinical features between different studies have been quite homogeneous. Reliability of clustering results and profiles has been studied widely, and there are also possibilities of statistical errors. Pusztai et al.[32] have pointed out the importance of array standardization. Arrays can contain cDNAs of variable lengths or small oligonucleotide sequences leading to differences in signal intensity.[32] There is also variability in the control tissue and sampling method used. Symmans et al.[33] have clearly demonstrated that transcriptional profiles from the same tumor differ according to sampling method, i.e., fine needle aspiration and core needle biopsy yield different gene expression profiles.

There are also some ethical problems. Not all patients want to know their gene expression profile, although it would be worth assessing because of increased clinical risk factors. Similarly, it is not perfectly certain that gene profiles do not change during melanoma progression, and it is not always safe or even ethical to take serial biopsies during disease progression only to detect gene profiles. Detection of gene profile has been shown to have a survival value in breast cancer,[20] but, so far, there is no evidence supporting gene or tissue arrays or proteomics as a source of any information, which would increase the survival of melanoma patients. Furthermore, Ntzani and Ioannidis[34] have shown in their analysis that only a limited number of the DNA microarray studies performed to address cancer outcome actually had prognostic value.

CONCLUSION

Gene and tissue arrays have multiplied the genetic and molecular information on melanoma in a few years. It is probable that multimarker analysis of primary tumors and archival lymph nodes will lead to molecular staging of patients with primary melanoma. Marker assays will also lead to wide use of oncogenomics, i.e., molecular profiles will be incorporated into therapy and clinical trials. However, these functional arrays require fresh or frozen tissue samples and this implication may lead to a change in sampling for pathological diagnosis. Tissue banks containing frozen tumor samples may comprise the basics for future personalized medicine.

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Melanoma, Familial

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Femke A. de Snoo
Leiden University Medical Center, Leiden, The Netherlands

Nicholas K. Hayward
Queensland Institute of Medical Research, Herston, Australia

INTRODUCTION

To date, two high-penetrance melanoma predisposition genes have been identified, *CDKN2A* and *CDK4*. However, because only a small fraction of melanoma families can be explained by mutations in these genes, the search for additional melanoma susceptibility genes continues. Of particular clinical concern is the frequently reported increased risk of melanoma in nonmutation carriers in *CDKN2A* or *CDK4* mutation-positive families. Increasingly, a number of environmental, phenotypic, and other low-penetrance genetic risk factors for melanoma are being recognized. Hence, familial melanoma is a complex disorder with many aspects still to be unraveled. Therefore, the usefulness of diagnostic DNA testing is still being questioned.

CLINICAL DESCRIPTION AND PREVALENCE

The incidence of melanoma is rising faster than all other cancers except lung cancer in women, currently varying between 5 (Western Europe) and 20 (Northern Europe) to over 50 (Queensland, Australia) cases per 100,000 per annum.[1,2] Familial clustering of melanoma was first described by Norris in 1820,[3] but it was not until the second half of the 20th century before others documented the familial occurrence of melanoma. Across several population-based studies, 1–13% of melanoma cases reported the occurrence of melanoma in at least one first-degree relative.[4] Hence, it is commonly accepted that melanoma predisposition is hereditary in $\sim$10% of all cases. But even in high-sun-exposure areas such as Queensland, Australia, less than 5% of melanoma probands report two or more first- or second-degree relatives affected with melanoma.[5]

Whereas susceptibility in small numbers of highly selected multiple-case melanoma families is consistent with autosomal dominant inheritance of a single major gene, segregation analysis in a large population-based sample of families failed to show a single major gene being responsible for melanoma transmission, possibly because of relatives' risk factors not being included in this analysis.[6] Thus, the bulk of familial clustering of melanoma may be better ascribed either to a codominant, a polygenic, or a recessive mode of inheritance.

In the absence of a characterized mutation there are no clear-cut criteria for ''familial melanoma,'' but as a working definition clinicians and researchers generally use this term to describe families in which there are at least three cases of melanoma. However, in low-sunlight areas such as northern Europe, familial melanoma is often defined when melanoma occurs in as few as two first-degree relatives. Additionally, individuals in whom multiple melanomas occur in the absence of a family history of the disease are of concern, because many of these cases have been found to harbor mutations in *CDKN2A*.[7] As will be illustrated below, clinical suspicion of a hereditary component should also arise when pancreatic cancer, uveal melanoma, or nervous system tumors occur in conjunction with melanoma in the same family.

RISK FACTORS

Risk factors for melanoma in general (often termed ''sporadic melanoma'') have either proven, or are most likely, to be also risk factors for familial melanoma. Apart from a family history of melanoma, the best known environmental risk factor for melanoma is sunlight exposure. This risk factor can, to a large part, explain the increase in melanoma incidence over recent decades and most probably also explains the geographical variance in penetrance figures for mutation-positive melanoma families (discussed later).

The phenotypic characteristics of fair skin (inability to tan), light hair (especially red) and eye color, extensive freckling, and high number of nevi (moles, particularly atypical) are all well-recognized risk factors for melanoma. Many have been shown to be independent risk factors, but their relative risks vary considerably among different studies. This variation is most probably due to differences in phenotypic definition. Elucidation of the

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genetic variants underlying these complex characteristics should lead to an even better defined phenotypic risk profile. Molecular analysis of one pigmentation gene, *MC1R*, a regulator of melanin production, has led to the identification of a genetic risk modifier for melanoma. Beyond the finding that certain *MC1R* variants are linked to the melanoma risk phenotypes of red hair, freckles, and fair skin, these variants also confer melanoma risk irrespective of the pigmentation phenotype.[8] Furthermore, *MC1R* variants have been found to increase melanoma penetrance in *CDKN2A* mutation-positive individuals.[9,10]

Of all the above-mentioned risk factors for melanoma, nevi (especially atypical) have by far the greatest clinical implication. Also apparent as precursor lesions both in sporadic and familial melanoma, many groups have reported the co-occurrence of a nevus phenotype with familial melanoma. Throughout the years familial melanoma has been referred to by many different names, which may reflect the difficulty to reach consensus on defining the associated nevus phenotype: B-K mole syndrome (initials of described families); dysplastic nevus syndrome (DNS), familial atypical multiple mole melanoma (FAMMM) syndrome, and atypical mole syndrome (AMS). Each of these definitions includes atypical nevi as the most predominant feature of the phenotype. Atypical nevi are defined as nevi that have a size of 5 mm or larger, have a flat component, and fulfill at least two of the following three criteria: 1) variegated pigmentation, 2) ill-defined border, and 3) irregular shape. The photographs in Fig. 1 illustrate melanoma and atypical nevi. Although both phenotypes are commonly coexpressed in familial melanoma, they are not always transmitted together.

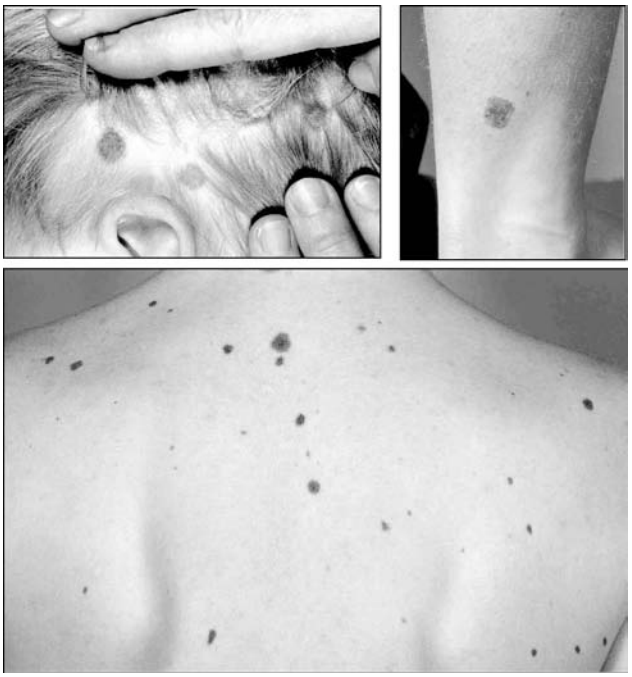

Fig. 1 Pigmented lesions on three different patients with familial melanoma or the atypical mole syndrome. The upper left picture shows several atypical nevi on the scalp, the lower picture shows the back of a patient with many common and atypical nevi, and the upper right picture shows a melanoma on the lower leg. (*View this art in color at www.dekker.com.*)

DIFFERENTIAL DIAGNOSIS (OTHER RELATED SYNDROMES CONTAINING MELANOMA)

Melanoma/Pancreatic Cancer Syndrome

Melanoma susceptibility has also been reported in conjunction with gastrointestinal cancer and breast cancer, and an excess of pancreatic carcinoma is frequently being observed. Comparison of *CDKN2A* mutation-positive and -negative melanoma families showed an increased risk of pancreatic cancer in the mutation-positive group.[11] Interestingly, the increased risk for pancreatic cancer seems to be restricted to only some of the *CDKN2A* mutation-positive families, implying other genes and/or environmental factors are responsible for modifying the penetrance of this tumor type. Disentanglement of these issues is of utmost importance because of the high mortality associated with this cancer and the lack of routine surveillance for it. Whether the correlation with other cancers is restricted to pancreatic cancer only or also involves different cancers remains to be answered by large-scale analysis of cancer history in melanoma families.

Cutaneous Melanoma/Uveal Melanoma Families

Another tumor sometimes described in familial melanoma kindred is uveal melanoma. Although the first *CDKN2A* mutation in such a family was recently published,[12] up to then no underlying gene defect has been found in uveal/cutaneous melanoma families. Genome-wide linkage scans are currently under way to localize additional predisposition genes in such families.

Melanoma/Nervous System Tumor Syndrome

Nervous system tumors are sporadically being described in melanoma families and multiple melanoma cases. In most of these the underlying genetic defect affects the p14ARF transcript of the *CDKN2A* locus (see below),

either exclusively or concomitantly with the p16 transcript.[7]

MOLECULAR GENETICS

The first identified melanoma susceptibility gene, located on chromosome 9p21-p13, has been known by many different names: *MTS1*, *INK4A*, *CDKN2*. The currently accepted gene nomenclature is *CDKN2A*, which stands for cyclin-dependent kinase inhibitor 2A. This locus was mapped by linkage analysis in families in which melanoma was considered the sole phenotype, rather than including a nevus phenotype as well.[13] By using different first exons, exon 1α and 1β, respectively, *CDKN2A* encodes for two proteins, p16INK4A (commonly referred to as p16) and p14ARF (alternative reading frame). Both proteins are tumor suppressors involved in cell cycle regulation. p16 inhibits phosphorylation (and thereby activation) of the retinoblastoma gene product (pRb) by *CDK4* and *CDK6*. p14ARF inhibits HDM2 degradation of p53. Thus, loss of function of either protein drives cell cycle progression through deregulation of either the pRb or p53 pathways (Fig. 2).

Most germline mutations in *CDKN2A* are missense mutations found in exons 1α and exon 2, some of which are recurrent. Because of the low frequency of mutations detected in familial melanoma, mutation analysis has been extended and has led to the finding of mutations in the 5′ untranslated region and intronic regions, where they affect splicing (e.g., Refs. [14–16]). Where analyzed, all but one of the recurring mutations within *CDKN2A* have been shown to be founder mutations. The exception to this rule is a 24-base-pair duplication at the beginning of the coding region, which results in the addition (or in some cases the deletion) of one repeat unit of a naturally occurring 24-base-pair tandem repeat. Haplotype analysis of families carrying this duplication has indicated that the mutation has arisen multiple times, presumably as a result of replication slippage.

Because of the sharing of exon 2, many mutations affecting p16INK4A also affect p14ARF. However, gene defects affecting only p14ARF have been described in some melanoma families[7] thus indicating that the ARF locus is a melanoma susceptibility gene in its own right.

A candidate gene search led to the finding of a second melanoma susceptibility gene, *CDK4*, on chromosome 12q14.[17] *CDK4* mutations have been reported in only three families worldwide, but whereas its role in familial melanoma overall is low, mutations of this gene have similar impact to those in *CDKN2A*.[18] Phenotypic characteristics of the families carrying *CDK4* germline mutations do not differ from *CDKN2A*-affected families, similarly showing a high penetrance for melanoma in mutation-positive family members and no strong correlation between carrier status and nevus phenotype.

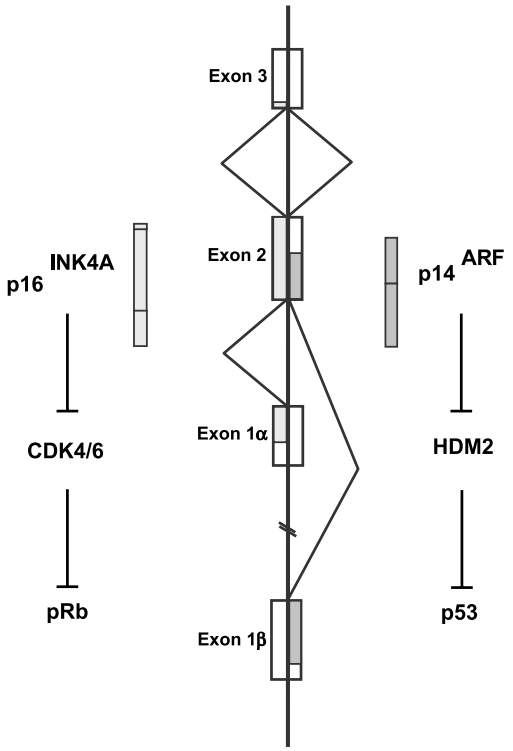

Fig. 2 The *CDKN2A* locus and its transcripts, p16 and p14ARF, generated by alternative splicing, along with their downstream targets. Boxes indicate exons, and colored sections the protein coding regions within them. (*View this art in color at www.dekker.com.*)

An on-line database[19] lists germline mutations of all disease loci implicated in familial melanoma.

Additional Melanoma Genes

Mutations in *CDKN2A* and *CDK4* account for only 5–50% of melanoma families. Many families in which no genetic defect has been found show linkage to chromosome 9p. Recent advances in mutation analysis of the *CDKN2A* gene suggest that some of these families will harbor mutations in the noncoding regions of the *CDKN2A* locus. However, several studies (including haplotype and loss of heterozygosity analyses) provide evidence for a second melanoma susceptibility gene residing centromeric of *CDKN2A*.

Considerable effort is being put into studies to identify other melanoma susceptibility genes. This effort has recently paid off with a publication showing convincing evidence for a melanoma susceptibility gene on chromosome 1p22.[20] Other nonreplicated linkage studies have

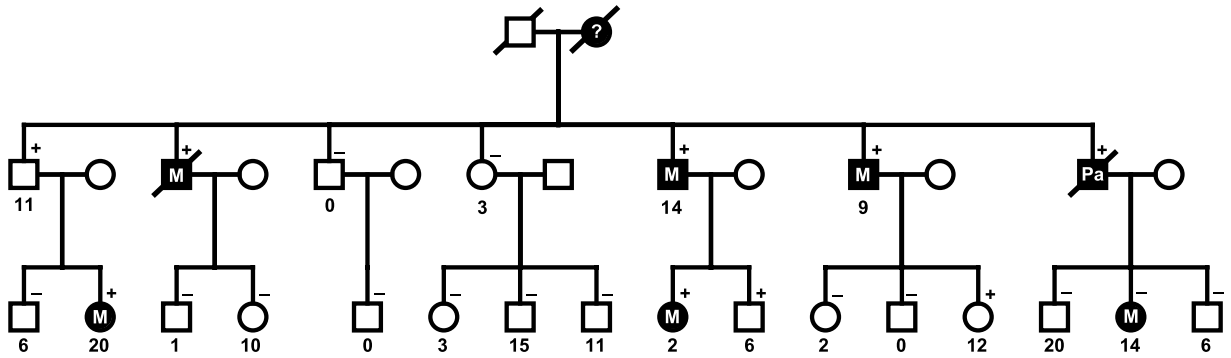

Fig. 3 Pedigree of a melanoma family, illustrating incomplete penetrance, the occurrence of pancreatic cancer, melanoma in a nonmutation carrier, and the lack of segregation of atypical nevi with carrier status. +=*CDKN2A* mutation-positive, −=*CDKN2A* mutation negative, M=melanoma, Pa=pancreatic carcinoma, ?=cancer of unknown origin. Numbers underneath individuals depict numbers of atypical nevi.

suggested the occurrence of melanoma predisposition genes on 1p36 and in the region of the HLA complex on 6p21.[21]

GENOTYPE–PHENOTYPE CORRELATIONS

The International Melanoma Genetics Consortium has estimated the penetrance of melanoma in *CDKN2A* mutation-positive family members between different geographical locations to range from 58% to 91% by the age of 80 years, with an average of 67%.[22] The broad confidence intervals make it impossible to provide precise melanoma risks. Mutations affecting only p16 compared to those affecting both p16 and p14ARF showed a trend (although not statistically significant) toward a higher penetrance in the latter.

Mutations affecting only p14ARF have been described in two melanoma families and an individual with multiple melanomas. Additionally, another multiple melanoma case and two other families have deletions of part of chromosome 9p that encompass both p16 and p14ARF coding sequences. Each of the latter and one of the p14ARF-only mutated families have cases of nervous system tumors.[7] These observations suggest that whereas both p16 and p14ARF predispose to melanoma, it is mutation of p14ARF that predisposes to nervous system tumors.

In families with an increased incidence of pancreatic cancer there is no clear correlation with the position or type of mutation in *CDKN2A*. Similarly, there is no obvious correlation between position (or type) of mutation within the gene and the development of nevi—this is particularly well highlighted by families with the same mutation being either extremely moley, or conversely, relatively free of nevi. One especially important observation is that abundance of nevi is not a predictor of *CDKN2A* mutation status. High numbers of nevi are also seen in nonmutation carriers within mutation-positive families (illustrated in the pedigree shown in Fig. 3).

MOLECULAR GENETIC TESTING, GENETIC COUNSELING, AND CLINICAL MANAGEMENT

Molecular genetic testing in familial melanoma is currently considered premature by the International Melanoma Genetics Consortium[23] for the following reasons. First, the mutation detection rate in melanoma families is very low. Second, risk estimates for mutation carriers are not well established (and have broad confidence intervals). In particular, the risk for other cancers, such as pancreatic cancer, is not clearly defined. Third, surveillance programs for mutation carriers have not yet been proven to be effective, questioning the medical benefit of the DNA test result. The last and most critical reason for not currently offering genetic testing in familial melanoma is the increased risk for melanoma in nonmutation carriers in *CDKN2A* mutation-positive families. Predictive DNA testing then should only be offered within the bounds of a clearly defined research protocol. In this setting, extensive genetic counseling addressing the limited value of testing in familial melanoma is a prerequisite. Such research protocols might offer possibilities to explore the still pending issues concerning the risk benefit and potential value of genetic testing for familial melanoma. They will need to address efficient screening protocols for melanoma and pancreatic cancer in the hope of leading to a decrease in mortality by earlier detection. Outside of such research investigations, clinical management of familial melanoma should be restricted

to recognition of high-risk individuals and subsequent yearly or half-yearly skin examination from age 10–12 onward. The strong decrease in life expectancy with increasing tumor thickness makes early recognition of melanoma through regular skin examinations the hallmark of clinical management.

CONCLUSION

Melanoma predisposition is hereditary in 10% of cases. Large international research projects have been set up to unravel many of the remaining issues concerning familial melanoma, which include the following:

- Identifying the causative gene defect in the majority of families.
- Determining precise risk estimates for melanoma and other cancers, especially pancreatic cancer, in mutation carriers.
- Attributing the degree and causes of the increased risk of melanoma in nonmutation carriers of mutation-positive families.
- Elucidating the underlying causes of the nevus phenotype and its risk implications.
- Assessing the efficacy of surveillance programs for melanoma and other cancers.

At present, the clinical management of familial melanoma should be restricted to recognition of the disease and subsequent regular skin examinations of high-risk individuals.

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Metabonomics and Its Role in Disease Diagnosis

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John C. Lindon
Jeremy K. Nicholson
Elaine Holmes
Imperial College London, London, U.K.

INTRODUCTION

The determination of the human genome generated much interest in using changes in levels of gene expression in individuals to discover the basis of disease and for the identification of new drug targets. Some significant advances have been made using this approach, and high-risk individuals can be identified. The ability to relate any changes seen to real conventional end points used in disease diagnosis remains elusive and much effort has been focused on the consequent protein level changes in tissues, cells, and biofluids (proteomics).

Metabonomics is another approach that provides data sets of similar high information content by systemic spectroscopic profiling of metabolites and metabolic pathways in whole organisms through study of biofluids and tissues. This results in complex multivariate data sets that require pattern recognition tools for interpretation, and the aim of these procedures is to extract biochemical information that is of diagnostic or other classification value. These identified substances become biomarkers that reflect actual biological events.

OVERVIEW

The main clinical and pharmaceutical areas where metabonomics is impacting include

- Validating animal models of disease, including genetically modified animals.
- Preclinical evaluation of drug safety, ranking of candidate compounds, assessment of safety in clinical trials and after product launch, evaluation of the effects of interactions between drugs, and between drugs and diet, and providing new measures of pharmaceutical efficacy.
- Improved diagnosis of human diseases, especially chronic and degenerative diseases.
- Better understanding of human population differences (epidemiological studies) and patient stratification for clinical trials and drug treatment (pharmaco-metabonomics).

Metabonomics yields time-dependent patterns of change in response to disease or drug effects. One role for metabonomics therefore is to direct timing of proteomic and genomic analyses to maximize the probability of observing ''omic'' biological changes that are relevant to functional outcomes.

Metabonomics, defined as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification,[1,2] bridges the gap between other -omics measurements and real-world end points. Metabolites can easily be identified and quantified, and changes can be related to health and disease, and are changeable by therapeutic intervention. There have been a number of reviews of metabonomics recently.[3,4]

METABONOMICS DATA ACQUISITION METHODS

The two principal methods used comprise ^{1}H nuclear magnetic resonance (NMR) spectroscopy and liquid-chromatography mass spectrometry (LC-MS). Nuclear magnetic resonance spectroscopy is nondestructive and provides detailed information on molecular structure, molecular dynamics, and mobility, especially in complex mixture analysis. Mass spectrometry is more sensitive than NMR spectroscopy and is used extensively for molecule identification, but in complex mixtures of very variable composition, the separation step increases variability. Most published studies on mammalian biology have used NMR spectroscopy, but LC-MS techniques with electrospray ionization are increasing in usage.

Typically, metabonomics is carried out on biofluids as these provide an integrated view of systems biology. The biochemical profiles of the main diagnostic fluids reflect both normal variation and the impact of drug toxicity or disease on single or multiple organ systems.[3] Urine and plasma obtained essentially noninvasively are appropriate for clinical trials monitoring and disease diagnosis. However, there is a wide range of fluids that can be, and have been, studied, including cerebrospinal, seminal,

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amniotic, synovial, digestive, blister and cyst fluids, and lung aspirates and dialysis fluids.[3]

A ^{1}H NMR spectrum of urine contains thousands of sharp lines from predominantly low-molecular weight metabolites (Fig. 1). The position of each spectral band (its chemical shift) gives information on molecular group identity. The splitting pattern on each band (J-coupling) provides knowledge about through-bond connectivity to nearby protons and molecular conformations. The band areas relate to the number of protons giving rise to the peak and hence to the relative concentrations of the substances in the sample. Absolute concentrations can be obtained if an internal standard of known concentration is added to the sample.

Plasma and serum contain both low- and high-molecular weight components, and these give a wide range of signal line widths. Broad bands from protein and lipoprotein signals contribute strongly to the ^{1}H NMR spectra, with sharp peaks from small molecules superimposed. Standard NMR pulse sequences can be used for spectral editing experiments. These are based on molecular diffusion coefficients or on NMR relaxation times and can be used to select only the contributions from macromolecules or the signals from the small molecule metabolites, respectively.

Nuclear magnetic resonance spectra take only a few minutes to acquire using robotic flow-injection methods, with automatic sample preparation involving buffering and addition of D_2O as a magnetic field lock signal for the

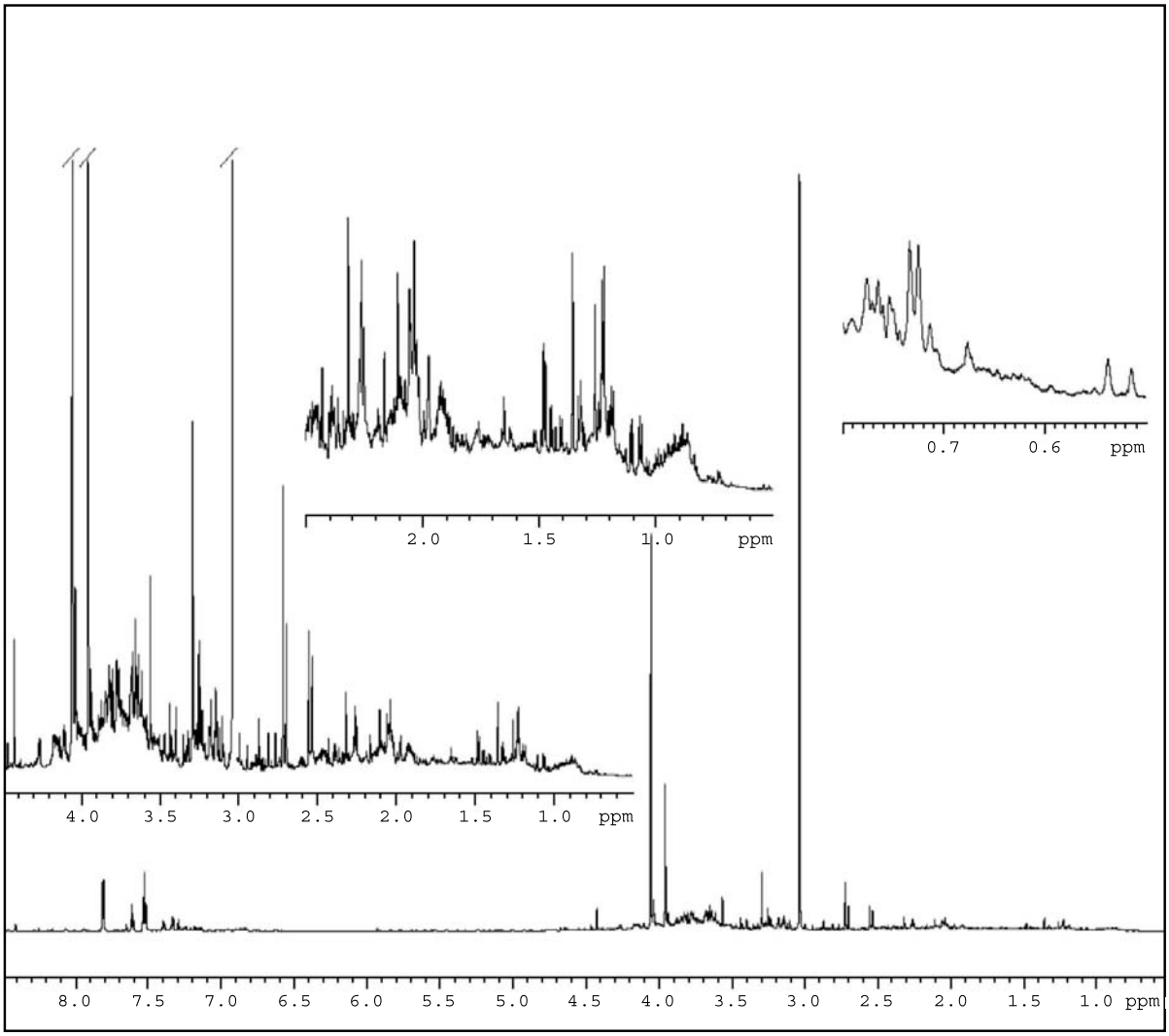

Fig. 1 ^{1}H NMR spectrum of human urine, with expansions showing the level of complexity. Peaks arise from different chemical types of hydrogen in the substances. The areas relate to molar concentrations, and the positions and splittings allow information to be obtained on molecular identity. The signal from water has been suppressed by an NMR procedure to avoid dynamic range errors in the detection process.

spectrometer. The large NMR signal from water is eliminated by the use of standard NMR solvent suppression methods. Using flow probes, the capacity for NMR analysis has increased and ~200 samples can be measured per day. Commercially available cryogenic NMR probes where the detector coil and preamplifier are cooled to around 20 K to provide up to a ×5 reduction in thermal noise. This has permitted the routine use of high-throughput natural abundance ^{13}C NMR spectroscopy of biofluids.

The use of mass spectrometry is increasing,[5] and for applications on biofluids, an HPLC chromatogram is generated with MS detection, using electrospray ionization (usually with both positive and negative ion chromatograms). At each point in the chromatogram, there is a full mass spectrum and so the data are three-dimensional (3-D), retention time, mass, and intensity, and this can be used as input to pattern-recognition (PR) studies.

The study of small pieces of intact tissues is possible using ^{1}H magic angle spinning (MAS) NMR spectroscopy.[6] Spinning the sample (~4–6 kHz typically) at an angle of 54.7° relative to the magnetic field reduces the line broadening effects seen in such heterogeneous, nonliquid samples. Studies have shown that diseased or toxin-affected tissues have characteristically different metabolic profiles to those taken from healthy organs. ^{1}H magic angle spinning NMR spectroscopy can also be used to probe metabolite dynamics and compartmentation. It can also be applied to in vitro systems such as tissue extracts or cell systems such as spheroids. A combined metabonomic analysis of different biofluids, tissue extracts, and intact tissues is possible, providing a comprehensive view of the biochemical responses to a pathological situation, an approach termed integrated metabonomics.

DATA ANALYSIS

The NMR spectrum of a sample can be considered an object in a multidimensional set of metabolic coordinates, the values of which are the spectral intensities at each data point. Similarity or differences between samples can then be evaluated using multivariate statistical methods or other pattern-recognition (PR) approaches.[7]

The general aim of PR is to classify an object based on identification of patterns in a set of experimental measurements or descriptors. It can also be used for reducing the dimensionality of data sets, e.g., using 2-D or 3-D mapping procedures, to enable easy visualization of any clustering of the samples. Data can be modeled using PR techniques so that the class of separate samples (a ''validation set'') can be predicted based on a series of mathematical models derived from original data or ''training set.''

Principal components analysis (PCA), one of the simplest techniques used extensively in metabonomics, allows the expression of most of the variance within a data set using a smaller number of factors or principal components. Each PC is a linear combination of the original data parameters with successive PCs explaining the maximum amount of variance possible, not accounted for by the previous PCs. Each PC is by definition independent of the other PCs. Conversion of the data matrix to PCs results in two matrices, scores and loadings. Scores are the new coordinates for the samples and may be regarded as the new variables, and in a scores plot, each point represents a single NMR spectrum. The PC loadings define the way in which the old variables are linearly combined to form the new variables and indicate which variables carry the greatest weight in transforming the position of the original samples from the data matrix into their new position in the scores matrix. In the loadings plot, each point represents a different NMR spectral region.

Unsupervised methods such as PCA are useful for comparing pathological samples with control samples, but supervised analyses that model each class individually are preferred where the number of classes is large. A widely used supervised method is partial least squares (PLS). This is a method which relates a data matrix containing independent (e.g., spectral) variables to a matrix containing dependent variables (e.g., variables describing the diagnosis) for those samples. Partial least squares can be used to examine the influence of time on a data set, which is useful for biofluid NMR data collected from samples taken over a time course of the progression of a pathological effect. Partial least squares can also be combined with discriminant analysis (DA) to establish the optimal position to place a discriminant surface which best separates classes.

BIOMARKER IDENTIFICATION

Identification of the metabolic biomarkers of a pathological situation can involve the application of the full range of analytical physical chemistry techniques, including mass spectrometry and NMR spectroscopy. The NMR spectra of urine and other biofluids, although they are very complex, allow many resonances to be assigned directly based on their chemical shifts, signal multiplicities, and by adding authentic material, and further information can be obtained by using spectral editing techniques.

Two-dimensional NMR spectroscopy can be useful for increasing signal dispersion and for identification of biomarkers. These include the ^{1}H–^{1}H 2-D J-resolved experiment, which yields information on the multiplicity and coupling patterns of resonances. Other 2-D experiments known as COSY and TOCSY provide ^{1}H–^{1}H

spin–spin coupling connectivities, thus giving information on which hydrogens in a molecule are close in chemical bond terms. Use of other types of nuclei, such as naturally abundant ^{13}C, ^{15}N, or ^{31}P, can help assign NMR peaks, and here 2-D correlation NMR experiments can also be obtained by the use of NMR pulse sequences that allow detection at the more abundant and sensitive ^{1}H nucleus. For example, these yield both ^{1}H and ^{13}C NMR chemical shifts of CH, CH_2, and CH_3 groups, useful again for identification purposes. There is also a sequence that allows correlation of protons to quaternary carbons.

It is also possible to separate substances of interest from a biofluid sample using solid-phase extraction or HPLC, and directly coupled chromatography-NMR spectroscopy methods can be used. The most powerful is HPLC-NMR-MS.[8] Other MS-based methods include MS^n for identification of fragment ions and FT-MS or TOF-MS for accurate mass measurement and derivation of molecular formulae.

APPLICATION TO PHYSIOLOGICAL EFFECTS

To understand biochemical alterations caused by disease, it is necessary to understand any underlying physiological sources of variation. Metabonomics has been used to determine the metabolic differences in cohorts of normal subjects and control laboratory animals. Many effects can be distinguished and characterized using metabonomics, including male/female differences, age-related changes, estrus cycle effects, diet, diurnal effects, interspecies differences and similarities, and the effects of gut microflora.[9]

Metabonomics has also been used for the phenotyping of mutant or transgenic animals that model human diseases and the investigation of the consequences of transgenesis such as the transfection process.[9] Metabonomic approaches can give insight into the metabolic similarities or differences between mutant or transgenic animals and the human disease processes that they are intended to simulate and hence their appropriateness for monitoring the efficacy of novel therapeutic agents.

APPLICATION TO DISEASE DIAGNOSIS

Many examples exist in the literature on the use of NMR-based metabolic profiling to aid human-disease diagnosis and most of the earlier studies have been reviewed[3] with a selection summarized below.

^{1}H NMR spectroscopy has been used to analyze the composition of the urine and plasma of a number of diabetic patients. There are marked elevations in the plasma levels of the ketone bodies and glucose, post-insulin withdrawal, and changes to lipoprotein composition. No other technique can provide a simultaneous assay on all three ketone bodies, glucose, the lipoproteins, and other important biochemicals in one fast and nondestructive test.

The levels of a variety of endogenous components in the synovial fluid aspirated from the knees of patients with osteoarthritis, rheumatoid arthritis, and traumatic effusions have been studied using NMR spectroscopy. The NMR spectrum of synovial fluid shows the signals of a large number of endogenous components, and correlations were reported between the disease states and levels of the *N*-acetyl signals from acute-phase glycoproteins and the levels and type of triglyceride.

The chemical composition of cerebrospinal fluid (CSF) gives a good indication of the health or otherwise of the central nervous system (CNS), and therefore biofluid NMR studies of CNS diseases have focused on CSF. Studies in this area include drug overdose, Reye's syndrome, and Alzheimer's disease.

A number of investigations involving kidney disease have been reported, including chronic renal failure, the uremic syndrome, glomerularnephritis, autosomal dominant polycystic kidney disease, and renal transplantation outcome.

Extensive biochemical assignments from the ^{1}H NMR spectra of seminal fluid and its component secretions, prostatic and seminal vesicle fluids, have been given, and the spectra derived from normal controls with those from patients with vasal aplasia (obstruction of the vas deferens leading to blockage of the seminal vesicles) and those with nonobstructive infertility have been compared.

One area of early use of biofluid metabolic profiling is in diseases caused by inborn errors of metabolism. Conventional diagnostic methods, including specific enzyme assays and gas chromatography/mass spectrometry, are sensitive but time-consuming. However, NMR spectroscopy of biofluids has been shown to be very powerful for the detection of inborn errors of metabolism. Most of the work has involved ^{1}H NMR studies on urine or serum.[10]

Metabonomics has been used to diagnose brain-tumor type based on NMR spectra of perchloric acid extracts of brain-tumor tissue,[11] classified as meningiomas, astrocytomas, glioblastomas, other glial tumors, medulloblastomas, metastases, and other tumors. Principal components analysis was used to reduce the data dimensionality and spectra were classified using neural networks. This gave $\sim$85% correct classification for meningiomas and nonmeningiomas. Classification of gliomas gave 62% correct within one grade. Other studies have used MAS NMR spectroscopy of involved and uninvolved tumor tissues, for renal cell carcinoma,[12] prostate,[13] and breast[14] tumors.

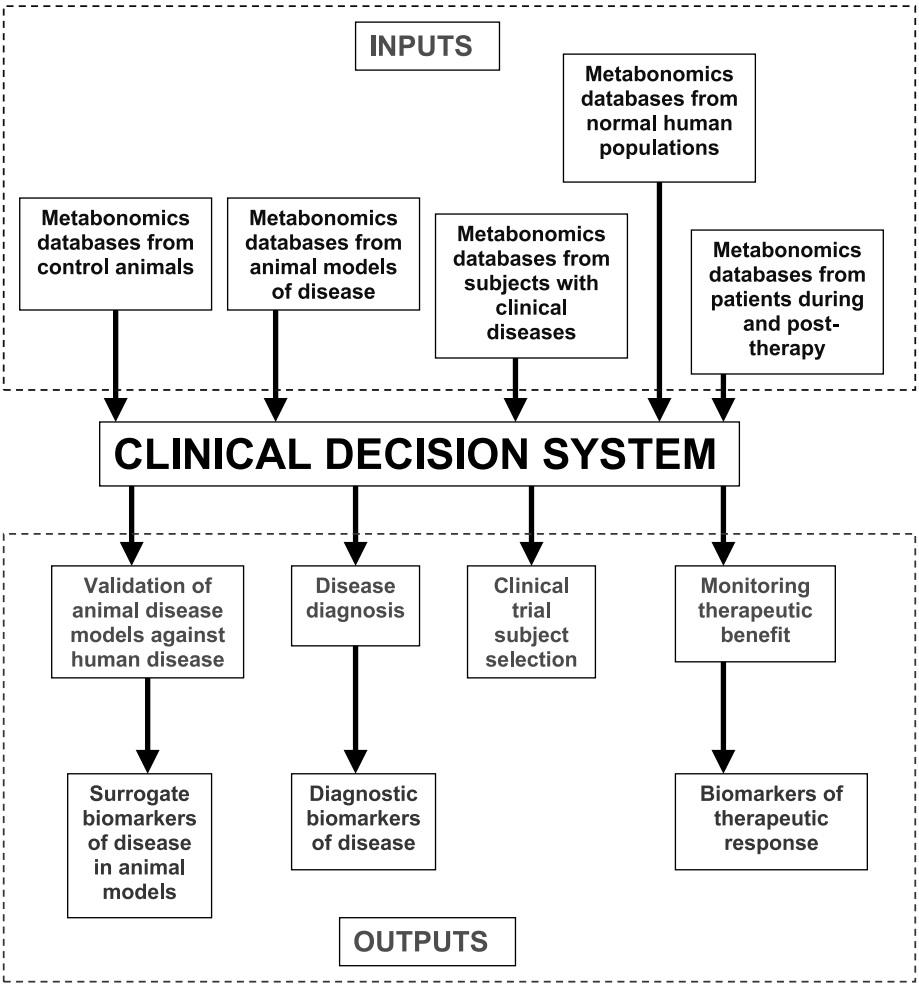

Fig. 2 Incorporation of metabonomics-based expert systems into clinical diagnosis for evaluation of disease models and the effectiveness of therapeutic procedures. (*View this art in color at www.dekker.com.*)

The ''gold standard'' diagnostic method for coronary heart disease (CHD) is X-ray angiography after injection of opaque dye into the blood stream. This is expensive and invasive with an associated 0.1% mortality and 1–3% of patients experiencing adverse effects. Recently, metabonomics has provided a method for diagnosis of CHD noninvasively through analysis of a blood serum sample using NMR spectroscopy.[15] Patients were classified, based on angiography, into two groups, those with normal coronary arteries and those with triple coronary vessel disease. Using a training set, the samples from the two classes were easily distinguished. The sample classes of a separate validation set were then predicted based on the model with a sensitivity of 92% and a specificity of 93%. The severity of the disease was diagnosed in patients with stenosis of one, two, or three of the coronary arteries. Separation of the three sample classes was evident although none of the wide range of conventional clinical risk factors that had been measured was significantly different between the classes.

CONCLUSION

There continues to be a need for advances in metabonomics analytical technologies both in NMR and MS. Nuclear magnetic resonance is likely to remain the method of choice for a broad impartial survey of metabolic profiles, especially given recent gains in sensitivity through the use of cryoprobe detectors. Mass spectrometry coupled to a separation stage is always likely to yield better detection limits for specific classes of metabolite.

It has proved possible to derive new biochemically based assays for disease diagnosis and to identify

combination biomarkers for disease, which can then be used to monitor the efficacy of drugs in clinical trials. Based on differences observed in metabonomic databases from control animals and from animal models of disease, diagnostic methods and biomarker combinations might be derivable in a preclinical setting. The use of databases to derive predictive expert systems for human disease diagnosis and the effects of therapy require compilations from both normal human populations and patients before, during, and after therapy. This approach is summarized in Fig. 2.

The ultimate goal of systems biology is the integration of data acquired from living organisms at all levels of molecular biology. In this respect, transcriptomics, proteomics, and metabonomics will all play an important role. Through the combination of these, and related approaches, will come an improved understanding of an organism's total biology, and with this better understanding of the causes and progression of human diseases, and the improved design and development of new and better targeted pharmaceuticals.

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Microarrays—Confocal Scanning

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Arthur E. Dixon
Savvas Damaskinos
Biomedical Photometrics Inc., Waterloo, Ontario, Canada

INTRODUCTION

Widefield confocal scanning is the imaging method of choice for microarrays. A confocal scanner rejects light from above and below the focal plane, thus rejecting fluorescence from the microarray substrate or from the aqueous buffer solution that is in contact with the array in some microarray formats. This article describes the principle of widefield confocal imaging and shows how this imaging technique fits the requirements for reading genetic and protein microarrays, and for tissue arrays. The sequence of events for reading arrays is described, and examples of both genetic microarray and tissue array images are presented.

CONFOCAL IMAGING

Three types of widefield confocal scanners are common for imaging microarrays. Scanning beam systems obtain a wide field of view using a telecentric f-theta laser scan lens combined with a slow-moving scanning stage;[1,2] scanning objective systems rapidly move a small objective lens across the width of the microarray while slowly moving the microarray in the perpendicular direction on a moving stage; and scanning stage systems use rapid stage motion to move the specimen under a stationary-focused laser beam. Confocal scanning laser microscopes can also be used, but their small field of view requires automated stage motion combined with image tiling to produce an image of the entire microarray. In addition to the problems of tiling separate subimages together, especially when the focus position may have changed from one subimage to the next, the boundaries of the subimages are exposed twice to the laser beam (and the corners four times), so that some fluorophore bleaching may occur at the edges of the images, resulting in poor quantification of probe spot intensity. The same problems arise in microarray readers based on CCD microscopes. Here the subareas are illuminated with a white light source, and nonconfocal images are collected using a CCD camera. A computer-controlled stage is used to move from one subarea to the next, and tiling is used to stitch the images together. In addition to multiple exposures of the fluorophores near the edges of the subareas, these systems have the additional disadvantages of collecting out-of-focus fluorescence from the glass substrate or an aqueous buffer solution (as they are not confocal).

Figure 1 shows a simple confocal microscope. Light from a point source (often a pinhole illuminated by a focused laser beam) passes through a beam splitter, expands to fill a microscope objective, and is focused to a tiny volume (at the focal point) inside the specimen (shown using solid lines). Light reflected (or emitted) from that point in the specimen is collected by the objective lens (a microscope objective) and is partially reflected to the right to pass through a pinhole to reach the detector. At the same time, light is reflected from parts of the specimen above the focal point (shown with dashed lines). This light is also collected by the objective lens and is partially reflected to the right, converging toward a focus behind the pinhole. Most of this light runs into the metal surrounding the pinhole and is not detected. Similarly, light reflected from parts of the specimen below the focal point (shown as dotted lines) converges toward a focus in front of the pinhole and then expands to hit the metal area surrounding the pinhole. Again, this light is blocked from reaching the detector. Thus the pinhole blocks light from above or below the focal point, so the detector output is proportional to the amount of light reflected back from only the parts of the specimen at the focal point. Images of the source pinhole and the detector pinhole formed by the objective lens are at its focal point (the source pinhole, detector pinhole, and the focal point are ''confocal'' with each other).

An image is collected by moving the specimen under the fixed laser beam in a raster scan (a scanning-stage microscope), by moving the beam using mirror scanners (a scanning-beam system), by moving the objective lens (a scanning-head system), or by moving the beam in one direction while moving the specimen in the perpendicular direction. Confocal images consist of sharp and empty areas; nonconfocal images consist of sharp and blurry areas. In a confocal microscope, light from out-of-focus parts of the specimen is rejected by the confocal pinhole, and it is this absence of out-of-focus information that allows three-dimensional images to be formed using a series of confocal slices.

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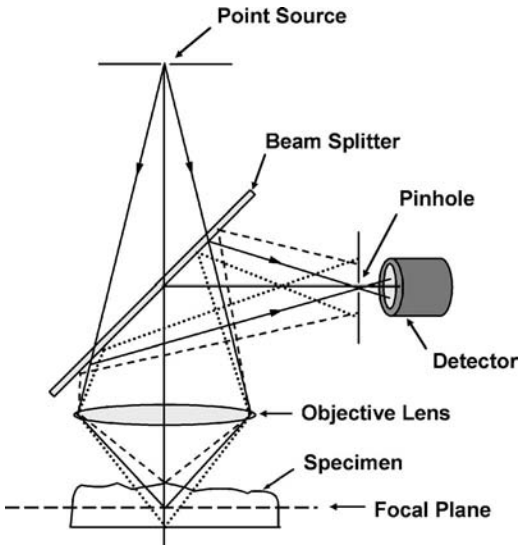

Fig. 1 Confocal laser microscope.

For many applications, an infinity-corrected confocal microscope is more useful than the simple microscope shown above. In an infinity-corrected mxicroscope (shown in Fig. 2), a parallel beam from a laser or other light source is focused by an infinity-corrected microscope objective onto a specimen at the focal plane. Light reflected from the specimen is collected by the microscope objective, and partially reflected by the beam splitter toward a detector lens that focuses the beam to pass through the detector pinhole to reach the detector. Just as before, light reflected (or emitted) from above or below the focal plane is rejected by the pinhole, and the microscope is confocal.

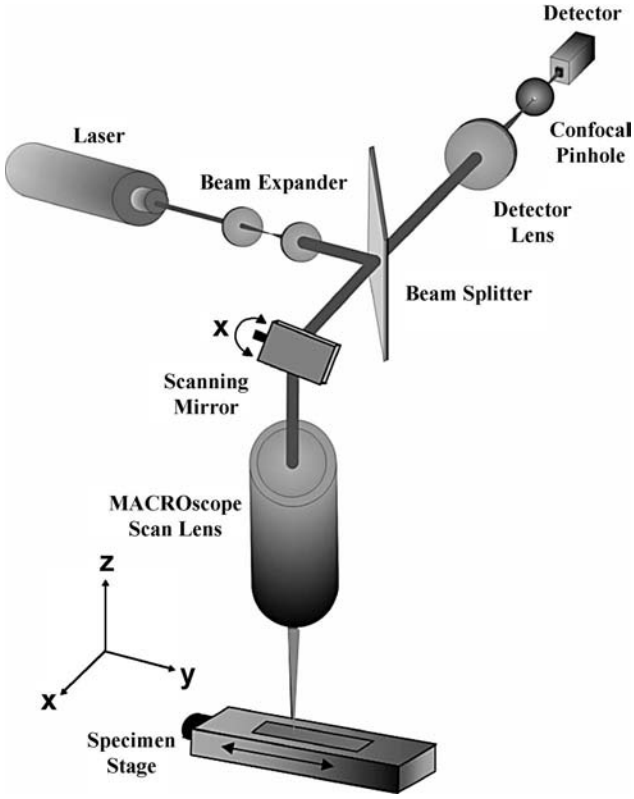

Fig. 3 Widefield confocal scanning laser MACROscope®.

A widefield scanning-beam confocal imaging system (MACROscope®) is shown in Fig. 3. Instruments based on this design behave very much like confocal scanning laser microscopes. All of the confocal scanning laser microscopy (CSLM) contrast mechanisms are available,

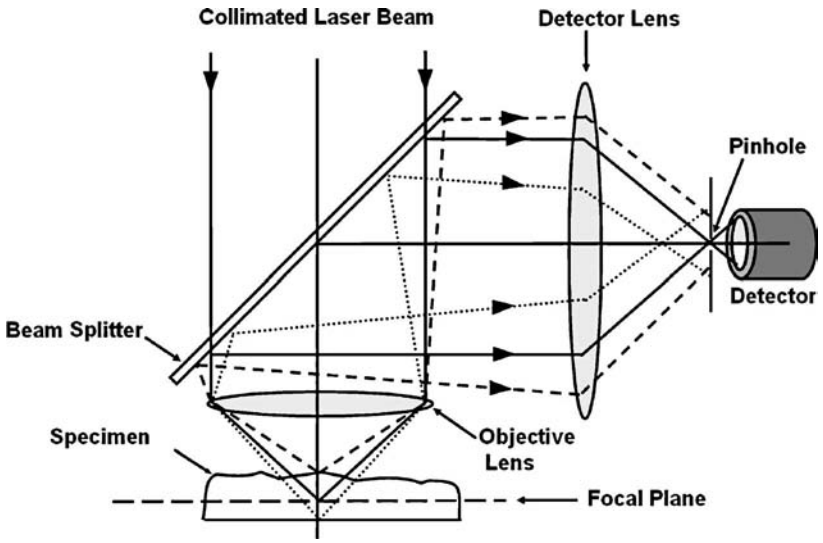

Fig. 2 Infinity-corrected confocal microscope.

but these instruments have a field of view that is about 20 times larger than a scanning laser microscope using a microscope objective with the same numerical aperture as the laser scan lens.

In Fig. 3, a beam expander expands the laser beam to match the diameter of the entrance pupil of the laser scan lens. This beam is reflected by a beam splitter toward a computer-controlled scanning mirror, situated at the entrance pupil position of a telecentric f-theta laser scan lens. The laser scan lens focuses the scanning beam on the specimen (usually on a microscope slide), which is mounted on a scanning stage. The scanning stage moves slowly in a direction perpendicular to the direction of the beam scan, such that the focused laser spot performs a raster scan across the specimen. Light reflected from, or fluorescence emitted by the specimen, is collected by the laser scan lens, passes back through the beam splitter, and is focused on a confocal pinhole in front of the detector. Light passing through the pinhole is detected. Photomultiplier tubes are often used as detectors because of their high speed, high sensitivity, and large dynamic range.

MICROARRAY IMAGING

For imaging DNA or protein microarrays in fluorescence, filters are added to the detection arm to block the reflected laser light, as well as fluorescence from other fluorophores on the specimen. For example, many microarrays use both Cy3 and Cy5. Cy3 is excited by a green laser (usually 532 nm), and Cy5 is excited with a red laser (often 635 nm). Although the red laser excites only Cy5, the green laser weakly excites Cy5 as well as strongly exciting Cy3. In addition, the emission spectra of Cy3 and Cy5 overlap slightly, so the reader manufacturer must choose filters carefully so there is no cross talk between detection channels. There may also be residual fluorescence from the substrate, which is minimized by confocal detection. Microarray scanners are available with up to four different laser sources, and multiple detection arms. Filter wheels containing a large number of filters allow sequential or simultaneous detection of several different fluorophores.

Several steps are required to image a microarray. First, the fluorophores used on the array are selected by the operator to enable the instrument to choose lasers and filter sets. A preview scan of the entire glass slide is performed for each fluorophore chosen, which allows the operator to choose the area to be imaged, and to set the detector gain and offset. The offset is set to minimize the background noise in the area of the image between the probe spots—this should be set to a small value, but not to zero. The gain is then usually set so that the brightest probe spots in the image are just below detector saturation. For some applications, the intensity of images from different fluorophores is adjusted by changing laser intensities. Finally, a high-resolution scan is performed on the area of the slide containing the microarray probe spots. For common microarrays with probe spots of 100 μm or more in diameter, a pixel setting of 5 μm works well. For best results, the pixel size should match the diameter of the focused laser beam. In some scanners, the smallest pixel size available is smaller than the focal spot diameter, so choosing this setting will not improve the image resolution, but it does act like frame averaging by making a larger number of overlapping intensity measurements inside the area of each probe spot.

The objective of these measurements is to analyze the fluorescence images to provide the user with a table of numbers (for each fluorophore) representing the integrated fluorescence intensity of all of the probe spots on the microarray. These numbers can then be transferred to an analysis program that matches the user's specific requirements. Image analysis comprises several steps. First, measurement circles in a grid pattern matching the microarray grid are placed around every probe spot in the image. Because the probe spots are not always centered exactly on the grid positions, the position of the measurement circles is adjusted to match the actual probe spot positions. The fluorescence intensity for each probe spot is calculated by integrating the fluorescence intensity of the pixels inside each measurement circle, and then subtracting the local background intensity calculated from pixels in the area just outside each circle. One number, representing the integrated fluorescence intensity for each probe spot and for each fluorophore, is stored in a table which is then available for the user's specific application software.

A trained operator can perform all of the steps described above to image a microarray like that shown in Fig. 4 in both Cy3 and Cy5, and analyze the images to produce tables of integrated intensities in an hour. A fully automated reader performs the same task in 10 min, and the only interaction with the operator is to load the microarray and specify which fluorophores are used. Ten minutes later, the operator can retrieve the output data files.

Figure 4A shows Cy3 and Cy5 images of a DNA microarray on a glass microscope slide, imaged with 5-μm pixels (and 5-μm focal spot size) on a DNAscope™ IV. The scan area was 20×70 mm. Figure 4B (left) shows a magnified image of one subgrid from the image in Fig. 4A, and Fig. 4B (right) illustrates the placement of measurement circles by the automated analysis routines in MACROview™. Note that the automated detection and placement algorithm has correctly ignored the smear of fluorescence at the edge of the picture and the dust particles in the image. Many grid placement algorithms require a bright fluorescent probe spot at the top left of

each subgrid (or at some other known position); this one does not.

Tissue microarrays and other tissue specimens on glass microscope slides are usually imaged in brightfield, using a white light source. These specimens require higher optical resolution than genetic microarrays on glass microscope slides, and in some cases submicron resolution is required. The tissue microarray shown in Fig. 5A was imaged on a DNAscope™ IV with a high-resolution (2-μm focal spot size) option, in a single scan. This is an epifluorescence image, excited by a green laser. Figure 5B is an expanded view of six of the tissue specimens.

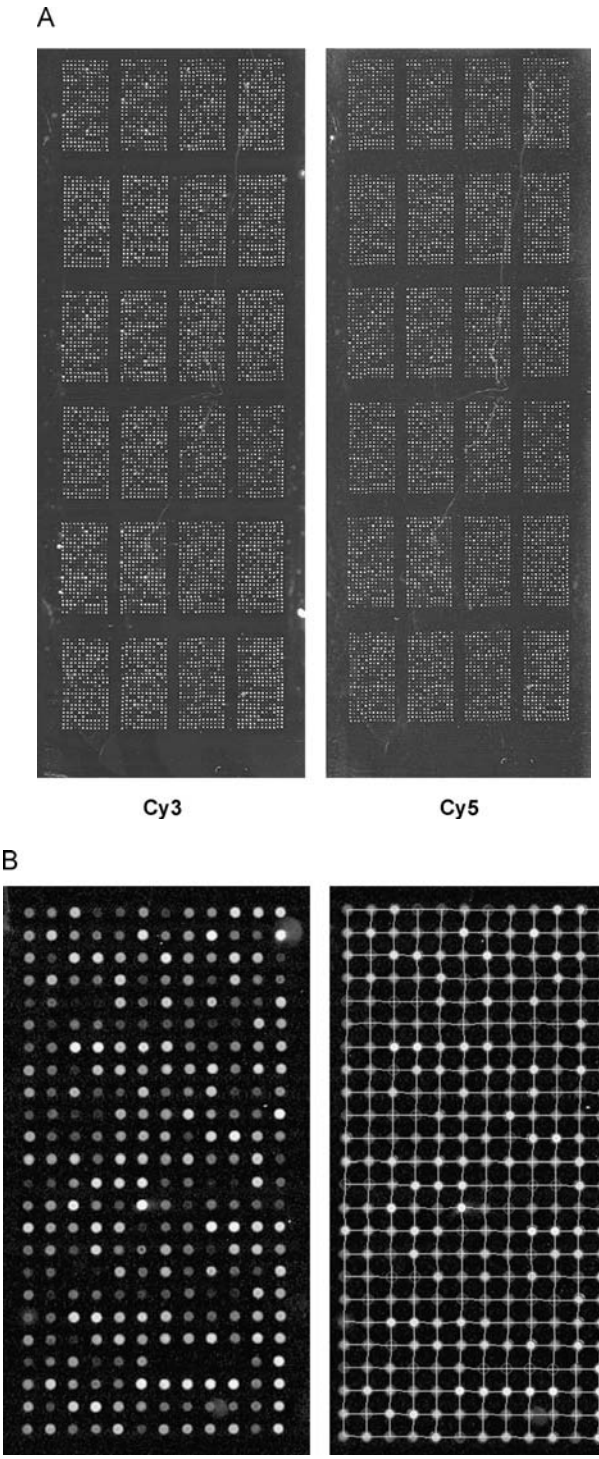

Fig. 4 (A) Fluorescence image of a genetic microarray on a glass microscope slide. Left is a Cy3 image; right is a Cy5 image. (B) Expanded view of one subarray from the image in (A). Measurement circles are shown around probe spots in the right image as placed by an automatic analysis algorithm.

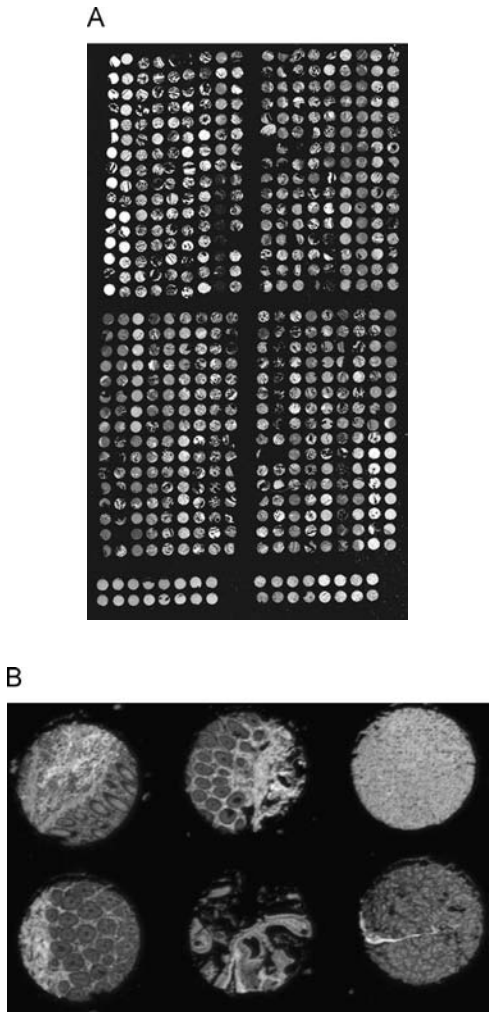

Fig. 5 (A) Epifluorescence image of a tissue array on a glass microscope slide. (B) Expanded view of six tissue specimens on the tissue array in (A).

CONCLUSION

Widefield confocal imaging provides the resolution, sensitivity, dynamic range, and background fluorescence rejection for fluorescence imaging of genetic and protein microarrays. The same instruments can also be adapted for imaging tissue arrays, both in brightfield and in fluorescence. We have reviewed the principles of confocal microscopy and the MACROscope® technology

on which the DNAscope™ is based. Images of DNA and tissue microarrays were shown, and the steps required to image and analyze a genetic microarray were described.

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Microarrays—Detecting DNA Copy-Number Changes

Beatriz Carvalho
Marjan Marije Weiss
Bauke Ylstra
Gerrit A. Meijer
VU University Medical Center, Amsterdam, The Netherlands

INTRODUCTION

Gross chromosonal abnormalities leading to gains and losses of (parts of) chromosomes are frequent events in the majority of cancers, especially in solid tumors. Microarray-based comparative genomic hybridization (array-CGH) is the most versatile technique currently available for detecting DNA copy number changes. We will review the array-CGH technique that, as the conventional comparative genomic hybridization (CGH), is able to detect DNA copy number changes throughout the whole genome in one single experiment, but with much higher resolution. Applications and future developments of array-CGH will also be discussed.

WHAT IS MICROARRAY-CGH?

Genomic instability is a hallmark of cancer development, and in the vast majority of human malignancies overt instability at the chromosomal level exists, frequently giving rise to unbalanced chromosome copy-number changes. For long, the possibilities for detecting these DNA copy-number changes were limited. Karyotyping required living cells, which severely hampered wide-scale clinical application of the technique outside the fields of hematological malignancies and congenital disorders. Fluorescence in situ hybridization allowed the analysis of only a very limited number of targets at a time, whereas DNA cytometry could only disclose the presence of gross genomic aberrations without any further specificity.[1] This situation has dramatically changed with the introduction of comparative genomic hybridization (CGH). Comparative genomic hybridization is a molecular cytogenetic technique that allows the genome-wide analysis of alterations in DNA sequence copy number.[2] Differentially labeled tumor and reference DNAs compete for hybridizing to normal metaphase chromosomes. The ratio between the two labels along the chromosome axis allows the mapping of gains/amplifications and deletions throughout the whole genome of a tumor (Fig. 1). Because for CGH no cell culturing is required, and even formaldehyde-fixed paraffin-embedded material can be used, this technique has become one of the most popular genome scanning techniques and has contributed to an enormous progress in the analysis and knowledge of chromosomal changes in solid tumors.[3,4] However, CGH is limited by the poor resolution of the metaphase chromosomes. Genomic alterations smaller than 5–10 Mb are not detected.[3] The recently developed CGH microarray technique overcomes this limitation[5,6] as the metaphase chromosomes are replaced by an ordered set of DNA fragments (typically 100–200 kb) of which the precise chromosomal location is known (Fig. 2). In this case the resolution is limited by the insert size and density of the DNAs spotted on the glass slide.[5–7] Thus, microarray-CGH has the ability of detecting small amplicons and deletions that potentially harbor specific oncogenes and tumor suppressor genes. As in conventional CGH, microarray-CGH allows the measurement of DNA copy-number aberrations by means of hybridization of two differentially labeled DNAs (the DNA to be tested against reference DNA). However, in array-CGH, because of the nature of the DNA spotted on the glass slides, it is possible to map the copy-number aberrations directly onto the genomic sequence. The DNA fragments spotted on the slide can be either large-insert genomic clones (cosmids, BACs, or PACs) or cDNA clones.[5,6,8] The last one identifies directly of which genes the chromosomal copy number has changed and allows the parallel comparison of the alterations in DNA copy number and gene expression in the same sample.[8] However, with cDNA clones fluorescent ratios do not reflect the chromosomal copy-number changes as precise, possibly due to cross hybridization effects.[8] Initially, arrays were spotted with fragmented DNA from the BACs/PACs.[5,6] As BACs are single-copy vectors, the yield of DNA obtained in culture is low, and for that reason big culture volumes were needed, and DNA density on the spotted arrays was low. Nowadays, PCR representations of the clones are spotted on the slide. This process fragments the DNA, allows small-scale

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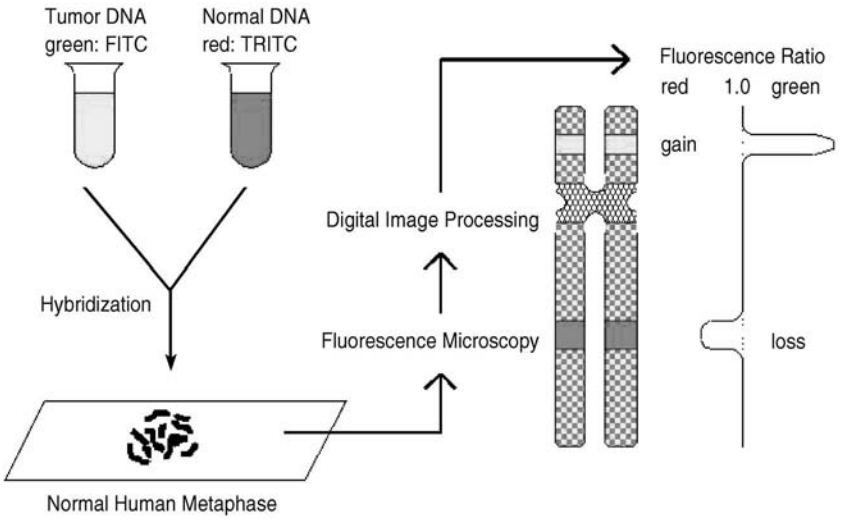

Fig. 1 Schematic representation of the comparative genomic hybridization (CGH) technique. Test and reference DNAs are differentially labeled and hybridized to metaphase spreads. After hybridization, the slide is washed and scanned. Images obtained are processed and ratios calculated.

DNA isolations, and allows high spotting concentration, which simplifies the process and enhances sensitivity.[9,10] Currently, genome-wide CGH arrays comprise generally about 3000 BAC and PAC clones, yielding a resolution of 1 to 1.5 Mb.[9,10] Just very recently, the first results of a 30-kb whole genome tiling path BAC array have been presented.[11] For cDNAs the number of clones is finite and will never be genome covering. Alternative strategies use arrays with spotted oligonucleotides (see below).

APPLICATIONS OF MICROARRAY-CGH

Array-CGH is nowadays the method of choice for studying DNA copy-number changes and has been successfully applied in the study of several tumor models.[12–25] Albertson et al.[26] showed how accurately array-CGH can define amplicon boundaries and identify peaks within the amplified regions, which lead to the localization of oncogenes involved in oncogenic process. In this way, CYP24 was identified as a candidate

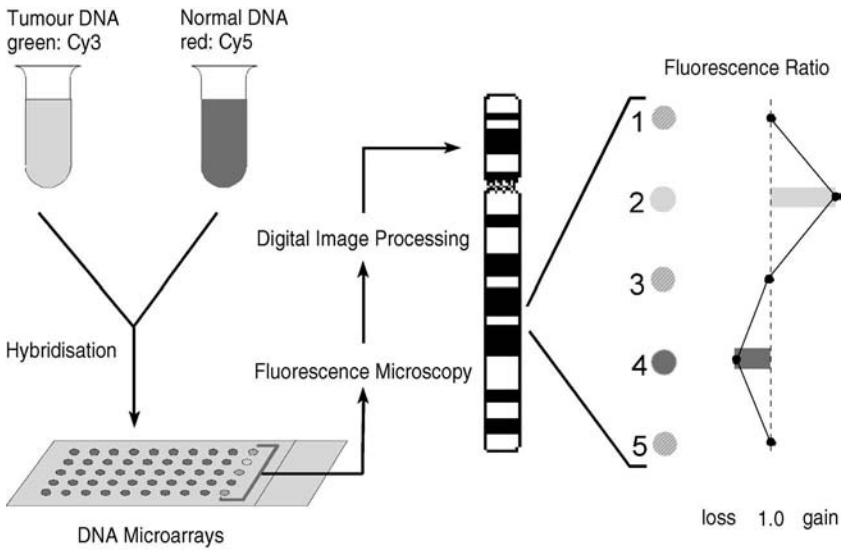

Fig. 2 Schematic representation of the microarray-CGH technique. Test and reference DNAs are differentially labeled and hybridized to genomic clones spotted on a slide. After hybridization, the slide is washed and scanned. Images obtained are processed and ratios calculated.

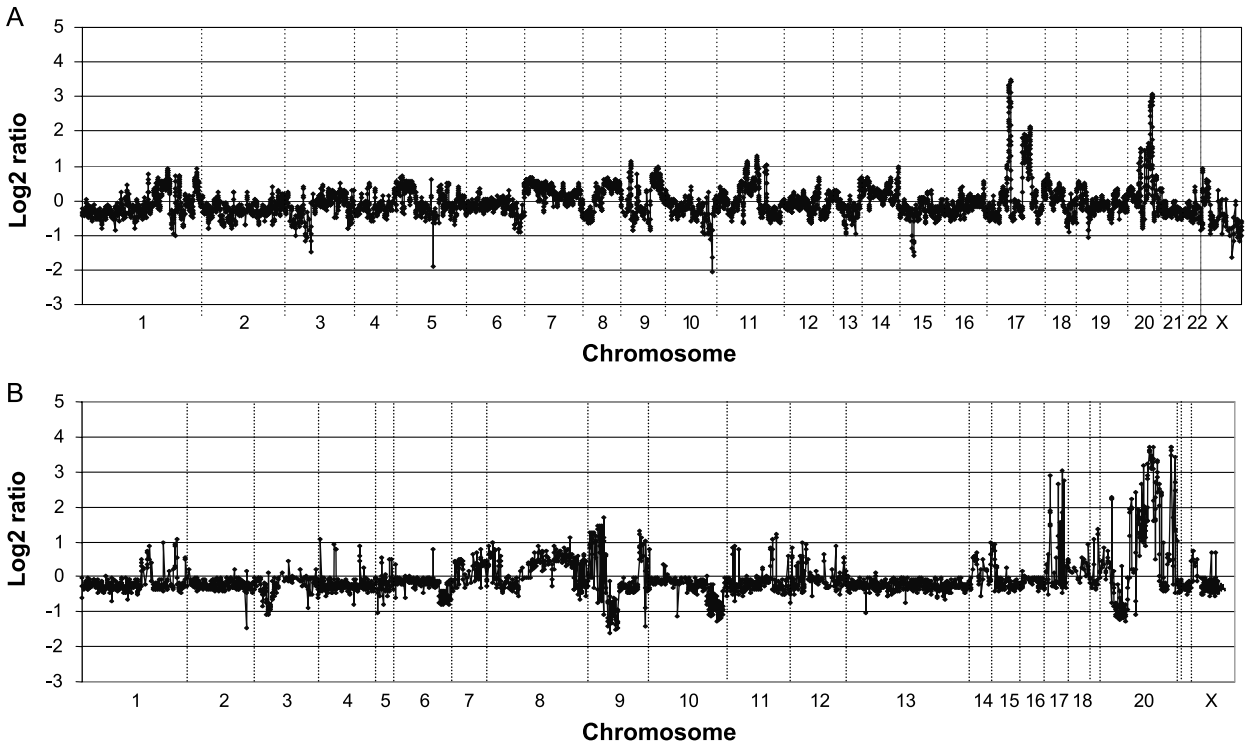

Fig. 3 Microarray-CGH profile of BT474 cell line (test), reference normal kidney DNA (A), oligonucleotides as probe DNAs spotted on the glass slide; moving average applied to the $\log_2$ ratio (B), BAC/PAC PCR representations as probe DNAs spotted on the glass slide. Vertical bars indicate spacing between chromosomes. (*View this art in color at www.dekker.com.*)

oncogene mapping to the amplified region at 20q13.2 in breast tumors.[26] This amplicon was also analyzed in stomach tumors by array-CGH, as 20q amplification is a frequent event in gastric carcinoma, and was narrowed down to a 800-kb segment.[27] Also, in a study of alterations of mouse islet carcinomas, which progress in a similar way as in human tumors, regions of LOH were further narrowed and new regions of copy-number loss and gain were defined.[28] The analysis of human genome sequences syntenic to the defined regions suggested several candidate tumor suppressor genes and oncogenes.[28] These studies show how powerful array-CGH can be in identifying the underlying genetic events of the chromosome aberrations, because of its high resolution and sensitivity.

Array-CGH is a strong technique for cancer diagnosis, prognosis, and therapy strategies.[29] In a study of 40 renal tumors, Wilhelm et al.[20] were able to correctly diagnose 33 of 34 malignant tumors and group together the benign lesions, showing that array-CGH is a feasible approach for cancer diagnosis. Cluster analysis of the microarray-CGH data in gastric cancer correlated with clinical parameters, e.g., lymph node status and survival,[17] which is important for prognosis and for decision in therapy strategies, as preoperative needle biopsies can be used diagnostically for the isolation of DNA for array-CGH. Analysis of the genome of occult micrometastatic cells was possible by array-CGH, leading to the identification of regions critically involved in metastasis, and thus providing means to identify in the future, patients who will benefit from therapies directed toward the eradication of occult micrometastatic tumor cells.[15] Array-CGH has been applied in the analysis of epigenetic alterations of tumors, by using CpG island microarrays,[30] and in the evaluation of the effect of mismatch repair competence on genomic alterations in cells that undergo selection for resistance to drug treatment.[31]

Array-CGH has also shown to be useful for diagnosis of other genetic diseases such as neurofiromatosis-2[7,32,33] and congenital aural atresia,[34] and for screening and detection of subtelomeric chromosome rearrangements in individuals with mental retardation or congenital anomalies.[35]

The applications of microarray-CGH are broad and will still increase as the need to understand diverse biological questions will continue to drive development of new uses of genomic arrays. Lin and collaborators[36] developed a physical mapping method based on radiation hybrids together with array-CGH that increases the speed and efficiency of positional cloning in somatic cell genetics. Array-CGH has also been applied in evolution studies by comparing DNA copy-number variation between humans and great apes.[37] Also, comparison of closely related

mycobacterium strains, by array-CGH, helps in the design of improved diagnostics and vaccines.[38] Moreover, array-CGH is sensitive enough to study dynamic processes, such as DNA replication. Khodursky et al.[39] traced the progression of the replication fork along the bacterial chromosome by using the relative abundance of replicated DNA over unreplicated DNA.

FUTURE DEVELOPMENTS

Developments and improvements of the array-CGH technique are taking place in order to have a robust technique that can be used in a regular basis in clinical applications. Algorithms that help to accurately define the breakpoints of chromosomal aberrations[40] as well as a fully automated quantification of the image data obtained in microarray experiments[41] have already been developed. Also, array-CGH using spotted oligonucleotides instead of BACs/PACs or cDNAs is currently being approached.[42] These oligos, which are currently used in expression profiling,[43] have the advantage of shortcutting the array production as there is no need for culturing and amplifying. Oligo-CGH has high accuracy in detecting amplifications, as with BAC-arrays (Fig. 3). It is also able to detect single-copy alterations, although with less accuracy, which requires further optimization.[42]

Finally, as the ultimate goal is to arrive to the gene that is affected by the chromosomal aberration, it is important to merge the array-CGH data with the expression data. Together, these two pieces of information will give a more complete picture of which genes and pathways are involved in the development of the disease.

CONCLUSION

In the past decade we have witnessed a fast evolution of comparative genomic hybridization as the tool for genome-wide analysis of DNA copy-number changes. Starting off as a research tool for cancer genetics mainly, it has now entered the field of clinical applications, a step facilitated by the arrival of microarray-based CGH.

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Microarrays—Electronic Microarrays

Elaine M. Weidenhammer
Jay Shaw
Nanogen, Inc., San Diego, California, U.S.A.

INTRODUCTION

DNA microarrays represent a novel technology that incorporates elements from the fields of microfluidics, microfabrication, molecular biology, chemistry, and bioinformatics. High-density arrays have changed the scope of genetic analysis by allowing researchers to examine thousands of genes in parallel, enabling whole-genome analysis in a single experiment. DNA microarrays, manufactured using photolithographic combinatorial synthesis techniques[1–4] or physical deposition,[5–7] have proven to be invaluable for gaining a genome-wide perspective for genotyping, haplotyping, and gene expression profiling experiments.

Test sites on high-density arrays are defined by the sequence of the DNA probe that is spotted or synthesized at a particular location. Target hybridization is mediated by controlling the temperature and salt concentrations of the hybridization and wash buffers. In contrast, active electronic microarrays are composed of individually controlled microelectrodes (test sites). These arrays use electric field control to drive the transport of charged molecules from the bulk solution to activated test sites, allowing addressing and hybridization reactions to occur within seconds.[8–10] Nucleic acids will concentrate only at the activated test sites; the remaining sites are unaffected and available for subsequent use. In addition to concentrating nucleic acids, electronic control can also be utilized to denature double-stranded DNA and to discriminate single base mismatches between target molecules and reporter probes. Electronic microarrays thus provide flexibility, enabling multiplexed analysis of different targets from multiple samples, and are particularly well suited to clinical diagnostics laboratories where flexibility, reproducibility, and accuracy are critical.

ELECTRONIC MICROARRAYS

Nanogen has commercialized a 100-test site array, the NanoChip® microarray, housed in plastic cartridges (NanoChip cartridge) that provide fluidic, electronic, and optical interfacing. A platinum microelectrode underlies each test site (Fig. 1A). An electrophoretic field is generated by applying a positive direct current (DC) bias to one or more microelectrodes and a negative bias to counter electrodes; negatively charged DNA molecules in the bulk solution are electronically transported, or addressed, to the activated test sites. By applying a negative DC bias to the microelectrodes, denaturation of double-stranded DNA and removal of mismatched or weakly hybridized reporter oligonucleotides can be achieved. Independent control of the test sites enables the user to electronically address samples in any configuration, allowing complete flexibility in assay design.

A gel permeation layer containing streptavidin is present over the array surface. This layer protects the DNA from electrochemical by-products during activation and enables retention of biotinylated target DNA sequences. Electronic activation not only concentrates the target over the test site, but also provides an electrochemical environment conducive to attachment of the biotinylated DNA to the activated test site. These features of the electronic microarray ensure that the biotinylated DNA will be present only at the designated sites, and allow sequential addressing of biotinylated DNA targets from multiple samples onto different test sites of an array without carry-over (Midwest Research Institute and Nanogen, internal results).

The NanoChip Molecular Biology Workstation is the instrument system in which NanoChip electronic microarrays are processed. Electronic addressing of samples is done in the ''loader'' component, which can process up to four NanoChip cartridges simultaneously. Fluorescent signal detection occurs in the ''reader'' component. Both the loader and reader are capable of performing fluidic and thermal processes. A computer interface controls the workstation and provides software for data analysis and management.

GENOTYPING ON ELECTRONIC MICROARRAYS

Detection of single-nucleotide polymorphisms (SNPs) on electronic microarrays is achieved via hybridization of allele-specific probes to biotinylated, amplified target DNA. Genomic DNA is extracted from the sample, and

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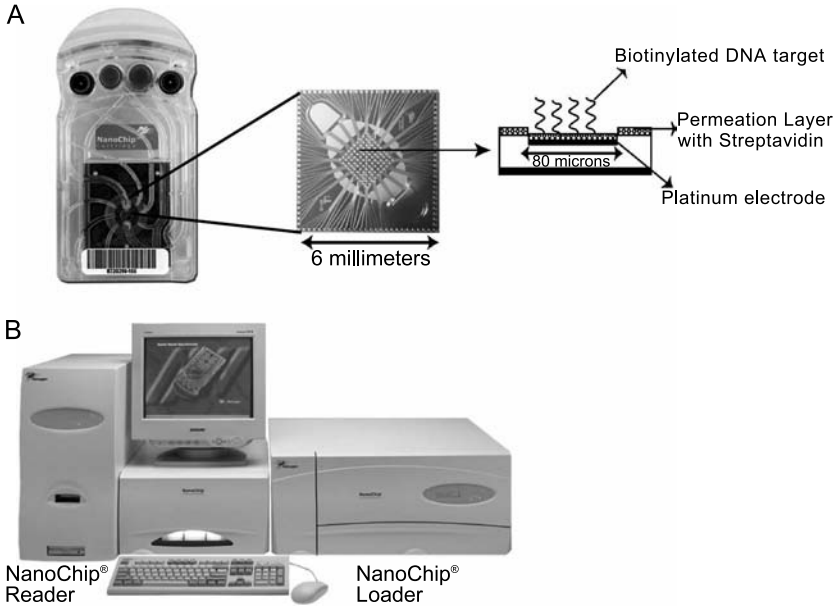

Fig. 1 (A) The NanoChip cartridge, NanoChip electronic microarray, and schematic cross section of an individual test site (not to scale). The NanoChip cartridge contains the microarray, fluidic channels and ports for automated addition of samples and reagents, and electronic interfaces. As depicted in the drawing of the test site, a permeation layer containing streptavidin covers the surface of the microarray, which enables attachment of biotinylated DNA targets. (B) The NanoChip molecular biology workstation. (*View this art in color at www.dekker.com.*)

the target sequence containing the region of interest is amplified by PCR. The use of one biotinylated amplification primer and one unmodified amplification primer generates a product that is biotinylated on one strand. A wide range of target size (from <100 base pairs to >1000 base pairs) can be accommodated on electronic microarrays. For genotyping analysis of two or more targets from a sample, multiple oligonucleotide primer pairs can be used in a single amplification reaction, which will generate multiple biotinylated DNA targets.

After the amplification reaction, desalting with size exclusion membranes is performed to facilitate electronic transport and remove unincorporated amplification primers. The biotinylated targets are then electronically addressed onto NanoChip cartridges. Software on the workstation enables the user to designate the test sites to be addressed for each sample. Electronic activation concentrates the biotinylated DNA in the sample to the test sites, where the DNA remains anchored via interaction with the streptavidin in the permeation layer. Unbound nucleic acids are removed by a series of fluidic washes before addressing the next sample. When multiple NanoChip cartridges are processed in a single loader run, cartridges are loaded in parallel, minimizing the time required for electronic addressing.

One method for sample genotyping uses fluorescently labeled reporter probes specific for the wild-type and variant sequences (Fig. 2). For target sequences containing significant secondary structure near the SNP, an unmodified ''stabilizer'' oligonucleotide can be used; this oligonucleotide hybridizes to the target sequence immediately adjacent to the nucleotide of interest (Fig. 2). Hybridization of the stabilizer next to a perfectly matched reporter probe generates a base-stacking energy component that, added to the Watson–Crick base-pairing energies, further stabilizes the reporter/target interaction. An alternative method utilizes chimeric ''discriminator'' oligonucleotides that contain a target-specific region, complementary to the wild-type or variant alleles, and one of two universal tail sequences. Two fluorescently labeled universal reporter probes, each complementary to one of the tail sequences, are used to detect the bound discriminators.

Whether allele-specific reporter probes or allele-specific discriminator oligonucleotides/universal reporter probes are utilized, genotype determination is achieved by monitoring the fluorescent signal remaining on each test site after the mismatched allele-specific oligonucleotides have been destabilized. Mismatched probes are removed by increasing the stringency of the environment, either thermally or electronically; perfectly matched probes will remain hybridized under the more stringent conditions. The NanoChip reader uses a two-laser system to detect the fluorescent signal remaining at each test site.

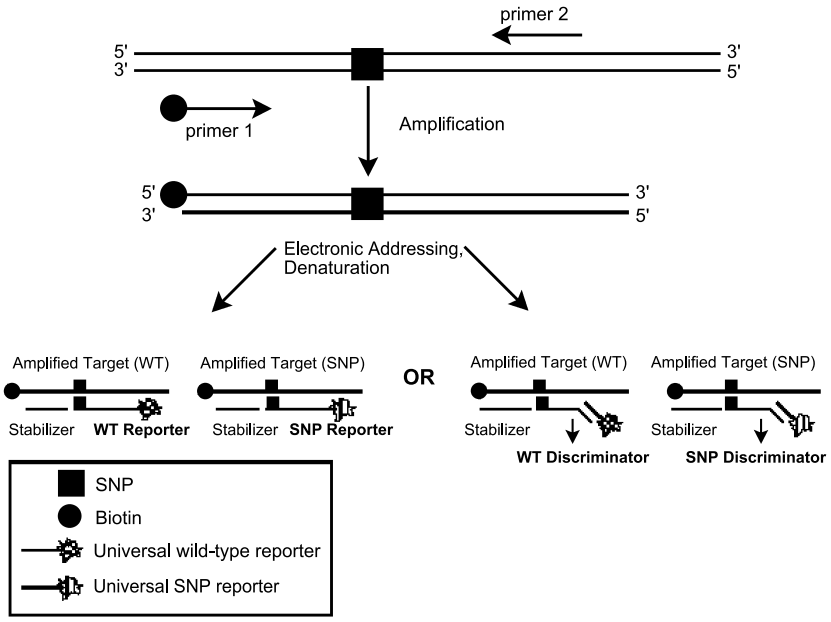

Fig. 2 Genotyping on electronic microarrays. The region of interest is amplified from genomic DNA by using one biotinylated amplification primer. After electronic addressing and denaturation, allele-specific reporter probes or allele-specific discriminator oligonucleotides/universal reporter probes are used for genotype determination (see text for detail). The use of stabilizer oligonucleotides is optional.

Predominance of fluorescent signal from one labeled reporter indicates a sample that is homozygous for the corresponding allele, whereas equivalent levels of fluorescent signal from both reporter oligonucleotides indicate a heterozygous genotype.

For multiplex genotyping, locus-specific discriminator (or reporter) pairs can be hybridized and discriminated sequentially (S.A. Williams, Children's Hospital of Orange County, personal communication).[11] Such analyses can be performed on a single DNA target containing multiple SNPs or on different DNA targets amplified from a single sample. As many as seven genotyping assays have been performed on a single 1.2-kb target (Dr. Dennis O'Kane, Mayo Clinic, personal communication). The ability to perform multiplex genotyping analyses greatly increases the capacity of the 100-site array, such that hundreds of genotypes can be generated without having to perform separate amplification and addressing reactions.[12–14]

CLINICAL DIAGNOSTIC APPLICATIONS

As correlations between genetic polymorphisms and disease are established, the need arises for robust, accurate methods to test for the presence of clinically relevant SNPs. Such testing may be performed to confirm the genetic cause of a clinical indication, establish risk profiles for specific diseases, or determine carrier status for heritable disorders in couples contemplating pregnancy. As the genetics underlying more complex disorders are understood, effective testing will require analysis of panels of SNPs. Electronic microarrays can be formatted to analyze multiple loci from multiple samples, and can be altered to incorporate new or different sets of markers as additional information becomes available. These features will be increasingly valuable in clinical research and clinical diagnostic laboratories as complex genetic diseases are assessed.

Several published reports provide examples of the utility of electronic microarrays for clinical diagnostics. Genotyping has been performed on the *TNFα* and *IL-4* cytokine genes;[15] on the prothrombin[16,17] and Factor V gene mutations,[17,18] which can lead to venous thrombosis; and on the *NAT1* and *NAT2* genes, which are involved in drug metabolism.[11,19] In addition, the NanoChip microarray has been used to genotype 362 samples for eight common mutations in the *MeCP2* gene, associated with RETT syndrome,[14] and to analyze 940 samples for 11 SNPs in the Factor VII, β-globin, and *RET* genes.[20] These studies demonstrate the flexibility and multiplexing capacities of NanoChip microarrays; they also illustrate the benefits of an open platform that enables researchers to tailor the arrays to their own experiments.

INFECTIOUS DISEASE APPLICATIONS

Microarray technology can have a broad range of applications in infectious disease. For example, Bekal

et al. examined the pathogenic potential of different *Escherichia coli* strains using conventional microarrays.[21] The DNA products from the PCR amplification of 105 virulence factors were spotted onto glass slides. Fluorescently labeled genomic DNA from different strains was hybridized to the arrays, allowing the detection of virulence genes and the potential to identify emerging pathotypes. In addition, detection and genotyping of rotaviruses have been performed with genotype-specific oligonucleotides immobilized on glass slides.[22] Standard microarrays have also been effective in the detection and subtyping of influenza viruses.[23,24]

These standard microarray experiments demonstrate the ability to query a sample against multiple strain- or genotype-specific probes—a primary goal in epidemiological studies. In the clinical laboratory setting, however, the testing situation may differ slightly. Patients typically present with symptoms that could be indicative of any one of a defined set of pathogenic agents. The goal of the clinical laboratory is to quickly identify which pathogen is responsible for the symptoms and to determine whether the pathogen is resistant to standard antibacterial or antiviral treatments. The number of patients to be examined varies seasonally and can be quite large in some months. For clinical testing of these patients, the ideal tool would enable the screening of multiple patient samples for a specific panel of pathogens, e.g., those causing respiratory illness. Electronic microarrays provide the flexibility required to perform these types of assays—multiple samples can be analyzed for the presence of a panel of pathogens, and genes conferring resistance can be analyzed simultaneously. The specific infectious agents being tested can be varied as necessary by substituting the amplification primers and reporter probes. Multiplex experiments using electronic microarrays have differentiated samples containing *E. coli*, *Pseudomonas aeruginosa*, or *Staphylococcus aureus*,[25] and have differentiated 12 species of mycobacteria in 270 patient samples (M. Sanguinetti et al., Universita Cattolica del S. Cuore, unpublished observations). The ability to use electronic microarrays to amplify and detect a panel of organisms and to quickly assess resistance should provide the clinical laboratory with a powerful tool for identifying the causative agents of a wide range of infectious diseases.

PHARMACOGENOMIC APPLICATIONS

Several benefits arise from incorporating genotyping analysis in pharmacogenomics studies. Genotyping results are not affected by diet, drug–drug interactions, or disease progression. Unlike phenotyping, test accuracy is not dependent on steady-state metabolism of the therapeutic. In addition, genotyping results are not dependent on the methodology used. By genotyping individuals suffering adverse drug reactions, specific genetic profiles associated with risk can be identified. Optimizing drug selection and dosing for poor and ultra metabolizers will not only result in increased effectiveness of treatment, but also reduce the need for multiple phenotypic evaluations.

The overall effectiveness of a given therapeutic will be determined by a combination of genetic loci encoding transporters, receptors, and metabolizing enzymes. The use of electronic microarrays to analyze panels of SNPs in such genes will allow greater accuracy in determination of the therapeutic effect and potential toxicity of therapeutics. Such a personalized panel represents the ultimate goal and promise of pharmacogenomics. Electronic microarrays can provide a valuable tool in the efforts to facilitate development of such panels and incorporate them into clinical practice.

CONCLUSION

Achieving maximum efficiency and cost-effectiveness on microarrays requires that all test sites provide useful information. Standard high-density arrays are, therefore, best suited for research studies requiring analysis of very large numbers of SNPs. Such discovery efforts provide valuable insight into markers of potential clinical utility. Those markers must then be clinically validated and, ultimately, incorporated into panels for diagnostic or prognostic use. The demands in a clinical environment differ from those in a research setting: multiple samples must be analyzed in a cost-effective, time-efficient way, and the methods used must be highly accurate and robust. Electronic microarrays are ideally adapted to these clinical applications.

Individual control of each test site allows DNA from different samples to be analyzed simultaneously on a single microarray. Unused test sites remain available for subsequent addressing, allowing utilization of all microarray sites regardless of the number of markers and samples being interrogated in any one experiment. The ability to analyze multiple SNPs from each sample provides the capacity necessary for development of complex genetic panels. Nanogen's open platform provides unique flexibility in assay design, allowing the user to reconfigure the panel of SNPs as additional information becomes available regarding the clinical utility of particular markers. The unique features of an electronic microarray not only provide the cost and time efficiencies required in a clinical laboratory setting, but also enable the validation and incorporation of new molecular diagnostic tests as new data are generated.

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Fabricating Microarrays

F

Mary Jane Cunningham
Dat D. Dao
Houston Advanced Research Center (HARC), The Woodlands, Texas, U.S.A.

INTRODUCTION

In 1987, Augenlicht et al. published the first paper on arrays and the use of gene expression profiles in typing cancers.[1] Almost two decades later, microarrays have been used as a tool in defining expression profiles in diseases as well as in compound discovery and development. Previous reviews have discussed the history and the application of microarrays.[2,3] This paper will cover printing microarrays, including the variety of printing surfaces, the immobilization techniques, the types of molecules printed, and the printing process itself. Topics which will not be covered will be other methods used in the microarray process: sample preparation, array hybridization, array scanning, image analysis and data analysis, and mining.

Microarrays are described as arrays of macromolecules (including proteins), tissue slices, cells, chemicals, or nanomaterials printed on a surface to mimic a ''micro'' environment. Typically, the materials are printed in hundreds or thousands covering 1–2 cm^2 of surface area. The advantage is that this large number of materials can be screened all at once. An investigator can sometimes repeat in one experiment what took other investigators years to evaluate. In addition, microarrays lend themselves well to producing data which allow the investigator to piece together a ''systems'' answer to the hypothesis. Many mathematical modeling algorithms and methods are now being used to try to ''reverse engineer'' a biological system or remap what pathways are involved based on the expression profiles obtained from microarrays.

MICROARRAYS

Traditionally, arrays have been categorized into different formats: conventional (cDNA and oligonucleotide), electrokinetic, fiberoptic, microfluidic, and more recently, applications in the nanotechnology field. The most popular format today is the conventional arrays, which will be discussed in detail here (Fig. 1). Within this paper, ''probe'' will refer to the macromolecule printed on the array surface and ''target'' will refer to the macromolecule derived from the sample and hybridized to the array. The other array formats have just recently been commercialized or are in development.

The conventional array format involves either printing macromolecules onto a surface or synthesizing the macromolecules in situ one building block at a time. The first technique, usually referred to as ''cDNA microarrays,'' is the printing of DNA onto a substrate. The initial papers describing this technique were derived from collaboration between the laboratories of Ron Davis and Pat Brown at Stanford University.[4] The second technique, usually referred to as ''oligonucleotide arrays,'' builds the macromolecular sequence in situ or at a specific location on the surface. Early applications used photolithography. A mask is put over the array surface to allow chemical reactions to take place only at specified sites. The reactions involve photolabile groups activated by light to free hydroxyl groups. The hydroxyl groups were then covalently bound with phosphoramidite-activated deoxynucleosides. The bound molecule is capped and oxidized. After rinsing, a new mask covers the array surface and a new set of reactions occur. With each round of reactions, an activated nucleoside is added and the oligonucleotide (''oligo'') is synthesized to approximately 25 nucleotide oligomers or ''25-mers.'' This method was originated by Stephen Fodor and David Lockhart at Affymetrix.[5] Two newer in situ methods have been developed: one which uses a digital micromirror to create a virtual mask for the photolithographic manufacturing process of microarrays[6] and another allows parallel synthesizing of several different types of molecules.[7] The first technology was initially developed at Texas Instruments and is being commercialized by NimbleGen and the second is being commercialized by Xeotron.

If an investigator wishes to set up his/her own arraying facility, cDNA microarrays are the easiest methodology to start with. (Here the authors are referring to printing microarrays of cDNAs or oligos by methods other than photolithography.) However, before starting, several choices of materials and processes are required.

The first choice is to decide which substrate the array will be printed on. Current choices are listed in Table 1

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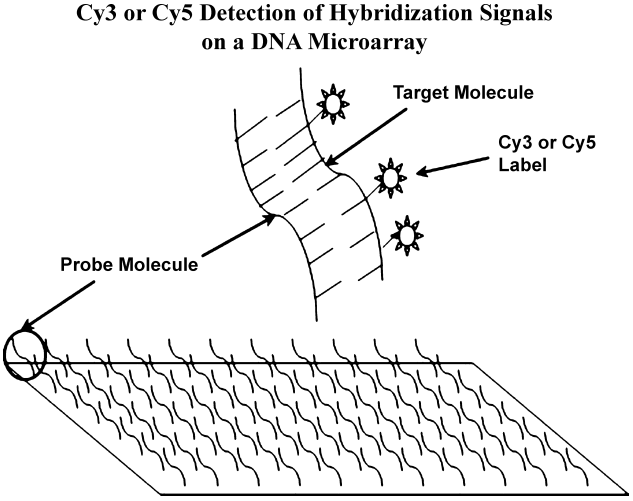

Fig. 1 The schematic depicts the hybridization reaction for cDNA or oligonucleotide microarrays. Briefly, the probes are oligonucleotides or expressed sequence tags (ESTs, actual form of cDNA used) printed onto a glass slide. The target molecule is the cDNA (fluorescently labeled with either Cy3 or Cy5) present in the hybridization solution. During the reaction, the fluorescently labeled cDNA in solution will hybridize to the probe on the glass surface with a homologous sequence.

and, traditionally, the most common substrates used are glass slides and nylon filters. Because of their larger surface area (several square centimeters), nylon filter arrays are sometimes referred to as ''macroarrays.'' Other surfaces listed in Table 1 are being used by formats just recently commercialized or in development, such as microfluidic arrays. Today, most conventional arrays use glass; however, it is extremely important that glass surfaces are cleaned prior to printing. Some preferred methods include ultrasonication[23] or washing with acid[10,12] or alkali.[24]

Table 1 Microarray substrate choices

Substrates	References
Beads	[8]
Compact discs	[9]
Fused silica slides	[10]
Gel pads (fixed to glass)	[11]
Glass slides	[4,5,12]
Nylon filters	[13,14]
Polypropylene film	[15]
Polystyrene microwell plates	[16]
Silicon wafer	[17–20]
Waveguides, planar	[21,22]

Table 2 Derivatizing agents for microarray surfaces

Modifications	Derivative	References
Agarose film	–	[27]
Gold substrates	–	[28]
Poly-lysine	–	[4]
Microporous polymer	Nitrocellulose	[24]
Silane	Aldehyde	[29]
Silane	Amine	[30,31]
Silane	Amine/diazotization	[32]
Silane	Amine/epoxy	[23]
Silane	Epoxy	[12,18–20]
Silane	Polyethylenimine	[25]
Silane	Self-assembly; aminosilanes	[10]
Silane	Self-assembly; alkylsiloxanes	[17]
Silane	Semicarbazide	[33]
Silane	Thiol	[34]

The second choice is whether or not to treat the substrate surface to further enhance the binding of the macromolecule or substance. Table 2 lists a variety of modifications which have been used and range from simply coating with poly-lysine[4] or using a multistep procedure with polyethylenimine.[25] The most common method is silanization and several silane derivatives are listed in Table 2. Some unique slide coatings are the use of microporous polymer, agarose file, and a deposition of gold substrates. Glass slides coated with amine, aldehyde, epoxy, thiol, or gold derivatives as well as a plain silylated form (CEL slides) are commercially available through TeleChem International, Inc.[26]

The third choice is a form of macromolecule to print. In the past, some investigators chose to print whole bacterial plasmids containing the cDNA insert.[1,13] More commonly today, cDNA is printed started with its double-stranded purified form. Recent references refer to the printing of long vs. short oligos; short oligos being 25-mers or less and long oligos being 40-mers or longer. Although factors, such as GC content and overall sequence similarity, are important and need to be considered for designing oligos to be printed,[35] several references have shown long oligos to be comparable in sensitivity and specificity to cDNA probes.[36,37]

Once the type of macromolecule is chosen, the quality of the printed macromolecules should be checked. One method is to check the PCR products (cDNA probes) by agarose gel electrophoresis. Any product which shows more than one band or does not show any product at all is not arrayed and can be substituted with a ''cleaner'' alternative product corresponding to the same gene. Also,

Fig. 2 Experimental prototype of a DNA Microarrayer: a Hamilton Microlab 2200 (Reno, Nevada) modified with a 32 Stealth printhead and SMP3 Micro Spotting Pins (TeleChem International, Inc., Sunnyvale, CA). Metal platters (Machine shop at HARC) have been fashioned to hold five slides during attachment, and will be set on top of the platform. The platform is capable of holding one microtiter plate and seven of these platters, allowing 35 slides to be printed in each round. (*View this art in color at www.dekker.com.*)

it is necessary to obtain an accurate quantification of the molecule to be arrayed. Yue et al. describes using an assay with PicoGreen.[38]

The fourth choice is whether to modify the macromolecule. Most cDNA probes are modified by adding an amino group at either the 3′- or 5′-ends.[4,12,19,20] Other modifications have included thiol,[10,28,31] disulfide,[34] or benzaldehyde groups;[33] although unmodified DNA probes have shown equal success.[32,37] In addition, some investigators have used chemical linkers to connect the modified surface with the modified probe. Yang et al. used a maleic anhydride linkage between a thiol-modified oligo and an aminosilane-modified surface.[31]

The fifth choice is the type of printing method to use. Printing methods have been divided into contact and noncontact methods.[39,40] The contact method uses microspotting devices to deliver the macromolecular solution to the printing surface. An example is depicted in Figs. 2 and 3. These devices deliver the solution by capillary action and tips are either disposed of or washed thoroughly before the next printing. Initial devices were hand-built using an *xyz*-axis gantry robot with the macromolecular solutions being delivered through stainless steel printing pins (Stanford University, National Institutes of Health). Pin heads are now available in ceramic (Apogent Discoveries, Hudson, NY) and tungsten (Point Technologies, Boulder, CO). Other delivery methods are capillaries, tweezers, or quills, and Holloway et al. describes several commercially available arrayers.[40]

Noncontact methods include inkjet printers which disperse solutions of macromolecules onto the array surface without the dispensing tool touching the array surface. Many of these devices use piezoelectric technology where an electrical current controls droplet formation to precise measurements.[19,41] Two current commercial vendors include Shimadzu Biotech (Pleasanton, CA) and PerkinElmer (Boston, MA).

After printing, several investigators recommend treating the printed surface to ensure a tight covalent bond and to lessen nonspecific binding during hybridization. The most common method is to cross-link the macromolecules to the surface with UV light.[32,37] This method has been used with glass slides as well as nylon filters. Other protocols bake the slides in a vacuum oven[4] and/or treat with succinic anhydride[4] or just treat with a mixture of succinic anhydride and acetic anhydride.[42]

Finally, it is recommended that a set of protocols be instituted to assess the quality of the printed slides. Microscopic examination of the slides with food coloring or hybridizing the slides with a labeled PCR primer (used to make the arrayed PCR products) has been used with random sampling, but these agents can destroy the active sites on the probes.[43] Other investigators have successfully used nondestructive means to assess quality, such as dCTP-Cy3 or SYBR Green dyes.[44,45]

Fig. 3 Experimental prototype of the DNA microarrayer: a close up view of a 32-pin dry station (Die-tech, San Jose, CA) that has been mounted to a specially designed wash station (Machine shop at HARC) secured to the base of the MicroLab 2200 between the two platforms. (*View this art in color at www.dekker.com.*)

CONCLUSION

Microarrays vary from the conventional formats of cDNA and oligonucleotide arrays to arrays now incorporating nanotechnology. The types of molecules and substances printed onto arrays also vary, and examples are cDNA probes, proteins, chemicals, cells, tissue slices, and nanomaterials. The easiest format for any investigator to use in their own laboratory from start to finish is the cDNA microarrays, which has been described in detail here. Several options need to be considered before starting and these choices include the printing substrate and its modification, the type of macromolecule and its modification, and the actual printing process. Very soon the newer formats of arrays may supercede the conventional ones emphasizing the need for more efficiency and less cost and continuing the revolution of compound discovery and development and finding disease cures.

ACKNOWLEDGMENT

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Microarrays—Standard Operating Procedures

Thorsten Forster
Peter Ghazal
The Scottish Centre for Genomic Technology and Informatics, University of Edinburgh Medical School, Edinburgh, U.K.

INTRODUCTION

Microarrays, or generally bioarrays, can be defined as the simultaneous assaying of a biological target sample interacting with a large number of biomolecule probes positioned in a gridlike layout on a physical substrate.

Like its forerunners dot blotting and cDNA filter arrays, microarray technology exploits complementary binding properties of biomolecules. As opposed to these forerunners, microarray technology is highly parallel in terms of the number of bioprobes that can be assayed simultaneously under a given experimental condition, and provides high throughput in terms of the number of experimental conditions that can be investigated in a relatively short time. The current level of microarray technology has been enabled through the combined use of robotics, miniaturization, labeling approaches, and image processing hardware and software.

Microarrays are redefining vast areas of experimental and applied sciences, from gene expression analysis to combinatorial chemistry and protein expression analysis. Although DNA microarray technology is now relatively mature and gene expression analysis a more widely used tool than protein expression analysis, it involves the assumption that mRNA levels correlate well with protein expression levels. However, because a gene may transcribe multiple mRNA sequences depending on splicing, polyadenylation or editing, and because translation of a sequence can lead to protein, which undergoes a variety of posttranslational modifications (such as phosphorylation, glycosylation, or lipidation), predictions of protein expression levels from mRNA expression are subject to some restrictions. Consequently, there has been an increasing interest in the direct, systematic measurement of protein expression levels.

DESCRIPTION OF TECHNOLOGY

Common features for all variations on this technology are the following:

- Large numbers of distinct probes are bound to a substrate in a gridded layout on a solid substrate.
- The level of abundance of each test analyte (target) can be assessed by the amount of labeled analyte specifically bound to its cognate probe on the surface of the chip.
- In general, target sample materials are labeled (there are direct and indirect methods), but in some cases the probe may contain the label.
- One target sample is labeled and assayed against one array, or two target samples are differently labeled and assayed against one array.
- After hybridization or other interaction, arrays are scanned to obtain images showing the amount of bound, labeled material for each probe on the array.
- Array images are subjected to image processing algorithms, which convert them to numerical signal levels for each probe.
- Signal correction and systematic adjustments (normalization algorithms) for nonbiological effects are performed before data analysis.
- Basic analysis of these data provides absent/present calls, semiquantitative levels of abundance or expression, and differential abundance or regulation between different experimental conditions.

Figure 1 shows a typical workflow for a cDNA microarray experiment, and Fig. 2 the most relevant differences when using protein microarrays.[1] A good overview can be found in Ref. [2].

VARIATIONS ON MICROARRAY TECHNOLOGY

The microarray platform used can be defined by the following factors:

- Probes can be double-stranded DNA (usually several thousand bases), single-stranded oligonucleotide sequences (~20–75 bases), antibodies, other proteins or biomolecules, chemical entities, and even tissue slices.
- Probes can be deposited robotically or by fluid mechanics; oligonucleotide sequences can also be

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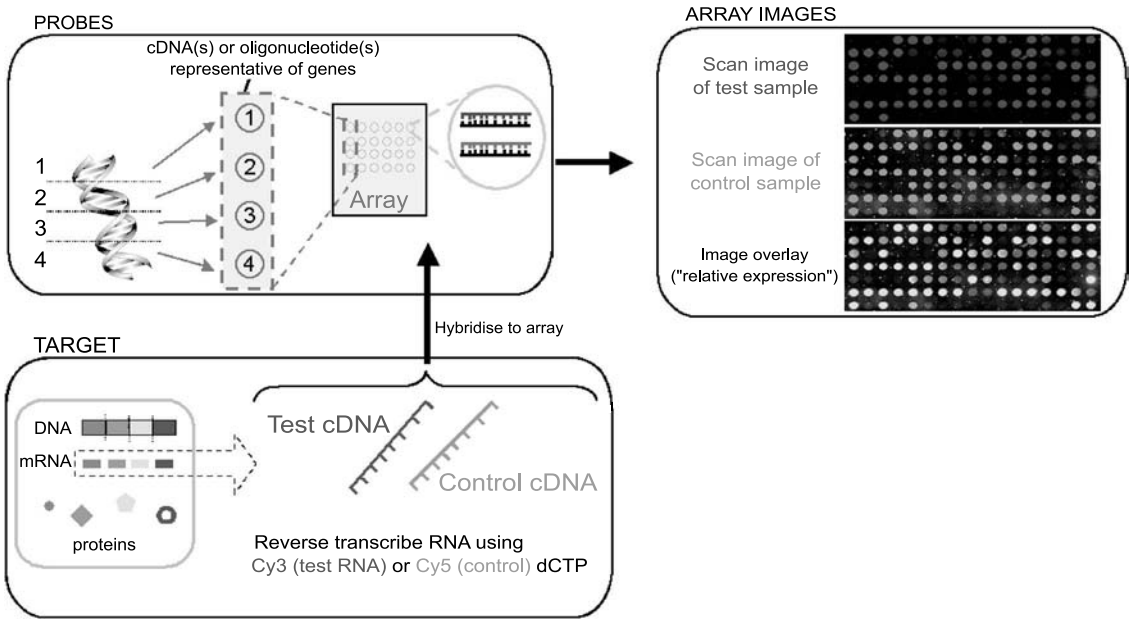

Fig. 1 Typical workflow for a cDNA microarray experiment. This example is based on oligonucleotide gene probes on a custom spotted array, using dual-dye cohybridization of two different samples on each array. Based on a genome of interest, probes are selected and synthesized. Using a robot, one can then arrange these probes into a grid on the substrate (in this case, a glass slide), where they remain bound to the surface by electrostatic forces, covalent cross-linking, or other means of attachment. On the other side of the process, biological samples are obtained from tissues or cell lines, followed by the extraction of total RNA and reverse transcription to cDNA. The last step also includes the dye-labeling step, and the sample is now referred to as target sample. In this case, a target sample from a control or reference condition has been dye labeled with Cy5, a target sample from an experimental or test condition with Cy3. These samples are then mixed and cohybridized to one array. This is followed by scanning the array at frequencies corresponding to both dye labels. The resulting false-color images can be obtained separately or as a composite image. Probes appearing red are more abundant (or higher expressed) in the experiment sample, probes appearing green are more abundant in the reference sample, and probes appearing yellow are equally abundant in both samples. (*View this art in color at www.dekker.com.*)

assembled in situ (e.g., using lithography methods) on the array.

- The most common protein/DNA array formats are glass slides (standard microscopy size) with various coatings or fully encapsulated cartridges containing the array.
- For single-target arrays, one target sample is labeled and assayed against one array. For dual-target arrays, two target samples are differentially labeled and assayed against one array.
- Labeling approaches include dyes, reflective or light-scattering particles, antibodies, or radioactive material.

ESSENTIAL ASPECTS OF MICROARRAY TECHNOLOGY

Laboratory Standards

Microarray experiments—both custom spotted and commercially available arrays—are susceptible to failure or data bias introduced by laboratory procedures. A very high level of laboratory protocols is therefore required. It has now frequently become a prerequisite for publication to record these steps at minimum information about a microarray experiment (MIAME) level set by the Microarray Gene Expression Data society (http://www.mged.org).

Laboratory protocols should cover precise instructions for sample acquisition and processing, array production and processing, the selection and synthesis of probes, labeling steps, the hybridization process, posthybridization processes, and storage. It is highly recommended that rigorous standard operating procedures be applied for both benchwork and data capture.[3]

Data Acquisition

Scanning and the conversion of resulting images (Fig. 3) into numerical data (‘‘quantification’’) is a source of technological data variability. Depending on the system used, scanning any array with inappropriate parameter values will either cause signal saturation or miss out low expressed probes. It is therefore better to scan each array more than once, with different parameter values, and to

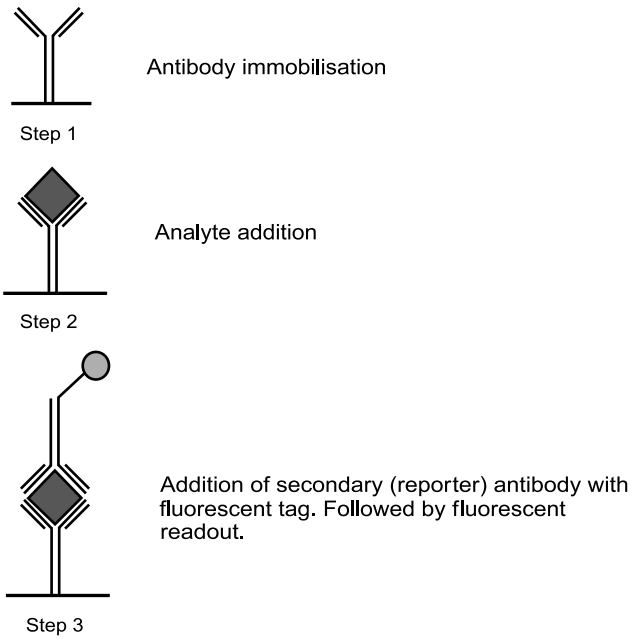

Fig. 2 Deposition and detection of a single probe on a protein array. The general concept of probe selection, probe deposition, target labeling, binding, and scanning are comparable to DNA microarrays, the main differences lying in the specific method of depositing and labeling proteins. In this case, the target sample or analyte is incubated onto the array before labeling the target by means of a specific antibody with a fluorescent tag. The analyte could also be labeled before incubation; this depends on user preference and the biological system under investigation. (*View this art in color at www.dekker.com.*)

select a best scan or use statistical approaches to combine the information from multiple scans. A standard protocol must be associated with this approach. This needs to cover suitable scanning parameters, a consistent quantification method, recommended level of manual fine-tuning for the grid alignment, and quality control steps such as the assessment of background noise.

Data Processing

It is most likely that despite use of stringent laboratory and data acquisition protocols, there are still systematic sources of variation or noise in the data that cannot be attributed to the biological sample differences under investigation. The use of data processing techniques[4–6] is not optional but required. Image data is associated with background noise (specks of dust, uneven hybridizations, etc.), which needs to be adjusted. Most array data are assumed to be on a scale that benefits from prior transformation of the data values. The term ''normalization'' in relation to microarray data is best defined as a collection of mathematical methods that are used to make target samples comparable by removing systematic effects introduced by dye-label incorporation differences, hybridization differences resulting in overall image ''brightness'' differences, and differences across an individual array caused by deposition of probes by different robotic print tips.

Background measured by the image processing software is usually removed by subtracting individual probe background from the probe signal value or by subtracting a local average background from each individual probe signal value. It is also recommended to define a detection-threshold limit for each array, which is the mean plus two standard deviations of negative control probes on the array, or another robust estimate for theoretically nonexpressed probes on the array.

On-chip signal ratios are most often log-transformed to base 2, which symmetrizes the distribution. To stabilize variance, transformations such as arc sinh are still being evaluated. For dual-target arrays, the currently most common method of normalization is the locally weighted scatterplot smoother (LoWeSS), which adjusts for the nonlinear differences between two dye labels on an array.[5] The effect of this can be seen in Fig. 4.

In many cases, the normalized log ratio is still subject to different levels of variance, and to make them comparable across a number of arrays, a second step can be included in the normalization, which adjusts variance rather than location of log ratios. One such method is the median absolute deviation scaling.[6]

Single-target arrays do not normally require any on-chip normalization like the above. The center (median) of the absolute signal intensities on the array is often scaled to match other arrays. Other common forms of normalization take into account specific print tip groups; that is, standard normalization methods are performed on physical subsets of the array rather than on the array as a whole. For further analysis, in particular cluster analysis, normalization also occurs on a per-gene level, which means that each gene's values across a number of arrays are adjusted to have a mean of 0 and a standard deviation of 1.

A special case of normalization is one that estimates the size of an effect rather than correct for it. Given a high enough level of replication, analysis of variance (ANOVA) can provide estimates for each effect (hybridization, dye label, print tip group, etc.) that has been included in the model; that is, the observed signal value is described by the level of signal that is due to unwanted sources of data variation and the signal differences that are due to real biological effects.[7]

Data Analysis

Microarrays produce an amount of data that has rarely been seen before in biological research. It is common to

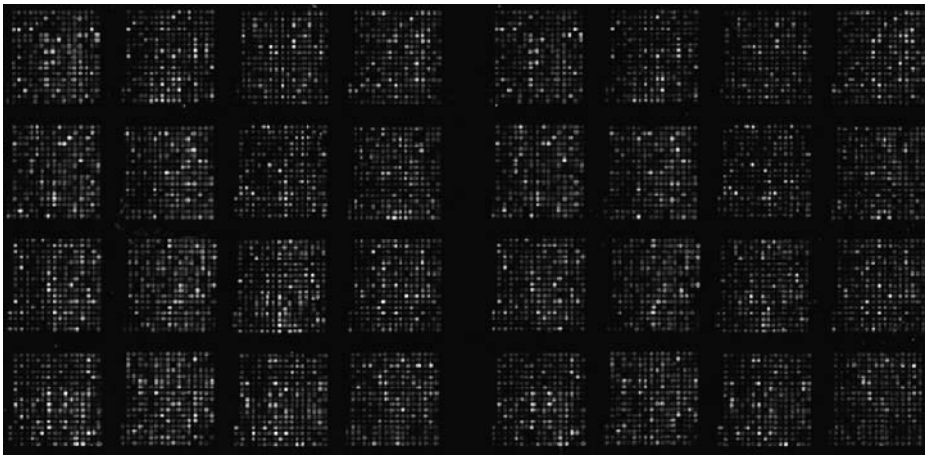

Fig. 3 Example of a false-color array scan image. The image is a composite of the image scanned for cy3 and the image scanned for cy5, resulting from the cohybridization of two target samples to one array. The original scan images are 16-bit grayscale; that is, signal intensities can range from 0 to 65536, and software converts this to color levels for display purposes. Each separate smaller grid within the entirety of the array consists of probes deposited by one of many robotic print tips. The ''gap'' in the middle of the array indicates that all probes have been printed onto the array twice. (*View this art in color at www.dekker.com.*)

use arrays containing 10,000–30,000 genes, and the use of multiple experimental conditions and replicate samples can easily produce millions of data points for an experiment. Analysis of the data is further complicated by the dimensionality of the data; that is, compared to ''normal'' data with many observations (e.g., patients) and few variables (e.g., weight and blood pressure) there are few biological samples and a large number of response variables—the probes. This poses statistical problems with analysis approaches.

Inference statistics such as *t* tests or Mann–Whitney *U* tests on a gene-by-gene basis effectively means hundreds or thousands of tests performed in parallel, which introduces a multiple-testing problem.[8] A proportion of all tests performed is liable to contain false positive results. This can be counteracted by adjustments to the computed *p* values; common approaches include, but are not limited to, Bonferroni and false discovery rate method.[8]

Following basic inference approaches to discover significant differentially expressed genes, the most common second form of analysis is exploratory, and a large part of this consists of a variety of clustering and classification algorithms.[9] These methods are statistically less rigorous, but often very relevant to the biologist. Interpretation of such results must be made with care, and subsequent result validation is required for hypothetical models of genetic regulation to stand.

The great potential of microarray technology is matched by potentially complicated experimental designs. This applies to statistical experiment design as well as experiment logistics. Experiment design[10–12] for microarray studies tends to be based on the principle of cohybridizing an experimental sample with a common reference sample (Reference Design). As opposed to a ''loop design'' where experimental samples are cohybridized in pairs, this provides less statistical power for the analysis, but better flexibility and extensibility of the study design. Studies designed around single-target platforms trade in reduced problems with differential label properties against error caused by different hybridization qualities and spot morphologies across arrays. Experiment design can also be driven by interest in either hypothesis- or data-driven approaches to analysis. The former requires high levels of biological replication to provide reliable results; the latter requires new mathematical approaches and potentially the integration of other sources of data.

Postanalysis Validation

Many microarray studies rely on a total of only tens of arrays to determine differences between multiple experimental conditions, and although the use of microarray technology as an ''interest filter'' for genes is perfectly valid, it needs to be supplemented[13] by follow-up studies with single-gene methods to confirm findings for individual genes or entire predicted genetic pathways.

APPLICATIONS

Microarray technology has proliferated into most areas of life sciences. It has found application in multiple organisms, multiple experimental conditions, and different biological or medical areas of interest such as cancer, immunology, or neurology. Microarrays enable researchers to identify genes that are differentially regulated in

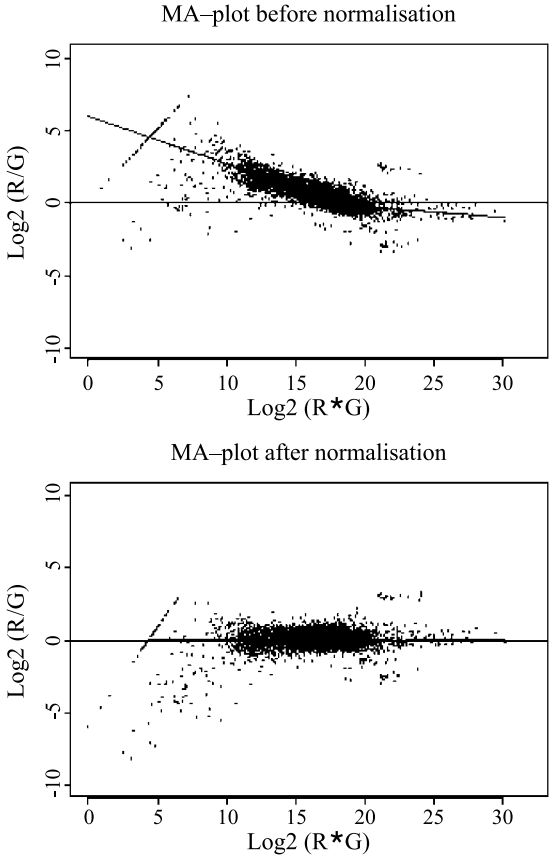

Fig. 4 MA plots before and after LoWeSS normalization. Before normalization, a systematic difference between the two dye labels on one array (R for ''red'' and G for ''green'') can be seen by the general shape of the data cloud diverging from the central line. In most experiments, one would expect only a subset of genes to be affected by different treatment conditions, and the most genes should therefore be centralized around zero in the horizontal (i.e., log ratio). The LoWeSS normalization adjusts the data cloud to match this assumption; the data cloud is straightened and centered around zero.

different experimental target samples, to semiquantitatively profile any experimental target sample, and to predict or diagnose disease by discovering new marker genes.[14,15] Microarrays also throw open the door to identifying cyclic behavior in time series experiments or to relate genes to one another based on similarities in their expression profiles across multiple conditions.[16] Special arrays for identifying splice variations[17] or single nucleotide polymorphisms[18] have also been used, and the number of practical applications for protein arrays[19] is increasing.

Looking at microarray data in isolation provides information on individual important genes and proteins in disease diagnostic and prediction, potential networks of gene interaction, new drug targets, and biological development. This is often enhanced by combining data from multiple sources; for example, using protein array data and cDNA microarray data from the same system can ultimately provide a systems-level understanding. Host and pathogen genomes can be interrogated simultaneously to gain a better understanding of their interactions.

DEVELOPMENTS AND CHALLENGES

There are a number of future key areas for this technology, some of which involve the maturing of this technology, and others that will likely lead to entirely new applications and discovery. Below are brief outlines for some of these developments.

System-level understanding is of course the ultimate goal, and requires building an understanding of how biological processes work from the molecular to organism level, and how changing factors (e.g., drugs) in the process may affect key pathway processes. Both DNA and protein microarrays provide only partial information, and there is a distinct need for a greater level of data *integration*.[20] This must combine data from various biological techniques, clinical data, and demographic data, as well as make better use of existing data of the same type, i.e., microarray data and gene/protein annotation data. The means to facilitate this are better *databases* and *ontologies*,[21] allowing one to directly link and analyze data from multiple formats and sources. *GRID technology* (sometimes referred to as ''Internet Version 2'') may be the technological base to accomplish this by providing data exchange formats and the required software and hardware architecture.

Microarray technology will also move further toward *miniaturization*, and ''nanoarrays'' for the detection of single molecules are a possibility, as are the deposition of entire genomes on smaller surface areas or the deposition of multiple probe/target combinations on one array substrate.

Irrespective of the substrate dimensions, there has also been a move toward using arrays for very *specialized applications*, such as the detection of splice variants or the fabrication of diagnostic chips containing only probes relevant for disease patterns, or selective neurological chips.

The current state of hybridize-now-analyze-later could soon be superseded by *real-time platforms*, which allow simultaneous hybridization events and data readout from the chip, which is closely coupled with conventional microelectronics. These platforms will be closer to point-of-care applications than current microarrays. They will almost certainly not rely on microscopy slides as substrates, but incorporate etched/channeled patterns in

materials with different properties. Despite these differences, they will still share purpose and gridlike layout with the current technology, and are likely to have a large impact in *chip*-based *medicine* of the future.

A further drive is toward more elaborate and good-quality *protein arrays*, which requires improvements in purification methods, spatial orientation on a substrate, and substrate adherence.[22,23] This is being researched, and protein arrays are getting ever closer to the level of comprehensiveness achieved by DNA microarrays, although they themselves are not faultless.

Independent of the technology used, methods of statistical and exploratory analysis are continuously being expanded, creating more powerful and, importantly, more robust results for biological interpretation. Machine learning, genetic networks, Bayesian methods, artificial intelligence, and other established mathematical approaches of analysis and *data mining* are being investigated for use in microarray research.

CONCLUSION

Independent of the platform used, microarray technology exhibits properties that cause complications in the analysis and interpretation of data. Data dimensionality as defined by large numbers of variables (probes) for small numbers of biological replicate samples is one such aspect, and the relative amount of technical and unwanted biological data variation is another. For these reasons, every aspect of a microarray experiment needs to be tightly controlled from the experiment design to laboratory work and data analysis. Standard operating procedures are a necessity, and where such methods exist and are adhered to, microarray technology is a very powerful tool opening new and promising avenues for further research.

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Microduplication Syndromes—17p11.2 Duplications, Proximal 15 Duplications, and Cat Eye Syndrome

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Heather E. McDermid
University of Alberta, Edmonton, Alberta, Canada

INTRODUCTION

The human genome contains numerous sites where the presence of complex low-copy repeated sequences leads to recurrent chromosomal rearrangements resulting in recognizable syndromes. Typically, mispairing and crossing over between adjacent direct repeats, resulting in the deletion of the intervening DNA, cause these syndromes. Such microdeletion syndromes include deletions of 22q11 (DiGeorge/velocardiofacial syndromes or DGS/VCFS), 7q11 (Williams syndrome), and 15q11–q13 (Prader–Willi/Angelman syndromes or PWS/AS) (reviewed in Ref. [1]). Theoretically, such nonallelic homologous recombination between repeats should also result in an equivalent number of reciprocal microduplications. However, microduplication syndromes are less well known, probably due to a combination of milder, less distinct phenotype which may be unrecognized as a particular syndrome, and the difficulty of detecting microduplications compared to microdeletions.

The classic case of reciprocal microdeletion/microduplication syndromes is that of the peripheral neuropathies of 17p12.[2] Deletion of a 1.5-Mb segment results in ''hereditary neuropathy with liability to pressure palsies'' (HNPP), whereas duplication of the same region results in Charcot–Marie–Tooth disease Type 1A (CMT1A). The rearrangements involve the 24-kb CMT1A-REP repeats and the change in dosage of the central gene PMP22 is thought to cause the peripheral neuropathies typical of both disorders. Details of these syndromes are covered elsewhere. Below are reviewed other microduplication syndromes, which include interstitial duplications such as CMT1A, as well as duplication through the presence of a supernumerary chromosome. The phenotype associated with such microduplication syndromes presumably arises due to the overexpression of one or more genes in the duplicated regions.

DUPLICATIONS OF 17p11.2

Recombination between a set of low-copy repeats in 17p11.2 causes the microdeletion syndrome known as Smith–Magenis syndrome (SMS). This disorder, with an estimated prevalence of 1/20,000, is characterized by mental retardation, neurobehavioral abnormalities, sleep disorders, speech and motor delays, midface hypoplasia, short stature, and brachydactyly.[3] The same deletion is seen in 90% of patients. Only recently has it been recognized that a reciprocal duplication also exists. The dup(17)(p11.2p11.2) syndrome is characterized by borderline to mild mental retardation, behavioral problems, short stature, dental anomalies, normal facies, and lack of major organ malformations[4,5] This syndrome typifies a milder phenotype that was not recognized as a syndrome until connected to the microduplication.

Nonallelic homologous recombination is thought to occur between the 200-kb SMS-REPs, which are direct repeats separated by $\sim$4 Mb.[6] A third SMS-REP exists with the SMS region, but because of its inverted orientation it does not usually participate in deletion/duplication formation. Interchromosomal and intrachromosomal exchanges appear to occur with equal frequency, as determined by microsatellite analysis of 16 dup(17)(p11.2p11.2) syndrome patients.[5] There appears to be no significant parent-of-origin bias among these 16 patients. The gene or genes involved in the dup(17)-(p11.2p11.2) syndrome are unknown. Recently, chromosome engineering in mice has resulted in mouse models for both deletion and duplication syndromes of the equivalent murine region of conserved synteny.[7]

DUPLICATIONS OF PROXIMAL 15Q

The region of chromosome 15q11–13 has long been known to be chromosomally unstable, being the site of deletions, duplications, triplications, translocations, inversions, and the breakpoints for supernumerary chromosomes. The resulting phenotype of such rearrangements is further complicated by the abundance of imprinted genes in the region. Thus, paternal deletions of the region result in Prader–Willi syndrome (PWS), whereas maternal duplications of the exact same region cause the phenotypically unrelated Angelman syndrome (AS) (both syndromes are described elsewhere). A series of complex low-copy repeats exist within the region and mark breakpoint sites.[8] The two most centromeric breakpoints,

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BP1 and BP2, form the proximal breakpoint of the PWS or AS deletions, and the common distal deletion breakpoint is known as BP3. Between the BP1/BP2 and BP3 breakpoints lies the PWS and AS critical region, or PWACR. The prevalence of this deletion is estimated at 1/10,000.

Duplications of this region take several forms. Interstitial duplications usually include the entire PWACR. Most paternal duplications are associated with normal phenotype, whereas maternal duplications are usually associated with developmental delay, delayed speech, autistic-like and other behavioral problems, and few or no dysmorphic features.[9] Interstitial duplications appear to share the same breakpoints with deletions, spanning the region between BP1/BP2 and BP3.[10] Both intrachromosomal and interchromosomal exchanges have been reported.

Interstitial triplications are also known to occur,[10,11] and may result in a more severe phenotype. Some triplications use some of the PWS/AS breakpoints, but they also break within and distal to the PWACR, implying that they may arise by a different mechanism than duplications. The duplication of the region distal to the PWACR may also contribute to the severity of the triplication phenotype.

Supernumerary marker chromosomes (SMC) represent a second basic type of duplication. The most common SMC is a bisatellited, isodicentric chromosome (Fig. 1) derived from proximal 15q and usually referred to as an inverted duplication 15 or inv dup(15). Because one centromere is usually inactivated, these chromosomes are more properly termed pseudodicentric. The inv dup(15) chromosomes are thought to arise through a U-type exchange between low-copy repeats present in opposite orientation. The inv dup(15)s exist in two basic sizes, although specific breakpoints may vary.[12] ''Small'' inv dup(15)s do not include the PWACR, break at BP1 or BP2, and are usually associated with normal phenotype, although some male carriers have been found to be infertile with azoospermia.[13] Such chromosomes are often familial and detected during routine prenatal diagnosis. ''Large'' inv dup(15)s include the PWACR, break at BP3 or more distally, and are associated with mild to severe mental retardation, autistic-like behavior, seizures, hypotonia, poor motor coordination, abnormal dermatoglyphics, and dysmorphic features such as strabismus. They are usually symmetrical (Fig. 1a), resulting in the presence of four copies of the PWACR [two on normal chromosome 15s, two on the inv dup(15)], and thus are more like the interstitial triplications. However, rare asymmetrical inv dup(15)s (Fig. 1b) have been reported,[12] resulting in only three copies of the PWACR.

It should be noted that there have been difficulties in the interpretation of some patients' karyotypes in the past, as duplications and triplications can be difficult to distinguish using microsatellite markers. Some patients with supernumerary chromosomes have also been found to have uniparental disomy of the normal 15s, leading to a PWS or AS phenotype. Many genes in the region have been well characterized, but while many duplication studies have determined the duplication size, few have determined whether these genes are actually overexpressed.

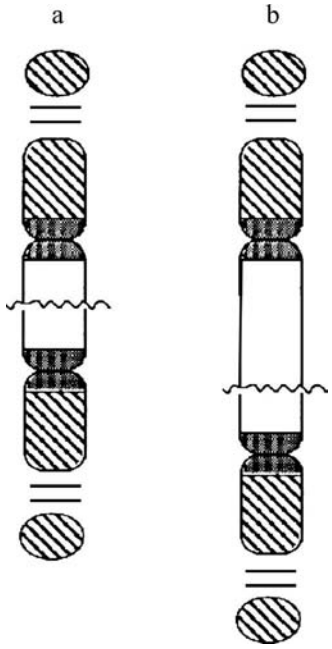

Fig. 1 Supernumerary bisatellited chromosomes, representing either inv dup(15) or inv dup(22). Such chromosomes can be symmetrical with the same breakpoint for each half (a) or asymmetrical with different breakpoints for each half (b). (Modified from Mears: Molecular Characterization of Duplications Associated with Cat Eye Syndrome. Ph.D. thesis; University of Alberta, 1995.)

The clinical features of the proximal 15q duplications are highly variable. Some of this variability may result from the form the duplication takes. It is possible that an interstitial duplication and duplication through a supernumerary chromosome of a similar region may not give the same phenotype. This could be due to possible differences in position effect (for instance, distance from a centromere) or changes in global imprinting of the region.

DUPLICATION OF 22Q11—CAT EYE SYNDROME

The inv dup(22), or CES chromosome, is associated with the clearly delineated disorder cat eye syndrome or CES (MIM #115470).[14] This syndrome is characterized by a

variety of congenital defects including ocular coloboma, anal atresia, preauricular tags/pits, heart defects (particularly the relatively rare total anomalous pulmonary venous return or TAPVR), kidney and skeletal defects, dysmorphic facial features, and mild mental retardation.[15] Cat eye syndrome is highly variable in severity and the number of features expressed, even within families, can range from severe to essentially normal. Cat eye syndrome is rare, with an estimated frequency in the range of 1/50,000 to 1/150,000 live births.

The typical CES karyotype is 47,+idic(22)(pter→q11.2), resulting in the presence of four copies of the CES critical region or CESCR. However, interstitial duplications of 22q11.2 have also been associated with CES.[16–18] Because of the small sample size of interstitial duplications and the extreme variability of the syndrome, it is unclear whether three copies of the region give a less severe phenotype than four.

The CESCR does not overlap with the DGS/VCFS deletion syndrome of 22q11, and thus they are not reciprocal syndromes (Fig. 2). However, the CES chromosome exists in several discrete sizes, some of which do include the DGS/VCFS critical region.[20] The CES region extends ~3 Mb from the centromere and is present in two copies in all inv dup(22). Some CES chromosomes also contain one or two copies of the adjacent 3-Mb region deleted in DGS/VCFS. The recurrent CES breakpoint regions occur in the same intervals as the proximal and distal 3-Mb VCFS/DGS deletion breakpoints.[20,21] Thus a Type I CES chromosome is symmetrical with two proximal breakpoints and no DGS/VCFS material (Fig. 2). Type II CES chromosomes contain one proximal and one distal breakpoint (Type IIa, asymmetrical) or two distal breakpoints (Type IIb, symmetrical), resulting in an additional one or two copies of the VCFS/DGS region. Interestingly, most reported Type II inv dup(22)s are asymmetrical, whereas most reported inv dup(15)s are symmetrical. An additional, less-frequent 22q11 breakpoint exists in the middle of the DGS/VCFS, which can also be used as a distal breakpoint.[22] All three breakpoint regions, each 1.5 Mb apart, harbor a similar low-copy repeat or LCR22.[23] The size of the inv dup(22) does not appear to have an obvious correlation to the severity of the phenotype, despite the fact that dosage-sensitive gene(s) must be present in the DGS/VCFS region.[20] However, the sample size of this study was small (10 patients).

The CESCR was further defined by studying a child with an unusual supernumerary double-ring chromosome 22 and all major physical features of CES.[24] The duplication extended from the centromere to the gene *ATP6E*, a distance of approximately 2 Mb (Fig. 2). Fourteen transcripts have been identified in the CESCR, as well as 12 mouse homologues in the region of conserved synteny on murine chromosome 6.[25,26] It is not yet known whether one or multiple genes are involved in CES. Unlike the inv dup(15), the study of the inv dup(22) is not complicated by the presence of imprinting.[27–29]

Like the inv dup(15), there are also inv dup(22)s that are associated with normal phenotype. These chromosomes presumably do not contain euchromatin, although their cytogenetic appearance is often not distinguishable from the CES chromosome. This is especially problematic when an inv dup(22) is found during prenatal testing, as there are currently no commercial fluorescence in situ hybridization (FISH) probes available to distinguish between the benign and CES chromosomes.

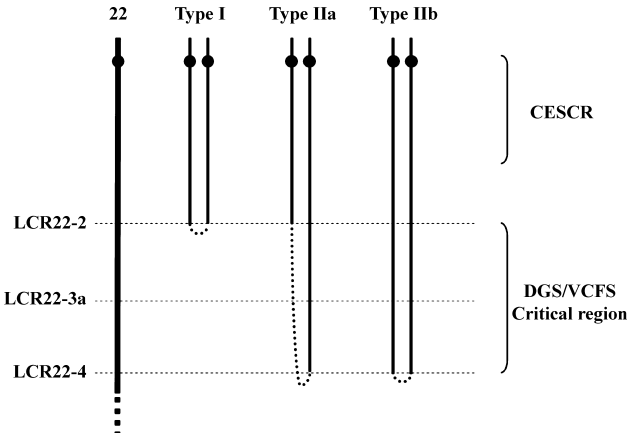

Fig. 2 Different types of inv dup(22), which contain no (Type I), one (Type IIa), or two (Type IIb) copies of the DGS/VCFS critical region. Breakpoints for both inv dup(22)s and the DGS/VCFS deletions are found in regions of low-copy LCR22 repeats. (Redrawn from Ref. [19].)

DIAGNOSIS OF MICRODUPLICATIONS

Microduplications in the form of SMCs can be identified by karyotyping followed by the identification of the chromosomal origin using FISH. However, interstitial microduplications are technically difficult to detect. Using FISH, a microdeletion can be detected as the absence of probe signal over the deleted chromosome. However, microduplications typically result in two superimposed signals, which are difficult to distinguish from a single signal. Confirmation of a microduplication usually requires analysis of FISH signals over high-resolution chromosomes or interphase nuclei. Commercial probes for microdeletion syndromes SMS and PWS/AS can be used to detect microduplications of these regions; however, no commercial probe is currently available for the CES-specific region. Other techniques such as microsatellite and dosage analysis are difficult to do in a clinical setting. The prevalence of both microduplications and

microdeletions in individuals with developmental delay and/or congenital malformations may soon become clearer, as quantitative analysis of the whole genome via comparative genomic hybridization (CGH) arrays becomes clinically applicable (reviewed in Ref. [30]). In this technique, fluorescently labeled DNAs from a patient and a normal individual are competitively hybridized to CGH arrays containing genomic clones from various regions of the genome, allowing differences in copy number of these regions to be detected.

CONCLUSION

The existence of reciprocal microdeletion/microduplication syndromes of 17p12 (HNPP/CMT) and 17p11.2 [SMS/dup(17)(p11.2p11.2) syndrome] suggests that other such reciprocal microduplication syndromes may exist but remain unrecognized. As more individuals are carefully checked for microduplications with more sophisticated techniques such as CGH arrays, the prevalence of such possible syndromes will become clear. It is particularly interesting to speculate for the DGS/VCFS region of 22q11, the site of the most common of the microdeletion syndromes with an incidence estimated at 1/4000 live births. The fact that a reciprocal 22q11 microduplication syndrome has so far gone unrecognized suggests that it is mild and highly variable. Considering the variability of the DGS/VCF deletion phenotype, which is currently recognized as at least three different syndromes, variability in the reciprocal duplications is highly likely. A mild and nondistinct phenotype is also suggested by the fact that the presence of the DGS/VCFS region on the CES chromosome does not appear to have an obvious effect on top of the CES phenotype. The one case reported to date with duplication of the DGS/VCFS region showed developmental delay and hypotonia, but the mother and grandmother with the same duplication appeared normal.[21]

Microduplications, like microdeletions, can shed light on developmental processes by revealing dosage-sensitive genes critical in developmental pathways. To this end, the genes in microduplication regions are being characterized and mouse models are being produced.

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Microplate Array Diagonal Gel Electrophoresis for SNP and Microsatellite Genotyping and for Mutation Scanning

Ian N. M. Day
Tom R. Gaunt
Xiao-he Chen
Rosalind H. Ganderton
Southampton University Hospital, Southampton, U.K.

INTRODUCTION

Electrophoresis continues to be widely used for diagnostic applications in molecular genetics, although many efforts are being made to develop liquid-phase and closed-tube systems. Throughput requirements are increasing in proportion to the number of diagnostically relevant genes, mutations, and polymorphic variants known. Many tests are polymerase chain reaction (PCR) based. The well-known industry-standard 96-well microplate has evolved to form the basis of many high-throughput approaches in the biosciences over the past 30 years and such 96 (and 384)-well formats are now frequently used for PCR. However, microplates are only permissive of liquid-phase reactions or solid-phase (binding) separations, whereas electrophoresis can derive information about parameters such as size, shape, and charge of molecular moieties as well as acting as a highly resolving separation approach for complex mixtures. In 1994, we described an electrophoresis system, microplate array diagonal gel electrophoresis (MADGE), which enables the simple conjunction of polyacrylamide or agarose gel electrophoresis in an open-faced 96-well microplate-compatible format. This permits checking of PCRs, allele-specific PCRs, and enzyme digests of PCRs. We have since developed 192-, 384-, and 768-well versions, ''dry'' formats, high-resolution formats for microsatellite analysis (''stretchMADGE''), and a thermal ramp (''meltMADGE'') approach for scanning for unknown mutations. Analysis software is also available. These developments are outlined below. Protocols and further links are available on our laboratory web server (Southampton Genetic Epidemiology Laboratories—http://www.sgel.humgen. soton.ac.uk).

MICROPLATE ARRAY DIAGONAL GEL ELECTROPHORESIS

In 1807, Ferdinand Frederic Reuss observed under a microscope the migration of colloidal particles in an electric field, perhaps the first electrophoretic separation. During the early 1970s, both agarose and polyacrylamide electrophoresis gel usage evolved to slab gel formats[1,2] much as they are still found in the laboratory today. Throughput requirements were not an issue at that time. Indeed, slabs were an advance from tube gels. For both tubes and modern capillaries, each ''lane'' is separate from any other and cross-referencing requires reliance on either an external frame of reference (e.g., time of elution) or an internal frame (e.g., coelectrophoresed mobility standards). Slabs give convenient alignment of tracks. Microplate array diagonal gel electrophoresis, in the process of achieving microplate compatibility, uses 96 (higher density in newer formats)-track origins (wells) with 8×12 array locations at the 9-mm pitch identical with microplates devised 30 years ago by Dynatech and now a highly established industry standard for liquid-phase operations.

The Original 96-Well Format

The original MADGE format[3] uses a two-dimensional plastic former in conjunction with one glass plate coated with gamma-methacryloxypropyltrimethooxysilane (sticky silane). The plastic former contains a 2-mm-deep, 100×150-mm rectangular ''swimming pool.'' Within the pool, there are ninety-six 2-mm cubic ''teeth'' (well formers) in an 8×12 array with 9-mm pitch directly compatible with 96-well plates. The array is set on a diagonal of 18.4° relative to the long side of the ''pool,'' which is parallel to the eventual direction of electrophoresis, giving gel track lengths of 26.5 mm (Fig. 1A). The gel former is placed horizontally, acrylamide mix poured into the pool, and the glass plate overlaid (Fig. 1B). After the gel has set, the glass plate is prized off, bearing its open-faced 96-well gel. It should be noted that although the diagonal-array approach may appear complex, many users have noted that this is the simplest gel preparation and sample tracking system they have ever used. Additionally, the human eye/brain is extremely good at pattern recognition and the small-scale user, once accustomed, is not obliged to use image

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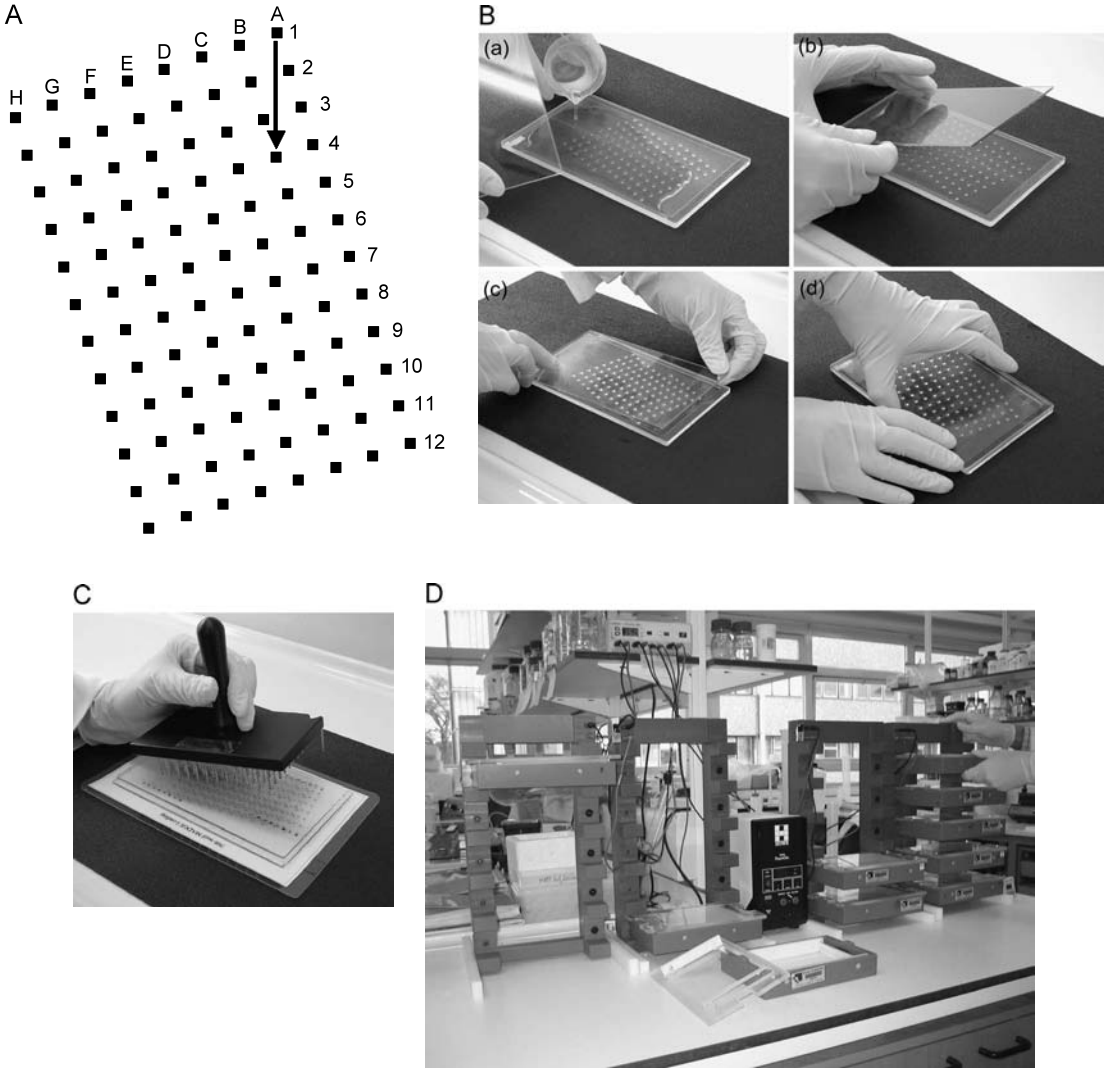

Fig. 1 Outline of components of MADGE system. (A) Schematic plan view of 96-well MADGE. Direction of electrophoresis is marked with an arrow relative to well A1. (B) Steps a–d for pouring a single 96-well PAGE MADGE gel. Gels can also be poured in batches in a purpose-built box, or gel mix can be poured through the small gap at one end into a preassembled sandwich of gel former and glass plate. (C) Ninety-six-pin passive replicator—essential for loading of high-density (384–768 well) MADGE gels. Either 8- or 12-channel pipettes or 96-pin devices can be used for 96-well gels. Air displacement rather than passive pins must be used for submerged gels. (D) ''Dry'' box gel stack frames and ''dry'' gel boxes for multiple ''dry'' box gel electrophoresis. (*View this art in color at www.dekker.com.*)

analysis software. A 26.5-mm track length (2×4 diagonal of array) such as in Fig. 1A or greater such as the 2×6 diagonal in Fig. 3 (see section below on higher-resolution MADGE), using polyacrylamide gel matrix, is efficient for many post-PCR analyses involving simple band pattern recognition. We also use agarose MADGE gels anchored on a hydrophilic plastic such as GelBond for checking long PCR products. Polyacrylamide MADGE gels are robust, reusable, directly stackable for storage or use, and fully compatible with industry-standard PCR microplates, thus minimizing procedural information transfers and enabling direct 96-channel pipetting. However, considerable advantage is gained even with 8- and 12-channel pipettes.

Short track length/run time, compactness, and scalability with hand-sized robust slab gels accessible for direct human interaction are additional benefits of MADGE. Startup costs are minimal, so ''third-world'' laboratories can also apply the system. Essentially, any analysis which can be reduced to short tracks (e.g., <3 cm) gains the throughput advantages. One technician can process

10–100 gels (960–38,400 tracks) per day according to the application and the MADGE implementation in use. Typical applications, in many laboratories worldwide, have been for allele-specific PCR and restriction digest checking post-PCR, for single-nucleotide genotyping in many samples in parallel in genetic epidemiological (''DNA bank'') research. Such MADGE-based approaches have been used extensively in our own laboratory (e.g., Ref. [4]) and in other laboratories (e.g., Refs. [5,6]). Another laboratory implemented complete genome mapping using high-throughput PCR from radiation hybrid cell lines combined with 96-well reusable MADGE-checking gels.

192-Well MADGE

For checking one or two bands, much of the usual MADGE track length remains unused. One general approach to single nucleotide polymorphism (SNP) calling is to use allele-specific PCR such as amplification refractory mutation system (ARMS):[7] one simple robust format uses two separate allele-specific amplifications, one testing for each allele, with a control (unlinked) amplicon also included in each reaction as a positive control. In this case, it is easiest to load one gel and recombine the two tracks of information about a given template into one ''virtual'' software track. This can be readily achieved by locating a second well halfway along the ''original'' MADGE track, giving two concatenated tracks of 12.25 mm (for 2×4 diagonal MADGE) in a 192-well gel.[8]

384-Well MADGE and 768-Well MADGE

384-well microplates have become an established new standard in higher-throughput genomics research laboratories for clone operations (arrayed libraries, gridding, spotting, storage, etc.) and for PCR. The difficulties in thin-walled plastics manufactured for the latter have been solved. 384-well microplates have wells in a 16×24, 4.5-mm pitch array. A diagonal turn of this array would result in MADGE wells and tracks too narrow (<1 mm) for manual access, but we have devised a 384-well MADGE format which combines four 96-well arrays in a linear (rather than tetradic) overlay. The 384-format locates the long axis of the array at 11.3° relative to the direction of electrophoresis and respectively give track lengths of 9.98 and 4.24 mm and track width of 1.5 mm. The $1.5 \times 1.5 \times 1.5$-mm wells can be accessed by human hand. However, the array is dense and confusing for 8- or 12-channel loading, but 96-pin passive or air displacement transfer is not a problem for human hand and eye. We have also derived a 768-well format from 384[9] in the same way that the 192-well format was derived from the original 96-well format (see above). Protocols for setting up 768-well gels are identical with those for 384-well gels. Unlike 96- and 192-well protocols, the 384-well protocol depends on the use of oil-free PCR, 96-pin manual transfers from 384-well PCR plate to gel, and use of dry rather than submerged gels to enable the use of passive pin transfers. However, this approach is also considerably more convenient. Combined with direct electrode contact in ''dry'' clamshell electrophoresis boxes which plug directly into contacts in a powered stacking frame and using 5–10 min electrophoresis times, very much higher throughputs can be achieved than with 96-well submersible systems. Components of this system are shown in Fig. 1C and D and a typical result from a 384-well gel is shown in Fig. 2. The present approach to machining of the gel formers is near its limit because the space between ''teeth'' is small, but other arrangements of teeth, or other approaches to gel former production, may enable yet higher densities. However, it is likely that this would force the transition from economy of startup and versatility of

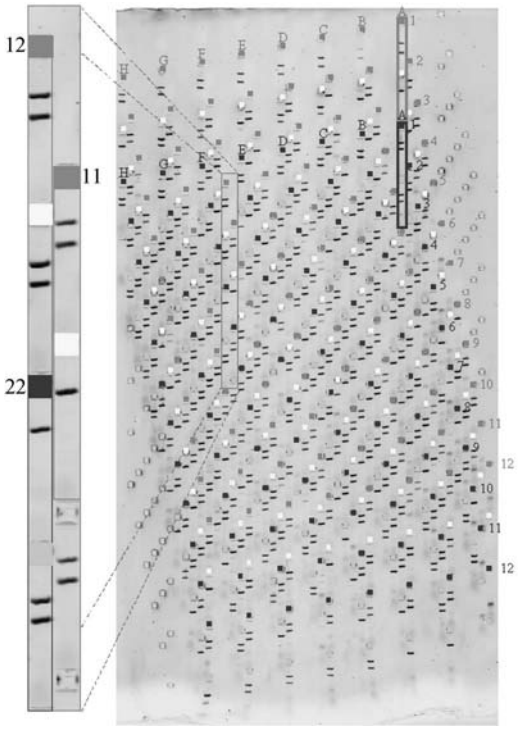

Fig. 2 384-well MADGE gel used for dual-reaction ARMS assay. On the main gel image the red rectangle indicates reactions a and b for sample A1, array 1, whereas the blue rectangle indicates reactions a and b for sample A1, array 2. The enlargement shows the three possible genotype calls as pairs of lanes with either two bands in each heterozygote or two bands in one and one in the other (for each category of homozygote). (*View this art in color at www.dekker.com.*)

the human operator to expensive hardware configured around a core mini–microscale format, and at this level, complete departure from industry-standard microplate format may be appropriate.

High-Resolution 96-Well MADGE (StretchMADGE) for Sizing Microsatellite Alleles

The following modifications enable resolution of bands with small (e.g., 1.5–5%) mobility differences (Fig. 3A):

- Increased track length on 2×6 diagonal instead of 2×4 diagonal.
- Introduction of internal molecular weight markers in every track.
- Sample dilution to avoid salt artefacts in the electrophoresis.
- Use of better resolving acrylamide derivatives such as duracryl.
- Thermostatic control of the electrophoresis.

This has enabled calling of minisatellite[10] and tetranucleotide and trinucleotide repeat multiallelic microsatellite polymorphisms.[11] We have been interested in using such sites *within* genes as linkage disequilibrium markers in association studies and wider utility can be anticipated. In our system, microsatellite amplicons are electrophoresed double stranded and there is the possibility that not only length polymorphic information but also internal sequence variation will be accessible by examining heteroduplex mobility.

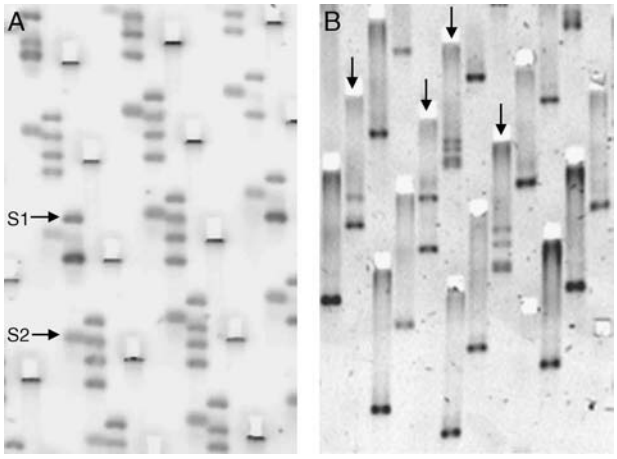

Fig. 3 StretchMADGE and meltMADGE. (A) StretchMADGE resolution of homoduplexes and heteroduplexes of a tetranucleotide microsatellite amplicon with five main alleles. Except in homozygotes, two resolving homoduplexes and two resolving or corunning heteroduplexes are observed. Homoduplexes are sized relative to flanking size markers (arrows) introduced into every track. Amplicon size is $\sim$260 bp, so one repeat size difference corresponds with approximately 1.5% band mobility difference. (B) MeltMADGE scanning of an amplicon for identification of unknown mutations. As explained in the text, heterozygotes for a mutation show a set of bands (tracks marked by arrows) instead of a single band, representing differential melting and hence differential mobilities during thermally ramped electrophoresis.

Temporal Thermal Ramp MADGE Electrophoresis (MeltMADGE) for Identification of Unknown Mutations

As MADGE involves a complex spatial configuration of sample wells and tracks, electrophoresis methods involving spatial gradients would be difficult to establish. Denaturing gradient gel electrophoresis,[12] which is used for identification of unknown mutations, involves a spatial gradient of denaturant. Polymerase chain reaction products heterozygous for a base change possess four moieties resolvable as separate bands. Consider a G to A transition. The homoduplex with a G–C base pair will migrate furthest in the gel because it will be the last moiety to denature with consequent reduction of migration velocity. The homoduplex with an A–T base pair will migrate almost as far. The heteroduplex with the relatively stable G–T mismatch will show a band of lower mobility. The heteroduplex containing the relatively unstable A–C mismatch will appear as the band nearest to the well. A thermal ramp in time can achieve a similar effect to a denaturing gradient in space and has the advantage of spatial homogeneity and hence applicability to the MADGE (or other array) formats. This forms the basis of our meltMADGE invention[13] and an early proof of principle example is shown in Fig. 3B.

We have previously described (for other use than meltMADGE) a purpose-built programmable real-time thermoregulated electrophoresis apparatus.[14] In brief, a $10 \times 10 \times 15$-cm electrophoresis tank contains buffer which is continuously mixed to ensure spatial thermal homogeneity, and the temperature is varied real-time by programmable software controlling heating and cooling systems and receiving feedback from temperature sensors in the tank. Overall, this is little more complex than a PCR machine, although for PCR low thermal mass for rapid temperature shifts is the objective, whereas for meltMADGE, the aim is high thermal mass for stability of small temperature shifts. The initial prototype accommodates 10–12 horizontal MADGE gels spaced in a carrier stack. Each 2-mm-thick gel is adherent to and supported by a 2-mm glass plate and is also covered by 2-mm glass, with spacing between each glass/gel/glass sandwich for buffer circulation. As buffer and hence thermal circulation is vigorous and the glass is both a good thermal conductor

and also quite thin, and the thermal ramps are relatively shallow, reasonable spatial thermal homogeneity is achieved, at least to within 0.1°C as determined by a reference platinum resistivity thermometer. Alternately, an electrophoresis box with a fast impellor, a rack for the gels, and a glass serpent taking the input from a programmable, recirculating water bath can be used. The latter are commercially available and the remainder is simple to build. At 10 MADGE gels per tank (96-well or 192-well) and 1–4 hr run time (faster if powerful cooling is available to counteract amperometric heating), it is feasible for one worker to optimize a number of amplicon assays in parallel, or to run several thousand scans (either of thousands of subjects for one amplicon or of several amplicons of matched melting characteristics for a few hundred subjects) per day. Assay optimization tends to be the limiting factor.

The reconfiguration of the denaturing gradient to have temperature as the dependent variable and time as the independent (controlled) variable opens a range of significant advantages:

1. It creates the option for high density arraying of wells and tracks (e.g., MADGE arrays), hence enabling high throughput of analyses.
2. Programmability of the gradient, which can be arbitrary and uses temperature rather than chemicals, is achievable easily in time. Arbitrary ramps (rather than simple linear gradients) would be very difficult in space. Gradients as an integral feature of the gel demand special gels. Programmability gives flexibility and convenience.
3. Potential for arbitrary ramps enables very short track lengths, and short run time (e.g., 1 hr) further enables high track density and hence throughput.
4. The use of ordered arrays (e.g., microplate format of MADGE) greatly simplifies data handling.
5. Arbitrary programming enables arbitrary resolution, thus reducing the analysis to simple pattern recognition rather than detailed relative mobility measurements. Pattern recognition evades the need for closely juxtaposed tracks or for internal markers. By contrast, the resolution of single-stranded conformation polymorphism (SSCP) is often small, cannot be programmed, and hence cannot be arbitrarily great. Thus advantages 1–4 cannot be realized with SSCP.

We have applied this system to a number of human genes both for studies of large case collections and for epidemiological research in population samples (BRCA1 in breast cancer risk; LDLR in familial hypercholesterolemia; MC4R-population research in obesity; APOE). Other assays relevant to the diagnostic laboratory are under development.

Identification of unknown mutations is an important aspect in genetic diagnostics. For mutations not known in advance, the choice at present is between direct sequencing, de novo scanning techniques to target sequencing more efficiently to appropriate regions, and ''chip'' technologies equivalent with direct resequencing by multiple parallel oligonucleotide hybridization.[15] For the identification of rare variants, important criteria are the degree of parallelism of samples possible, the number of bases analyzed per ''run,'' and the total number of bases an individual can scan per day. For heterozygote sequencing in humans, the difficulties of accurate base calling (essential for every base to avoid excessive numbers of false positive calls) may restrict sequencing as a first-line approach.[16] Even as sequencing improves, reagent requirements and need for high-resolution electrophoresis introduce significant cost issues. Chip technologies, while promising, have low capability for sample parallelism; a chip is expensive ($5000–10,000) for any selected sequence and also requires an optimization process; and the approach will demand high capital expenditure on chip-reading hardware. Scanning technologies are much used, but intense activity to develop improved approaches reflects the importance and shortfalls in this field. Such technologies generally rely on electrophoresis and give some information about the mutation from the resultant band pattern. MeltMADGE enters the field alongside SSCP,[17] denaturing gradient gel electrophoresis (DGGE),[12] and denaturing HPLC.[18] Sensitivity to all base changes has been a concern with SSCP. Denaturing gradient gel electrophoresis, although used in diagnostics laboratories on account of good sensitivity to most if not all base changes, is laborious and has low throughput. Denaturing HPLC has recently become prevalent but incurs high capital costs and high consumables costs, both tenfold greater than for the other techniques. In effect, meltMADGE should be considered as a convenient, high-throughput format of DGGE. Our comparisons with other techniques (to be published) show good sensitivity to most base changes, given appropriately planned and optimized assays.

CONCLUSION

There is intense activity to systematize the genomic, genetic, transcriptomic, and proteomic descriptions of organisms. A supplement to *Nature Genetics* ''The Chipping Forecast'' considered in detail the shift from hypothesis-led to descriptive hypothesis-forming research in the biosciences.[19] The systems that we have described here incur no substantial capital costs. Reagent costs are minimal and, in general, gel-based assays represent well-understood, widely available, adaptable methods. The

system is thus readily accessible to almost any standard laboratory. With a capacity up to 1 million tracks per day for minimal investment, the achievable throughputs for low-resolution electrophoresis by ''dry'' gel PAGE MADGE match the current shift of philosophy in biosciences research toward higher throughput, systematic description. Similarly, the throughputs with meltMADGE are two orders of magnitude greater than the currently favored approaches of SSCP and denaturing HPLC. Microplate array diagonal gel electrophoresis and melt-MADGE may meet the criteria of economy, sample parallelism, convenience of setup, and accessibility to large and small laboratories alike, and may thus find an important role in diagnostic, as well as research, applications. Future applications might also include enzyme, protein, RNA, protein–DNA, and other categories of biomolecular analysis.

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Microsatellite Instability

Wolfgang Dietmaier
University of Regensburg, Regensburg, Germany

Arndt Hartmann
University of Basel, Basel, Switzerland

INTRODUCTION

Microsatellites (MS) are repetitive stretches of genomic DNA consisting of mono-, di-, tri-, tetra-, and pentanucleotide repeats. Tens of thousands of MS are distributed throughout the genome. The length of MS repeats in individuals of a species tends to be highly polymorphic making them suitable as genetic markers. For this reason, MS analysis has become a valuable tool in forensic medicine, paternity testing, tissue typing, and molecular diagnostics. Because of their simple sequence structure, MS are usually located in regions of DNA that are not transcribed or translated. However, a growing number of coding mono-, tri-, and tetranucleotide repeats are reported to be involved in cancer or neurodegenerative disorders. Because of their repetitive nature, MS sequences are prone to alteration during DNA replication as a result of polymerase slippage and/or misalignment of template strands. In normal cells the mismatch repair (MMR) system detects and repairs small DNA alterations such as missense mutations and small insertions and deletions. A defective MMR system may result in shortened or extended microsatellite sequences (microsatellite instability, MSI), ultimately accumulating genomewide mutations affecting fundamental cellular processes such as signal transduction, cell growth, apoptosis, or DNA repair.

Microsatellite instability occurs in about 15% of all colorectal cancers (CRC) and is a hallmark of the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. Inactivating germline mutations in MMR genes will predispose patients of HNPCC families to colorectal and other cancers (e.g., tumors of the endometrium, ovary or upper urinary tract). Promoter hypermethylation of MMR genes may epigenetically cause MSI in sporadic CRC. Microsatellite instability tumors exhibiting such ''mutator phenotype'' differ from microsatellite-stable (MSS) tumors in their molecular, histopathological, and clinical characteristics. Microsatellite-stable tumors are characterized by aneuploidy and frequent large chromosomal deletions or amplifications, a feature referred to as chromosomal instability (CIN). In contrast, colorectal MSI tumors are commonly diploid with few chromosomal alterations. They typically arise in the right colon, are poorly differentiated, are of mucinous type, and show extensive intra- and peritumoral lymphocytic infiltration. Patients with MSI CRC tend to have a favorable clinical prognosis and are reported to respond better to chemotherapeutical treatment than patients with MSS CRCs.

DIFFERENT TYPES OF MSI

Microsatellite instability was first described nearly simultaneously by three groups in 1993.[1–4]

There are two types of MSI in colorectal tumors: highly frequent MSI (MSI-H) is defined by a MSI frequency of >40% using a specified microsatellite marker panel. In contrast, tumors with a frequency of MSI of <40% are referred to as MSI-low tumors (MSI-L).[5] Mononucleotide markers such as BAT25, BAT26, and BAT40, which are almost monomorphic in germline DNA, show the highest mutation rates in tumors of the HNPCC spectrum. Alterations in these markers are associated very specifically with deficient MMR genes *MSH2*, *MLH1*, *MSH6*, and *PMS2* in CRC. In contrast, the MSI-L phenotype is mostly restricted to dinucleotide markers and those with longer repeat units. A standardized, sensitive, and specific marker panel for detection of MSI in CRC was recently defined[5] and is currently recommended as the consensus marker panel for molecular HNPCC diagnostics in CRC.[6]

The mutation spectrum (e.g., *KRAS* mutation, *TP53* mutation, LOH at 5 q, 17 p, and 18 q) and the clinicopathological features of MSI-L and MSS tumors are quite similar but differ from that in MSI-H cancers which show relatively open mutations in genes with coding repetitive sequences (e.g., *TGFBR2*, *BAX*). It has recently been suggested that MSI-L arises as a consequence of epigenetically silencing the *O*-6-methylguanine DNA methyltransferase (*MGMT*) gene through promoter hypermethylation leading to promutagenic methylguanine adducts which are not sufficiently eliminated and thereby overcharging the MMR system.[7]

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MICROSATELLITE INSTABILITY IN HNPCC AND IN SPORADIC CRC

The MSI-H phenotype is a defining feature of HNPCC patients and is a result of predisposing constitutional mutations in the MMR genes *MSH2*, *MLH1*, *MSH6*, *hPMS2*, and *hMSH3*.[8] Most mutations were found in *MSH2* and MLH1 and less frequently in *MSH6, hPMS2*, and *hMSH3*. Germline mutations in MMR genes predispose not only to CRC but also to endometrial, upper urinary tract, and ovarian malignancies. Small intestinal and gastric cancers as well as tumors of the biliary tract are also seen in HNPCC, but they are relatively rare events. Highly frequent MSI is not restricted to HNPCC but occurs in approximately 12% of sporadic CRC with epigenetically and biallelically silenced MLH1 genes through promoter methylation in the vast majority of cases.

Hereditary and sporadic MSI-H CRCs share several clinicopathological characteristics. These include right-sided location, poor differentiation, a medullary or mucinous phenotype, and extensive lymphocytic tumor infiltration, but specific characteristics for HNPCC and sporadic tumors exist as well.[9–12] Hereditary nonpolyposis colorectal cancer is more common in males and occurs in younger patients compared to sporadic MSI-H colorectal carcinomas. Hereditary nonpolyposis colorectal cancers show more frequently Crohn's-like lesions and peritumoral lymphocytes than sporadic MSI-H CRCs. In contrast, sporadic CRCs are more likely to show two or more tumor subclones with diverse grades of differentiation than HNPCC tumors.[10] Adenomas from HNPCC patients are more often of the MSI-H phenotype than sporadic adenomas.

In addition, different molecular pathways seem to be involved in the carcinogenesis of sporadic and familial CRC. There are disruptions of the *wnt* pathway and frequent *KRAS* mutations in HNPCC cancers and/or cell lines, whereas sporadic MSI-H tumors show little evidence of *APC*-mutation, *CTNNB1* (beta-catenin) mutation or abnormal CTNNB1 immunolocalization, and *KRAS* mutation.[13–16] Little evidence, however, exists regarding these alterations in sporadic MSI-H CRC.[15,16]

CLINICAL OUTCOME OF PATIENTS WITH MSI-H COLORECTAL TUMORS

Considering the different molecular and histological features of tumors with microsatellite instability and chromosomal instability (see introduction) one could expect that cancers emerging from MSI-H and CIN pathways would also differ in clinically relevant characteristics. Several studies investigating MSI-status and prognosis show that survival of patients with the MSI-H phenotype is longer than that of patients with MSS cancers. However, some conflicting reports let the National Cancer Institute workshop conclude that MSI-H has not been demonstrated to be an independent predictor of prognosis.[6] These results may be due to both inconsistent definitions of MSI before 1998 and retrospective investigation of small patient cohorts. In a relatively large population-based study, the clinical outcome of MSI-H and MSS patients was assessed and associated with classic clinical prognostic factors. Highly frequent MSI was independently associated with prolonged survival in patients without adjuvant chemotherapy.[17] In another study, MSI-H correlated with lower tumor stage indicating that either MSI-H contributes to improved survival independently of other prognostic factors or MSI-H is independently predictive of lower pathological stage, thus contributing to the improved survival through tumor downstaging.[18] The reason why MSI-H improves clinical outcome is not clear yet. One can assume that the MSI-H typical mutation spectrum, i.e., small genomewide mutations, leads to mutation within coding repeats in genes of cell surface proteins thereby provoking an intense lymphocytic infiltrate and attack of the tumor.[19]

MICROSATELLITE INSTABILITY—PREDICTOR OF ADJUVANT TREATMENT RESPONSE

The different tumor characteristics could be used to develop improved and more individualized chemotherapy in the future. There are promising data that MSI-H and MSS CRC respond differently to specific classes of chemotherapeutic agents. There is evidence that the effective treatment with methylating agents such as *N*-methyl-*N*′-nitro-nitrosoguanidine, *N*-methyl-*N*-nitrosourea, and their chemotherapeutic analogs, streptozocin and temozolomide, requires functional MMR. Tumor cell lines with defective *MSH2*, *MSH6*, *MLH1*, or *PMS2* genes as well as *MSH2*-deficient mouse embryonic cells are highly resistant to these drugs compared to the corresponding MMR-proficient cell lines. It is suggested that the absence of MGMT alkylated guanine will lead to incorrect incorporation of thymine into the newly synthesized strand. This mismatch will be recognized by MMR proteins, which, after a further replication round, leads to apoptosis by a not-yet-understood mechanism. This process seems to be interrupted in MSI-H tumors with inactivated MMR resulting in ''damage tolerance'' which should be considered in therapy stratification.

Another widely used chemotherapeutic drug targeting DNA is cisplatin leading to intrastrand DNA cross-links between adjacent purines and activation of DNA repair.

As the nucleotide excision repair (NER) system is an important part of this cellular response, it is suggested that a highly effective NER system leads to resistance or reduced sensitivity of tumors to cisplatin treatment by efficient removal of DNA adducts. Mismatch repair also seems to affect the sensitivity of tumors to cisplatin treatment, but in a different way. Cell culture experiments revealed that tumor cell lines with defective MSH2 or MLH1 proteins show a twofold resistance to cisplatin compared to MMR proficient cell lines.[20] There are several clues that MMR may contribute to the toxicity of cisplatin in the same way it mediates the action of methylating agents. It has been shown that MMR proteins (MSH2/MSH6, MLH1) can be recruited by cisplatin adducts,[20–22] and MSH2 specifically recognizes 1,2-intrastrand cross-links between two adjacent guanine residues which are induced by cisplatin treatment. It has been shown in vitro that MutS-alfa binds to duplex oligonucleotides containing a 1,2-diguanyl intrastrand cross-link with a thymine opposite to 3′-guanine in the cross-link. This situation corresponds to O^6-methylG-T mismatch activating MMR processes and activation of apoptosis if MMR proteins are intact. Thus MSI-H tumors will respond neither to alkylating nor to platinating agents and should be treated by different drugs.

The value of MSI-H as a molecular predictor of response to 5-fluorouracil (5-FU)-based adjuvant chemotherapy has recently been addressed in several studies. The first indication of the importance of MSI-H in the response to 5-FU-based agents came from in vitro studies showing that colon cancer cell lines with defective MMR were more resistant to 5-FU.[23]

Interestingly, clinical studies showed controversial results regarding the response of MSI-H tumors to adjuvant 5-FU treatment. A clear benefit of 5-FU treatment in stage III CRC (Dukes C) with MSI-H phenotype was shown in a retrospective, nonrandomized, hospital-based cohort of 656 CRC patients.[24] A striking survival benefit from adjuvant chemotherapy for patients with right-sided tumors and patients with MSI-H tumors was found. Highly frequent MSI was an independent predictor of improved survival in multivariate analyses. This finding was confirmed by another study demonstrating a favorable outcome in patients treated with adjuvant fluorouracil-based chemotherapy for stage III CRC with MSI-H and retention of heterozygosity of chromosome 18 q.[25,26] An even more significant benefit of adjuvant chemotherapy was demonstrated in MSI-H cancers carrying mutations in the transforming growth factor β1 receptor type II gene (*TGFBR2*). The TGFB1 pathway plays an important role in tumor suppression, because it leads to inhibition of tumor proliferation by blocking the cell cycle in late G1. The TGFB1 pathway is also known to be inactivated in most MSI-H tumors as a consequence of mutations within a coding repeat, and unregulated cell proliferation may thus lead to increased susceptibility to chemotherapy. However, an adverse effect of MSI on the response to adjuvant 5-FU treatment was recently reported. This study showed that randomized patients with stage II or stage III MSI-H colon cancer do not benefit from 5-FU-based adjuvant treatment and may even be harmed.[17] Further prospectively controlled and population-based studies are needed to clarify whether MSI will affect the response to adjuvant chemotherapy.

CONCLUSION

Highly frequent MSI (MSI-H) as a result of defective MMR affects about 15% of all CRC. Highly frequent MSI tumors differ from microsatellite-stable tumors in a number of molecular and clinicopathological features. Approximately 3% of CRC show MSI-H due to predisposing constitutional mutations in MMR genes. This group of patients is referred to as hereditary nonpolyposis colorectal cancer, HNPCC. Familial and sporadic MSI-H tumors are largely similar in their molecular characteristics, but differ in some clinicopathological features. Patients with MSI-H cancers respond differently to various chemotherapeutic treatment regimens compared to MSS CRC patients. Further prospective and population-based studies are needed for a better and individualized therapy stratification of CRC.

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Molecular Beacons and Other Hairpin Probes

Natalia E. Broude
Boston University, Boston, Massachusetts, U.S.A.

INTRODUCTION

Stem-loop (hairpin) DNA oligomer probes provide increased specificity of target recognition as compared with linear DNA probes. Invention of molecular beacons, a variant of stem-loop oligonucleotides, which are able to fluoresce upon hybridization but are ''dark'' in the absence of the target, made possible real-time monitoring of hybridization process. A variety of robust assays using diverse, structurally constrained oligonucleotides have been developed that exploit endless number of formats and detection methods.

Hairpin DNA oligonucleotides are also used as different components in many DNA amplification methods providing higher specificity and lower background. Thus, these structured probes are an indispensable tool in modern biotechnology and diagnostics.

ENHANCED SPECIFICITY OF HAIRPIN DNA PROBES

Specificity of probe/target hybridization is a crucial factor determining efficiency of most nucleic acid-based methods used in diagnostics and biotechnology. Hybridization specificity is determined as a match-versus-mismatch discrimination: binding to sites that differ from the perfectly complementary sequences even by a single base pair substitution is characterized by a substantial free-energy penalty.[1] If the free-energy penalty is high enough, a set of conditions (so-called stringency conditions) can be found where perfect complexes will be considerably more stable than the complexes containing mismatches.

Stem-loop DNA probes are single-stranded oligonucleotides containing a sequence complementary to the target that is flanked by self-complementary target-unrelated termini. Thermodynamic analysis of hybridization characteristics of linear and stem-loop DNA probes proved that it is a general feature of structurally constrained probes to distinguish mismatches over a larger range of temperatures or other experimental parameters comparing to unstructured probes.[2,3] Thus, stem-loop DNA probes allow for a wider window of stringency conditions, which provide better match/mismatch discrimination.

MOLECULAR BEACONS

Molecular beacon (MB) is a stem-loop DNA oligonucleotide, which carries a fluorophore and a quencher at the 3′- and 5′-ends[4,5] (Fig. 1A). In the absence of the target, these molecules form closed stem-loop structures in which fluorophore and quencher are in close proximity—this results in fluorescence quenching. In the presence of the DNA or RNA target, molecular beacon forms a complex with it, which brings apart the fluorophore and the quencher. Once the fluorophore and quencher are spatially separated, the fluorescence develops under illumination and quantitatively reports on the presence of the target (Fig. 1A).

Molecular beacons, being inactive in absence of the target, do not require purification of the hybridization product from the excess MB and thus provide simple, homogeneous (close-tube) format of the assay and, consequently, a possibility of real-time hybridization monitoring. This feature of MBs made them a tool of choice in many applications. They were used for detection of single nucleotide polymorphisms, in quantitative PCR, in isothermal amplification, as DNA microarray-immobilized probes and biosensors, and as antisense probes for detecting RNAs in vivo (for reviews, see Refs. [6,7]). Recent developments were directed toward simplification of the MB approach and creation of the ''label-free'' optical biomolecular sensors. For example, immobilization of fluorophore-labeled MBs on a gold surface allowed using the surface as a quencher, thus eliminating the need in quenching moiety in the beacon structure.[8] Fully unlabeled stem-loop DNA probes were used as electrochemical DNA sensors capable of detecting femtomoles of DNA by electrochemical signal rather than by optical signal.[9]

Fluorescence monitoring allows quantitative kinetic analyses of the conformation changes of molecular beacons. Therefore they have also become a useful tool in studies on conformational changes of DNA under various conditions or caused by various types of reagents

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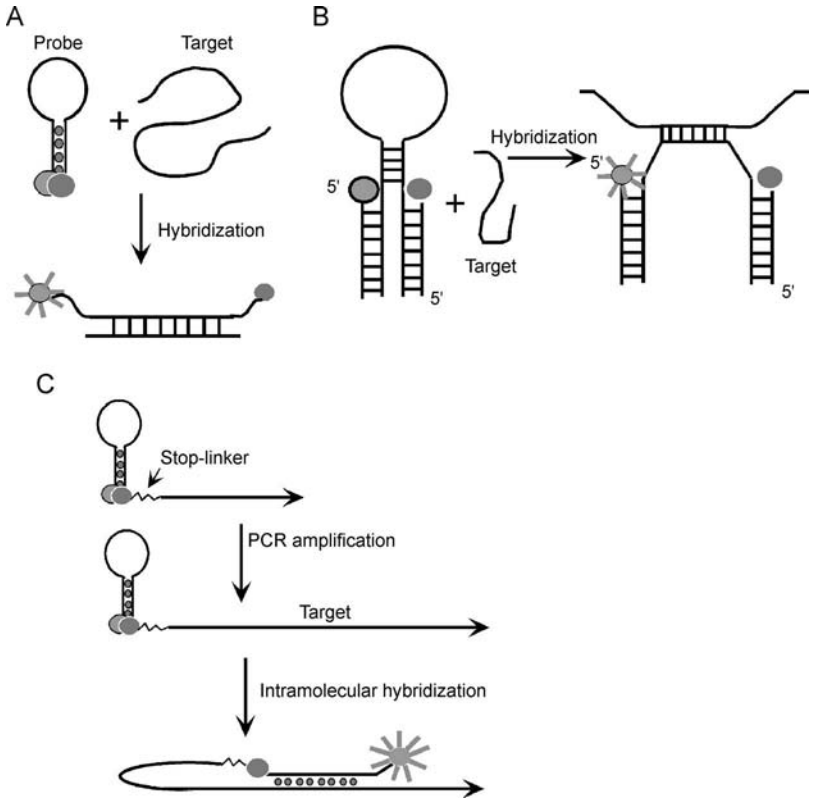

Fig. 1 Outline of different assays using molecular beacons. (A) Molecular beacon is in a closed (''dark'') form in the absence of the target, and a fluorophore (red) and a quencher (gray) are in a close proximity, which quenches fluorescence. Upon hybridization with the target, the beacon changes its conformation, which results in spatial separation of the fluorophore and quencher. As a result, bright fluorescence reveals the presence of a target. (B) Tripartite molecular beacons with universal arms and varying loop provide flexibility in design and reduced cost. (C) Schematics of the beacon-like Scorpion probe approach. Incorporation of a stem-loop-shaped probe into the PCR primer allows unimolecular target detection, which ensures faster kinetics and higher stability of the complex as compared with bimolecular reaction. (*View this art in color at www.dekker.com.*)

interacting with nucleic acids. These studies include real-time monitoring of DNA cleavage caused by enzymes or chemicals, protein–DNA interaction studies, and studies of various dye interactions with duplex DNA. Conformational studies of single-stranded DNAs as well as kinetic and thermodynamic characteristics of triplex formation have also been performed using molecular beacons.[6]

Structural Variants of Molecular Beacons

There are several derivatives of ''classical'' MBs, which differ from the conventional stem-loop DNA oligonucleotides. First, it was shown that the linear DNA oligonucleotides without self-complementary termini (stemless) but bearing fluorophore and quencher at the 5′- and 3′- ends could work as MBs.[10] Later, the same was reported for the peptide nucleic acid (PNA) oligomers.[11] PNAs are DNA mimics with pseudopeptide (polyamide) backbone instead of sugar–phosphate one in DNA. Apparently, the flexibility of the sugar–phosphate and polyamide backbones of DNA and PNA, respectively, in combination with a strong hydrophobic interaction between the fluorophore and the quencher, keeps these structures preferably in a closed form in the absence of a target.[11,12]

Specificity, Sensitivity, and Reproducibility

Molecular beacons exhibit exceptional sequence specificity because of the favorable structure energetics. They are used in combination with different amplification technologies, such as polymerase chain reaction (PCR) and rolling circle amplification (RCA), which allows real-time amplification monitoring. Fluorescence detection is very sensitive: an increase of a signal over the background in the presence of the target up to two orders of magnitude can be achieved. Thus sensitivity of PCR and RCA in the presence of molecular beacons can be as high as single molecule in case of PCR and 10–100 molecules in case of RCA. Another important feature of MBs is the

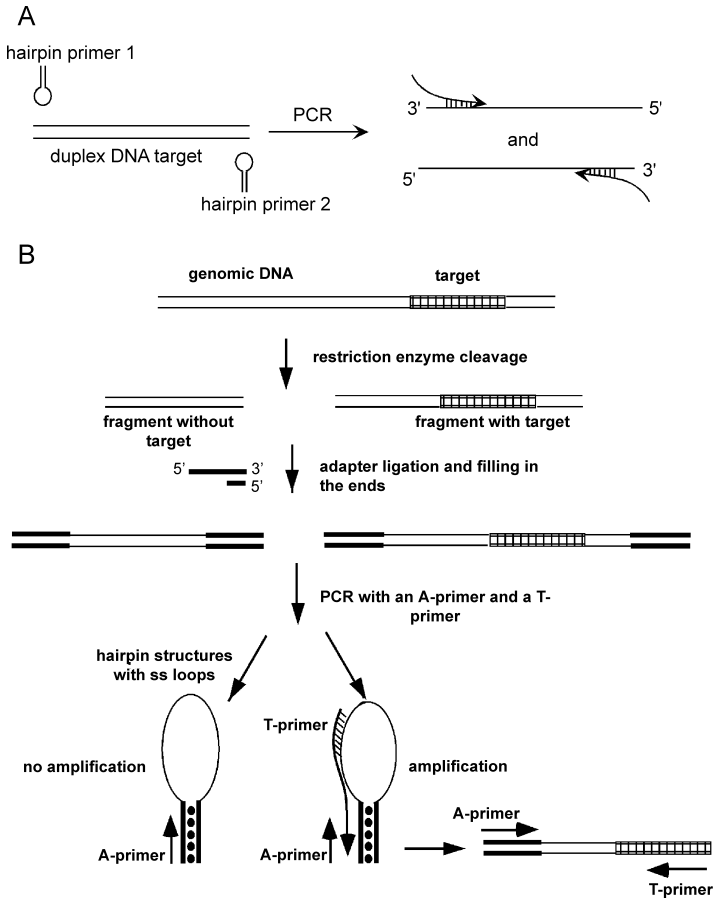

Fig. 2 Examples of application of stem-loop DNA structures in amplification methods. (A) Hairpin-shaped primers allow hot-start PCR because they anneal to the target only at high temperature upon denaturing of the stem. (B) The PCR suppression approach. Genomic PCR is digested with a restriction enzyme and ligated with specially designed adapters. During the PCR, only the DNA fragments containing the target are efficiently amplified, while the vast number of the target-unrelated fragments remains intact.

possibility of multiplex analysis by using MBs with different fluorophores.

Advantages and Limitations

The major advantage of MBs is the possibility of real-time hybridization monitoring. This provides simple close-tube format of the assay, which reduces chances of contamination and false positives. Among other advantages of hairpin probe application in biotechnology and diagnostics are an endless number of formats and detection methods.

Current limitations of MB technology include the high cost of MB synthesis and their instability in the presence of DNA polymerases with exonuclease activities. The efforts, therefore, in this respect are directed toward simplification and/or generalization of the beacon design. An example of simplification of MB design is the use of MBs immobilized on a gold surface, which serves as a quencher.[8] Tripartite MBs with universal parts carrying fluorophore and quencher and sequence-specific oligonucleotides allow flexibility in MB design[13] (Fig. 1B). This approach also brings down the MB cost. Application of chemically modified MBs, which are not cleavable by exonucleases (e.g., PNA beacons or 2-*O*-methyl-deoxy containing MBs), is a way to overcome instability of MBs in the presence of exonucleases.[11,14]

Combination of Molecular Beacon Approach with Other Technologies

Molecular beacons have been incorporated in many detection techniques using DNA amplification with the aim of real-time process monitoring. However, the area of MB applications is much wider and is not limited by DNA amplification methods. Catalytic MBs represent a next generation of molecular probes with the potential to amplify signals and thus to detect nucleic acid targets without PCR amplification.[15] These MBs are DNA constructs that combine the features of molecular beacons and hammerhead-type deoxyribozymes with RNase

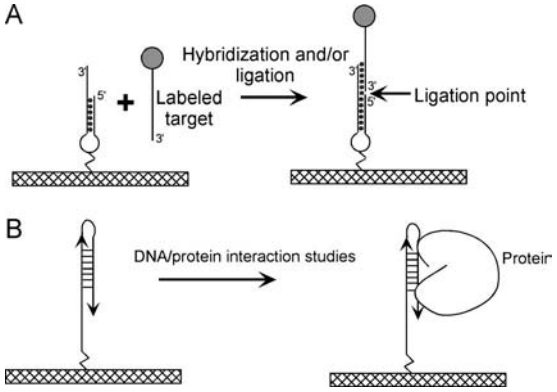

Fig. 3 Outline of two major approaches for application of immobilized stem-loop DNA oligonucleotides. (A) Sequence-specific capturing and mutation detection using immobilized stem-loop probes. (B) DNA microarrays with hairpin probes for DNA/protein interaction studies. (*View this art in color at www.dekker.com.*)

activity, which are located on two different modules.[15] In the absence of a target, the beacon hybridizes with the deoxyribozyme module. When the target is present, the beacon changes its conformation and allows the substrate (a stemless fluorogenic oligonucleotide) to hybridize with the deoxyribozyme module. The deoxyribozyme cleaves the substrate, which results in increasing fluorescence, substrate dissociation, and beacon hybridization with the deoxyribozyme. Thus, the cycle is repeated. This approach is just in the development stage, but in the proof-of-principle experiments, it was shown that it initiated catalytic events.[15]

The combination of PNA-based technology with molecular beacons opened the possibility of targeting duplex DNA without prior denaturation. PNA openers are short PNA oligomers, which invade duplex DNA and locally expose a single-stranded region within duplex DNA forming a P-loop complex. Locally denatured DNA region serves as a unique target for sequence-specific binding of DNA or PNA probes forming PD- or PP-loops, respectively.[16] If the probes are DNA or PNA beacons, they are capable of real-time reporting on cognate targets located within the preopened double-stranded DNA.[11,12]

USE OF STEM-LOOP OLIGONUCLEOTIDES IN DNA AMPLIFICATION AND MICROARRAY TECHNOLOGY

Stem-Loop Structures in DNA Amplification

Stem-loop DNA constructs are also used for different purposes in both thermocycling and isothermal DNA amplification (Fig. 2). For example, stem-loop primers allow hot-start PCR and thus increased specificity of amplification.[17,18] Scorpion probes are designed to serve simultaneously as a PCR primer and as a molecular beacon.[19] During PCR, when the primer is extended and the target is synthesized, the stem loop unfolds and the loop sequence hybridizes intramolecularly with amplified target, thus developing fluorescence (Fig. 1C). The scorpion primer approach uses a unimolecular mechanism of probe–target hybridization, which ensures faster kinetics and higher stability of the probe–target complex.[19]

Design of the large stem-loop constructs from DNA restriction fragments by ligation of special GC-rich adapters forms the basis of the PCR suppression (PS) technology (Fig. 2B). To perform suppression PCR, genomic DNA is digested with a restriction enzyme and ligated with an adapter with a high GC content.[20,21] As a result, long self-complementary ends flank each single-stranded DNA fragment. These self-complementary termini form duplexes during each PCR annealing step, so that the fragments adopt large stem-loop structures, which makes PCR with the adapter–primer (A-primer) alone relatively inefficient. This effect is called PCR suppression (PS). However, the PCR is efficient with two primers, A-primer and target-primer (T-primer). In this case, only DNA fragments with the target are efficiently amplified on the background of all other fragments without a target. Several variants of PS approach were used in subtractive hybridization, cDNA and genomic differential displays, and in multiplex PCR (see Ref. [6] for a review). Hairpin structures were also used for elimination of PCR errors from amplified DNA.[22] Specially designed primers forming stem loops allow also isothermal amplification of DNA; this method is designated as loop-mediated amplification (LAMP).[23,24]

Stem-Loop Structures in DNA Microarray Technology

Hairpin DNA probes can be immobilized on the surface of microarrays and used in hybridization experiments for mutation detection (Fig. 3A)[25] and DNA/protein interaction studies (Fig. 3B).[26] Kinetic analysis confirmed better performance of stem-loop structures as compared with linear oligonucleotides.[27]

CONCLUSION

Stem-loop oligonucleotide constructs are widely used in molecular biology, genomics, and diagnostic biotechnology. Two major factors are responsible for such broad applications of these DNA constructs: 1) enhanced specificity of the probe–target interaction and 2) the possibility of close-tube real-time monitoring formats.

There is no doubt that many new applications of hairpin probes will be developed in the future. Especially useful will be the methods that may allow direct detection of target molecules without prior amplification.

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Mucopolysaccharidosis Types IIIA and IIIB

M

Clare E. Beesley
University College London, London, U.K.

INTRODUCTION

Mucopolysaccharidosis (MPS) type III or Sanfilippo syndrome encompasses a group of four lysosomal storage disorders that are characterized by the inability to breakdown the glycosaminoglycan (GAG), heparan sulfate. Glycosaminoglycans are complex polysaccharides that are an important component of connective tissues and the extracellular matrix and heparan sulfate is an integral part of cell surfaces. Deficiency of one of the degradative enzymes results in the accumulation of partially degraded GAGs inside lysosomes, which leads to cell death and ultimately organ dysfunction. Each of the four Sanfilippo subtypes, A, B, C, and D, is caused by the deficiency of a different enzyme in the same degradative pathway for heparan sulfate: heparan-*N*-sulfatase (EC 3.10.1.1), alpha-*N*-acetylglucosaminidase (EC 3.2.1.50), acetyl-CoA *N*-acetyl transferase (EC 2.3.1.3), and *N*-acetylglucosamine-6-sulfatase (EC 3.1.6.14), respectively. The incidence of MPS III has been estimated to be approximately 1 in 58,000 live births. All four Sanfilippo subtypes are autosomal recessive disorders but the genes have only been identified in types A, B, and D. The acetyl-CoA *N*-acetyl transferase enzyme that is deficient in type C patients is membrane-bound and this property has hindered its isolation and subsequent cloning. For subtype D, a total of 14 patients have been described worldwide and genetic defects have only recently been reported. The majority of Sanfilippo patients have subtypes A or B and it is these subtypes that will be focused on in this article.

CLINICAL DESCRIPTION AND DIAGNOSIS

Clinical Description

Clinical symptoms are similar in both Sanfilippo subtypes. They usually occur after 2 years of apparently normal development and include hyperactivity, aggressive behavior, delayed development (particularly in speech), sleep disturbances, coarse hair, hirsutism, and diarrhea. There are only relatively mild non-CNS manifestations and this may be the reason why Sanfilippo patients present later than other MPS patients. Following the hyperactive and aggressive phase there is then a period of progressive mental retardation with death usually between the second and third decade of life. In both Sanfilippo A and B a small number of more slowly progressive forms with later onset have been described.[1,2] An adult Sanfilippo A patient was recently described who presented with cardiomyopathy at 45 years and, interestingly, had no neurological symptoms.[3]

Biochemical Diagnosis

A deficiency of heparan-*N*-sulfatase (HNS) or alpha-*N*-acetylglucosaminidase (NAGLU) in Sanfilippo A and B patients, respectively, leads to the accumulation of partially degraded heparan sulfate inside lysosomes. This accumulation is progressive and leads to hypertrophy of the lysosomal system and subsequent urinary excretion of GAGs. Quantitative analysis of urinary GAGs by precipitation with dimethylmethylene blue[4] is the initial step in the biochemical diagnosis, followed by two-dimensional electrophoresis to identify the individual GAGs.[5] Patients with Sanfilippo syndrome show a raised urinary GAG level and abnormal excretion of heparan sulfate and heparin-like component. It is impossible to differentiate between the four Sanfilippo subtypes by the urinary GAG pattern so specific enzyme assays are carried out on leukocytes or fibroblast cells,[5,6] to determine the subtype and make a definitive diagnosis.

MOLECULAR GENETICS

Sanfilippo A

The gene encoding HNS is situated on chromosome 17q25.3, contains eight exons and extends over 11 kb of DNA. It produces a mRNA transcript of $\sim$3.1 kb, which encodes a protein of 502 amino acids with a predicted molecular mass of 54.67 kDa.[7,8] The reaction catalyzed by HNS is the removal of the *N*-linked sulfate group from the nonreducing terminal glucosamine residues of heparan sulfate. Recombinant HNS produced in Chinese hamster ovary (CHO) cells exists in two forms, a major species of 62–64 kDa (precursor) and a minor species of 56–58 kDa

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(mature). Both forms have the same size and N-terminus as the comparable forms isolated from liver.[9] The native molecular mass of HNS as determined by fast-performance liquid chromatography (FPLC) is 115 kDa so it exists as a dimer inside lysosomes.[10] Heparan-*N*-sulfatase has five potential *N*-glycosylation sites and all are used, with three high mannose or complex oligosaccharide chains and two with tri- and/or tetracentenary complex-like and biantennary chains.[11] At least one mannose group will be phosphorylated because mannose-6-phosphate groups on all soluble lysosomal enzymes are used to transport the enzymes from the *trans* Golgi to the lysosome via mannose-6-phosphate receptors. Heparan-*N*-sulfatase is a member of a highly conserved group of sulfatases that undergo a common and unique posttranslational modification necessary for catalytic function. This process involves the conversion of an active site cysteine residue to formylglycine and is catalyzed by the C(alpha)-formylglycine generating enzyme (FGE). The gene for FGE (*SUMF1*) was recently cloned and shown to be defective in multiple sulfatase deficiency patients, in whom the activity of all sulfatases is reduced.[12,13]

Sanfilippo B

The gene encoding NAGLU is situated on chromosome 17q21.1, extends approximately 8.2 kb, and contains six exons. The cDNA is 2.7 kb long and encodes a protein of 743 amino acids that has a predicted molecular mass of 82 kDa.[14,15] Alpha-*N*-acetylglucosaminidase acts as an exoglycosidase on the nonreducing end of alpha-*N*-acetylglucosaminide residues of heparan sulfate. Two forms of NAGLU have been purified from human placenta with apparent molecular weights of 77 and 80 kDa, representing the mature and precursor forms, respectively.[14] There are seven potential glycosylation sites and it has been predicted that the site at residue 272 is occupied and carries a phosphorylated carbohydrate side chain necessary for lysosomal targeting.

Mutation Analysis

Following the characterization of the *HNS* and *NAGLU* genes, many mutations have been identified in Sanfilippo A and B patients. The initial step is to perform polymerase chain reaction (PCR) amplification on the coding exons and intron/exon boundaries and numerous sets of primers have been published. Initially single-stranded conformation polymorphism (SSCP) analysis was used to screen the PCR products for possible mutations. Nowadays, all of the PCR fragments are purified and sequenced directly, either by radioactive methods or by fluorescent sequencing technology. Many different mutations have now been identified and both disorders show extensive genetic heterogeneity.

Sanfilippo A

To date, 70 mutations have been identified in Sanfilippo A patients. These consist of 50 missense, 4 nonsense, 8 deletions, 7 insertions and 1 splice site mutation. Some mutations have been found in more than one patient whereas others are unique to individual families. Some mutations have been found at high frequencies in particular populations. The R245H, R74C, 1079delC, and S66W were the most frequent mutations in the Dutch (56.7%),[16] Polish (56%),[17] Spanish (36.5%),[18] and Italian (33%)[19] populations, respectively. Several non-disease-causing polymorphisms have been identified including R456H, which has a high frequency of 55% in the normal Australian population.[20] A study of four polymorphic markers in the Spanish Sanfilippo A patients with the 1079delC mutation showed that they had a conserved haplotype, suggesting a common origin for this mutation.[18]

Sanfilippo B

To date, 99 mutations have been identified in Sanfilippo B patients. These consist of 60 missense, 8 nonsense, 16 deletions, 12 insertions and 2 splice site mutations. Again some mutations have been found in more than one patient whereas others are unique to individual families. In contrast to Sanfilippo A, common mutations have not been identified in patients of different national origins. However, in a recent study of Greek Sanfilippo B patients, three mutations were found at a high frequency, with mutations Y140C, H414R, and R626X accounting for almost 70% of the mutant alleles.[21]

Genotype/Phenotype Correlation

The relationship between the severity of a monogenic disorder and the mutations is called the genotype/phenotype correlation. If the mutation causes the production of a truncated protein either by a nonsense mutation or by a deletion or insertion (frameshift), this is predicted to be severe and the protein nonfunctional. Patients homozygous for these types of mutations are predicted to be severely affected and this is true in both Sanfilippo A and B. The effect of missense mutations on the protein is more difficult to predict because some will allow the production of functional enzyme albeit at lower levels and

others will not. It depends on the function of a particular amino acid within the protein as some will have a more critical role than others. This can only be investigated by expression studies where the mutation is generated in a normal copy of the gene, transfected into mammalian cells, such as CHO, and the resulting enzyme assayed for activity. All missense mutations should be investigated in this way, firstly to confirm their pathogenicity and secondly to determine which ones produce residual activity. Without performing such a procedure it is possible that a rare polymorphism could be reported as a mutation. By carrying out further studies such as immunoprecipitation of mutant enzyme and cellular localization by confocal microscopy, more information can be obtained about the effect of the mutation on the processing and transport of the enzyme. This will increase our understanding of the effect of the mutation on enzyme function and further our knowledge of the disease process. The majority of missense mutations reported in Sanfilippo A and B have been found in severely affected patients either in homozygous form or in combination with a truncating mutation so it can be deduced that they have a serious effect on enzyme function. Only a small number of attenuated, late onset patients have been described in Sanfilippo A and B, and, as predicted, they all have at least one missense mutation, which will be responsible for the residual enzyme activity. The effect of polymorphisms on enzyme activity still requires investigation in Sanfilippo A and B.

TREATMENT

As previously mentioned, soluble lysosomal enzymes are synthesized in the *trans* Golgi network and transported via mannose-6-phosphate receptors to the lysosome. Research into the process has enabled this sorting mechanism to be utilized to target recombinantly expressed replacement enzyme to deficient lysosomes. This treatment is now available for some lysosomal storage disorders, such as Fabry and MPS I, but recombinant enzyme is unable to cross the blood–brain barrier. As neurological disease is the major symptom in Sanfilippo patients, this form of treatment is unsuitable. Bone marrow transplantation has been performed in a few Sanfilippo A and B patients but has proven unsuccessful, even when carried out before development of clinical symptoms.[22] Gene therapy is currently being investigated using a variety of viral vectors and a number of animal models exist for Sanfilippo A and B including mice (IIIA, IIIB), dogs (IIIA, IIIB), and emu (IIIB). Such models can be used to investigate different viral vectors and different methods of delivery such as direct injection into the brain. However, many problems need to be overcome before gene therapy becomes a viable form of treatment for single gene disorders. One of the major problems of introducing recombinant enzyme, however it is produced, into patients that have no functional enzyme is the immunological response to the ''foreign'' protein. This may be worse in those patients who have no cross-reactive material. Most patients with missense mutations will probably produce full-length protein that is nonfunctional, so will not develop antibodies to the recombinant protein. Therefore it is a prerequisite that the genotype of all patients undergoing treatment is known. At present, the only form of treatment for Sanfilippo patients is palliative care.

CONCLUSION

Over the last 8–9 years, there have been major advances in understanding the genetics of Sanfilippo A and B. Many mutations have been described, some of which are common, but the majority of which are unique to individual families, making both Sanfilippo A and B genetically heterogeneous. The disease-causing mutations can be identified in the majority of patients diagnosed with Sanfilippo A and B, and accurate carrier detection is available for other family members. Mutation screening in several countries has highlighted the prevalence of particular mutations in different populations and this is important in initial screening programs. By characterizing the genotype in many patients we are able to understand more about the genotype/phenotype correlation in Sanfilippo disease. By analyzing the mutations we can learn more about the function of the enzyme and understand more about the pathogenesis of Sanfilippo disease. Knowing the genotype will be a prerequisite for any form of future therapy and we may be able to predict from the genotype whether there is likely to be any adverse reaction to recombinant enzyme. However, no treatment will be available for Sanfilippo A and B until there is a means by which replacement enzyme can cross the blood–brain barrier in order to treat the major neurological symptoms.

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Multiplex Real-Time PCR

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Maurice M. Exner
Quest Diagnostics Nichols Institute, San Juan Capistrano, California, U.S.A.

INTRODUCTION

The polymerase chain reaction (PCR) has been adapted for a myriad of applications involving the amplification of nucleic acid sequences. This technology often involves a specific detection methodology to analyze the products of a reaction. The advent of real-time detection systems, which detect amplified products concurrently with the amplification process of the reaction, has enabled the creation of a system that can provide accurate, quantitative results without cumbersome post-amplification steps. Multiplex real-time PCR technology is simply a variation of the standard reaction methodology in which there are multiple amplification reactions that take place in a single reaction vessel. The ability to combine reactions together provides a number of benefits. Not only does it allow for multiple target detections, which saves time, effort, and money, but it also provides a means for enhanced experimentation. For instance, with the ability to discriminate different targets in a reaction, comparative or relative quantitation of targets can more accurately be achieved, and positive controls can be coamplified with targets of interest to monitor the efficiency of each individual reaction. There are a number of different chemistries available that allow for multiplex real-time amplification and discrimination of targets. Each chemistry and each multiplexing strategy has its advantages as well as its own technical challenges. However, if these challenges can be overcome, this ''simple'' variation of a standard PCR reaction turns into an extremely powerful tool for clinical diagnostics.

MULTIPLEX REAL-TIME POLYMERASE CHAIN REACTION METHODOLOGY

Multiplex PCR is the amplification of multiple targets, or the capability of a system to amplify multiple targets, within the same reaction vessel. ''Real time'' refers to the fact that the detection of the amplified product is taking place in real time or during the actual process of the amplification reaction. Therefore multiplex real-time PCR involves the simultaneous amplification, detection, and discrimination of multiple amplified targets in a single reaction. Real-time PCR and multiplexing methodologies, when combined, provide a flexible research or clinical tool that can be specifically tailored to interrogate nucleic acid sequences and to provide a researcher with experimental answers to a variety of questions. Flexibility is a product of the variety of chemistries available to perform real-time PCR, in conjunction with the numerous strategies available to combine reactions in a multiplexed fashion.

The detection of real-time PCR products is based on increases in fluorescence that are apparent only when amplified products are present. Real-time PCR instrumentation uses a light source to produce an excitation wavelength that activates fluorescent molecules within the reaction. Following the interaction between the fluorescent molecule or probe and the target amplicon, fluorescent emissions can be observed and monitored. The specific method by which products in a reaction are discriminated depends on the particular detection chemistry being utilized. Some of the most commonly utilized chemistries or fluorescence detection schemes for real-time PCR detection include: 1) 5′ nuclease or ''TaqMan'' probes;[1] 2) hybridization probes or fluorescence resonance energy transfer (FRET) probes;[2] 3) molecular beacons;[3] 4) minor groove binding probes;[4] and 5) double-stranded DNA binding dyes (SYBR green).[5]

Each different detection chemistry has specific characteristics that lend to different multiplexing strategies. Multiplexing is generally achieved by using multiple primer and/or probe sets in a reaction, although there may also be cases where a single primer set is used with multiple probes, or a single probe could be used with a number of different primer sets. The amplified products generated can be discriminated by means of melting curve analysis,[6] by an analysis and discrimination of fluorescent emissions,[7] or by a combination of the two. When the different strategies are combined with the variety of available chemistries, a vast number of permutations and possibilities are created. The strategy utilized will depend on the information desired by the researcher.

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DISCRIMINATION OF POLYMERASE CHAIN REACTION PRODUCTS

Fluorescent Emission Discrimination

The discrimination of multiple targets that are amplified within a reaction can be performed using a differential color analysis; that is, multiplexing is accomplished by using a different ''color'' fluorophore for each particular probe.[7] By scanning the fluorescence emission wavelengths observed in a reaction vessel, and by separating the observed emissions according to the discrete wavelengths produced by each particular fluorophore, PCR products can be selectively identified. The number of different targets that can be discriminated in a single reaction is dependent on the excitation and emission wavelengths that the instrumentation is able to produce and monitor, along with the availability of fluorophores with distinct emission spectra.

Melting Temperature Analysis

A melting temperature (Tm) analysis measures the dissociation rates of hybridized nucleic acids, and there are two main methods by which this technique can be used to differentiate PCR products. Either the dissociation of a specific probe molecule from a target strand can be analyzed[8] or the temperature at which an entire amplicon molecule denatures can be determined.[6] When using specific probes (hybridization probes or minor groove binding probes), Tm discrimination is achieved based on the hybridization affinities of the probes for the target amplicon. Following thermocycling, the probes are allowed to anneal to the target amplicons. Upon hybridization of the probes to their specific targets, fluorescence is produced. The reaction temperature is then gradually increased, and fluorescence emissions are continually monitored. When a probe reaches its Tm, it dissociates from the amplicon and fluorescence ceases. Probes used for multiplexing can be designed with different lengths and different GC contents such that each has a distinguishable melting temperature. Besides being used to differentiate completely different targets, Tm analysis using specific probes can also be used to interrogate single nucleotide polymorphisms (SNPs). Targets with a single base pair mismatch with a probe sequence can readily be distinguished from targets with complete homology to the probe. The combination of colorimetric differentiation with Tm analysis creates the potential for multiplex detection and analysis of a large number of SNPs in a single reaction.

Melting temperature (Tm) analysis that does not involve the use of specific probes is based on the Tm of an entire double-stranded amplicon. In this case, fluorescence is a product of molecules such as SYBR green that bind to double-stranded DNA and fluoresce when they are bound to the DNA double helix. Therefore at relatively low temperatures that permit DNA duplexes to remain stable, fluorescence will be observed, but when temperatures are increased and the double-stranded DNA molecules denature, the SYBR green will no longer bind to the nucleic acids, and fluorescence is no longer detectable. This strategy for Tm analysis will produce a characteristic melting curve that is based on the length, sequence, and GC content of each amplified product. However, it should be noted that using SYBR green lacks specificity because sequence-specific probes are not utilized. Consequently, genetic variability of target sequences from different patients makes identification of an amplified product, based on Tm of the amplicon, somewhat inconsistent.

MULTIPLEX ASSAY DESIGN

There are a number of inherent challenges involved in designing and running multiplex real-time PCR assays. First and foremost is the problem with unequal amplification efficiencies for different products. This may be a result of differences in primer and probe binding affinities for their targets, different primary and secondary structures of target regions, and interaction or cross-reactivity between oligonucleotides within a reaction. These differences lead to competition for reagents such as nucleotides and the DNA polymerase, and this competition will lower the reaction sensitivities of the less efficient reactions. One simple way to decrease competition is to increase the concentrations of reaction components, such as dNTPs or the polymerase.[9] Choosing primers and probes with very similar length, GC content, and hence Tm values can also minimize variation in reaction efficiencies. Competition can be further abrogated by using limiting amounts of primers, such that the primer supply for a reaction is exhausted before the amplification is able to outcompete less efficient reactions and consume a majority of the reagents. This technique is very successful when using a controlled reaction, such as when an internal positive control amplification is multiplexed in the reaction. However, for other target amplifications, especially with unknown or low concentrations of target, a decrease in primer concentration may decrease the reaction efficiency and sensitivity.

Another form of cross-reactivity that exists in multiplexing, besides interactions between oligonucleotides, is with respect to overlap in the fluorescent spectra of the fluorophores being used. Fluorophores must carefully be chosen such that there are sufficient discrete wavelengths

Table 1 Examples of multiplex real-time PCR methodologies and corresponding applications

Chemistry	Target differentiation method	Application	Ref.
5′ nuclease probe	Color	Pathogen detection/differentiation	[10]
5′ nuclease probe	Color	Genotyping/allelic discrimination	[11]
5′ nuclease probe	Color	Normalization of pathogen concentration to human genomic DNA concentration	[12]
5′ nuclease probe	Color	Detection of sarcoma specific fusion transcripts	[13]
Hybridization probes	Tm analysis	Pathogen detection/differentiation	[14]
Hybridization probes	Color	Relative quantitation of tumor marker gene expression	[15]
Hybridization probes	Color	SNP detection/genotyping	[16]
Molecular beacons	Color	Pathogen detection/differentiation	[7]
Molecular beacons	Color	Pathogen detection/differentiation with the inclusion of an internal positive control	[17]
SYBR green	Tm analysis	Pathogen detection/differentiation	[18]
SYBR green	Tm analysis	Detection of leukemia specific fusion transcripts	[19]
Minor groove binding probes	Color	SNP detection/genotyping	[20]

that separate the emission spectra. This is particularly critical with increasing degrees of multiplexing in a single tube. The constant generation of new fluorescent dyes, as well as the use of nonfluorescent quenchers (used with 5′ nuclease probes, molecular beacons, or minor groove binding probes), has increased the number of different targets that can be discriminated in a reaction. However, considering that there is significant crossover in wavelengths of the emission spectra for most commonly used fluorophores, it is currently practical to distinguish only three or four different colors within a reaction.

When distinguishing between amplified targets on the basis of melting temperatures, salt and magnesium concentrations must be optimized, and Tm values of the probes must be carefully chosen. Because secondary structures and interactions between reaction components cannot always be accurately predicted using computer programs, optimization of reaction conditions and the choice of a proper probe sequence must often be determined empirically. Despite the fact that in theory, dozens of different products can be discriminated in a single reaction by Tm, in reality, for routine clinical assays, a maximum of three or four products is all that can easily be discriminated. This is because of the overlap in Tm ranges; Tm values are relatively consistent, but nevertheless, run to run reaction variability makes it such that a range of at least $\pm 2^{\circ}C$ must be allowed between products to ensure that no cross-reactivity is observed.

APPLICATIONS

There are a multitude of different uses for which multiplex real-time PCR can be employed. In a clinical setting, this technology has been most prominently used in the infectious disease field, in which case it is highly beneficial to be able to simultaneously identify and detect multiple pathogens from a single specimen. This approach has become commonplace in many laboratories, with assays that can be designed to be qualitative or quantitative. Quantitative multiplex reactions may involve amplification of multiple targets that are quantified using external calibration curves. Alternately, relative quantification can be calculated using products that are coamplified in the same reaction. This method typically compares or normalizes the target of interest to a calibrated standard or to a housekeeping gene. This relative quantitation is also an example of another separate use for multiplexing. With respect to relative quantitation, greater accuracy is achieved using multiplexing because identical reaction conditions are present for both targets being compared (they are in the same reaction tube).

The addition of an internal positive control (IPC) to a reaction is another common use for multiplexing in a clinical setting. The inclusion of an IPC monitors the efficiency of each individual PCR reaction. The IPC can be included at the point of specimen collection to monitor sample stability, it can be added just prior to nucleic acid extraction to monitor extraction efficiency, or it can be added solely to the PCR mastermix to determine whether inhibitory substances are present following extraction.

In addition to infectious disease applications, multiplex real-time PCR is also extensively used in the fields of molecular genetics and oncology. For instance, multiplex techniques are used to detect multiple SNPs in a single reaction and to do allelic discrimination experiments. In

addition, relative quantitation is used for gene dosing determinations, and it is also used to monitor gene expression; reverse-transcriptase PCR can be used on mRNA to determine the expression levels of tumor markers or to determine the response of specific genes to drug therapy. Some examples of real-time PCR applications are listed in Table 1. This is but a small sampling of the many uses for multiplex PCR, but a more complete listing and description is beyond the scope of this work.

CONCLUSION

Although multiplex real-time PCR can generally be described as the amplification and detection of multiple targets in a single reaction tube, there is tremendous variation with respect to the methods used to perform this technique and how the experimental data are utilized. By combining different available chemistries and different multiplexing strategies, the technique can be tailored for use in a number of different applications, from SNP analysis to multiple pathogen detection, and from oncology and molecular genetics to infectious disease uses. Presently, multiplex real-time technologies are limited only by the available instrumentation and chemistries, and it is currently practical to discriminate three to four targets in a single multiplex reaction. The combination of Tm determinations and multiple-colored fluorophores gives the potential for the discrimination of many more targets, and this could be further expanded using technology such as microarrays. However, the expansion to microarrays and to greater degrees of multiplexing has yet to be achieved on a level that is satisfactory for routine clinical testing. Nevertheless, multiplex real-time methods that are currently available are already changing the diagnostic field, as they are providing more rapid and accurate results, and they are allowing scientists to answer clinical questions that were unable to be answered before the arrival of this technology.

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Murray Valley Encephalitis Virus

David W. Smith
Annette K. Broom
The Western Australian Centre for Pathology and Medical Research and The University of Western Australia, Nedlands, Western Australia, Australia

John S. Mackenzie
Curtin University of Technology, Shenton Park, Western Australia, Australia

INTRODUCTION

Murray Valley encephalitis virus (MVEV) is a member of the Japanese encephalitis serocomplex of the genus *Flavivirus* of the family Flaviviridae and is closely related to both Japanese encephalitis (JEV) and West Nile (WNV) viruses. It is endemic in tropical areas of Australia and causes severe encephalitis with high mortality and morbidity. The earliest recorded outbreaks of arboviral encephalitis occurred along the east coast of Australia between 1917 and 1925, and was then given the name Australian X disease. It was not named Murray Valley encephalitis until an outbreak occurred in that region in 1951, at which time MVEV was isolated. A major outbreak centered on the Murray Valley region then occurred in 1974, with cases subsequently being identified in all mainland states of Australia. Although the vast majority of these cases were due to MVEV, some were found to have been caused by another flavivirus, Kunjin (KUNV). The term Australian encephalitis was coined to include disease caused by either virus, but due to confusion over that terminology, it is now preferred that they are called MVEV encephalitis or KUNV encephalitis.

NATURAL HISTORY

The incubation period is not well established, but is in the range of 1–4 weeks.[1,2] The majority of infections are asymptomatic or nonspecific. Very few cases of non-encephalitic illness have been described, as it is likely that most are undiagnosed or unreported. Two cases were identified during an epidemic in Western Australia (WA) in 2000. The illness consisted of headache, myalgia, and backache with or without recorded fever.[3] Both had a spontaneous recovery.

Only about 1:500 to 1:1000 infected individuals develop encephalitis.[2] In children, MVEV encephalitis presents as fever of 1–2 days duration, almost always with convulsions.[2,4] Reduction of mental state and respiratory failure may follow. Some recover rapidly, whereas others progress to more severe disease characterized by involvement of central brain structures, brain stem, and, possibly, the spinal cord. In adults, the encephalitis begins with headache, fever, and altered mental state.[2,4] Tremor may be apparent on examination and cranial nerve palsies may develop.[4] The course varies from rapid recovery to a prolonged illness with respiratory paralysis or even death.[2–4] Mortality is about 12% in adults, confined to those aged 50 years or over, compared with about 25% in children, particularly those aged less than 2 years (Table 1). Approximately 40% of survivors have neurological residua varying from mild cranial nerve palsies to spastic quadraparesis. Severe residua are much more likely in those over 50 or under 2 years old. Long-term follow-up is available for only a small number of cases, so other late sequelae may not be recorded.

The CT scan is usually unremarkable or shows nonspecific cerebral edema, and EEGs show nonspecific changes.[2,4] MRI scans in late disease have been reported to show thalamic or gray-matter destruction.[5] The CSF shows a variable leukocyte pleocytosis, usually with lymphocyte predominance and a raised protein.[2,4]

TREATMENT

Treatment is supportive, and access to respiratory support is important in the survival of severe cases, which is believed to account for the improvement in outcomes between the outbreaks in the 1950s and 1974.[2] There are no data on specific interventions for MVEV encephalitis. However, the disease and pathology are very similar to encephalitis due to JEV and WNV virus, and it is reasonable to extrapolate from those. Based on the experience with JEV encephalitis,[6] steroids are unlikely to be of

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Table 1 Outcome of encephalitis due to Murray Valley encephalitis virus for cases reported between 1978 and 2001 in Western Australia, the Northern Territory, and South Australia

	Number	Mortality	Severe neurological residua	Minor neurological residua	Normal
Adults $\geq$50 years old	9	3	2	1	3
Adults <50 years old	17	0	1	6	10
All adults	26	3	3	7	13
Children $\leq$2 years old	22	6	8	2	6
Children >2 years old	3	0	0	1	2
All children	25	6	8	3	8

Source: From Refs. [2–4,21].

value, although dexamethasone can be used to reduce intracranial pressure if needed. Alpha-interferon showed some promise for JEV encephalitis, but a recent placebo-controlled trial did not find any benefits in children.[7] There has been interest in intravenous immunoglobulin for treatment of WNV encephalitis, but there is limited human experience.[8]

ECOLOGY OF MVEV

The virus is maintained in a cycle involving water birds and mosquitoes (primarily *Culex annulirostris*) in enzootic foci within the north of WA and adjacent area of the northern half of the Northern Territory (NT).[9] Surveillance using sentinel chicken flocks has demonstrated MVE activity during the tropical wet season (February to July) in these areas in most years.[9] There is some evidence to suggest that the incidence of MVEV has increased in recent years which may be associated with dam building on the Ord River in the Kimberley region and the establishment of large water impoundments.[10]

Activity outside the enzootic areas follows heavy rainfall and flooding, although not invariably, indicating that other factors are also important.[11] When spread occurs, infected migratory water birds or possibly wind-blown infected mosquitoes carry the virus into the flooded areas and set up local epizootic mosquito–bird cycles of infection.[12] A subsidiary mechanism for generating epizootic activity is reactivation of vertically transmitted MVEV that survives in desiccation-resistant eggs of species such as *Ochleratus tremulus* (formerly called *Aedes tremulus*).[13] Spread can be extensive and lead to introduction or reintroduction of MVE into normally arid areas further south in WA or in Central Australia.[3,13] This mechanism is believed to have been the source of the epidemics that occurred in the heavily populated areas of southeastern Australia in 1974,[14] and for the temporary reintroduction of the virus into that area in 2001.[13,15] Little is known about the role of tropical areas of northern Queensland in the maintenance of MVEV in Australia, but virus has been recovered from mosquitoes in this area.[9,16] It has also been isolated from mosquitoes and a fatal human case in Papua New Guinea (PNG).[9,17]

EPIDEMIOLOGY OF MVEV INFECTION IN AUSTRALIAN POPULATIONS

In the endemic areas in the north of WA and the NT, seropositivity rates are very high among indigenous communities, with most being infected before the age of 10 years,[18] indicating that there is often sufficient activity to lead to human infections. In areas of regular epizootic activity, seroprevalence is lower but very high seroconversion rates may occur across epidemic seasons.[19] Areas with uncommon or rare epidemic activity have very low population infection rates, being less than 5% in the Murray Valley area 7 years after their last epidemic.[20]

Cases of MVEV encephalitis occur in most years in the enzootic areas of northern WA and the NT.[2,9] (Fig. 1). There is no evidence to indicate that there is any age difference in susceptibility to infection,[19] and patterns of clinical disease reflect the risk of exposure and susceptibility. Therefore in the endemic areas, encephalitis most often occurs in young indigenous children or in adults who are new residents or tourists in the area.[2,4] Epidemic activity causes occasional encephalitis cases further south in WA in the Pilbara and Gascoyne regions,[2] as well as northern Queensland.[9] Rarely, wider spread results in human cases in the southern half of WA and normally arid areas of inland WA and Central Australia.[3,21] Encephalitis in these epidemic areas mainly occurs in adults, presumably reflecting their higher risk of mosquito exposure.

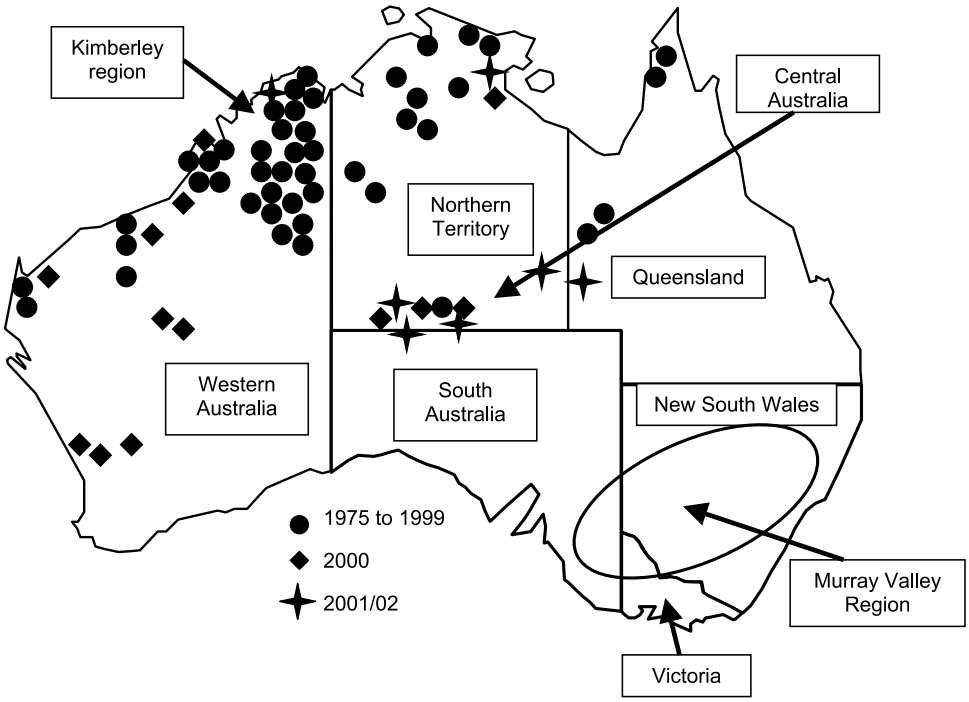

Fig. 1 The distribution of reported cases of encephalitis due to MVEV acquired in Australia between 1975 and 2002. This only includes cases where a definite or likely location of infection could be determined. Most cases occurred in the Kimberley region of Western Australia (WA) and the top end of the Northern Territory. In 2000 and 2001, there was southerly spread in WA and reappearance of disease in Central Australia. Sentinel chicken surveillance picked up MVEV activity in the Murray Valley area of New South Wales in 2001, but without human cases.

MOLECULAR GENETICS

There are two genetic lineages of the virus in Australia. The first includes the original isolate of MVEV, and that lineage is thought to have caused the epidemics on the east coast (including part of the 1974 epidemic) after which it disappeared. The second lineage appeared in the north of WA in 1969 and was responsible for part of the 1974 outbreak. Since then this lineage has been responsible for nearly all MVEV activity in Australia, with less than 2% sequence divergence across time and geographic areas in the NS5 region.[9] However, recent data based on the E gene sequence do indicate that there are minor strain variations over time, but not across different geographical areas (Johansen, C.A., personal communication). This is consistent with there being a single enzootic focus, most likely in the north of WA, which is the source of MVEV throughout Australia, and that evolution of the virus occurs within that enzootic focus.

One early isolate (OR156) from the north of WA contained additional uridine residues resembling two PNG isolates[22] from 1956 and 1966, suggesting that other

Table 2 Yield of diagnostic tests for MVE encephalitis for human cases tested at the Western Australian Centre for Pathology and Medical Research between 1978 and 2001

	Proportion positive for samples collected at the following days after onset of symptoms			
	≤3	4 to 7	>7	All
Serum IgM	6/9	12/14	14/14	32/37
CSF IgM	7/8	3/6	3/3	13/17
CSF PCR	3/5	0/2	0/1	3/8

IgM in serum and cerebrospinal fluid (CSF) detected by indirect immunflourescent antibody method. Polymerase chain reaction (PCR) was a nested reverse transcription PCR directed at 3′ noncoding region sequence.

lineages exist in PNG and possibly the eastern Indonesian archipelago. The E gene sequence data show that recent PNG isolates cluster with isolates from the Kimberley region of WA prior to 1984, but are different from the more recent Australian isolates. Therefore, there may be regular movement of the virus between the north of WA and PNG. Little is known about the distribution of MVEV in PNG and its role in human disease.

DIAGNOSIS

The virus has rarely been cultured from human material, and the viremia is likely to be short-lived. Therefore, while MVEV will grow in mosquito cell lines and suckling mouse brain, cell culture methods have not been useful in diagnosis of infections.

Most diagnoses are made serologically. Paired sera taken in the first few days after onset and 2–3 weeks later will show rising antibody by HI test, EIA, or IFA, although the HI is the least sensitive and may rise late or, rarely, not at all. IgM is present in nearly all patients at the time of presentation. In the WA series, IgM was detected in serum in most cases within 1 week of onset of illness, and in all cases beyond that, whereas CSF IgM was positive in about 75% (Table 2). Following infection serum IgM may remain positive for months, so that the presence of IgM, in the absence of virus detection of a rise in IgG, can only be taken as possible evidence of recent infection.

Antibody will cross-react with other flaviviruses, particularly Kunjin and JE which may also cause encephalitis in the same geographical area. The specificity of antibody can be determined by neutralization tests or epitope-blocking EIA.[23] However, even with specific tests, misleading results may occur in patients who have had a previous flavivirus infection. In these circumstances, antibody to the previously infecting flavivirus may rise before antibody to the currently infecting virus.[24]

Detection of MVEV-RNA in CSF can be achieved using a nested PCR directed to the 3′ untranslated region of the genome, and it is a useful investigation where early samples are available at a time when serology may still be negative (Table 2).

MOLECULAR METHODS FOR MVE SURVEILLANCE

Traditionally, flavivirus surveillance has been carried out by mosquito trapping using carbon dioxide light traps, transport of the trapped mosquitoes to a central laboratory on dry ice, and then speciating, counting, and processing of mosquitoes for virus isolation. This produces excellent information about vector species, infection rates, and the types of viruses they carry[16] provided it is practical to perform this at a time when MVE is likely to be active. Unfortunately, MVE activity occurs over large and sparsely populated areas of Australia, and peak activity occurs during the wet season when flooding may restrict access to many areas. This has meant that mosquito trapping for virus isolation is often not achievable during periods of high virus activity. Sentinel chicken surveillance, using fortnightly or monthly bleeds to check for seroconversion, has proven to be valuable in monitoring MVEV activity within endemic and epidemic areas.[15] However, it does not provide data about virus strains and is not useful for some other flaviviruses of interest such as JEV and dengue virus. It has been shown that a seminested RT-PCR was able to reliably detect JEV in dead mosquito pools,[25] and this is likely also to be suitable for MVEV. This shows considerable promise as a robust surveillance technique that can detect multiple flaviviruses quickly, using dried mosquitoes that can be sent by routine transport mechanisms or by post.

CONCLUSION

Murray Valley encephalitis virus is an important virus that causes serious disease within Australia and has the potential to lead to major epidemics in heavily populated areas, as has occurred in the past. Molecular studies have supported epidemiological data indicating that it exists in an enzootic focus in the north of Western Australia and that this acts as a source for MVEV throughout Australia. Polymerase chain reaction-based methods are valuable in supplementing serological diagnosis and are now being developed as tools for identification and characterization of flaviviruses in mosquitoes.

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Mycobacteria, Atypical

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Chand Wattal
Sangeeta Joshi
Sir Ganga Ram Hospital, New Delhi, India

INTRODUCTION

Mycobacterial disease, an ancient scourge of mankind, still remains an enigma to medical world. The scourge, once thought to have been conquered with the advent of BCG vaccine, is still desired a lot to be known. Its atypical variety known as nontuberculous mycobacteria (NTM) has come to the fore as the burden of geriatric population is increasing because of diseases such as AIDS and various modern treatment modalities for organ transplant or oncology therapies, which are being used more frequently, creating a pool of immunocompromised population susceptible to this infection. Besides this, minimal-access laparoscopic surgeries have exposed our population to the hazards of the infections caused by the environmental microbes earlier unknown to cause fulminant infections. Similarly, waterborne NTM, once thought to be of no consequence, is increasingly recognized as a cause of various skin/soft tissue infections following a surgical procedure.

Currently, more than 80 species of NTM have been described. If an organism takes 7 days or more to grow, it is slow growing (types 1, 2, and 3), whereas the ones growing in <7 days are rapid growers (type 4).

EPIDEMIOLOGY AND PREVALENCE

Most of the NTM have been isolated from water and soil,[1] e.g., species of the *Mycobacterium avium* complex, *Mycobacterium marinum*, *Mycobacterium kansasii*, *Mycobacterium xenopi*, *Mycobacterium simiae*, and rapid growers (Table 1).[2] Most common NTM associated with nosocomial disease are *Mycobacterium fortuitum*, *Mycobacterium chelonae*, and *Mycobacterium abscessus*. *M. xenopi* requires a temperature of 28°C or more to grow and has been recovered from hot water and hot water taps within hospitals.[3]

Airborne environmental NTM may result in respiratory disease, whereas ingestion may be the source for cervical lymphadenitis and, in AIDS patients, may lead to a disseminated disease. Soft tissue and skin infections are likely a result of direct inoculation of NTM. It is not known whether NTM disease has a latent period or not. Because NTM disease is not notifiable, the worldwide estimates of incidence and prevalence may not be accurate. A study conducted by Centers for Disease Control and Prevention (CDC, Atlanta, GA 30333, U.S.) from 1991 to 1992[4] demonstrated a dramatic increase in numbers of NTM, especially *M. avium* complex.

CLINICAL PRESENTATION

Nontuberculous mycobacteria are classified depending on the clinical syndrome associated with it (Table 2).[5]

Pulmonary Disease

Predisposing factors[6] for the development of pulmonary disease include alcoholism, bronchiectasis, cyanotic heart disease, cystic fibrosis, prior mycobacterial disease, pulmonary fibrosis, smoking, and chronic obstructive lung disease. Radiological features include thin-walled cavities, parenchymal infiltrates, and pleural involvement. High-resolution computed tomography of the chest shows a combination of multifocal bronchiectasis and nodular parenchymal disease.[7]

Lymphadenitis

In children (1–5 years), localized nontender lymphadenitis is seen involving the submandibular, submaxillary, cervical, or preauricular lymph nodes[8] which may enlarge, rupture, and form sinuses. Almost 80% NTM lymphadenitis are due to *M. avium* complex.

Localized Skin and Soft Tissue Infection

Most species of NTM have been described causing cutaneous disease[9,10] (Table 2) or nosocomial skin and soft tissue infection, postinjection abscess, surgical wound infections, and infections because of long-term intravenous or peritoneal catheters. *M. chelonae* causes disseminated nodular skin disease in immunocompromised patients. *M. marinum* causing ''swimming pool granuloma'' or ''fish tank granuloma'' gets implanted while cleaning fish tanks or handling fish. Solitary papules get

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Table 1 Runyon's classification of NTM

Type	Characteristics	Common organisms
Type 1	Slow grower, photochromogen	*M. kansasii*, *M. marinum*, *M. simiae*, *Mycobacterium asiaticum*
Type 2	Slow grower, scotochromogen	*Mycobacterium scrofulaceum*, *Mycobacterium szulgai*, *Mycobacterium gordonae*, *M. ulcerans*
Type 3	Slow grower, nonchromogen	*M. avium–intracellulare*, *Mycobacterium malmoense*, *Mycobacterium terrae*, *Mycobacterium haemophilum*, *M. ulcerans*, *M. xenopi*
Type 4	Rapid grower	*M. fortuitum*, *M. chelonae*, *M. abscessus*, *Mycobacterium smegmatis*

formed that progress to ulceration and scar formation. *Mycobacterium ulcerans* causes disease in children and young adults, producing necrotic lesions of skin and underlying tissue.[9] In our experience, five cases (four wound infections following a laparoscopic surgery and one injection abscess) of *M. chelonae* (three isolates) and *M. fortuitum* (one isolate) were isolated which were sensitive to imipenem, amikacin, and ciprofloxacin.

Infections of Bursae, Joints, Tendon Sheaths, and Bones

Most of the NTM[9] can cause chronic granulomatous infection of the tendon sheaths, bursae, joints, and bones (Table 2). *M. abscessus* is a common cause of posttraumatic wound infection.[10]

Disseminated Disease

In immunosuppressed patients (AIDS with CD4 cell count <50 cells, posttransplant, long-term steroid use, malignancies), disseminated disease because of various NTM has been described (Table 2).[9,10] The disease can present as fever of unknown origin in case of *M. avium* complex[11] or as multiple localized abscesses or nodules. The organism can be isolated from blood or bone marrow more so if *M. avium*.

DIAGNOSIS

There are no specific skin tests standardized for NTM.

Microscopic Examination

Because nontuberculous mycobacteria are transiently present in the sputum, three or more sputum samples should be evaluated for acid-fast bacilli. Bronchial washing is a better sample. A fine needle aspiration performed from the lymph nodes may yield NTM on cultures and show granulomatous changes on cytology. The two commonly used stains are the Ziehl–Neelsen stain (sensitivity 50–60%) and auramine–rhodamine (sensitivity 80%).

Culture

Culture still remains the gold standard in diagnosing mycobacterial infections,[12] which, besides being more

Table 2 Clinical syndromes associated with NTM

Syndrome	Common	Less frequent
Pulmonary disease	*M. avium* complex, *M. kansasii*, *M. abscessus*, *M. xenopi*, *M. malmoense*	*Mycobacterium celatum*, *M. simiae*, *M. smegmatis*
Lymphadenitis	*M. avium* complex	*Mycobacterium scrofulaceum*, *M. malmoense* (in Europe), *M. abscessus*, *M. fortuitum*
Skin soft tissue disease	*M. marinum*, *M. ulcerans*, rapid growers	*M. haemophilum*, *M. smegmatis*, *M. kansasii*
Bone and joint infection	*M. avium* complex, *M. marinum*, rapid growers, *M. kansasii*	*M. haemophilum*, *M. smegmatis*, *M. scrofulaceum*
Disseminated infection HIV-positive	*M. avium*, *M. kansasii*	*Mycobacterium genavense*, *M. haemophilum*, *M. xenopi*, *M. fortuitum*
Disseminated infection HIV-negative	*M. abscessus*, *M. chelonae*	*M. marinum*, *M. kansasii*, *M. haemophilum*
Catheter-related infections	*M. fortuitum*, *M. abscessus*, *M. chelonae*	*Mycobacterium mucogenicum*

Table 3 Culture methods for mycobacteria

Method	Medium	Manufacturer
Conventional methods		
Egg-based solid media	Lowenstein Jensen medium	
Agar-based media	Middlebrook 7H10, 7H11 medium	
Broth media	Middlebrook 7H9, Dubos Tween albumin broth	
Automated/semiautomated methods		
Biphasic medium	Septichek system	Becton Dickinson
Radiometric	BACTEC 460 system	Becton Dickinson
Nonradiometric	MB/BacT system	BioMerieux
Fluorescence	MGIT, BACTEC 9000MB	Becton Dickinson

sensitive than staining techniques, also allows identification and antimicrobial sensitivity known.[13] The decontamination methods used for processing samples for *Mycobacterium tuberculosis* (MTB) can also be used for NTM. Commonly used media and automated and semiautomated methods are mentioned in Table 3. For maximal recovery within 4–6 weeks, one liquid and one solid media are recommended. In general, recovery rate does not differ considerably among the different automated systems but is higher and shorter (10–14 days) than the solid media.

Identification

Species identification is performed by conventional biochemical tests along with their growth characteristics.[14] The ρ-nitro-α-acetylamino-β-hydroxy propiophenone (NAP) test can be used to differentiate NTM from the MTB complex directly from the BACTEC broth. High-performance liquid chromatography and gas–liquid chromatography can be used to speciate and analyze the mycobacterial cell wall fatty acids. Nucleic acid probe kits have been used to directly amplify *M. tuberculosis* and *Mycobacterium avium intracellulare* from MB/BacT and BACTEC cultures using nucleic acid sequence-based amplification assay (NASBA, bioMerieux)[15] and acridinium–ester-labeled DNA probes[16] with a sensitivity and specificity of nearly 100%. The biomass obtained in MB/BacT is superior to BACTEC for direct molecular assays.

MOLECULAR METHODS

For rapid diagnosis, two nucleic acid amplification tests (NAA), the amplified *M. tuberculosis* direct test (MTD) (bioMerieux) and AMPLICOR (Roche) *M. tuberculosis* test, have been cleared by the Food and Drug Administration (FDA) for direct use in smear-positive respiratory samples, whereas enhanced MTD (Genprobe, bioMerieux) is also approved for smear-negative respiratory cases. A number of molecular methods have been used to differentiate between *M. tuberculosis* and NTM and also for speciating NTM.

Nucleic acid sequence-based amplification assay, a MTD type of assay, has been used for the detection of *M. avium*, *Mycobacterium paratuberculosis*, and *Mycobacterium intracellulare* using a set of primers and probes[15] directly on sputum sample. Restriction enzyme analysis of the amplified segment of the gene encoding for the 650 kDa mol. wt. heat shock protein and polymerase chain reaction with single universal primer was used to identify MTB complex and species identification of NTM.[17] Bhattacharya et al.[18] have developed a multiplex PCR based on amplification of 165-, 365-, and 541-bp target fragments to distinguish between strains of MTB complex and NTM.

Hall et al.[19] evaluated the Microseq 500 system and could identify 98.3% of the ATCC strains and 90.1% of the clinical isolates to the species/group level. The 5′ exonuclease fluorogenic PCR assay[20] had a 100% positive predictive value to detect mycobacteria in clinical samples. Smear-positive samples had a sensitivity of 90.3% as compared with smear-negative (49.2%). The INNO-LiPA Mycobacteria and GenoType Mycobakterien (Hain Diagnostica) are DNA strip assays and have identified 89.4% and 95.1% of mycobacterial isolates, respectively.[21]

A new technology involving high-density oligonucleotide arrays or ''DNA chips'' can be used to examine large amount of DNA sequences. The nucleotide sequence diversity in 10 mycobacterial species from 121 mycobacterial isolates was examined by Gingeras et al.[22] by analyzing the rpoB oligonucleotide array hybridization patterns. The use of this array allowed simultaneous detection of mutations that confer rifampicin resistance as well as species identification.

DRUG SUSCEPTIBILITY OF NONTUBERCULOUS MYCOBACTERIA

Drug susceptibility methods for NTM are not standardized. However, MB/BacT and radiometric BACTEC method, agar proportion method, broth microdilution, and E test have been used for this purpose. Standard first-line antitubercular agents are tested along with clarithromycin, amikacin, streptomycin, quinolones, and sulfonamide.

M. avium Complex

M. avium complex (MAC) is usually resistant to the first-line antituberculous agents. Patients with previous exposure to macrolides and those who develop MAC while receiving macrolides for its prophylaxis should have their initial isolate tested for macrolide susceptibility. Minimal inhibitory concentration (MIC) of >32 μg/mL is the recommended breakpoint for resistance.[6]

M. kansasii

All initial isolates should be tested against rifampicin. Resistance usually develops during treatment. A resistant isolate could be tested against ciprofloxacin, ofloxacin, clarithromycin, ethambutol, streptomycin, and sulfonamide.

Routine antituberculous agents are not tested for rapidly growing mycobacteria. The drugs tested include amikacin, cefoxitin, fluoroquinolones, doxycycline, clarithromycin, tobramycin, imipenem, and sulfonamides. Susceptibility tests for other slow-growing NTM may include isoniazid, rifampin, ethambutol, ciprofloxacin, and clarithromycin. Analysis of mutations in the emb B region could predict intrinsic and acquired resistance to ethambutol in case of NTM.

TREATMENT

M. avium Complex

The newer macrolides are the mainstay of therapy for MAC pulmonary and disseminated disease (Table 4). Intermittent streptomycin for the first 2–3 months of therapy for the first 8 weeks has also been used. For disseminated disease in AIDS, treatment regimens should include clarithromycin or azithromycin and ethambutol and a third agent rifabutin can be used, but drug interactions exist between rifabutin and protease inhibitors. Prophylaxis for MAC, with either azithromycin (1200 mg once weekly) or clarithromycin (500 mg twice daily, BD), is now recommended for all AIDS patients with CD4 cell counts <50 cells/mm^3.[23] Rifabutin (300 mg/day) or a combination of azithromycin (1200 mg once weekly) plus rifabutin (300 mg/day) has been proven to be effective.

M. marinum

Surgical excision of the skin lesions or electrodessication with or without adjuvant ATT is the most common treatment. The organism is uniformly resistant to isoniazid. Other drugs have been referred to in Table 4.

Rapidly Growing Mycobacteria

M. fortuitum, *M. chelonae*, and *M. abscessus* are resistant to the antituberculous agents. Depending on the susceptibility pattern, treatment with amikacin, ciprofloxacin, cefoxitin, and clarithromycin is recommended (Table 4).

For treatment of pulmonary disease because of other NTM, see Table 4.

Table 4 Treatment of NTM

Species	Drug	Duration
M. avium complex	Clarithromycin (500 mg, BD)+ethambutol (15 mg/kg)+rifampicin (600 mg) or rifabutin (300 mg)	Culture-negative for 12 months. In HIV-positive patients, treatment is for life.
M. kansasii	Rifampicin (600 mg)+isoniazid (300 mg)+ethambutol (15 mg/kg)	18 months, culture negative for 12 months
M. marinum	Clarithromycin (500 mg, BD) or minocycline or doxycycline (100 mg) or rifampicin (600 mg)+ethambutol (15 mg/kg)	3 months
M. fortuitum, *M. chelonae*, and *M. abscessus*	Amikacin (10–15 mg/kg)+cefoxitin (12 mg/day) or clarithromycin (500 mg, BD)	Cutaneous and disseminated—6 months; pulmonary—2 weeks
M. malmoense	Clarithromycin (500 mg, BD)+ethambutol (15 mg/kg)+rifabutin (300 mg)+streptomycin	18–24 months
M. szulgai, *M. xenopi*, *M. simiae*	Isoniazid, rifampicin, ethambutol, and streptomycin	18–24 months

CONCLUSION

To conclude, atypical mycobacteria have come to stay with us and it is important to keep a high index of suspicion. To do so, it becomes mandatory to isolate and identify. The role of molecular assays is clearly defined as a supportive one and cannot be viewed in isolation as the only evidence of infection. Molecular assays cannot be recommended as the first-line routine assays for the diagnosis of mycobacterial disease because of its cost and exacting technique implications.

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Mycobacterium leprae

Diana L. Williams
Thomas P. Gillis
National Hansen's Disease Programs, Baton Rouge, Louisiana, U.S.A.

INTRODUCTION

Advances in molecular technologies have brought forth new diagnostic procedures for rapid, specific, sensitive, and cost-effective identification of *Mycobacterium leprae* and drug-resistant strains. These tools have made possible the specific and early diagnosis of leprosy which is critical for initiating appropriate treatment to hasten a cure and prevent progressive nerve damage. Genomics of both man and *M. leprae* are providing new information regarding disease susceptibility as well as markers for strain typing *M. leprae*. Annotation of *M. leprae*'s genome has provided insight into why the leprosy bacillus is an obligate intracellular parasite and has provided the basis for future experimentation to better understand its pathogenicity.

LEPROSY

Leprosy (Hansen's Disease) is a chronic infectious disease caused by infection with *Mycobacterium leprae* affecting the skin, mucous membranes, and peripheral nerves.[1] The predilection of *M. leprae* for peripheral nerves can result in nerve damage leading to sensory and motor paralysis which in turn can cause deformities of the hands and feet and blindness. It is estimated that 2–3 million patients suffer from irreversible nerve damage as a long-term consequence of *M. leprae* infection and that approximately one-fourth of the 621,000 new cases in 2003[2] will suffer from some form of nerve damage.

Leprosy exhibits a spectrum of clinical forms resulting from the infected patient's ability to attack and destroy *M. leprae*.[1] At one extreme of the spectrum is the localized form of disease, tuberculoid leprosy (TT), in which very few bacteria are observed in infected tissues as a result of partially effective cell-mediated immunity (CMI). At the opposite end of the spectrum is the disseminated form of the disease, lepromatous leprosy (LL), in which large numbers of *M. leprae* are observed due to ineffective CMI. Patients whose disease appears neither TT nor LL fall in an intermediate category called borderline leprosy. In approximately 50% of leprosy patients under treatment, acute inflammatory reactions develop that are immunologically mediated responses to *M. leprae*.[1] Type 1 reactions are seen in patients with significant T-cell immunity to *M. leprae* and in extreme cases may result in necrosis with abscess formation in nerve trunks with irreparable loss of nerve function. Type 2 reactions, or erythema nodosum leprosum (ENL), are characterized histologically by a vasculitis and a neutrophilic infiltrate superimposed on a lepromatous infiltrate. Chronic episodes of ENL can lead to nerve damage and eventual loss of nerve function. Both reactions can be managed with anti-inflammatory agents such as steroids or in the case of ENL, thalidomide.

Identification of the host genes that influence host susceptibility/resistance enables a greater understanding of disease pathogenesis. Genetic linkages with leprosy have been recognized for years.[3,4] For example, twin studies in India demonstrated higher concordance rates for leprosy among monozygotic twins (60–85%) than dizygotic twins (5–20%).[3] Recent developments in genome-wide linkage analysis have identified three major susceptibility loci that are linked to and associated with leprosy susceptibility.[4] Fine mapping of these chromosomal regions is now required to better understand the contribution derived from these putative susceptibility genes.

ETIOLOGICAL AGENT

M. leprae is a nonmotile, nonspore-forming, microaerophilic, acid-fast-staining bacterium that usually forms slightly curved or straight rods. *M. leprae* has never been grown on artificial media but can be maintained in axenic cultures in a metabolically active state for a few weeks.[5] As a result, propagation of *M. leprae* has been restricted to animal models including the armadillo, normal, and athymic rodents.[1] These systems have provided the basic resources for genetic, metabolic, and antigenic studies of the bacillus. Growth of *M. leprae* in mouse foot pads also provides a tool for assessing

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viability and for testing drug-susceptibility of clinical isolates.[5,6]

Genome and Transcriptome

Annotation of *M. leprae*'s genome has rekindled basic investigations into aspects of metabolic, biochemical, and pathogenic potential of this obligate intracellular pathogen of humans. Comparison of *M. leprae*'s genome with that of its close relative *Mycobacterium tuberculosis* suggests that *M. leprae* has undergone an extreme case of reductive evolution.[7,8] This is reflected in its smaller genome (3.3 Mb for *M. leprae* vs. 4.4 Mb for *M. tuberculosis*) and a major reduction in G+C content (66% for *M. tuberculosis* to 58% for *M. leprae*). *M. leprae* contains 1605 open reading frames (ORFs), encoding potentially functional proteins compared to 4000 ORFs in *M. tuberculosis*. Proteomic studies, using two-dimensional SDS polyacrylamide gel electrophoresis analysis, with *M. leprae* predict 391 proteins[9] as compared to approximately 1800 proteins in *M. tuberculosis*.[10] One of the most striking features of *M. leprae*'s genome is that it possesses 1116 inactivated genes, genes lost through mutation (pseudogenes), compared to six pseudogenes in *M. tuberculosis*.[7] In addition, a large number of genes have been entirely deleted from the genome. The result of this massive gene loss leaves *M. leprae* with only 50% of its genome encoding functional genes as compared to 90% functional genes in *M. tuberculosis*.

The largest functional groups of genes in *M. leprae* were found to be involved in the metabolism and modification of fatty acids and polyketides, transport of metabolites, cell envelope synthesis, and gene regulation.[7,8,11] Downsizing of the genome has resulted in the elimination of entire metabolic pathways, their regulatory circuits, and accessory functions, resulting in a pathogen with very specific growth requirements.

While comparative genome analysis provides useful clues to identify deficits in general cellular metabolic potential and cellular composition, it offers only a starting point from which functional studies can ensue. Because of the inability to cultivate *M. leprae* axenically, purification of large quantities of bacterial proteins for analysis is extremely limited. Transcriptional analysis of *M. leprae* genes provides a perspective that is complementary to protein analysis by identifying actively transcribed genes and therefore those genes essential for optimal growth during infection in the host. Therefore an efficient method for purifying *M. leprae* RNA has been developed and transcripts for several genes have been identified directly from skin biopsy tissues of multibacillary leprosy patients.[12] With the development of a complete genomic array for *M. leprae*, the elucidation of the functional transcriptome should be forthcoming.

A major limitation exists for studying the impact of specific genes on *M. leprae*'s metabolism and pathogenesis. Because of the inability to cultivate *M. leprae* axenically, genetic systems for creating gene knockout mutants have not been developed. To overcome this limitation, mycobacterial surrogates such as, *Mycobacterium smegmatis*, have been used to test the function of several *M. leprae* genes.[13,14] While this is a powerful approach for studying *M. leprae* genes, caution must be taken in interpreting data generated using surrogate genomics because *M. leprae* genes are being expressed in a mycobacterial system that has a wealth of other genes not found in *M. leprae*.

Metabolism and Physiology

The primary reasons for investigating the metabolic aspects of *M. leprae* has been to determine whether special media could be formulated to support in vitro growth of the bacilli and to learn more about metabolic pathways that could potentially be exploited for developing new antileprosy drugs. Early work provided a picture of a bacterium with some basic anabolic and catabolic pathways needed for survival in the host, but a thorough assessment of *M. leprae*'s metabolic potential was still lacking.[15] With the completed sequencing and annotation of *M. leprae*'s genome, an improved version of *M. leprae*'s metabolic capabilities exists.[7,8]

Annotation of the genome identified genes showing that *M. leprae* has the capacity to generate energy by oxidizing glucose to pyruvate through the Embden–Mayerhoff–Parnas pathway supporting earlier biochemical observations. Acetyl-CoA from glycolysis enters the Krebs cycle producing energy in the form of ATP. In addition to glycolysis for energy production, genome analysis as well as biochemical studies in *M. leprae* and *M. tuberculosis* suggests that these organisms rely heavily upon lipid degradation and the glyoxylate shunt for energy production. In this regard *M. leprae* contains a full complement of genes for B-oxidation but very few genes as compared to *M. tuberculosis* capable of lipolysis. Acetate as a carbon source has been lost to *M. leprae* as genes such as acetate kinase, phosphate acetyltransferase, and acetyl-CoA synthase are pseudogenes. Overall, *M. leprae* has much fewer enzymes involved in degradative pathways for carbon and nitrogenous compounds as compared to *M. tuberculosis*. This is reflected in the paucity of oxidoreductases, oxygenases, and short-chain alcohol dehydrogenases, and their probable regulatory genes. Other major

problems associated with metabolism for *M. leprae* exist in that the bacilli have lost anaerobic and microaerophilic electron transfer systems, and the aerobic respiratory chain is severely curtailed making it impossible for *M. leprae* to generate ATP from the oxidation of NADH. In contrast to the reduction in catabolic pathways, anabolic capabilities of *M. leprae* appear relatively unharmed. For example, complete pathways are predicted for synthesis of purines, pyrimidines, most amino acids, nucleosides, nucleotides, most vitamins, and cofactors. The maintenance of these anabolic systems suggests that the intracellular niche that *M. leprae* finds itself may be highly restrictive for these compounds or transport systems for them do not exist.

Cell Wall Chemistry and Related Genes

A great deal has been learned about the nature of the mycobacterial cell wall through biochemical and genetic manipulation of cultivable strains such as *M. tuberculosis*, *Mycobacterium avium*, *M. smegmatis*, and *Mycobacterium bovis* BCG. Similar approaches with *M. leprae* have been meager by comparison, but basic chemical studies have concluded that the cell wall is a covalently linked peptidoglycan–arabinogalactan–mycolic acids complex similar in composition to all mycobacterial cell walls.[16,17] The peptidoglycan of *M. leprae* contains meso-diaminopimelic acid as do other mycobacteria; however, *M. leprae* contains the unusual substitution of glycine for alanine in the bridging tetrapeptide. The muramic acids of the peptidoglycan in *M. leprae* are modified with *N*-glycolyl as opposed to the more common *N*-acetyl substitution. Coupled to the peptidoglycan through a disaccharide linkage is the arabinogalactan component of the outer cell wall. Linear arrays of D-arabinose–D-galactose polymers with appendages of D-arabinose make up this layer lying external to the peptidoglycan. Mycolic acids are esterified to the terminal arabinoses of the arabinan chains completing the bulk of the cell wall. A rich array of intercalating lipoglycans, phosphotidylinositol mannosides (PIM), lipomannans (LM), and lipoarabinomannans (LAM) are also present. Finally, the dominant lipid in the cell wall which engenders *M. leprae* with immunological specificity is phenolic glycolipid I (PGL-I). Recent studies suggest that PGL-1 is involved in the interaction of *M. leprae* with the laminin of Schwann cells suggesting a role for PGL-1 in peripheral nerve/bacilli interactions.[18]

Annotation of the *M. leprae*'s genome and comparative genomic studies with other bacterial genomes has produced insight into the putative genes needed to direct the synthesis of this complex biopolymer.[11] Most of the genes necessary to build the peptidoglycan–arabinogalactan–mycolate polymer appear to be present in the *M. leprae* genome and fit a reasonable strategy for its construction. A few exceptions are two genes involved in polyprenyl–phosphate synthesis (*dxs-II*, *idi*), a gene (*fabH*) involved in meromycolate synthesis, and a glycosyltransferase gene (*pimB*) involved in the biosynthesis of PI, PIM, LM, and LAM. It should be noted that much of this comparative work, while speculative, provides an important framework from which to investigate the authenticity of these putative pathways.

MOLECULAR TOOLS

Diagnosis of Leprosy

Early diagnosis and effective treatment of leprosy are essential for reducing the risk of neurological complications resulting in progressive and irreversible nerve damage. Confounding the diagnosis is the increased presence of other mycobacterial infections of the skin. As *M. leprae* is not cultivatable, culture amplification with subsequent phenotypic identification of *M. leprae* is not feasible. In addition, there are no approved blood tests or skin test reagents for leprosy diagnosis that are used on a routine basis. Therefore differentiation of leprosy from other cutaneous granulomatous diseases is routinely based on characteristic histopathological features and demonstration of acid-fast bacilli by microscopy.[1]

Rapid molecular-based assays have been developed for detection of *M. leprae* directly from patient specimens using available genetic data (for review, see Ref. [19]). These assays have been based primarily on the use of polymerase chain reaction (PCR) amplification of an *M. leprae*-specific gene fragment and identification of the *M. leprae* DNA fragment in the lesion. Many different genes (e.g., *hsp18*, *ag36*, and *groEL1*), noncoding repetitive sequences (RLEP), and 16S rRNA (DNA or RNA) of *M. leprae* have been utilized in these assays. On the basis of extensive assessment of these tests in field studies, PCR-based techniques have shown a specificity of essentially 100% and a sensitivity ranging from 34% to 74% in patients with paucibacillary forms of the disease to greater than 90% in patients with multibacillary forms of the disease. Automation of PCR-based techniques has allowed their implementation in many reference laboratories, chiefly in leprosy endemic countries. Therefore PCR can provide an excellent adjunct to clinical and histopathological diagnosis of leprosy. It is anticipated that information gained from these tests will be useful to

the overall public health strategy for identifying and treating early leprosy.

Diagnosis of Drug-Resistant *M. leprae*

Treating infectious diseases with combinations of effective antibiotics is a proven method of limiting the emergence and spread of new or existing antibiotic-resistant pathogens. Current multidrug therapies (MDT) formulated for leprosy[1] are based on this principle and appear to be effective at both minimizing the development of drug resistance and reducing the prevalence of leprosy in many areas of the world.[2] However, over the last several years, drug resistance has been observed for *M. leprae* (for review, see Ref. [20]). Leprosy presents a very special problem for the detection of resistance because of the inability to culture *M. leprae* axenically. The current method for determining the activity of drugs against *M. leprae* is the mouse foot pad inoculation assay.[6] While this assay gives definitive information pertaining to the susceptibility of an *M. leprae* isolate to standard antileprosy drugs, it is cumbersome, requiring up to a year to obtain results.

Mutations in *M. leprae* genes associated with the development of resistance to key antileprosy drugs, including rifampin, dapsone, and ofloxacin, have been identified.[20] The β-subunit of the RNA polymerase, encoded by *rpoB*, is the target for rifampin, a derivative of rifamycin S. Missense mutations within an 81-bp region of *rpoB*, referred to as the rifampin resistance determining region (RRDR), have been associated with rifampin resistance in *M. leprae*. The dihydropteroate synthase, encoded by *folP-1*, is the target for dapsone, a sulfone. Missense mutations within a region of *folP-1*, referred to as the sulfone resistance determining region (SRDR), have been associated with high-level dapsone resistance in *M. leprae*. The GyrA subunit of the DNA gyrase, encoded by *gyrA*, is the target for ofloxacin, a fluoroquinolone. Mutations within a region of *gyrA*, referred to as the quinolone resistance-determining region (QRDR), have been associated with ofloxacin resistance in *M. leprae*. Mechanisms of resistance to the other effective antileprosy drugs including clofazimine and minocycline have not been elucidated primarily because of the inability to isolate sufficient numbers of resistant organisms for investigation.

The above information has been used as the basis for development of rapid, molecular assays for detecting mutations associated with rifampin-, dapsone-, or ofloxacin-resistant *M. leprae* directly from patient's specimens.[20] These assays are based on the use of PCR to amplify a fragment of an *M. leprae* drug target gene (RRDR, SRDR, or QRDR) and identification of missense mutations associated with drug resistance in these fragments using mutation detection methodologies. Polymerase chain reaction-based mutation detection assays which have been developed for drug susceptibility testing of *M. leprae* in clinical specimens include PCR-direct DNA sequencing, PCR-single-strand conformation polymorphism analysis (PCR-SSCP), PCR-heteroduplex analysis (PCR-HDA), and PCR-solid phase reverse-hybridization analysis. These assays demonstrate high specificity and sensitivity for detecting drug-susceptibility patterns of *M. leprae* from clinical specimens. The use of these molecular assays for limiting the spread of drug-resistant *M. leprae* is currently being evaluated as an integral component of an overall public health strategy for controlling leprosy.

Strain Identification

Understanding the epidemiology of leprosy is a prerequisite for effective control of the disease. As *M. leprae* cannot be cultured in vitro, it has been virtually impossible to assess exposure, onset of infection, and various aspects of disease progression. As a consequence, the sequence of events which must occur for successful transmission of leprosy is poorly understood. Genetic markers may hold the key to establishing species and strain-specific markers for assessing exposure to *M. leprae* and tracing transmission patterns. These tools should be helpful for improving our understanding of the epidemiology of leprosy. Over the last two decades, a wide range of molecular tests have been applied to reveal genotypic variation of *M. leprae*. Results of initial studies suggested that the genome of *M. leprae* was highly conserved based on restriction fragment length polymorphism (RFLP) analysis of *M. leprae* isolates using a combination of restriction enzymes and probes, and sequencing the internal transcribed spacer region of the 16S–23S rRNA operon yielded no polymorphic DNA sequences.[21–23] A polymorphic structure in the *pol*A gene[24] and variation in a GACATC repeat in the *rpo*T gene[25] have been described, but the value of these elements for differentiating *M. leprae* appears to be limited. In completing the sequence of the *M. leprae* genome, Cole et al.[7] identified several tandem repeats that could prove useful for discriminating *M. leprae* strains. Recently, Shin et al. reported evidence for diversity among *M. leprae* obtained from several patient biopsy samples in the Philippines, based on the frequency of TTC repeats located downstream of a putative sugar transporter pseudogene.[26] In addition to the TTC locus, in silico analysis of the genome sequence indicates that *M. leprae* has several other tandem repeat loci which may provide the genetic diversity necessary for creating a typing scheme capable of answering important questions related to the epidemiology of leprosy.

CONCLUSION

Over the past several years, *M. leprae*'s genetic information has been used to identify markers for the development of rapid, highly specific, and sensitive assays for the diagnosis of leprosy and drug-resistant leprosy directly from patient specimens. Genetic markers for susceptibility to leprosy have been identified, making the development of molecular assays for assessment of risk to this disease feasible. In addition, molecular markers for strain identification have been identified. Together, these assays will potentially aid in the elimination program by providing rapid diagnostic and epidemiological tools for leprosy. In addition, the availability of genomic information has opened new areas of leprosy research that will increase our knowledge of the metabolic, physiological, and pathogenic lifestyle of *M. leprae*, and aid in the identification of novel targets and skin test reagents for early diagnosis of leprosy.

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Mycoplasma spp. and *Ureaplasma* spp.

Florian Daxboeck
University of Vienna, Vienna, Austria

INTRODUCTION

Mycoplasma spp. belong to the class of mollicutes (derived from the Latin mollis=soft and cutis=skin, indicating that these organisms lack a cell wall). Mollicutes have evolved regressively from gram-positive bacteria (*Clostridia*). Within the class of mollicutes, the family Mycoplasmataceae includes the genera *Mycoplasma* and *Ureaplasma*, which represent the most important mollicutes in human medicine. The genome size of Mycoplasmataceae is small, ranging from 577 to 1380 kbp (*Mycoplasma* spp.) and from 760 to 1140 kbp (*Ureaplasma* spp.). The limited genomic information implies the extensive nutrient requirements of the organisms when cultured in vitro and the inability to survive outside their natural hosts. To date, complete genomes of *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma penetrans*, and *Ureaplasma urealyticum* have been sequenced.

Although some *Mycoplasma* spp. are capable of invading human cells, close attachment to the host cells without invasion is typical. Diagnosis of infections with Mycoplasmataceae is challenging because of their fastidious growth in vitro. Standard single-step polymerase chain reaction (PCR) assays have been established for most human-pathogenic Mycoplasmataceae (Table 1). In addition, for the most clinically relevant species, advanced PCR-based methods such as real-time PCR or nucleic acid sequence-based amplification (NASBA) have been constructed.

When *Mycoplasma* spp. or *Ureaplasma* spp. cause infections in their main mucosal habitat (e.g., respiratory tract or urogenital tract), copy numbers above the sensitivity level of a standard single-step PCR are expectable. When a screening for colonization is performed, and notably when the pathogens are to be detected in other anatomical sites, methods providing superior sensitivity should be applied.

M. PNEUMONIAE

M. pneumoniae is an important causative agent of upper and lower respiratory tract diseases. The most frequent extrapulmonary manifestations include encephalopathy, Guillain–Barré syndrome, perimyocarditis, and arthritis. Overall, these manifestations occur in less than 10% of patients, mostly in association with respiratory tract disease. There is no evidence of a predisposition of immunocompromised patients for severe or extrapulmonary *M. pneumoniae* infection.

When diagnosis of *M. pneumoniae* infection is based on serology from serum, assays (e.g., ELISAs) for separate detection of the early-appearing IgM (children) or IgA (adults) should be combined with the detection of all specific antibodies (microparticle agglutination assay). The most important antigen of *M. pneumoniae* is the attachment protein P1 (169 kDa). In patients with respiratory tract disease, molecular methods are useful to assist serological diagnosis. Molecular detection of *M. pneumoniae* is especially useful in patients with suspected mycoplasma central nervous system disease, perimyocarditis, arthritis, and other extrapulmonary manifestations. With the exception of the urogenital tract, detection of *M. pneumoniae* by PCR from sites outside the respiratory tract is always diagnostic. Because of the possibility of immunologically mediated disease, negative PCR results must be interpreted with caution. Detection of low quantities of *M. pneumoniae* in the throat can reflect a past illness.

It is important to note that 1 cfu (colony forming unit) or 1 ccu (color changing unit) of *M. pneumoniae* does not correspond to one genome equivalent, e.g., one single organism. In contrast, 1 cfu is estimated to be 10–1000 bacterial cells, and 1 ccu is estimated to be 10–100 organisms.[1] Importantly, formation of conglomerates of the bacteria in biological samples can limit the sensitivity of ultrasensitive detection methods.

Polymerase chain reaction was first applied for *M. pneumoniae* diagnosis in 1989 by Bernet et al. (target sequence: ATPase operon gene). To date, a variety of additional primer pairs targeting the genes encoding for the P1 protein and the 16S rRNA have been published.[2,3] A kit for PCR detection of *M. pneumoniae* is commercially available. Single-step PCR assays usually have a sensitivity of 10^2–10^3 genome copies/ml when applied to clinical samples.[4] Hybridization of PCR products is generally thought to increase the sensitivity of PCR compared with gel electrophoresis and ethidium bromide staining alone, but discordant findings have been pub-

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lished.[5] The sensitivity can be increased by 10^2 using nested PCR. However, false positive results because of cross-contamination remain a major concern when this method is applied. From a technical point of view, nested PCR has been overcome by real-time PCR assays.[6] The advantages of real-time PCR include high sensitivity, monitoring and semiquantitative assessment of the amplified product during PCR, and a reduction of hands-on time. Quantification of mycoplasmas in the respiratory tract may be useful because recent work has shown that patients with acute infection harbor greater quantities of the pathogen in respiratory secretions (10^2 and 10^4 ccu/mL) than convalescent patients. Low (and unpredictable) copy numbers may be present in nonrespiratory sites.

RNA amplification techniques are a promising perspective in *M. pneumoniae* diagnosis. The theoretical advantage of RNA-based mycoplasma diagnosis is the higher sensitivity because a large number of rRNA copies ($>10^3$) is present in each cell. In addition, the detection of this nucleic acid is indicative of the presence of viable mycoplasmas. A reverse transcriptase (RT-) PCR for detection of *M. pneumoniae* has been developed.[7] In addition, the nucleic acid sequence-based amplification technique (NASBA) has been applied to the pathogen both in a conventional and in a real-time format.[8] The sensitivity of these assays is indicated with 5–50 ccu. A NASBA kit for detection of *M. pneumoniae* is commercially available.[9] In one case, amplification of eubacterial 16S rDNA and subsequent sequencing of the amplicon enabled diagnosis of *M. pneumoniae* infection in a case of osteomyelitis, in which pus cultures had been repeatedly negative.[10]

An ELISA kit for direct antigen detection and a hybridization assay using a ^{125}I-labeled DNA probe complementary to the rRNA of *M. pneumoniae* are commercially available. These methods show a sensitivity of 10^4 cfu/mL and have been replaced by amplification-based methods.

MYCOPLASMA HOMINIS

M. hominis is a common inhabitant of the urogenital tract of healthy adults, with colonization rates of up to 20%. The pathogen is associated with pelvic inflammatory disease (PID), bacterial vaginosis, spontaneous abortion, and postpartum fever. In addition, *M. hominis* can cause posthysterectomy wound infections, endocarditis, abscesses, septic arthritis, and suppurating infections of hematomas. Immunocompromised patients are at higher risk for extragenital infection. Kits for the isolation of this species and *U. urealyticum* (including antibiotic susceptibility testing) are commercially available, but PCR is more sensitive. A PCR assay targeting the 16S rDNA has been shown to provide a sensitivity of 10–100 genome copies (under optimized conditions).[11]

M. GENITALIUM, *MYCOPLASMA FERMENTANS*, AND *M. PENETRANS*

M. genitalium is associated with nongonococcal urethritis (NGU), cervicitis, and endometritis. The pathogen has also been recovered from joints (in patients with arthritis) and from the respiratory tract, whereby the pathogenetic role in the latter site is uncertain. Like *M. fermentans* and *M. penetrans*, *M. genitalium* has been investigated intensively in the early 1990s because of its suspected role in the pathogenesis of AIDS. This ability of *Mycoplasma* spp. could not be demonstrated unequivocally, but immunocompromised patients are at risk for colonization and infection with these mycoplasmas. *M. genitalium* shows a high degree of similarity with *M. pneumoniae* with regard to cell morphology (flask shape and the terminal tip-like attachment structure) and antigens. These two pathogens may be present concomitantly in respiratory, urogenital, and synovial fluid samples. In these cases, isolation of the pathogen is problematic because *M. pneumoniae* shows a similar colony morphology and the same susceptibility to antimicrobial agents (except thallium acetate, which inhibits selectively the growth of *M. genitalium*). Primer pairs targeting the sequences encoding for the MgPa protein and the 16S rRNA have been published, whereby the latter assay has been shown to be more sensitive.[12] A real-time PCR assay based on amplification of the *M. genitalium* 16S rRNA gene with a sensitivity of 10 copies/reaction has been developed.[13]

M. fermentans has been shown to cause arthritis and acute respiratory distress syndrome (ARDS). The pathogenic potential of *M. penetrans* is not fully elucidated; however, bacteremia in an immunocompetent patient has been described. Both pathogens have been implicated in the pathogenesis of chronic fatigue syndrome. PCR primers targeting the 16S rDNA (*M. fermentans*) and the 16S–23S rDNA spacer region (*M. penetrans*) have been described.[14,15]

U. UREALYTICUM AND *UREAPLASMA PARVUM*

U. urealyticum causes nongonococcal urethritis (NGU) and septic arthritis in immunocompetent and immunocompromised patients. The pathogen is of special importance in newborns, where it may cause bloodstream infection, pneumonia, and central nervous system disease. The clinical significance of *U. parvum*, the

second human-pathogenic member of the genus established in 2002, remains to be determined. PCR primer targeting the 16S rDNA of *U. urealyticum* (which show no cross-reaction with the *U. parvum* genome) has been described.[11]

SIMULTANEOUS DETECTION OF SEVERAL MOLLICUTE SPECIES

As mollicutes usually cause diseases of the respiratory or the urogenital tract, where the spectrum of pathogens to be considered is large, considerable interest has been focused on the simultaneous detection of several mollicutes, or mollicutes and other pathogens, by the same assay. Although the regions V2 and V3 of the 16S rDNA display sufficient interspecies differences for species-specific detection, conserved sequences of the 16S rDNA gene enable detection of most *Mycoplasma* spp., *Ureaplasma* spp., *Spiroplasma* spp., and *Acholeplasma* spp. by a single assay using the primer pair GPO-1/MGSO.[9] All mollicutes, which have been isolated from humans, are listed in Table 1.

A recently described seminested PCR based on this assay using the additional primer My-ins provides improved sensitivity. This assay has been shown to allow amplification of *M. genitalium*, *M. hominis*, *Mycoplasma faucium*, *U. urealyticum*, and *U. parvum* specific sequences.[16]

A variety of assays for simultaneous detection of mollicutes and other pathogens have been described, e.g., a multiplex real-time PCR assay to detect *M. pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila* in respiratory samples.[17] In addition, a multiplex RT-PCR for the detection of *M. pneumoniae* and eight additional respiratory pathogens has been constructed.[18] Simultaneous detection of *M. hominis* together with the bacterial vaginosis-associated pathogens *Lactobacillus* spp. and *Gardnerella vaginalis* by real-time PCR has been described.[19]

When cell cultures are to be tested for mycoplasma contamination, a convenient alternative to PCR (using GPO-1/MGSO) is a commercially available ELISA kit for antigen detection.

MOLECULAR TYPING OF *MYCOPLASMA* SPP. AND *UREAPLASMA* SPP.

Molecular typing of *Mycoplasma* spp. and *Ureaplasma* spp. is rarely indicated, but outbreaks of respiratory

Table 1 Mollicutes infecting/colonizing humans

Genus	Species	Site of colonization		Clinical relevance	Published PCR primer sequences
		Oropharynx	Genitourinary tract		
Mycoplasma	*salivarium*	Yes	No	•	Yes
Mycoplasma	*orale*	Yes	No	•	Yes[a]
Mycoplasma	*buccale*	Yes	No	?	No
Mycoplasma	*faucium*	Yes	No	?	No
Mycoplasma	*lipophilum*	Yes	No	?	No
Mycoplasma	*pneumoniae*	Yes	?	•••	Yes
Mycoplasma	*hominis*	Yes	Yes	•••	Yes
Mycoplasma	*genitalium*	Yes	Yes	••	Yes
Mycoplasma	*fermentans*	Yes	Yes	••	Yes
Mycoplasma	*primatum*	No	Yes	?	No
Mycoplasma	*spermatophilum*	No	Yes	?	No
Mycoplasma	*pirum*	?	?	?	No
Mycoplasma	*penetrans*	No	Yes	•	Yes
Mycoplasma	*arginini*	?	?	•	Yes[a]
Mycoplasma	*felis*	?	?	•	No
Mycoplasma	*canis*	?	?	•	No
Ureaplasma	*urealyticum*	Yes	Yes	•••	Yes
Ureaplasma	*parvum*	?	Yes	?[b]	Yes
Acholeplasma	*laidlawii*	Yes	No	?	Yes[a]
Acholeplasma	*oculi*	?	No	?	No

Graduation of clinical relevance: •—anecdotal reports; ••—human infection is rare and/or restricted to patients at risk; •••—well-established, frequently encountered pathogen.

[a]Primer sequences for in vitro studies have been constructed but not applied to clinical specimens.

[b]*Ureaplasma parvum* has been described only recently (2002), so that a considerable clinical relevance cannot be ruled out.

Source: Ref. [22].

disease because of *M. pneumoniae* or *M. hominis* infections following surgery may warrant the application of these techniques. Pulsed field gel electrophoresis (PFGE) is regarded as gold standard in molecular typing. The disadvantages of PFGE are that the method is time-consuming and cost-intensive. More convenient alternatives, which have been applied to *M. pneumoniae*, include random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analysis.[20] In addition, serological typing has been applied to this pathogen. Arbitrarily primed (AP-) PCR has been used to investigate the transmission of *M. hominis* and *U. urealyticum*.[21]

CONCLUSION

Although all human-pathogenic mollicutes are cultivable using special media, PCR provides superior diagnostic accuracy. Diagnosis of *M. pneumoniae* infection can be based on serology, whereas PCR is preferable for other *Mycoplasma* spp. and *Ureaplasma* spp. When these pathogens are to be detected in their natural mucosal habitat, copy numbers above the sensitivity level of standard single-step PCR assays can be expected, especially when they cause clinically significant infection. PCR-based methods of superior sensitivity (e.g., real-time PCR and NASBA assays) are useful for detection of low copy numbers of the pathogen in atypical sites. Common methods for molecular typing (PFGE, RAPD, and AP-PCR) have been applied to some *Mycoplasma* spp. and to *U. urealyticum*, whereby these versatile techniques can be applied to any human-pathogenic mollicutes.

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Myelodysplastic Syndrome—Risk Class Analysis by Microarray

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Wolf-Karsten Hofmann
J. W. Goethe University, Frankfurt, Germany

INTRODUCTION

Myelodysplastic syndromes (MDS) are clonal disorders characterized, initially, by ineffective hematopoiesis and, subsequently, by frequent development of acute myeloid leukemia (AML). Peripheral blood cytopenias, in combination with a hypercellular bone marrow exhibiting dysplastic changes, are the hallmarks of MDS. In 1982, the French–American–British (FAB) Cooperative Group classified five subentities of MDS: refractory anemia (RA), refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-T), refractory anemia with ringed sideroblasts (RARS), and chronic myelomonocytic leukemia (CMML). This classification based on morphological criteria was recently revised, resulting in the World Health Organization (WHO) classification that includes, in addition, specific chromosomal changes.

In addition to FAB and WHO classifications, the initial chromosomal aberration, the age of patient, and the number and severity of cytopenias are most important to evaluate the prognosis of MDS as summarized in the International Prognostic Scoring System (IPSS). The median survival of MDS patients according to this classification ranges from 6 years for low-risk patients to 6 months for high-risk patients. The value of IPSS for assessing individual prognosis in patients with MDS has been demonstrated in several further studies.

OVERVIEW

The recently available microarray technology is a powerful new tool to assess the expression of a large number of genes in a single experiment. The most important use of array technology in medical science is to characterize global gene expression profiles, which are specific for disease subgroups or have prognostic value (e.g., for disease progression). The aim of those studies is to accelerate the identification of diagnostic and prognostic markers, and to identify novel tumor-specific markers for certain malignant diseases.

One of the groundbreaking studies[1] using oligonucleotide microarrays showed that global gene expression analysis can distinguish with high accuracy between leukemic blasts from patients with either AML or acute lymphoblastic leukemia (ALL). Subsequently, a number of studies[2–4] analyzing different kinds of tumor entities including malignant lymphomas or solid tumors (e.g., breast cancers carrying mutations of *BRCA1* and *BRCA2*) were performed by demonstrating the power of microarray analyses to correlate specific gene expression profiles with well-established classification/prognostic systems.

TECHNIQUE OF GLOBAL GENE EXPRESSION ANALYSIS

$CD34^+$ Cell Selection and Nucleic Acid Preparation

Fresh bone marrow is processed immediately after aspiration to select the $CD34^+$ cells within the next 2 hr. $CD34^+$ cells are purified by high-gradient magnetic cell separation (MACS) using superparamagnetic streptavidin microparticles. Yield and purity of the positively selected $CD34^+$ cells are evaluated by flow cytometry.

Total RNA is extracted using TRIzol (Life Technologies, Grand Island, NY) according to the manufacturer's protocol with minor modifications. In addition, RNA is purified by the RNeasy system (Qiagen, Inc., Valencia, CA) following the manufacturer's advice. The quality of RNA is critical for any microarray experiment. To assure that the gene expression measured by microarray assay is not affected by degradation of the RNA extracted from the purified $CD34^+$ cells, the Bioanalyzer system (Agilent, Waldbronn, Germany) is used to evaluate the quality of the RNA. Figure 1 shows the Bioanalyzer pattern of a high-quality RNA compared with a pattern that is related to degradation of RNA.

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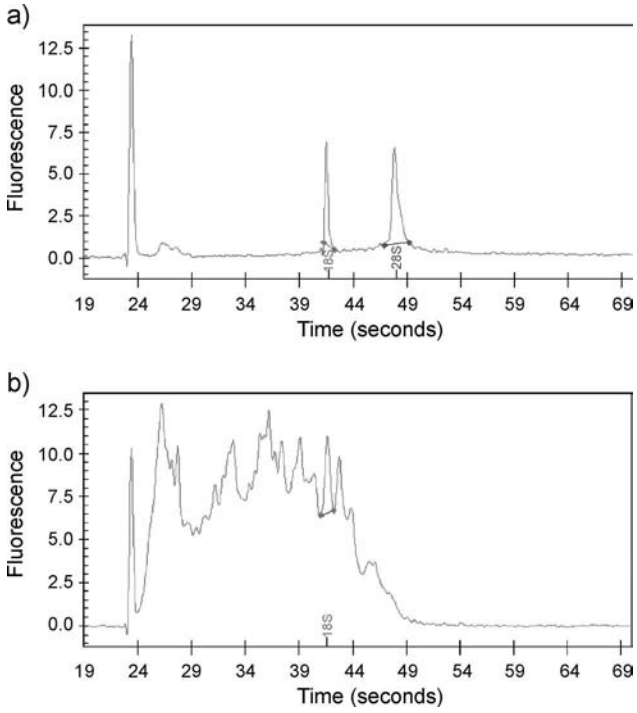

Fig. 1 Quality of RNA from $CD34^+$ cells as determined by the Bioanalyzer system. (a) Analysis image of a high-quality total RNA extracted from $CD34^+$ cells. Beside the marker spike (left), the 18S and 28S spikes (right) are clearly separated. There is no significant degradation of RNA. (b) Image of a degraded total RNA. Beside the marker spike, there is no clear separation of the 18S and/or 28S fraction demonstrating the high level of RNA degradation. (*View this art in color at www.dekker.com.*)

Oligonucleotide Microarrays and Data Analysis

The GeneChip system (Affymetrix, Inc., Santa Clara, CA) can be used for oligonucleotide microarray analysis of $CD34^+$ MDS cells. The detailed protocol for sample preparation and microarray processing is available from www.affymetrix.com. Because of the limited number of $CD34^+$ cells and the low content of RNA in these hematopoietic stem cells, a double in vitro transcription technique (''nanogram-scale assay'') has been established. To assay 50 ng of total RNA, the standard Affymetrix target amplification protocol has been modified by using first-round cRNA product to generate a double-stranded cDNA that is then used for a second round of in vitro transcription for synthesis of the biotinylated cRNA. GeneChip image analysis is performed using the Microarray Analysis Suite 5.0 (Affymetrix). Data are analyzed by the GeneSpring™ software version 5.1 (Silicon Genetics, Inc., San Carlos, CA).

Class membership prediction[5,6] refers to the process of statistically dividing clinical samples (e.g., bone marrow cells) into reproducible classes that have similar gene expression behaviors or properties. Using a ''training set'' a list of predictive genes that can be used to discriminate one group from other groups of samples (diseases) exclusively according to their gene expression profile is selected. In a second analysis, the predictive genes are used to analyze a ''test set'' of additional samples whose classes are currently not known to place such samples into already known classes.

PREDICTION OF DISEASE RISK USING $CD34^+$ CELLS FROM PATIENTS WITH MDS

The clinical diagnosis of typical MDS according to FAB/WHO[7,8] criteria is often straightforward and presents no difficulty. Although the diagnosis may be suspected based on the history and the peripheral blood findings, morphological examination of the bone marrow and cytogenetics are essential to establish the final diagnosis and to estimate the prognosis of the disease. The latter may be difficult and requires additional genetic information, which can hopefully be obtained from global gene expression data in MDS.

We and others[9,10] have shown that characterizing the cellular and molecular defects of clinical samples from patients with MDS by global gene expression analysis requires the analysis of a freshly isolated and homogenous population of bone marrow cells. Because expression of CD34 is a marker for hematopoietic stem cells, experiments to detect abnormalities have focused on $CD34^+$ cells from patients with MDS. The first description of MDS-specific genes that were found to be overexpressed in $CD34^+$ cells from patients with MDS compared with leukemic cells from patients with AML was published in 2001.[10] Twenty genes [including delta-like 1 (DLK1), a secreted protein that has epidermal growth factor motifs] were selected by direct comparison of expression data in MDS vs. AML. By hierarchical cluster analysis, it could be demonstrated that the selected genes can be used to clearly distinguish the MDS samples from the AML samples.

We have used two independent data sets created from a total of 19 patients with MDS to predict the risk groups of those patients at the time of initial diagnosis. Using the first data set (11 patients, samples 275–286; Fig. 2a), we identified by class membership prediction 11 genes whose expression can be used to differentiate with high accuracy between low-risk MDS, high-risk MDS, and normal individuals (samples 271–274; Fig. 2a).

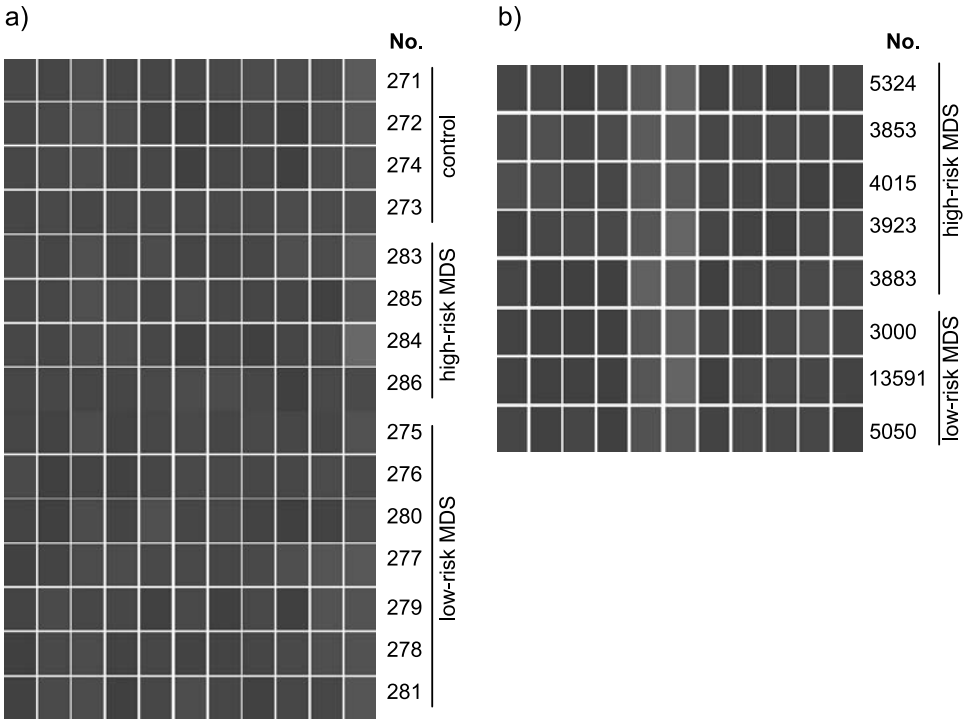

Fig. 2 Identification of genes expressed in $CD34^+$ marrow cells, which can distinguish between low-risk MDS, high-risk MDS, and healthy individuals. (From Ref. [9].) (a) Results represent the analysis by hierarchical clustering with Spearman's confidence correlation of 15 samples of $CD34^+$ bone marrow cells. Eleven genes were selected to predict the class membership of each of the samples. Vertical: Each of the samples. Horizontal: Each of the 11 predictive genes. (b) Validation of the 11 predictive genes used to distinguish between high-risk and low-risk MDS by gene expression profiling. The 11 predictive genes were used for clustering analysis in a second data set obtained from $CD34^+$ cells from patients with low-risk and high-risk MDS (test set). Two clusters corresponding to the IPSS classifications of ''high risk'' and ''low risk'' were found. No misclassification of any of the samples of the test set occurred, demonstrating the power of the selected genes for risk group prediction. No., sample number. Color code: blue, low expression; red, high expression. The intensity of the color reflects the reliability of the expression data. (*View this art in color at www.dekker.com.*)

To verify the power of the genes selected by class membership prediction, we used the 11 genes for hierarchical clustering with Spearman's confidence correlation (Fig. 2a). We generated three clusters. All of the controls (normal $CD34^+$ cells) were in one cluster (with a maximum of two subclusters). Furthermore, all of the high-risk MDS and low-risk MDS were separated in different clusters with a maximum of three subclusters.[9]

In a second experiment, we have used the 11 predictive genes to do clustering analysis in a independent data set obtained from $CD34^+$ cells from patients with low-risk and high-risk MDS (samples 3000–13591; Fig. 2b). The aim of this additional experiment was to evaluate the predictive power of the 11 selected genes in new series of patient samples. As shown in Fig. 2b, we found two clusters corresponding to the IPSS[11] classification ''high risk'' and ''low risk.'' No misclassification of any of the samples of the test set occurred, demonstrating the power of the selected genes for risk group prediction.

CONCLUSION

Microarray analysis can detect gene expression profiles that are strongly associated with different risk groups in MDS. Therefore we believe that the prognosis of this disease may be predicted by using gene expression analyses of purified $CD34^+$ cells. We have shown that microarray analysis can be used with small amounts of RNA, which can be obtained from cells during routine diagnostic bone marrow aspiration. This method may facilitate the making of therapeutic decisions in cases where the diagnosis and/or risk evaluation is not possible based on morphological and classical cytogenetic data. The results from a number of studies using $CD34^+$ cells as well as other cell types (e.g., granulocytes) from patients with MDS are pending. Furthermore, such analysis of global gene expression provides new insights into alterations of cellular pathways in early hematopoietic stem cells in MDS.

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Myotonic Dystrophy

M

Josef Finsterer
Krankenanstalt Rudolfstiftung, Wien, Austria

INTRODUCTION

Myotonic dystrophy, also termed dystrophia myotonica (DM), is an autosomal dominant, progressive, multisystem, myotonic disorder, of which the key feature is delayed muscle relaxation (clinical myotonia) because of muscle fiber hyperexcitability from spontaneous or induced runs of repetitive muscle fiber action potentials (electrical myotonia).[1] DM is genetically heterogeneous and is subdivided into type-1 (DM1) and type-2 (DM2). Although there are aspects that differentiate DM1 and DM2, many features overlap, so that only molecular genetic investigations unambiguously distinguish them (Table 1).[2]

CLINICAL DESCRIPTION

DM1

Ninety percent of DM1 patients present at adulthood with delayed muscle maturation, distal muscle weakness, wasting, myotonia, cataracts, cardiac abnormalities, smooth muscle dysfunction, insulin resistance, daytime sleepiness, testicular atrophy (low reproductive fitness), ''difficult'' personality, neuropsychiatric disturbances, and frontal balding.[1] Ten percent of the patients present at infancy with hypotonia (floppy infant), oromotor dysfunction, tent-shaped mouth, feeding and respiratory insufficiency (diaphragmatic hypoplasia), arthrogryposis, and mental retardation in those who survive until adulthood (congenital DM).[1] All manifestations show a progressive course. Usually, creatine kinase is elevated. Muscle biopsy shows type 1 predominance, centrally located nuclei, severe fiber atrophy with nuclear clumps, hypertrophic and angulated fibers, and occasionally, necrotic fibers, fibrosis, or fat deposits. Cardiac involvement comprises conduction defects (mostly HV prolongation) and, less frequently, primary cardiomyopathy (wall thickening, hypertrabeculation, dyskinesia, and reduced myocardial tissue velocities). Cerebral MRI shows large Virchow–Robin spaces, white matter lesions, and atrophy. There may also be megacolon, megarectum, and delayed gastric emptying without dyspeptic symptoms. Some patients have hypo-IgG and, in some cases, thyrotropin deficiency. Phenotype severity and age at onset vary greatly between patients.

DM2

Although less severe, DM2 closely resembles adult-onset DM1 with common features, including progressive weakness, myotonia, cardiac arrhythmias, cataract, male hypogonadism, insulin insensitivity, and hypogammaglobulinemia.[2] The most frequent manifestations are muscle pain, muscle stiffness, myotonia, and weakness.[2] Muscle pain is frequently fluctuating or episodic. Weakness characteristically affects the neck flexors, elbow extensors, deep finger flexors, hip flexors, and hip extensors. Facial and ankle dorsiflexion weakness is uncommon. Mild muscle wasting occurs in -10% of the cases.[2] Creatine kinase is typically mildly elevated. Muscle biopsy shows similar features as in DM1, but preferentially type 2 fiber atrophy. Cataracts of the posterior, subcapsular, and iridescent type develop between age 30 and 50 years.[2] Cardiac manifestations comprise palpitations, intermittent tachycardia, and episodic syncope, which frequently increase with age. Frequent ECG abnormalities are atrioventricular or intraventricular block. Some patients develop fatal arrhythmias. Idiopathic cardiomyopathy is rare. Cerebral MRI may demonstrate atrophy but less severe than in DM1. γ-Glutamyltransferase may be elevated.

MOLECULAR GENETICS

DM1

DM1 is caused by an unstable CTG expansion in the 3′ untranslated region of the dystrophia myotonica protein kinase (*DMPK*) gene on chromosome 19q13.3 (Table 1).[1] Affected subjects are usually heterozygous for the expansion. Only six homozygous subjects have been reported.[2] In affected subjects the repeat length varies between 50 and 5000. Alleles with 50–80 repeats are

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Table 1 Genetic differences/similarities between DM1/DM2

Genetic aspects	DM1	DM2
Transmission	Autosomal dominant[a]	Autosomal dominant[a]
Gene locus	19q13.3	3q21.3
Mutated gene	*DMPK*	*ZNF9*
Gene product	Protein kinase	Zinc finger protein 9
Function	Signal propagation[b]	RNA-binding protein[b]
Type of mutation	CTG expansion	CCTG expansion
Number of expanded repeats	50–5000	75–11000
Localization of mutation	3′ Untranslated region	Intron-1
Gene dosage	Hetero/homozygous	Homozygous
Somatic cell heterogeneity	+	++
Congenital cases	+	−
Intergenerational repeat-size variation	+	++
Anticipation	+	−
Reversions in offsprings	+	++
Increase of repeat size with age	+	+
Transcribed into RNA	+	+
Translated	−	−
Intranuclear RNA-foci	+	+
Premutation differences mother/offspring	+	Unknown
Premutation differences father/offspring	+	Unknown

[a]Disease despite normal allele.
[b]Assumed.

classified as protomutation with a mild or asymptomatic phenotype.[3] Alleles between 38 and 49 repeats, designated as premutation, do not manifest clinically, but may expand to the full mutation range in subsequent generations. In the normal population the repeat length ranges between 4 and 37. The normal allele is usually stable upon transmission. Occasionally mutations occur, causing a transition from a large normal allele to a small, expanded allele (transition mutation). One factor that prevents DM1 from dying out is segregation distortion (alleles with 19–37 repeats are preferentially transmitted to offsprings than alleles with 4–18 repeats). The expanded *DMPK* allele is transcribed but not translated. It produces RNA transcripts containing long tracts of CUG repeats, but no dysfunctional protein. Mutant mRNA is not exported to the cytoplasm but retained within multiple nuclear foci. The amount of *DMPK* in the cytoplasm is presumably reduced, as it can be translated only from the normal allele (haploinsufficiency).

A hallmark of the CTG expansion is its high intra-individual, interindividual and intergenerational variability in somatic and germline cells. Expansion instability is thought to arise from its capacity to form long hairpin loops during replication, leading to folded structures, and the involvement of various DNA-repair mechanisms. Several models try to explain how an encounter between folded structures and the replication apparatus leads to expansion, including the following: Rad52 and Rad54, involved in DNA strand break repair (homologous recombination), strongly destabilize long CTG repeats, enabling expansion. Msh2 (major component of the mismatch repair system) stabilizes anomalous slipped strand or loop end structures thereby promoting incorporation of further repeats.[4] DNA Pkcs is involved in nonhomologous end joining.[4] So far, however, it remains unknown how these mechanisms affect repeat instability.[4] Once in the disease-associated range, the expansion size progressively increases in subsequent generations (anticipation), although reversion back to a normal size may also occur (retraction). The CTG expansion continues to expand throughout life, causing age-dependent somatic mosaicism. During embryogenesis the CTG expansion remains fairly equal in various tissues, but postnatally marked variability emerges in different tissues. CTG expansions appear to continue to increase even in non-dividing cells. The repeat instability is tissue specific, with muscle cells and myocardiocytes containing larger expansions than blood leukocytes. Premutation alleles are generally more stable during transmission through the female than the male germline.[3] Small protomutations are very unstable in the male germline and highly biased toward expansion.[3] Congenital DM patients have CTG expansions >1000 and inherit the disease almost invariably from their mother, reflecting a sex difference in the dynamics of the repeat instability during gametogenesis. Rarely, fathers transmit congenital DM1. When the repeat

size varies between sibs, differences tend to be greater when the affected parent is the father.

Immunohistological investigations localize *DMPK*, an 80-kDa protein kinase, most highly expressed in the skeletal muscle and heart, to the neuromuscular junction, terminal cisternae of the sarcoplasmic reticulum, and gap junctions. *DMPK* consists of a leucine-rich repeat, a catalytic domain, an α-helical coil-coiled region, and a transmembrane-spanning tail.[5] The *DMPK* function is not yet fully understood.[6] *DMPK* has been implicated in modulating calcium homeostasis and initial events concerning excitation–contraction coupling and was associated with the heat shock protein myotonic dystrophy protein kinase-binding protein.[6] Additionally, the specific regulation of *DMPK* expression and activity during myocyte differentiation suggests a functional implication of *DMPK* in the generation or maintenance of myotubes.[6] *DMPK* is reduced in the DM1 muscle.

Mechanisms to explain the deleterious effect of mutant *DMPK* are the following:

1. Chromatin conformation alteration in the vicinity of the *DMPK* gene, resulting in partial suppression of neighboring genes, like the dystrophia myotonica-associated homeobox protein (DMAHP or SIX5) or immunodominant peptide N59. Suppression of DMAHP may contribute to the development of cataracts.[7]
2. Haploinsufficiency, resulting in shortage of the functionally available enzyme.
3. Altered cellular *DMPK* location.
4. Disturbed distribution and transport of the expanded mRNA transcripts.
5. Toxic effect of mutant mRNAs by sequestering essential RNA-CUG-binding proteins.There is evidence that rather a gain of function for RNA than haploinsufficiency plays the prominent pathogenetic role.[7] Gain of function involves a transdominant effect (both alleles are poorly processed) on RNA-CUG-binding proteins, such as CUG-binding protein 1 (CUGBP1), elav-like RNA-binding protein 3 (ETR3), muscleblind proteins (MBNL, MBLL, and MBXL), and PKR.[7] Gain of function is substantiated by recruitment of muscleblind proteins into ribonuclear inclusions in fibroblasts and muscle cells from DM1 and DM2 patients, which clearly interact with expanded CUG repeats. mRNA accumulation presumably skews alternative splicing also of unrelated specific pre-RNAs,[8] because RNA CUG induces reiteration of and reversion to embryonic splicing patterns, mediated by CELF proteins. CUGBP1 does not extensively bind expanded CUG repeats in vitro, although some studies detected CUGBP1 within ribonuclear inclusions.

Targets of abnormal splicing are mRNAs for:

1. The insulin receptor. Insulin resistance may be due to missplicing of exon 11 skipping isoform pre-mRNA.
2. Cardiac troponine-T. Splicing regulation of troponine transcripts is abnormal.
3. Tau protein. Altered stoichiometry of the exon 2 skipping tau protein isoform could explain behavioral and cognitive alterations.
4. Myotubularin-1. Muscle-specific isoform is reduced and an abnormal transcript appears in differentiated DM1 myocytes.
5. Muscle-specific chloride channel ClC-1. Loss of ClC-1 mRNA and protein after inappropriate regulation of ClC-1 alternative splicing because of CUGBP-1 overactivity results in hyperexcitability as a result of reduced transmembrane chloride conduction.[7]
6. MyoD. This transcription factor, required for the differentiation of myoblasts during muscle regeneration, by activating differentiation-specific genes via binding E-boxes, is reduced in cells containing mutant *DMPK* transcripts.[8] All these findings suggest abnormal regulation of alternative splicing to be fundamentally involved in the pathogenesis of DM1. There is also evidence for a delay in muscle maturation, possibly because of a retarded rate of myoblast fusion to multinucleated myotubes.[6]

DM2

DM2 is caused by an unstable CCTG expansion in intron-1 of the *ZNF9* gene.[9] Allele expansions range from 75 to 11,000 repeats (mean 5000; (Table 1). Because the CCTG repeat is too large to amplify by PCR, all expansion-positive individuals appear to be homozygous and are thus indistinguishable from unaffected truly homozygous controls. Family studies may distinguish true homozygotes from expansion carriers. Failure of the expanded allele to amplify, also referred to as ''blank allele,'' suggests a non-Mendelian inheritance pattern and provides evidence that a family carries a DM2 expansion or that paternity was misinterpreted.[2] Expansion sizes in the blood of affected children are usually shorter than in their parents. Most affected American families can trace an affected ancestor to Germany or Poland and all are of European descent.[2]

ZNF9, also referred to as cellular nucleic acid-binding protein, contains 7 zinc-finger domains and is thought to be an RNA-binding protein. It is broadly expressed, with the highest expression in heart and skeletal muscle. Mutant *ZNF9* RNA accumulates in numerous nuclear foci, similar to those in DM1, but with lower stability of the probe–target complex. Whether nuclear RNA foci contain the entire unprocessed *ZNF9* transcript is unknown. As in

DM1, intranuclear foci bind specific RNA-binding proteins, leading to altered splicing of the insulin receptor and CLC1.[2]

MOLECULAR GENETIC TESTING

DM1

Small expansions are determined by standard PCR. Large expansions are refractory to PCR and are traditionally identified by Southern blotting by hybridization of restriction-digested genomic DNA. For Southern blotting, genomic DNA is digested with *Eco*R1 and then separated according to size by agarose gel electrophoresis. After denaturation and neutralization, DNA fragments are transferred onto a membrane, which is then randomly probed with ^{32}P-labelled cDNA25. Blots are then washed and exposed at -80°C for 5–10 days.

DM2

In a first step, PCR amplification is performed across the CCTG repeat.[9] For genomic Southern analysis, DNA is digested with *Bso*B1 or *Eco*R1.[2] Because of the size and somatic instability of the CCTG repeat, Southern analysis fails to detect expansions in 20% of the known carriers.[2] Expanded alleles appear as single discrete bands, multiple bands, or smears. Contrary to other expansion disorders, *ZNF9* expansions are almost always less intense than normal alleles.[2] To improve detection of CCTG repeat expansions, a repeat assay has been developed, amplifying the region containing the CCTG expansion by use of the primers CL3N58-D R (5′-GGCCTTATAACCATG-CAAATG-3′), JJP4CAGG (5′-TACGCATCCGAGTTT-GAGACGCAGGCAGGCAGGCAGGCA-GG-5′), and JJP3 (5′-TACGCATCCGAGTTTGAGACG-3′) followed by Southern analysis of the PCR products probed with an internal probe. By using a PCR primer that primes from multiple sites within the CCTG repeat, expansions are detected by the presence of a smear of products with a molecular weight higher than in control lanes.[2] To ensure specificity, repeat assay PCR products are transferred to a nylon membrane and probed with an internal oligonucleotide probe.[2] The repeat assay cannot be used to determine the CCTG repeat length.

GENOTYPE–PHENOTYPE CORRELATION

DM1

Conflicting results indicate a generally poor genotype–phenotype correlation.[3] Only disease severity and age at onset correlate positively with the CTG expansion size.[1] At least in patients with CTG expansions > 1000, the risk of intraventricular conduction delay increases with increasing expansion size. Various other measures of disease severity correlate positively with the CTG repeat size in leukocytes, although the range of disease severity associated with a particular repeat size is broad.

DM2

Because of the somatic instability of the expansion (-9000 repeats in the blood of a single patient), a final assessment of phenotype–genotype correlations is not possible. At least the CCTG expansion size increases with age and age at onset.[2]

PREVALENCE

DM1

DM1 is one of the most common inherited neuromuscular disorders with a worldwide prevalence of 2.1–14.3 per 100,000.[1] The prevalence is unexplainably increased in isolates in Canada (Saguenay—Lac-Saint-Jean region, prevalence: 189 per 100,000), Sweden, and Iceland. The worldwide incidence of DM1 is 1:8000.

DM2

By pooling the data of all publications since 1994, approximately 500 DM2-patients have been identified. Although the exact incidence and prevalence is unclear, it is considered to be similar to DM1, based on the clinical similarity between DM1/DM2. The incidence of DM2 is estimated to be 1:20000. DM2 is equally frequent in both genders.

DIFFERENTIAL DIAGNOSES

Differential diagnoses that have to be considered before diagnosing DM are other myotonic syndromes, facioscapulohumeral muscular dystrophy, oculopharyngeal muscular dystrophy, limb-girdle muscular dystrophy, congenital muscular dystrophy, distal muscular dystrophy, hereditary inclusion body myositis, congenital myopathy, endocrine myopathy, motor neuron disease, and mitochondrial encephalomyopathy.

MANAGEMENT

So far, there is no causal treatment. Physiotherapy is recommended as soon as weakness or myotonia occurs. Orthoses may be helpful to stabilize knees or ankles. Treatment with nonsteroidal antiinflammatory drugs, carbamazepine, or corticosteroids ameliorates muscle pain in single DM1 patients. If myotonia predominates in DM1, mexiletine, phenytoin, or carbamazepine may be beneficial, but no data supporting its effectiveness in DM2 are available. For muscle cramps, quinine sulfate may be tried. However, if cardiac conduction defects are present, antiarrhythmic medication should be avoided. Modafinil reduces daytime sleepiness and improves mood in DM1 patients. Testosterone or growth hormone increases muscle mass but fails to improve muscle strength. As soon as the diagnosis is established, patients should undergo cardiological examinations. Reinvestigation is indicated if baseline investigations are highly abnormal or if patients become cardiologically symptomatic. At least an ECG should be recorded annually. In case of arrhythmias, appropriate therapy is essential. Patients should be regularly evaluated for thyroid dysfunction. If indicated, hormone replacement therapy should be initiated. Diabetes requires adequate therapy. Cataracts may require surgical treatment at some point. Noninvasive ventilatory support is effective in nocturnal hypoventilation. Because single DM1 patients experience respiratory depression in response to benzodiazepines, opiates, or barbiturates, aggravation of myotonia by depolarizing muscle relaxants or malignant hyperthermia-like manifestations during anesthesia, general anesthesia should be carried out with caution and the postoperative period needs careful attendance. In DM1 patients, survival to higher ages is markedly reduced. In DM2, progression is slow, and prognosis and life expectancy are more favorable than in DM1. Life expectancy may be reduced only in single cases, mainly if there are malignant rhythm abnormalities.

GENETIC COUNSELING

DM1

Genetic counseling of an affected proband or an asymptomatic mutation carrier aims to clarify their genetic status and relies on the family history and genetic testing. In case of an affected proband it is essential to investigate the parents, sibs, and offsprings of the proband. Offsprings of a DM1 patient carry a 50% chance of inheriting the mutant allele. The chance of a female DM1 patient of having an affected child increases with the severity of the maternal phenotype. A mother who has had a previous child with congenital DM has 20–40% risk to have another affected child. Before prediction testing of an at-risk asymptomatic adult is carried out, an affected family member should be tested. Asymptomatic children at risk for adult-onset DM1 should not be tested. In counseling a person with DM1 it is prudent to mention the small possibility of transmitting congenital DM. Genetic counseling is generally hampered by the high instability of the CTG expansion and the poor genotype–phenotype correlation.

DM2

Generally, genetic counseling is complicated by the highly unstable CCTG expansion and the uncertain genotype–phenotype correlation. CTG expansions in the blood of affected children are usually shorter than in their parents.[9] Males have a minor probability than females to transmit the mutation to their offspring. If DM1 is suspected, probands should undergo genetic testing as the golden standard of diagnosing this condition. DNA analysis is also applicable in prenatal testing. However, if abortion is not desired, prenatal testing should not be performed.

CONCLUSION

DM is genetically heterogeneous and comprises at least two disorders (DM1 and DM2). DM1 and DM2 are clinically similar, progressive multisystem, myotonic disorders due to a CTG repeat expansion in the *DMPK* gene (DM1) or to a CCTG repeat expansion in the *ZNF9* gene (DM2). Both expansions show high somatic, interindividual, and intergenerational instability and uncertain genotype–phenotype correlation. Both expansions are transcribed but not translated. Transcripts of the mutant alleles, harboring large CUG and CCUG expansions, are retained within nuclear foci. Suggested pathomechanism is considered to be a transdominant effect on the function of RNA-binding proteins, rather than haploinsufficiency. CUG-binding proteins regulate alternative splicing of proteins such as troponine-T, insulin receptor, ClC-1, myotubularin-1, tau protein, myo-D, and presumably various other mRNAs. Genetic counseling is hampered by the high expansion variability. Therapy of DM1 and DM2 is symptomatic. Preliminary results show that ribozyme-mediated cleavage of mutant *DMPK* mRNA reduces nuclear foci and partially restores function of the insulin receptor.[10] Other promising therapeutic concepts rely on the delivery of antisense RNA, which is capable of inhibiting mutant *DMPK* transcripts. Paralleles between the *ZNF9* gene mutation and the *DMPK* gene mutation indicate that RNA microsatellite expansions in a noncoding portion of a gene,

transcribed but untranslated, are pathogenic and cause multisystem disease.

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Nanotechnology-Based Lab-on-a-Chip Devices

N

K. K. Jain
Jain PharmaBiotech, Basel, Switzerland

INTRODUCTION

Scientists and engineers have borrowed miniaturization, integration, and parallel-processing techniques from the computer industry to develop laboratory devices and procedures that fit on a wafer or microchip. Biochip is a broad term indicating the use of microchip technology in molecular biology and can be defined as arrays of selected biomolecules immobilized on a surface. A microarray is a collection of miniaturized test sites arranged on a surface that permits many tests to be performed simultaneously, or in parallel, in order to achieve higher throughput. The sample spot sizes in microarray are usually less than 200 μm in diameter. The trend to miniaturization continued with the introduction of microfluidic technology. To meet the increasing needs, the density and information content of the microarrays is being improved. One approach is fabrication of chips with smaller, more closely packed features—ultra-high-density arrays, which will yield:

- High information content by reduction of feature size from 200 to 50 nm.
- Reduction in sample size.
- Improved assay sensitivity.

NANOTECHNOLOGY

The process of miniaturization in biochip technology has been revolutionized by the introduction of nanotechnology—a term used for the creation and utilization of materials, devices, and systems through the control of matter on the nanometer-length scale, i.e., at the level of atoms, molecules, and supramolecular structures. The term is applied for the construction and utilization of functional structures with at least one characteristic dimension measured in nanometers (a nanometer is one billionth of a meter (10^{-9} m). Nanotechnology is applied for healthcare when it is referred to as nanobiotechnology. The topic is discussed in more detail elsewhere.[1] Nanobiotechnology has also been applied to molecular diagnostics and referred to as nanodiagnostics.[2] The assigned title of the article ''lab-on-a-chip'' indicates focus on nanofluidics. However, several nanotechnologies shown in Table 1 are used for molecular diagnostics and some of these can be applied in the form of nanochips.

Microarrays and Nanoarrays

Nanoarrays are the next stage in the evolution of miniaturization of microarrays. Whereas microarrays are prepared by robotic spotting or optical lithography, limiting the smallest size to several microns, nanoarrays require further developments in lithography strategies used in nanotechnology and include the following:

- Electron beam lithography.
- Dip-pen nanolithography (DPN).
- Scanning probe lithography.
- Finely focused ion beam lithography.
- Nanoimprint lithography.

Protein Nanoarrays

High-throughput protein arrays allow the miniaturized and parallel analysis of large numbers of diagnostic markers in complex samples. This capability can be enhanced by nanotechnology. Dip-pen nanolithography technique has been extended to protein arrays with features as small as 45 nm, and immunoproteins as well as enzymes can be deposited. Selective binding of antibodies to protein nanoarrays can be detected without the use of labels by monitoring small (5–15 nm) topographical height increases in atomic force microscopy (AFM) images.

Protein Nanoarrays (BioForce Nanosciences, Ames, IA, USA) contain up to 25 million spots per square centimeter. They can be used to detect protein–protein interactions. BioForce's NanoReader uses a customized AFM to decipher molecules on a NanoArray chip. The company was awarded a grant in 2002 by the U.S. Department of Defense for breast cancer research titled, ''Protein Nanoarrays for Studying Malignant Progression in Breast Cancer Cell Lines.'' The project constructed a nanoscale protein array platform and used it as a basic research tool to study alterations in cellular signaling pathways that accompany breast cancer disease progression.

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Table 1 Nanotechnologies with potential applications in molecular diagnostics

Nanotechnology on a chip
Microfluidic chips for nanoliter volumes: NanoChip
Optical readout of nanoparticle labels
NanoArrays
Protein nanoarrays
Nanoparticle technologies
Gold particles
Nanobarcodes
Magnetic nanoparticles: Ferrofluids, supramagnetic particles combined with MRI
Quantum dot technology
Nanoparticle probes
Nanowires
Nanopore technology
Measuring length of DNA fragments in a high-throughput manner
DNA fingerprinting
Haplotyping
Cantilever arrays
Multiple combined tests (such as protein and DNA) to be performed on the same disposable chip
Prostate-specific antigen binding to antibody
DNA nanomachines for molecular diagnostics
Nanoparticle-based immunoassays
DNA–protein and nanoparticle conjugates
Nanochip-based single-molecular interaction force assays
Resonance light scattering technology
Nanosensors
Living spores as nanodetectors
Nanopore nanosensors
Quartz nanobalance DNA sensor
Probes encapsulated by biologically localized embedding (PEBBLE) nanosensors
Nanosensor glucose monitor
Photostimulated luminescence in nanoparticles
Optical biosensors: Surface plasmon resonance technology

Source: From Ref. [1].

Microfluidics and Nanofluidics

Microfluidics is the handling and dealing with small quantities (e.g., microliters, nanoliters, or even picoliters) of fluids flowing in channels the size of a human hair (approx. 50 μm thick) or even narrower. Fluids in this environment show very different properties than in the macro world. This new field of technology was enabled by advances in microfabrication—the etching of silicon to create very small features. Microfluidics is one of the most important innovations of biochip technology. Typical dimensions of microfluidic chips are 1–50 cm^2 and have channels 5–100 μm. Usual volumes are 0.01–10 μL but can be less. Microfluidics is the link between microarrays and nanoarrays as we reduce the dimensions and volumes.

Microfluidics is the underlying principle of lab-on-a-chip devices, which carry out complex analyses, while reducing sample and chemical consumption, decreasing waste and improving precision and efficiency. The idea is to be able to squirt a very small sample into the chip, push a button and the chip will do all the work, delivering a report at the end. Microfluidics allows the reduction in size with a corresponding increase in the throughput of handling, processing, and analyzing the sample. Other advantages of microfluidics include increased reaction rates, enhanced detection sensitivity, and control of adverse events.

Drawbacks and limitations of microfluidics and designing of microfluidic chips include the following:

- Difficulties in microfluidic connections.
- Because of laminar flows, mixing can only be performed by diffusion.
- Large capillary forces.
- Clogging.
- Possible evaporation and drying up of the sample.

Applications of microfluidics include the following:

- DNA analysis.
- Protein analysis.
- Gene expression and differential display analysis.
- Biochemical analysis.

NANOTECHNOLOGY-BASED LAB-ON-A-CHIP

Nanotechnology on a chip is a new paradigm for total chemical analysis systems. The ability to make chemical and biological information much cheaper and easier to obtain is expected to fundamentally change healthcare, food safety, and law enforcement. Lab-on-a-chip technology involves micrototal analysis systems that are distinguished from simple sensors because they conduct a complete analysis; a raw mixture of chemicals goes in and an answer comes out. Sandia National Laboratories (Albuquerque, NM, USA) is developing a handheld lab-on-a-chip that will analyze for airborne chemical warfare agents and liquid-based explosives agents. This development project brings together an interdisciplinary team with areas of expertise including microfabrication, chemical sensing, microfluidics, and bioinformatics. Although nanotechnology plays an important role in current efforts, miniaturized versions of conventional

architecture and components such as valves, pipes, pumps, separation columns are patterned after their macroscopic counterparts. Nanotechnology will provide the ability to build materials with switchable molecular functions which could provide completely new approaches to valves, pumps, chemical separations, and detection. For example, fluid streams could be directed by controlling surface energy without the need for a predetermined architecture of physical channels. Switchable molecular membranes and the like could replace mechanical valves. By eliminating the need for complex fluidic networks and microscale components used in current approaches, a fundamentally new approach will allow greater function in much smaller, lower-power total chemical analysis systems.

A new scheme for the detection of molecular interactions based on optical readout of nanoparticle labels has been developed. Capture DNA probes were arrayed on a glass chip and incubated with nanoparticle-labeled target DNA probes, containing a complementary sequence. Binding events were monitored by optical means, using reflected and transmitted light for the detection of surface-bound nanoparticles. Control experiments exclude significant influence of nonspecific binding on the observed contrast. Scanning force microscopy revealed the distribution of nanoparticles on the chip surface.[3]

BioForce Nanosciences has taken the technology of the microarray to the next level by creating the ''nanoarray,'' an ultraminiaturized version of the traditional microarray that can actually measure interactions between individual molecules down to resolutions of as little as 1 nm. Here 400 nanoarray spots can be placed in the same area as a traditional microarray spot. Nanoarrays are the next evolutionary step in the miniaturization of bioaffinity tests for proteins, nucleic acids, and receptor–ligand pairs. On a BioForce NanoArrayT, as many as 1500 different samples can be queried in the same area now needed for just one domain on a traditional microarray.

Microfluidic Chips for Nanoliter Volumes

Nanoliter implies extreme reduction in quantity of fluid analyte in a microchip. The use of the word ''nano'' in nanoliter (nL) is in a different dimension than in nanoparticle, which is in nanometer (nm) scale. Diagnostic companies with ''nano'' in their name may have other technologies. For example, Nanolytics Inc. with lab-on-chip based on nanoliter microfluidics and Nanogen with NanoChip based on microelectronics.

Chemical compounds within individual nanoliter droplets of glycerol were microarrayed on to glass slides at 400 spots/cm^2 in another technique.[4] Using aerosol deposition, subsequent reagents and water were metered into each reaction center to rapidly assemble diverse multicomponent reactions without cross contamination or the need for surface linkage. This proteomics technique allowed the kinetic profiling of protease mixtures, protease–substrate interactions, and high-throughput screening reactions. From one printing run that consumes <1 nmol of each compound, large combinatorial libraries can be subjected to numerous separation-free homogeneous assays at volumes 103–104 smaller than current high-throughput methods. The rapid assembly of thousands of nanoliter reactions per slide using a small biological sample (2 mL) represents a new functional proteomics format implemented with standard microarraying and spot-analysis tools.

NanoChip

NanoChip (Nanogen, Inc., San Diego, CA, USA) is a microelectronic chip format suitable for rapid single nucleotide polymorphism (SNP) analysis and genetic dissection of complex phenotypes. In beta testing, the NanoChip system has been shown to provide accuracy equal to or better than DNA sequencing and other methods for SNP confirmation. Unlike other systems, the NanoChip system uses electronically accelerated hybridization under very low-salt conditions, potentially avoiding problems with DNA conformation and secondary structures, whereas most sequencing and primer extension technologies require high-salt conditions. The NanoChip system allows the user to array and analyze DNA on its NanoChip cartridges in user-selected formats in a single day with walkaway automation. The NanoChip system integrates advanced microelectronics and molecular biology into a platform technology with broad commercial applications in the fields of biomedical research, genomics, medical diagnostics, genetic testing, and drug discovery.

NanoPro™ System

The NanoPro™ System (BioForce Nanosciences, Inc.) consists of three separate components:

1. The NanoArrayer™ embodies proprietary instrumentation and methods for creating a broad spectrum of NanoArray™-based biological tests. This device places molecules at defined locations on a surface with nanometer spatial resolution. The arrays of molecules (NanoArrays™, see below) are unique to BioForce and can only be created with a NanoArrayer™.
2. NanoArrays™ are ultraminiaturized biological tests with applications in many areas. The company's first NanoArray™ products are presently being evaluated for commercial utilization by potential users and are

targeted toward the proteomics/genomics and diagnostics markets. These products include a custom nucleic acid NanoArray™ for RNA expression profiling as well as virus detection NanoArray™ called the ViriChip™. NanoArray™ chips are for protein expression profiling and immunodiagnostics.
3. The NanoReader™ is a customized AFM optimized for reading NanoArray™ chips. Using the AFM as a readout method optimizes analysis, including no requirement for secondary reporter systems (no fluorescence, radioactivity, or enzyme-linked detection), reductions in materials used (several thousand molecules per test), and increased sensitivity (ultimately single molecule detection).

Use of Nanotechnology in Microfluidics

Techniques such as nanoimprinting are used to construct large arrays of nanoscale grooves with efficiency and speed. Now such grooves can be sealed with similar ease to form nanofluidic channels. Laser-assisted direct imprint technique has been described by scientists at Princeton University that allows the construction of millions of enclosed nanofluidic channels side by side on a single substrate, which is ideal for such parallel processing.[5] By sputter-depositing silicon dioxide at an angle onto an array of prefabricated nanochannels imprinted into the surface of a biopolymer substrate, not only is an effective and uniform seal formed over the entire array, but the channels are further narrowed down to 10 nm, from an initial width of 55 nm. The authors point out that this process could be easily used for narrowing and sealing micro- and nanofluidic structures formed by other patterning techniques. By minimizing the hollow space in such structures, it could help surpass existing limitations in the spatial resolution of these techniques.

A chip-scale maze for combing out strands of DNA and inserting them into nanoscale channels was made using standard, inexpensive lithographic techniques.[6] Their ''gradient nanostructure'' might be used to isolate and stretch DNA molecules for analysis, e.g., to look for bound proteins such as transcription factors along the strand. Such molecules would be obscured in normal solution because DNA, like any other linear polymer, collapses into a random coil as a featureless blob. The strands can, in principle, be straightened by feeding them into channels just a few tens of nanometers wide, using nanofluidic techniques.

Researchers from Oak Ridge National Laboratory (Oak Ridge, TN) have found a way to fabricate flexible tubes whose diameters are 100 nm, i.e., 10 times narrower than those used in today's microfluidic systems.[7] The tubes could be used to make stacked, interconnected fluidic networks designed to shunt fluids around biochips that sense and analyze chemicals. The researchers discovered the method when they noticed that depositing a certain type of polymer into tiny silicon grooves caused the polymer at the tops of the grooves to close across the gaps, forming tubes. They realized the process could be used to make tiny networks of tubes for use in microfluidics. The method is also compatible with conventional processes for making chips and can be used to integrate the networks with electronic chip components.

Nanoparticle Protein Chip

At the Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB (Stuttgart, Germany), a new type of protein chip is being developed based on protein-binding silica-nanoparticles. The surface of this minute particle with a diameter of less than one ten-thousandth of a millimeter can be configured with many different capture proteins. The particles configured in this way are then applied to silicon carriers in thin, even layers. After contact is made with a sample, the chips can be analyzed using state-of-the-art mass spectrometry, MALDI-TOF mass spectrometry. Knowing the masses of the bound proteins provides a direct indication of their identity.

Biosensor Nanochips

Biosensors incorporate a biological sensing element that converts a change in an immediate environment to signals that can be processed. Nanotechnology-based biosensors have been reviewed elsewhere.[8] Nanomaterials are exquisitely sensitive chemical and biological sensors. As their surface properties are easily modified, nanowires can be decorated with virtually any potential chemical or biological molecular recognition unit, making the wires themselves analyte independent. The nanomaterials transduce the chemical binding event on their surface into a change in conductance of the nanowire in an extremely sensitive, real-time, and quantitative fashion. Boron-doped silicon nanowires (SiNWs) have been used to create highly sensitive, real-time, electrically based sensors for biological and chemical species.[9] The sensors can be electronically gated to respond to the binding of a single molecule. Prototype sensors have demonstrated detection of nucleic acids, proteins, and ions. These sensors can operate in the liquid or gas phase, opening up an enormous variety of downstream applications. The detection schemes use inexpensive low-voltage measurement schemes and detect binding events directly so there is no need for costly, complicated, and time-consuming labeling chemistries such as fluorescent dyes or the use of bulky and expensive optical detection systems. As a result, these sensors are inexpensive to manufacture and

portable. It may even be possible to develop implantable detection and monitoring devices as biochips based on these detectors.

CONCLUSION

It is now obvious that direct analysis of DNA and protein could dramatically improve speed, accuracy, and sensitivity over conventional molecular diagnostic methods. However, DNA, RNA, protein, and their functional subcellular scaffolds and compartments are in the nanometer scale, the potential of single molecule analysis approach would not be fully realized without the help of nanobiotechnology. Advances in nanotechnology are providing nanofabricated devices that are small, sensitive, and inexpensive enough to facilitate direct observation, manipulation, and analysis of a single biological molecule from a single cell. This opens new opportunities and provides powerful tools in fields such as genomics, proteomics, molecular diagnostics, and high-throughput screening. Given the inherent nanoscale of receptors, pores, and other functional components of living cells, the detailed monitoring and analysis of these components will be made possible by the development of a new class of nanoscale probes. The most important area of application will be in point-of-care diagnostics. Important indications will include cancer diagnosis and detection of infectious disease agents, particularly in bioterror attacks. Future trends in diagnostics will continue in the miniaturization of biochip technology to nano range. There is also a trend to build the diagnostic devices from bottom up starting with the smallest building blocks.

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NEBcutter—A Program to Cleave DNA with Restriction Enzymes

Janos Posfai
R. J. Roberts
Tamas Vincze
New England Biolabs, Inc., Beverly, Massachusetts, U.S.A.

INTRODUCTION

NEBcutter is a computer program offering services to research laboratories that employ nucleic acid technologies. The software provides bioinformatics tools for experiments where DNA molecules are manipulated with restriction enzymes. Type II restriction enzymes recognize specific four- to eight-nucleotide-long patterns in DNA, and introduce cuts into the strands at predictable positions.[1,2] The specificity and precision of cleavage make these enzymes useful in a wide array of applications. Gene cloning, detection of point mutations, probing of repeat sequences, genome mapping, and characterization of genetic variations[3–5] are a few of these. The NEBcutter program supports such applications by providing routines for the systematic analysis of restriction site distributions within defined DNA sequences. The service is accessible over the web, through a link on http://www.neb.com.

The discovery of restriction enzymes and the accumulation of significant amounts of sequenced DNA prompted the development of the first restriction digest analysis computer programs.[6,7] Since then, hundreds of new restriction enzymes have been characterized. Today, the authoritative restriction enzyme database, REBASE,[8] lists over 3500 type II entries, with almost 250 different specificities. The amount of sequenced DNA has expanded equally rapidly. The GenBank[9] collection of DNA sequences now contains over 30 billion bases, including complete genome sequences of dozens of species (human, mouse, fruitfly, yeast, bacteria, and so on). Substantial changes in computer power and computing environments occurred during this time as well. Advanced algorithms,[10] network access, linked databases, and graphic presentations have become standard. In this new environment, NEBcutter provides tools for state-of-the-art restriction enzyme digest analysis.

METHODS

Analysis starts by specifying a target DNA sequence. Many popular vector and phage sequences (such as pBR322, lambda, T7, and so on) are directly accessible for processing. Alternatively, DNA sequences can be pasted into an entry form, or uploaded from local user files. Sequences of GenBank entries can also be loaded simply by specifying their accession numbers. The program distinguishes between linear and circular sequences, and handles them according to their topology. Occasional ambiguities in the input sequence are permitted; these are resolved by putting special marks on hits that are conditional on the specific sequence. Analyzing inputs with runs of N-s would be meaningless because the runs could be sites for any enzyme, and so such inputs should be avoided. Sizes of target sequences are currently limited to 300 kb.

The program determines the cut sites for a repertoire of restriction enzymes. This repertoire is selectable and may be New England Biolabs-marketed enzymes, all commercially available specificities or all specificities ever characterized. Users can add hypothetical enzymes with fictitious sites to the list manually. NEBcutter keeps its internal data structures up to date by connecting daily to REBASE and by fetching the latest information about restriction enzymes and specificities.

Results from a digest are presented in schematic figures, summary tables, and detailed textual pages. In the default setting, the site distribution of enzymes with single cuts is displayed (Fig. 1). One-click options can bring up the distributions of double or triple cutters. One can also switch to a display of custom digests with particular enzyme(s). Cut sites sensitive[11] to the mammalian CpG methylation, or to typical *E. coli* methylation are highlighted on the output. Color-coded symbols distinguish cuts with blunt ends from cuts with 5′ and 3′ extensions. The user can zoom on selected regions, all the way to the actual sequence. Circular sequences need to be linearized (with a single click) before engaging this zoom option.

In many applications, locations of gene flanking cut sites are of particular interest. To find them, NEBcutter places arrows on the output to indicate where the genes of the input sequence are, assuming that GenBank-style coordinate annotations are available. When the input is a plain, unannotated sequence, NEBcutter displays open reading frames (ORFs) according either to its own internal

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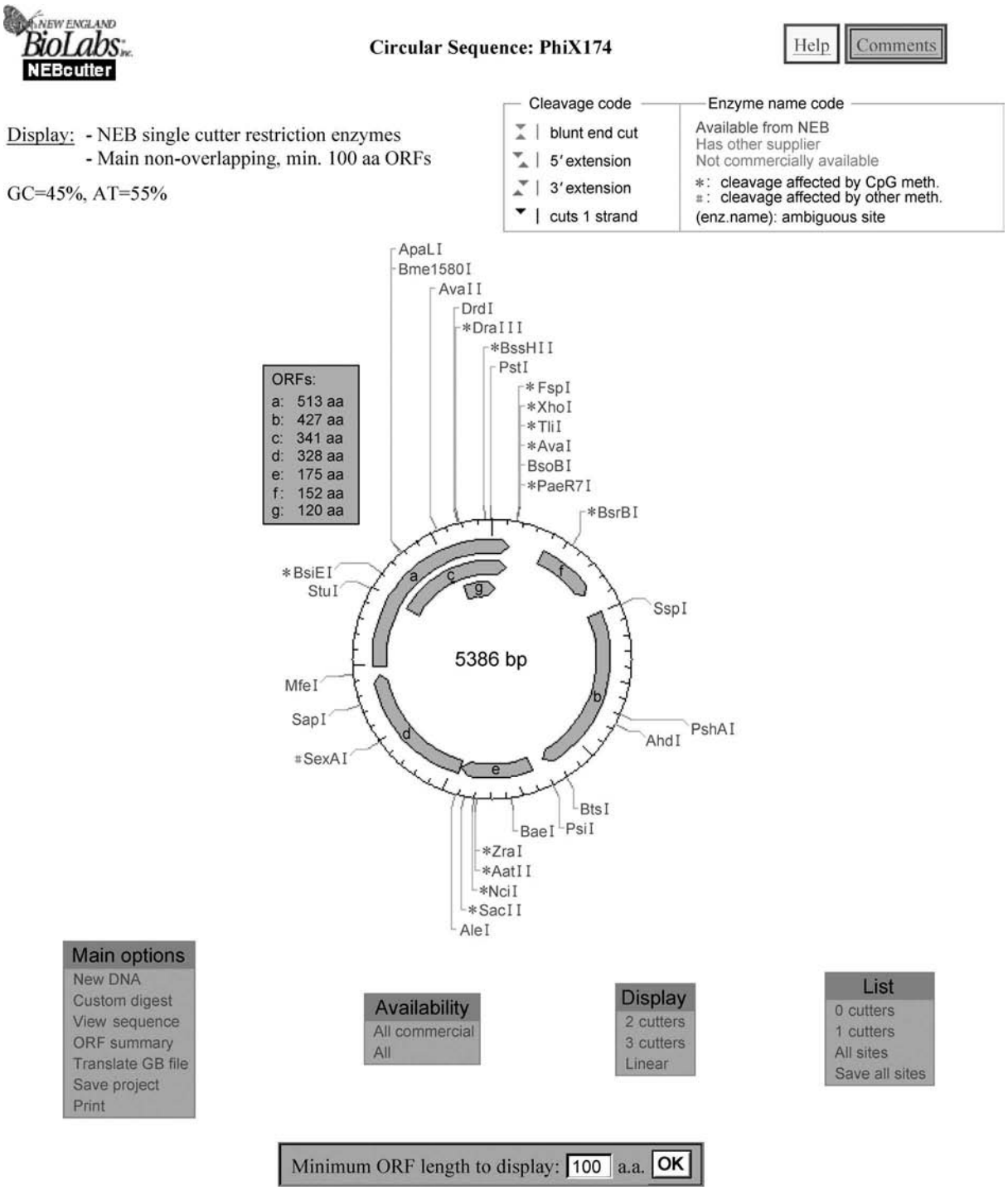
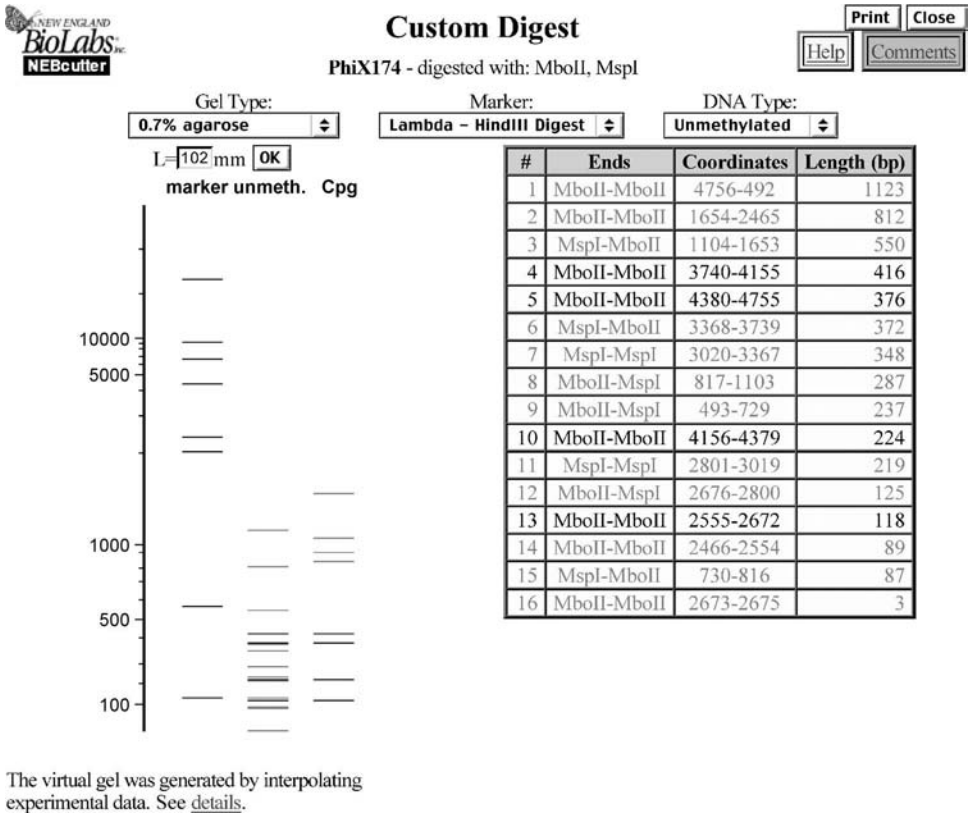


Fig. 1 Restriction digest analysis results for phage PhiX174. Sites for enzymes with single cuts in the circular 3.4-kb sequence are displayed. Methylation-sensitive sites are marked with symbols. Color coding distinguishes blunt end cuts from ones that create overhangs. Grey arrows indicate the genes. Menu options for further actions are available at the bottom of the web page. (*View this art in color at www.dekker.com.*)

ORF finder routine, or to manually entered and edited data. Clicking on an arrow brings the represented ORF into focus. On this page, sites that flank the ORF are indicated. ORF-specific silent mutagenesis analysis can also be run. This module computes silent mutations that would turn previously noncutting enzymes into cutters.



#	Ends	Coordinates	Length (bp)
1	MboII-MboII	4756-492	1123
2	MboII-MboII	1654-2465	812
3	MspI-MboII	1104-1653	550
4	MboII-MboII	3740-4155	416
5	MboII-MboII	4380-4755	376
6	MspI-MboII	3368-3739	372
7	MspI-MspI	3020-3367	348
8	MboII-MspI	817-1103	287
9	MboII-MspI	493-729	237
10	MboII-MboII	4156-4379	224
11	MspI-MspI	2801-3019	219
12	MboII-MspI	2676-2800	125
13	MboII-MboII	2555-2672	118
14	MboII-MboII	2466-2554	89
15	MspI-MboII	730-816	87
16	MboII-MboII	2673-2675	3

Fig. 2 Virtual gel for the custom double digest of PhiX174 with the restriction enzymes *Mbo*II and *Msp*I. A lambda *Hin*dIII digest marker lane is included to scale the gel. Separate lanes simulate runs for unmethylated and CpG-methylated targets. Fragments are ordered in the table for the unmethylated DNA experiment. (*View this art in color at www.dekker.com.*)

From the results of calculated hypothetical digests, the program is able to simulate how the fragments would separate in various gel electrophoresis experiments (Fig. 2). A reference lane with selected marker fragments can be added to the view. Additional lanes will demonstrate the effects of possible DNA methylations.

With only a few clicks, NEBcutter will execute typical restriction digest analysis tasks. For more sophisticated tasks, sets of options are available. On-line context-specific help is accessible from every page. Pop-up windows show the basic information for every reported site. Direct links into REBASE are provided and detailed information about particular restriction enzymes is only a click away. Similarly, direct links connect to on-line enzyme ordering from New England Biolabs. Management tools enable users to save and later retrieve their own projects. User feedback is encouraged and facilitated via a built-in e-mail interface.

Software and Algorithms

The program runs on a Sun Ultraspark-II server, under SunOS 5.7 operating system. NEBcutter is a suite of cooperating routines, where program modules execute different tasks. Calculations and queue management are implemented in C (gcc version 2.96) modules. Calls to functions of the GD library perform the visualization tasks. User interfaces are HyperText Markup Language (HTML) pages, generated dynamically by PHP scripts and tested extensively for compatibility with Netscape and Microsoft Explorer browsers. Connections are open for these standard browsers via an Apache web server.

The program localizes all restriction sites in a single pass over the target sequence with a finite state automaton[12] algorithm. With this algorithm, execution time increases only in a linear fashion as the input sequence length increases. In the neighborhoods of sequence ambiguities, the program switches to a brute force algorithm.

When identifying ORFs, the program considers maximal length segments from start to stop, assuming standard codon assignment and bacterial (nonsplicing) DNA sequences. Overlapping ORFs are pruned by a heuristic algorithm, which favors longer ORFs over shorter ones.

Gel run simulations are based on mobility data from our own laboratory experiments, or from the gel manufacturer's manual (www.elchrom.com/technic/manuals/man007.asp).

Cubic spline curve interpolation[13,14] yields intervening data points.

Data Handling and Data Security

Suspended NEBcutter sessions can be resumed. Using the default settings, the program stores all project data (sequence, enzyme selection, display settings, and manual entry) on the server and deposits an associated session code on the user's computer. When the user later reconnects, these session codes are returned to the server, data from the user's earlier projects are retrieved, and results of those sessions are reconstructed. The identifier codes are randomly generated, unique numbers, and are stored in browser cookies. Each code is account-specific, so its use requires that the subsequent connection is made through the same log-in account. Another consequence is that people sharing an account also share each other's data. This data sharing can be avoided by using the ''delete project'' button on the main menu at the end of a session. Alternatively, users can block NEBcutter's save-and-resume feature by checking the appropriate menu option on the interface. Completely disabling a browser's use of cookies is another way of increasing data privacy.

The second method of saving and resuming NEBcutter sessions is activated through a menu option. With this option, project data are returned to the user and stored in a local data file, named by the user. To restart the project later, the user needs to upload this file. This second method is designed for reactivation of an analysis after an indefinite suspension of a session. The first method is intended for temporary interruption of a session. After 2 days of inactivity, server-stored session data files are deleted automatically.

By the time a session is reactivated, new restriction enzymes may have been added to the database, or the content of REBASE may have changed in other ways. Suspended sessions can still resume analysis with the original repertoire of enzymes, which makes the reconstruction of earlier results possible.

Concurrent users of NEBcutter do not notice each other, and they cannot access each other's data. For the purposes of troubleshooting, resource allocation, and optimization, NEBcutter administration routines monitor user activity, maintain log files, and compile usage statistics.

Graphics Output

NEBcutter gives users full access to the digest figures they create. The figures can be exported into graphic files in several standard formats. Resolution of the GIF raster format is selectable, and preview versions of vector graphics EPS format figures are adjustable. The graphic images are of high quality and are ready for inclusion in publications.

Supplementary Materials

Results from genomewide restriction enzyme digests have been precompiled and posted at http://www.neb.com/~posfai. For all prototype enzymes, site frequencies and densities, as well as average fragment lengths, appear here for mammalian, plant, and insect chromosomes, as well as for total genomic sequences. Site frequencies in one genome can be compared to site frequencies in other genomes, to frequencies predicted by first-order Markov models,[15] or to frequencies in randomized sequences. Both the Markov models and the randomized sequences are based on actual genome dinucleotide counts.

CONCLUSION

Restriction enzymes are invaluable tools of DNA engineering and analysis; they are used in many applications, both in research and in molecular diagnostic laboratories. The NEBcutter program provides tools for the design and interpretation of experiments that employ such enzymes. The software is freely available over the web, from the New England Biolabs web site. The program is aimed at the bench scientist, and has intuitive, easy-to-use interfaces. To assure that results are current, NEBcutter connects to REBASE directly, and updates its internal restriction enzyme data structures daily. An important and distinguishing feature of our software is its ability to consider the effects of base methylation—the potential interference with cleavage.

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Neisseria meningitidis

Martin J. Callaghan
Dominic F. Kelly
Andrew J. Pollard
University of Oxford, Oxford, U.K.

INTRODUCTION

Neisseria meningitidis is a gram-negative diplococcus and is a member of the family Neisseriaceae within the order Eubacteria. This family includes the genera *Neisseria*, *Moraxella*, *Kingella*, and *Acinetobacter*. The genus *Neisseria* includes two human pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitidis* (the meningococcus). The meningococcus is enveloped by a polysaccharide capsule, which is essential for pathogenicity. Differences in the immunological reactivity of the capsule define 13 serogroups of which five (A, B, C, W135, and Y) are most commonly associated with invasive meningococcal disease.

EPIDEMIOLOGY

The epidemiology of *N. meningitidis* has been reviewed by Cartwright.[1] Humans are the sole natural host for the meningococcus, which is carried asymptomatically as part of the commensal nasopharyngeal microflora in approximately 10% of the general population. The meningococcus may occasionally cause invasive disease by traversing the nasopharyngeal mucosa into the bloodstream, where it multiplies rapidly. Although invasive behavior is poorly understood and unpredictable in the individual, it appears to be related to pathogen characteristics (e.g., serogroup, clonal lineage), host factors (e.g., age, smoking, immunological abnormalities, concurrent viral infections), and environmental factors (e.g., overcrowding, local climatic factors). The majority of disease occurs in the under 5 years age groups with a peak incidence in the 6–12 months age group. The United States and Europe experience an endemic rate of disease mainly caused by serogroup B, C, and Y meningococci (one to five cases per 100,000 yearly), punctuated by occasional hyperendemic or geographically localized outbreaks. In China and sub-Saharan Africa, epidemic and pandemic diseases caused by serogroup A, and, recently, serogroup W-135 meningococci, are common (up to 500 cases per 100,000) with major epidemics occurring every 5–10 years.

SPECTRUM OF DISEASE

The most characteristic presentations of meningococcal disease are septicemia and meningitis. Septicemic disease is usually accompanied by a hemorrhagic rash, and can be rapidly fatal with disseminated intravascular coagulation and multiorgan failure. More unusual presentations include low-grade septicemia with maculopapular rash, cellulitis, pneumonia, septic arthritis, and osteomyelitis.

DIAGNOSIS

In a patient with suspected meningococcal disease, diagnosis can be made by using microbiological culture of blood, cerebrospinal fluid, or aspirates from the hemorrhagic rash. Sensitivities of approximately 30% have been reported for diagnosis by blood culture.[4] These low levels of diagnostic sensitivity may be related to collection of blood and cerebrospinal fluid (CSF) after the first dose of antibiotics has been administered, as meningococci are rapidly killed following a single dose of intravenous penicillin. In the United Kingdom and some countries in Europe, antibiotics are given in primary care to reduce the potential for deterioration of the patient's condition during transport to the hospital.

Recently, diagnostic sensitivity has been improved by the introduction of polymerase chain reaction (PCR)-based molecular diagnostic methods into clinical practice. Blood or CSF can be taken from a suspected clinical case and meningococcal infection can be confirmed by identification of meningococcal gene sequences, which can persist in these fluids up to 4 days after the start of antibiotic treatment. Whereas molecular characterization utilizes genes with significant variation to distinguish different clones, clinical diagnosis requires the identification of genes conserved across all meningococci. In the United Kingdom, sensitive and specific assays have been developed using the *ctrA* gene, encoding a protein involved in capsular synthesis and transport.[2] This gene is specific to *N. meningitidis*, and common to all meningococcal serogroups. During recent clinical evaluation of

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95 children with probable meningococcal disease, 82 (88%) had a positive PCR, whereas 32 (33%) were culture-positive.[3] No false negative PCR results were obtained with this protocol because all culture positive cases were confirmed by PCR. False negatives were observed in a previous *ctrA* PCR assay using different oligonucleotide PCR primers on serum or plasma samples.[4] Whereas false positive results have been observed in an assay based on the IS1106 insertion sequence,[5] which may be found in other microorganisms, no false positives have been observed to date with the *ctrA* PCR assay.

A further advance has been the development of a single-tube multiplex, real-time PCR assay for detection of all the organisms forming the major differential diagnosis of significant childhood sepsis and meningitis in Europe and North America.[6] This uses fluorescent dye-labeled sequence-specific probes for the simultaneous detection of the *N. meningitidis* capsular transport gene (*ctrA*), *Haemophilus influenzae* capsulation gene (*bexA*), and *Streptococcus pneumoniae* pneumolysin gene (*ply*). As soon as meningococcal infection is confirmed, the serogroup of the infecting organism can be determined. A PCR-based assay is used to detect the *siaD* gene, which encodes the serogroup-specific sialyltransferase enzyme, enabling differentiation of serogroup B, C, Y, and W135 organisms.[7,8] This assay is less sensitive in terms of detection of meningococci than the *ctrA* PCR assay. Serogroup A bacteria can be identified by PCR detection of the *mynA* gene, which encodes an enzyme involved in synthesis of the serogroup A capsular polysaccharide.[9]

MANAGEMENT

Prehospital treatment with intramuscular penicillin is recommended where disease is suspected, because deterioration of the patient's condition can be rapid. Although meningococci with intermediate resistance to penicillin have been described, they have not been associated with treatment failure. In practice, before a diagnosis is established, most patients will be treated with a third-generation cephalosporin such as ceftriaxone to give broad-spectrum antibiotic cover where the causative organism is not yet identified. There are no reports of resistant meningococci for this antibiotic.

The severe nature of meningococcal disease means that good supportive management of respiratory and cardiovascular function are essential. Admission to an intensive care unit is often required, and the focus on such supportive care has significantly improved mortality over the last 10 years.[10] A significant number of survivors are left with disabilities relating to limb amputation, skin damage, and neurological impairment.

PCR-based techniques have been of most importance in the fields of public health and epidemiology. The improved sensitivity of case ascertainment allows a more accurate measurement of the level of meningococcal disease in the human population, which is important for public health decision making.

CHARACTERIZATION OF MENINGOCOCCI

Effective public health countermeasures against meningococcal disease, such as vaccination programs, rely on the identification of meningococci, the ability to track temporal and geographical spread of meningococci, and the ability to relate locally and globally obtained isolates.[11] Furthermore, characterization of the genetic diversity of meningococcal isolate collections has provided insights into the structure of meningococcal populations, and the evolutionary mechanisms that shape them.[12] A range of techniques have been used to characterize meningococci, including differences in the electrophoretic mobility of cytosolic enzymes or the DNA sequence diversity of the meningococcal genome. However, methods relying on differences in the immunological reactivity of cell surface components are in most widespread use (Fig. 1).

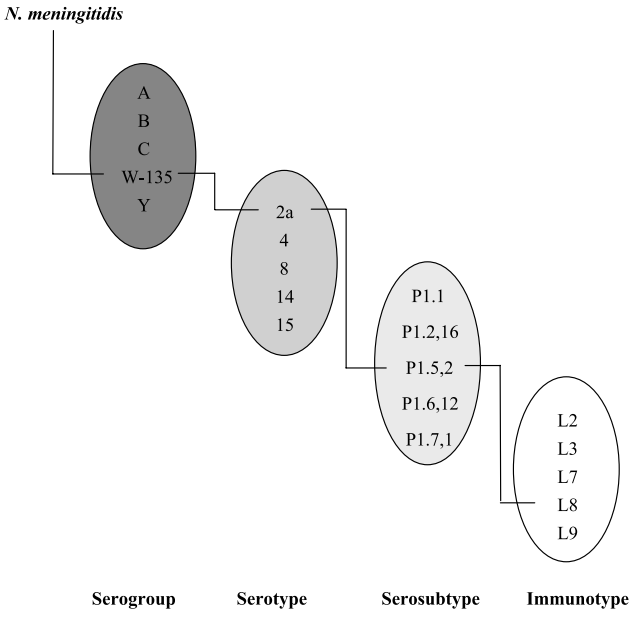

Fig. 1 Serological characterization of *N. meningitidis*. *N. meningitidis* can be further subdivided by the immunological reactivity of its capsular polysaccharide (serogroup), PorB protein (serotype), PorA protein (serosubtype), and LPS (immunotype). The figure describes the serological characterization of a meningococcal isolate with the classification: W-135:2a:P1.5,2:L8. (*View this art in color at www.dekker.com.*)

Immunological Characterization of the Meningococcus

In addition to determination of the meningococcal serogroup by the immunological reactivity of the capsular polysaccharide, more detailed characterization can be achieved by detection of antigenic variation of ''subcapsular'' components of the meningococcal outer membrane (Fig. 2).[13] Meningococci produce two porin proteins, PorA and PorB, which form trimeric pores in the meningococcal outer membrane. The antigenic variation in the PorB and PorA monomers is used to define the meningococcal serotype and serosubtype, respectively. The meningococcal surface is also coated in lipooligosaccharide (LPS), an amphipathic, phosphorylated glycolipid. This is composed of a membrane-anchoring lipid, a backbone to which core saccharides are attached, which are in turn linked to structurally variable oligosaccharide moieties. Variation in the oligosaccharide moiety of the surface LPS is used to define the meningococcal immunotype.

An inherent limitation of immunological characterization is the antigenically variable nature of the targeted surface components.[14] Such variation means that new reagents are continually required to identify previously uncharacterized variants.[15] A further problem is the dynamic nature within individual bacteria of expression of the structures to which serological typing reagents are targeted. Switching of antigenic types by horizontal genetic exchange of the genes encoding them, or involved in their synthesis, also poses problems for the reliability of immunological characterization techniques.[16] Some carried meningococci even lack the genes necessary for capsular synthesis and transport, thus precluding serogroup determination.[17]

Molecular Characterization of the Meningococcus

Molecular techniques offer improvements over immunological methods in both specificity and sensitivity. Techniques include ribotyping, pulsed-field gel electrophoresis (PFGE), analysis of repetitive elements, or DNA sequence determination of individual loci.[18] These techniques are particularly useful in detecting ''microevolution'' of rapidly evolving genes among related meningococci and for understanding their short-term epidemiology.[19] For example, DNA sequence-based analysis of *porB* gene polymorphisms has led to improvements in the discrimination between immunologically similar meningococci and in defining the relationships among them.[20]

For longer-term analysis of meningococcal epidemiology and population structure, ''multilocus'' characterization techniques are more useful. These techniques index neutral, slowly evolving variation at widely distributed genetic loci around the meningococcal chromosome, such as those encoding cytoplasmic ''housekeeping'' enzymes involved in central metabolic processes. As such, these methods are more representative of the genomic sequence

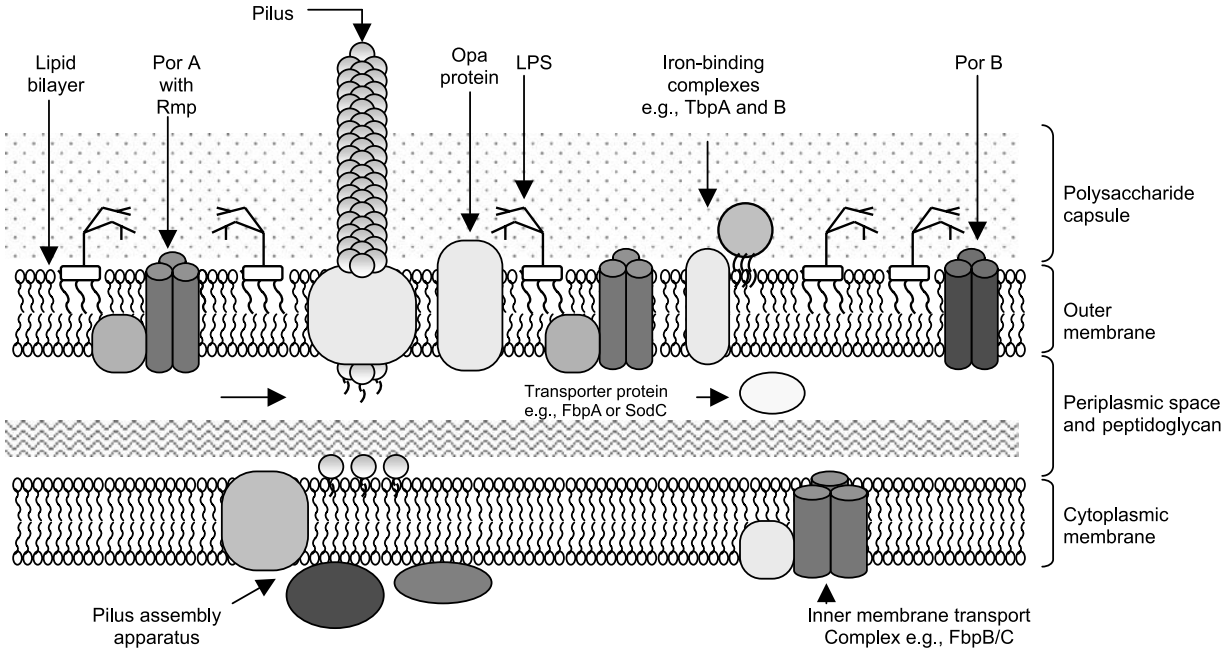

Fig. 2 Schematic representation of meningococcal surface structures. (Morley, S.L.; Pollard, A.J. Vaccine prevention of meningococcal disease. Vaccine **2002**, *20*, 666–687. Copyright 2002, with permission from Elsevier.)

Table 1 Protein vaccine candidates that might offer protection against serogroup B disease

Vaccine candidate	Stage of development
Outer membrane vesicle	
Finlay Institute	Licensed in some South/ Central America countries
NIPH	Completed efficacy studies in teenagers
RIVM	Immunogenicity studies in various age groups
Purified outer membrane proteins	
Transferrin-binding protein B (TbpB)	Preliminary clinical studies in volunteers
Neisserial surface protein A (NspA)	Preclinical research
Transferrin binding protein A (TbpA)	Preclinical research
Ferric enterobactin receptor (FetA)	Preclinical research
Recombinant PorA	Preclinical research
Peptides from PorA	Preclinical research
T-cell-stimulatory protein A (TspA)	Preclinical research
Genome-derived antigens	Various stages

Adapted from Feavers, I.M. Meningococcal Vaccines and Vaccine Developments. In *Meningococcal Vaccines*; Pollard, A.J., Maiden, M.C.J., Eds.; Humana Press: Totowa, New Jersey, 2001; 1–22.

of individual isolates. Multilocus enzyme electrophoresis (MLEE)[21] exploits the differences in the electrophoretic mobilities caused by genetic variation of such enzymes. The resultant electrophoretic profile is used to assign an electrophoretic type (ET), and isolates with indistinguishable electrophoretic profiles are assigned to the same ET. Phylogenetic analysis of MLEE data has enabled the definition of meningococci into genetically related groups, commonly referred to as clonal lineages, known as subgroups (serogroup A), and clusters or complexes (serogroups B and C).

More recently, multilocus sequence typing (MLST)[11] has been introduced, which adopts a similar approach to MLEE except that determination of the sequence of seven gene fragments encoding housekeeping enzymes are used to collect genetic data directly, rather than by inference from the phenotypic differences of expressed proteins. This has allowed MLST to provide a much finer level of isolate characterization than MLEE. Unique combinations of the alleles at the seven loci are known as sequence types (STs), and related sequence types (those which have at least four identical loci to a known central genotype) are known as clonal complexes. Since its development in the late 1990s, MLST has become widely accepted as the standard method of meningococcal isolate characterization because of its discriminating power and the unambiguous nature of DNA sequence determination, which does not vary between different equipment, reagents, or operators involved in its preparation. Rapid electronic exchange and analysis of MLST data in conjunction with epidemiological information regarding the isolates from which the data were obtained can be achieved by internet connection to a central electronic database (http://neisseria.org/nm/typing/mlst). Multilocus analysis of meningococci isolated from asymptomatic carriage and also from cases of invasive disease has indicated that meningococcal populations are extremely diverse, being composed of many clonal lineages; however, most invasive disease can be attributed to a limited number of lineages, and these have been termed the hyperinvasive lineages.[12,22]

CONCLUSION: GENOMICS AND VACCINATION

Despite the advances associated with molecular techniques, meningococcal disease remains a globally significant health problem, and the prospect of comprehensive vaccination remains elusive. Whereas vaccines based on the capsular polysaccharide are either licensed or under development against meningococci associated with serogroups A, C, and Y, as well as W-135, attempts to develop a polysaccharide-based serogroup B vaccine have proven unsuccessful. This fact is especially relevant as much of the meningococcal disease in Europe and North America is caused by meningococci associated with the serogroup B capsule. A number of alternative approaches to the development of serogroup B meningococcal vaccines are currently in development,[14] and these are summarized in Table 1. The recent completion of three meningococcal genomes [(http://www.sanger.ac.uk/Projects/N_menigitidis/); Refs. [23,24]] has revolutionized the process of vaccine development and the application of genomic approaches such as reverse vaccinology to this resource has already led to the identification of novel vaccine candidates.[25]

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Nested PCR and Multiplex Nested PCR

David Tarragó-Asensio
Ana Avellón-Calvo
National Center for Microbiology, Madrid, Spain

INTRODUCTION

Since the polymerase chain reaction (PCR) was described in 1985 many variations of the initial technique have been developed to improve the sensitivity, specificity, cost-effectiveness, and time consumed. The sensitivity rates are crucial in diagnostic PCR systems, especially those involving labile or not abundant microorganisms in clinical specimens. One of the methods currently employed to increase sensitivity and specificity is the nested PCR (nPCR). This technique consists in the utilization of two consecutive PCRs, each containing a different pair of oligonucleotides. Paradoxically, the high sensitivity of the method often results in a handicap when reagent or sample contamination is present. On the other hand, multiplex PCR approach has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. Although the technique has been shown to be a valuable method for the identification of viruses, bacteria, fungi, and/or parasites, it is really useful for the screening of clinical samples involved in syndromes and infections such as acute respiratory tract infections, genital infections, and meningitis and encephalitis. Multiplex and nested PCR have experienced a wide development overall in the field of infectious diseases caused by viruses.

NESTED POLYMERASE CHAIN REACTION

Principle

Nested PCR (nPCR) consists in the consecutive performance of two PCRs usually involving less than 45 cycles each. The larger fragment produced by the first reaction is used as template for the second PCR. The system is allowed if necessary to be preceded by a retro-transcription reaction (RT-nPCR). The performance of two individual reactions achieves a considerable improvement of the sensitivity and specificity (Fig. 1). Several reasons explain the fact that PCRs of 30 cycles each (first- and second-round PCR) improve the yield compared to a single reaction performing 60 cycles. It is known that DNA polymerases can reduce its activity after a number of high-temperature cycles.[1] Besides, the denaturalization step on amplicons from the first PCR is more effective than on the initial template because of their smaller size.[2] The specificity of the nPCR system is additionally improved, because the chance of unspecific primer hybridization is dramatically low.

nPCR Special Features

Primers

The first PCR contains external pairs of primers, whereas the second includes two primers that are internal to the first primer pair. The primers employed should be different in both reactions (Fig. 1); although there is a possibility of employing one of the first-round primers into the second reaction, designing a seminested PCR, however, sometimes results in unspecific amplifications. If during the primer design process it is not possible to choose four different conservative regions, the use of sense or antisense partially overlapping sequences is an allowed alternative. The hybridization temperatures of each pair of primers may be different, but it is convenient to be similar in order to make, if necessary, cross-primer reactions that permit to evaluate each individual primer function.

Biochemical conditions

The final biochemical conditions of both reactions should be experimentally determined individually as in one reaction PCR.

Template

Only a small amount of the first amplification fragment (1–3 μL) is necessary to serve as template for the nested reaction.

Avoiding nPCR Contamination

The extremely high sensitivity of the nPCR system often results in problems, due to contaminations coming from

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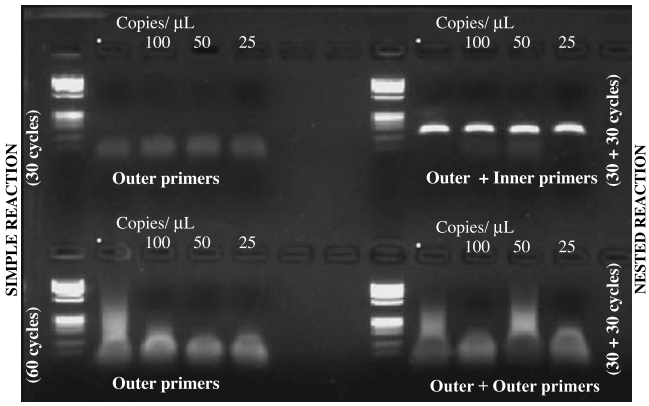

Fig. 1 The figure is based on a generic adenovirus-specific nPCR as described by Avellón et al.[4] On the left, simple PCR using the outer primers, with 30 (above) and 60 (down) cycles. On the right above, nPCR using outer and inner primers as the published method (sensitivity estimated of around 10 to 100 copies/mL). On the right down, nPCR employing outer primers in both reactions. The adenovirus amplification band (168 nt) is found only in the assay reproducing the published method.

previously amplified sequences. Those sequences can be carried on the skin surface of the operator or on laboratory materials, and can be found at the laboratory facilities. When a previously amplified sequence is included by chance into the reaction mixture, it results in a false-positive reaction. The use of negative controls can check the validity of the assay, but the possibility of individual contamination is almost impossible to be ruled out. Because of that, a wide routine precaution should be taken to avoid contaminations, including the following:

1. Sample aliquots: at least two different sample aliquots should be made and stored at a laboratory outside the PCR laboratory. A positive result should be always confirmed in a second aliquot.
2. Reagent preparation should be performed in a ''clean'' area separated from the rest of the laboratory, preferably in a flow laminar cabinet. First and nested PCR reactions should likewise be performed at separate laboratories, into distinct laminar flow cabinets and employing different supplies of pipettes, pipette tips, Eppendorf tubes, and any common reagent as distilled water or PCR buffer. Nucleic acid extraction procedures can be done in the same area and with the same materials as the first reaction activities. Finally, the detection process should be carried out in a specific laboratory. Summarizing, to perform nPCR we need four separate laboratories: a reagent laboratory, a nucleic acid extraction and first PCR laboratory, a nested laboratory, and an amplification detection laboratory.
3. Polymerase chain reaction operators should be meticulously trained before the initiation of their activity. They are encouraged to wear disposable gloves in each PCR area, changing them frequently, especially when leaving a laboratory to enter another. Equally, they should wear protective clothing in each area.
4. Positive control DNA plasmids should be carefully prepared at a limit of 1 or 2 logs higher than the expected limit of detection, to avoid being the origin of contamination.

MULTIPLEX PCR

Principle and Troubleshooting

The technical benefit of multiplex PCR over conventional PCR is that in multiplex PCR more than one target sequence can be simultaneously amplified by including more than one pair of primers in the PCR reaction. The optimization of multiplex PCRs may result in some difficulties and the main one is the preferential amplification of certain specific targets. The presence of more than one primer pair in the PCR reaction increases the chance of obtaining nondesirable amplification products, primarily because of the formation of primer dimers. Nonspecific products may be amplified more efficiently than the desired target producing impaired rates of annealing and extension. Trial-and-error approach may have to be used when testing several primer pairs, because there are no means to predict the performance features of a putative primer pair even among those that satisfy the general parameters of primer design. Special attention to the homology of primers with their respective target sequences, their GC content, and concentration, and obviously avoiding inter- and intraprimer homology have to be considered. All the primer pairs in a multiplex PCR should enable similar amplification efficiencies for their respective target. Polymerase chain reaction selection is defined as a mechanism that favors the amplification of certain templates due to the properties of the target or the target's flanking sequences. The choice of primers has been shown to be crucial to avoid PCR selection. A primer length of 18 bp or larger and a GC content around 40% and with identical or very close optimum annealing temperatures should not display any significant homology either internally or to one another could be assayed. Optimization of PCR components such as PCR buffer constituents, dNTPs, enzyme concentrations, and PCR additives, such as bovine serum albumin, dimethyl sulfoxide, glycerol, or betaine, has been reported to be useful in multiplex PCRs. These additives may act to avoid the stalling of DNA polymerization, which may occur through the formation of secondary structures within regions of

template DNA during the extension process. Such cosolvents may also act as destabilizing agents, reducing the melting temperature of GC-rich sequences, or as osmoprotectants, increasing the resistance of the polymerase to denaturation. The primer pairs must be inclusive for as many strains of the target microbe as possible, and depending on the amplicon detection method, their targets are easily resolvable. The latter may be achieved by using primer pairs that result in PCR products that can be separated and clearly visualized using gel electrophoresis or hybridization probes with maximum specificity.

Many multiplex PCRs developed before utilized a nested strategy. Nesting significantly increases the sensitivity of the assay but also increases the chance of false-positive results due to contamination and may also complicate automation.

CLINICAL APPLICATIONS OF NESTED AND MULTIPLEX PCR IN DIAGNOSTIC MICROBIOLOGY

Applications in Qualitative PCR

The nPCR systems are currently widely employed in both qualitative, simple[3,4] or multiplex,[5–13] and quantitative[14,15] diagnostic tests, as well as in PCR systems employed in the identification of viruses.[16–19] Regarding the above mentioned qualitative nPCRs, the primer design allows to detect from 10 to 100 genome copies per microliter of any adenovirus[4] or lyssavirus,[3] and can be performed from a variety of samples including cerebrospinal fluid (CSF), urine, stools, respiratory swabs, conjunctival swabs, or brain necropsies. These nPCRs can be included in multiplex systems, especially adenovirus nPCR,[12] as the range in hybridization temperatures and biochemical conditions is very high.

The first nested multiplex PCR for detection and typing of herpesviruses (HSV-1 and -2, VZV, CMV, HHV-6, and EBV) was applied to CSF from patients with meningitis, encephalitis, and other clinical syndromes.[5] This assay was further modified to include a reverse transcription step and primer pairs to detect enterovirus cDNA.[6,7] Utilizing equimolar concentrations of primers aligning the 3′ ends with one of two consensus regions within the herpesvirus DNA polymerase gene and the 5′ ends with the related or nonrelated sequences of each agent to be amplified, the first round of amplification yielded a 194-bp fragment indicating the presence of herpesvirus. The second round of amplification utilizing primer mixtures contained nonhomologous and type-specific primers selected from different regions of the aligned DNA polymerase genes of human herpesviruses producing a product with a different size for each related virus. These studies demonstrate the utility of this multiplex RT-nPCR for the detection of enteroviruses and herpesviruses in CSF samples from patients with various neurological manifestations and the usefulness of the technique in patient management and design of antiviral therapy. Additionally, a multiplex nPCR method was developed for the simultaneous detection and typing of all human lymphotropic herpesviruses described to date, including EBV, CMV, HHV-6, variants A and B, HHV7, and HHV8.[8]

A multiplex nPCR was also developed for differentiating simultaneously the DNA of polyomaviruses JC, BK, and SV40.[9] In the first amplification step the same set of primers were used to amplify a conserved DNA region of the large T antigen gene of JCV, BKV, and SV40. The second round of multiplex nPCR was carried out using a set of primers designed to render products of different sizes for each related virus. Cerebrospinal fluid was examined from AIDS patients with clinical and neuroradiological evidence of progressive multifocal leukoencephalopathy and from AIDS patients with other neurological alterations. Urine specimens from bone marrow transplant recipients affected by hemorrhagic cystitis were also tested. The results obtained suggest that the assay is a good tool for supporting the diagnosis of polyomavirus infection and could be used for epidemiological purposes and in other studies in order to define better the role of polyomaviruses in human disease. Other multiplex nPCR systems described allow the simultaneous detection of parainfluenza virus types 1 to 4,[10] measles virus, rubella virus, and parvovirus B19[11] or phlebovirus.[13]

Applications in Quantitative PCR

There are a wide variety of tests for the quantitative estimation of viruses. New methods to quantitate small amounts of DNA polyomavirus[14] and CMV[15] in clinical specimens were described based on nPCR. The method, a nested competitive polymerase chain reaction (ncPCR), is able to quantitate between 10 and 10^5–10^6 copies per tube of virus DNA and showed good reproducibility when clinical samples were analyzed. Throughout the whole procedure, an internal standard (mimic) competes for the primers with the target DNA. A solution containing 2×10^{10} DNA copies/ml is used as target sequence to generate a standard curve. Signals of both bands are analyzed by computerized densitometry. The original number of DNA copies present in clinical specimens is determined by interpolating the logarithmic signal ratio of amplified products into the standard curve.

Applications in Serotyping, Genotyping, and Subgrouping Viruses

Multiplex PCR has been shown to be a powerful and cost-effective tool for typing and subtyping virus strains in different epidemiological studies.

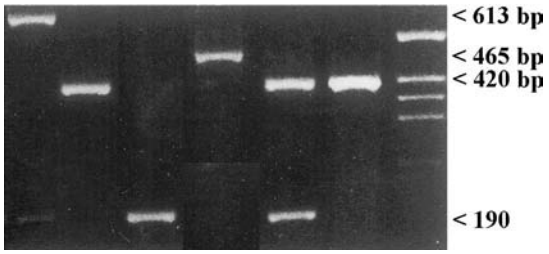

Fig. 2 gB Genotyping of clinical strains of CMV. From left to right lanes: gB2 (613 bp), gB 1 (420 bp), gB3 (190 bp), gB4 (465 bp), gB1 and gB3, gB1, molecular weights in base pairs. (*View this art in color at www.dekker.com.*)

For example, CMV gB was genotyped directly from clinical specimens by using multiplex nested PCR (M-nPCR) technology.[16] Specifically, a pair of primers to conserved regions of all gB genotypes within the gB gene (gB and gpUL55) was used for primary amplification. A mixture of nested primers to specific and conserved regions of each gB genotype was used for secondary PCR amplification, yielding amplicons of different sizes for each gB genotype that could easily be differentiated by agarose gel electrophoresis (Fig. 2). Using the above assay 40 serum specimens and 26 cerebrospinal fluid (CSF) samples, which had previously tested positive for CMV in 66 AIDS patients with different CMV disease conditions, were gB genotyped. Other nPCR systems are based on amplification and subsequent sequencing of the fragment for molecular analysis.[17–19] These systems allow genotyping and molecular analysis from small quantities of nucleic acid contained in clinical samples, without the need for cellular culture.

CONCLUSION

Nested and multiplex PCR have proven to be a valuable tool for the detection, quantification, and differentiation (subgrouping, subtyping, and genotyping) of viruses and other infectious agents.

ARTICLES OF FURTHER INTEREST

Multiplex Real-Time PCR, p. 855
Parvovirus B19, p. 976

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Neuroblastoma—Clinical Implications of Real-Time PCR

Jörg Dötsch
Wolfgang Rascher
University of Erlangen, Nürnberg, Germany

Holger Christiansen
University of Erlangen, Marburg, Germany

INTRODUCTION

This review summarizes present knowledge on the clinical implications of real-time polymerase chain reaction (PCR), a novel fluorescence-based method for the quantitation of gene expression and gene copy number variations, as well as gene amplification and locus haploinsufficiencies in neuroblastomas. Particular emphasis is placed on precision and accuracy and the comparison of TaqMan® real-time PCR with other techniques and devices for quantitative real-time PCR, and more traditional methods for the quantitation of gene expression such as competitive quantitative PCR. Finally, applications with potential clinical implications, such as the detection of minimal residual disease (MRD) or the assessment of tumor malignancy, are discussed.

TECHNICAL DESCRIPTION

Principles of TaqMan PCR

The use of the TaqMan reaction has been described in a number of original and review articles.[1,2] This approach makes use of the 5′ exonuclease activity of the DNA polymerase (AmpliTaq Gold®). Briefly, within the amplicon defined by a gene-specific PCR primer pair, an oligonucleotide probe labeled with two fluorescent dyes is created, designated as TaqMan probe. As long as the probe is intact, the emission of the reporter dye (i.e., 6-carboxy-fluorescein, FAM) at the 5′ end is quenched by the second fluorescence dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3′ end. During the extension phase of PCR, the polymerase cleaves the TaqMan probe, resulting in a release of reporter dye. The increasing amount of reporter dye emission is detected by an automated sequence detector combined with a special software (ABI Prism 7700 Sequence Detection System; Perkin-Elmer, Foster City, CA). The algorithm normalizes the reporter signal (R_n) to a passive reference. Next, the algorithm multiplies the standard deviation of the background R_n in the first few cycles (in most PCR systems, cycles 3–15, respectively) by a default factor of 10 to determine a threshold. The cycle at which this baseline level is exceeded is defined as threshold cycle (C_t) (Fig. 1). C_t has a linear relation with the logarithm of the initial template copy number. Its absolute value additionally depends on the efficiency of both DNA amplification and cleavage of the TaqMan probe. The C_t values of the samples are interpolated to an external reference curve constructed by plotting the relative or absolute amounts of a serial dilution of a known template vs. the corresponding C_t values.

In most cases of mRNA quantitation, gene expression has to be related to housekeeping genes that are expressed relatively stable throughout the cells. One major general difficulty in the use of housekeeping genes for the determination of mRNA transcript ratios is the possibility that their expression might also be altered by coexpression of pseudogenes and environmental changes (e.g., by hypoxia in case of glyceraldehyde-3-phosphate dehydrogenase). Pseudogenes may be eliminated using primer combinations that are intron spanning. However, unidentified influences cannot be dealt with as easily. Therefore, we suggest the use of at least two housekeeping genes for quantitation of mRNA expression. However, this aspect clearly needs further evaluation.

A different approach to quantify gene expression is the use of an external standard with a known gene expression or gene amplification. Although these preconditions are difficult to meet for mRNA quantitation, a reliable external standard can easily be obtained for N-MYC amplification at the DNA level because healthy volunteers usually do not carry more than two copies of the genomic sequence per cell in their peripheral blood.

Alternative Real-Time PCR Methods

There are other methods for real-time PCR not relying on exonuclease cleavage of a specific probe to generate a fluorescence signal. One of them, the LightCycler® System (Roche Molecular), makes use of so-called

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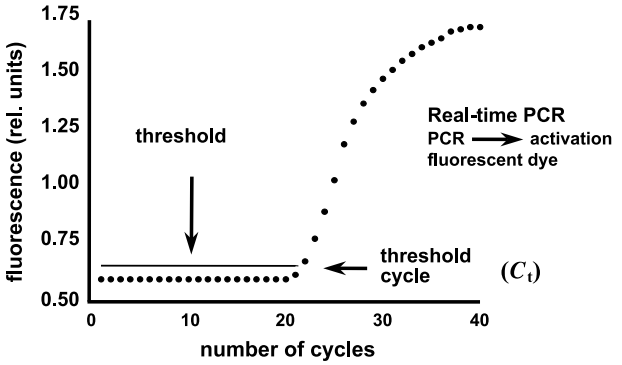

Fig. 1 Sketch of the principle of TaqMan PCR for the quantitation of gene expression. By measuring the amplicon concentration in the early exponential phase of the PCR reaction, the exhaustion of reagents is avoided. (From Ref. [3].)

''hybridization probes.''[4,5] Like exonuclease probes, hybridization probes are used in addition to PCR primers. But unlike the first, hybridization probes combine two different fluorescent labels to allow resonance energy transfer. One of them is activated by external light. When both probes bind very closely at the DNA molecules generated by the PCR amplification process, the emitted light from the first dye activates the fluorescent dye of the second probe. This second dye emits light with a longer wavelength, which is measured every cycle. Thus, the fluorescence intensity is directly correlated to the extent of probe hybridization and, subsequently, is directly related to the amount of PCR product. A major advantage of the LightCycler is the very short time of a PCR run. However, the TaqMan system allows to analyze a higher number of samples at one time and, at least theoretically, might be more accurate as there is an internal reference dye to monitor minor variations in sample preparation.

Apart from exonuclease and hybridization methods for real-time PCR, there are other options, including hairpin probes, hairpin primers, and intercalating fluorescent dyes. Hairpin probes, also known as molecular beacons, contain reverse complement sequences at both ends that bind together, whereas the rest of the strand remains single-stranded, creating a panhandle-like structure. In addition, there are fluorescent dyes at both ends of the molecule, a reporter and a quencher similar to the TaqMan probes. In the panhandle-like conformation, there is no fluorescence because the fluorescent reporter at one end and the quencher at the other end of the probe are very close to one another.[6] As the central part of a molecular beacon consists of a target-specific sequence, both ends are separated from each other when this part of the molecule is bound to the PCR product and a fluorescence signal can be emitted from the reporter dye. Hairpin primers, also named ''Amplifluor Primers®'' (Intergen), are similar to molecular beacons, but fluorescence is generated as they become incorporated into the double-stranded PCR product during amplification. Another very simple technique to monitor the generation of PCR product in a real-time fashion is the use of intercalating dyes, such as ethidium bromide and SYBR Green I®, which do not bind to single-stranded DNA but to the double-stranded PCR product.[5,7] However, hairpin primers and intercalating dyes do not offer the high specificity of the probe-based techniques and a positive signal might even be generated by primer dimers.

ROBUSTNESS, LIMITATIONS, AND PITFALLS IN THE USE OF REAL-TIME PCR

The use of TaqMan PCR may be particularly difficult if gene expression at a low level is to be quantified. One major pitfall in this context is the accidental determination of genomic DNA when RT-PCR is intended. There are various approaches to meet this problem: It is always useful to select primer combinations that are intron spanning.[8] If there is no possibility to select intron-spanning primers, RNA samples may be pretreated with DNAse. However, this measure should not be chosen routinely and may also be deleterious if only small amounts of RNA that are partially destroyed as well are present.[9]

One other difficulty is the high degree of technical expertise that is required to achieve as low a variation coefficient and as sensitive a measurement as possible. It could be shown that the degree of technical expertise may alter the gene level that is measured by up to 1000-fold.[9]

Because real-time PCR is highly sensitive, the risk of having interference with minor contamination is quite considerable. However, the risk of false-negative results must not be underestimated because post hoc PCR steps are not ''visible'' to the degree that is provided by the more traditional methods for gene quantification.[10]

RELIABILITY AND SPECIFICITY OF REAL-TIME PCR

The use of TaqMan real-time PCR for the quantification of gene expression in neuroblastoma tissues has been shown to be at least as reliable as the application of other quantitative PCR techniques such as competitive PCR.[11] Whereas the expression of highly expressed genes, such as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, is well correlated between the two methods for the determination of genes with lower expression such as neuropeptide Y (NPY), TaqMan PCR is much more sensitive than competitive PCR in neuroblastoma tissues.[11] In addition, the spectrum of linear measurements for real-time PCR is in the range of 10^6, in contrast to 10^2 in competitive RT-PCR. Finally, a considerably

higher number of samples per day can be measured by real-time PCR (up to 400 measurements). In comparison to the Northern blot assessment, only a minimal fraction of mRNA is necessary to quantify gene expression by real-time PCR.[1]

CLINICAL APPLICATIONS OF REAL-TIME PCR APPLICATION IN NEUROBLASTOMAS

Quantitation of Prognostic Markers in Neuroblastoma Tissue

A number of biochemical markers have been investigated on the gene level as to their relevance in relation to patient prognosis. Among these are N-MYC, NPY, and somatostatin 2 gene expression.[12] The first two will be discussed in detail.

N-MYC

N-MYC amplification in neuroblastomas is of utmost interest with regards to the prognosis of disease. In fact, in clinical practice, N-MYC gene expression correlates with both advanced disease stage and rapid tumor progression.[13]

Several methods have been used for the detection of N-MYC detection mainly based on Southern or dot blot, quantitative PCR and fluorescent in situ hybridization techniques. However, the use of most PCR methods is restricted by the fact that end-point measurements are used for quantitation. Therefore, Raggi et al.[14] introduced a TaqMan real-time base method for the determination of N-MYC amplification in neuroblastomas. The authors demonstrate a precise assay with an interassay coefficient of variation of 13% and an intraassay coefficient of variation of 11%. The threshold cycle for the detection of N-MYC correlates in an inverse linear way with the logarithm of the input of genomic DNA molecules. There is a good linear relationship between the N-MYC amplification measured by TaqMan real-time PCR and competitive PCR. Using Kaplan–Meier survival curves, the authors showed that the amplification of N-MYC as assessed by TaqMan real-time PCR is closely linked to cumulative survival as this has already been demonstrated with several other techniques for the quantitation of N-MYC amplification.

Similar results were obtained by others using the LightCycler approach for the quantitation of N-MYC amplification.[15]

NPY

The detection of NPY mRNA expression in neuroblastomas has been of particular interest because NPY is known to correlate with the differentiation and malignancy of neuroblastomas. NPY is released into the circulation and may reflect the tumor burden of an individual neuroblastoma patient.

Consequently, NPY gene expression has been quantified by using a number of different techniques for quantitation such as Northern blot, competitive RT-PCR, and, lately, TaqMan real-time PCR.[11]

There is a good linear relationship between NPY mRNA expression as measured by quantitative competitive PCR and TaqMan real-time PCR. However, in the lower concentration range, only real-time PCR is able to discriminate different NPY mRNA levels. This finding suggests that the more traditional competitive PCR might not be able to detect differences in the expression of less abundant genes in neuroblastoma tissues. Definitely, with a limited amount of cells, real-time PCR would be the more desirable method to use.

MRD

Lately, the application of real-time PCR has focused on the detection of MRD of neuroblastoma. A number of groups used tyrosine hydroxylase—the key enzyme for the synthesis of catecholamines as a marker for the detection of neuroblastoma cells in the blood and bone marrow.

One group depicts that as few as 70 transcripts per milliliters can be measured. There were no false-negative samples in control samples.[16] Similar results with regards to the use of tyrosine hydroxylase for the detection of MRD were reported by another group, which described the sensitivity of the assay as $1{:}10^6$ white blood cells.[17] Again, real-time PCR was found to be more sensitive than qualitative PCR in the detection of MRD.[18] However, one pitfall in the use of tyrosine hydroxylase might be its association with a higher degree of differentiation of the neuroblastoma. Therefore, less differentiated tumors might be detected at higher threshold levels, or could even be missed.

A different surrogate parameter for the quantitation of MRD in neuroblastoma patients is GD2 synthase—the key enzyme for the synthesis of β1,4-*N*-acetylgalactosaminyltransferase. The measurements proved to have clinical relevance in evaluating adjuvant therapy with anti-GD2 antibodies for bone marrow purging in neuroblastoma by detecting a subclinical level of tumor cells in bone marrow aspirates and was predictive of long-term outcome.[19,20]

CONCLUSION

The recent studies that have used real-time PCR for the quantitation of target genes in neuroblastoma tissues

and for the detection of MRD provide encouraging data for the use of this technique introduced only 7 years ago. Apart from a high degree of precision, practical advantages of real-time PCR, such as easy handling, rapid measurements, and a broad linear range for the measurements, have to be highlighted.

From a diagnostic point of view, one interesting aspect for the future might be the use of semiautomated or automated real-time devices for the assessment of gene expression and amplification in neuroblastomas, putting into consideration the relatively easy and little time-consuming way of measurement. Real-time PCR might help to quantify more prognostic markers, expression of genes involved in multidrug resistance, and genes related to tumor invasion and metastasis. There are first reports on the use of multiplex real-time PCR, a development that might certainly facilitate diagnostic procedure in neuroblastoma diagnostics in the coming years.

Disease management as such might initially not be altered by a new method for the determination of genetic neuroblastoma markers. However, if this relatively novel method becomes available more widely (e.g., as a consequence of dropping costs for this still-very-expensive technique), the number of prognostic markers examined might help to improve therapeutic strategies in the long run.

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Neurofibromatosis Type 1

Bruce R. Korf
University of Alabama at Birmingham, Birmingham, Alabama, U.S.A.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder that affects approximately 1 in 3500 individuals throughout the world. It displays a high rate of new mutation and complete penetrance. Diagnosis is based on clinical criteria, but molecular testing is now available on a clinical basis. Insights into pathophysiology based on understanding the *NF1* gene and the cellular pathway in which it participates are beginning to suggest possible avenues of therapy, with several clinical trials currently under way.

CLINICAL DESCRIPTION

NF1 is a progressive multisystem disorder, which includes some features that occur early in life and others that do not appear until later. It displays a wide range of variable expression, including differences within the same family. The hallmark feature is the neurofibroma, a benign tumor that arises from the nerve sheath.[1] There are two major types of neurofibromas: focal growths and plexiform neurofibromas. Focal neurofibromas arise as discrete nodules at some point along a nerve. They may occur at the nerve root, along the length of a nerve, or at a nerve ending. They may be visible as growths that protrude above the surface of the skin, or cause a swelling due to subcutaneous growth. Dermal neurofibromas often present with a purplish discoloration to the overlying skin. They are soft and painless, but may have significant cosmetic impact. Internal neurofibromas are usually asymptomatic, but some can cause symptoms of nerve compression such as pain or sensory loss, or may invade the spinal canal and cause cord compression. Focal neurofibromas are rarely present in young children, but gradually increase in number over the years. Tumors often first appear or grow during puberty, or in women, during pregnancy. The numbers of neurofibromas vary widely in individuals, from a few small tumors to uncountably many.

Plexiform neurofibromas consist of a diffuse enlargement of multiple fascicles of a nerve. Some extend over a considerable distance and include multiple nerve branches. Plexiform neurofibromas may be associated with overgrowth of soft tissues, causing significant cosmetic deformity and functional impairment. Most, if not all, occur congenitally. The tumors tend to grow during the early childhood years, and often remain static in size thereafter. Plexiform neurofibromas that occur near the surface of the body may invade the skin, causing thickening and palpable cords of tumor. Some are associated with irregular hyperpigmentation of the overlying skin.

NF1 usually presents with pigmentary features. Multiple café-au-lait macules begin in early childhood, and skin-fold freckles appear between 3 and 5 years of age.[2] Iris Lisch nodules are melanocytic hamartomas that are useful in diagnosis but are clinically silent.[3] Other common nontumor manifestations include skeletal dysplasia, macrocephaly, vascular dysplasia, and learning disabilities. Skeletal dysplasia most often affects long bones, particularly the tibia.[4] Tibial dysplasia presents in infancy and creates risk of fracture. Orbital dysplasia occurs in association with trigeminal plexiform neurofibroma. Children with NF1 tend to be short and have relative or absolute macrocephaly.[5] Vascular dysplasia consists of nodular proliferation within the arterial wall and may cause arterial stenosis or hemorrhage due to dissection.[6] Learning disabilities occur in at least 50% of affected individuals and include both verbal and nonverbal problems.[7] There may also be attention deficit disorder and neuromotor problems.

Individuals with NF1 are at increased risk for malignant neoplasms.[8] The most common is optic glioma, affecting approximately 15%.[9] Most of these tumors are asymptomatic, but progressive growths can impair vision or cause neuroendocrine disturbance. Gliomas can occur elsewhere in the brain, especially the brainstem. Malignant peripheral nerve sheath tumors are sarcoma-like lesions that arise from neurofibromas (usually plexiform neurofibromas), with a lifetime risk around 10%. Other malignancies that are associated with NF1 include juvenile myelomonocytic leukemia and rhabdomyosarcoma.

DIFFERENTIAL DIAGNOSIS

NF1 is one of a set of disorders subsumed under the term ''neurofibromatosis.'' The others are NF2 and schwannomatosis. The features of these disorders are compared in

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Table 1 Comparison of features of the three neurofibromatoses

	NF1	NF2	Schwannomatosis
Inheritance	Autosomal dominant Complete penetrance	Autosomal dominant Complete penetrance	Autosomal dominant Incomplete penetrance
Frequency	1:4000	1:40,000	Very rare
Features	Neurofibromas; café-au-lait macules; learning disabilities; skeletal dysplasia	Vestibular schwannomas; other schwannomas; meningiomas; ependymomas; cataract	Schwannomas
Gene/Protein	*NF1*—chromosome 17 Neurofibromin	*NF2*—chromosome 22 Merlin/schwannomin	Unknown
Function	GTPase-activating protein	Cytoskeletal protein	Unknown

Table 1. The clinical distinction between NF1 and the others is usually relatively easy to establish: only NF1 is associated with multiple café-au-lait macules, neurofibromas, optic glioma, etc. Both NF2 and schwannomatosis are characterized by multiple schwannomas, with the vestibular schwannoma being the hallmark of NF2. Clinical diagnostic criteria for NF1 are presented in Table 2.[10] Many of the features are age dependent, so the diagnosis is often not established at first evaluation of a young child. There are several variant forms of NF that need to be considered; these are also described in Table 2.

MANAGEMENT

There is no definitive medical treatment for NF1; management is currently limited to surveillance and symptomatic treatment of symptomatic manifestations. Life expectancy is decreased on average, with disease-related mortality due to malignancy and vascular accidents.[11] Affected individuals should be seen on a regular basis by a physician familiar with the disorder. Children should be followed by an ophthalmologist for evidence of optic glioma. Neurofibromas can be removed

Table 2 Diagnostic criteria for NF1 (top) and variant forms of NF1 (bottom)

- At least six café-au-lait macules
  5 mm prepuberty
  15 mm postpuberty
- Skin-fold freckles
- Two or more neurofibromas/one plexiform neurofibroma
- Two or more iris Lisch nodules
- Optic glioma
- Characteristic skeletal dysplasia
  Tibia
  Orbit
- Affected first-degree relative

Variant form	Features
Familial multiple café-au-lait spots	Autosomal dominant multiple cafe-au-lait spots with no other signs of NF1; some families allelic to NF1, others not
Spinal neurofibromatosis	Multiple spinal nerve root neurofibromas, but no other NF1 features; may be allelic to NF1 or NF2
Watson syndrome	Pulmonic stenosis, developmental delay, café-au-lait spots; allelic to NF1
Segmental NF	Features of NF1 confined to a restricted body region; most likely due to mosaicism for *NF1* mutation

An individual who fulfills any two criteria can be diagnosed as being affected.

Table 3 Major types of *NF1* gene mutation and applicable approaches to mutation detection

Type of mutation	Technique(s)
Whole gene deletion	FISH, microsatellite analysis for hemizygosity
Multiexon deletion or small insertion	RT-PCR analysis of RNA; Southern analysis; sequence analysis of cDNA
Splicing mutation	Analysis of cDNA for size, followed by sequencing; sequencing of intron–exon borders
Stop mutation/frameshift	Protein-truncation assay followed by sequencing, or direct sequencing of cDNA or genomic DNA
Missense mutation	Sequencing of cDNA or genomic DNA
Chromosome rearrangement	Chromosomal analysis

surgically, although regrowth of plexiform lesions is common. Optic gliomas that are asymptomatic do not need to be treated, but symptomatic lesions are treated with chemotherapy (vincristine/carboplatin). Children should be followed for the occurrence of learning disabilities and attention deficit disorder, both of which are managed by standard means. Affected individuals should be counseled regarding the warning signs of malignancy, particularly unexplained pain or sudden growth of a neurofibroma. Clinical trials are now underway toward development of nonsurgical treatments of neurofibromas, particularly plexiform neurofibromas. Clinical trials are tracked on the website http://www.nf.org/clinical_trials.

GENETIC COUNSELING

NF1 is inherited as an autosomal dominant, with complete penetrance but variable expressivity. An affected individual has a 50% chance of transmission of NF1 to any offspring, although severity cannot be predicted. Approximately 50% of cases occur sporadically due to apparent new mutation. Unaffected parents of a sporadically affected child face a risk of recurrence based on germline mosaicism, although this risk is low.

MOLECULAR GENETICS

The *NF1* gene is located on chromosome 17 and encompasses more than 300 kb of genomic DNA with 60 exons. The protein product is referred to as ''neurofibromin,'' a 2818 amino acid protein. Neurofibromin includes a GTPase-activating protein (GAP) domain, which regulates the conversion of Ras-GTP to Ras-GDP. Neurofibromin therefore appears to be a regulator of Ras-mediated intracellular signaling.[12] The *NF1* gene behaves as a classic tumor suppressor. Affected individuals are heterozygous for *NF1* mutations, but both alleles are mutated in tumor cells. The ''tumor cell'' of the neurofibroma is the Schwann cell, but much of the bulk of the lesion consists of other heterozygous cells, including fibroblasts and mast cells, recruited to the lesion by cytokines yet to be identified. Malignant growths are likely to arise from the acquisition of additional genetic changes, such as loss of function of p53. It is unclear whether some of the nontumor manifestations, such as learning disability, also arise from a tumor suppressor mechanism, or whether haploinsufficiency explains any of these lesions.

MOLECULAR GENETIC TESTING

Until recently, there was no reliable molecular diagnostic test for NF1, so diagnosis was limited to application of clinical criteria. Although it is usually easy to establish a clinical diagnosis of NF1, there are several situations in which molecular testing would be useful. The clinical diagnosis often is uncertain in young children with multiple café-au-lait spots or an affected parent, because other features such as skin-fold freckles or neurofibromas may not occur until a child is older. Individuals with atypical presentations, such as isolated plexiform neurofibromas, may also benefit from molecular testing. Finally, molecular diagnosis offers the possibility of prenatal testing by amniocentesis, chorionic villus sampling, or preimplantation testing.

The major challenges posed in molecular diagnosis are the large size of the gene and the fact that mutations can be distributed anywhere along the gene. There are no major ''hotspots'' or recurrent mutations. Mutation analysis requires use of a set of complementary techniques to detect the various types of mutations, including complete gene deletion, intragenic deletion or duplication, frameshift, stop mutation, splicing mutation, and the rare missense mutation[13] (Table 3). Genotype–phenotype correlations have been difficult to establish. The one clear correlation is that individuals with complete gene deletion tend to have a severe course, with developmental delay, dysmorphic features, and large numbers of neurofibromas.[14] The possibility of mosaicism must be considered in those with signs of the disorder in whom

no mutation is found; study of affected cells, such as Schwann cells from a neurofibroma, may be necessary to identify a mutation in individuals with mosaicism.

CONCLUSION

Neurofibromatosis type 1 is a relatively common single gene disorder with widely varied, progressive features. Management is currently focused on clinical diagnosis, surveillance, symptomatic treatment, anticipatory guidance, and genetic counseling. Since the identification of the *NF1* gene much has been learned about pathophysiology, indicating that the gene functions as a tumor suppressor. Genetic testing is now a clinical reality, and clinical trials have begun, raising hope of development of nonsurgical treatments in the near future.

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Neuronal Ceroid Lipofuscinoses

Nanbert Zhong
New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

INTRODUCTION

Neuronal ceroid lipofuscinoses (NCLs), also known as Batten disease, comprise eight recessive and one dominant form, as well as several rare and atypical variants (Table 1) that are as yet unclassified. They are characterized by intralysosomal accumulations of ceroid lipopigments as the hallmark, which can be detected as granular osmiophilic deposits (GRODs), or curvilinear (CV), fingerprint (FP), or rectilinear (RL) profiles by electron microscopy (EM). In typical cases, GRODs are present in NCL_1, CV in NCL_2, and FP in NCL_3. However, the profiles can vary, and mixed profiles can occur.[1]

The incidence of NCLs has been estimated to be approximately 0.1–7 in 100,000 births in the general population, and as high as 1 in 12,500 births as a result of founder effects in genetically isolated populations such as the Finnish population.[1] With this number still growing, NCLs are becoming one of the most common neurodegenerative disorders of childhood.[2]

Although efforts at gene therapy, either in vivo or ex vivo, and protein replacement are making progress, presently, there is no effective treatment available for NCLs. The outcome of the disease is usually fatal within a few years after the onset of clinical symptoms.[3] Early definitive diagnosis of NCLs and characterization of genetic deficiency in the affected family are the primary intervention approaches. Carrier identification and prenatal diagnosis are the only ways currently available to prevent NCLs.

NCLs ARE GENETICALLY HETEROGENEOUS

Genetic studies have identified six genes—CLN_{1-3}, $CLN_{5,6}$, and CLN_8—underlying NCL_{1-3}, $NCL_{5,6}$, and NCL_8, respectively (Table 1). The function of each gene is yet unclear, although two lysosomal peptidases, palmitoyl-protein thioesterase 1 (PPT1) and tripeptidyl peptidase 1 (TPP1), have been characterized. The CLN_3-encoded protein, Battenin; the CLN_6-encoded protein, CLN6p; and the CLN_8-encoded protein, CLN8p are predicted to be transmembrane proteins. The CLN_5-encoded protein, CLN5p, was determined to be a lysosomal soluble protein, even if it contains hydrophobic segments. A total of 151 mutations have been identified, including 41 at locus CLN_1, 52 at CLN_2, 31 at CLN_3, 4 at CLN_5, 22 at CLN_6, and 1 at CLN_8 (http://www.ucl.ac.uk/ncl). Heterogeneity, both phenotypically and genotypically, has been demonstrated. CLN_1 mutations can result in clinical onset at infantile, late-infantile, or adult age; however, late-infantile onset can be caused by mutations of CLN_2, CLN_3, CLN_5, CLN_6, and CLN_8 genes.[2,4–6]

KEY PROCEDURES FOR DIAGNOSING NCLs

Clinical Assessment

Clinical features of NCLs include rapid progressive deterioration of vision, seizures, development of mental retardation, movement disorders, and behavioral changes.[7] Brain atrophy as documented by imaging studies, including magnetic resonance imaging (MRI), is most pronounced supratentorially and in the brainstem. Cerebellar atrophy, which results in enlargement of the fourth ventricle without concomitant cerebral atrophy, may be an early sign of NCLs.[8,9]

Practically, the progressive development of signs and symptoms is variable over time. Having a clear diagnostic algorithm (Fig. 1) will facilitate the clinical workup and characterization of the genetic deficiency. A good clinical evaluation, by which each individual examiner is capable of assigning an accurate Clinical Coding Score (CCS)[2] to any NCL patient, should be applied in the neurological assessment. The symptoms that should be evaluated include age at onset, first/initial symptoms at onset, and clinical course such as vision loss/blindness, learning and speaking disabilities, difficulty/inability to walk, involuntary movements/lack of coordination, rigidity/increased muscle tone, behavioral problems, and seizures/convulsions. A neuropsychological assessment for NCL patients may include WISC-IV,[10] WRAML,[11] and NEPSY,[12] in addition to the standard Mental Status Examination.[13]

Laboratory Diagnostic Strategies

Pathological studies using EM to identify ceroid lipopigmental inclusions were, and still are, the standard

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Table 1 NCLs comprise a group of neurodegenerative diseases characterized by ceroid lipopigments

OMIM	Genetic classification	Historic name of the disease	Age at onset	Clinical classification[a]	EM of ceroid lipopigment[b]	Chromosome location	Gene or locus	Ethnicity
256730	NCL_1	Santavuori–Haltia	Infantile, late infantile, juvenile, adult	INCL	GROD	1p32	CLN_1	Global, Finland
204500	NCL_2	Jansky–Bielschowsky	Late infantile, infantile, juvenile	LINCL	CV or mixed	11p15	CLN_2	Global
204200	NCL_3	Vogt–Spielmeyer or Batten	Juvenile	JNCL	FP or mixed	16p12	CLN_3	Global
204300	NCL_4	Kufs	Adult	ANCL	Mixed	*Unknown*	CLN_4[c]	Global
256731	NCL_5	Finnish variant	Late infantile	fLINCL	FP, CV, or RL	13q31	CLN_5	Finland, Europe
601780	NCL_6	Portugal/gypsy/ Indian variant	Late infantile	pLINCL	CV, FP, or RL	15q21	CLN_6	Global
N/A	NCL_7	Turkish variant	Late infantile	tLINCL	FP or mixed	*Unknown*	CLN_7[c]	Turkish (?)
600143	NCL_8	Northern epilepsy	Late infantile	EPMR[d]	CV- or GROD-like	8p23	CLN_8	Global, Finland
162350	NCL_9	Parry-type Kufs	Adult	dANCL	Mixed	*Unknown*	CLN_9[c]	Global
N/A	NCL_{10}	Unclassified variants	Late infantile	Unclassified	Mixed	*Unknown*	CLN_{10}[c]	Global

N/A = not available; (?) = unconfirmed.
[a]INCL = infantile NCL; LINCL = late-infantile NCL; JNCL = juvenile NCL; ANCL = adult NCL.
[b]GROD = granular osmiophilic deposit; CV = curvilinear; FP = fingerprint; RL = rectilinear.
[c]Gene is not yet identified.
[d]Progressive epilepsy with mental retardation.

procedure for making the initial differential diagnosis from other neurological disorders.[7] Whenever possible, EM should be carried out at the initial medical center for a patient believed to have an NCL. If the EM results are positive for lipopigment inclusions, further genetic characterization should be pursued. If the initial EM findings are negative, then the EM study should be repeated with different tissues every 3–6 months.

Deficiency of PPT1 or TPP1 has been determined in various tissues such as lymphocytes, lymphoblasts, fibroblasts, brain, and amniotic cells, and chorionic villi in NCL_1[14,15] or NCL_2.[16,17] Testing the activities of PPT1 or TPP1 may help to identify or exclude NCL_1 or NCL_2 patients. Testing of enzymatic activity is the choice method for carriers, for whom the information of familial DNA mutation is not available. However, if the familial mutations have been identified, molecular testing of the mutation(s) would be preferred to determine the carrier status.

Molecular testing of NCLs consists of two phases: clinically based testing for common mutations, and research-based testing for rare or novel mutations. Conducting molecular analyses of CLN_{1-3} genes will allow identification of the majority of NCL patients/families, which account for >85–90% of clinically recognized NCL cases in the general population with mixed ethnic backgrounds. Five common mutations in CLN_{1-3} genes, which are c.223A→G (Y109D) and c.451 C→T (R151X) in CLN_1, IVS5-1G→C (splicing error) and c.636 C→T (R280X) in CLN_2, and deletion of a 1.02-kb genomic fragment involving exons 7 and 8 in CLN_3, have been characterized.[18] Testing for these mutations may provide a sensitivity ranging from 66% for NCL_1, to 75% for NCL_2, to 78% for NCL_3, among clinically referred cases.[5,19] Mutation of the 2-bp deletion in exon 4 of the CLN_5 gene accounts for 90% of NCL_5 cases,[20] and the mutation c.70C→G (R24G) in CLN_8 was seen in all NCL_8 cases.[21] No single mutation in the CLN_6 gene could be characterized as common in the heterozygous population, but the mutation c.214G→T (E72X) in exon 3 has been found in 91% of NCL_6 families from Costa Rica.[22–24] A total of 144 ''private'' or ''familial'' mutations identified in CLN_{1-3}, $CLN_{5,6}$, and CLN_8 genes can be detected in research laboratories. NCL patients with a specific ethnic background (Fig. 1b) may present with one of the NCL variants encoded by the $CLN_{5,6}$ or CLN_8 gene. Therefore, mutation screening should be extended to these genes.

Molecular technology involved in the genetic analysis of common mutations (Table 2) for NCL_{1-3}, NCL_6, and NCL_8 includes the amplification refractory mutation system (ARMS) and allele-specific primer extension (ASPE).[18,25] DNA sequencing of polymerase chain reaction (PCR)-amplified genomic fragments (PCR

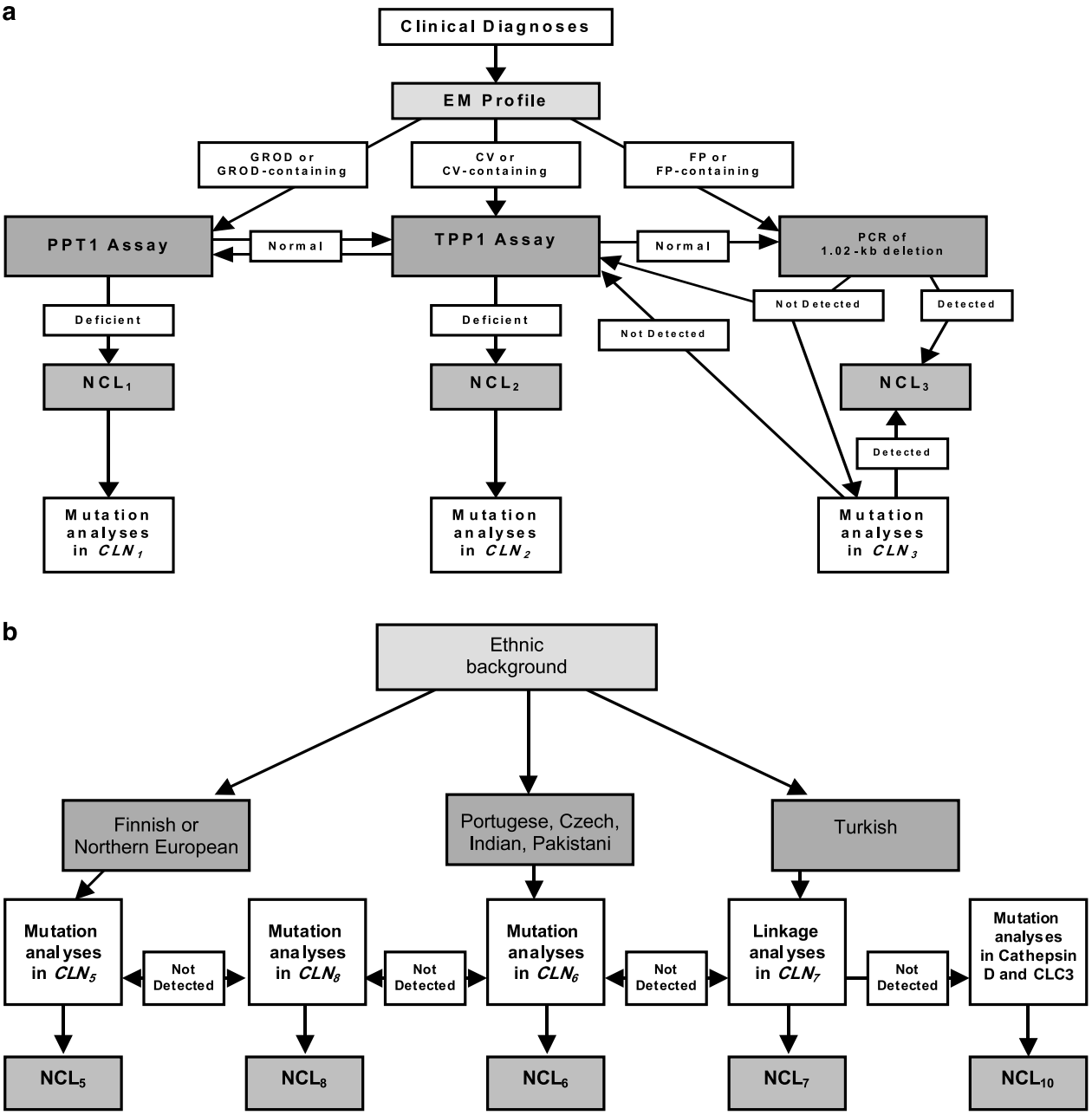

Fig. 1 (a) Diagnostic algorithm for $NCL_{1–3}$. Following clinical assessment, EM studies would provide ultrastructural information guiding the assays of either PPT1 or TPP1, and DNA deletion at the *CLN*$_3$ gene. If enzyme deficiency is demonstrated, mutation analyses will be followed at the *CLN*$_1$ or *CLN*$_2$ gene. (b) Diagnostic algorithm for $NCL_{5–10}$. Following molecular studies on *CLN*$_{1–3}$, the patient should be subjected to further analyses in genetic loci of *CLN*$_{5–10}$. NCL_9 is not included because of dominant inheritance, which can be recognized clinically. (*View this art in color at www.dekker.com.*)

sequencing), including six fragments for *CLN*$_1$, four for *CLN*$_2$, six for *CLN*$_3$, and five for *CLN*$_6$, is used as a golden standard procedure to screen rare or familial mutations for $NCL_{1–3}$ (Table 3). The PCR sequencing is also applied in NCL_6.[24] Although detailed information regarding primer sequences and PCR conditions is provided in this entry, the laboratory that performs the procedures should be aware that each individual protocol ought to be optimized, especially the annealing temperatures and the final concentration of Mg^{2+} in the PCR reactions. Validating the molecular protocols before genetic testing is offered because a clinical test is highly recommended.

Table 2 Molecular testing of common mutations for NCLs

Molecular approach	Genetic locus	Common mutation	Primer	Sequence (5′→3′)	T_a (°C)[a]
ARMS	*CLN*$_1$	c.223A→G	223F[b]	ggagttgtaagtgagttatag	57
			223A	ccttacctccatcagggt	
			223G	ccttacctccatcagggg	
		c.451C→T	451F	ttctcacagtgccttgtgc	57
			451C	gctctctcctgggcatcg	
			451T	gctctctcctgggcatca	
	CLN$_2$	c.6363C→T	636R[b]	ggtggtaaggaattgaggac	64
			636C	ccctctgtgatccgtaagc	
			636T	ccctctgtgatccgtaagt	
		IVS5-1G→C	IVS5R	ggtggtaaggaattgaggac	64
			IVS5-1G	agcctgacttctccctacag	
			IVS5-1C	agcctgacttctccctacac	
ASPE	*CLN*$_3$	1.02-kb deletion	1.02F	cattctgtcacccttagaagcc	56
			1.02NL[c]	gggggaggacaagcactg	
			1.02Mut[d]	ggacttgaaggacggagtct	
ARMS	*CLN*$_5$	2-bp deletion	2-bp R	gaaacatgttcaaccaaatggc	60
			2-bp NL	ataggtaaagggatttcttcatatg	
			2-bp Mut	gataggtaaagggatttcttcatg	
	CLN$_6$	c.214G→T	214R	gccctgggacagtaccttga	60
			214G	gcagctggtattccctctcg	
			214T	gcagctggtattccctctct	
	CLN$_8$	c.70C→T	70R	gatgcatgcacttacctttaag	63
			70C	actatgcatcctgggggatcc	
			70G	actatgcatcctgggggatcg	

[a]T_a = annealing temperature.
[b]F = forward; R = reverse.
[c]NL = normal allele.
[d]Mut = mutant allele.

CONCLUSION

Making a definitive diagnosis is a great challenge for health professionals dealing with NCLs. Accomplishment of the entire diagnostic procedure may require months, years, or decades before genetic approaches are applied, whereas enzymatic and molecular tests need only days or weeks. It is recommended that genetic tests be pursued when a patient shows progressive neurological symptoms, on account of not only time concerns, but also economic concerns. However, the molecular tests need to include more loci/mutations or to employ cutting-edge technology, such as microarray-based hybridization or capillary-based electrophoresis, to increase detection sensitivity.

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Table 3 Molecular screening of rare mutations for NCLs

Molecular approach	Genetic locus	Rare mutation	Primer	Sequence (5′→3′)	T_a (°C)[a]
PCR sequencing	*CLN*$_1$	Exon 1	In[b]0-51466F[c]	ctcggccagcggggtctgg	60
			In1-52100R	tcatatagcccagccgcagc	
		Exon 2–4	In1-56293F	gataatgctgtttgaggcctcag	63
			In4-57729R	aagtctcttcctatgtctccagc	
		Exon 5	In4-59003F	gatgaggatgctaaggatgaag	61
			In5-59797R	gccaggcgtagtggcgcatg	
		Exon 6–7	In5-68262F	ggattacaggtgtgagccactg	63
			In7-70422R	agtgctgggattacaggcatgag	
		Exon 8	In7-71602F	gctgacctctgtactcaacagtg	61
			In8-72211R	ccatattggacatgagtcaggtg	
		Exon 9	In8-74591F	taatactcaggacaaactgcatta	60
			In9-76322R	ctacccatttcctgaggcctg	
	CLN$_2$	Exon 1–3	E1-754F[d]	cttgggaacccataaacaggacc	65
			In3-2039R	tctgacatgatcgccatcccatg	
		Exon 4–6	In3-2892F	ggaaagcaatgaatgagggcaag	63
			In6-3807R	caggtggtaaggaattgaggaca	
		Exon 7–9	In6-3782F	cagtgtcctcaattccttaccac	63
			In9-4749R	gaaaggtgtgtggttccacctg	
		Exon 10–13	In9-5064F	ctccagcaagacctgggctata	64
			In13-6249R	ccaacagggcagaataagggac	
	CLN$_3$	Exon 1–2	E1-458F	cctgccaatagatcattcccg	60
			In2-1568R	tcacttccctcttctcatgcc	
		Exon 3–4	In2-3452F	cccaacattgaatgaggatgg	60
			In4-4428R	tcaggcaaggaccaggtgag	
		Exon 5–8	In4-5209F	cctggaagctctgcggtcta	60
			In6-6772R	ctgaccttaggcgatctgcc	
		Exon 9	In8-8716F	cttagaggcagtgagctacc	58
			In9-9085R	ccataaccagtacactcactc	
		Exon 10–13	In9-10203F	tgcgtggtctggttaattgtg	60
			In13-11034R	caagattgcgctactgcactc	
		Exon 14–15	In13-15028F	ggaggccacctctccttccc	63
			In15-15798R	gagaaatctgctgacatacctttc	
	CLN$_3$	Exon 1	In0-105721F	gagaggagacaggactggag	60
			In1-107352R	cagacagagaaggaaacgcgg	
		Exon 2	In1-117930F	gacctctttgtggctaatctc	59
			In2-118221R	ggagaagtgacttgtctaagg	
		Exon 3	In2-122052F	gacaagtccgttacctcctctg	60
			In3-122652R	ccttctacctgaacacctctc	
		Exon 4–5	In3-124746F	tcacatactgtgacagaggtgg	62
			In5-125545R	gagctcacagtgcctttacag	
		Exon 6–7	In5-126797F	gtaaactgtgacgtggcactc	60
			In7-128637R	actcatgctctcggtctctggtta	

[a]T_a = annealing temperature.
[b]In = intron.
[c]F = forward; R = reverse.
[d]E = exon.

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Nipah Virus

Michael S. Bronze
Ronald A. Greenfield
University of Oklahoma, Oklahoma City, Oklahoma, U.S.A.

INTRODUCTION

Nipah virus (NiV) is a recently described zoonosis responsible for an epidemic of severe, febrile encephalitis in Malaysia and Singapore. Taxonomically, NiV is a member of the Paramyxoviridae family, Paramyxovirinae subfamily, and *Henipavirus* genus. There were 265 reported cases that resulted in 105 deaths. Ninety-two percent of the reported cases in Malaysia involved patients employed in pig-farming industries. The primary risk factors for infection were the presence of sick or dying pigs on farms and exposure to pigs displaying signs of NiV infection. Human to human transmission is thought to be uncommon. Affected patients presented with fever, headache, dizziness, and prominent neurological features, including changes in mentation and consciousness and signs of brainstem dysfunction. Pathologic examination of the brain revealed prominent vasculitis and thrombosis, neuronal degeneration, and nuclear and eosinophilic cytoplasmic inclusions. Treatment with ribavirin improved mortality in affected patients, and the epidemic was controlled after the culling of nearly 1 million pigs.

EPIDEMIOLOGY

The outbreak of NiV encephalitis began in September 1998 in Malaysia and subsequently spread to other regions of pig farming and then to Singapore. The outbreak was preceded by a respiratory illness in pigs and was originally thought related to Japanese encephalitis virus (JEV). However, the infection did not abate with mosquito control measures and JEV vaccination strategies, and all but a few persons were seronegative for anti-JEV IgM antibodies.[1] The 265 reported human cases likely represent an underestimate of the total number of human infections. The number of infected individuals who experienced asymptomatic seroconversion or became only mildly ill is not precisely known.[2] Asymptomatic infection was serologically identified in 6–17% of those tested in a case-control study evaluating risk factors for NiV infection.[3]

Epidemiological studies revealed that the primary risk for transmission to humans was exposure to sick pigs or fresh pig products. Only 8% of persons with NiV encephalitis had no reported contact with sick pigs, pig urine, or feces.[3] An additional risk factor included employment as an abattoir worker; however, employment as a pork seller or being part of the military effort responsible for culling pigs as part of an eradication campaign carried low risk for acquisition of infection. Human to human transmission was thought to be uncommon.[4]

Based on similarities to Hendra virus (HeV), a related *Henipavirus*, flying foxes (fruit bats) were evaluated as a natural reservoir of NiV. The presence of neutralizing antibodies against NiV was detected mainly in Island flying foxes (*Pteropus hypomelanus*) and Malayan flying foxes (*Pteropus vampyrus*), and NiV has been detected in the urine of Island flying foxes.[5] Neutralizing antibodies have not been routinely found in other animal species. These observations led to the hypothesis that pigs acquire the virus by consuming fruits partially eaten by bats that release the virus in their secretions.[2] Infection in humans is thought to be a dead-end infection.

VIROLOGY AND PATHOGENESIS

Viruses of the Paramyxoviridae family include a number of highly contagious human and animal pathogens, including measles and mumps viruses. NiV is a single-stranded, negative sense nonsegmented RNA virus that is protein encapsidated, helical in shape, and is 120–500 nm in diameter.[6] By electron microscopy, the virus is pleomorphic in appearance and the nucleocapsid has a ''herringbone appearance,'' as seen in Fig. 1.[7] The entire length of the NiV genome is 18,246 nucleotides, which is 12 nucleotides longer than HeV, and demonstrates 70% nucleotide homology in the open reading frames (ORFs) and 44–66% homology in the 5′ and 3′ noncoding regions with HeV.[2,6] It shows broad tropism for a number of cell lines, as is common for Paramyxoviruses. In culture, the virus infects cells through pH-independent membrane fusion mediated by its F and G glycoproteins, forms syncytia, and the cytopathic effect consists of multinucleated giant cells, vacuolation, and apoptosis.[7]

The NiV genome contains multiples of 6 nucleotides conforming to the ''rule of 6'' of other Paramyxovirinae,

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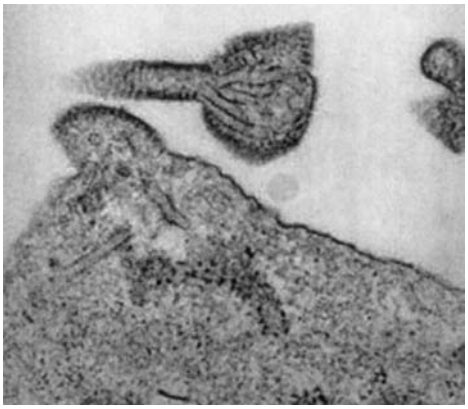

Fig. 1 Electron micrograph of Nipah virus. [From Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia.]

thereby allowing for greater efficiency of genome replication.[6,8] Additionally, the genome contains 6 major genes (in order from 3′ to 5′ end) as summarized in Table 1: N (nucleocapsid), P/V/C (phosphoprotein/V protein/C protein), M (matrix), F (fusion), G (attachment glycoprotein), and L (large polymerase).[9,10] The NiV 3′ leader sequence is 55 nucleotides long and is identical to other Paramyxovirinae, and there is a 33-nucleotide trailer at the 5′ end. Whereas the 3′ leader sequence is nearly identical to HeV, the NiV 5′ trailer is significantly longer and differs by 3 nucleotides.[6] The N gene of NiV is 2242 nucleotides long and contains two major predicted ORFs. The larger of the two (1599 nucleotides) encodes for a 532 amino acid protein, and the smaller ORF encodes for a 72 amino acid protein that is not found in the HeV N gene.[10] The ORF of the mRNA for the N gene of NiV shares 78% homology with that of HeV, whereas homology in the nontranslated 5′ and 3′ regions is 67% and 41%, respectively.[10] The N protein is the most abundant protein in the Nipah virion and has an apparent molecular mass of 58,000 Da.[9] Genomic RNA forms complexes with N to produce the nucleocapsid.

The mRNA for the P/V/C gene of NiV is predicted as 2702 nucleotides and contains ORFs that encode the P, V, and C proteins.[10] The extent of homology of the ORF between NiV and HeV is approximately 70% for the P gene, 88% for the V gene, and 85% for the C gene.[9,10] The P protein is the largest protein encoded by the P/V/C genes of NiV and has a calculated molecular mass of 78,301 Da.[9] C and V proteins may regulate transcription and replication. The V protein is thought to facilitate the evasion of host cell interferon by inactivating interferon signaling by direct inhibition of STAT1 and STAT2, which are interferon-responsive transcription factors.[11]

The mRNA for the M (matrix) gene is predicted at 1359 nucleotides in length and contains an ORF of 1059 nucleotides that encodes a 352 amino acid protein that has a molecular mass of 39,928 Da.[10] The HeV and NiV M proteins are 77.1% homologous and share significant sequence homology with M proteins of morbilliviruses. The ORF of the F gene is 1641 nucleotides in length, and the mRNA for the entire gene is predicted to be 2337 nucleotides. The F protein is thought to be a type I transmembrane protein whose membrane spanning domain occurs at amino acids 489–518 and is synthesized as an inactive precursor (F_0) that is cleaved by host proteases forming F_1 and F_2.[6] The ORF that encodes the putative attachment glycoprotein (G) of NiV is 2543 nucleotides in length and has 83.3% homology with that of HeV.[12] Both the F and G proteins are membrane glycoproteins that mediate viral fusion and attachment to host cells. Both the G and F proteins are targets for host neutralizing antibodies, suggesting a potential role for the F and G proteins as vaccine constructs.[12]

Nipah virus demonstrates significant tropism for endothelial and neuronal cells in humans and for neuronal and respiratory tract cells in pigs.[13] It is not fully known where initial replication occurs, but it is assumed that either lymphoid or respiratory sites are involved as with other Paramyxoviruses such as measles virus.[2] Viremia probably spreads the virus to other tissues. The endothelium may represent a secondary site of viral replication. Vasculitis is a key element in the pathogenesis of NiV infection, and the presence of viral antigens within endothelial and smooth cells supports the importance of vascular tropism in the pathogenesis of the infection.

Wong et al.[14] have recently reported an animal model of NiV infection that appears similar to acute infection in humans. In this study, golden hamsters (*Mesocricetus auratus*) were challenged either intraperitoneally or intranasally, and the investigators evaluated the clinical course, transmission of NiV to uninfected animals housed with challenged animals, and pathology in infected animals. Uninfected animals housed with challenged animals did not develop clinical signs or seroconversion.

Table 1 Molecular characterization of nipah virus

Gene	Length of gene[a]	Predicted protein size[b]	Molecular weight of protein (Da)
N	2242	532	57,993
P[c]	2704	709	78,301
M	1359	352	39,827
F	2337	546	60,233
G	2543	602	67,038

[a]Length in nucleotides as predicted from mRNA.
[b]Length in amino acids predicted from the ORFs.
[c]P gene includes ORFs for P, V, and C proteins. The predicted protein listed is the P protein only. The V protein of NiV is predicted to be 454 amino acids, while the C protein is 166 amino acids and has a M_r of 19,735 Da.
Source: Refs. [6,9,10].

Virus was identified or isolated by PCR or culture from urine, brain, lung, kidney, liver, and spleen, but not from blood. The authors speculated that the absence of detectable virus in the blood may be related to successful clearance of circulating virus by the immune system or by transport of virus particles out of the circulation by infected blood leukocytes. The role of endothelial cells in clearing virus from the circulation is not fully known. Pathological features resembled those of human infection, suggesting that this model may be beneficial in unraveling the pathogenesis of NiV in humans and as a tool to test antiviral and vaccine strategies designed to treat or prevent infection.[14]

CLINICAL FEATURES

The majority of patients reported contact with pigs. Following a short incubation period, most patients presented with fever, headache, dizziness, vomiting, and reduced level of consciousness.[15] Meningismus was not a common finding. In their series, Goh et al.[15] identified prominent neurological findings, especially in those with a reduced level of consciousness. Common neurological features included segmental myoclonus, cerebellar signs, seizures, and nystagmus, whereas bilateral ptosis, dysarthria, and dysphasia were less common features. The majority of patients were healthy prior to hospitalization with comorbid diseases present in approximately 12%.[15]

Laboratory features included thrombocytopenia and elevated hepatic transaminases in approximately one-third. The most common abnormalities of the cerebrospinal fluid were elevation of the white blood cell count and protein levels. Magnetic resonance imaging revealed multiple, discrete high signal intensity lesions disseminated throughout the brain with >10 lesions occurring in 70% of patients.[16]

The pathological findings of fatal human NiV encephalitis have been reviewed.[17] The major histopathologic findings included systemic vasculitis with extensive thrombosis and organ necrosis, especially in the central nervous system, lungs, and spleen, and endothelial cell necrosis and syncytial giant cell formation. Eosinophilic cytoplasmic and nuclear inclusions, resembling those in other *Paramyxovirus* infections, were seen. Viral antigens were particularly prominent by electron microscopy and immunohistochemistry in endothelial and smooth muscle cells and in neurons, linking vasculitis, and thrombosis to the pathogenesis of the infection.[17]

Diagnosis of NiV infection can be confirmed by detection of type-specific antibodies in cerebrospinal fluid or serum. Serum IgM capture enzyme-linked immunosorbent assay (ELISA) was positive in 65% within 4 days after onset of infection and in 100% by day 12, and IgG ELISA was positive in 100% by day 25.[2] Current ELISAs have 98.4% specificity but may have lower sensitivity.[18] Virus isolation requires biosafety level 4 laboratories, and virus has been isolated from cerebrospinal fluid, urine, and throat and nasal secretions.[17] NiV has tropism for a number of cell culture systems and grows well in Vero cells, forming syncytia. Demonstration of NiV can be accomplished using immunostaining, neutralization assays with specific antisera, polymerase chain reaction, electron or immunoelectron microscopy, or by ultrastructural in situ hybridization using positive or negative sense riboprobes.[7,18] Diagnostic reverse transcriptase-polymerase chain reaction (RT-PCR) assays are available through the Centers for Disease Control and Prevention (CDC) and can detect NiV sequences from tissues of CSF.[18] Diagnostic RT-PCR assays have been based on the sequences of the P and N gene coding regions.

Infection with NiV resulted in severe encephalitis with mortality reaching 40%.[1,2,15] The mean time from onset of illness until death was approximately 10 days, and death was usually related to severe brainstem involvement.[15] Factors associated with poorer prognosis included older age, lower Glasgow coma scores, the presence of segmental myoclonus, impaired brainstem function (manifested by abnormal doll's eyes and pupillary reflexes, vomiting, hypertension, and tachycardia), seizures, areflexia, and abnormal hepatic transaminases and platelet count at time of hospitalization.[15] Of those who survive, 15% of patients have residual neurologic deficits including persistent vegetative state, residual cognitive impairment, and cerebellar disabilities.[15] Interestingly, some patients with NiV infection presented with relapsing encephalitis and late-onset encephalitis.[19] Clinically, relapsing or late-onset encephalitis resembled the acute encephalitis and was thought to represent persistence of NiV.

Ribavirin, a broad-spectrum inosine monophosphate dehydrogenase inhibitor, was shown beneficial in treating patients with NiV encephalitis in an open label trial.[20] As compared with historical controls, treated patients had statistically lower mortality (32% versus 54%). By multivariate analysis, treated patients appeared to have less residual neurological deficits, although this did not reach statistical significance.[20]

Control of the epidemic was ultimately achieved by culling over 1 million pigs from 900 affected farms thought to represent the remaining reservoir of NiV. This posed a significant financial impact on the Malaysian and Indonesian economies and raises concern about the use of NiV as a bioterrorism agent to achieve economic disruption as well as cause disease. A surveillance program has been established to serologically monitor pig farms for evidence of NiV infection. Recently, Guillaume et al.[21] have reported preliminary data on a recombinant vaccinia virus expressing NiV G and F genes. These

vaccines protected hamsters against death following NiV challenge infection, but did not evoke sterile immunity, as evidenced by demonstration of increased levels of neutralizing antibodies after the challenge in all vaccinated animals.[21]

CONCLUSION

The outbreak of NiV infections in Malaysia is an excellent example of how a novel pathogen can significantly impact human and animal health and the economies of certain geographic regions. However, the recognition of the clinical illness, identification of NiV, and subsequent sequencing of its genome have revealed the impressive power of modern virology and clinical medicine to identify and define a pathogen and develop potential treatment and preventive strategies, thereby limiting its potential impact.

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NIST Reference Materials for Medical Testing

Catherine D. O'Connell
John P. Jakupciak
National Institute of Standards and Technology, Gaithersburg, Maryland, U.S.A.

INTRODUCTION

Biomarker discovery and validation is in great demand by the pharmaceutical and healthcare industries. The translational component of the human genome information to clinical use is only now beginning to appear. This effort is largely being lead by the pharmacogenomics and toxicogenomics communities in the development of new drugs and in determining their safety and efficacy. Standardized controls or reference materials will be essential for the acceptance of data collected from these new technology platforms by regulatory agencies.

NIST REFERENCE MATERIALS PROGRAM

It is essential to have high-throughput[1] and global scanning[2] methods that are low-cost and reliable clinical platforms to analyze large cohort studies, which statistically validate potential DNA, RNA, and protein assays. The National Institutes of Standards and Technology has developed expertise in measuring genomic instability of DNA repeat elements, the measurement of mutations in nuclear-encoded genes, and the measurement of DNA sequence variations in the mitochondrial genome. This is due, in part, to the development of a measurement program for human identification in conjunction with the Department of Justice.[3,4] In addition to examining current practices and assessing specific causes of measurement error, NIST has developed standard reference materials (SRMs) for optimizing the reproducibility of measurements for restriction fragment-length polymorphism (RFLP) and short tandem repeat (STR) alleles, and mitochondrial DNA sequences used by forensic community. In total, five SRMs are currently available for this purpose, with two additional materials underway.

For the NIST measurement programs, we followed the ACMG guidelines including the recommendation that validation studies incorporate well-characterized samples and positive and negative controls, thus providing evidence of generic and specific sequence amplification.[5]

FRAGILE X SYNDROME

Genetic instability of triplet repeat sequences is an initial step in the etiology of hereditary neurodegenerative diseases and the cause of fragile X syndrome, the most common form of inherited mental retardation. This new paradigm of genetic disease, the transmission of expanded triplet repeat sequences, is associated with approximately 15 other human hereditary diseases.[6,7] Fragile X syndrome is caused by the progressive expansion of CGG repeats in the *FMR1* gene, and there is a direct correlation between the number of CGG repeats and the frequency of inheriting an expanded CGG tract. The *FMR1* gene in normal individuals harbors between 5 and 37 CGG repeats, and three other size categories are recognized that confer clinical information. Full mutations (>230 repeats) are associated with disease, whereas premutations (55–200 repeats) provide at-risk predictive information. The fourth category includes repeat lengths that provide inconclusive diagnostic and predictive information (45–54 repeats) and is generally referred to as the gray zone. Accurate measurements of the length of the CGG tract are critical to the clinical diagnostics community.

The National Institute of Standards and Technology (NIST) has established a quantitative measurement program for trinucleotide repeats[8] through the development of accurate protocols and reference materials for the analysis of the *FMR1* gene after polymerase chain reaction (PCR) amplification of the CGG repeats. Amplification is a commonly used technique for *FMR1* gene analysis and is faster than the traditionally used Southern blot. However, interlaboratory comparisons on the accuracy of amplifying CGG repeats have shown tremendous variation. This is because, in part, of the secondary structure properties of triplet repeats and the lack of standardized protocols. We have determined the metrics in need of standardization for fragile X repeat measurements and have reported the specific measurement variability associated with slab-PAGE and capillary electrophoresis (CE) separations, interlane variability, intercapillary variability, and the variability associated with the amplification of CGG repeat elements.

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As reported,[8] the measurement errors associated with slab gels ranged from 0.05 to 0.35 standard deviation (SD). There was also little variation between gels and amplification reactions. The CE measurements were more precise with SD between 0.02 and 0.29. This measurement error is equivalent to one nucleotide of a single triplet repeat. The accuracy in determining the number of CGG repeats was in agreement for the normal and gray-zone length alleles using both slab-PAGE and CE. However, accuracy decreased for the measurement of larger alleles, where repeat size varied ±3 repeats for measuring 85–95 repeats and ±4 repeats for measuring 105–110 repeats, our largest amplified allele. The size of the CGG repeat element in each sample was confirmed by automated fluorescent DNA sequencing.

Summary

The complexity of the various CGG-containing alleles, the variable severity of disease manifestations, and the marked heterogeneity of clinical presentation in carriers are hallmarks of fragile X syndrome. As the risk of reoccurrence is high in the family and carrier relatives, accurate diagnosis of at-risk individuals, even before classic signs of disease manifest, is important. Accurate methods to measure CGG repeat length, and reference materials to distinguish alleles within the different size categories, are important for diagnosis and counseling of affected families. Although CE measurements had better precision than slab-gel measurements, anomalous rapid electrophoretic mobility of repeats occurs in proprietary gels. Hence NIST has developed a control material consisting of an allelic DNA ladder to enable accurate size determinations across platforms. This material, SRM 2399, will be released in late 2004.

TP53

The TP53 tumor suppressor gene is associated with numerous pathways that maintain genetic stability and control cell life and death. Normally, p53 protein levels are very low; however, the levels of p53 change over time. P53 levels fluctuate as cells adapt to pathway-initiated stimuli and p53 feedback systems.[9] Activation of p53 occurs when cells are stressed or damaged. For example, DNA single-strand breaks caused by ionizing radiation are sufficient to trigger up-regulation of p53. Damaged cells have a greater risk of becoming cancerous, thus p53 protein inhibits the cell cycle resulting in programmed cell death. The normal p53 protein does not function in human cancers when the gene is inactivated as a result of mutations. Hence p53 appears to be a critical element in the protection against tumorigenesis.[10]

Almost all mutations in the p53 gene reduce the p53 protein's capability to activate transcription. The majority of p53 mutations that occur in human tumors are located in the sequence-specific DNA binding domain.[11] Hence mutations in the *p53* gene disrupt basic cellular function. PCR-based cancer diagnosis of the status of the *p53* gene requires detection of both common and rare mutation(s) that occur throughout the gene. Moreover, amplification efficiencies of mutant and wild-type sequences are unequal and associated with the distance from the primer to the point mutation.[12] For sensitive detection of TP53 mutations, reliable detection technologies and reference materials for PCR mutation detection systems are needed to monitor both the accuracy of detection and the possible introduction of errors during PCR. Renewable reference materials, such as the NIST TP53 mutation panel,[2] serve to normalize the data collected in different laboratories using different primers and analytical platforms.

The renewable reference panel contains the single-base substitutions most commonly found in human cancers, as well as three that proved difficult to detect by scanning technologies.[2] The panel consists of 12 plasmid clones derived from human genomic DNA containing a wild-type *TP53* gene, 11 of which contain a specific mutation in exons 5–9; the 12th is wild type. This panel accounts for 30% of reported TP53 cancer-associated mutations.[13] We evaluated the panel using single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), denaturing high performance liquid chromatography (DHPLC), and exon sequencing, four of the most commonly used methods for mutation detection. Following exon-specific amplification, the amplicons of all 12 clones were analyzed by SSCP, DGGE, DHPLC, and sequencing. SSCP detected 75% of the mutations in the reference panel, as expected. The heteroduplex technologies, DGGE and DHPLC, as well as fluorescent sequencing, positively identified 100% of the reference mutations.

Summary

The reference panel for the detection of TP53 mutations was created from site-directed mutagenesis and cloned into a renewable plasmid vector. Validation of the panel was performed by direct DNA sequencing and with common mutation scanning technologies.

Our results demonstrate that these reference materials and methods can be used in the clinical diagnosis of TP53 mutations in heritable and somatic disease. They may also be useful in the validation of other mutation detection technologies. The panel provides internal quality control and lowers the inherent error of measurements by ensuring that protocol optimization can always detect the mutations contained in the panel. Hence each diagnostic laboratory

can adjust its ''in-house'' protocols to obtain expected and normalized results that can be readily shared and easily compared between different diagnostic laboratories using different analytical techniques.

MITOCHONDRIA

A major focus to improve health-care outcomes for patients with solid tumors is the early detection of disease.[14] This includes identification of early cancer biomarkers and the validation of both detection technologies and clinical utility. As one of three Biomarker Validation Laboratories of the Early Detection Research Network (EDRN), NIST evaluates analytical methods for the identification of biomarkers associated with the early detection of cancer, and develops high-throughput assays/technologies for clinical applications. Validated biomarkers can be reliably used by laboratories to detect cancer in asymptomatic patients at risk for a specific cancer, make a diagnosis once symptoms appear, or monitor cancer patients for recurrence.

Mitochondrial dysfunction has been demonstrated by progressive decline of oxidative phosphorylation capacity of muscles, liver, heart, and brain.[15] The impaired bioenergetic function of the mitochondria has been associated with carcinogenesis in human tissues.[16] Mutations have been detected in both coding and noncoding regions of the mitochondrial genome in cancer and thus may correlate with disease status and serve as a biomarker.[14] Little data exist to explain the mechanism of how mitochondrial mutations and dysfunction predispose individuals to cancer(s). It is likely that energy depletion and free radical generation triggered by alterations in mitochondrial genotype underlie the basis of tumorigenesis.

Recently, we developed a high-throughput DNA sequencing protocol for the detection of mitochondrial DNA mutations (sequence variants) in cancer.[17] The protocol was compared with currently available protocols and technology platforms to assess their utility in identifying mitochondrial sequence variants associated with lung cancer. Primary lung tumors and patient controls (blood) were used to isolate mtDNA, which was analyzed by slab gel and capillary electrophoresis and DNA resequencing microarrays.

Mitochondrial DNA was fully sequenced from both blood and tumor obtained from 12 individuals with lung cancer. Using a nested protocol and M13 primers for sequencing, the CE protocol developed was able to obtain 97–100% sequence coverage for the forward strand and 90–100% sequence coverage for the reverse strand. Tumor-specific sequence variants were identified in 6 of 12 clinical samples from lung cancer patients.

Although many nucleotide changes were detected, our study demonstrates that 45% of mutations do not alter the amino acid sequence. In this limited study, individuals with lung cancer that were of the same age had strikingly different mitochondrial genome signatures, suggesting that these variants are cancer-associated changes. Although the sample set is small, we note that no correlation could be made with respect to presence or number of sequence variants and either tumor type or patient age from which the tumor sample was derived. For example, one individual, age 82, contained 4–7 sequence variants, while two others, age 78 and 84, contained no or one sequence variation between somatic and germ line.

Two SRMs have been developed for validation of mitochondrial sequence changes.[18,19] SRM 2392 is used to confirm homoplasmic mitochondrial DNA sequence of the entire mitochondrial genome. SRM 2394 is used to detect and quantify unique heteroplasmy within the mitochondria present in a population of cells.

Summary

These results demonstrate the advantages of fluorescent sequencing for the identification of both heteroplasmic and homoplasmic sequence variants in small quantities of human tissues and bodily fluids. In addition, cost analysis revealed that automated CE is cheaper than microarray resequencing in its currently available format. CE high-throughput analysis of the entire mitochondrial genome from lung cancer patients detected sequence variants in Stage I, Stage III, and Stage IV tumors, suggesting that mitochondrial instability could serve as a biomarker for early detection. Because mitochondrial mutations, unlike nuclear genes, are not restricted by cancer type, the identification of mtDNA sequence variants may serve as a universal fingerprint for other cancers and mitochondrial-linked diseases.

CONCLUSION

The National Institute of Standards and Technology (NIST) compares, evaluates, and validates DNA (and other biologic) markers for clinical diagnostics. Where applicable, we develop high-throughput techniques for both solid tumor and noninvasively (i.e., serum) collected sample analysis. We incorporate broad-based consensus via interdisciplinary NIST-sponsored workshops into the development of reference material(s). In general, NIST activities in fragile X, TP53, and mitochondrial variants provide specialized technical and scientific expertise for measurement needs, thus fostering economic and clinical development.

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Certain commercial equipment, instruments, materials, or companies are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation nor endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are the best available for the purpose.

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Nucleic Acid Sequence-Based Amplification (NASBA) and Transcription-Mediated Amplification (TMA)

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Margareta Ieven
University Hospital of Antwerp, Edegem, Belgium

Katherine Loens
University of Antwerp, Wilrijk, Belgium

INTRODUCTION

The qualitative and quantitative detection of RNA is clinically important. Reverse transcription of RNA followed by polymerase chain reaction (PCR) is a two-step reaction, which can be inconvenient; furthermore, it may be difficult to differentiate mRNA from contaminating genomic or proviral DNA. This review presents alternative RNA amplification strategies and describes the data published in studies that have used this methodological approach. Nucleic acid sequence-based amplification (NASBA) (bioMérieux, Boxtel, The Netherlands) and transcription-mediated amplification (TMA) (Gen-Probe Inc., San Diego, CA; Bayer Diagnostics Division, Tarrytown, NY; Chugai Diagnostics Science, Tokyo, Japan) amplify RNA under isothermal conditions. The constant temperature maintained throughout the amplification reaction allows each step of the reaction to proceed as soon as an amplification intermediate becomes available. Products of TMA and NASBA are single-stranded and thus can be applied to detection formats by using probe hybridization without any denaturation step.

TECHNICAL ASPECTS

Using an RNA template, NASBA and TMA amplify RNA via the simultaneous action of three enzymatic activities: an RNA polymerase activity, a reverse transcriptase (RT) activity, and RNase H activity. The synthesis of cDNA is primed by specifically designed primers, one end of which is a target-specific sequence, while the other end contains a promoter for the RNA polymerase. The RT synthesizes an RNA–DNA hybrid, the RNase H digests the RNA component, and the RT synthesizes double-stranded DNA; finally, the RNA polymerase produces numerous RNA copies (Fig. 1). An excellent overview of the NASBA amplification principle and primer and probe design rules is given by Deiman et al.[1]

NASBA and TMA were originally applied for specific RNA amplification, but the protocol is adaptable for DNA amplification as well, by changing template denaturation, primer design, and sample extraction.

Because the amplification product is single-stranded RNA, it can easily be detected by hybridization with sequence-specific probes. Different techniques can be used: enzyme-linked gel assay,[2] electrochemiluminescence (ECL),[3] and real-time detection by molecular beacons.[4–7]

MULTIPLEX NASBA AND TMA

Greijer et al.[5] designed a multiplex real-time NASBA using molecular beacon probes labeled with three different fluorophores to quantify human cytomegalovirus (CMV) IE1 mRNA by competitive coamplification of wild-type and calibrator RNA. A simultaneous detection of late pp67mRNA in whole-blood samples of CMV-infected lung transplant patients was also obtained. Despite the somewhat lower sensitivity of the real-time NASBA compared with the conventional NASBA, the simultaneous quantification of IE1 and detection of pp67 RNA was reproducible and accurate. Loens et al.[6] also developed a multiplex real-time NASBA for the simultaneous detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila* RNA in respiratory specimens by using three fluorophores. They also found a slightly lower sensitivity of the multiplex real-time assay compared to the conventional ECL detection on spiked respiratory samples.

The Procleix human immunodeficiency virus-1/hepatitis C virus (HIV-1/HCV) assay (Gen-Probe Inc.) detects both HIV- and HCV RNA in 1 tube.[8] The source of the reactivity can be determined by discriminatory assays using virus-specific probes. The HIV-1/HCV assay and the discriminatory assays are able to detect 10–13 copies of HIV-1/mL with 95% detection rates, and can detect 30 copies/mL of HCV with 95% sensitivity. Furthermore,

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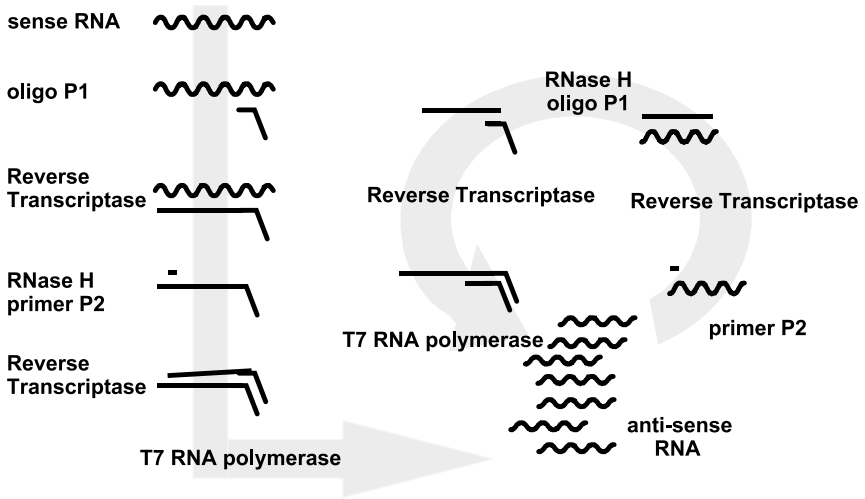

Fig. 1 NASBA amplification.

the assay can be used with high sensitivity and specificity for the screening of donor blood.[9]

Gaydos et al.[10] evaluated the performance of the APTIMA Combo 2 assay (Gen-Probe Inc.) for the simultaneous detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in endocervical swabs and first-catch urine specimens (FCU) from females, and compared the results with patient infected status, available by using other commercial nucleic acid amplification techniques. Sensitivity and specificity rates for *C. trachomatis* in swabs were 94.2% and 97.6%, respectively, and 94.7% and 98.9%, respectively, in FCU. Sensitivity and specificity for *N. gonorrhoeae* in swabs were 99.2% and 98.7%, respectively, and in FCU, 91.3% and 99.3%, respectively.

ADVANTAGES AND DISADVANTAGES

One advantage of NASBA and TMA compared to PCR is that they are continuous, isothermal processes that do not require a thermocycler. The constant temperature maintained throughout the amplification reaction allows each step of the reaction to proceed as soon as an amplification intermediate becomes available. Thus, the exponential kinetic of the amplification process, which is caused by multiple transcription of RNA copies from a given DNA product, is intrinsically more efficient than DNA amplification methods, which are limited to binary increases per cycle.[11]

RNA is the genomic material of numerous viruses. The application of an RNA-based amplification technique offers advantages compared to a DNA-based amplification technique: no additional RT step is required, thus saving time and reducing the risk of contamination.

NASBA and TMA can be targeted at ribosomal RNA; thus they offer a diagnostic advantage because bacterial rRNA is present in multiple copies/cell and it also implies biological activity, whereas DNA is present at a much lower number of copies. Therefore the likelihood of initiating amplification is greater when rRNA is targeted instead of DNA. Furthermore, the assays could be used in viability studies.

There are also a number of disadvantages. The specificity of the reactions might be lower because the enzymes used are not thermostable, and so that the reaction temperature may not exceed 42°C without compromising the reaction. However, the specificity rate is increased by additional hybridization with target-specific probes. Finally, the length of the amplified RNA target sequence should be in the range of 120–250 nucleotides. Shorter and longer sequences are amplified less efficiently.

Furthermore, RNA integrity and amplification inhibitors are also the main causes of concern for NASBA, TMA, RT-PCR, and other RNA amplification procedures. Stability of RNA may be affected during collection, processing, and storage of specimens prior to its isolation. The addition of RNase inhibitors to the clinical specimens, such as guanidine thiocyanate, is required to preserve RNA integrity. Some studies suggest that quality control of buffers for storage of clinical material is critical when RNA is to be analyzed.[2]

To overcome part of this problem and to improve the reproducibility of the in-house developed NASBA standardized reagents, the ''NucliSens Basic Kit'' (bioMérieux) is now commercially available. It contains all the necessary reagents for 1) nucleic acid release and inactivation of RNases and DNAses; 2) silica-based extraction of nucleic acids; 3) NASBA reagents; and 4) reagents for electrochemiluminescent detection including the ECL probe, and

paramagnetic particles to link the capture probe for detection. The primers and the target-specific biotinylated capture probe are to be synthesized for each target.[12]

QUANTITATIVE NASBA AND TMA APPLICATIONS

Quantitative nucleic acid amplification tests are gradually introduced into the laboratory. These assays are used for determination of the viral load of patients infected with blood-borne viruses such as human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV).

HIV-1 RNA: Using the NucliSens HIV-1 QT (bioMérieux),[13,14] between 25 and $>5\times10^6$ copies/mL of HIV-1 RNA can be detected with a 95% detection rate of 176 copies/mL, by using 1 mL of EDTA, citrate, or heparin plasma as a sample. Murphy et al.[13] compared the Roche COBAS AMPLICOR MONITOR version 1.5 (Roche Molecular Systems), the NucliSens HIV-1 QT with extractor, and the Bayer Quantiplex Version 3.0 (Bayer) for quantification of HIV-1 RNA in 460 plasma specimens from HIV-1 infected patients. They observed that NucliSens showed 100% specificity and the best sensitivity with an input of 2 mL. In another recent study, the same specificity was displayed by the NucliSens HIV-1 QT assay.[14] In a comparison with the ultrasensitive AMPLICOR HIV-1 Monitor version 1.0 assay (Roche Molecular Systems), the NucliSens and the AMPLICOR assays were equivalent in detecting HIV-1 RNA (concentrations of 10^3–10^5 copies/mL), although at lower RNA concentrations, the NucliSens assay was more sensitive.

Recently, a real-time NASBA was described for the quantification of HIV-1 isolates.[7] All HIV-1 groups and subtypes could be detected and quantitated with equal efficiency. The throughput of the assay was high, with 96 samples amplified and detected within 60 min.

The performance of the Gen-Probe HIV-1 viral load assay was compared to that of widely used commercial HIV-1 RNA assays by using a panel of primary isolates from Kenya.[15] The Gen-Probe HIV-1 viral load assay and the Roche AMPLICOR HIV-1 MONITOR test version 1.5 were both sensitive for the quantitation of the Kenyan isolates, which represent subtypes A, C, and D. For the majority of viruses, the Gen-Probe HIV-1 viral load assay was more sensitive than the Roche AMPLICOR HIV-1 MONITOR version 1.0, the Bayer Quantiplex HIV RNA 3.0 assay, and an in-house RT-PCR method.

The conserved 5′NCR of HCV has been used as a target for the development of qualitative and quantitative ECL-based NASBA assays for genotypes 1a, 1b, 2, 3, 4, and 5.[16] When different assays were compared, the HCV NASBA-QT assay was over 10 times more sensitive than the bDNA assay, while the quantitative results of both assays were highly concordant. The HCV NASBA-QT assay was comparable in sensitivity with the HCV MONITOR assay (Roche Molecular Systems), but the latter yielded consistently lower values.[16]

The qualitative COBAS AMPLICOR HCV version 2.0 PCR assay (Roche Molecular Systems) and the VERSANT HCV RNA qualitative assay (Bayer) were compared for analytical sensitivity and clinical performance.[17] The calculated limit of detection for HCV TMA was 6 IU/mL. To compare clinical performance, 300 specimens were evaluated [112 samples indeterminate in an anti-HCV enzyme immunoassay (EIA) and HCV PCR negative, 79 EIA positive and HCV PCR negative samples, and 105 EIA and HCV PCR positive samples]. For these groups, the interassay accordance ranged from 96.2% to 100%. HCV TMA demonstrated an excellent concordance with HCV PCR on clinical samples.

Quantitative assays for determination of the viral load of cytomegalovirus (CMV) might be important, but are still under development and have to be further evaluated in clinical studies.

NASBA and TMA may have an increased sensitivity compared to PCR-based methods.[15,18] In patients chronically infected with HCV who are undergoing antiviral therapy, sustained virologic response is suggested by viral clearance by end of treatment. However, some individuals relapse after achieving apparent viral clearance by end of treatment. Residual serum HCV RNA was detected by TMA in end of treatment samples from 34.6% of patients who had achieved apparent viral clearance by PCR.[18] Such new sensitive assays could help redefine end-of-treatment response and assist in the antiviral management of HCV infection.

OTHER NASBA AND TMA APPLICATIONS

CMV mRNA: Although it is not the most sensitive tool available, NucliSens CMV pp67 test (bioMérieux) specifically detects only the clinically relevant stages of infection, and was shown to have the best specificity and positive predictive value for disease development.[19]

Recently, quantitative NASBA assays have been described for the detection of IE1 and pp67 mRNA expression in lung transplant recipients, and for the quantitative detection of IE1, pp67, and immune evasion genes US3, US6, and US11 in CMV-infected cells.[20] Greijer et al. concluded that effective antiviral treatment was reflected by a rapid disappearance of pp67 mRNA, confirming the clinical utility of the assay.

The BDProbeTec ET *Mycobacterium tuberculosis* Complex Direct Detection Assay (DTB) (Beckton Dickinson Biosciences, Sparks, MD) was compared with the enhanced *M. tuberculosis*-amplified Direct Test (AMTDII) (Gen-Probe Inc.), acid-fast staining and culture, with the combination of culture and clinical diagnosis as the

gold standard.[21] After resolution of the discrepant results, the sensitivity and specificity rates, as well as positive and negative likely ratios for AMTDII were 88%, 99.2%, 110, and 0.11 for respiratory specimens and 74.3%, 100%, 740, and 0.26 for extrapulmonary specimens, respectively. The corresponding values for DBT were 94.5%, 99.6%, 235, and 0.05 for respiratory specimens and 92.3%, 100%, 920, and 0.07 for extrapulmonary specimens, respectively. Differences between both assays were associated with the presence of inhibitors.

A sensitive and specific NASBA assay was developed for the amplification and detection of *M. pneumoniae* 16S rRNA in respiratory specimens.[2] The sensitivity of this NASBA was comparable to that of a PCR targeted at the P1 adhesin gene. A commercially available NASBA NucliSens Basic Kit® assay[12] and a real-time NASBA assay for the detection of *M. pneumoniae* 16S rRNA in respiratory specimens were developed by the same group.[4] The intrarun variability coefficients for the real-time detection of specimens spiked with 50, 500, and 5000 color changing units (CCU) of *M. pneumoniae* were 26.4, 8.1, and 4.6 respectively; the interrun variation coefficients for the same inputs were 34.8, 10.7, and 13.9, respectively. The intrarun variation coefficients for the electrochemiluminescence detection of 50, 500, and 5000 CCU were 61.8, 45.3, and 28.2, respectively; the interrun variation coefficients for the same inputs were 57.7, 78.2, and 42.6 respectively. Loens et al. concluded that real-time NASBA, in comparison with ECL detection, shows high concordance in sensitivity and specificity with a clear advantage for the real-time technology regarding handling, speed, and number of samples that can easily be tested in a single run. Furthermore, the real-time NASBA assay requiring less manipulations and producing results without postamplification processing reduces the potential risk for product carryover.

Goessens et al.[22] compared the Gen-Probe transcription-mediated amplification assay (AMP CT), the LCx assay (Abbott Laboratories, Chicago, IL), and the Roche COBAS AMPLICOR assay for the detection of *C. trachomatis* in urine samples. Sensitivities and specificities were calculated by using the following gold standard: true positive if two or more techniques yielded a positive result. The sensitivities of LCx, COBAS AMPLICOR, and AMP CT were 84%, 93%, and 85%, respectively. Specificity exceeded 99% for all three assays.

CONCLUSION

An increasing number of NASBA- and TMA-based assays—such as those for HIV-1 and HCV genomic RNA, CMV mRNA assays, as well as *C. trachomatis* and *M. tuberculosis* rRNA assays—containing all the necessary components required for isolation, amplification, and detection have become commercially available. They are user-friendly and easy to use in clinical settings.

For various other applications, specific primers and probes have been designed that can be used in combination with the NucliSens Basic Kit, which contains all the components except for the specific primers and probes. These assays need further evaluation in clinical settings.

At present, ECL is the most sensitive method for the detection of the NASBA amplicon. An increasing number of real-time applications are being evaluated, even in multiplex formats. A major advantage of the use of molecular beacons is the ability to carry out real-time detection during amplification, which shortens the overall time of the assay, reduces the risk of contamination, and also provides information about the kinetics of the reaction.

Quantitative assays will become increasingly important, especially for the follow-up of patients, infected with blood-borne viruses such as HIV and HCV, who are under antiviral treatment.

Because NASBA and TMA have been shown to be unaffected by sensitivity and specificity problems associated with other isothermal techniques, it seems likely that their unique features will make this a technology that will be widely used in research and diagnosis. As the processes become more refined and standardized, both assays may displace or supplement current methods for rapid clinical laboratory diagnosis.

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ARTICLES OF FURTHER INTEREST

Automated Nucleic Acid Extraction, p. 93
Molecular Beacons and Other Hairpin Probes, p. 846
RNA Storage, p. 1172

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Oligonucleotide Design for PCR Primers and Microarray Probes

N. Sergeev
A. Rasooly
United States Food and Drug Administration–Center for Devices and Radiological Health, Silver Spring, Maryland, U.S.A.

K. E. Herold
University of Maryland, College Park, Maryland, U.S.A.

INTRODUCTION

Hybridization between molecules of single-stranded DNA, including short sequences (i.e., oligonucleotides), is a key step in many molecular biology technologies including polymerase chain reaction (PCR) and DNA microarrays. The design of the oligonucleotides for these two applications is the subject of this article. Many of the design considerations are overlapping between the two applications.

OVERVIEW

DNA hybridization methods exploit the base-pairing properties that enable one strand of DNA to recognize its specific complementary strand to form the double-stranded DNA molecule. Thus on a microarray chip, a single strand of DNA tethered to a surface (a probe) can readily hybridize with its single-stranded complement even in a complex mixture of many other DNA molecules. When the target molecules are fluorescently labeled, the resulting signal at a particular location on the array establishes the existence of the complimentary sequence in the target.

Polymerase chain reaction (PCR) is another technology where DNA hybridization plays a central role. In PCR, the initial hybridization is between the oligonucleotide primers and the target DNA to be amplified. The PCR reaction produces large numbers of copies of a defined DNA fragment. The primers bind to a complementary sequence and initiate extension of the adjacent DNA regions using DNA polymerase. Primer design is one of the key determinants for the specificity and the yield of the reaction. Robust PCR primer design requires that the melting temperatures of the two primers must be closely matched.

Thus the melting temperature is one of the primary design variables in both of these technologies. The melting temperature, T_m, of a DNA duplex is defined as the temperature where one-half of the nucleotides are paired with their complement and one-half are unpaired. T_m is typically measured as the midpoint of the spectroscopic hyperchromic absorbance shift during DNA melting. T_m depends mainly on the DNA GC base content, cation concentration of the buffer, and DNA double-strand length. Currently, the most accurate prediction of T_m for oligonucleotide DNA uses the thermodynamic nearest-neighbor (NN) model. The NN model for nucleic acids assumes that the stability of a given base pair depends on the identity and orientation of neighboring base pairs. The NN model basic assumption is that probe free energy can be calculated from the enthalpy and entropy of all NN pairs. NN calculations for T_m prediction are useful for microarray design and for the selection of PCR primers and hybridization probes.

MELTING TEMPERATURE PREDICTION

The nearest-neighbor melting temperature thermodynamic model[1] is represented by

$$T_m = \frac{\Delta H}{\Delta S + R \ln C_t} + 16.6 \log_{10}[x_{salt}]$$

where

$$\Delta H = \sum_i n_i \Delta H_i + \Delta H_{\substack{\text{init} \\ \text{GC}}} + \Delta H_{\substack{\text{init} \\ \text{AT}}} + \Delta H_{sym}$$

$$\Delta S = \sum_i n_i \Delta S_i + \Delta S_{\substack{\text{init} \\ \text{GC}}} + \Delta S_{\substack{\text{init} \\ \text{AT}}} + \Delta S_{sym}$$

and where ΔH_i and ΔS_i are the enthalpy and entropy terms associated with the $n-1$ pairs of neighboring nucleotides contained in the sequence of interest. R is the universal gas constant (1.987 cal/mol K). C_t is the total molar concentration of single strands when oligonucleotides are self-complementary or it is equal to 1/4 of this concentration in the case of non-self-complementary

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sequences. The model assumes an equal concentration of the complementary strands.

POLYMERASE CHAIN REACTION PRIMER DESIGN

The task of designing PCR primers[2] starts from the identification of the target sequence (amplicon) of interest. It is assumed here that the target sequence is known from previous work. The region near each end of the target is then analyzed for promising primer sequences. Variables at this stage include the starting location and primer length. The base composition and particular sequence of each potential primer impact the ability of the primer to perform well through their influence on secondary variables including melting temperature and nonspecific annealing.

The melting/annealing temperature of a primer can be predicted from the nearest-neighbor model described above. The first requirement is that the primer pair must have closely similar annealing temperatures because extension of both strands occurs simultaneously under the same reaction conditions. The primers, being small and mobile, outcompete the complementary target strands and anneal forming a short double-stranded section with a long 5′ single-strand overhang. The annealing temperature chosen for the PCR reaction is usually 5–10°C less than the melting temperature of the primer pair to promote stable hybridization between the primers (which typically exist in excess numbers) and the amplicon to obtain high yield in each cycle.

The choice of a very low annealing temperature often leads to nonspecific annealing where the primers hybridize with mismatches at the wrong location on the single-stranded target. Nonspecific hybridization can lead to unexpected amplicons and competition with the desired target for reaction constituents. This provides a design incentive to use longer primers that have a higher annealing temperature. However, the requirement of longer primers often complicates primer design because of increased opportunities for primer hybridization to other primers (primer dimers) or hybridization between sections of a primer (secondary structure such as hairpins).

To avoid primer dimers, complementarity can be detected at design time by aligning the primers which is a simple task for short sequences. The stability of a primer dimer can be predicted by calculating its melting temperature. In most cases, it is best to simply choose a primer set that does not have such complementarity, although short complementary regions have such a low melting temperature that they usually do not present a problem.

Hairpins, where a single-stranded molecule loops back and anneals with itself (because of an inverted repeat), can form in single-stranded DNA and interfere with primer annealing. Such structures can be more stable than similar length annealing between separate molecules because the loop stabilizes one of the ends. Hairpins formed with highly stable ends can sometimes exhibit slow melting kinetics which implies that they may exist in a hairpin state even after a typical PCR denaturing protocol at 94°C. If a fraction of the primers are bound up as hairpins, the yield of the PCR reaction will be reduced. Hairpin potential can be predicted based on sequence analysis.

Nonspecific annealing of primers to the target at locations other than the exact match can cause reductions in yield of the PCR reaction. Potential annealing sites can be predicted and ranked by alignment of the primers to the target sequence. Because primer extension will only occur when the 3′-end is hybridized, 3′-end stability at mismatched locations is the most important consideration. For mismatch sites where the 3′-end is matched, the primer sequence must exhibit sufficient mismatches in the rest of the sequence to destabilize the hybridization. A related issue is that the preferred 3′-end sequence is CTA or CAT and the worst choice is CGG. This is based on the fact that mismatch stability increases $G:T \leq G:A < T:T \leq G:G < A:A < C:C < C:T < C:A$.[3] Thus a 3′-end structure such as CGG provides relatively stable hybridization with a wide range of mismatch conditions and can thus promote nonspecific amplification.

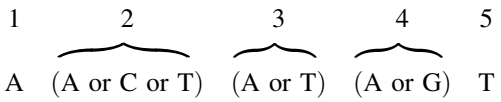
DESIGN OF DEGENERATE SEQUENCE OLIGONUCLEOTIDES

In some DNA microarray-based applications, ‘‘universal’’ primers, which enable amplification of several related amplicons simultaneously, can significantly reduce the number of reactions needed and thus save cost and time. The design of universal PCR primers relies on multiple sequence alignment (alignment of multiple sequences that inserts gaps into the individual sequences to align conserved sequences in the same column) which is used to identify sequences of highly conserved regions. Instead of developing individual primer sets for each of a group of paralogous genes or alleles of a single gene, universal primers can be designed to amplify several such genes simultaneously. Identification of the resulting amplicons by DNA microarrays allows discrimination of a range of genes using a single PCR reaction, as demonstrated for amplification and identification of 16 Staphylococcal enterotoxins simultaneously.[4]

As a rule, these universal primers are designed from conserved regions of related genes. In many cases, there

are several positions within these conserved regions that exhibit variation. Universal primers can be designed, based on such regions, as a mixture of all the sequence permutations represented by the variation at each location. Such primers are termed degenerate and are described using the International Union of Pure and Applied Chemistry (IUPAC) base nomenclature.

For example, the sequence AHWRT represents a degenerate primer 5 bases long with degeneracy at positions 2–4 where H represents A, C, or T, W represents A or T, and R represents A or G.



During synthesis of this degenerate primer, a mixture of the indicated phosphoamidites is added into the reaction at the variable positions; for example, the second base in the example sequence can be A, C, or T. The synthesis results in a mixture of 5-nt-long primers instead of a single sequence. The degeneracy of the final primer mixture is the product of the degeneracy at each base position (degeneracy = 12 for the 5-nt example).

Degenerate primers have a range of melting temperature which can be calculated using the nearest-neighbor model. The lower limit of the range typically corresponds to the sequences formed by low melting bases (A–T) and can be recommended as the annealing temperature in initial experiments. A second concern regarding degenerate primers is concentration of the primers in a PCR reaction. The concentration of each individual sequence in a degenerate primer mixture is the total concentration divided by the degeneracy. Thus the primer concentration must be increased by a factor equal to the degeneracy to achieve the proper primer concentration for each individual primer in the mixture.

High values of degeneracy are impractical because of issues associated with primer concentration and the increased potential for primer dimers. Several rules can be applied to decrease the degeneracy of a primer designed for a particular set of conserved sequences. First, a few weakly destabilizing mismatches such as G:T can be ignored because they do not disrupt the duplex stability greatly. Second, universal nucleotide analogs can be used, such as inosine, which pairs stably with any of the four nucleotides. Because degenerate primers are actually mixtures of oligonucleotides, the experimental conditions (annealing temperature, buffer, magnesium chloride, primer concentration) should be optimized experimentally, similar to the procedure for multiplex PCR (PCR reaction which multiple DNA regions amplified simultaneously using multiple primers).

MICROARRAY PROBE DESIGN

From a probe design perspective, there are two types of oligonucleotide microarrays utilizing short (up to 25 nt) or long (up to 60 nt) probes. Long probes are major successors of cDNA-s and PCR amplicons utilized for expression profiling and whole genome comparisons, whereas short probes are good for single nucleotide polymorphism analysis genotyping and identification of microbial pathogens.

Selection of proper probes is one of the key steps for obtaining consistent and reliable data from microarray hybridization experiments. The main goals in probe design are common for all platforms and can be summarized as high specificity (complementary to the unique region of the sequence of interest) and uniform hybridization properties for all of the probes in the array. Attainment of these design goals provides accurate detection and quantification of the target over a large dynamic scale.

To satisfy these design goals, the typical range of GC content should be between 40% and 60% to avoid the presence of long GC clusters that could cause unspecific binding. Repetitive motifs or regions consisting of the same consecutive bases may result in slippage along the target resulting in unspecific hybridization. Self-annealing sites allowing hairpin formation have the potential to reduce the number of available probes for hybridization. Common practice is to eliminate secondary structure concerns by using denaturing solutions during the spotting step (50% DMSO) and/or high stringency conditions for the hybridization step (20–50% formamide or 55–65°C).

Whereas these rules of probe design are reasonable and straightforward, still, there is no simple model for a priori prediction of hybridization intensities that produces consistent estimates of hybridization behavior. Several parameters such as probe GC content, length, base order, and even kind of terminal nucleotides, as well as size, type (DNA or RNA), and secondary structure of the target could influence both the thermodynamics and kinetics of probe-target duplex formation. Additionally, hybridization conditions (temperature, time, ionic strength, concentration of the probe and the target, the presence of denaturing agents or detergents) and washing conditions (temperature and ionic strength) contribute to final signal intensity on microarrays.

One of the most promising approaches for probe design is based on the nearest-neighbor model described above. This model postulates that the stability of DNA–DNA duplexes mainly depends on the composition and orientation of neighboring base pairs. The duplex stability is correlated with a value of target-probe hybridization free energy (ΔG), which can be calculated from entropy and enthalpy of each possible matched nearest-neighbor

pair. Despite the fact that values used in the nearest-neighbor model were obtained for hybridization in solution, calculated free energies of duplexes demonstrate a clear correlation with the hybridization intensities on microarrays. Probes with similar ΔG values of duplex formation are found to bind their corresponding targets with equal intensity.

Methods for probe synthesis and immobilization on a chip surface must be taken into account when discussing quantitative reliability of DNA-microarray data. Recent advances in technology allow synthesis of high-density arrays of probes (up to 10^6 probes/cm^2) directly on a chip surface using photolithography. However, the chemistry of the in situ synthesis has a finite yield such that in a 25-step reaction with 99% yield at each step, the resulting yield of probes with the correct sequence is only 78% ($0.99^{25}=0.78$). Alternative array manufacturing approaches such as ink-jet printing and robotic spotting each produce microarrays with different hybridization properties.

Microarrays of probes attached to a chip surface by either their 3′- or 5′-ends appear to have nearly identical sensitivity, specificity, dynamic range, and reproducibility. At the same time, probes synthesized on a chip surface in $3' \rightarrow 5'$ direction or attached to a glass slide through 3′-ends cannot be utilized in many common types of enzymatic modifications such as primer extension, ligation, and flap cleavage.

Selection of proper length of probes is dictated by the experiment objective. In general, long probes (50–60 nt) produce more intense hybridization signal than shorter ones but possess lower specificity. Intensity of the hybridization signal rises rapidly for probe lengths in the range of 17–50 nt and then reaches a plateau. The presence of a linker (typically 6–9 carbon atoms) between the chip surface and probe sequence facilitates hybridization for short probes that increases hybridization signal and results to higher sensitivity.

Single nucleotide polymorphisms (SNP) have increasing importance in both clinical and diagnostic roles. When designing a probe, some additional design rules should be applied for the best resolution of perfect match (PM) and single base pair mismatch (SBM) situations. In general, mismatch stability (T_m or ΔG of the duplex) is dependent on the length of probe, nature of the mismatch, mismatch position along the probe, and nearest neighbors of the mismatch. For shorter probes, the destabilizing effect of a mismatch is more dramatic. A reasonable compromise between specificity and sensitivity of SBM analysis can be achieved by using shorter probes (18–20 nt). The type of a mismatch is even more important than its location along the probe.

For example, in case of a G:A mismatch, reliability of the assay can be increased by switching to the antisense strand. The resulting C:T mismatch has a higher destabilizing effect, and the decrease in fluorescence intensity is more readily detected. Mismatch location is also an important concern in designing probes for SNP detection. In general, mismatches located near the ends of a probe have smaller destabilizing effect on the duplex. As a rule, ultimate and penultimate mismatches cannot be reliably detected even after optimization of hybridization and washing conditions.

POLYMERASE CHAIN REACTION PRIMERS AND PROBES FOR ANALYSIS OF CONSERVED SEQUENCES

Gene families are groups of closely related genes having similar DNA sequences which evolved from a single ancestral gene and may code for similar products. For example, Staphylococcal enterotoxins (SEs) are a family of 17 major serological types of heat-stable enterotoxins that are one of the leading causes of gastroenteritis resulting from consumption of contaminated food. These toxins share similar DNA sequence and similar biological activities. Analysis of gene families using multiple sequence alignment is important for both phylogenetic research as well as such practical applications as detection and identification of clinically relevant genes.

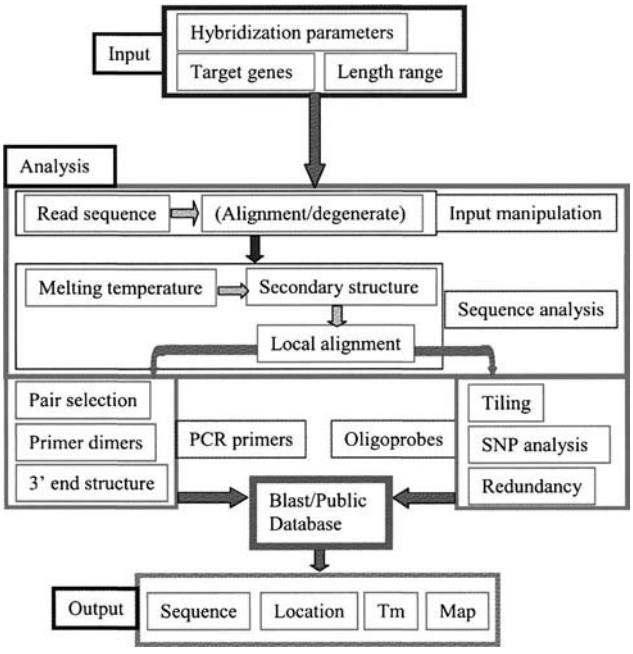


Fig. 1 Outline of computer program for oligonucleotide design. The algorithm includes three modules: input, sequence analysis, and output. (*View this art in color at www.dekker.com.*)

For the design of universal PCR primers and gene-specific microarray probes for analysis of gene families and conserved sequences, there is a need to identify conserved regions flanking variable regions among all sequences. The conserved regions may be used to design universal primers for simultaneous amplification of the variable regions from multiple target organisms. These genetically divergent regions may be used to design gene-specific oligonucleotide probes to discriminate among the target sequences.

The selection of these sequences follows the same basic rules as the selection of oligonucleotides for simple PCR and microarray applications with the additional constraints that the conserved regions between divergent sequences are frequently limited regions of the genes and the use of universal primers implies that the primers have a range of melting temperatures.

ALGORITHM FOR OLIGONUCLEOTIDE DESIGN

Software for oligonucleotide design is commonly available[5,6] and generally has input, sequence analysis, and output modules. An outline of such an algorithm for oligonucleotide design is shown in Fig. 1.

In the input module, the user specifies the target sequence and various parameters for the desired oligonucleotides including melting temperature range, salt concentration, and the length range. Other input parameters (not shown) may include weighting factors for the various design constraints under consideration. The analysis module first carries out input manipulation, reading the target sequences and performing multiple alignment of sequences if the genes analyzed are part of a gene family or include conserved domain. The alignment allows design of PCR primers for amplification of multiple conserved sequences and the development of oligonucleotides that can either detect all conserved sequences or distinguish among conserved sequences.

A first element of the sequence analysis is the T_m calculation for each candidate oligo using the nearest-neighbor model. Each sequence is then scored for potential secondary structures, such as hairpins and self-complementary regions, to limit self-annealing. Alignment between the candidate oligo and the target (local alignment) is performed to look for and avoid other hybridization sites.

The sequence analysis module may perform additional analysis steps for oligonucleotides to be used as PCR primers, including pair selection of forward and reverse primers using all of the permutations of the candidates. Amplicon length is calculated for each primer pair along with the melting temperature of each primer. Additional rules for primer selection such as 3′-end requirements can be applied at this point to find preferred primer pairs. For oligonucleotides to be used as probes, SNP analysis and selection of overlapping primers (tiling) may be performed.

Finally, the output module produces a list of oligonucleotides with their sequences, location on the target sequence, and melting temperature, and may include a map of the target sequence indicating the position of the oligonucleotides.

CONCLUSION

Applications of oligonucleotides as PCR primers and microarray probes have expanded dramatically in recent years. The design of oligonucleotides for these applications involves a large number of considerations starting with the melting temperature and including a number of competing criteria. Each application has unique aspects making it impossible to cover the subject of oligonucleotide design in full generality. The key design issues are summarized in this article and discussed in terms of simple applications with reference to a software-based design approach that allows the user to balance the competing design criteria. This design approach has proven very successful but the final test of a well-designed oligonucleotide must be performed using the molecules themselves because biological systems exhibit more complex behavior than can be fully captured in software.

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Onchocerca volvulus

Thomas R. Unnasch
University of Alabama at Birmingham, Birmingham, Alabama, U.S.A.

INTRODUCTION

Onchocerciasis, or river blindness, has historically been one of the most important causes of infectious blindness. It is endemic to sub-Saharan Africa and six countries of Central and South America. The disease, although not fatal, results in severe socioeconomic disruption of affected communities. Onchocerciasis is caused by infection with the nematode *Onchocerca volvulus*. *O. volvulus* is an obligate parasite of humans, which is notable because most *Onchocerca* are parasites of ungulates. The sexual stages of the parasite exist in the human host. The adult females produce large numbers of larvae (known as microfilaria), which distribute themselves throughout the dermis of an infected individual, although they are also capable of invading other organs, such as the eye. The parasite is transmitted between infected humans by vector black flies of the genus *Simulium*. When a black fly takes a blood meal from an infected individual, microfilaria are ingested along with the blood. The microfilaria undergo two molts to form infective larvae, or L3, which migrate to the head capsule of the fly. When an L3-infected fly feeds, the L3 are introduced. The L3 then undergo two more molts to produce adult parasites, completing the life cycle. The prepatent period, or the time between the introduction of the L3 into a human host and the final development of a fertile female, is 18–24 months.

Three internationally supported programs to eliminate onchocerciasis as a public health problem have been initiated in the past 30 years. These included the Onchocerciasis Control Programme in West Africa (OCP), which was instrumental in eliminating blinding onchocerciasis as a public health problem in 11 countries of West Africa. The OCP conducted active operations in the period of 1975–2002. The OCP primarily relied on a strategy of vector control supplemented in later years by ivermectin distribution, the single drug known to be safe and effective for the treatment of onchocerciasis. Two programs, the African Program for Onchocerciasis Control (APOC) and the Onchocerciasis Elimination Program in the Americas (OEPA), are currently active. Both solely rely on ivermectin distribution. The goal of this article is to review the development of DNA-based diagnostics for *O. volvulus* and their application both to onchocerciasis control and to biogeographical studies.

DEVELOPMENT OF DNA-BASED DIAGNOSTIC TESTS FOR *O. VOLVULUS*

Development of DNA-based detection methods for *O. volvulus* began in the 1980s before the development of the polymerase chain reaction (PCR). Using conventional screening approaches, several DNA probes were identified that exhibited varying specificities.[1] Some were specific for parasites of the genus *Onchocerca*,[2] while others were specific for *O. volvulus*.[3,4] One probe (pFS-1) was developed that was specific for parasites endemic to the rain forests of West Africa.[5] DNA sequence analysis revealed that all of these probes were related, having been derived from a tandemly repeated DNA sequence family specific to the genus *Onchocerca*,[6] designated the O-150 repeat family. Further analysis of the O-150 repeat family revealed that its variation was constrained, and that the members could be grouped into subfamilies in which the members were nearly identical. Some subfamilies were found in all members of the genus *Onchocerca*. Others were specific for *O. volvulus* or other *Onchocerca* species, and some were found only in certain isolates of *O. volvulus*.[6] These results explained the observed specificity of the previously isolated DNA probes and permitted the rational design of new oligonucleotide probes.[6]

The repeated nature of the O-150 repeat made it particularly amenable to the development of a PCR assay.[7] In the initial PCR assays, degenerate primers were used to amplify all of the subfamilies of the O-150 repeat and the products distinguished on the basis of hybridization to oligonucleotide probes.[7] Subsequently, primer pairs were developed that specifically targeted a subfamily of the O-150 repeat that was found only in *O. volvulus*, resulting in an *O. volvulus*-specific PCR.[8]

Before the development of the O-150 PCR, the gold standard for the diagnosis of *O. volvulus* was microscopic detection of microfilaria in small skin biopsies (skin snips) collected from infected individuals. The sensitivity

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of the O-150 PCR ranged from 90% to 100% when compared to the microscopic skin-snip assay.[9–11] The specificity of the O-150 PCR was 100% when tested on individuals never exposed to *O. volvulus*. In one study, amplification was also obtained from 13/34 skin-snip-negative individuals residing in an *O. volvulus* endemic area, suggesting that the O-150 PCR of skin-snip-derived DNA might be more sensitive for the detection of *O. volvulus* than microscopic examination.[9] However, a similar study found the O-150 PCR to be no more sensitive than microscopy when care was taken to thoroughly examine the snip.[10] Taken together, these results suggested that the O-150 PCR assay was 100% specific for the detection of *O. volvulus* and at least as sensitive and perhaps more sensitive than the skin-snip assay.

The original O-150 PCR assays involved the detection of products following amplification and Southern blotting followed by oligonucleotide hybridization.[7] Subsequently, enzyme-linked immunosorbent assay (ELISA)-based[12] and chromatographic (test strip) methods were also developed.[13,14] Both methods were found to have comparable sensitivities and both were at least as sensitive as gel electrophoresis.[12–14] The ELISA-based method allows the investigator to define a rational cutoff for defining positive samples. The test strip methods, although not permitting the assignment of a quantitative cutoff, do not require the use of any sophisticated equipment, bringing the O-150 PCR one step closer to field applicability.

One of the disadvantages of the skin snip is that collection of skin biopsies requires the use of a sclerodermal punch. These instruments are expensive and must be sterilized between uses. The painful nature of the test also limits its acceptance among affected communities. Thus attempts have been made to use the O-150 PCR to detect parasite DNA in samples that can be collected by less invasive means. Toé et al.[15] demonstrated that parasite DNA could be detected in superficial skin scrapings using the O-150 PCR. The sensitivity of this assay was high when compared to microscopy (92%) but lower when compared to O-150 PCR amplification of skin-snip DNA. The O-150 PCR has also been used to detect *O. volvulus* DNA in the urine of infected individuals.[11] Although successful, the sensitivity of the urine-based PCR was quite low (14%).

Perhaps the most important application of the O-150 PCR has been for the identification of *O. volvulus* L3 in black flies. The overall goal of both the OCP and OEPA is to eliminate transmission of *O. volvulus.* In the pursuit of this goal, it was necessary to determine the level of transmission. The most efficient way to accomplish this was by examining vector black flies for L3 rather than monitoring for new infections. This is because it is difficult to monitor infection incidence in underdeveloped areas where populations are mobile and medical records are incomplete. In addition, the long prepatent period (18–24 months) means that new human cases provide a snapshot of transmission that is at least 18 months old.

Traditionally, transmission monitoring has been accomplished through dissection of individual black flies. However, there are two drawbacks to dissection. The first is that throughout much of West Africa, *O. volvulus* is sympatric with *Onchocerca ochengi*. Both *O. volvulus* and *O. ochengi* are transmitted by the same black flies, and their L3 are morphologically indistinguishable. This means that it is difficult to obtain an accurate estimation of the level of transmission for *O. volvulus* using dissection in areas where both parasites are found. This difficulty was overcome through the development of the O-150 PCR and of species-specific oligonucleotides capable of distinguishing *O. volvulus* and *O. ochengi*. A second disadvantage to dissection is that it becomes increasingly inefficient as the prevalence of infected flies declines, as occurs in the face of an effective control program. The high sensitivity of the O-150 PCR for the detection of *O. volvulus* DNA opened the possibility that this assay might be used to screen pools of black flies, permitting the efficient simultaneous screening of large numbers of flies. Therefore, the O-150 PCR was adapted to permit its use on pools of flies, and a method was developed to predict the prevalence of infection in the vector population based on the proportion of negative pools found and the number of flies contained in each pool.[16] This method was evaluated in field studies in Africa[17] and the Americas[18] that compared infection rates estimated by dissection and pool screen PCR. In all cases, the PCR results were indistinguishable from the prevalence of infection as estimated by dissection.

Recently, the pool screen PCR technique has been used in a countrywide study in Ecuador to estimate the effect of long-term ivermectin distribution on *O. volvulus* transmission.[19] The data from this study documented a dramatic reduction in *O. volvulus* transmission as a result of long-term ivermectin distribution, and demonstrated that in one focus transmission had been completely suppressed. This demonstrated that the O-150 PCR could be used to assist control programs in monitoring transmission in areas where the intensity of transmission was low, and also provided evidence that under certain circumstances, *O. volvulus* transmission could be eliminated through ivermectin distribution alone.

THE O-150 REPEAT FAMILY AS A TOOL TO STUDY RELATIONSHIPS AMONG *O. VOLVULUS* POPULATIONS

Since the 20th century, it had been known that onchocerciasis exhibited two distinct clinical presentations in West Africa. In the rainforest bioclime, a nonblinding

form of the disease existed where onchocercal blindness was rare, even in hyperendemic areas. In the savanna bioclime, a blinding form of the disease was endemic, where onchocercal blindness was common. Epidemiological, entomological, pathological, biochemical, and immunological studies all supported the hypothesis that this difference was linked to the fact that two distinct strains of the parasite were endemic to the rainforest and savanna bioclimes of West Africa. Before the development of the O-150 PCR, a DNA probe derived from the O-150 repeat sequence family (pFS-1) was identified that hybridized only to parasite isolates collected from the forested regions.[5] Following the development of the O-150 PCR, this probe was used to classify PCR products from villages from West Africa that exhibited blinding or nonblinding epidemiological disease patterns. The results demonstrated an almost perfect correspondence of the probe classification and disease pattern.[20] This suggested that the differences in disease pattern were due to genetic differences in the parasite populations and that the O-150 PCR, when used in conjunction with strain-specific probes, was capable of distinguishing the two strains.

The discovery that the O-150 repeat family was variable enough to differentiate the forest and savanna strains of *O. volvulus* in West Africa suggested that it might also be used as a tool to study the relationships among other populations of *O. volvulus.* For example, strain-specific probes developed to classify West African parasites have been used in studies of *O. volvulus* from Uganda,[21] which suggested that Ugandan parasites might form a population that was distinct from those found in West Africa. Similarly, extensive analyses were carried out on the O-150 families from West African parasites and those from Sudan and the Americas.[22,23] The results demonstrated that, as expected, parasites from the savanna and forest bioclimes of West Africa were statistically different from one another. Parasites from Southern Sudan were statistically indistinguishable from those in the West African savanna in keeping with the fact that onchocerciasis is endemic throughout the wide savanna zone spanning the entire continent of Africa from east to west. In contrast, parasites from the Abu Hamad focus of onchocerciasis in Northern Sudan were distinct from both West African strains.[23] The Abu Hamad focus is isolated from all other foci of *O. volvulus* by the desert, and the vector there is a distinct species of *Simulium damnosum* s.l. Parasites from Guatemala and Brazil were found to be distinguishable from the forest strain of West African *O. volvulus* but were indistinguishable from savanna strain parasites.[22] This finding supported the hypothesis that *O. volvulus* was introduced into the Americas as a result of the trade in African slaves, as the majority of the slaves imported into both Guatemala and Brazil originated from the savanna areas of West Africa.

CONCLUSION

As the sections above demonstrate, the O-150 PCR has been established as a sensitive and specific tool for the detection of *O. volvulus*. The assay has been adapted to detect infections both in humans and vector black flies, and it has been exploited by onchocerciasis control programs in both Africa and the Americas. The variable nature of the O-150 repeat also makes it a useful tool for differentiating parasite populations and for conducting biogeographical studies.

Several improvements could still be made to increase the utility of the O-150 PCR. For example, automated and microtiterplate-based DNA purification techniques may improve the throughput of the assay and may increase the number of flies that may be tested in a given pool, decreasing the marginal cost of the assay. Furthermore, previous studies have demonstrated that the O-150 PCR may be used to measure the filaricidal effects of various experimental drugs.[24] However, ivermectin, the only drug currently approved for treatment of onchocerciasis, kills *O. volvulus* microfilaria but has less of an effect on the adult form of the parasite. Thus, it is difficult to measure adult viability in the face of ivermectin treatment, something that is necessary to determine when an *O. volvulus*-infected individual has become infection free and treatment may be stopped. It is possible that use of the O-150 PCR to detect *O. volvulus* DNA in tissues other than skin might be used for this purpose.

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Orthopoxviruses—Monkeypox, Cowpox, *Vaccinia*, Camelpox, Mousepox

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M. Sofi Ibrahim
James D. Mellott
The United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, U.S.A.

INTRODUCTION

One of the most remarkable achievements in medical history is the eradication of smallpox, a once devastating disease that plagued mankind for millennia. Although the *Variola* virus, the causative agent of smallpox, is the only *Orthopoxvirus* specific for humans, other zoonotic *Orthopoxvirus* species, e.g., buffalopox, cowpox, monkeypox, and *Vaccinia* viruses, can cause disease in humans. Concerns have been raised that *Variola* may reemerge or other orthopox viruses may be rendered more pathogenic by genetic engineering. Reemergence of *Variola* or genetically altered chimeras would pose serious public health threats of global proportions. The recent occurrence of the first monkeypox infection in the Western Hemisphere clearly demonstrates the ease with which zoonotic *Orthopoxvirus* infections can cross geographic boundaries and species barriers. Evidence exists that altering *Orthopoxvirus* genome can enhance host susceptibility to infection. For example, it has recently been demonstrated that engineering interleukin-4 gene into the mousepox (ectromelia) virus genome induced lethal infection in genetically resistant and vaccinated mice.

MORPHOLOGY, TAXONOMY, AND GENOMIC ORGANIZATION

Orthopox viruses are oval, brick-shaped particles with geometrically corrugated outer surface. The particles' sizes range from 220 to 450 nm in length and 140 to 260 nm in width. The outer envelope consists of a lipoprotein layer embedding surface tubules and enclosing a biconcave core. The core contains the viral DNA and core fibrils, and is surrounded by the core envelope and a tightly arranged layer of rod-shaped structures known as the palisade layer. Between the palisade layer and the outer envelope exist two oval masses known as the lateral bodies.

Genus *Orthopoxvirus* is one of the 11 known genera in the viral family Poxviridae, which comprises two subfamilies: Chordopoxvirina and Entomopoxvirinae. Subfamily Chordopoxvirinae contains eight genera: *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, *Suipoxvirus*, and *Yatapoxvirus*, and at least 36 species that are associated with vertebrates. Subfamily Entomopoxvirinae contains three genera: *Entomopoxvirus* A, B, and C, and 25 species that are associated with insects.

Genus *Orthopoxvirus* contains 11 known species: buffalopox, camelpox, cowpox, ectromelia, monkeypox, rabbitpox, raccoonpox, taterapox, *Vaccinia*, *Variola*, and volepox viruses and two tentative species: skunkpox and Uasin Gishu viruses.

Orthopox viruses contain double-stranded DNA genomes whose 5′ and 3′ ends are closed, forming terminal loops. The genomes of camelpox, cowpox, ectromelia, monkeypox, *Vaccinia*, and *Variola* viruses have been sequenced and are published in GenBank. The genome size of these viruses varies from 177,923 bp (*Vaccinia* Ankara) to 224,499 bp (cowpox Brighton). The genome size of camelpox Kazakhstan, ectromelia Moscow, monkeypox Zaire-1996, and *Variola* Bangladesh-1975 viruses are 205,719, 209,771, 196,858, and 186,103 bp, respectively. The 5′ and 3′ termini contain inverted terminal repeats (ITR) whose sizes vary considerably in different species. The size of ITR in camelpox, cowpox, ectromelia, monkeypox, *Vaccinia*, and *Variola* viruses are 7736, 9710, 9413, 6378, 12,068, and 725 bp, respectively. The number of genes also varies in different species. The number of genes of camelpox, cowpox, ectromelia, monkeypox, *Vaccinia*, and *Variola* genomes are 212, 234, 176, 193, 266, and 191, respectively.

REPLICATION AND INFECTIVITY

Orthopoxvirus genomes encode about 176 to 266 proteins, including a number of enzymes and factors that are necessary for self-replication and maturation. The central region of the genome contains highly conserved genes that are essential for viral replication, and the terminal

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regions contain less conserved genes that are important for virus–host interactions. Although it is well known that replication occurs in the cytoplasm, it is not clear how or if host cell nuclear factors are involved in viral replication or maturation.

Viral replication begins with attachment of viral particles to host cell surface, most likely through cell receptors, e.g., epidermal growth factor in the case of *Vaccinia*, and involves expression of early, intermediate, and late genes. Initial uncoating occurs during entry, followed by synthesis of early mRNAs which are translated to facilitate further uncoating and transcription of intermediate mRNAs. Intermediate mRNAs, in turn, are translated to allow transcription of the late mRNAs. The late mRNAs are translated into structural and enzymatic components of the virions. These components, along with DNA concatemers that are formed during the early phase of replication, are assembled into genomic DNA and packaged into immature virions, then infectious intracellular mature virions (IMV). The last step of viral replication is the acquisition by IMV of virus-altered Golgi membranes and plasma membranes, and formation of infectious extracellular mature virus (EMV).

The newly replicated progeny exits the lysed cells and invades other cells, repeating the cycle. The entire replication cycle is completed within about 12 hr. Within a few hours after infection, the virus takes over the cell machinery, inhibiting cellular DNA, RNA, and protein synthesis, and causing cell death.

Although orthopox viruses have highly similar morphological and genomic characteristics, they have distinct host preferences and vary considerably in their infectivity and pathogenicity.

Variola virus is specific for humans, but it has also been shown experimentally to cause lethal infections in nonhuman primates.[1] The severity of human infections depends on the host immune response and the viral strain. Infections with *Variola* major induce severe, generalized, or systemic disease with fatality rates up to 40%, whereas infections with *Variola* minor cause a milder form of the disease with case fatality rates of 1% or less.

Vaccinia virus has no definitive host, but it has been isolated from camels, buffalos, cows, pigs, and rabbits.[2] *Vaccinia* has been used in humans as a vaccine against smallpox infections because it produces mild, localized infections; however, it can also induce life-threatening complications in immune-compromised individuals.[3]

Cowpox virus is probably maintained in rodent reservoirs in nature, but can occasionally infect humans, elephants, cattle, cheetahs, cats, and other animals.[2] Human infections with cowpox were known for centuries, but have been brought to public attention since the early vaccination attempts by Edward Jenner. Although most infections with cowpox produce localized lesions, severe infections were reported.[4]

Monkeypox virus was first identified in laboratory-maintained cynomolgus monkeys.[5] The virus is believed to have been circulating for a long time in numerous animal hosts, particularly squirrels, in central and western Africa. Human infections with monkeypox virus were first recognized in Zaire and later in Liberia and Sierra Leone.[6] The disease caused by monkeypox virus closely resembles smallpox, but the pustular rash is mostly localized in the neck and groin areas. Most infections occur by direct contact with infected animals. Person-to-person transmission rarely occurs.

Mousepox (ectromelia) virus was first recognized as a mouse pathogen in 1930. Its pathogenesis differs from other orthopox viruses in that the virulent strains induce severe hepatitis in mice. It is highly infectious and can easily decimate mouse colonies in laboratories and breeding facilities. Vaccinating mice with the *Vaccinia* confers protection and has been used to control infections in mouse colonies.[7] No human infections have been reported due to ectromelia. However, the virus was useful as model to study the molecular basis of *Orthopoxvirus* virulence despite the fact that the mouse disease differs significantly from smallpox.

MOLECULAR TESTING

Many phenotypic and genotypic methods involving virological, immunological, and molecular approaches have been used to identify *Orthopoxvirus*. The molecular approaches, including DNA sequencing, polymerase chain reaction (PCR), restriction fragment-length polymorphism (RFLP), real-time PCR, and microarrays, are more sensitive and specific than the virological and immunological approaches. Of these approaches, sequencing provides the highest level of specificity for species or strain identification but current sequencing techniques are not yet practical as rapid diagnostic tools in most laboratories. Restriction fragment-length polymorphism analysis[8] and microarray genotyping[9] also provide high levels of specificity, and when combined with PCR, these approaches can offer high levels of sensitivity as well. Real-time PCR methods provide exquisite levels of sensitivity and specificity.[10,11] The basic concept behind real-time PCR is the measurement, by fluorescence detection, of the amount of nucleic acids produced during every cycle of the PCR. Several detection chemistries, e.g., intercalating dyes (SYBR Green), hydrolysis probes [5′ nuclease or Taqman, minor groove-binding proteins (MGB)], hybridization probes [fluorescence resonance energy transfer (FRET)], and molecular

beacons, are used. Several instruments for real-time PCR, e.g., the ABI 7700, Smart Cycler, LightCycler, RAPID, Opticon, MX 4000, Rotor-Gene, and others, are commercially available. When combined with portable analytical platforms such as the Smart Cycler or LightCycler, real-time PCR systems can be readily deployed to field sites for rapid testing.

In the following section, we describe an approach for genome typing of orthopox viruses by using long polymerase chain reaction (LPCR) amplification, followed by RFLP analysis.

GUIDELINES ON PERFORMING LPCR–RFLP ANALYSIS

Processing *Orthopoxvirus* Samples

Successful performance of LPCR–RFLP analysis requires pure and undegraded DNA roughly 40–150 kb in length. There are numerous commercial nucleic acid purification technologies for a variety of sample types, e.g., QIAamp Genomic DNA (Qiagen) and AquaPure DNA (Bio-Rad). These methods involve cell lysis and protein denaturation followed by DNA precipitation or fractionation by reversible binding to an affinity matrix. They have been used to purify high-quality DNA from *Orthopoxvirus* cultures and clinical specimens.

Primer Design

Ideally, LPCR primers length should be 20 to 29 bases with no hairpins or 3′ complementarity, and with T_m between 60°C and 68°C in 85 mM salt. Several software programs for primer design are available commercially, such as Primer Express (ABI Biosystems) and Oligo (National Biosciences), among many others. To amplify as many orthopox viruses as possible, consensus or degenerate primers should be designed. Table 1 shows examples of consensus primers designed to amplify eight overlapping segments spanning about 98% of *Orthopoxvirus* genomes.

LPCR–RFLP Performance and Analysis

Amplifying large DNA fragments requires high-fidelity DNA polymerase enzymes, e.g., TAKARA LA (Takara). The conditions for LPCR amplification require optimizing primer, magnesium ion, and enzyme concentrations as well as thermal cycling number and timing. In a typical LPCR reaction protocol, 1–100 ng of template DNA is amplified in the presence of 1 μM of each primer, 300 μM of dNTPs, 2.5 mM $MgCl_2$, and 1.5 U of TAKARA LA polymerase. The amplified LPCR products are purified on agarose gels, then are digested with a restriction enzyme, e.g., *Cla*I. The digested DNA fragments are then electrophoresed on 6% polyacrylamide gel for a constant period of time at constant voltage. A reference DNA marker is run along with the digested DNAs in the first, middle, and last lanes to help identify the size of the restricted DNA fragments. After staining with ethidium bromide, the restriction pattern is visualized and photographed with a digital camera.

Standardization of electrophoretic conditions, i.e., using the same lot of precast polyacrylamide gel, and applying the same voltage, e.g., 50 V for the same period of time, e.g., 6.5 hr for each run, is very important to obtain reproducible banding patterns. The positions for all DNA fragments in each restriction pattern are determined and digitized by fingerprinting software, e.g., Dendron (Solltech). The program assigns a molecular weight value for each DNA fragment in each restriction pattern using the reference DNA marker. A similarity coefficient (S_{AB})

Table 1 LPCR primers and their 5′ and 3′ coordinates in relation to *Variola* genome (GenBank LL22579)

Segment	Forward primer	Reverse primer
1	1,297/5′–GCTCCGTTGCATCAGTTCTG–3′/1,316	17,296/5′–GGATGTGGTCCGTTGTTGG–3′/17,278
2	16,778/5′–GAYGCATCTTATGGATCCCA–3′/16,797	35,810/5′–TAACGCMGACACATCGTGTG–3′/35,791
3	35,737/5′–CCGTCCAGATGCGAGAGAAT–3′/35,756	56,840/5′–CTCCRTTYGGTTGGTTGACG–3′/56,821
4	55,915/5′–RCCGATCGCAGTTAGAACGG–3′/55,934	79,733/5′–CGGAACACCAATGYGGYGAC–3′/79,714
5	79,541/5′–CTRCCGCAAGCTATTAGGCG–3′/79,560	103,460/5′–GGAAGAGTRGTGTTCGCTCC–3′/103,441
6	103,272/5′–GCCGGTGTAACATTCTCCAC–3′/103,291	126,608/5′–CGRAGGTAACCCATGCGAAC–3′/126,589
7	126,028/5′–TCGAACGAAGRCCTCCTTCG–3′/126,047	151,555/5′–GGTYCCGGAKTCGCGATTTC–3′/151,536
8	150,516/5′–CRTGTACCGACRCACTWGAG–3′/150,535	184,518/5′–RCACCTTCAGATRCCGATCC–3′/184,499

is calculated for every pair of restriction patterns using the absolute difference/total area formula:

$$S_{AB} = 1.0 - \frac{\sum_{i=1}^{k} |a_i - b_i|}{\sum_{i=1}^{k} (a_i + b_i)}$$

where a_i and b_i are the intensities of fragment position i in the restriction patterns A and B, and k is the total number of possible positions. If only fragment position is used in calculations (without regard to the fragment intensity), a and b are set to either 1 or 0 for presence or absence of a fragment at a given position. If two restriction patterns are completely different, then $S_{AB}=0$; if they are identical then $S_{AB}=1$; and if half of the total fragments have an identical fragment in both patterns, then $S_{AB}=0.5$. A dendrogram, graphically representing the relationships between multiple patterns, is constructed from a matrix of similarity coefficients by using the unweighted pair group method.[12] The pairs with the highest similarity values are grouped together with the similarity coefficient corresponding to the branch point on the dendrogram. The remaining branch points are calculated in order of descending similarity coefficients. When more than two restriction patterns are considered for a branch point, the branch point is calculated as the average of all the similarity values involved.

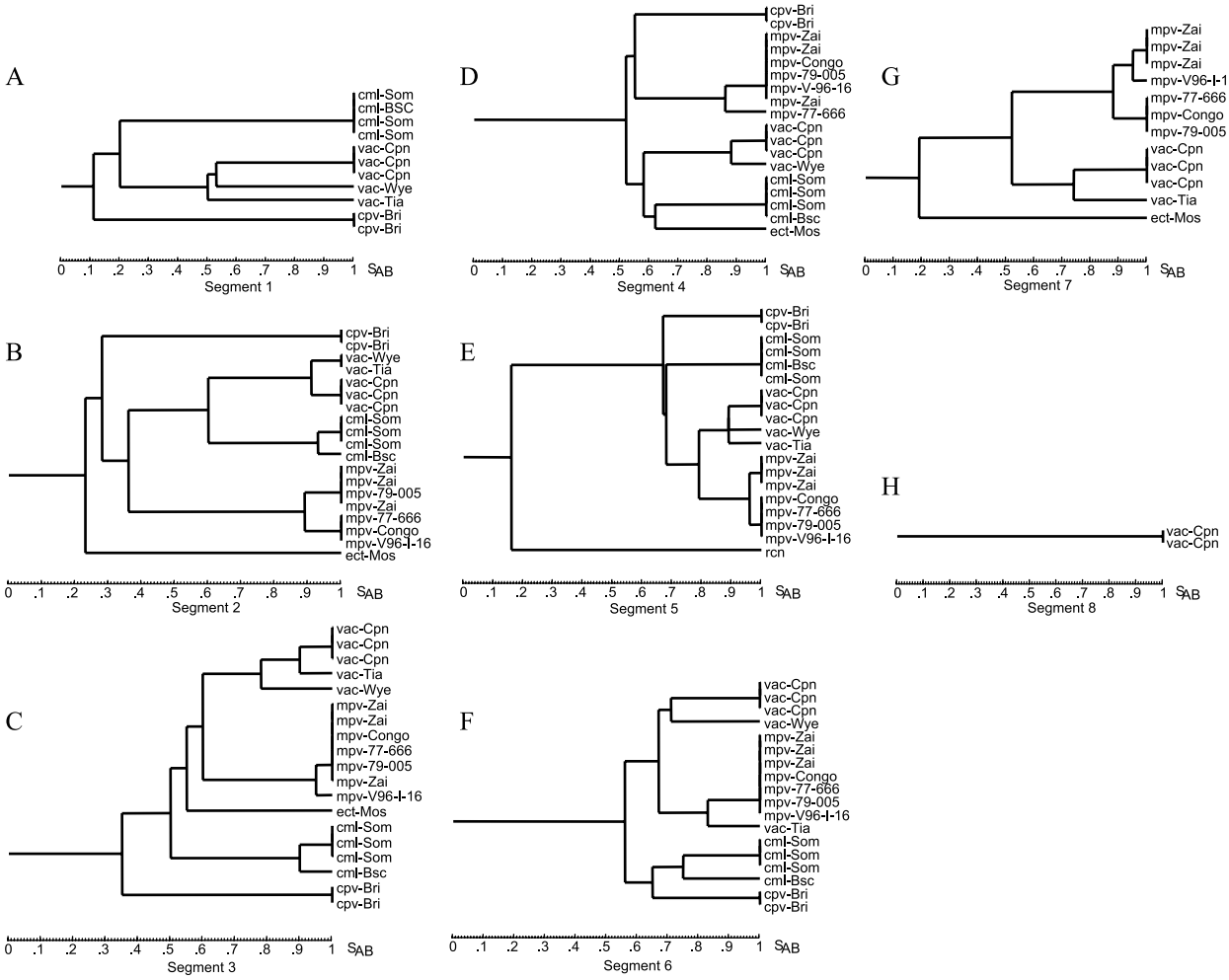

Fig. 1 A–H. Dendrograms of the restriction fragment-length polymorphism (RFLP) profiles of camelpox Somalia (cml-Som), camelpox BSC-40 (cml-BSC), cowpox Brighton (cpv-Bri), ectromelia Moscow (ect-Mos), monkeypox 77-666 (mpv-77-666), monkeypox 79-005 (mpv-79-005), monkeypox Congo-8 (mpv-Congo), monkeypox V96-I-16 (mpv-V96-I-16), monkeypox Zaire (mpv-Zai), raccoonpox (rcn), *Vaccinia* Copenhagen (vac-Cpn), *Vaccinia* Tian-Tan (vac-Tia), and *Vaccinia* Wyeth (vac-Wye) viruses of segments 1 to 8. The similarity coefficient S_{AB} values are indicated on the horizontal axes.

Figure 1 illustrates one LPCR–RRFLP approach using the LPCR primers listed in Table 1 and following the guidelines described above. The experiments were carried out with genomic DNAs from different strains of camelpox, cowpox, ectromelia, cotia, monkeypox, raccoonpox, and *Vaccinia* viruses. As shown, it was possible to classify every sample within each species. The S_{AB} ranges of the different strains within a species were 0.53–1, 0.89–1, 0.78–1, 0.86–1, 0.89–1, 0.71–1, and 0.74–1 in segments 1, 2, 3, 4, 5, 6, and 7, respectively. The lowest S_{AB} value within the same species (0.53) was observed with *Vaccinia* Copenhagen and *Vaccinia* Tian Tan in segment 1. The S_{AB} ranges of different species were 0–0.31, 0.15–0.67, 0.21–0.7, 0.43–0.63, 0.13–0.86, 0.31–0.83, and 0.31–0.61 in segments 1, 2, 3, 4, 5, 6, and 7, respectively. Because only one species, *Vaccinia* Copenhagen, was amplified in segment 8, it was not possible to estimate the S_{AB} values with other species or strains, but the S_{AB} value for the different replicates of the same strain was 1.0. These results suggest that closely related species and strains can be grouped and identified using any of the eight segments. However, because lower S_{AB} values were obtained with the flanking segments 1, 2, 7, and 8 than with the central segments 3, 4, 5, and 6, we concluded that genotyping with the terminal segments can be useful for strain differentiation whereas genotyping with the central segments can be useful for species differentiation.

CONCLUSION

All members of genus *Orthopoxvirus* share common morphological and biological characteristics notwithstanding the broad spectrum of their mammalian host repertoire that includes rodents, domesticated and feral mammals, humans and nonhuman primates. They differ from other double-stranded DNA viruses in that they are self-reliant in their replication strategy, as they replicate exclusively in the cell cytoplasm using a set of specialized enzymes, transcription, and replication factors. There are 11 recognized *Orthopoxvirus* species, five of which (buffalopox, cowpox, monkeypox, *Vaccinia*, and *Variola* viruses) are known to cause human disease with varied severity depending on the viral strain and host's immune response. Changes in certain protein genes or insertion of foreign genes into the genome may influence virulence.[13,14] Furthermore, *Vaccinia* virus, which has been used for years as a vaccine against smallpox, is still widely used as an expression vector for a variety of genes from numerous infectious agents. For these, and other reasons, there have been concerns that members of the *Orthopoxvirus* genus may emerge as natural, incidental, or deliberate health threats. Therefore, the development of rapid, sensitive, and specific methods for identification and classification of these viruses is critically important to prepare and deter against their potential resurgence. Among the modern methods for rapid, sensitive, and specific identification of orthopox viruses are real-time PCR, RFLP analysis, and microarray analysis. A method using LPCR–RFLP analysis is described to illustrate the utility of this approach in whole-genome typing of *Orthopoxvirus*. Using this approach, and other new approaches, could discriminate *Orthopoxvirus* species and strains, and possibly identify genetically mutated or altered viruses.

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Osteogenesis Imperfecta

Anna Gajko-Galicka
Medical University of Bialystok, Bialystok, Poland

INTRODUCTION

Osteogenesis imperfecta (OI) is an autosomal dominant genetic disorder of connective tissues, which is also known as ''brittle bone disease'' because of the susceptibility of the affected individuals to fracture from the mildest trauma. It exhibits a broad range of clinical severity, ranging from multiple fracturing and perinatal death to a mild form that may elude clinical detection. Biochemical and molecular genetic studies have shown that the vast majority of affected individuals have mutations in the either the *COL1A1* or *COL1A2* gene that encodes the chains of type I procollagen—the major structural protein of bone. The relationship of specific mutations to severity is an exceedingly difficult task. Most patients with mild type I OI produce structurally normal collagen in reduced amounts because of null *COL1A1* allele. The structural mutations in patients with types II, III, and IV affect connective tissues through a dominant negative mechanism, in which the presence of the mutant chain results in defective extracellular matrix. Prenatal diagnostic techniques have allowed the early detection of this disorder, particularly in families in which the molecular defect is already known. Because OI is an incurable genetic disease, two alternative approaches for gene therapy are being investigated: one is replacement of mutant cells with normal cells through bone marrow transplantation, and the second involves the suppression of mutant genes by introducing ribozymes into the cells to change a structural defect in collagen to a quantitative defect in normal collagen with mild phenotype. Types V, VI, and VII are not associated with type I collagen gene defects. However, for now, treatment of patients by bisphosphonate therapy can improve bone mass and improve quality of life in all types of OI.

PREVALENCE AND CLINICAL DIVERSITY

OI is an autosomal genetic disorder characterized by brittleness of bones and susceptibility to bone fractures from very mild trauma. Fractures may be rare or frequent (more than 200 prior to puberty), and bone fragility may be accompanied by reduced life span and various connective tissue abnormalities: short stature, blue sclerae, joint laxity, dentinogenesis imperfecta, easy bruising, and hearing loss. OI represents extreme variation in severity from lethal perinatal to barely detectable. These clinical features, along with radiological and genetic criteria, were used to separate four major clinical types of OI.[1] The patients affected with the mildest (type I) OI have blue sclerae, osteopenia, slight growth retardation or normal height, and premature deafness; dentinogenesis imperfecta is less common. Fractures are not commonly observed at birth, but begin with ambulation and decrease following puberty. Type II is the lethal perinatal form; infants exhibit multiple intrauterine fractures of the ribs and long bones. They have a relatively large and soft cranium, short limbs, and a narrow thoracic cavity; death usually results from pulmonary insufficiency. Type III is the progressively deforming form with multiple fractures, short stature, and severe dentinogenesis imperfecta. Children with type III may die of respiratory problems in infancy; surviving patients require multiple orthopedic rodding procedures and wheelchairs for mobility. Type IV OI is characterized by phenotypical variability, from severe to mild bone deformity, normal sclerae, variable short stature, and possible brittle teeth. The Sillence classification of OI[1] is being expanded to include a greater range of subgroups of patients. Patients with type V, VI, and VII OI exhibit moderate to severe bone fragility. Type V is characterized by three distinctive features: the presence of hypertrophic callus formation at fracture sites, calcification of interosseous membranes between the bones of the forearm, and the presence of a radio-opaque metaphyseal band immediately adjacent to the growth plates upon X-ray.[2] The distinctive features of type VI OI are the fishscale-like appearance of the bone lamellae and the presence of excessive osteoids.[3] The characteristic feature of type VII is a rhizomelic shortening of the humerus and femur.[4]

In its various forms, OI probably affects about 1 in 5000 to 1 in 10,000 individuals of all ethnic origins.

MOLECULAR GENETICS

Types I–IV OI are associated with mutations in the *COL1A1* and *COL1A2* genes that encode the two $\alpha 1$(I)

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and one α2(I) chains, respectively, of the type I collagen trimer (Database of Human Type I and Type III Collagen Mutations: http://www.le.ac.uk/genetics/collagen). Type I collagen is the major structural protein of bones, skin, ligaments, tendons, and most other connective tissues. It is synthesized as a soluble procollagen, containing globular propeptides at the ends of each chain. Cleavage of propeptides generates the collagen that self-assembles into fibrils, which are the major source of mechanical strength of connective tissues and the template for matrix deposition and mineralization in the bones. The chains comprising the triple-helical domain of type I collagen are constructed from repeating Gly–X–Y triplets, in which X and Y can be any amino or imino acid (except for tryptophan or cysteine). Glycine, with its small side chain, is essential at every third position for triple-helix formation. The most common mutations (about 85%) are single-base substitutions in the triple-helical domain that change a codon for a glycine to a codon for another amino acid with a charged, polar, or bulky side chain. Replacement of glycine by serine, cysteine, alanine, valine, aspartic acid, glutamic acid, arginine, and tryptophan has been identified, with serine being the most common and tryptophan being exceedingly rare. Another substantial group of mutations (about 12%) consists of single-exon splicing defects, and the remaining mutations include nonsense substitutions, frameshift or in-frame deletions, and insertions. The nonsense substitutions and frameshift mutations have not been detected in the *COL1A2* gene. The genetic defect underlying types V and VI OI remains to be elucidated, as it does not appear to be associated with collagen type I mutations. In the case of type VII OI, the existence of extended affected families has enabled the chromosome location of the gene defect to be mapped to chromosome 3p22–24.1.[5]

GENOTYPE–PHENOTYPE CORRELATION

Although more than 250 different mutations have been characterized in OI, a mechanism by which individual mutations cause various degrees of skeletal abnormalities is not well understood. Many investigators have attempted to correlate the type of mutation, the gene in which the mutation occurred, and the location of the mutation with phenotypical consequences. Mutations in the *COL1A1* gene resulting in one null allele and in the synthesis of half the normal amount of functional proα1(I) chains have mild clinical consequences in individuals with type I OI. The phenotypical effects of mutations that result in the generation of abnormal type I procollagen molecules are more deleterious than those of null mutations. The structural mutations affect connective tissues through a dominant negative mechanism, in which the presence of the mutant chain in the extracellular matrix directly disorganizes and weakens the matrix. Therefore the extent of mutant chain incorporation into extracellular fibrils can play a role in the clinical outcome. In general, bulky residues are more deleterious than small residues, but the existence of lethal substitutions by serine and alanine indicates that the region in which the substitution occurred is also important. Mutations in *COL1A2* can alter up to 50% of the procollagen molecules, whereas similar mutations in *COL1A1* can alter up to 75% of the newly synthesized molecules. It has also been proposed that the position of a mutation along the α1(I) chains may be a crucial factor, with mutations closer to the carboxyl end resulting in a more severe phenotype than mutations near the amino terminal end of the chain.[6] However, there are many exceptions to these rules. Because the collagen triple helix is not a uniform structure and exhibits regional differences in its stability and ability to accommodate mutations, a regional model has been postulated for the relationship of genotype to phenotype.[7] Examination of the lethal clusters regularly occurring along the α2 chain revealed a striking alignment with the binding regions of proteoglycans. The genetic background and modifier genes as well as environmental or epigenetic factors may affect the expression of phenotype.

DIAGNOSIS

The diagnosis of OI is usually made based on clinical findings alone. The presence of fractures, together with short stature, bone deformity, blue sclerae, dentinogenesis imperfecta, or a family history of OI, is usually sufficient for diagnosis. However, it is important to rule out diseases and other reasons that can cause fractures of bones in children. Most patients with types I–IV OI have private mutation; therefore extensive analysis of the *COL1A1* (18,000 bp) and *COL1A2* (38,000 bp) genes must be undertaken to define the mutations. Most investigators have developed a two-step experimental strategy to detect mutations. In the first step, an attempt is made to localize a region in collagen gene that may contain a mutation by procedures such as analysis of the type I collagen synthesized by cultured skin fibroblasts and cyanogen bromide peptide mapping. After the region containing the mutation is identified, the mutation is defined by sequencing cDNA or genomic DNA from the region. However, only some mutations cause overmodification of collagen chains, resulting in delayed electrophoretic mobility that can be detected by analysis of the protein, and no current procedure for scanning mRNA or DNA for mutations has been shown to detect all single

base substitutions. An automated procedure of cDNA sequencing for the procollagen chains with sequencing primer information was described.[8] In the diagnosis of type I OI with no detectable biochemical abnormality of type I collagen, a 4-bp insertion polymorphism in the 3′ untranslated region of the *COL1A1* gene is highly informative for null allele testing and can be used in prenatal diagnosis.[9]

GENETIC COUNSELING

In general, OI is an autosomal dominant condition, resulting either from inheritance of a mutant gene from an affected parent, or from a new dominant mutation; autosomal recessive cases of OI are very rare (type VII). Some children suffer from OI as a result of mosaicism in a parent who shows little or no clinical abnormality but carries the mutation in a small number of gonadal and somatic cells. The risk of recurrence of OI in families with genetic mosaicism is approximately 7% with each pregnancy.

PRENATAL DIAGNOSIS

Prenatal diagnosis is of concern in families at risk for recurrence of OI where there is a risk for the birth of an affected child because of parental germline mosaicism, or in a family affected with an autosomal dominant form of OI. Four techniques are used at present for prenatal diagnosis. High-resolution ultrasound scan of fetal limb length and morphology can reliably identify fetuses with type II OI by 14–16 weeks' gestation, and some fetuses with the progressive deforming variety of OI (type III) by 18–20 weeks' gestation. In the absence of fracture or significant bowing, ultrasound cannot be used to identify fetuses with the milder forms of OI, even in families with a positive history of OI. If mutant type I collagen has been identified in cultured fibroblasts from an affected parent or child, then mesenchymal cells cultured from chorionic vilus biopsies taken at about 10–11 weeks' gestation, which synthesize collagen comparable to dermal fibroblasts, can be used for diagnostic studies.[10] Because OI represents a private mutation in most families, mutational analysis of DNA from chorionic vilus or amniotic fluid cells can be used only in pregnancies where the molecular defect has been previously identified. In large families, linkage studies may identify the gene and the allele, which contain the mutant sequence, thus permitting prenatal diagnosis by haplotype analysis of fetal DNA and can be used in pregnancies at risk for all types of OI.

CLINICAL MANAGEMENT

The management of OI focuses on minimizing fractures and maximizing function in all aspects of the child's life. Until recently, the correction of deformities, intramedullary rodding of long bones, orthotic support, muscle strengthening, and mobility devices, such a wheelchairs, were the mainstays of treatment.[11] The recombinant human growth hormone has been used to augment growth and bone mass. The greatest potential currently resides in bisphosphonate therapy. Bisphosphonates as potent inhibitors of bone resorption have been shown to be highly effective in improving bone mass in children with severe forms of OI.[12] As a consequence, the fracture rate was reduced significantly and quality of life increased in all patients. However, in long-term therapy, bone turnover is suppressed to levels lower than those in healthy children, and the consequences of chronically low bone turnover in children with OI are unknown.

THERAPEUTIC APPROACHES

The finding that marrow stromal cells (MSCs) infused into irradiated transgenic mice with OI were engrafted in bones and contributed to the formation of osteoblasts led to a trial of bone marrow transplantation in children with type III OI.[13] The trabecular bone histological examination showed changes indicative of new dense bone formation; increase in bone mineral content and reduction in the frequency of fractures were also reported. The goal of gene therapy of dominant negative disorders such as OI must be to shut off the expression of the mutant allele, creating a functionally null allele. Selective suppression of the expression of the mutant allele in connective tissues could modulate the clinically severe forms of OI into the biochemical equivalent of mild type I OI. The specificity of mutant allele suppression observed in the experiments with the use of antisense oligonucleotides appears to be insufficient for therapeutic trials. The use of ribozymes is an alternative and particularly attractive approach of antisense therapy.[14] The specificity of their action is greatly improved because they can cut only RNA molecules containing the specific cleavage site. For OI, about 25% of the causative type I collagen point mutations generates a novel ribozyme cleavage site. Information on the required level of suppression can be obtained from studies of mutant type I collagen expression levels in bones of clinically mild mosaics. The use of MSCs as vehicles to deliver therapeutic genes to bones is being evaluated in laboratory and animal studies. In this approach, bone marrow is harvested and, ex vivo, mesenchymal stromal cells are isolated, expanded in numbers,

genetically modified, and then reinfused into the donor.[15] The next step in the development of somatic gene therapy is the use of recently created mice with a point mutation in the *COL1A1* gene, which truly mimics the most common forms of OI.[16]

CONCLUSION

The underlying defects of clinical heterogeneity in individuals with OI as well as the relationship between genotype and phenotype are still not completely understood. Current medical treatment with bisphosphonates aims to improve bone mass and is independent of both phenotype and genotype. However, bisphosphonates have a prolonged half-life in bones, and their safety and efficacy have not been established in controlled trials in children. The heterogeneity of mutations in type I collagen genes poses many problems in planning new strategies for OI treatment. Specific treatment will need to be directed to each affected individual's unique mutation. The continued creation of true animal models of OI and the evaluation of each intervention are essential in the identification of an effective and safe therapy.

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p53 Status of Tumors: Diagnostic, Prognostic, and Therapeutic Exploitation

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Apollonia Tullo
Istituto di Tecnologie Biomediche Sezione di Bari CNR, Bari, Italy

Anna Maria D'Erchia
Dipartimento di Biochimica e Biologia Moleculare, Università di Bari, Bari, Italy

Elisabetta Sbisà
Istituto di Tecnologie Biomediche Sezione di Bari CNR, Bari, Italy

INTRODUCTION

Over the past several years, knowledge of molecular mechanisms regulating normal and aberrant cell growth leading to tumor formation has been enhanced. Although human cancers are biologically and pathologically quite distinct, it is surprising that perturbation of the p53 pathway occurs in most, if not all, types of human cancers, highlighting the central role of p53 in tumor development. p53 inactivation determines a condition of genetic instability, justifying the subsequent susceptibility to acquire mutations of different other genes. Alterations to *p53* gene appear to have little or no prognostic value for patients treated by surgery alone, but are associated with worse survival for patients treated with chemotherapy. Studies have begun to identify markers predicting whether a tumor will respond to a particular chemotherapy. The ultimate goal is to prospectively identify patients who should receive chemotherapy and to tailor the treatment to the molecular profile of the tumor and the patient. Such an approach has the potential to dramatically improve response rates. The role of new biomarkers, such as p53, seems to be very promising in predicting prognosis and sensitivity to chemotherapy, thus representing the first step toward a better definition of therapeutic strategies according to the molecular characterization of individual patients. Treatments that depend on the p53 pathway require the scanning of the entire *p53* gene and the identification of any type of mutation. Several diagnostic strategies have been developed to identify mutations in the *p53* gene; among these, automated direct sequence analysis remains the most precise method for high-throughput screening of a wide range of mutations. The improvement of the emerging chip technology will represent the most advanced alternative. Novel strategies are emerging for the treatment of tumors that have *p53* mutations. There are several approaches leading to the restoration of the normal p53 function: the most direct one involves gene therapy used to transfer the wild-type *p53* gene to cancer cells that express mutant *p53*; other ambitious strategies aim to convert mutant p53 protein into its wild-type form by using small peptides, or by introducing drugs that disrupt the interaction between viral and cellular proteins and p53; and another angle of attack is provided by the stimulation of the host's immune response to mutant *p53*.

We report an overview of the *p53* status in tumors, major methods for screening *p53* mutations, and therapeutic approaches suggested for restoring p53 function.

P53 ONCOSUPPRESSOR GENE

The first role of p53 to be discovered was in tumor generation. *p53* gene has the features of a recessive oncosuppressor in its wild-type form, and it can be a dominant oncogene in its mutated form. Mutations in the *p53* oncosuppressor gene occur in most of human cancers, and regulation of the protein is defective in a variety of others (for review see Ref. [1]).

Recently, two proteins, p73 and p63, have been identified as members of the *p53* gene family. Unlike *p53*, both *p73* and *p63* are rarely mutated in human cancers, and their involvement in tumor generation is still to be clarified.[2]

The *p53* gene (20 kbp) is located in a single copy on the short arm of chromosome 17 and contains 11 exons interrupted by 10 introns. It codes for a protein of 393 amino acids consisting of at least four functional and regulatory domains: the N-terminus contains the transactivation domain; the central domain is responsible for sequence-specific DNA binding; the oligomerization domain ensures assembly of p53 into conformationally active tetramers; and the C-terminus contains a negative regulatory domain whose posttranslational modification may play an important role in modulating the specific activity of p53.

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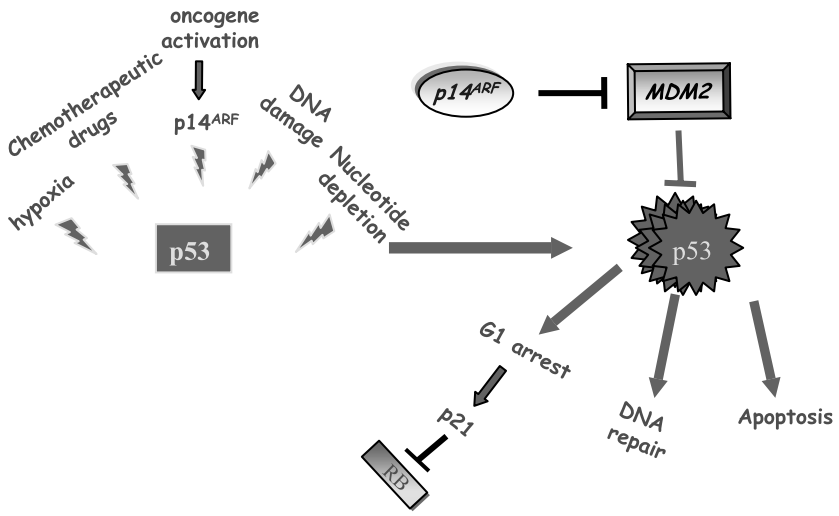

Fig. 1 The p53 pathway. p53 is activated by different stresses and induces the expression of several genes involved in cell growth inhibition, DNA repair, and apoptosis. p14ARF inactivates MDM2 and stabilizes p53. (*View this art in color at www.dekker.com.*)

The biochemical activity involves its ability to bind DNA in a sequence-specific manner and to act as a transcription factor. The p53 protein is extensively posttranslationally modified by phosphorylation and acetylation, which affect its sequence-specific DNA binding and transcriptional activities. In normally growing cells, p53 protein levels are very low. In response to different cellular stresses, such as DNA damage, oxidative stress, radiations, hypoxia, and so on, p53 is stabilized by covalent and noncovalent modifications, succeeding specific protein–protein interactions that, in turn, rapidly increase p53 protein levels and induce the expression of genes involved in cell cycle arrest to allow repair processes, or, failing that, to induce the expression of genes promoting apoptosis (Fig. 1).[3] Genotoxic damage, also caused by chemotherapy or radiotherapy, induces p53 overexpression to control the rate of proliferating damaged cells, thus triggering the mismatch repair or apoptotic pathway.

The activity of p53 is regulated primarily through control of protein stability. One of the principal regulators of p53 stability is MDM2, which not only binds the transactivation domain of p53, inhibiting its ability to function as a transcription factor, but also targets p53 for degradation. The factor, which can inactivate MDM2 and stabilize p53, is the $p14^{ARF}$ tumor-suppressor gene product that binds directly to MDM2, thereby inhibiting p53 proteolysis (Fig. 1).

p53 PATHWAY IN SPORADIC TUMORS

The occurrence of *p53* mutations ranges from 20% to 60% in sporadic solid tumors (Table 1). *p53* mutations may also be inherited in families with a predisposition to multiple tumors as in the Li–Fraumeni syndrome. Different tumors show different mutation patterns according to mutation type and location. Furthermore, some mutations are typical of certain geographical areas (Asian countries) and depend on exposure to environmental mutagens (e.g., aflatoxin B1). Moreover, single nucleotide polymorphisms (SNPs) have been proposed to play a role in tumor genesis (e.g., Arg72Pro).

The International Association of Cancer Registries (IARC database) report 18,585 somatic and 225 germline

Table 1 *p53* mutations in sporadic tumors

Tumor	*p53* mutation (%)
Esophagus	49
Ovary	48
Colorectum	44
Head and neck	41
Pancreas	39
Lung	39
Skin	36
Bladder	33
Stomach	31
Brain	27
Liver	27
Breast	25
Uterus	23
Soft tissues	16
Bones	15
Hematological	15
Prostate	14
Kidney	14
Testis	12
Thyroid	12
Cervix/uterus	6

Source: http://www.iarc.fr/p53/Index.html.

mutations in the *p53* gene with over 1700 different mutations (http://www.iarc.fr/p53/Index.html).[4] The most frequent are missense mutations, which are scattered throughout the 11 exons as well as at splice junctions. Although most mutations are in exons 5–8, which are involved directly in DNA binding, over 13% of mutations occurs outside. Moreover, inactivation of p53 may occur without a change in its sequence. Mutations in the genes involved in the p53 pathway may affect p53 function without affecting p53 structure or protein expression. Both viral and cellular proteins bind p53 and modulate its activity.

MDM2 and $p14^{ARF}$ are the principal cellular proteins that interact with p53, determining its stability. It was found that MDM2 is overexpressed in different tumor types such as sarcomas, breast, brain, bladder, lung cancers, and leukemia. In these tumors, MDM2 overexpression and *p53* mutations are mutually exclusive, suggesting that either mechanism may inactivate p53.

$p14^{ARF}$ is frequently deleted in cancers with a wild-type *p53* gene, providing strong evidence for a role played by this protein as an upstream regulator of p53 function in some tumor types. In addition, mutations in different enzymes, such as kinases and acetylases, which activate p53, could also disrupt p53 function. Mutations in downstream p53 effectors involved in cell cycle arrest and apoptosis could lead to a p53 ''null'' phenotype even if the *p53* sequence is wild type.

It has been found that the toxicity and efficacy of many of the current treatments are also profoundly affected by the activity of the p53 pathway. Most striking was the observation, using the same colorectal cancer cell type, that 5-fluorouracil-induced apoptosis requires wild-type p53, but not its principal target gene $p21^{WAF1}$, whereas adriamycin-dependent apoptosis could be inhibited by the $p53–p21^{WAF1}$ axis.[5] In ovarian tumor, *p53* status is a strong predictor of response to platinum-based chemotherapy. Patients whose tumors have p53 mutations show fewer chances to achieve a complete response following platinum-based regimens compared with patients without *p53* mutations. Conversely, experimental and clinical data seem to show that paclitaxel enhances apoptosis through a p53-independent pathway. Such observations highlight the usefulness of *p53* gene profiling as a form of cancer diagnosis and genotyping in defining the best therapeutic treatment for each patient.[6]

Treatments depending on the p53 pathway require the scanning of the entire *p53* gene and the identification of any type of mutation, not only in the *p53* gene.

p53 MOLECULAR DIAGNOSIS

Most studies of p53 have defined mutations by using immunohistochemistry (IHC), even if the use of IHC as an indirect measure of *p53* status gives ambiguous results.

Later, several molecular methods are developed for rapid prescreening of *p53* mutations, which do not give any information on the nature of the mutation. They can be grouped as either conformation-based techniques or base mismatch recognition techniques (for review, see Ref. [1]).

The conformation-based procedures for mutation detection are single-strand conformation polymorphism (SSCP), heteroduplex analysis (HA), denaturing gradient gel electrophoresis (DGGE), and modifications of DGGE: temperature gradient gel electrophoresis (TGGE) and constant gradient gel electrophoresis (CDGE). These techniques utilize the property that DNA fragments containing a sequence alteration have altered mobility under certain conditions of gel electrophoresis compared with a control.

Base mismatch recognition methods exploit the ability of chemicals or proteins (e.g., endonuclease V, RNase, or MutS and MutY mismatch repair proteins) to recognize mismatched bases in DNA heteroduplexes. The single-strand specificity of RNase has similarly been utilized to digest RNA:DNA or RNA:RNA (nonisotopic RNase cleavage assay or NIRCA) heteroduplex.

In recent years, the previously described technologies have been significantly simplified and automated to make them less time-consuming, and they have been coupled together, producing a variety of methodological approaches for *p53* mutation detection. The possibility of binding heteroduplex and oligonucleotides arrays to solid and semisolid supports has led to automation. Moreover, the possibility to detect DNA fragments of different sizes by capillary electrophoresis (CE) instead of labor-intensive gel electrophoresis, miniaturization (chip technology), as well as the use of fluorophores instead of dangerous reagents have all drastically reduced the drawbacks of the above methods and have improved their sensitivity (100%) and throughput.

Moreover, the increased efficiency of DNA sequencing analysis is an important goal in modern biology. Direct sequence analysis is the most precise method for determining *p53* mutation status (100% specificity). Automated sequencing has streamlined sequence data production and acquisition.

Another recent approach, pyrosequencing, has shown accurate results for *p53* mutation detection. Pyrosequencing is a nonelectrophoretic real-time DNA sequencing method that uses luciferase–luciferin light release as the detection signal for nucleotide incorporation into a target DNA.

Advances in genomics, proteomics, bioinformatics, and nanotechnology have recently increased exponentially. The integration of these scientific fields with multiplexed assays allows the development of devices that undoubtedly enable higher throughput both in screening of mutations and sequencing determination: the chip technology.

The high-density oligonucleotide arrays (DNA chip), attached to a solid glass or nylon matrix, allow the rapid scanning of any gene to determine the zygosity (i.e., discrimination between homozygotes and heterozygotes) for all possible allelic variations in patient samples (mutations and polymorphisms) and expression studies. Dedicated instrumentation and software allow fluorescence detection, data acquisition, and analysis of hybridization patterns.

THERAPEUTIC APPROACHES RELATED TO p53 STATUS

As testified by the extensive bibliography, *p53* is used as a tool for better diagnosis and prognosis, and also for development of new cancer therapies (Ref. [1]; for review, see Ref. [7]). There are several approaches for restoring normal p53 function, depending on the status of the gene. If the *p53* gene is mutated, the most direct approach is gene therapy, in which normal *p53* gene is introduced back into tumor cells using mostly replication-defective adenoviruses that deliver a human p53 cDNA sequence driven by strong viral promoters. One significant limitation of this gene transfer approach is the inability to properly regulate gene expression after gene transfer. A second potential complication is that most *p53* mutations found in human cancers are not null mutations, but rather encode mutant versions of the p53 protein that may have unwanted activities such as a gain of function. However, several studies are exploring new vectors that have modified tissue and cellular tropism.

Another example of targeting cancer cells containing a high level of mutant p53 protein or without p53 uses an adenovirus hybrid, called ONYX-015, engineered to kill cells with mutant p53, but not wild-type p53. Briefly, the human adenoviruses infect quiescent cells and induce them into the S-phase of the cell cycle so that viral DNA replication can proceed. The E1A protein of human adenoviruses, which binds pRB (retinoblastoma oncosuppressor gene) and other related proteins, is largely responsible for this entry into the S-phase. The E1B adenovirus gene encodes a 55-kDa protein that binds and inactivates the cellular p53 protein. In the ONYX-015 mutant adenovirus, the E1B gene product has been inactivated by mutation so that the virus cannot replicate in normal cells with a functional p53 protein. It has been reported that injection of this mutated virus into human p53-deficient solid tumors caused a significant regression of the tumor.

An alternative tumor targeting strategy exploits the loss of wild-type p53 function with regard to its dual role in the transcriptional regulation of gene expression. In fact, p53 can positively regulate the expression of target genes involved in cell growth inhibition or induction of apoptosis, but it is also able to suppress the transcription of other genes having a canonical promoter. Compelling evidence has indicated that such repressing activity is an important component of the tumor-suppressor function of p53. This gene therapy maximizes the expression of a potential therapeutic gene in tumors while simultaneously downregulates the same gene in normal cells. This system should be able to incorporate a number of different therapeutic genes, including prodrug-activating genes and immunomodulators. This approach makes use of two constructs. In the first construct, the potential therapeutic gene is placed under the control of the human heat shock protein 70 (Hsp70) gene promoter, which is upregulated in tumor cells in a more advanced stage and with poor prognosis. In addition, this promoter is activated by several p53 mutants and is repressed by wild-type p53. In this way, the therapeutic gene is selectively expressed only in tumoral cells with a mutated *p53*. In normal cells, residual expression of the therapeutic gene is repressed by using the second construct that drives a transcriptional repressor, or an antisense of the therapeutic gene under the control of a promoter activated by wild-type p53.

Other ambitious strategies aim to convert mutant p53 protein into its wild-type form by using small peptides, or by introducing drugs disrupting the interaction between MDM2 protein and p53.

The study of the allosteric and structural classes of mutant p53 protein has permitted researches to obtain the design of small peptides, which promote the stability not only of the wild-type p53, but also of mutant p53 to maintain an active conformation. Different classes of mutants require different rescue strategies, and before using activating drugs, it is crucial to define the type of *p53* mutation.

Another attractive area of therapeutic development is the discovery of molecules that mimic the function of gene products whose synthesis is induced by p53. In particular, attention of researchers is focused on two principal genes: the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$ and the proapototic gene *bax*.

If *p53* is wild type in a tumor and p53 pathway is ablated, therapeutic strategies are addressed to the activation of endogenous *p53* gene. Many therapeutic strategies have focused on MDM2, the principal negative regulator of the p53 pathway. By making use of MDM2 antisense, it is possible to inhibit MDM2 expression and to activate the p53 apoptotic response in tumor. Moreover, natural peptides have been found to bind MDM2 20 times more efficiently than p53, thus preventing the interaction between MDM2 and p53. In addition, several general inhibitors of transcription and the CDK inhibitor, roscovitine, are potent activators of p53-dependent transcription when delivered at moderate doses by selectively decreasing the expression of MDM2. Recently, it has been found that another small molecule, leptomycin

B, which inhibits the CMR1 nuclear exportin, is a potent activator of the p53 response. It can kill neuroblastoma cells in a p53-dependent manner while inducing only a reversible growth arrest in nontransformed cells.

CONCLUSION

Because p53 alterations are so frequent in human tumors, the detection of *p53* mutations and the analysis of the p53 pathway should be a useful diagnostic tool for rational drug or therapy development. Indeed, cancers presenting mutations of *p53* tend to be more aggressive in resisting chemotherapy and are less respondent to most protocols than cancers of similar tissues expressing wild-type *p53*. The repair of p53 pathways in cancer cells holds tremendous promise for the development of new, safer, more selective and effective cancer treatments.

The ultimate goal of identifying prognostic indicators is that they might eventually guide oncologists in the design of appropriate treatment regimens. The principal therapeutic strategies described here will have merit in the clinic and some of them have already been found to have successful therapeutic effects. Future progress will require a precise diagnosis of the p53 pathway status of individual tumors.

ACKNOWLEDGMENTS

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Padlock Probes

David Y. Zhang
Josephine Wu
Mount Sinai School of Medicine, New York, New York, U.S.A.

INTRODUCTION

The padlock probe or circularizable oligonucleotide probe (C-probe) is a unique molecule that offers significant advantages over conventional oligonucleotide probes. A closed circular structure forms through ligation of its ends after hybridization onto a target, and locks onto its target due to helical turns formed between complementary sequences of the target and the padlock probe. The nature of padlock and stringent requirement for ligation make this probe especially useful for in situ hybridization and detection of single-nucleotide polymorphisms (SNPs).

TECHNICAL DESCRIPTION

Oligonucleotide probes provide a useful method for the detection of target nucleic acids by the formation of a double-helical structure between complementary sequences based on stringent requirements of Watson–Crick base pairing, making hybridization extremely specific. First described by Kool in 1991,[1] the padlock probe or C-probe is a uniquely designed oligonucleotide probe containing three regions: two target complementary sequences located at the 5′ and 3′ termini and an interposed generic linker region.[2,3] Once the padlock probe hybridizes to a target, its 5′ and 3′ ends are juxtaposed (Fig. 1A) and a closed circular molecule is then generated by incubating the padlock probe–target complex with a DNA ligase. The resulting closed circular molecule is locked on its target due to the helical turns formed between the complementary sequences of the target and the padlock probe.[2] The permanently bound padlock probe allows for stringent washing to remove unbound components, thereby enhancing assay signal-to-noise ratios. Therefore, the advantage of the padlock probe is that it offers greater specificity and less background when compared to current conventional probes. Furthermore, once the padlock probe hybridizes to a specific target sequence, it can then serve as a template for linear (rolling circle amplification, RCA) or exponential (ramification amplification method, RAM) amplification and significantly increases the specificity and sensitivity of signal detection.

Recently, two interesting methods were presented for catenation of padlock probes to double-stranded DNA (dsDNA) sequences. Escudé et al.[4] demonstrated the formation of a triplex complex between a dsDNA and a circularizable probe through the interaction between polypurine–polypyrimidine sequences in the dsDNA target and padlock probe. The advantage of the triplex padlock probe is that it allows for binding of the padlock probe to dsDNA without predenaturation. Kuhn and colleagues designed two peptide nucleic acid (PNA) probes that are complementary to the opposite strand of padlock probe binding site.[5] These PNA ''openers'' can invade the dsDNA and may facilitate binding of the padlock probe to target DNA. Furthermore, they prevent the padlock probe from shifting laterally, resulting in more specific localization of the signal intracellularly.

The padlock probe ranges from 70 to 125 nucleotides long with two target complementary regions, each ranging from 15 to 30 nucleotides, and a linker region ranging from 30 to 75 nucleotides.[2,3,6,7] The padlock probe can be readily synthesized with most DNA synthesizers. The 5′ end of the probe must be phosphorylated and the 3′ end must contain a hydroxyl group; both are required by DNA ligase. Addition of a phosphate group to the 5′ end is usually achieved by incubation with a polynucleotide kinase in the presence of ATP or during chemical synthesis.[8]

Successful ligation of the two ends depends on two factors: both ends must bind adjacent to each other and no mismatch should exist at the ends, especially the first 1–3 nucleotides at its 5′ and 3′ ends (Fig. 1B).[2,8] A variety of ligases can be used for this reaction, such as T4 DNA ligase, Taq ligase, Ampligase, and T4 RNA ligase.[9] However, ligation efficiency can reach as high as 90% with T4 DNA ligase.[5,8] Furthermore, Nilsson et al.[2] demonstrated the ligation specificity of T4 DNA ligase depended on increasing the NaCl concentration. Whereas NaCl concentrations between 50 and 150 mM result in optimal ligation activity, at 250 mM NaCl T4 DNA ligase was shown to correctly distinguish a C/G match from notoriously difficult T/G mismatches, resulting in a greater than 1000-fold difference in ligation rates. Although T4 DNA ligase is efficient for ligation of DNA probe onto a DNA target, ligation of a DNA probe

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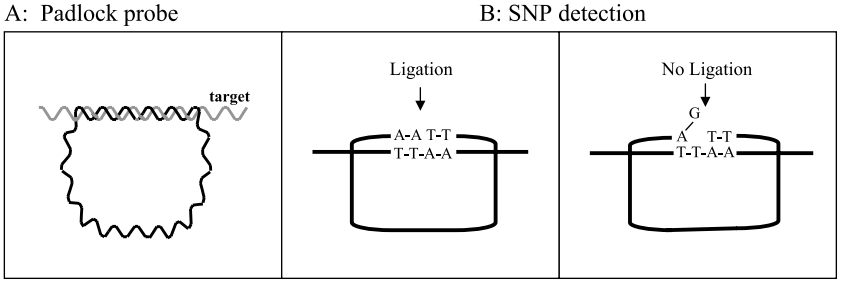

Fig. 1 Schematic representation of the padlock probe. (A) A padlock probe hybridizes to its target through its complementary regions and helical turns to form a closed circular probe. This results in the interlocking of the padlock probe onto the target. (B) Only when the ends of the padlock probe perfectly match with its target can ligation occur, thus allowing detection of a single-nucleotide polymorphism.

onto an RNA target is very poor.[8] However, the ligation efficiency can reach 80% by addition of $MnCl_2$, low NaCl concentration, low ATP concentration, and high concentration of T4 DNA ligase.[8,10] The stringent requirement of ligation allows detection of single nucleotide differences between target DNA and probe (Fig. 1B).

Application

As a result of its high specificity, the padlock probe has found clinical and research applications in several genetic assays, including in situ hybridization and SNP detection. The padlock probe was initially designed to analyze chromosomes in a cell undergoing interphase or metaphase.[2] Because of its ability to lock onto target DNA, a glass slide can be washed under stringent conditions (above the melting temperature), therefore lowering background signal and improving detection sensitivity. With the aid of fluorescent reporter molecules, such as biotin or digoxigenin, the bound padlock probes attached to interphase or metaphase chromosomes can be visualized under a microscope.[2] Two fluorescent-labeled padlock probes have been used to detect human centromeric alpha satellite DNA of chromosome 13 and 21, which contains multiple repeats.[11] Two chromosomes were individually visualized under a microscope. Furthermore, the padlock probe can detect a single nucleotide difference in two satellite sequences.[11] Although the padlock probe alone is adequate to detect a repeat motif, it is insufficient to detect a single-copy gene sequence in situ because of low signal intensity.

Single-nucleotide polymorphisms of genomic DNA are considered to be the most common form of genetic variations, occurring in approximately every 250–2000 bases. With the availability of large SNP databases, detection of specific sets of SNPs will allow for identification of disease-associated polymorphisms to correlate therapies with clinical outcomes and drug effect as well as parentage and forensic testing. The padlock probe offers a significant advantage to detect SNPs. For ligation to occur, both ends must perfectly hybridize to the target at juxtaposition (Fig. 1B). The discrimination of SNPs by ligation was approximately 1:100 to 1:1000 in a mixture of mutant alleles in an excess of wild-type alleles.[12] For RNA targets, the discrimination by ligation ranged from 20- to 200-fold.[10]

CONCLUSION

The padlock probe represents the next generation of probe-based hybridization with the ability of direct target detection. The padlock probe improves the binding affinity and hybridization stringency and allows more efficient SNP discrimination. With the use of different fluorescent-labeled padlock probes, multiplex padlock assays could greatly increase throughput in genotyping and permit highly sensitive and specific measurement of gene expression. The padlock probe and its amplifications (i.e., rolling circle amplification and ramification amplification) allow for broad applications in clinical testing, research laboratories, and drug discovery. Clinically, it can be used for detection and identification of infectious agents, cancer screening and diagnosis, neonatal genetic diagnosis and parentage, and forensic testing.

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Paraoxonase (PON1) Gene Polymorphisms

Lucio G. Costa
Clement E. Furlong
University of Washington, Seattle, Washington, U.S.A.

INTRODUCTION

Paraoxonase-1 (PON1) (EC 3.1.8.1 or EC 3.1.1.2) is a high-density lipoprotein (HDL)-associated serum enzyme whose primary physiological role is to protect low-density lipoproteins (LDLs) from oxidative modifications. PON1 can also hydrolyze organophosphorus (OP) compounds, including commonly used insecticides, and its name derives from one of its most commonly used in vitro substrates—paraoxon. More recently, in addition to its role in lipid metabolism and, hence, in cardiovascular disease and arteriosclerosis, PON1 has also been shown to be involved in the metabolism of lactones and cyclic carbonates. Early studies of enzymatic activity in serum indicated a bimodal or trimodal distribution in Caucasian populations. Two main polymorphisms in the coding region, as well as five in the 5′ regulating region, have been characterized. The Q192R polymorphism determines the catalytic efficiency of hydrolysis of some substrates, and certain promoter polymorphisms, in particular C-108T, contribute to the level of expression of PON1. Recently, additional polymorphisms in the coding region, 5′ regulatory region, and PON1 introns have been reported.

STRUCTURE AND POLYMORPHISMS OF PON1

The PON1 cDNA encodes a protein of 355 amino acids from which only the amino-terminal methionine residue is removed during secretion and maturation.[1] The retained leader sequence is required for the association of PON1 with HDL particles, and, indeed, PON1 is entirely associated with HDL in human serum. PON1 protein is synthesized mostly in the liver, from which it is released by a docking process whereby HDL particles transiently associate with the cell membrane and remove PON1 from the membrane. Physical mapping placed the human *PON1* gene on chromosome 7 q21–22. Two polymorphisms are present in the PON1 coding sequence: a Glu (Q)/Arg (R) substitution at position 192, and a Leu (L)/Met (M) substitution at position 55.[2] These coding region polymorphisms have been studied for effects on catalytic efficiencies of hydrolysis of specific substrates. The L/M polymorphism at position 55 has not been found to affect catalytic efficiency, but has been associated with plasma PON1 levels, with PON1 M55 individuals having, on average, lower PON1 levels.[3,4] This association appears to result primarily from linkage disequilibrium of the M55 allele with the inefficient −108T promoter polymorphism.[4] However, the Q/R polymorphism at position 192 significantly affects the catalytic efficiency of PON1 in a substrate-dependent manner.[5] Five additional 5′ regulatory region polymorphisms of the *PON1* gene have been characterized: −108TC, −126GC, −162GA, −832GA, and −109CG.[4,6] The −108TC polymorphism has the largest effect of these 5′ regulatory region polymorphisms on PON1 expression, with the −108C allele generating, on average, approximately twice the level of plasma PON1 as the −108T allele.[4] Recently, 2 additional coding region polymorphisms (I102V in Finns and R160G in Chinese), 8 new 5′ regulatory region polymorphisms, and more than 140 polymorphisms in PON1 introns have been identified. New functional coding region polymorphisms have been described and characterized.[7] An illustration of the *PON1* gene with its polymorphisms known to date is given in Fig. 1.

The gene frequencies of some of these polymorphisms have been determined. The $PON1_{Q192}$ gene frequency ranges from 0.75 for Caucasians of Northern European origin to 0.31 for some Asian populations.[8] Gene frequency for $PON1_{L55}$ ranges from 0.57 in Caucasian populations to 0.99 in an Oji–Cree population. Several studies have shown that the L_{55} and R_{192} alleles are in strong disequilibrium, with >90% of the R_{192} allele having L at position 55.[8]

THE IMPORTANCE OF DETERMINING PON1 STATUS

In recent years, the importance of determining an individuals' PON1 status has been repeatedly highlighted.[7,9–13] The term *PON1 status* includes both the $PON1_{192}$ genotype as well as PON1 activity. The concept was prompted by examination of a number of epidemiological studies, which examined only the $PON1_{192}$ and $PON1_{55}$ genotypes, without considering the large

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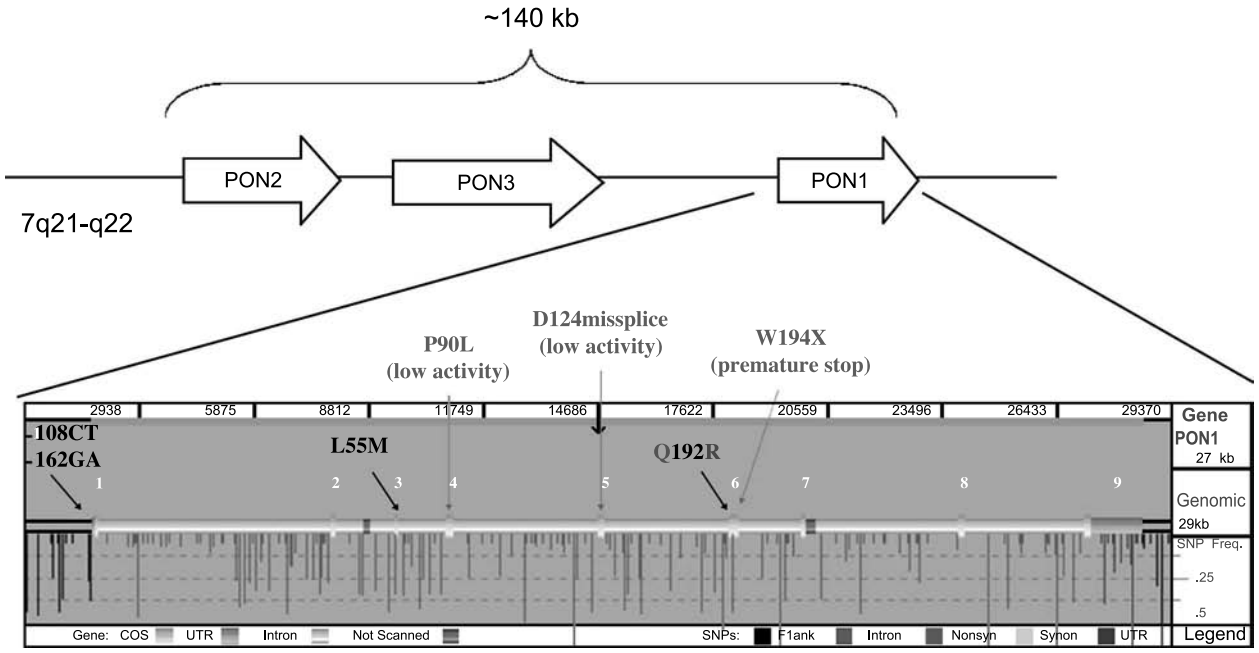

Fig. 1 The *PON1* gene is located on human chromosome 7, near the genes encoding PON2 and PON3. The expanded *PON1* gene diagram shown illustrates the locations and frequencies of single nucleotide polymorphisms (SNPs) that have been identified in the *PON1* gene, with SNP locations indicated by vertical lines below the gene structure diagram. The numbered boxes correspond to the nine exons found in *PON1*. The length of each line indicates the frequency of that SNP in the population. In addition to the previously identified SNPs that result in amino acid substitutions at L55M and Q192R, 166 new SNPs were identified by the UW/FHCRC Variation Discovery Resource (Seattle SNPs). The PON1 SNP diagram at the bottom was created at the SeattleSNPs web site (http://pga.gs.washington.edu/), using the GeneSNPs public web resource (http://www.genome.utah.edu/genesnps) available from the Utah Genome Center. (*View this art in color at www.dekker.com.*)

variability of PON1 protein levels within each genotype (Fig. 2). Indeed, PON1 levels are at least as important as genotype in determining rates of clearance of oxidized lipids, as well as determining resistance to organophosphate toxicity.[10] By plotting the rates of diazoxon hydrolysis against paraoxon hydrolysis at high salt concentration (2 M NaCl) for individuals in a population, the subjects are clearly divided into three groups: individuals homozygous for $PON1_{Q192}$, heterozygotes ($PON1_{Q/R192}$), and individuals homozygous for $PON1_{R192}$; in addition, information of PON1 activity levels is also obtained (Fig. 2; Ref. [9]). The procedure can be easily carried out in 96-well microtiter plate readers capable of following continuous enzyme kinetics at the appropriate wavelength (for details, see Ref. [14]). For this assay, plasma from blood collected in heparin or citrate can be used, but not ethylenediaminetetraacetic acid (EDTA) plasma, because EDTA irreversibly inhibits PON1. The accuracy of this PON1 status approach to determine the $PON1_{192}$ functional genotype has been verified by direct genotyping using a polymerase chain reaction (PCR) method,[2,14] and has been found to be close to 100%. Individuals with discrepant assignments have been found to have one dysfunctional PON1 allele.[15] For example, several individuals were genotyped as 192 Q/R heterozygotes by PCR, but the functional enzyme analytic assay followed by the sequencing of their *PON1* genes demonstrated that one or the other PON1 alleles were defective.[15] Methods for genotyping other polymorphisms in the 5′ promoter region (e.g., −108TC), as well as newly discovered polymorphisms in the coding region (e.g., W194X), have also been described.[14]

ROLE OF PON1 IN ORGANOPHOSPHATE TOXICITY

Several in vitro experiments have shown that PON1 hydrolyzes a number of organophosphates—among these, paraoxon, chlorpyrifos oxon, and diazoxon; active metabolites of the insecticides parathion, chlorpyrifos, and diazinon; as well as nerve agents sarin, soman, and VX. The effects of the $PON1_{192}$ polymorphism may be substrate-dependent, as the $PON1_{Q192}$ isoform hydrolyzes diazoxon, sarin, and soman more rapidly than $PON1_{R192}$

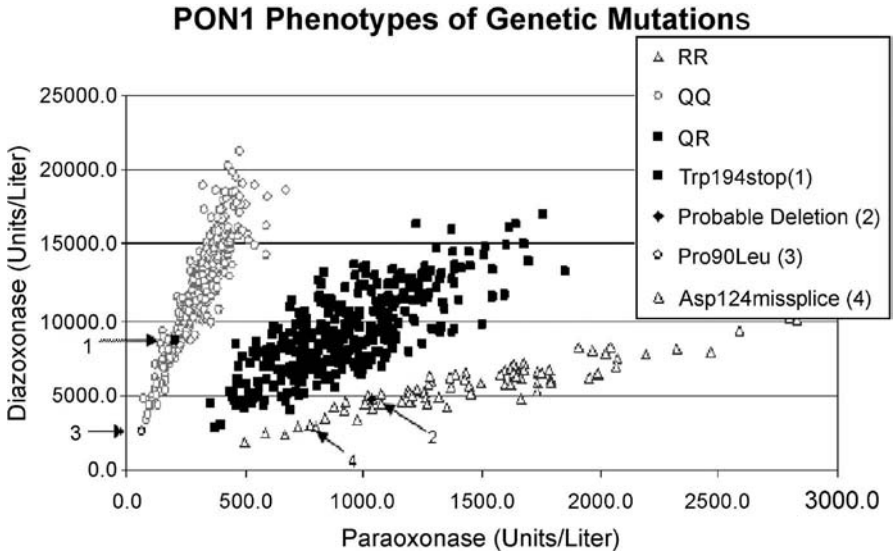

Fig. 2 Determination of PON1 Status. Plot of diazoxonase vs. paraoxonase activities in the plasma of carotid artery disease cases and controls, coded for PON1 Q192R genotype (determined by PCR). Note that the two-substrate assay provides an accurate inference of $PON1_{192}$ genotype as well as the level of plasma PON1 activities (PON1 status). Because it is a functional analysis, it provides a 100% accurate determination of the functional genomics of PON1 status. Newly discovered SNPs explain why some individuals have lower PON1 activity than would be predicted by Q192R genotype alone. Individuals 1–4 were genotyped as heterozygotes (Q/R192); however, their enzyme analysis indicated homozygosity for Q or R. Complete sequencing of their *PON1* genes revealed mutations in one allele, resulting in only one alloform of PON1 in their serum. The mutations revealed are noted in the figure. (Note the large variability in PON1 levels, even among individuals of the same Q192R genotype). (From Refs. [7], [9], and [15].)

in in vitro assays.[5] However, $PON1_{R192}$ is more efficient at hydrolyzing paraoxon and chlorpyrifos oxon than the Q isoform. The existence of polymorphisms in PON1 that confer different hydrolyzing abilities toward OPs, as well as different levels of expression, has long suggested that certain individuals may be more sensitive to OP toxicity.

Animal studies have provided evidence for the role of PON1 in modulating OP toxicity, and have clarified the role of $PON1_{192}$ polymorphism. Initial studies showed that administration of exogenous PON1 to rats or mice would confer protection against the acute toxicity of various OPs (reviewed in Refs. [11] and [16]). Further evidence was provided by studies carried out in PON knockout ($PON1^{-/-}$) mice and PON1 transgenic (TG) mice expressing either the human $PON1_{R192}$ or the $PON1_{Q192}$ allele. As predicted, $PON1^{-/-}$ mice have drastically increased sensitivity to the toxicity of chlorpyrifos oxon and diazoxon, but not to paraoxon.[17,18] Administration of purified human $PON1_{R192}$ or $PON1_{Q192}$ to $PON1^{-/-}$ mice indicated that both isoforms were equally protective toward diazoxon toxicity and equally ineffective in protecting against paraoxon toxicity, whereas $PON1_{R192}$ offered better protection than the $PON1_{Q192}$ isozyme toward chlorpyrifos oxon.[18] These in vivo findings were explained by results from kinetic analyses of substrate hydrolysis by purified human PON1 isoforms under physiological conditions. The catalytic efficiencies of both isoforms for diazoxon were similar, whereas that of $PON1_{R192}$ was higher than $PON1_{Q192}$ for chlorpyrifos oxon. In case of paraoxon, even though the $PON1_{R192}$ was more efficient than $PON1_{Q192}$, its overall catalytic efficiency was very low, indicating that PON1 does not play a significant role in detoxifying paraoxon in vivo.[18] Studies in TG mice have supported these findings by indicating that the toxicity of chlorpyrifos oxon is greatly reduced in $hPON1_{R192}$ TG mice, but not in $hPON1_{Q192}$ TG mice compared with $PON1^{-/-}$ mice.[19] Current work is exploring the role of PON1 in modulating the effects of OPs on gene expression in the brain by microarray analysis. Although these studies clearly indicate that the $PON1_{192}$ genotype and the level of PON1 expression modulate OP toxicity in animal models, direct confirmation in humans of the relevance of PON1 status in determining relative sensitivity to OP toxicity needs to be further investigated. The limited number of studies aimed at testing this hypothesis in humans has been recently summarized,[16] but more studies are needed, where better indications of the level and nature of exposure and the consequences of exposure are documented. Nevertheless, results of animal studies have also pointed out the potential therapeutic use of PON1 in treating individuals for exposure to OP insecticides or nerve agents. Engineering recombinant PON1 variants with high catalytic efficiencies toward specific compounds

may indeed prove to be a useful addition to the treatment of OP intoxication.[10]

PON1 AND CARDIOVASCULAR DISEASE

In recent years, PON1 has taken center stage in research endeavors aimed at identifying possible risk factors for cardiovascular disease.[10–12,20] The influence of PON1 and its polymorphisms on cardiovascular disease appears to be related to its ability to inhibit LDL and HDL oxidation.[21,22] Overall, in vitro studies have shown that PON1 has esterase-like, peroxidase-like, and phospholipase-like activities that inhibit the formation of proinflammatory oxidized phospholipids, or, perhaps more correctly, degrade them once they are formed.[11] Studies in TG animals have added strong evidence for a role of PON1 in the development of arteriosclerotic lesions. $PON1^{-/-}$ mice fed a high-fat diet exhibited larger aortic atherosclerotic lesions than wild-type mice.[17] On the contrary, TG mice expressing human PON1 (L55, Q192) had significantly decreased arteriosclerotic lesions.[23] In agreement with these in vivo findings, HDL isolated from $PON1^{-/-}$ and hPON1 TG mice had no ability or had an increased ability, respectively, to prevent LDL oxidation in vitro. Additional studies have shown that $PON1_{Q192}$ is twice as efficient as $PON1_{R192}$ in reducing the total lipid peroxide content of human arteriosclerotic lesion homogenates.[22]

A large number of population studies have tested the association of $PON1_{Q192R}$ and $PON1_{L55M}$ polymorphisms with coronary heart disease (CHD). Among these studies (for more details, see Refs. [10] and [11]), about half yielded significant results, where the 192R allele was consistently associated with CHD. The $PON1_{R192}$ and $PON1_{55L}$ alleles are in strong linkage disequilibrium, and the $PON1_{55L}$ genotype predicted CHD in several studies, including one in which $PON1_{192}$ genotype did not predict disease. However, the $PON1_{55L}$ polymorphism is in linkage disequilibrium with the T-108C promoter polymorphism, which is correlated with higher plasma PON1 concentrations, hence the importance, as pointed out earlier, of determining each individual's PON1 status, which encompasses both plasma PON1 levels as well as $PON1_{192}$ genotypes.[9] Indeed, PON1 phenotype has been shown to be a better predictor of vascular disease than the $PON1_{192}$ or $PON1_{55}$ genotype,[13] a finding that has been confirmed in other studies (e.g., Refs. [15] and [24]).

PON1 AND DRUG METABOLISM

Evidence exists that PON1 may be involved in the metabolism of pharmaceutical drugs, in which case the PON1 status of an individual would be relevant to a drug's effectiveness and/or side effects. The diuretic spironolactone is hydrolyzed by PON1, with similar efficiency by the Q and R isoforms.[25] However, the hypocholesterolemic drugs mevastatin, lovastatin, and simvastatin, previously reported to be hydrolyzed by PON1, are instead metabolized by PON3.[12] The antibacterial prodrug prolifloxacin is activated by PON1, with $PON1_{R192}$ displaying higher activity. However, glucocorticoid γ-lactones are inactivated by PON1. Although evidence is still limited, novel classes of so-called prodrugs may be developed by incorporating a lactone or cyclic carbonate moiety into their molecules, which can be inactivated or bioactivated in vivo by PON1.[11,12,26]

CONCLUSION

The *PON1* gene, located on chromosome 7, displays several polymorphisms in the coding and promoter regions that affect the catalytic ability of the protein as well as its level of expression. Both aspects need to be considered when investigating the role of PON1 in organophosphate toxicity, cardiovascular heart disease, and other diseases as well as drug metabolism. Additional areas of current and future research should elucidate the contribution of environmental, dietary and lifestyle factors to the modulation of PON1 expression, as well as the relationship between PON1 and *PON2* and *PON3*, two related genes located on chromosome 7.[10,12] Furthermore, nothing is yet known about the contributions of the recently described >150 polymorphisms in modulating PON1 message splicing efficiency and thereby PON1 levels.

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Parkinsonism, Autosomal Dominant

Dominic Thyagarajan
Flinders University of South Australia, Bedford Park, Australia

INTRODUCTION

In this article, I review recent findings in genetic parkinsonian syndromes, focusing on those showing autosomal dominant inheritance. The finding of genes for recessive and dominant syndromes is providing insights into the pathophysiology of common, sporadic Parkinson's disease. These findings have again stimulated renewed interest in the Lewy body, thought to be the pathological ''hallmark'' of the sporadic disease. I conclude by reviewing what the genes in autosomal dominant parkinsonian syndromes mean for a diagnostic and conceptual approach to Parkinson's disease.

GENES AND PARKINSONISM

Parkinsonism is a syndrome of rest tremor, rigidity, bradykinesia, loss of postural reflexes, and the freezing phenomenon.[1] Most often, it is a manifestation of Parkinson's disease (PD), which is characterized neuropathologically by brainstem Lewy bodies. Recent epidemiological and molecular data suggest that this is not one disorder, but is comprised of at least several disorders with genetic, environmental, or mixed etiologies. Genes play a complex role in PD. Most twin studies, which are powerful tools in dissecting genetic from environmental contributions to the disease, have discounted a genetic influence in PD. However, it is difficult to draw solid conclusions from them because of methodological shortcomings.[2] The largest of them, an unselected population-based cross-sectional twin study, has shown similar concordance for PD in monozygotic (MZ) and dizygotic (DZ) twin pairs with onset >50 years, but a striking concordance in MZ pairs with onset <50 years.[3] This is consistent with a strong genetic component in young-onset disease. This does not support a strong genetic component in late-onset disease, but a large population-based and geneological study in Iceland[4] showed that 560 patients with late-onset PD were significantly more related to each other than were subjects in matched groups of controls. The risk ratio (RR) for PD was 6.7 [95% confidence interval (CI), 4.3–9.6] for siblings, 3.2 (95% CI, 1.2–7.8) for offspring, and 2.7 (95% CI, 1.6–3.9) for nephews and nieces of patients with late-onset PD. The study claimed that shared environmental influences in late-onset PD are not the explanation because the relatedness extended beyond the nuclear family and spouses was not at increased risk. However, the different RR values for siblings and offspring are consistent with shared environmental factors in early life. [^{18}F] dopa positron emission tomography (PET) studies in MZ and DZ twin pairs discordant for PD[5] showed reduced striatal uptake not only in the affected twin but also in the clinically unaffected twin, indicating that concordance for nigral pathology in twins with PD is higher than that suggested by clinical twin studies.

Most familial aggregation studies indicate a familial component in PD, but have not adequately compared early-onset vs. late-onset disease.[6] A recent community-based study, in which 221 probands had onset <50 years and 266 had onset >50 years, found significantly greater risk in siblings (RR of 7.9; 95% CI, 2.5–25.5), but not parents (RR of 1.7; 95% CI, 0.9–3.3), of early-onset cases; however, the RR in parents and siblings of late-onset cases was similar (roughly 2–3), confirming a familial component in both early-onset and late-onset PD and further suggesting a recessive genetic influence in early-onset disease.[6]

Dominantly Inherited PD

Golbe et al.[7] described the first large kindred of autosomal dominant PD with autopsy verification in 1990 and later reported a clinical genetic analysis of 60 individuals in five generations. The kindred originated from Contursi, a hill town in the Salerno province of the southcentral region of Italy. There was severe neuronal loss and Lewy bodies in the substantia nigra, typical of PD. Mean age at onset was 46.5 years—15 years younger than that of PD in the community—and the disease had a rapid course, averaging 9.7 years from onset to death. Contrary to observations in the initial report of the pedigree, rest tremor was frequent, present in 58.1%. Most of the affected individuals responded well to levodopa and exhibited its usual motor complications. Most also developed cognitive impairment toward the end of the illness. At the time, a number of other pedigrees of dominantly inherited parkinsonism had been described, some with clinical and neuropathological features similar to sporadic Lewy body PD and some with none.[8]

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α-Synuclein Mutations (PARK1 and PARK4)

PARK1: missense mutations in the α-synuclein gene

In 1996, genetic markers on chromosome 4q21–q23 were found to be linked to the PD phenotype in the Contursi pedigree and, a year later, a mutation causing the disease in the kindred and three other unrelated Greek families was found—A53T in exon 4 of the gene encoding the presynaptic protein α-synuclein.[9] The mutation, denoted PARK1, was almost fully penetrant, but there was phenotypic variability (e.g., isolated postural tremor and dementia with Lewy bodies in the Contursi kindred). The same mutation has been subsequently identified in a handful of other families of Mediterranean origin,[10] and, in some instances, additional clinical features were described,[10] such as the presence of myoclonus, central hypoventilation, and orthostatic hypotension. A second mutation in α-synuclein, A30P, has since been found in autosomal dominant PD in a German family,[11] but large surveys in Caucasian[12] and other ethnic groups[13] have indicated that alterations in the α-synuclein gene are rare in sporadic and familial PD, confined to only few families and are possibly the result of founder mutations. However, a case–control study in PD has shown that noncoding variability in the synuclein gene is a risk factor for the development of PD.[14]

PARK4: triplication of the α-synuclein gene

Genetic and clinical heterogeneity in autosomal dominant parkinsonism was evident soon after α-synuclein gene mutations were reported. In a four-generation Iowan kindred of suspected Prussian or Irish descent, originally described in 1962 and in detail by Muenter et al.,[15] linkage to 4q21–q23 was excluded and segregation with a 4p15 haploptype was found, denoted PARK4.[16] This family is probably related to one described by Waters and Miller[8] and Muenter et al.[15] This disease has an asymmetrical, early-onset (mean 33.5±8.5 years), and rapidly progressive course with death within 4–11 years. Levodopa-responsive parkinsonism with bradykinesia, rigidity, resting tremor, stooped posture, loss of postural reflexes, and marked early weight loss are the initial manifestations. Motor complications of levodopa develop early. In the terminal stages, there is often global dementia with rigidity, stupor, and myoclonus. Autonomic features occur. Except for a somewhat later onset of dementia, most of these clinical features (i.e., early onset, rapid progression, rest tremor, levodopa responsiveness, levodopa motor complications, and dementia) are similar to those in the Contursi kindred. Within the Iowan pedigree, there is a separate phenotype consisting of mild late-onset parkinsonism and also several cases of essential tremor in whom there was segregation with the 4p haplotype.[16] Pathologically, limbic areas and pigmented nuclei are severely involved with nerve cell loss, gliosis, and widespread Lewy bodies, many of them in the cerebral cortex.[15] The changes are more extensive than in sporadic PD, and the pattern of neurochemical abnormalities is different—pronounced decrease of choline acetyl transferase in the frontal and temporal cortices and hippocampus, and a severe depletion of striatal dopamine affecting the caudate and putamen equally, rather than interregional and subregional loss maximal in the caudal putamen. In contrast, members of the Contursi kindred have a distribution of Lewy bodies and neuronal loss restricted more to the substantia nigra and nucleus basalis of Meynert. An interesting twist emerged when an unaffected member of the Iowan pedigree who did not share the 4p15 haplotype later developed the disease, prompting a second genomewide search at higher resolution, which refocused attention on the synuclein locus.[17] Reevaluation of the 4p linkage data revealed a sample swap. Resequencing of the α-synuclein gene revealed no mutations, but use of intragenic markers suggested multiple α-synuclein alleles. Quantitative real-time polymerase chain reaction (PCR) yielded gene-dosage data consistent with triplication of α-synuclein in affected individuals, confirmed by fluorescence in situ hybridization (FISH). The triplicated region is between 1.6 and 2.04 Mb in size, and carriers of the triplicated region have four functional copies of the α-synuclein gene and a doubling of the gene dosage in an estimated 17 genes. It seems likely that parkinsonism in the Iowan kindred is related to the gene dose effect of α-synuclein. Interestingly, the α-synuclein triplication segregates only with parkinsonism, not postural tremor.[17] The PARK4 classification has become confusing, and, no doubt, nomenclature will evolve as more genes are characterized.

Other evidence for genetic and clinical heterogeneity in dominant parkinsonism: PARK3, PARK5, and PARK10

Linkage to 2p13 in several families of Northern European descent[18,19] (PARK3), an I93M mutation in exon 4 of the ubiquitin carboxy-terminal hydrolase (UCH-L1) in a German family[20] (PARK5), confirmation of linkage to a locus 12p11.2-q13 (PARK10),[21,22] and a susceptibility locus on 1p32 (PARK10)[23] have indicated further genetic and clinical heterogeneity in dominant parkinsonism. The features are summarized in Table 1.

Diagnostic Considerations

Commercial testing for autosomal dominant parkinsonism is not well developed, partly because the known mutations

Table 1 Autosomal dominant parkinsonism

Condition	Gene	Mutation type	Chromosome	Penetrance	Onset and course	Population	Pathology	Clinical	Detection
PARK1	α-Synuclein	Missense A53T, A30P	4q21.3	~100%	Late 40s, rapid	Isolated Mediterranean and German families; not in sporadic or familial PD	Lewy bodies more apparent in substantia nigra and nucleus basalis of Meynert; cortical Lewy bodies in some	Postural and rest tremor, myoclonus, central hypoventilation, orthostatic hypotension, L-dopa responsiveness, L-dopa motor complications, eventual dementia in most	Sequencing RFLP analysis
PARK4	α-Synuclein	Triplication	4q	~100%	30s, rapid	Iowan kindred of suspected Irish or Prussian descent	Widespread Lewy bodies, including cortex	Similar to PARK1 Subgroups with later onset and essential tremor	Quantitative RT-PCR FISH
PARK5	UCH-L1	Missense I93M	4p14		Similar to sporadic PD	Single German pedigree; not in 500 controls; not in other AD families or sporadic PD,[24,25]	NA	Similar to sporadic PD; rest tremor with asymmetrical onset	RFLP analysis
PARK3	Unknown	NA	2p13	40%	Similar to sporadic PD	Several Northern European families	Similar to PD	Similar to sporadic PD	Linkage only
PARK8	Unknown	NA	12p11.2–q13.1	Low	Similar to sporadic PD	21 Caucasian-dominant PD pedigrees; large dominant Japanese family; two Caucasian families from Western Nebraska and Canada (German origin)	Nigral degeneration *without* Lewy bodies in Japanese family[21]	Similar to sporadic PD	Linkage only
PARK10	Unknown	NA	1p32	Low	Similar to sporadic PD	51 Icelandic families with more than one patient with PD	NA	Late-onset PD	Linkage only

RFLP, restriction fragment-length polymorphism; RT, reverse transcription.

are rare, the search for novel mutations in known genes involves more sequencing or a mutation detection assays, or the gene itself is not known. The two known point mutations in the α-synuclein gene may be conveniently detected by restriction endonuclease digestion of PCR products: the A53T mutation creates a novel *Tsp*45 I restriction site in exon 4[9] and the A30P mutation creates a new *Mva*I site in exon 3.[11] In the *UCH-L1* gene, the I53M change, found only in a single family so far, introduces a new *Bsm*F1 site.[20]

A diagnostic assay for the triplication of the α-synuclein gene is more complex and would involve FISH or quantitative real-time PCR.[17] Although large studies in various populations have shown the lack of utility of screening, even in most families with apparent dominant inheritance of PD,[12] if an α-synuclein mutation is found, the significance is great because the penetrance is practically 100%.

WHAT DO GENES IN AUTOSOMAL DOMINANT PD TELL US ABOUT PATHOGENESIS?

The cause and pathogenesis of PD are incompletely understood, but rare mutations in familial cases (Table 1) are beginning to unravel the variety of cellular processes involved in the accumulation of Lewy bodies and dopaminergic cell death. It is not possible to discuss this fully without reference to the genetic discoveries in autosomal recessive parkinsonism, which have occurred in parallel with the finding of dominant genes and are outside the scope of this article. The subject has been recently reviewed.[26,27] Derangements in protein handling seem to be central in the pathogenesis of PD (Fig. 1). α-Synuclein is a small protein characterized by imperfect repeats (KTKEGV) distributed through most its amino-terminal half of the polypeptide that also includes a hydrophobic middle region and an acidic carboxy-terminal region. Its function is not established but it is believed to modulate synaptic vesicle turnover and synaptic plasticity. In vitro, it polymerizes into ~10-nm fibrils, which are a major component of nigral Lewy bodies and Lewy neurites in PD and other forms of parkinsonism. It is unfolded in its native state but becomes structured on binding to lipid membranes. In the unfolded form, it is degraded by proteosomes in a ubiquitin-independent manner. In diseases where it aggregates, it forms a β-sheet structure. α-Synuclein promotes mitochondrial dysfunction and oxidative stress—another central aspect in the pathogenesis of PD.[28] Oxidative stress may further promote aggregation and lead to Lewy body formation. α-Synuclein knockout mice are phenotypically normal and show no abnormalities of dopaminergic function, and α-synuclein mutations are likely to be gain-of-function mutations rather than loss-of-function mutations. Although none of the transgenic animal models replicates all features of the human disease, they do show neurodegeneration associated with abnormal accumulation of detergent-insoluble α-synuclein and abnormal proteolytic processing of α-synuclein, particularly in the

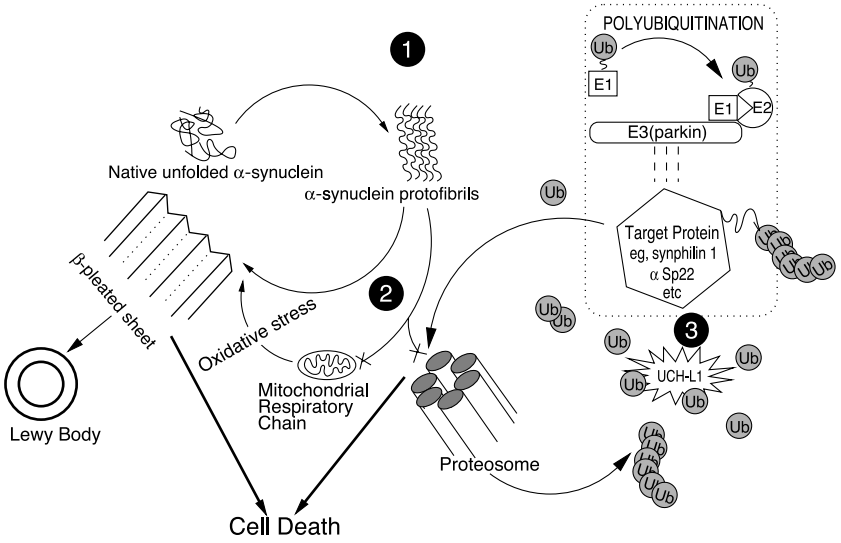

Fig. 1 A simplified hypothetical model for the pathogenesis of Parkinson's disease. (From Refs. [26] and [27].) In PARK1, mutated α-synuclein appears to aggregate more readily in transgenic models. The protofibrils appear to inhibit the proteasomal degradation of proteins (site 2). PARK4 may induce neurodegeneration by a simple gene dosage effect (site 1). Mutations in UCH-L1 may interfere with polyubiquitination of protein in the cell, or may cause aggregation of the UCH-L1 itself. The proteosomal system and its relevance for autosomal recessive forms of parkinsonism (e.g., due to parkin mutations) are outside the scope of this article.

A53T mutant. In the case of the α-synuclein triplication, simple overexpression of the protein may be responsible for accumulation and neurodegeneration. Overexpression of human wild-type α-synuclein in mice produces selective decrements in DA nerve terminals in the striatum. Oxidative ligation of dopamine to α-synuclein leads to the accumulation of the α-synuclein protofibril, which may explain the relative selectivity of neurodegeneration in the dopaminergic system even though α-synuclein is widely expressed through the brain. In PARK5, the reduced catalytic activity of UCH-L1 may affect the cleavage and turnover of the protein substrate, leading to aggregation of substrate and, as a seed, of other aggregation-prone abundant proteins. Alternatively, the I93M substitution may render UCH-L1 itself prone to aggregation.[20]

CONCLUSION

Mutations in and triplication of α-synuclein, a ubiquitous synaptic protein and major component of Lewy bodies, have been found in autosomal dominant parkinsonism closely resembling sporadic PD clinically and neuropathologically. Abnormal processing and aggregation of this protein are now center stage in the pathogenesis of PD. Other loci indicate that PD, like historical concepts of fever, is not a single disorder, but several (Table 1). The study of rare mutations in individual families has shifted attention to the causal influence of genes. Ultimately, genetic studies will provide insights into the treatment and prevention of this common neurodegeneration.

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Parvovirus B19

Timm C. Harder
Landeslabor Schleswig-Holstein, Neumuenster, Germany

Peter Rautenberg
University Clinics Schleswig-Holstein, Campus Kiel, Kiel, Germany

INTRODUCTION

Clinical manifestations of parvovirus B19 infections vary with the immunological and hematological status of the patient. In healthy children and adults, parvovirus B19 causes a self-limiting anemia, whereas in individuals with chronic hemolytic anemia and in immunocompromised patients, it can exacerbate an already existing anemia. Infection of the fetus may result in fetal loss or nonimmune *hydrops fetalis*. Recently, B19 has been implicated in other nonhematological disorders.

TYPE OF PATHOGEN

The *Erythrovirus* genus of the Parvoviridae family embraces the human parvovirus B19 as its type species as well as several simian parvovirus species, all of which reveal a narrow host range and replicate in erythroid progenitor cells. Parvoviruses are nonenveloped icosahedric particles that are 18–26 nm in diameter.

MOLECULAR CHARACTERIZATION

Genome Structure

The 5594-nucleotide, single-stranded DNA genome of parvovirus B19 is flanked by identical palindromic inverted terminal repeats of 382 nucleotides at each end of the genome, which are required in cis for replication. Transcription of two main open reading frames (ORFs), which encode one nonstructural protein (NS1) and two virion polypeptides (VP1 and VP2), is initiated at the *p6* promoter on the extreme left end of the plus-strand genome (Table 1). At least nine differentially spliced transcripts are generated.

Replication Cycle

Parvovirus B19 binds to cells expressing the glycosphingolipid blood group antigen P and requires the presence of $\alpha_5\beta_1$ integrin as a coreceptor for cell entry.[1] Replication is confined to cells that cycle through the S-phase. This restricts the spectrum of fully permissive host cells mainly to erythroid precursors in the bone marrow and liver of the fetus, and to erythroid progenitors postnatally. The viral transactivating NS1 protein regulates both transcription and replication. It also mediates a multistep cell cycle arrest, which is mainly confined to the G_1 phase.[2]

NS1 induces apoptosis, possibly because of its nucleoside–triphosphate binding motif, which triggers pathways also used by tumor necrosis factor-α (TNF-α).[3] Apoptosis contributes to loss of erythroid progenitors. Heterologous NS1 transactivation also involves IL-6 expression, which may have triggering effects on various inflammatory and autoimmune disorders associated with B19 infection.

The minor (VP1) and major (VP2) virion polypeptides are generated from alternatively spliced, partially overlapping mRNA, and autoassemble into virions of 60 capsomeres into which either a negative-stranded or a positive-stranded viral genome in approximately equal ratios is packaged. Replication is unusually efficient, producing virus loads of 10^8–10^{13} particles per milliliter of blood during an acute primary infection. The function of the phospholipase A2-like activity in the N-terminal region of VP1 (VP1u) remains unclear, although it may play a role in host cell entry, nuclear transport, and release of mature virions through phospholipase A2-specific secretory pathways. Interestingly, release of cellular phospholipase A2 is stimulated by IL-6, which in turn is upregulated by NS1 in infected cells.[4]

PREVALENCE

Transmission and Epidemiology

Parvovirus B19 is transmitted mainly by droplets via the respiratory route, but contaminated blood products and vertical transmission from mother to fetus represent further important pathways. The incubation period, as defined by the appearance of rashes, is 13–18 days and

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Table 1 Properties of plus-strand genomic parvovirus B19 DNA

Genome region	Location (nucleotide)[a]	Function	Protein properties
5′ Palindrome	1–382	*cis* element required for replication and packaging	
5′ p6	144–434	Sole active promoter	
NS1	615–2630	Nonstructural transactivating protein; regulates transcription and replication	ORF3, 671 aa[b], 77 kDa
VP1	2623–4968	Minor virion protein (ca. 4%)	ORF 1, 781 aa, 84 kDa
	aa 1–227	Unique sequence stretch of VP1, principal neutralization target	
	aa 130–195	α_2-Phospholipase motif	
	aa 577–677	P-antigen-binding site	
VP2	3304–4968	Major virion protein (ca. 96%)	ORF 1, 554 aa, 58 kDa
	aa 350–450	P-antigen-binding site	
Encoding 7.5 kDa	2089–2305	Unknown	ORF 1, 72 aa, 7.5 kDa
Encoding 11 kDa	4910–5171	Unknown	ORF 2, 87 aa, 11 kDa
Encoding X 9 kDa	2873–3015	Unknown	ORF 2, 81 aa, 9 kDa
3′ Palindrome	5212–5594		

[a]Numbering based on GenBank accession no. AF162273 (isolate HV).
[b]aa, amino acids.

signals the decline of infectivity. The infectious period starts 7 days before onset of rash. Clusters of acute primary infection occur in spring and early summer, with regional epidemics recurring every 4–5 years.[5]

The prevalence of B19-specific IgG antibodies increases with age to about 80% in the elderly. The annual seroconversion rate among susceptible women of childbearing age is estimated to be 1.5%, increasing about 10-fold during epidemics.[6] The prevalence of B19 DNA in blood donors varies between 1:200 and 1:35,000.[5] Current virucidal procedures applied to blood products fail to prevent the transmission of B19 virus. However, B19 loads of $<10^{4.0}$ genomes per milliliter of blood are regarded as unlikely to be contagious.[7]

Molecular Epidemiology

B19 viruses exhibit a remarkably high degree of sequence conservation, with a <2% variation between temporally and geographically widely separated isolates. However, the VP1u fragment has been reported to vary by up to 8% in chronically infected patients.[8] This might reflect evasion of neutralization as one mechanism of persistence because VP1u is predominantly involved in the formation of neutralization-relevant epitopes.

Recently, several genetically distinct B19-like parvovirus isolates were obtained from patients in France. Servant et al.[9] have proposed distinguishing between three genotypes [a classical B19-like (genotype 1), a Lali-like genotype (genotype 2), and a V9-like genotype (genotype 3)], which are equidistantly related to each other (differences of up to 14.2% across the whole genome) and were shown to cocirculate. Although the clinical spectrum of the newly discovered genotypes appears to resemble that of the B19 lineage, explicit differences in the *p6* promoter region (up to 25.2%) point toward distinct cell tropisms. The high ratio of synonymous vs. nonsynonymous mutations characterizing these genotypes indicates ancient separation and long-term evolution of these viruses.[9]

CLINICAL ASPECTS

Immunocompetent Hosts

Infection in immunocompetent hosts is asymptomatic in about 25% of cases. During childhood, the clinical hallmark of acute B19 infection is an exanthematous malar rash (''slapped cheeks''), also referred to as *erythema infectiosum* or fifth disease (to be distinguished from measles, rubella, scarlet fever, and varicella). In persons with normal hematopoiesis, parvovirus B19 induces a clinically unapparent self-limited transient anemia and reticulocytopenia.[10] Apoptotic death of nonpermissive megakaryocytes due to overexpression of NS1 protein[11] occasionally leads to thrombocytopenia, especially in children.

Symmetrical peripheral arthropathies affect about 60% and 30% of acutely infected adult females and males, respectively. In the majority of cases, clinical symptoms usually resolve within 4 weeks because of a classical Th1 response, which aids in clearing B19 virus through VP2-specific IgM and high-affinity neutralizing VP1-specific

IgG antibodies.[5] However, in some cases, B19 DNA can be detected in plasma—albeit only by the most sensitive amplification techniques—for months and even years after symptoms have receded.

Immunity following uncomplicated recovery is believed to ensure life-long protection. Whether B19 infection plays a causal, putatively HLA-restricted role in chronic joint diseases and various other vasculitic and neurological disorders is still being debated.[12] In individuals with elevated red blood cell turnover rates (e.g., sickle cell anemia, congenital erythrocyte membrane defects, and enzymopathies), parvovirus B19 infection may lead to a transient aplastic crisis (TAC) that can prove fatal if not treated promptly.[5,10]

Infection During Pregnancy

About 30–50% of women of child-bearing age are susceptible to B19 infection. The annual seroconversion rate in susceptible women of child-bearing age is estimated to be 1.5%. The risk of infection is highest in women with infected children in the household.[6,13]

The risk of fetal loss appears to be highest (9–15%) in mothers infected during the first 20 weeks of gestation. The highest risk of fetal loss coincides with the period of highest fetal hepatic hematopoietic activity in the second trimester.[13–15]

The risk of *hydrops fetalis* developing 2–6 weeks after primary maternal infection varies between 0% and 3%. About two thirds of all cases shows remission either spontaneously or after intrauterine transfusion of blood containing B19-neutralizing antibodies.[13,16] The presence of virions in the nuclei of cardiac myocytes suggests that, in addition to B19 fetal anemia, direct cardiac involvement plays a role in the pathogenesis of B19-induced *hydrops fetalis*.[12]

Cardiac Manifestations

Cardiac myocytes are fully B19-permissive only in the fetus. However, B19 DNA has also been detected in P-antigen-bearing endothelial cells and myocytes of the heart in patients with myocarditis or dilated cardiomyopathy who otherwise have no signs of viremic B19 infection.[17–19]

Infection in Immunodeficient Patients

Life-threatening pure red cell aplasia (PRCA) is observed in patients who are unable to mount appropriate titers of neutralizing antibodies against B19 because of severe congenital or acquired immunodeficiencies. In most cases, a chronic persisting course, which demands therapeutic intervention, develops.[10] Recent findings indicate an increased risk of kidney and heart allograft rejection in transplant patients with nonacute, low-level parvovirus B19 DNA (<1000 genomes per milliliter of blood), which is potentiated by simultaneous infection with the human cytomegalovirus.

DIAGNOSIS

Diagnostic measures depend on the immune status of the patient and the clinical picture. As a rule, detection and staging of B19 infection are best achieved by combined

Table 2 Methods for detection of parvovirus B19 DNA in clinical material

Method	Indication	Sensitivity/detection limit[a]	Reference
DNA hybridization	Large-scale quantitative screening	ca. 10^6 (1 pg)	[23]
In situ hybridization	Noncanonical symptoms (e.g., myocarditis)	Single cells	[12]
Single-round PCR (agarose gel, ethidium bromide)	Initial individual diagnosis, screening	10^5	[24]
Single-round PCR-EIA (DIG-labeled or DNP-labeled probes)	Large-scale screening (plasma pools, factor concentrates)	1.6×10^3 (colorimetric); 6×10^2 (chemoluminometric)	[7,25]
Nested multiplex PCR (agarose gel, ethidium bromide)	Differential diagnosis of exanthematic viral infections	ca. 10^2	[26]
Quantitative real-time PCR (TaqMan and LightCycler, internal standard)	Initial individual diagnosis of chronic/relapsing infections; follow-up studies, therapeutic guidance; contagiosity estimates	10^2–10^9(linear quantification range)	[21,27]

[a]All values represent genome equivalents per milliliter (gEq/mL); 0.65 gEq equals approximately 1 IU (WHO standard).

testing for B19-specific IgM (and IgG) and DNA in serum.[20] Various commercially available formats for antibody detection are available, all based on recombinant VP1/VP2 proteins. Elaborate qualitative and quantitative DNA amplification techniques for detection of B19 DNA abound (Table 2). However, being tailored to detection of B19 genotype 1, they might require adaptation to the newly discovered genotypes 2 and 3. Quantification of B19 DNA, now based on a World Health Organization (WHO) standard, is appropriate to guide therapeutic planning and to assess the contagiosity of pooled plasma and other blood products. In situ detection of B19 DNA is indicated if noncanonical forms of B19-associated disorders, such as myocarditis and chronic joint disorders, are suspected.[12]

MANAGEMENT

Treatment Options

Virus-neutralizing antibodies contribute to clearance of B19 virus in both acute and persistent infections, and are currently the only safe treatment option for TAC and PRCA. In iatrogenically immunosuppressed patients with B19 infection, transient relaxation of the immunosuppressive regimen often enables virus clearance by the patient's invigorated immune system.[5,21]

Prevention and Control

No licensed vaccine for humans is available. Phase I clinical studies of recombinant baculovirus-expressed parvovirus B19 virus-like particle vaccines with an enriched content of VP1 have been promising.[22] Screening of blood products is advisable before use in high-risk patients.

RECOMBINANT B19 VECTORS

Recombinant B19 virus is highly efficient at transducing cells of the erythroid lineage. It is also capable of low-level transduction of nonerythroid P-antigen-bearing cells.

CONCLUSION

Parvovirus B19, which generally provokes an acute but mild, self-limiting disease in children, can cause life-threatening disorders and chronic infection in immunocompromised patients or patients with congenital hemolytic disorders. There is mounting evidence that B19 virus is an emerging pathogen for viral myocarditis and dilated cardiomyopathy. Currently available elaborate molecular B19 diagnostic techniques need to be modified to factor the sequence variation of the two recently discovered new human *Erythrovirus* genotypes. Quantification of the B19 virus load can help to guide therapeutic planning for immunocompromised patients and to facilitate the assessment of the contagiosity of B19-contaminated blood products.

ARTICLE OF FURTHER INTEREST

Real-Time PCR, p. 1117

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Patent Aspects in Proteomics and Genomics

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Hans Wilhelm Meyers
Patent Attorneys von Kreisler Selting Werner, Köln, Germany

INTRODUCTION

Without patent protection, no successful commercialization of a new technical entity can take place. In particular, this proves true in the highly competitive field of biotechnology. This contribution describes the current state of the patentability of issues in Genomics and Proteomics.

NATURE OF PATENTS

The basic principle underlying patent protection is simple: Only the patentee is allowed to use and practice the invention for commercial exploitation. The duration of a patent is generally 20 years from the date of application. A patent is limited to the territory of the state in which it was granted.

Contrary to widespread conventional wisdom, a patent does not grant the patentee a right to practice the invention, but only to exclude others from doing so. Statutory regulations may interfere with the patentee's freedom to practice his invention.

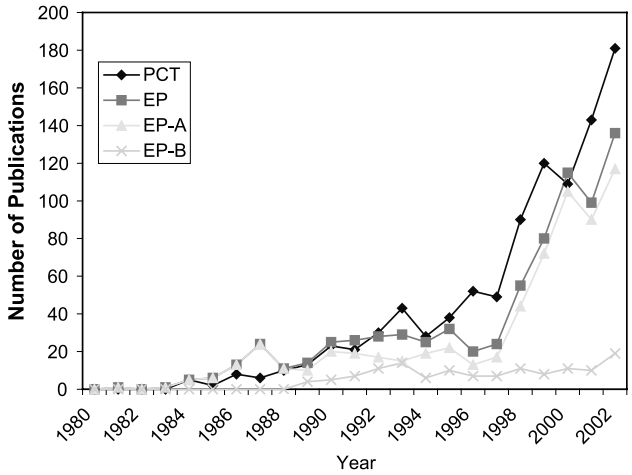
The patentee's so-called ''freedom-to-operate'' may be further restricted by the existence of other patents. It often happens that a first patentee holds a patent for a basic invention, e.g., the polymerase chain reaction (PCR) method, which is the subject of further research and development activities resulting in patents for improvements of the basic invention, e.g., diagnostic kits using PCR. If the patent for such an improvement is held by someone other than the first patentee, then the second patentee cannot practice the improvement without permission of the first patentee. Likewise, the first patentee cannot carry out the improvement without an arrangement with the second patentee.

SIGNIFICANCE OF PATENTING

Research results in the field of genomics and proteomics frequently have considerable relevance in medical or pharmaceutical applications. For example, a new drug has to cope with many obstacles before it is granted permission for use in the human setting. According to reliable estimates, the downstream process of developing a drug until it achieves regulatory approval costs about $500–800 million. Imitation of scientific and technical results in any field is legal, so long as no issues of unfair competition come up. The only possibility to protect, e.g., a new drug or an application of a known substance in the pharmaceutical field, is to obtain patent protection. In the absence of patent protection, competitors can adopt the results and commercialize the drug without incurring the expense of drug development costs.

A further issue is commercial exploitation of results of basic research, e.g., finding of a target such as a receptor or an enzyme having relevance in patho-physiology or potential application in diagnostics. Such entities are often discovered and validated by universities or nonprofit scientific institutes. Also, these institutions try to obtain return on investment and have to cooperate with large companies having the financial resources for screening ligands interacting with the target for drug development. It goes without saying that these institutions, in particular, need patent protection for their research results.

The relevance of patenting in the field of proteomics and genomics is illustrated by the following figure[a]:



The figure depicts the number of patent applications containing the keyword ''genomi'' starting 1980 until May 2003. PCT means patent application published as International Patent Application; EP-A means Patent applications published by the European Patent Office (EPO); EP-B refers to the number of published granted European Patents

[a]*View this art in color at www.dekker.com.*

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and EP is the sum of the foregoing. Patent applications are published 18 months after first filing (priority).

PATENTABILITY REQUIREMENTS: NOVELTY, INVENTIVE STEP, INDUSTRIAL APPLICABILITY

Novelty

Novelty is a patentability essential which is well-defined in the patent laws of most of the member states of the World Trade Organization. Nothing can be patented that is not new. Although novelty is basic to patentability, different concepts of novelty exist throughout the different patent systems worldwide. The most straightforward is that of ''absolute novelty,'' which is, e.g., applied by the European Patent Convention (EPC) in Art. 54 EPC.

The definition of the state of the art given by the EPC is very broad. It is held to comprise everything made available to the public via written or oral description, by use, or in any other way, before the date of filing of the European patent application (or the priority application). The state of the art, according to EPC, is not locally restricted. When examining the claims of the patent application with regard to their novelty, the European Patent Office will consider a prior publication of the invention irrespective of whether the publication took place in one of the member states of the EPC or elsewhere.

In contrast to the EPC, the statutory standard for novelty in the United States, according to 35 U.S.C. section 102, gives a negative definition of novelty and grants a grace period of one year, in which a publication of the invention by the inventors will not be considered relevant to novelty.

The different definitions of novelty throughout these important patent systems have very significant consequences. An invention may be novel in the United States, even if it lacks novelty in the terms defined by the EPC.

How Is Novelty Assessed by the Patent Authorities?

An invention is novel when its subject matter, as defined in the claims, was not disclosed prior to the first filing date of the invention. The claims are a listing of features defining the invention in certain categories and normally in generalized terms, which have to be supported by the description or specification. Patent categories include devices, substances, processes, methods of use, to name the most important ones. For example, if a publicly available reference discloses each and every feature of an invention, i.e., a claim, then the invention is not novel. The reference can be a publication in oral, written, and electronic (or any other) form.

However, if more than one reference is necessary to identify each and every feature of a claim, then the subject matter of the claim is novel.

Inventive Step

While novelty is a well-defined issue, the question of the presence of an inventive step (EPC terminology) is often more striking. When novelty has been acknowledged by an examiner, a patent will only be granted if the subject matter is also not obvious over prior art. Article 56 EPC reads as follows:

> An invention shall be considered as involving an inventive step if, having regard to the state of the art, it is not obvious to a person skilled in the art.

The respective U.S. regulation section 103 (a) reads as follows:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

How Is Inventive Step Assessed by the Patent Authorities?

The references cited by an examining authority have to be assessed in view of the so-called ''person skilled in the art.'' The skilled person represents an individual who knows all references that have ever been published in any language, but who lacks the creativity in posing and solving objects or problems in a particular technical field. Also, a team of individuals can be addressed as ''person skilled in the art,'' particularly in borderline technical fields.

The question of inventive step is more complicated and ambiguous than the question of novelty. Inventive step considers several issues—e.g., whether the invention uses well-known technologies; whether the invention solves a known problem, only in a different way, via techniques which are known as such or the level of the skill of the artisan.

Inventive step must be proved by the applicant in case the patent examiner or a third party in an opposing procedure raises the issue of lack of inventive step in the invention. An indication of inventive step is an unexpected result proven by comparative examples or improved properties of a new substance. Thus, if a new peptide or

protein shows improved properties over the wild-type protein, an inventive step can be acknowledged.

Requirements of Enablement, Written Description, and Utility

Apart from the essentials of ''novelty'' and ''nonobviousness,'' the invention has to be described in such a manner as to comply with both the written description and the enablement requirement. These requirements are contained in 35 U.S.C. section 112. Similar regulations also exist in other patent laws, as exemplified by the Articles 83 and 84 EPC.

An underlying rationale of the patent system is to grant a patentee an exclusive right for commercialization of an invention, provided, however, that the invention is sufficiently disclosed. Consequently, the purpose of the enablement requirement is to assure that other parties skilled in the art can practice the full scope of the claimed invention without undue experimentation. Claims are typically drafted in broad terms to prevent competitors from circumventing a patented invention. The claims must be sufficiently supported by the patent specification, i.e., claim breadth of the claims and the disclosure (enablement) in the description of the invention should correspond with each other. Otherwise, obstacles in the patent prosecution process will become inevitable and even if a patent is finally issued, it could still be ruled invalid while in the process of being enforced.

To satisfy the written-description requirement, the invention shall be described in sufficient detail so that a person of ordinary skill in the art would recognize that the inventor was in possession of the claimed invention at the time of filing. The requirements of written description and enablement are distinct from each other. This implies that although a patent specification may sufficiently enable a person skilled in the art to practice the invention, it may still be ruled to have failed to comply with the written-description requirement. The case ''The Regents of the University of California vs. Eli Lilly and Co.''[1] provides an example for the significance of the written-disclosure requirement. The disclosure contained the nucleotide sequence of a rat proinsulin cDNA and a general method for obtaining the corresponding human cDNA. The patent claims were broad in scope, covering not only the rat cDNA but also the vertebrate, mammalian, and human cDNA. The latter ones were held invalid because the specification did not provide an adequate written description. In summary, the general tenor in U.S. decisions is that it is not sufficient for obtaining sound patent protection to merely disclose a wish or a plan for obtaining the claimed invention.

In addition, the patent systems typically require that the claimed invention must have a utility (as set forth in 35 U.S.C. section 101) or must be susceptible of industrial application (as set forth in Art. 57 EPC). With regard to genetic patenting, the European Patent Convention explicitly states the industrial application (e.g., function) of a sequence or a partial sequence of a gene must be disclosed in the patent application (see Rule 23e (3) EPC).

WHAT CAN BE LICENSED?

If a result of research in proteomics or genomics shall be licensed, in general a license agreement is concluded. The subject of a license agreement can be know-how or subject matter of an Intellectual Property Right (IPR), in most cases a patent or patent application. Therefore, it is recommended to file a patent application before starting negotiations.

Typically, targets, substances having physiological effects, methods, and technologies, can be subjects of license agreements. Whereas substances having physiological effects are candidates for drug development, so that classical license agreements can be concluded, agreements concerning targets often contain the so-called ''reach-through'' clauses. In the classical case, a license is granted to the drug developing party, which in turn pays royalties and/or down-payments to the licensor. Royalties are based on turnover figures achieved by commercialization of the drug. Targets are research tools for discovering drugs interfering with the target. A particular license agreement can contain clauses obliging the licensee to pay a royalty amount based on turnover figures related to any drug discovered and developed by using the target.

WHAT CAN BE PATENTED IN THE FIELD OF GENOMICS AND PROTEOMICS?

At present, there is no general patent system available under which regime a patent with worldwide extension can be achieved. However, the World Trade Organization (WTO) has issued an international regulation concerning intellectual property rights binding WTO member sates. This regulation is named Agreement On Trade-Related Intellectual Property Rights (TRIPS). TRIPS Article 27 says ''[. . .] patents shall be available for any inventions, whether products or processes, in all fields of technology, provided that they are new, involve an inventive step and are capable of industrial application.''

However, the second and third paragraphs relativize this very general statement by stating

> Members may exclude from patentability inventions, the prevention within their territory of the commercial exploitation of which is necessary to protect order in the public or morality, including to protect human, animal or plant life or health or to avoid serious prejudice to the

> environment, provided that such exclusion is not made merely because the exploitation is prohibited by their law. Members may also exclude from patentability: (a) diagnostic, therapeutic and surgical methods for the treatment of humans or animals; (b) plants and animals other than microorganisms, and essentially biological processes for the production of plants or animals other than nonbiological and microbiological processes. However, Members shall provide for the protection of plant varieties either by patents or by an effective sui generis system or by any combination thereof.

Therefore there are still significant differences in the patent systems of the world, although the TRIPS agreement helps to harmonize the different systems in the future by founding a basis to start with. However, the field of biotechnology, besides the software industry, is still the area most affected by any exceptions of the broad definition according to TRIPS Art 27 (1). The statutes of the European Patent Convention (EPC) contain exclusions from patentability as mentioned in TRIPS Art. 27 (2) and (3). The European Patent Office (EPO) grants patents which are, after grant, administered by the contracting states chosen by the patent applicant. The European Commission issued a Directive 98/44, which was adopted by the European Patent Organization.

One of the most significant feature of the Directive refers to the provisions pertaining to the patentability of biological material. Among other things, it was regulated that an isolated nucleic acid of human origin, an isolated gene of human origin, an isolated protein of human origin, parts of the human body in isolated state, cells of human origin, as well as compartments of cells of human origin could be patented as long as these subject matters were fulfilling all other patentability requirements such as novelty, inventive step, industrial applicability, and sufficiency of disclosure. The requirement of sufficient disclosure of a protein or nucleic acid or gene is only fulfilled if a function of these entities is disclosed. The Directive also regulates that the human body, at various stages of its development, including germ cells, is not patentable. This includes a sequence or a partial sequence of a gene if no function is disclosed. The Directive identifies four classes of inventions to be unpatentable. These include, but are not limited to, processes for cloning human beings; processes for modifying the germ line genetic identity of human beings; use of human embryos for industrial or commercial purposes and processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal (this description was adopted from the oncomouse decision).

Before the Directive was issued, the EPO granted patents on transgenic animals following the decision of the Board of Appeals T 19/90 (Oncomouse Decision).[2] Also, transgenic plants are patentable as long as the respective patent does not encompass a plant variety. The Directive has not yet been transformed into national law by eight EU member states, e.g., Germany, Italy, The Netherlands.

The situation in the United States was not as difficult because higher life forms were regarded as patentable. The application concerning the Harvard Mouse issued to US Patent 4,736,866 on April 12, 1988. It issued without difficulty following the ''oyster'' case, Ex Parte Allen (Board of Appeals and Interference, April 3, 1997[3]), which held that polyploid oysters are nonnaturally occurring manufactures or compositions of matter, falling within the definition of invention. Furthermore, subject matter directed to plants and seeds are routinely allowed by the U.S. Patent and Trademark Office, and in general, higher life forms are considered patentable subject matter within the United States. Similarly, in Japan, higher life forms, both plant and animal, are considered allowable subject matter.

Although the patenting issues have widely been resolved as far as the granting proceedings are concerned, there is still an ongoing debate on ethical issues involved with patenting of living material or material derived from living material. Also, little is known-as of yet, how civil courts will handle patent infringement lawsuits concerning biological inventions.

CONCLUSION

Today, most of the basic problems involved in patenting in the field of Genomics and Proteomics are resolved. In all significant industrial regions, a reasonable patent protection is available for inventions concerning substances, compositions of matter, processes, and methods, both in the pharmaceutical as well as the diagnostic industries.

DISCLAIMER

The purpose of this article is to highlight the importance and to explain the general issues of patenting in biotechnology, in particular Genomics and Proteomics. It does not contain definite legal or patent advice and should not be used as a substitute for the advice of your lawyer or patent attorney.

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PCR and RT-PCR Analysis in Archival Postmortem Tissues

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Serena Bonin
Francesca Petrera
Giorgio Stanta
International Center for Genetic Engineering and Biotechnology (ICGEB) and University of Trieste, Trieste, Italy

INTRODUCTION

Most tissues from biopsy, surgery, or autopsy origin are usually fixed and paraffin embedded as a routine procedure in hospital departments of pathology. Tissue storage is a fundamental step in the usual clinical procedure for histopathological investigations. The traditional method of tissue preservation is the fixation of tissues, mostly in formalin, followed by paraffin wax embedding. In this way, biochemical, molecular, and structural integrity is ensured also for future retrospective analyses, because there is no further chemical degradation in paraffin. Usually, these tissues have been stored in the archives of pathology departments for decades. Among the archive tissues, postmortem tissues obtained from autopsy represent an important resource for the study of rare diseases, neuropathology, or molecular epidemiology. For these studies in fact, the analysis of both pathological and normal tissues as control is fundamental. The protocols of tissue storage normally suggest the fixation step in buffered formaldehyde solution in the dark for about 24 hr before paraffin wax embedding. This procedure is habitually maintained for biopsy and surgical samples, but autopsy tissues are usually fixed for longer periods of time. Various factors, such as postmortem interval or the type of fixative and the fixation time, could affect the quality and utilization of nucleic acids from archive tissues. Extensive degradation of nucleic acids is often found in archival tissues older than 20 years because nonbuffered formaldehyde solution was frequently used in the past. The most widely used fixative is neutral formalin, but for special purposes other fixatives may be used in histopathology laboratories. Of these, Bouin's solution and acetone can compromise the PCR amplification efficiency.[1] Longer fixation times could also induce unsuccessful PCR amplification due to enhanced degradation of nucleic acids. Of the parameters involved in the degradation of nucleic acids, the postmortem interval is the most important factor affecting the successful extraction of high-molecular weight DNA from frozen autopsy tissues.[2] The delay between death and tissue collection is referred to as the postmortem interval (PMI). Commonly, a cadaver is refrigerated at 2–4°C within a few hours of death; an autopsy may not be performed for legal reasons before 4 to 36 hr or more have passed, depending on different rules and conditions in use.[2]

Generally, the amount of degraded DNA in frozen autopsy tissues is correlated directly with the duration of the postmortem period.[3] Otherwise, in the same type of tissue PMI does not appear to affect significantly the recovery of total RNA or mRNA.[2] Even if formalin fixation compromises the quality and intactness of nucleic acids, it has already been demonstrated that it is possible to recover and analyze DNA[4] and RNA[5,6] from formalin-fixed and paraffin-embedded postmortem tissues. The use of PCR-based techniques does not require intact nucleic acids for amplification, therefore even an increased degradation may not affect the outcome of the analysis. In our experience, in postmortem tissues, fixed in nonbuffered formalin, DNA fragments longer than 90 bp cannot be amplified. As a consequence of DNA degradation, only very short sequences could be analyzed in postmortem tissues. However, for longer fragment analysis it is possible to perform a partial restoration and reconstruction of DNA length.[4,7] With this procedure it is possible to fill in the DNA breaks and amplify around 300 bp of DNA sequence from autopsy tissues.

As already reported[5,8] it is also possible to extract and analyze RNA from formalin-fixed and paraffin-embedded tissues from autopsy tissues.[5,6] Usually, in routinely treated formalin-fixed material the available fragments of RNA range between 100 and 200 bp. Analysis of RNA obtained from postmortem, formalin-fixed, paraffin-embedded tissues showed higher levels of degradation resulting in an amplification length of not more than 100 bp.

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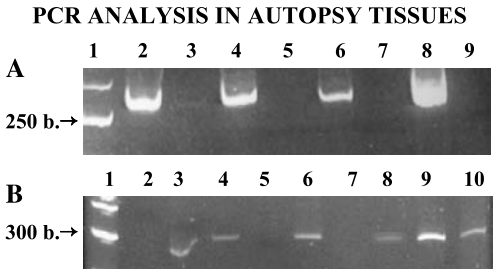

Fig. 1 Polymerase chain reaction products of DNA obtained from autopsy archive tissues. (A) Postmortem sample analysis for the apolipoprotein E gene (287 bp). Size marker (lane 1), postmortem samples with the restoration and denaturation steps (lanes 2, 4, 6, and 8), the same postmortem samples without the restoration step (lanes 3, 5, 7, and 9). (B) Postmortem sample analysis for the TTR gene (291 bp). Size marker (lane 1), postmortem samples with the restoration treatment and denaturation step (lanes 2–10).

NUCLEIC ACIDS EXTRACTION

The fixation of tissue samples in formalin leads to extensive cross-linking of all tissue components. As a result, the nucleic acids isolated from these specimens are highly fragmented. The extent of fragmentation depends on the tissue type and the condition of fixation. This fragmentation is enhanced in postmortem paraffin-embedded tissues. No modifications of the usual protocol of nucleic acid extraction are needed using postmortem archive tissues. For any type of formalin-fixed, paraffin-embedded tissue there is the possibility to easily isolate single cells or subpopulations of cells by microdissection. Methods for the extraction of DNA[4,9] and RNA[5,8] from archive tissues propose the use of single or multiple 6–8-μm histological sections from the paraffin blocks. The first step in the extraction protocols from paraffin-embedded tissues is the elimination of paraffin. Paraffin is soluble in organic solvents such as xylene. After the deparaffinization, tissues are then washed with ethanol several times to completely eliminate residual xylene, as even small amounts could block the activity of the enzymes used in the next steps.[8] To obtain DNA and RNA for further analysis it is necessary to digest the tissue sections by proteinase K or other proteolytic enzymes to remove the cross-linked proteins. The difference in the protocol for the isolation of RNA and DNA from archival autopsy tissues is the composition of the lysis buffer and proteinase K concentration as previously reported.[4,5,8,9] To purify nucleic acids from proteinase K and proteolysis residues, an extraction with phenol/chloroform is needed, for DNA we use phenol–Tris and for RNA phenol–H_2O. Final samples are obtained by alcohol precipitation using glycogen as a carrier.[4,5,8,9] The concentration of nucleic acids is measured at the absorbance of 260 nm.

RESTORATION TREATMENT AND DNA ANALYSIS

It is possible to amplify even longer DNA sequences of about 300 bp from postmortem formalin-fixed and paraffin-embedded tissues utilizing a simple restoration treatment. The restoration method is based on the fact that DNA degradation is connected with random single-strand breaks. To obtain longer stretches of DNA, a pre-PCR treatment is performed. It consists of a DNA rehybridization step in a specific buffer at 55°C[4] followed by a DNA polymerization at 72°C for 20 min. This single-cycle PCR at the elongation temperature (72°C) restores the nicks in the rehybridized DNA using the other corresponding strand as a template.[4] Because of the random position of the nicks, the fragment reconstruction gives sequences long enough for successive PCR analysis. The treated samples are then normally stored at −20°C until specific PCR analyses. To obtain a PCR amplification with restored DNA in postmortem archive tissues, a denaturation step at 95°C is required before PCR amplification, because in the restoration itself the double-stranded DNA is strongly linked with a more stable secondary structure.[4] As reported in Fig. 1A, the PCR amplification in untreated necropsy tissues was unsuccessful, but in reconstructed and denaturated ones the amplification fragments are evident. The fact that the PCR amplification was obtained in formalin-fixed, paraffin-embedded biopsy specimens (Table 1), even without the restoration procedure, confirms that more extensive DNA degradation is confined to postmortem tissues. This simple restoration/denaturation procedure can overcome the problem of DNA degradation in the analysis of necropsy archive tissue. This could eventually result in better suitability of archival DNA for genetic investigations and would overcome the limit for longer-size PCR fragments for specific needs.

Table 1 Polymerase chain reaction amplification of DNA of autopsy and biopsy origin

Amplicon	Restored and denatured autopsy DNA	No restored biopsy DNA
ApoE[a] (287 bp)	4/4	4/4
TTR1[b] (291 bp)	6/9	9/9
TTR1 (339 bp)	1/9	9/9

[a]Apolipoprotein E.
[b]Human prealbumin gene.

Table 2 Reverse transcriptase polymerase chain reaction amplification of β-actin RNA of autopsy and biopsy origin

β Actin amplicon size	Autopsy RNA	Biopsy RNA
77 bp	4/4	4/4
100 bp	4/4	4/4
120 bp	1/4	4/4
170 bp	0/4	4/4

RNA ANALYSIS

Reverse transcriptase polymerase chain reaction (RT-PCR) has become one of the most widely applied techniques in biomedical research. The ease with which the technique permits a specific mRNA detection and quantification has been a major asset in the molecular investigation of disease pathogenesis. Disease imbalances in the expression of specific mRNAs can be sensitively and quantitatively determined by RT-PCR. Reverse transcriptase polymerase chain reaction also offers some opportunities in diagnostic methods such as the detection of RNA viruses. Different studies have shown the possibility of analyzing RNA extracted from autopsy formalin-fixed, paraffin-embedded tissues[5,10] by RT-PCR analysis. In most of the protocols RNA is firstly transcribed in cDNA and subsequently amplified by PCR. The major concern related to archive postmortem tissues is the enhanced degradation of RNA. Usually, in routinely treated formalin-fixed material of biopsy or surgery origin the amenable fragments of RNA range between 100 and 200 bp. Analysis of RNA obtained from postmortem formalin-fixed, paraffin-embedded tissues showed a higher level of degradation (Table 2). As reported in Fig. 2, amplicons that had been successfully amplified were not longer than 75–100 bp (Fig. 2A and B). In fact, for fragments nearly 150 bp long, the RNA amplification was obtained only in a few samples (Fig. 2C and D). To obtain a successful RT-PCR analysis and maximize the amplification of degraded RNA it is better to amplify short sequences, of about 70–100 bp. It is also possible to perform quantitative RT-PCR analysis using RNAs obtained from necropsy formalin-fixed and paraffin-embedded tissues. Several variables need to be controlled in gene-expression analysis, such as the amount of starting material, enzymatic efficiencies, and differences between tissues. The quantification of RNA from postmortem formalin-fixed, paraffin-embedded tissues presents specific problems that cannot be resolved with a competitive analysis because of RNA degradation in the preserved samples. Because the degradation is presumably random, the quantification in these tissues cannot be resolved using competitive analysis with internal standards. The comparison of different template preparations (standard vs. pathological template) introduces errors. The sample fragmentation due to fixation severely distorts the absolute quantification. In relative quantification the level of the mRNA of interest is related to the amounts of housekeeping genes; these are supposed to be constant both in the tissue and in the pathological situation of interest. As a result, relative quantification is preferred and strongly recommended in postmortem archival tissues. Performing a relative calculation eliminates all distortions due to fixation artefacts or sample impurity. Accurate normalization of gene-expression levels is an absolute prerequisite for reliable results. The purpose of normalization is to remove the sampling differences in order to identify real gene-specific variation. For RT-PCR there is a

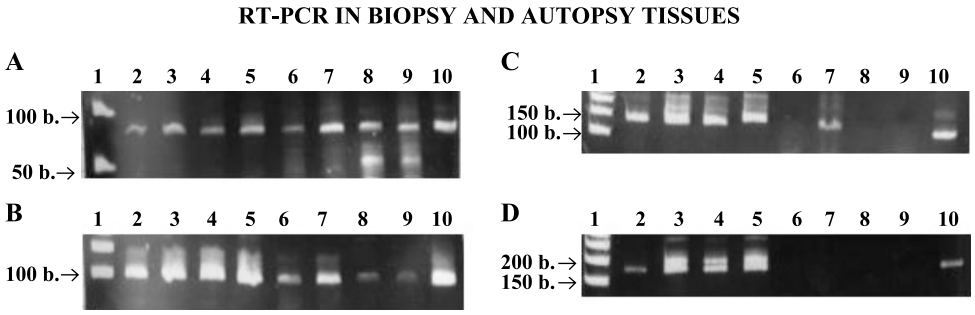

Fig. 2 Reverse transcriptase polymerase chain reaction products in formalin-fixed, paraffin-embedded tissues of autopsy and biopsy origin for β-actin gene. (A) RT-PCR analysis for β-actin gene (77 bp). Size marker (lane1), biopsy samples (lanes 2–5), autopsy samples (lanes 6–9), positive control (RT-PCR analysis in RNA obtained from HepG2 cells) (lane 10). (B) RT-PCR analysis for β-actin gene (100 bp). Size marker (lane1), biopsy samples (lanes 2–5), autopsy samples (lanes 6–9), positive (lane 10). (C) RT-PCR analysis for β-actin gene (120 bp). Size marker (lane1), biopsy samples (lanes 2–5), autopsy samples (lanes 6–9), positive control (lane 10). (D) RT-PCR analysis for β-actin gene (170 bp). Size marker (lane1), biopsy samples (lanes 2–5), autopsy samples (lanes 6–9), positive control (lane 10).

general consensus on using a single control gene for normalization purposes. In more than 90% of the cases reported in the literature,[11] GAPDH, β-actin, 18S, and 28S rRNA were used as a single control gene. Different authors have already reported that housekeeping gene expression can vary considerably, so the validity of the conclusions is highly dependent on the control used.[11] Vandesompele et al. strongly recommend the use of at least three control genes for calculating the normalization factor in order to remove nonspecific variation of target genes. Nowadays, there are several analytical approaches to measure the amount of RT-PCR product in archival tissues, including real-time RT-PCR,[12] membrane hybridization,[13,14] capillary electrophoresis,[14,15] PCR-ELISA.[16] All of them are suitable for relative quantification in paraffin-embedded tissues of necropsy origin reducing the amplicon size to less than 100 bp.

CONCLUSION

Formalin-fixed and paraffin-embedded tissues of autopsy origin are an important source for molecular analysis especially in rare diseases, neuropathology, and molecular epidemiology studies because of the availability of either normal or pathological specimens. The major difficulty in using these tissues is the extensive degradation of nucleic acids. The development of a simple treatment for DNA restoration and quantitative RT-PCR opens the autopsy archives for molecular biology analysis. The possibility of performing molecular genetic analysis in autopsy tissues will improve diagnostic procedures and will enable a large number of research studies on various diseases. Retrospective analysis of these archival tissues would provide the means to correlate molecular findings with the response to treatment and clinical outcome. In addition, these findings will guide future prospective studies in analyzing native or freshly frozen tissues.

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PCR Chemiluminescent Immunoassay (PCR-CLEIA)

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Aldo Roda
Mara Mirasoli
Patrizia Pasini
Simona Venturoli
Monica Musiani
Marialuisa Zerbini
University of Bologna, Bologna, Italy

INTRODUCTION

Polymerase chain reaction (PCR) is a well-established, powerful diagnostic tool that provides rapid, highly sensitive, and specific DNA or RNA detection. The demand for equally rapid and sensitive methods to achieve detection and semiquantitative information on PCR products has brought about the coupling of PCR to enzyme-linked immunosorbent assay (ELISA). These assays involve hybridization of amplicon with specific oligonucleotide probes (thus enhancing the specificity of the method) and detection of the hybridization product with suitable enzyme-labeled antibodies. A further advance of the technique involves the use of chemiluminescence as detection system, yielding PCR chemiluminescent enzyme immunoassay (PCR-CLEIA). This further improves the rapidity and sensitivity of the technique and lets us envisage future developments toward miniaturized and automated high-throughput methods.

In this paper, the principle of the method, applications, and future trends of PCR-CLEIA are presented.

DISCUSSION

PCR, with its extraordinary sensitivity, is the method of choice for the detection of nucleic acids present in very low concentrations in biological specimens. Diagnostic applications of this technique mainly concern analysis of gene amplification, expression in tumors, and expression of etiological agents in human infections. Assays that combine PCR amplification and the immunological chemiluminescent detection of PCR products (PCR-CLEIA) provide high specificity, sensitivity, objectivity, and rapidity. In particular, the use of chemiluminescence as a detection system provides superior assay performance. Chemiluminescence is a versatile analytical tool that offers several advantages over other detection principles, including high detectability, high selectivity, wide dynamic range, and rapidity.[1,2] Thanks to the high sensitivity of chemiluminescence detection, very small volumes of reagents and samples can be used, which results in lower assay costs and the possibility of exploiting high-density formats such as 384-well and 1536-well microtiter plates. This last feature, combined with rapidity, renders chemiluminescence detection suitable for the development of high-throughput methods.

PCR-CLEIA can be very useful in understanding the progression of the disease, monitoring the success of therapy, and evaluating the potential risks of transmission of pathogens. Moreover, PCR-CLEIA can be very useful in the diagnosis of infectious diseases that can persist in the presence of a small number of infectious agents, and in distinguishing low-level innocuous infections from those which may be of clinical relevance.

PCR-CLEIA is based on the following:

1. Direct incorporation of a labeled nucleotide during PCR amplification reaction [mainly by incorporation of digoxigenin (Dig)-labeled dUTP].
2. Hybridization of labeled amplified products with biotin-labeled probes specific for the target (alternatively, the PCR product can be biotin-labeled by performing the amplification reaction in the presence of a biotin-labeled dUTP or primer; in this case, hybridization will be performed with either a Dig-labeled or an enzyme-labeled probe).
3. Capture of hybridized amplicons onto streptavidin-coated white or black microtiter plates (alternatively, the probe can be immobilized onto the solid phase, then the labeled amplicon can be added and hybridization can be performed directly into the wells of the microtiter plate).
4. Detection of immobilized hybridized amplicons by anti-Dig antibodies conjugated to peroxidase or alkaline phosphatase (this step is not necessary in case an enzyme-labeled probe is used).

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5. Addition of chemiluminescent substrates with high sensitivity and wide dynamic range.
6. Measurement of chemiluminescent signals by microplate luminometers or imaging systems and correlation to amounts of amplified products.

A schematic representation of the principle of the method is shown in Fig. 1.

With PCR-CLEIA, semiquantitative information on target DNA or RNA is obtained. Moreover, results evaluation is objective, whereas conventional PCR requires a more subjective interpretation; at low DNA concentration, interpretation of results may be doubtful. Unlike conventional PCR, PCR-CLEIA is automatable, able to process simultaneously a large number of samples in reasonable time, and can be applied to DNA or RNA quantification. The chemiluminescent substrate used in amplicon detection permits a large-scale range of measurements compared with colorimetric or fluorometric detection.

To develop a standardized PCR-CLEIA, different steps have to be optimized.

Optimization of PCR-CLEIA Method

Streptavidin-coated microtiter plates able to efficiently adsorb biotinylated nucleic acids are commercially available. To exclude high background luminescence emitted by plate formats, preliminary measurement of phosphorescence emission intensity after exposure to ambient light of the microtiter plate can be performed.

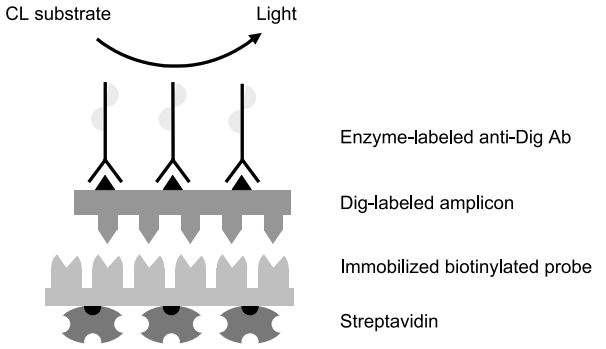

Fig. 1 Microtiter plate hybridization and immunodetection scheme. Schematic representation of the microtiter plate immunochemiluminescent hybridization assay of PCR products: the Dig-labeled PCR product is hybridized with biotin-labeled specific oligoprobes; hybridized amplicons are captured onto streptavidin-coated microtiter plates, then detected by means of anti-Dig antibodies and chemiluminescent detection. CL, chemiluminescent; Dig, digoxigenin; Ab, antibody.

The optimal concentration of target-specific probe has to be determined. PCR products, obtained from serial dilutions of the original sample, have to be hybridized with different concentrations of probe and, subsequently, captured onto the microtiter plate. The chemiluminescent signal intensity from each well is then measured. The oligoprobe concentration that allows to obtain the calibration curve with the lowest limit of detection and the highest sensitivity (slope of the curve) has to be chosen. Usually, no further improvement is obtained by increasing the oligoprobe concentration.

Direct incorporation of a labeled nucleotide during amplification reaction is preferred because it increases the sensitivity of the assay with respect to extension labeling primers.

To overcome differences in DNA or RNA extraction and amplification efficiencies between independent samples, results can be normalized based on a suitable housekeeping gene control.

To enhance the reproducibility of the method, quality control should be performed by including a minimum of two negative controls and two positive controls in each assay.

PCR-CLEIA provides semiquantitative information on the target sequence. However, more accurate quantitative information can be obtained by adding an internal standard consisting of a competitor sequence of target DNA, which can be internally mutagenized. The coamplification of target and internal standard competitor sequences, using the same set of primers and the same physical–chemical parameters, allows the same efficiency of amplification and permits the construction of competitive titration curves for the quantitation of the target DNA.

Limit of Detection

Limits of detection of the PCR-CLEIA methods have to be determined using different concentrations of target nucleic acids. The products of amplification, once hybridized with the immobilized specific probes, are analyzed and the limit of detection for each amplicon is determined as the chemiluminescent signal significantly above cutoff. The criteria for defining the cutoff value and the positivity of samples have to be determined for each protocol based on the expected variability of the results.

Specificity

Serial dilutions of samples containing known numbers of copies of target genomes and negative controls (unrelated genomes) have to be PCR-amplified and hybridized with

target-specific probes. A positive signal has to be detected when target genome PCR amplicons are hybridized with the specific probes. A signal within background range (negative signal) has to be detected when PCR amplicons from unrelated genomes are set for hybridization with target-specific probes.

Precision

To investigate the reproducibility of the PCR-CLEIA method, at least two positive and two negative reference samples have to be amplified, and then assayed with type-specific immobilized oligoprobes in triplicate in three independent assays. Intraassay coefficient of variation (CV) lower than 5% and interassay CV lower than 10% are desirable.

Applications of PCR-CLEIA

PCR-CLEIA has been developed and applied to the detection of PCR-amplified enterotoxin A gene from *Clostridium perfringens* in artificially contaminated ground beef.[3] A biotinylated primer pair was designed for the amplification of a fragment of the *C. perfringens* enterotoxin A gene. PCR-amplified products were detected by hybridization ELISA protocols by applying a streptavidin capture step for the hybridized PCR products to an internal Dig-labeled probe using chemiluminescent detection with Lumiphos 530TM as substrate.

Quantification of circulating human cytomegalovirus (HCMV) in the plasma of severely leukopenic patients was also achieved by PCR-CLEIA through the detection of biotinylated PCR products of the HCMV UL50 region using HCMV-specific enzyme-labeled probes and automated chemiluminescence detection able to reveal 100 HCMV genomes per milliliter of plasma.[4]

A sensitive PCR-CLEIA assay was developed and applied to quantitative detection of enteroviruses in environmental samples.[5] Following reverse transcription (RT), viral cDNA was labeled with Dig-dUTP during the PCR amplification step. The labeled PCR products were then hybridized with enterovirus-specific biotinylated probe, captured in streptavidin-coated microtiter wells, and detected by an anti-Dig peroxidase conjugate using a chemiluminescent substrate and automated measurement. The assay was able to detect 0.01 plaque-forming units (PFU)/mL, proving 10–100 times more sensitive than colorimetric detection or dot blot hybridization.

PCR-CLEIA has proved to be a sensitive and versatile method for the analysis of human and murine cytokine mRNA expression. Indeed, Dufour et al.[6] were able to quantify mRNA for five porcine cytokines: interferon (IFN)-gamma, interleukin (IL)-2, IL-4, IL-10, and IL-18 on peripheral blood mononuclear cells. The main features of the methodology were: RT to obtain DNA sequences, PCR and detection of amplicons for all cytokines simultaneously, cytokine quantification in relation to a housekeeping gene control (glyceraldehyde-3-phosphate dehydrogenase, or GAPDH), detection of amplicons by ELISA using a chemiluminescent substrate with high sensitivity and wide dynamic range, and automation of the detection system for analysis of a large number of samples. This highly sensitive quantitative RT-PCR-CLEIA was able to detect 100–200 cytokines mRNA copies per 7.5×10^4 cells.

Immunochemiluminescent PCR detection of varicella zoster virus (VZV) DNA from the cerebrospinal fluid of 287 patients with meningitis, encephalitis, or other neurological diseases or symptoms improved considerably the detection rate of the VZV–PCR product compared with agarose gel electrophoresis.[7]

Hepatitis G virus (HGV) was recently identified as a new member of the family Flaviviridae, but its clinical significance is still unclear. Because no immunoassay for the diagnosis of HGV is available, to facilitate the detection of the viral genome by mass screening in the clinical laboratory, a sensitive RT-PCR assay with ELISA detection was developed. Sequences within the 5′-noncoding region and within the putative NS5a region were independently amplified in the presence of Dig-dUTP and were detected by hybridization with biotinylated capture probes bound to a streptavidin-coated matrix. Semiquantitative detection via chemiluminescence was performed in microtiter plate format machines. At least 8×10^2 genome equivalents per milliliter of serum using both primer pairs could be detected.[8]

The PCR-CLEIA methods described above were developed using the conventional 96-well microtiter plate format. However, other assay formats can be explored, thanks to the use of chemiluminescence detection, thus enhancing the performance of the method.

For example, using multianalyte assay formats and type-specific probes, PCR-CLEIA can also be easily applied to the simultaneous identification of the different genotypes of pathogens and can therefore be a valid tool to study the prevalence and potential pathogenicity of single genotypes. This is possible, thanks to the use of chemiluminescence as detection system, which allows both quantification and localization of the hybrids captured onto the plate.[9] In particular, to allow multianalyte binding assays, we have developed a novel microtiter plate containing 24 main wells, each divided into seven subwells, and we explored its clinical potential by developing a PCR-CLEIA for simultaneous detection and typing of seven high-oncogenic-risk human

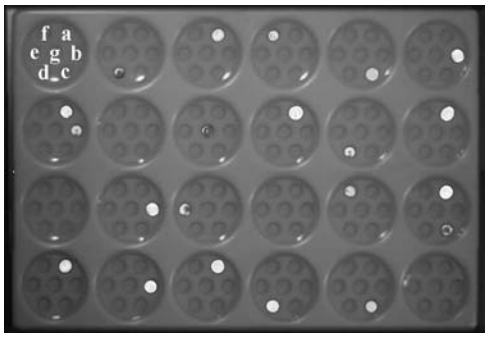

Fig. 2 Multititer plate figure. Analysis of clinical samples [$n=24$; 5 negative and 19 positive (five for HPV 16, three for HPV 18, two for HPV 31, three for HPV 33, one for HPV 35, two for HPV 45, one for HPV 58, one for HPV 16 and 18, and one for HPV 16 and 31)] performed on the novel microtiter plate format for multianalyte assays. Overlay image of the plate live image and the chemiluminescent signal. White letters indicate HPV genotypes detected in the subwells of each main well, based on the immobilized type-specific oligoprobe positions: (a) HPV 16; (b) HPV 18; (c) HPV 31; (d) HPV 33; (e) HPV 35; (f) HPV 45; (g) HPV 58.

papillomavirus (HPV) DNAs in one well. The assay is based on consensus PCR amplification of a conserved sequence of 30 HPV genital genotypes and on the typing of seven HPV DNA using the novel microtiter plate format. Thanks to the possibility of immobilizing seven type-specific capture oligoprobes in separate positions within the same well, typing was performed simultaneously by hybridization followed by a chemiluminescent immunoassay in one well and with one aliquot of the amplification reaction product. The chemiluminescence signal was imaged using an ultrasensitive charge-coupled device (CCD) camera and the light output intensity of each internal subwell was measured. The method proved to be specific and allowed the detection of 50 copies of genome for HPV 16, 18, 33, and 58, and 100 copies of genome for HPV 31, 35, and 45. Intraassay and interassay coefficients of variation of the method were 5.6% and 7.9%, respectively. This new assay format offers advantages in terms of multitest simplification and reductions in reagent volumes and analysis time. The same principle could be applied to the development of single-tube panels of tests for other pathological conditions.

Figure 2 shows the results obtained by analyzing 24 clinical samples with the multianalyte PCR-CLEIA assay, developed using the novel microtiter plate format. In particular, the overlay image of the plate live image and the chemiluminescent signal is shown.

Future trends for PCR-CLEIA mainly involve the development of miniaturized and high-throughput assay formats. With respect to this, we are now developing a PCR-CLEIA on 384-well microtiter plates, which allows to greatly increase the amount of information obtained in one assay. The use of smaller assay volumes allowed us to significantly reduce the amount of samples and reagents, thus providing savings. This format allows the screening of 20 samples for as many as 15 HPV genotypes per sample in one assay and with one 50-μL amplification product aliquot. Moreover, thanks to the high density of the format, it is possible to produce calibration curves for each genotype and to obtain semiquantitative information on the viral load of positive samples. It is worth noting that this assay format provides optimal performance only when chemiluminescence is used as a detection system, thanks to its high detectability even in low volumes.

The use of robotic samples and reagent handling systems will enhance the throughput of the assay and allow the operator to control the analytical steps and the reproducibility of the overall procedure.

CONCLUSION

PCR-CLEIA allows rapid, specific, and sensitive detection of target DNA or RNA sequences. The use of chemiluminescence as a detection system provides optimal performance in terms of sensitivity and linear range of the method, and increases rapidity with respect to conventional spectrophotometric detection. Furthermore, chemiluminescence allows the use of innovative assay formats, such as multianalyte or high-density microtiter plate, thus further enhancing throughput of the method.

Future trends of this technique involve the development of miniaturized and fully automated assays, in particular the use of microarray and microchip technologies, which allow the execution of numerous multianalyte assays in very small sample and reagent volumes and short times. However, at present, these systems are mostly applied to screening purposes because quantification accuracy of each microspot is hampered by the absence of physical separation and cross-talk phenomena.

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Personalized Medicine

K. K. Jain
Jain PharmaBiotech, Basel, Switzerland

INTRODUCTION

Personalized medicine simply means the prescription of specific therapeutics best suited for an individual based on pharmacogenetic, pharmacogenomic, and pharmacoproteomic information. Several other terms are used to describe individualization of treatment and include tailored therapy, predictive medicine, and genomic medicine. Individualized treatments that have been used in medicine prior to the discussion of the concept of personalization include blood transfusion, tissue transplantation, and cell therapy. Tissue typing based on genetic markers is used to match the transplant to the recipient individual, and patient's own tumor cells are used in some cancer cell therapies. This concept has now been extended to pharmaceuticals. It is now recognized that patients with the same disease respond differently to drugs depending on their genetic constitution and other factors so that there are marked variations in efficacy and safety of the same drug for different patients.

BASICS OF PERSONALIZED MEDICINE

Important basics of personalized medicine are:[1]

- Pharmacogenetics is a term recognized in pharmacology in the pregenomic era and concerns the study of influence of genetic factors on action of drugs.
- Pharmacogenomics is the application of genomics to drug discovery and development. It involves the study of mechanism of action of the drugs on the cells as revealed by gene expression patterns.
- Pharmacoproteomics is the application of proteomics to drug discovery and development. Subtyping patients on the basis of protein analysis may help to match a particular target-based therapy to a particular marker in a subgroup of patients. Pharmacoproteomics may be considered an extension of genomics and partially overlaps pharmacogenomics.

There is a degree of overlap between pharmacogenetics and pharmacogenomics. The two terms are considered by some as similar but one must recognize the distinction as shown in Table 1.

MOLECULAR DIAGNOSTICS IN RELATION TO PERSONALIZED THERAPY

Several molecular diagnostic technologies[3] are used including single nucleotide polymorphism (SNP) genotyping, haplotyping, gene expression studies by biochip/microarrays, and proteomics. Integration of molecular diagnostics with therapeutics will be important.[4] Relation of molecular diagnostics to personalized medicine is shown in Fig. 1.

Single Nucleotide Polymorphisms

Potential uses of SNP markers include drug discovery and prediction of adverse effects of drugs. Single nucleotide polymorphisms have the following relation to an individual's disease and drug response:

- SNPs are linked to disease susceptibility.
- SNPs are linked to drug response.
- SNPs can be used as markers to segregate individuals with different levels of response to treatment (beneficial or adverse) in clinical settings.

Haplotypes

An alternative approach to SNP genotyping is haplotyping.[5] Gene-based haplotypes are composed of the nucleotides that occur at SNP positions on a single chromosome at the locus of a single gene. Haplotypes are the most precise markers possible for a given gene because they contain all the variations in a gene. Haplotyping is a way of characterizing combinations of SNPs that might influence response and is considered to be a more accurate measure of phenotypic variation. However, SNP-based tests have greater power when the number of causative SNPs (a subset of the total set of SNPs) is smaller than the total number of haplotypes. One limitation of haplotyping is that haplotypes need to be determined for each individual, as SNPs detected from a pool of DNA from a number of individuals cannot yield haplotypes. Clinical trials using haplotyped individuals are the first genetically personalized medical treatments.

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Table 1 Pharmacogenetic vs. pharmacogenomic studies

Feature	Pharmacogenetics	Pharmacogenomics
Focus of studies	Patient variability	Drug variability
Scope of studies	Study of sequence variations in genes suspected of affecting drug response	Studies encompass the whole genome
Methods of study	SNP, expression profiles, and biochemistry	Expression profiling
Relation to drugs	One drug and many genomes (patients)	Many drugs and one genome
Examination of drug effects	Study of one drug in vivo in different patients with inherited gene variants	Examination of differential effects of several compounds on gene expression in vivo or in vitro
Application relevant to personalized medicine	Patient/disease-specific healthcare	Drug discovery and development or drug selection

Source: Modified and expanded from Ref. [2].

BIOCHIP/MICROARRAYS FOR PERSONALIZED MEDICINE

Biochips play an important role in the development of personalized medicine. Various applications are:[6]

- For storage of the patient's genomic information
- SNP genotyping
- Genetic screening for detection of mutations
- Gene expression profiling
- Diagnosis and prognosis of cancer
- In drug safety for pharmacogenetics and toxicogenomic studies
- Monitoring of pathogens and resistance in infections
- Stratification of patients in clinical trials

Protein chips will be particularly useful for clinical implementation of personalized medicine. Profiling proteins on biochips will be useful for distinguishing the proteins of normal cells from early-stage cancer cells, and from malignant metastatic cancer cells. In comparison with the DNA microarrays, the protein microarrays/chips

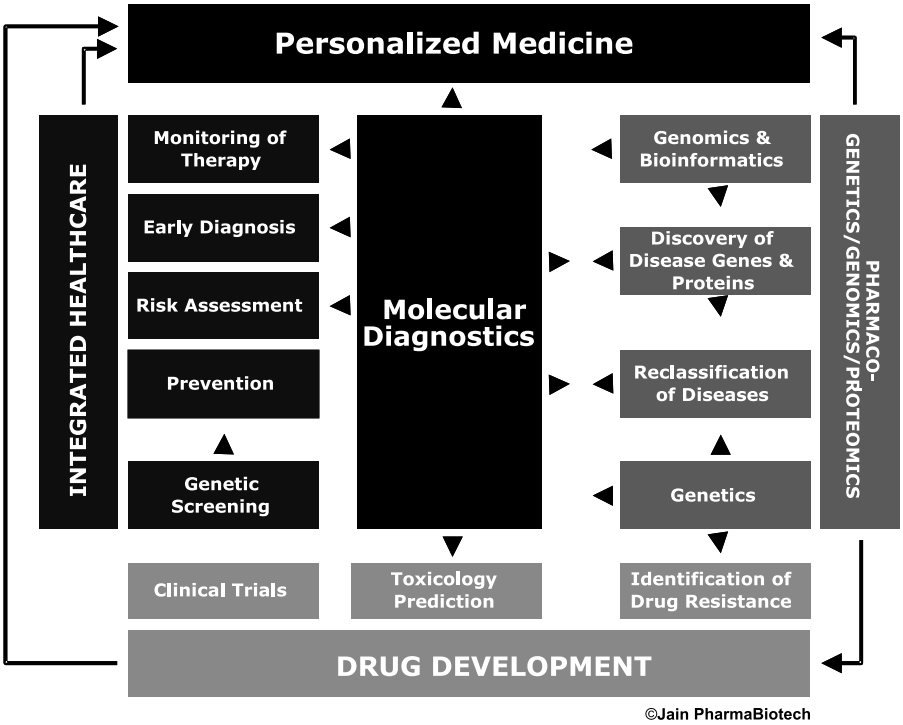

Fig. 1 Molecular diagnostics and personalized medicine. (Reproduced from Jain, K.K. Molecular Diagnostics, Jain PharmaBiotech Publications, Basel, 2004.) (*View this art in color at www.dekker.com.*)

offer the possibility of developing a rapid global analysis of the entire proteome leading to protein-based diagnostics and therapeutics.

Diagnostic applications of biochips in healthcare require a time-consuming and expensive validation process but provide the greatest area of potential growth with an emphasis on the point-of-care and personalized medicine. A further refinement of biochip technology using nanoparticles will enable SNP mapping point-of-care hand-held diagnostic devices and biomarker-based drug development as a basis for personalized medicines.

PHARMACOGENETICS

Pharmacogenetics has a threefold role in the pharmaceutical industry, which is relevant to the development of personalized medicines:[7]

1. For study of the drug metabolism and pharmacological effects
2. For predicting genetically determined adverse reactions
3. Drug discovery and development and as an aid to planning clinical trials

It is of considerable importance to know the metabolic status of an individual, particularly when using drugs with a narrow therapeutic range. Differences in metabolism of drugs can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Inter- and intra-individual variability in pharmacokinetics of most drugs is largely determined by variable liver function as described by parameters of hepatic blood flow and metabolic capacity. Among the factors affecting these parameters are genetic differences in metabolizing enzymes. The most important of these is P450.

Cytochrome P450

The cytochrome P450 (CYP450) enzyme system consists of a large family of proteins, which are involved in the synthesis and/or degradation of a vast number of endogenous compounds such as steroids, cholesterol, vitamins, and retinoic acid, as well as the metabolism of exogenous toxins. Cytochrome P450 enzymes can alter, abolish, or enhance drug metabolism. Genotyping or phenotyping can now be recommended as a complement to plasma concentration determination when aberrant metabolic capacity (poor or ultrarapid) of CYP2D6 substrates is suspected. The current rapid developments in molecular genetic methodology are expected to provide new tools for prediction of the activity of the various drug-metabolizing enzymes. Because cytochrome P450s play key roles in regulating important physiological processes, they are also attractive targets for drug discovery. Inhibitors of P450 enzymes are used clinically or are under evaluation for treatment of a number of diseases. AmpliChip CYP450 microarray (Roche Diagnostics) is commercially available for identification of polymorphisms in CYP2D6 and CYP2C19.

Genotyping

Currently, the most significant polymorphisms in causing genetic differences in Phase I drug metabolism are known, and therapeutic failures or adverse drug reactions caused by polymorphic genes can be predicted for several drugs. Patients are being genotyped in clinical trials. Genotype-based drug dose adjustment information can be useful when the drug is introduced into clinical practice and would enable the dose adjustment for individualized therapy.

Due to the rapid development of cost-effective methods for genotyping and the need to genotype only once in the lifetime of a patient, it would be advisable to include the genotype in the patient's record. It is also desirable to include the genotypes of transport proteins and drug receptors, which can reveal highly predictive genetic information. This would provide the physician with valuable information to individualize the treatment. Besides development of personalized medicines, the impact of genotyping on medical practice would shift the emphasis from present diagnosis-based treatment to detection of disease prior to clinical manifestation and preventive treatment with appropriate medicine and a dose that is most effective and safest for an individual.

ROLE OF PHARMACOGENOMICS IN PERSONALIZED MEDICINE

Molecular genetic methods may be applied both for genetic profiling (polymorphisms, mutations, etc.) of cohorts and for monitoring and guidance of therapies. Such tests can be used by the pharmaceutical companies to help identify suitable subjects for clinical trials, aid in interpretation of clinical trial results, find new markets for current products, and speed up the development of new treatments and therapies. However, only a few pharmacogenomic tests are commercially available and it remains to be seen what impact these will have on the market and on healthcare in general.

Pharmacogenomics in Drug Discovery

Pharmacogenomic analysis can identify disease susceptibility genes representing potential new drug targets. This can lead to novel approaches in drug discovery for individualized application of therapy. It may help focus effective therapy on smaller patient subpopulations which, although demonstrating the same disease phenotype, are characterized by distinct genetic profiles. In other words, personalization of drugs may start at the discovery stage.

Pharmacogenomics in Drug Development

Current applications of pharmacogenomics in drug development include prospective genotyping in Phase I trial to ensure that a subject population is representative with respect to drug metabolism phenotypes. Inclusion and exclusion of patients may be based on genotypes. The banking of genetic material from later stage trials for retrospective studies on drug response is becoming more frequent, but is not yet standard in the industry. Retrospective studies using collections of DNA that supply medical information on specific disease types, drug response, and ethnic composition could build a foundation for the evolution of medicine from diagnosis and treatment toward prediction and prognosis which are important components of integrated personalized medicine.[8]

PHARMACOPROTEOMICS

There is an increasing interest in proteomics technologies now because DNA sequence information provides only a static snapshot of the various ways in which the cell might use its proteins whereas the life of the cell is a dynamic process. Pharmacoproteomics will play an important role in the development of personalized medicine because proteomics-based characterization of multifactorial diseases may help to match a particular target-based therapy to a particular marker in a subgroup of patients. Individualized therapy may be based on differential protein expression rather than a genetic polymorphism. Advantages of using pharmacoproteomics are:

- Pharmacoproteomics is a more functional representation of patient-to-patient variation than that provided by genotyping.
- Because it includes the effects of posttranslational modification, pharmacoproteomics connects the genotype with the phenotype.
- Pharmacoproteomics could increase the predictability of early drug development and identify noninvasive biomarkers of toxicity or efficacy.[9]

ROLE OF BIOINFORMATICS IN PERSONALIZED MEDICINE

The massive amount of information generation by the Human Genome Project, detection of SNPs, and proteomic data would require bioinformatic tools for cataloguing and analysis of information. Bioinformatics will integrate various technologies and sources of information to facilitate the development of personalized medicine as shown in Table 2. Bioinformatic tools will facilitate informed therapeutic decision making by the physicians.

EXAMPLES OF PERSONALIZED THERAPIES

There are numerous examples where currently available genomic technologies are applied to individualize the treatment to patients who are likely to benefit from a

Table 2 Role of bioinformatics in the development of personalized medicine

Role of bioinformatics in molecular diagnostics as applied to personalized medicine
Analysis and classification of gene expression profiles
Analysis of single nucleotide polymorphisms
Computational diagnostics
Diagnosis of subtype of a disease to select the probability of success of optimal treatment
Genetic screening
Role of bioinformatics in pharmacogenomics
Genotyping for stratification of clinical trials
Selection of targets in pharmacogenomics-based drug discovery
Use of pharmacogenomic data to develop rational therapies
Role of bioinformatics in pharmacogenetics
Analysis of the role of polymorphisms in interindividual variations in drug response
Use of computational tools for predicting drug metabolism, toxicity, and efficacy
Integration of pharmacogenetic data with clinical outcomes to facilitate diagnosis
Linking of pharmacogenetic data to literature on adverse reactions and drug–drug interactions
Role of bioinformatics in pharmacoproteomics
Analysis of data from protein microarrays
Measurement of protein expression
Use of search engines for proteomic databases

Source: From Jain, K.K. Personalized Medicine. Jain PharmaBiotech Publications, Basel, 2004.

Table 3 Examples of personalized therapies

Approach to therapy	Comments
Genotyping as a guide to therapy for AIDS	Enables the monitoring of viral load and detection of resistance to treatment in personalized protocols for patients
Autologous cell vaccine is prepared from the patient's tumor and is injected back into the patient	The patient's immune system is better able to recognize, locate, and combat remaining cancer cells
HER-2 gene amplification and overexpression in breast cancer and can be detected by HER-2 breast test (Vysis' PathVysion)	This is an aid in the assessment of breast cancer patients who will respond to treatment with trastuzumab (Genentech's Herceptin)
A validated test that can identify a subgroup of hypertensive patients that should be treated with ACE-inhibitors as first line of treatment	This subgroup (30%) will show a much better response to ACE-inhibitors than the remaining population

certain therapy. There are many potential applications in cancer, genetic disorders, and infections. A few examples are shown briefly in Table 3.

ETHICAL AND SOCIAL ASPECTS OF GENETIC PROFILING

Genetic profiling for pharmacogenetic purposes is less likely to raise objections than genetic testing for primary disease risk assessment. Nevertheless, ethical issues concerning patient confidentiality, possible misuse of genotyping data, and possible harm to patient's social and insurability status need to be resolved before the implementation of pharmacogenetics in healthcare. Holding sensitive information on someone's genetic makeup raises questions of privacy and security and ethical dilemmas in disease prognosis and treatment choices. After all, polymorphisms relevant to drug response may overlap with disease susceptibility, and divulging such information could jeopardize an individual. On the other hand, legal issues may force the inclusion of pharmacogenetic information into clinical practice. Once the genetic component of a severe adverse drug effect is documented, doctors may be obliged to order the genetic test to avoid malpractice litigation.

FUTURE OF PERSONALIZED MEDICINE

Future medicine will be practiced in an environment in which molecular classification of diseases based on genomic analysis will replace the one based on symptoms. Molecular diagnostics to predict the results of therapy will be based on patient's genomic profile. There is still a considerable amount of work to be done before personalized medicine is established in clinical practice. Challenges facing development of personalized medicine are:

- One limitation to the application of pharmacogenomic techniques to personalized medicine is that current knowledge on phenotype–genotype correlation is based on statistical observations that are not totally verifiable on the individual level.
- Not all the treatments can be personalized.
- There is an initiative from the biopharmaceutical industry but little support has come from governments or healthcare organizations.
- There is a great need for education of the health professionals and patients on the advantages and limitations of personalized medicine.
- Ethical, legal, and social problems need to be addressed.
- Management of huge amounts of data needed whereas current bioinformatic manpower is limited.
- Technologies required for implementation of personalized medicine still need refinement.

A thorough understanding of the principles and applications of pharmacogenomics, the most important basic of personalized medicine, will be an indispensable part of the future of drug therapy in clinical practice. Personalized medicine scenario in the year 2010 for prevention could be as follows:

A patient would need to provide only a buccal smear sample in the physician's office for DNA analysis. This will provide information about predisposition to several diseases. This analysis may eventually be performed for a very reasonable cost as the genome chip becomes commercially viable. By identifying genetic predisposition to disease, the physician will focus on risk assessment and develop a comprehensive personalized plan to modify risk factors, and initiate preventive strategies.

Personalized medicine scenario in the year 2010 for treatment could be as follows:

A patient presents in the physician's office with certain symptoms and signs. The patient carries a biochip with record of his/her genetic information. The clinical diagnosis is supplemented with point-of-care molecular diagnostics. The treatment prescribed is personalized according to the patient's genetic makeup and the subtype of the disease and is linked to a diagnostic test.

CONCLUSION

Technological advances, along with new insights into the molecular pharmacology of medications and the functional consequences of polymorphisms in the human genome, are providing the tools needed to elucidate genetic determinants of drug response and translate functional genomics into personalized medicine.[10] Considerable evidence has now accumulated to show that the use of pharmacogenomics and pharmacogenetics as a basis of personalized medicine is feasible. Personalized medicines are available for a few disorders. Knowledge gained from pharmacogenomics and pharmacogenetics is being used to improve the use of currently available medicines and to discover and develop new medicines for specific groups of diseases. Advantages of personalized medicine for patients can be summarized as follows:

- Effective and specific therapies
- Less risk of adverse effects
- No time lost in trial and error with ineffective drugs
- Lower cost of treatment
- Facilitate development of preventive healthcare

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Peutz–Jeghers Syndrome

William M. Grady
Andrew M. Kaz
University of Washington Medical Center, Seattle, Washington, U.S.A.

INTRODUCTION

It is estimated that greater than 5% of all colorectal cancers (CRCs) can be directly linked to a specific, inherited allele. The ''polyposis syndromes,'' which are further subdivided into adenomatous and hamartomatous syndromes, comprise approximately 1% of these hereditary cancers.[1] The adenomatous polyposis syndromes consist of familial adenomatous polyposis (FAP) and attenuated FAP. The hamartomatous polyposis syndromes predominantly include Peutz–Jeghers syndrome (PJS), juvenile polyposis, and Cowden syndrome. The genetic etiologies responsible for significant subsets of these disorders have been previously identified.[2]

Peutz–Jeghers syndrome (PJS) is a rare, inherited syndrome characterized by gastrointestinal polyposis and mucocutaneous pigmentation. The histologically distinct polyps of PJS occur throughout the GI tract, but are most consistently found in the jejunum, ileum, and duodenum. Individuals with PJS are at risk for numerous intestinal and extraintestinal malignancies, including small bowel, stomach, breast, and gonadal tumors.[3] In the late 1990s, the genetic etiology of PJS was described by two groups of investigators.[4,5] Germline mutations in a serine/threonine kinase designated *STK11* (also known as *LKB1*), which functions as a tumor suppressor, are likely responsible for roughly half of PJS cases.[6]

This chapter discusses the clinical and genetic aspects of PJS, including the function of the *STK11* gene product, and lists the specific mutations that have been described to date. Special attention is paid to available genetic testing and current surveillance recommendations.

CLINICAL FEATURES OF PEUTZ–JEGHERS SYNDROME

Peutz–Jeghers syndrome is an autosomal dominantly inherited condition with an incidence of approximately 1 in 200,000 births.[2] This rare syndrome is characterized by hamartomatous polyposis of the GI tract, mucocutaneous hyperpigmentation, and a significantly increased risk of developing intestinal and nonintestinal malignancies. Symptoms attributed to PJS are often absent at the time of diagnosis, although patients can present early in life with gastrointestinal emergencies including intussusception, small bowel obstruction, and GI bleeding.[7] The hamartomatous polyps are located in the small bowel (particularly jejunum) in upward of 80% of patients, in the stomach in 40%, and in the colon or rectum in 40%.[1] Peutz–Jeghers syndrome polyps are composed of nonneoplastic tissue with a noticeably distorted architecture. Histologically, they are distinguished by normal epithelium covering frondlike projections that are composed of smooth muscle branching from the muscularis mucosa to the polyp head.[7] This pattern is often designated ''arborization.''

In addition to the characteristic hamartomas that occur in the gastrointestinal tract, pigmented macules along the vermillion border and in the interdigital spaces occur in individuals affected by PJS. Amplified numbers of melanocytes at the epidermal–dermal junction, containing increased basal-cell melanin, account for the blue to brown macules seen in PJS patients. Most commonly affected are the lips, the buccal mucosa, the skin around the eyes and nose, and the digits. The great majority of individuals with PJS (>95%) have evidence of increased melanin deposition. Although the macules on the lips and skin can fade over time, the buccal lesions typically persist.[8]

Requirements for Clinical Diagnosis

According to guidelines set forth by Giardiello et al., PJS can be diagnosed if the following criteria are met: the presence of histopathologically confirmed hamartomatous polyps, plus two or more additional criteria, including a positive family history, mucocutaneous hyperpigmentation, or small-bowel polyps.[9] Peutz–Jeghers can generally be differentiated from other similar syndromes by clinical criteria. Commonly confused conditions are juvenile polyposis (no pigmentation; hamartomas with different histology), mixed hereditary polyposis syndrome (no pigmentation; adenomatous and hyperplastic polyps), and Carney complex (pigmentation present; no GI polyps).[3,7]

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Peutz–Jeghers Syndrome and Cancer Risk

Peutz–Jeghers syndrome is a hereditary cancer-susceptibility syndrome, as evidenced by the development of malignancies in the great majority of patients during their lifetimes, the early age of cancer diagnosis in these family members compared to the general population, and the occurrence of cancers in related individuals in these families. Although the polyps in PJS patients are restricted to the gastrointestinal tract, the malignancies that arise in these families can be found in a variety of organ sites, including the gastrointestinal tract. The gastrointestinal malignancies can occur anywhere in the mucin-secreting portion of the luminal GI tract as well as in the pancreas. Interestingly, although foci of dysplasia can be found in larger Peutz–Jeghers polyps, the hamartomatous polyp of Peutz–Jeghers syndrome is typically a benign neoplasm. Most gastrointestinal cancers are believed to arise from coexisting adenomatous polyps. Nongastrointestinal tumors occur in the skin, thyroid, lung, breast, cervix, ovary, and testis. Of note, the mucocutaneous lesions of PJS are not premalignant.[1]

In regard to the magnitude of the cancer risk in PJS patients, Giardiello et al. reported a cumulative lifetime cancer risk in individuals with PJS of 93% in a recent meta-analysis.[10] These investigators also described the risk of developing pancreatic cancer in individuals with Peutz–Jeghers syndrome to be 100-fold greater than the general population.[9] Boardman et al. observed a 9.9-fold relative risk increase for all cancers; the risk was greatest for neoplasms of the gastrointestinal tract (RR = 151) and breast (RR = 20.3).[11] In another series of PJS patients, Lim et al. observed a 37% cancer risk by age 65 (47% risk in those carrying a *STK11* mutation), and a relative risk of 9.9 (13.2 with a *STK11* mutation)[12] (Table 1).

Neoplasms of the female, and rarely male, genital tract have also been observed with increased frequency in individuals with PJS. These lesions include ovarian sex cord tumors with annular tubules (SCTAT), mucinous ovarian neoplasms, and adenoma malignum of the cervix.[6]

Table 1 Risk of cancer in Peutz–Jeghers syndrome

Site	Relative risk	Reference
All cancers	10–15	[10–12]
Esophagus	57	[10]
Stomach	213	[10]
Small intestine	520	[10]
Colon	84	[10]
Pancreas	100–132	[9,10]
Lung	17	[10]
Breast	14–20	[10–12]
Ovary	27	[10]
Uterus	16	[10]

GENETICS OF PEUTZ–JEGHERS SYNDROME

Peutz–Jeghers syndrome is inherited in an autosomal dominant manner. Birth prevalence rates have been reported anywhere from 1:25,000 to 1:280,000. The disorder can occur in any racial or ethnic group.[3] Penetrance in PJS appears to be variable, and genotype–phenotype correlations are currently poorly characterized. In families with clinically evident PJS, some members appear to demonstrate only mucocutaneous pigmentation whereas others have both pigmentation and polyps. Approximately half of probands have an affected parent, and the other half have no family history of PJS, suggesting the presence of either genetic heterogeneity or modifier genes that can modulate the effects of mutant *STK11*.[3,13] The proportion of individuals who carry de novo mutations is currently unknown.

Amos et al. recently described the risk to family members after the identification of a proband with PJS. Fifty percent of probands will have an affected parent, although family history may appear negative because of lack of signs or symptoms of PJS, or the early death of a parent due to an unrelated cause. Siblings of the proband have a 50% risk of PJS if one parent is affected; if neither parent is affected, the risk appears to be minimal. Children of PJS patients have a 50% chance of inheriting the disorder if a positive family history or a *STK11* germline mutation is identified. The risk to the proband's offspring cannot be accurately determined if the proband has a negative family history or undetectable *STK11* mutation.[3]

STK11 AND PEUTZ–JEGHERS SYNDROME

In 1997, two loci for PJS were identified. The first was mapped to chromosome 19p13.3, near marker D19S886, by genomic hybridization and linkage analyses.[4] Subsequently, a second locus was described on 19q13.4 in the vicinity of D19S891.[14] The following year, two groups pinpointed the PJS gene on 19p13.3. Hemminki et al. noted truncating germline mutations on chromosome 19 in multiple patients with PJS. The gene, called *STK11* (or *LKB1*), is a serine/threonine kinase with strong homology to the Xenopus serine/threonine kinase XEEK1.[15] Jenne et al. described five separate germline mutations in *STK11* in a three-generation Peutz–Jeghers family and concluded *STK11* mutations are responsible for the development of

PJS in at least a subset of PJS families.[16] *STK11* germline mutations have been described in up to 100% of PJS families in some studies, although in other series, *STK11* mutations were absent in 33% to 42% of individuals with clinical manifestations of PJS.[17,18] Boardman et al. analyzed five PJS probands and 23 individuals with sporadic PJS, and found *STK11* mutations in only two and four patients, respectively. Thus PJS appears to demonstrate genetic heterogeneity, and it is likely that other loci will be implicated in this disease.[13]

The Function of the *STK11* Gene Product and Its Role as a Tumor Suppressor Gene

STK11 contains 10 exons spanning 23 kb and is expressed in all human tissues.[16] It encodes a protein kinase that is likely a tumor suppressor, as loss of the normal *STK11* allele has been described in the polyps of PJS patients with germline mutations in the other allele.[15] Peutz–Jeghers syndrome therefore became the first cancer-susceptibility condition described due to inactivation of a protein kinase.

Indeed, Mehenni et al. illustrated that mutant STK11 proteins expressed in COS7 cells demonstrated little or no protein kinase activity when compared with wild-type *STK11* and proposed that STK11's ability to phosphorylate other proteins was related to its role as a tumor suppressor.[19] The consequences of the deregulated kinase activity observed in STK11 mutant appear to be on the regulation of both cell proliferation and apoptosis. Tiainen et al. demonstrated that reconstituting cancer cells that carry mutant *STK11* with wild-type *STK11* resulted in a G_1 cell cycle arrest and the suppression of cell proliferation demonstrating the role of *STK11* as a tumor suppressor gene.[20] STK11 has been shown to regulate cell cycle progression through the induction of the cyclin-dependent kinase inhibitor p21 by a p53-dependent mechanism.[21] In addition to p53-dependent effects on cell cycle arrest, Karuman et al. illustrated the STK11 protein can physically associate with the p53 protein and probably regulates p53-dependent apoptosis.[22] STK11 normally translocates from cytoplasm to nucleus during apoptosis and is significantly upregulated in pyknotic intestinal cells, whereas polyps from PJS patients have been shown to lack STK11 by immunostaining. Thus deficient apoptosis regulation may be an important factor in the formation of intestinal polyps in PJS patients.[22] Consistent with the importance of cellular localization in the function of wild-type *STK11*, some mutations in *STK11* have been shown to affect the normal subcellular localization of STK11. For example, an SL26 mutation found in a PJS family, which results from a small in-frame deletion, produces a protein that retains its kinase activity but only resides in the nucleus, whereas normal STK11 is found in both cytoplasm and nucleus.[23]

Additionally, as demonstrated by Ylikorkala et al., STK11 likely participates in the vascular–endothelial growth factor (VEGF) pathway. Mice with targeted *Stk11* disruption died in midgestation, with evidence of neural tube defects and vascular anomalies. These phenotypes were correlated with noticeably increased levels of VEGF mRNA.[24] Bardeesy et al. generated $Lkb1^{+/-}$ mice and observed the formation of Peutz–Jeghers polyps in these mice and also found that cells from these mice showed loss of culture-induced senescence and modulation of factors related to angiogenesis, extracellular matrix remodeling, and cell adhesion.[25] Finally, other groups have found that STK11 binds to and regulates Brg1, a protein responsible for inducing cell cycle arrest. The inability of *STK11* mutants to mediate Brg1-dependent growth cessation may correlate with the development of the Peutz–Jeghers syndrome.[26]

Specific *STK11* Mutations

Mutations in the *STK11* gene typically lead to truncation of the protein product.[27] DNA sequencing of tissues from individuals with Peutz–Jeghers syndrome has led to the elucidation of over 100 mutations. Stenson et al. in the Human Gene Mutation Database have collected and categorized all 108 reported *STK11* mutations as of March 5, 2004, as follows: 40 missense/nonsense substitutions, 13 splicing substitutions, 30 small deletions, 10 small insertions, 3 small indels, 8 gross deletions, 1 gross insertion and duplication, and 3 complex rearrangements[28] (Table 2).

Table 2 Currently identified mutations in *STK11*

Type of mutation	Total number of mutations
Nucleotide substitutions (missense/nonsense)	40
Nucleotide substitutions (splicing)	13
Small deletions	30
Small insertions	10
Small indels	3
Gross deletions	8
Gross insertions and duplications	1
Complex rearrangements (including inversions)	3
	Total = 108

Source: Adapted from Ref. [28].

GENETIC TESTING FOR *STK11*

Genetic testing for germline *STK11* mutations is currently offered at four clinical laboratories.[29] DNA specimens isolated from peripheral blood or the buccal mucosa can be tested utilizing either genetic sequencing of the entire coding region, mutation scanning (e.g., SSCP, DHLPC, DGGE, etc.), or mutation analysis (i.e., assessment for specific mutations). The variability in the types of STK11 mutations identified in PJS families to date suggests that no single currently available mutation analysis technique is likely to be 100% accurate in identifying *STK11* mutations. In addition, at least a subset of PJS families appear to have pathogenic mutations outside of the coding region for the gene, suggesting some mutations will be missed with routine methods used currently in the clinical labs that offer testing.[28] One laboratory also offers prenatal diagnosis. Assaying for loss STK11 expression by immunostaining PJS polyps or cancers has also been proposed as a diagnostic method; however, the accuracy of STK11 immunostaining for the detection of STK11 germline mutation carriers is unknown at this time. In fact, Wei et al., demonstrated that the expression of STK11 in polyps is heterogeneous, even in polyps from a single patient. Furthermore, some polyps and cancers contained both nuclear and cytoplasmic expression of STK11, whereas others showed no evidence of STK11 expression.[30] Thus immunohistochemistry cannot be recommended as an effective adjunct to genetic testing at this time.

The detection rate of *STK11* germline mutations using genetic analysis is variable and depends upon whether a tested individual has a positive family history of PJS. Approximately 70% of individuals tested by sequence analysis who have a positive family history will harbor disease-causing mutations of *STK11*.[3–5,14] If a specific *STK11* mutation has been previously identified in an affected family member, other family members can be tested with accuracy approaching 100%.[1] The detection rate of *STK11* mutations appears to be lower in individuals without a known family history, ranging from 20% to 70%.[3,13]

In general, genetic testing is informative in two situations: 1) to predict whether an ''at-risk'' asymptomatic individual has inherited PJS already clinically diagnosed in a family member; or 2) to confirm the diagnosis of PJS in a patient where the clinical diagnosis is uncertain.[31] Testing of at-risk, asymptomatic family members (which typically involves pre- and posttest genetic counseling) might provide useful information relating to age of onset of PJS, as certain mutations may predispose to earlier development of the syndrome.[3] Additionally, patients harboring distinct *STK11* mutations might have an elevated cancer risk.[12] Testing children for PJS (which can become clinically manifest early in life) is similarly available after an *STK11* mutation has been identified in an affected family member. In all of these cases, genetic testing may allow individuals and their physicians to devise strategies regarding treatment and future cancer surveillance. Prenatal testing for PJS is currently available, although uncommonly performed.

SURVEILLANCE OF PEUTZ–JEHGERS PATIENTS

Because the lifetime cancer risk is significantly elevated in PJS patients compared to the general population, an aggressive cancer surveillance program has been proposed by several groups. In principle, such programs will prevent certain gastrointestinal malignancies and detect other cancers at an earlier stage, which should improve cancer-related mortality. These guidelines, which have not been formally adopted by professional medical organizations, are empiric and based upon expert opinion because of the lack of evidence-based clinical trial data.[1]

Recommendations from the St. Mark's Polyposis Registry, which has been modified by other groups, include the following: annual pancreatic ultrasound exams; annual pelvic ultrasound exams in females, and testicular ultrasound exams in males; biennial upper gastrointestinal endoscopic exams beginning at age 10; biennial colonoscopic exams beginning at age 25; and biennial small bowel X-ray studies starting at age 10. Any gastrointestinal polyps detected should be removed endoscopically and those polyps that cannot be endoscopically resected should be removed surgically via laparotomy with intraoperative endoscopy.[6] In addition, mammography should begin at age 25 and be repeated every 5 years until age 35, then every 2 years until age 50, followed by yearly exams after 50 years of age. Pap smears are recommended every 3 years.[7,32] Family members of PJS patients should be enrolled in surveillance programs if they have clinical evidence of PJS or a detectable *STK11* mutation.

CONCLUSION

The clinical description of Peutz–Jeghers syndrome has evolved over time and now can be defined specifically at the genetic level in a substantial subset of PJS patients. Currently, PJS can be defined as an inherited hamartomatous polyposis syndrome, associated with mucocutaneous lesions and an increased predisposition to numerous gastrointestinal and nongastrointestinal malignancies.

Many cases of PJS occur as a consequence of germline mutations in the *STK11* gene, a serine/threonine kinase that appears to be an important nuclear kinase involved in cell growth and apoptosis pathways. Genetic testing for *STK11* germline mutations is currently available on a clinical basis.

Finally, despite the recent advances in our understanding of PJS, *STK11* mutations do not account for all cases of PJS, and it is likely that additional genes or loci remain to be identified. In addition, further studies of *STK11* genotype/phenotype correlations are required to determine the role of allelic heterogeneity on the clinical manifestations of *STK11* germline mutations. These studies promise to lead to more accurate application of genetic screening and cancer surveillance strategies to PJS family members.

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Plasmodium spp.—Detection by Molecular Techniques

Gabriella A. Farcas
Kevin C. Kain
University of Toronto, Toronto, Ontario, Canada

INTRODUCTION

Malaria incidence is increasing, accounting for an estimated 300 to 500 million cases and 3 million deaths annually.[1] In the Americas, malaria incidence has risen dramatically, with as much as a 400% increase in reported cases in the last 20 years.[2] At least 30,000 travelers from industrialized countries are reported to contract malaria each year,[1] and despite treatment between 1% and 4% of those who acquire *Plasmodium falciparum* malaria will die. This fatality rate can climb to 20% or higher in those patients who develop severe malaria or those who are elderly.

MALARIA DIAGNOSTIC METHODS

A variety of diagnostic methods exist to identify the four malaria species and determine parasite burden in clinical samples; however, each method has advantages and disadvantages. Providing rapid and reliable malaria diagnosis continues to be a challenge for laboratories in both endemic and nonendemic countries. The utility and accuracy of the methods described below depend upon the level of technical expertise, training, and quality control as well as on the availability of necessary equipment.

Microscopy

Despite significant advances in the field, microscopic detection of parasites on Giemsa-stained blood smears remains the reference standard for malaria diagnosis in laboratories around the world, as it has been for over a century. It is generally available and relatively inexpensive, and it allows not only the identification of malaria species, but also the assessment of parasitemia. However, the staining and interpretation process is labor intensive and requires considerable expertise, particularly for accurate species identification at low parasitemia or when mixed infections occur. The ability to maintain the required level of expertise in malaria diagnostics is problematic, especially in peripheral medial centers in countries where the disease is not endemic.[3]

Alternative microscopic approaches such as those involving the use of fluorescent dyes (e.g., acridine orange), alone or after centrifugation (QBC; quantitative buffy coat), may be more sensitive but less specific for the diagnosis of *P. falciparum*, yet these methods are not reliable for species identification and require specialized equipment.[4]

Immunochromatographic Antigen Detection Tests

The World Health Organization has recognized the need for simple, cost-effective, and accurate diagnostic tests for malaria which could overcome the expertise-associated limitations of microscopy as well as the changing patterns of accepted morphology of malaria species due to drug pressure, strain variation, or approaches to blood collection.[5]

Nonmicroscopic malaria-diagnostic devices rely on the detection of malaria antigen, such as parasite histidine-rich protein 2 (e.g., NOW ICT *Pf/Pv*™, PATH Falciparum Malaria IC™, and Parasight F™), or parasite LDH (e.g., OptiMAL™) in whole blood. The underlying principle of immunochromatographic procedures utilize conjugated monoclonal antibodies against the malaria antigen of choice as an indicator of infection.

Histidine-rich protein 2 (HRP-2) is a *P. falciparum*-specific antigen, therefore assays limited to the detection of HRP-2 are unable to detect other human *Plasmodium* species. A newer generation of rapid diagnostic assays (RDA) use HRP-2 as well as aldolase, a pan-malarial antigen, to identify nonfalciparum infections, yet they remain unable to differentiate between the nonfalciparum species.

Parasite LDH (pLDH)-based assays take advantage of the species-specific isomers of this enzyme from the parasite glycolytic pathway. Parasite LDH-based RDAs have a *P. falciparum*-specific antibody and two pan-malarial antibodies, which recognize nonfalciparum pLDH.

Compared to microscopy and PCR, RDAs range widely in sensitivity and specificity. One of the latest HRP-2-based assays was 96% sensitive for pure *P. falciparum* and 94% for mixed *P. falciparum* infections

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and 84% sensitive for nonfalciparum infections with an overall specificity of 96%.[6] However, for both falciparum and vivax infections, sensitivity fell as parasitemia declined.[6] In a recent evaluation of returning travelers in Berlin, the OptiMal test (a pLDH assay) was found to be 76.2% sensitive and 99.7% specific.[7] In another study, the OptiMAL assay failed to detect malaria infection in 15% of cases.[8] The sensitivity for detection of *P. falciparum* infection was 87%, and for *P. vivax* was 79%. Similar to HRP-2 tests, the sensitivity of the pLDH tests decreased significantly with lower parasite densities (<500/μL).[8]

False negative and false positive results do occasionally occur with these devices, even at high parasitemias, possibly due to prozone effect (false negatives), the possible lack of or alteration in the *hrp 2* gene (false negatives),[9] or the presence of rheumatoid factor (false positives).[10] Importantly, if the clinical suspicion of malaria remains high despite a negative RDA result the assay should be repeated within 12 to 24 hr[9] and these assays should be accompanied by thick and thin blood smears.

Despite some inherent limitations, evidence suggests that rapid malaria diagnostic devices may represent a useful adjunct diagnostic tool to microscopy in a clinical setting while definitive results are sought from a reference laboratory.

Molecular Methods—Radiolabeled Probes

As an alternative approach to the detection and species identification of malaria, species-specific probes were developed in the late 1980s and early 1990s. Hybridization of radiolabeled oligonucleotide probes to complementary species-specific regions of the abundant and stable parasite small ribosomal subunit RNA (ssRNA)[11] seemed promising for the detection of the four species of human Plasmodia. This method of detection is not commonly used as a diagnostic tool.

Molecular Methods—Polymerase Chain Reaction (PCR)

Nested PCR

Polymerase chain reaction-based diagnostic methods for malaria represent a major advancement, surpassing microscopic methods both in sensitivity and specificity. Polymerase chain reaction can detect as few as 1–5 parasites/μL of blood (<0.0001% of infected red blood cells) compared to 50–100 parasites/μL using microscopy or RDAs.[12–14] Furthermore, PCR can readily detect mixed-species infections and may be automated allowing processing of large number of samples.

The multicopy 18S (small subunit) rRNA genes of *Plasmodium* spp. that infect humans have been demonstrated to be highly stable and conserved. Assays to detect them have displayed no cross-reactions to human DNA or other human pathogen DNA/RNA including nonhuman *Plasmodium* spp.[11,13,14] The 18S gene of *Plasmodium* is an ideal molecular target for malaria parasite identification, both for the abundance of the DNA gene targets and because it is composed of a mosaic of conserved and variable regions, allowing the amplification of sequences from samples using primers that are conserved within every member of the genus *Plasmodium*.[15,16] Identification to the species level, however, requires an additional round of PCR, a nested PCR, with species-specific primers.

Although this nested-amplification procedures is highly sensitive and specific, it is more cumbersome and expensive than single PCR assays. Also, because it is an open system (i.e., open transfer of amplicons between tubes) there is an inherent risk of contamination. Amplified products can be detected by gel electrophoresis, Southern or slot blotting followed by hybridization with DNA probes or by a colorimetric detection procedure with an enzyme-labeled antibody and chromogenic substrate in a microtiter format. The detection of amplification products by chemiluminescence hybridization or enzyme immunoassays in microplate format offers better potential for standardized commercial assays and automation.[12,17–19]

Real-time PCR

Despite their superior sensitivity and specificity over microscopy, traditional PCR, and particularly nested PCR, methods[14] are labor intensive with turnaround times that are generally too long for routine clinical application. Moreover, these are open systems that require considerable pre- and postsample handling and therefore special efforts need to be employed in order to prevent false positive assays. Real-time quantitative PCR technology has the potential to overcome these limitations, offering a simple, time-effective, and quantitative diagnostic option. Using DNA binding dyes, such as SYBR green, molecular probes, or hybrids labeled with fluorescent probes, real-time PCR can detect and quantify amplicons in as little as 40 min.

The closed amplification vessels utilized in this system minimize the need for excessive sample handling, particularly when compared to nested reactions, and eliminate additional post-PCR sample handling steps normally required for amplicon detection. These features

significantly reduce the potential for sample contamination. When the real-time assay is combined with the available robotic sample preparation/nucleic acid extraction modules, the process may be fully automated. This combined with their ease of use and rapid turnaround times makes these assays well suited to routine diagnostic laboratories.

Although a number of in-house real-time assays for malaria diagnosis have been developed, few meet the good manufacturing practices (GMP) standards for commercialization. One commercially available real-time PCR assay for malaria diagnosis has recently been evaluated.[20] It contains reagents and enzymes for the specific amplification and detection of a species-conserved 140-bp region of the *Plasmodium* 18S rRNA genes of all four human malaria species. In addition to the hybridization probe used for amplicon detection, the assay contains a second heterologous amplification system to identify potential PCR inhibition in samples. Four quantification standards with known concentrations of cloned gene copies per microliter (equivalent to 70 to 70,000 genome copies per microliter) are used as known positive controls and to generate a standard curve to assess parasite burden.

Direct correlations between parasitemia, as determined by microscopy, and gene copy number, as determined from the standard curve, are somewhat confounded by the multicopy nature of the rRNA genes, by the variable numbers of these genes within each species, and by the presence of multinucleate schizont stages. However, these two approaches represent alternative methods of quantifying parasite burden within a sample and additional clinical trials will be required in order to determine which method is more predictive of clinical outcome.

One limitation of the current generation of real-time assay is their inability to differentiate between the four *Plasmodium* species. In its current form, a positive assay would need to be accompanied by a malaria smear or nested PCR in order to ascertain the *Plasmodium* spp. involved, unless the assay is used primarily as a screening test or to exclude malaria in blood products. The high cost of this state-of the-art technology places real-time PCR-based assays out of reach for developing countries. However, diagnostic laboratories may find the rapid turnaround time, quantitative results, high sensitivity, and the excellent negative predictive value (allowing one to exclude malaria in a patient) to have potential impact on patient care.

Improved real-time PCR malaria diagnostic devices are underway. These utilize fluorescence resonance energy transfer (FRET) technology to differentiate species based on differing melting curve profiles. With a quantification component and an internal control component, this new generation of diagnostic tools will yield sensitive and specific results in under an hour.

Mass Spectrometry

A novel blood-based malaria diagnostic approach has recently been published.[21] Ultraviolet laser desorption mass spectrometry (LDMS) is based on the detection of hemozoin (malaria pigment) formed by the parasites during the intraerythrocytic growth phase. As all four human malaria species catabolize host hemoglobin releasing hemozoin in blood, this by-product becomes a pan-malarial diagnostic biomarker. The LDMS detects the heme within the hemozoin, but not heme bound to intact hemoglobin with a sensitivity of 100 parasites per microliter, comparable to that of an average microscopist. A semiquantitative relationship seems to exist between heme signal and parasitemia, rendering this a quantitative diagnostic assay. Upon further field validation, this could become a powerful, low-cost tool (<U.S.$0.05) for rapid (<5 min) and high-throughput malaria screening by nonspecialists. Although a field-portable version of this assay is currently under development, this assay is limited by its inability to distinguish between *Plasmodium* species.

Microarrays

The publication of the *Plasmodium* genome offers much opportunity in the field of malaria diagnostics. Although at present an expensive technology suited only to reference laboratories, microarrays may play an important role in future infectious disease diagnostics. Species-specific malaria diagnosis will be combined with genetic markers for other febrile infectious diseases to further enhance the diagnostic screening process of patients.

CONCLUSION

Polymerase chain reaction-based assays are increasingly replacing microscopy as reference standards for malaria diagnostic, molecular epidemiological, and treatment studies. The disadvantages and limitations of this procedure are being recognized and addressed in the new generation of PCR-based diagnostic assays such as real-time PCR. Implementation of techniques such as real-time PCR can streamline the routine molecular diagnostic laboratory and can take greater advantage of the recent advances in gene discovery and drug-resistance mutations. As it becomes more affordable and more standardized, microarray technology may well become the future

of molecular detection, characterization, and diagnosis of malaria.

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Plasmodium spp.—Detection of Drug Resistance by Molecular Techniques

Gabriella A. Farcas
Kevin C. Kain
University of Toronto, Toronto, Ontario, Canada

INTRODUCTION

The increase in malaria incidence worldwide is due, in large part, to the development and spread of drug-resistant malaria parasites. Resistance has emerged to virtually all classes of antimalarials, although at very different rates. For the oldest antimalarials [quinine (Q) and artemisinin derivatives], resistance has been slow to develop and treatment failure with these drugs is not an operational problem in most malaria-endemic areas. Until recently, there was no confirmed artemisinin resistance; however, Kyle[1] and Peters[2] recently generated a stable isolate of artemisinin-resistant *Plasmodium falciparum* malaria. Although resistance to artemisinin has not yet been reported in natural infections, the use of this drug and its derivatives is associated with a high rate of recrudescence after monotherapy; therefore it must be used in combination with longer-lasting antimalarials.[3]

For newer synthetic antimalarials, resistance has developed much faster, sometimes at an alarming rate. Chloroquine (CQ) was long considered the prophylactic and therapeutic agent of choice for *P. falciparum.* Chloroquine-resistant (CQR) falciparum malaria first emerged in Southeast Asia and South America over half a century ago, and subsequently spread to almost all malaria-endemic areas except Central America, above the Panama Canal, Haiti, and parts of China and the Middle East.[4] CQ resistance occurs both in *P. falciparum* and *Plasmodium vivax*, but the former accounts for most of the global disease burden. CQR *P. vivax* malaria is mostly concentrated in Oceania (Papua New Guinea, Vanuatu, and Indonesia) but has also been confirmed in South America.[4–7] As of yet, no confirmed resistance has been documented for *Plasmodium malariae* or *Plasmodium ovale.*

The advent of CQ resistance led to the development of other synthetic antimalarials including sulfadoxine pyrimethamine (SP), mefloquine (MQ), halofantrine, and atovaquone (ATQ)–proguanil (Malarone™). SP is still used as first-line therapy for treatment of falciparum malaria in much of sub-Saharan Africa and South America, despite evidence for escalating resistance in these regions.[7] MQ is now commonly used in many regions of Southeast Asia, Africa, and South America, and remains an efficacious antimalarial in most endemic regions on the borders of Thailand, with Myanmar and Cambodia where multidrug-resistant falciparum malaria has been the norm since the late 1980s.[4] ATQ–proguanil is the latest antimalarial to be approved for the treatment of falciparum malaria in many western countries. Although presently very efficacious for the treatment of *P. falciparum* in all endemic regions including areas of multidrug resistance, ATQ–proguanil resistance has now been documented in a limited number of cases, primarily travelers returning from Africa.[8,9]

DETERMINANTS OF ANTIMALARIAL RESISTANCE

Many factors may potentially contribute to the establishment and spread of drug-resistant malaria.[10] At the molecular level, drug resistance may arise because of several different mechanisms, including changes in drug accumulation within the parasite, alterations in drug efflux mechanisms, and secondary point mutations within parasite enzymes and other drug targets. Single-point mutations within drug targets often render parasites more ''fit'' under drug pressure. These mutational events are accelerated in the presence of subtherapeutic drug dosing, particularly in the case of drugs with long half-lives.

DETECTION TECHNIQUES

Resistance to conventional antimalarials is escalating, and novel strategies to monitor for resistance are needed. There are no simple bedside methods for assessing antimalarial drug susceptibility. Therapeutic response to antimalarials can be assessed in vivo by using the World Health Organization (WHO) treatment protocols,[11] as well as in vitro by measuring the parasite's sensitivity by inhibition of growth or maturation of blood stages. Both of these strategies have a number of limitations.[12] More recently, a number of studies have begun to elucidate the

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genetic basis of drug resistance. This information has been translated into molecular assays to characterize malaria isolates for genetic markers of drug resistance. These studies have now been extended, suggesting that these biomarkers can be useful in predicting treatment failures and that they can be used to detect and track global patterns of drug-resistant malaria.[12]

RESISTANCE MARKERS

Molecular markers for drug resistance identified to date include *pfcrt* polymorphisms associated with CQ drug resistance; *dhfr* and *dhps* polymorphisms associated with SP resistance; and *pfmdr1* polymorphisms putatively associated with modulating resistance to CQ, MQ, Q, and artemisinin.[4]

Chloroquine and Quinine

CQ acts by disrupting heme metabolism in the digestive vacuole of *P. falciparum*. CQR parasites survive by preventing the accumulation of CQ within the vacuole; however, the precise mechanism by which this occurs is unknown.[13]

Polymorphisms in two genes of the *P. falciparum* genome have been implicated in molecular CQ resistance studies. The *pfcrt* gene is located on chromosome 7 and codes for PfCRT, a vacuolar membrane transporter protein. Although a number of polymorphisms in this gene are associated with CQ resistance, the substitution of threonine for lysine at position 76 has been shown to be most closely associated with in vivo resistance.[14,15] This association comes with a caveat, namely that some chloroquine-sensitive (CQS) malaria strains also harbor this mutation, suggesting that K76 is required for CQ resistance but that other *pfcrt* polymorphisms must also be involved. However, in vivo outcomes are also clearly influenced by the level of underlying immunity (premunition) to malaria in the individual being treated, and this may limit the ability of mutations in *pfcrt* to predict in vivo outcome following CQ therapy.[16,17] However, the wild-type sequence does reliably predict successful treatment outcome with CQ.

The second *P. falciparum* gene implicated in CQ resistance, *pfmdr1*, is located on chromosome 5 and encodes the p-glycoprotein homologue (Pgh1). The aspartic acid-to-tyrosine point mutation at codon 86 has been associated with CQ resistance, as have a number of other polymorphisms in this gene (Asp 1042, Tyr 1246, Phe 184, and Cys 1034). However, these mutations have not consistently been associated with in vivo CQ resistance, although they may well modulate susceptibility to CQ, MQ, Q, and halofantine.[18] Furthermore, genetic cross-experiments between CQS and CQR parasite lines and parasite transfection studies have not supported a direct role for *pfmdr1* mutations in CQ resistance; rather, these studies have identified mutations in a digestive vacuole transmembrane protein, PfCRT, as causally linked to CQ resistance.[15]

There have been suggestions that *pfmdr1* mutations associated with CQ resistance may also account for reduced parasite susceptibility to Q; however, this needs further confirmatory studies.[19,20]

Mefloquine

The mechanisms of action and resistance to MQ have not been fully elucidated. The *pfmdr1* gene has been investigated as a molecular marker for MQ resistance, but evidence remains controversial. It was suggested that both variations in gene copy number and point mutations at positions 86, 184, 1034, 1042, and 1246 of the *pfmdr1* gene may confer drug resistance, but these findings have been challenged. Some investigations have reported increased sensitivity with the Tyr 86 mutation in *pfmdr1*, whereas others have found no effect.[18] To date, field studies do not consistently support a direct role for these mutations in vivo treatment outcomes.[7]

Sulfadoxine Pyrimethamine (Fansidar™)

The molecular basis of resistance for SP is perhaps the best characterized. Sulfadoxine and pyrimethamine act synergistically, with the former inhibiting dihydroptoate synthase (DHPS) and the latter inhibiting dihydrofolate reductase (DHFR), both of which are enzymes involved in folate synthesis. Point mutations at five *dhps* codons (Gly 437, Glu 540, Gly 581, Ala 436, and Ser 614) have been implicated in resistance to SP by decreasing the binding affinity of the enzyme. Mutations in *dhfr* associated with SP resistance include Ile 51, Arg 59, and Leu 164, with Asn 108 representing the key mutation for SP resistance. The degree of SP resistance increases in a stepwise fashion in response to progressive accumulation of these mutations. In recent in vivo studies in Cameroon and Kenya, the triple DHFR mutation at codons 108, 59, and 51 was associated with early treatment failure,[21] suggesting that these could be useful markers for predicting the therapeutic effectiveness of SP in a given area.[22,23] Of interest, there may be some overlap between existing SP resistance and emergence of resistance to newer antifolate combination drugs such as chlorproguanil dapsone (LAPDAP).[24] Hence, screening of *dhfr* and *dhps* mutations may also provide molecular data to guide the regional implementation of LAPDAP programs.

Atovaquone–Proguanil (Malarone)

ATQ and proguanil operate synergistically. ATQ is a 2-hydroxynaphtoquinolone that disrupts electron transport in the parasite mitochondria at the cytochrome *bc*1 complex and collapses membrane potential, without affecting the host mitochondria.[25,26] Proguanil significantly enhances the ability of ATQ to collapse mitochondrial membrane potential, and the combination drug should slow the emergence of AVQ-resistant parasites. However, mutations in the cytochrome *b*1 gene have appeared and are associated with ATQ/Pro resistance in vitro and in vivo. A *P. falciparum* isolate from a Thai patient with recrudescence after ATQ therapy showed a mutation at codon 268 with a tyrosine-for-serine substitution and an increased IC_{50} of almost 10,000-fold. A small number of ATQ/Pro treatment failures have now been reported primarily in nonimmune patients. Most have involved a N268S mutation, although one patient isolate contained a tyrosine-for-asparagine substitution at the same position.[8,9,27,28] ATQ resistance parasites generated in vitro may possess additional mutations in cytochrome *b*, including mutations at positions 133, 272, and 280, although these mutations impart lower levels of resistance in vitro and have not been observed in patient isolates to date.[29]

MOLECULAR TECHNIQUES FOR THE DETECTION OF ANTIMALARIAL DRUG RESISTANCE

Mutation analysis for antimalarial drug resistance currently includes a variety of strategies including PCR amplification followed by direct sequencing, single-strand conformation polymorphism (SSCP) assays, PCR followed by restriction fragment length polymorphism (RFLP) assays, allele-specific amplification assays, and real-time assays to detect point mutations conferring resistance.

In PCR-RFLP analysis, restriction enzymes are used to cut the PCR product at a specific location, proximal to the mutation site, such that wild-type and mutation-containing fragments display differing digestion patterns on agarose or polyacrylamide gel electrophoresis. SSCP is another gel mobility strategy used to detect single-point mutations in amplified DNA. PCR is used to amplify the region of interest and the resultant DNA is separated as single-stranded molecules by electrophoresis on a nondenaturing polyacrylamide gel. Wild-type and mutant single-stranded DNA fold differently, affecting their motility rate through the gel. The mutations are detected as new bands on autoradiograms (radioactive detection), by silver staining of bands or the use of fluorescent PCR primers that are subsequently detected by an automated DNA sequencer (nonradioactive detection).

Real-time PCR using hybridization probes to identify mutants based on specific melting curve patterns of the amplicon is also being developed.[30] The wealth of genetic information afforded by the completion of the *Plasmodium* genome will certainly help identify new drug targets, further our understanding of the molecular basis of drug resistance, and facilitate the creation of rapid assays for the detection and surveillance of drug-resistant malaria.

CONCLUSION

The predictive value of molecular markers of resistance is best assessed in the context of in vivo field trials using the WHO in vivo model. The association of molecular markers with in vivo resistance is confounded by several factors. First, in vivo studies are often carried out in regions where malaria is hyperendemic and adaptive immunity (premunition) exists. Such immunity may confound the interpretation of drug efficacy because premunition may facilitate clearance of drug-resistant parasites. Second, technical issues may confound interpretation. Many studies fail to check for drug levels to ensure that adequate drug absorption has occurred, thereby overestimating the number of resistant infections. In addition, studies often fail to use techniques to ensure that treatment failures are true recrudescences rather than reinfections.[31,32]

Most current models of resistance assume single-gene models; however, resistance may be multigenic particularly with respect to 4-aminoquinoline drugs such as MQ, and implicated genes and mutations still require further in vivo validation. In contrast, ATQ and SP mechanisms of action are better defined, allowing for a clearer understanding of the role of mutations in key proteins such as CYTb and DHFR/DHPS in mediating resistance. Ultimately, the use of validated genetic markers of drug resistance will facilitate molecular surveillance and global monitoring for emerging drug-resistant malaria, and will potentially serve in predicting treatment outcome.

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Platelet Antigen Genotyping

Evelyne Kretzschmar
Heiner Trobisch
Institute for Transfusion Medicine, Duisburg, Germany

INTRODUCTION

Alloantibodies against platelet antigens play an important role in immune-mediated platelet disorders. There are essentially three clinical conditions caused by platelet alloantibodies: platelet transfusion refractoriness, neonatal alloimmune thrombocytopenia (NAIT), and posttransfusion purpura. In NAIT, fetal thrombocytopenia is caused by maternal alloimmunization against antigens on fetal platelets, a condition which may result in life-threatening complications. Typing of the human platelet antigens can be useful for the diagnosis and treatment of these patients. Typing of human platelet antigens is also important for the study of the association of human platelet antigens with an increased probability of developing various diseases. Recently, several studies indicated that polymorphisms underlying platelet antigens might represent genetic risk factors for coronary thrombosis.

In the past two decades platelets were characterized by serological antigen capture assays, such as the monoclonal antibody immobilization of platelet antigens. The introduction of polymerase chain reaction (PCR) technology for amplification of platelet-specific mRNA made the characterization of platelet alloantigens possible at the molecular level and opened the doors for the development of new typing techniques. All but one of the different allelic forms of the human platelet antigens were the result of single nucleotide polymorphisms (SNPs) in the genes encoding the relevant platelet proteins (see below).

In this review, an overview of various genotyping techniques will be presented. New techniques which make human antigen genotyping even more convenient will also be discussed.

HUMAN PLATELET ANTIGENS

Nomenclature

Historically, multiple names existed for the human platelet antigens. In 1990, the Platelet Serology Working Party of the International Society of Blood Transfusion (ISBT) and the International Committee for Standardization in Haematology (ICSH) suggested a numerical terminology which has been widely accepted.[1] The term HPA was introduced to indicate *h*uman *p*latelet *a*ntigen. By convention, ''system'' is assigned to an HPA where both SNP and alloantibodies have been identified. The different antigens are numbered in the order of their discovery. The high-frequency allele of a system is named first (=a) and its low-frequency allele second (=b). The ''W'' after the antigen name is added when alloantibodies for only one allele have been defined. Six biallelic alloantigen systems (HPA-1 to -5 and HPA-15) and the low-frequency antigens HPA-6W to HPA-16W have been described. Table 1 lists HPA types, previous designations, associated nucleotide and amino acid changes, the frequency of each antigen in Caucasians, and the corresponding glycoproteins. Phenotype frequency varies significantly within populations. The Platelet Nomenclature Committee of the ISBT and the International Society of Thrombosis and Haemostasis (ISTH) developed, in 2003, a new nomenclature system.[3] The Platelet Nomenclature Committee maintains a web site with the latest changes, as well as with the positions for the newly assigned single nucleotide polymorphisms.[3]

Most platelet alloantigenic determinants characterized so far were formed by single amino acid substitutions induced by a point mutation of the respective gene. One exception is the rare platelet alloantigen HPA-14W which was formed by one amino acid deletion (Lys611del) of platelet GPIIIa (Table 1).

Some of the ''platelet-specific'' antigens have been detected on other cells as well. Therefore antigens are now called ''platelet associated'' and are divided into two groups: ''platelet-nonspecific'' antigens which are shared by platelets and many diverse cell types, and the ''platelet-specific'' antigens which are uniquely expressed by platelets.

Localization on Platelet Membrane Glycoproteins

The platelet membrane glycoproteins GPIa, GPIbα, GPIbβ, GPIIb, GPIIIa, and GPI-linked CD109 have been

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Table 1 Human platelet antigens and their polymorphisms

Antigen[a] (previous designation)	Phenotype frequency in Caucasians [%][b]	Nucleotide[c]	Amino acid	Glycoprotein location (synonym)
HPA-1a (Zw^a, PlA^1)	97.9	T196	Leu33	GP IIIa (β_3)
HPA-1b (Zw^b, PlA^2)	28.8	C196	Pro33	
HPA-2a (Ko^b)	>99.9	C524	Thr145	GP Ibα
HPA-2b (Ko^a, Sib^a)	13.2	T524	Met145	
HPA-3a (Bak^a, Lek^a)	80.95	T2622	Ile843	GP IIb (α_{IIb})
HPA-3b (Bak^b)	69.8	G2622	Ser843	
HPA-4a (Yuk^b, Pen^a)	>99.9	G526	Arg143	GP IIIa (β_3)
HPA-4b (Yuk^a, Pen^b)	<0.1	A526	Gln143	
HPA-5a (Br^b, Zav^b)	99.0	G1648	Glu505	GP Ia (α_2)
HPA-5b (Br^a, Zav^a, Hc^a)	19.7	A1648	Lys505	
HPA-6W (Ca^a, Tu^a)	0.7	G1564	Arg489	GP IIIa (β_3)
		A1564	Gln489	
HPA-7W (Mo^a)	0.2	C1267	Pro407	GP IIIa (β_3)
		G1267	Ala407	
HPA-8W (Sr^a)	<0.01	C2004	Arg636	GP IIIa (β_3)
		T2004	Cys636	
HPA-9W (Max^a)	0.6	G2603	Val837	GP IIb (α_{IIb})
		A2603	Met837	
HPA-10W (La^a)	<1.6	G281	Arg62	GP IIIa (β_3)
		A281	Gln62	
HPA-11W (Gro^a)	<0.25	G1996	Arg633	GP IIIa (β_3)
		A1996	His633	
HPA-12W (Iy^a)	0.4	G141	Gly15	GP Ibβ
		A141	Glu15	
HPA-13W (Sit^a)	0.25	C2531	Thr799	GP Ia (α_2)
		T2531	Met799	
HPA-14W (Oe^a)	<0.17	1929_1931		
		delAAG	Lys611del	GP IIIa (β_3)
HPA-15a (Gov^b)	60.2	C2108	Ser703	CD 109
HPA-15b (Gov^a)	80.5	A2108	Tyr703	
HPA-16W (Duv^a)	<1.0	C517	Thr140	GP IIIa (β_3)
		T517	Ile140	

[a]References are listed in Ref. [2].
[b]Phenotype frequencies adopted from: http://www.nibsc.ac.uk.
[c]The old nucleotide positions are listed. For newly assigned nucleotide positions, see Ref. [3].
Source: Modified after Ref. [2].

identified as carriers of platelet alloantigenic determinants. Some of these glycoproteins are part of the integrin family and are membrane glycoprotein heterodimers consisting of noncovalently associated α and β subunits. Two platelet membrane receptors that figure prominently in the antigenic profile of platelets, the cohesion receptor GP IIb–IIIa, now called $\alpha_{IIb}\beta_3$, and the collagen receptor GP Ia–IIa ($\alpha_2\beta_3$), are integrins. The GP Ib–IX–V receptor, as another carrier of antigenic epitopes, is an integrin as well. The cohesion receptor is expressed up to 80,000 times on the platelet surface and mediates the common cohesive pathway with adhesive proteins such as the von Willebrand factor and fibrinogen. The platelet collagen receptor plays a major role in the adhesion of platelets to collagens. The Ib–IX–V complex functions as a receptor for von Willebrand factor and enables platelets to interact with blood vessel walls under physiological or stress conditions. Cartoons of GP IIb–IIIa, GP Ia–IIa, and the receptor complex GP Ib–IX–V with major HPA sites are shown in Fig. 1. Their CD nomenclature, their Genbank accession numbers, and their corresponding HPAs are listed in Table 2.

DETECTION OF HUMAN PLATELET ANTIGENS

Many studies have reported successful molecular detection of various HPAs. These techniques are based upon

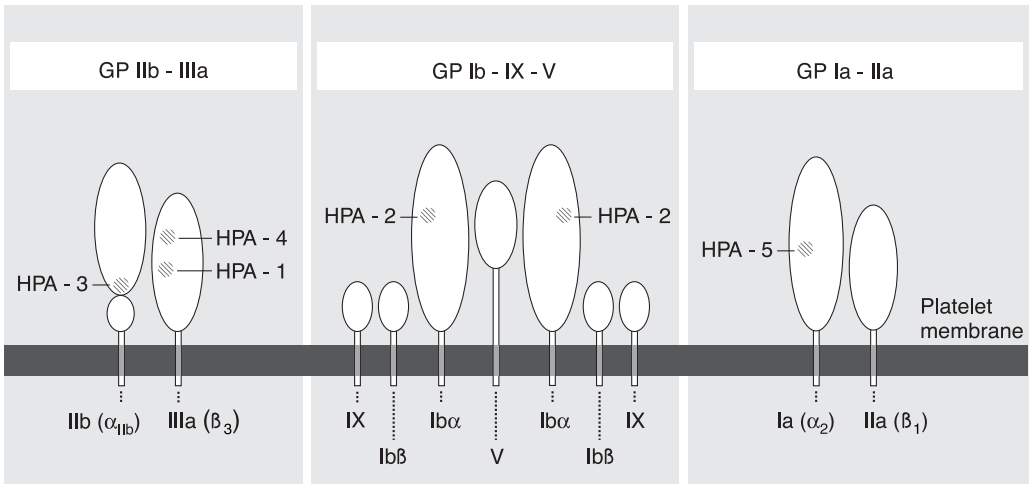

Fig. 1 HPA-1 to -5 on glycoprotein receptors. Three receptor complexes on the platelet membrane with HPA-1 to -5 sites (hatched areas) are shown schematically: the platelet cohesion receptor GP IIb–IIIa, the receptor complex GP Ib–IX–V, and the platelet collagen receptor GP Ia–IIa. The GP Ib–IX–V complex exists on the platelet surface with a suggested stoichiometry of 2:2:2:1 (GP Ibα/GP Ibβ/GP IX/GP V).

conventional PCR where post-PCR steps are required. These conventional techniques will be discussed first and will be followed by discussion of newer techniques not requiring post-PCR steps.

Allele- or Sequence-Specific PCR (SSP-PCR)

Polymerase chain reaction with sequence-specific primers (SSP-PCR) is currently the most widely used technique for HPA genotyping. This approach is based on the principle that a 3′ mismatched nucleotide in a primer-template hybrid will prevent extension during PCR, therefore inhibiting DNA amplification. That is, when the 3′ nucleotide of a primer is complementary to the sequence at the site of allelic variation, a PCR product will be generated. However, when the 3′ nucleotide of the primer is mismatched, the amplification of the template will not occur or will proceed with very low efficiency. For the detection of each HPA allele, two sets of primers are designed, each set containing an allele-specific primer and a common primer. After gel electrophoresis of the PCR product, DNA is stained and analyzed under UV light. The HPA genotype is then determined by the absence or presence of a DNA fragment. As an internal control for the success of the PCR reaction, an additional pair of primers for the amplification of the human growth hormone gene or another gene is usually added to each reaction.

Table 2 Glycoprotein receptors with their subunits and HPAs

GP receptor complex	Subunit composition (synonym)	CD nomenclature[a]	HUGO[b]	Genbank accession number	Antigen (HPA)
GP Ib–IX–V	GP Ibα	CD42B	GP1BA	NM 000173	2
	GP Ibβ	CD42C	GP1BB	NM 000407	12W
	GP IX	CD42A	GP9	NM 000174	
	GP V	CD42D	GP5	NM 004488	
GP IIb–IIIa	GP IIb (α_{IIb})	CD41	ITGA2B	NM 000419	3; 9
	GP IIIa (β_3)	CD61	ITGB3	NM 000212	1; 4; 6W; 7W; 8W; 10W; 11W; 14W; 16W.
GP Ia–IIa	GP Ia (α_2)	CD49B	ITGA2	NM 002203	5; 13W
	GP IIa (β_1)	CD29	ITGB1	NM 133376	
CD109	CD109	CD109	CD109	NM 133493	15

[a]CD nomenclature: http://www.ncbi.nih.gov/prow/.
[b]Human gene nomenclature: http://www.gene.ucl.ac.uk/nomenclature.

Various HPAs have been genotyped by SSP-PCR.[4,5] More references are cited in Ref. [6]. Efforts have been made to standardize the PCR conditions for detecting different polymorphisms. A consensus protocol for genotyping of HPA-1 to -5 and HPA-15 can be found on the platelet immunology pages in the Haematology Division section of the National Institute for Biological Standards and Control web site: http://www.nibsc.ac.uk.

Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) analysis has been implemented for genotyping of platelet antigens in many laboratories. This technique exploits the fact that single nucleotide substitutions may create or abolish a unique enzyme cleavage site. This makes possible allelic discrimination. Genomic DNA is first amplified by PCR using forward and reverse primers flanking the polymorphic region and then subjected to restriction enzyme digestion. The fragments are separated by electrophoresis, stained and analyzed under UV light. The genotype is then interpreted from the restriction pattern.

Polymerase chain reaction–restriction fragment length polymorphism analysis is simple to perform but requires the extra post-PCR step of digestion of the amplified DNA fragment. However, digestion can be incomplete and results in a homozygous individual could be falsely interpreted as heterozygous. Therefore, to avoid undetected incomplete enzyme activity, the amplified DNA fragment should be designed with an additional site for the same restriction enzyme.

Literature for RFLP typing of many HPAs are listed in Ref. [6].

Allele-Specific Oligonucleotide Hybridization

Allele-specific oligonucleotide (ASO) hybridization has been used in several of the initial studies investigating the molecular basis of the HPA alloantigens. This technique utilizes a generic PCR amplification with primers flanking the specific SNP, immobilization on a nylon membrane, and hybridization to labeled allele-specific oligonucleotide probes. Under appropriate hybridization and wash conditions, these probes bind only to their complementary sequence and are able to distinguish single nucleotide differences. The PCR products bound to an allele-specific probe are then detected using a luminescent or chromogenic substrate. Interpretation of the HPA types is based on the presence or absence of positive reactions. Allele-specific oligonucleotide hybridization has been used to detect various HPAs (references are cited in Ref. [6]).

A variation of the ASO technique is the reverse dot-blot hybridization.[7] The ASOs are covalently bound to a nylon membrane and the labeled amplification products are simultaneously hybridized to the probes.

Oligonucleotide Ligation Assay

In the oligonucleotide ligation assay (OLA), gene segments with the polymorphic sites are amplified in a single multiplex PCR. Aliquots of the PCR product are then incubated with pairs of labeled allele-specific probes complementary to contiguous sequences. One probe contains the allele-specific base. Where both probes are bound by complementary sequences, they can be covalently linked by a thermostable DNA ligase to produce a double-labeled product. This double-labeled product can then be detected by an enzyme-linked immunoabsorbent assay (ELISA). If the allele-specific base is absent from the template DNA, annealing of the allele-specific probe is incomplete and ligation cannot occur. No product can then be detected. Oligonucleotide ligation assay does not require the temperature-sensitive hybridization and washing steps used in the ASO technique. The ELISA makes the OLA system suitable for automation. HPA-1 to -5 have been typed with this system (reference in Ref. [8]).

Single-Strand Conformational Polymorphism

The mobility of single-strand DNA in nondenaturing polyacrylamide gels depends on size and tertiary conformation. Single-base changes alter the tertiary conformation such that the single-strand DNA shows a different mobility in the polyacrylamide gels. This is exploited in single-stranded conformational polymorphism (SSCP) to analyze HPA polymorphisms. The method has been used to genotype HPA-1 to -5 and HPA-10W (references are listed in Ref. [8]). The SSCP electrophoresis requires precise temperature control as any variation in temperature can affect single-strand DNA migration.

Preferential Homoduplex Formation Assay

The preferential homoduplex formation assay (PHFA) is based on DNA strand competition between a double-labeled amplicon (a reference double-stranded DNA with each strand separately labeled) and the unlabeled DNA sample. During hybridization with a precisely controlled temperature gradient, preferential formation of a homoduplex occurs more often than a heteroduplex. When

reference DNA and test DNA are identical, heteroduplex formation is favored. If the sequences are not identical, there will be little reduction in the double-labeled population. The reformed double-labeled DNA is immobilized on a microtiter well and detected by ELISA system (for references, see Ref. [8]).

Melting Curve Analysis on the LightCycler®

The new LightCycler PCR technology measures the match of a hybridization probe with the polymorphic region of DNA by melting curve analysis and allows simultaneous detection of both HPA alleles. The LightCycler (http://www.roche-applied-science.com/lightcycler-online/) is a microvolume fluorometer integrated with a thermal cycler that combines rapid thermal cycling for PCR with real-time fluorescence monitoring. First, PCR is performed with two hybridization probes which are labeled with different fluorophores at the adjoining ends. One probe is designed to hybridize to the polymorphic sequence of the PCR templates, and the other hybridizes one to five nucleotides apart on the adjacent region. When these two probes anneal to the PCR template, they are in close proximity, and fluorescence energy transfer (FRET) occurs between the two fluorophores. The emitted fluorescence measured by the LightCycler monitors the amplification process in real time. After the amplification is completed, melting curve analysis allows genotyping. For melting curve analysis, the temperature is lowered below annealing temperature and then slowly increased. At a certain temperature, the probes melt off the DNA template, causing decreased fluorescence. The melting point can then be determined using an algorithm. When probe and PCR product match completely, the melting point is relatively high. A single point mutation, however, makes hybridization weaker and results in a lower melting point. Thus alleles can be easily discriminated. HPA typing on the LightCycler has been accomplished for HPA-1 to -5 and HPA-15.[9–11]

TaqMan® Assay

The TaqMan technology is also based on fluorescent probes. This technique takes advantage of the 5′ nuclease activity of Taq polymerase. TaqMan probes have a reporter dye at the 5′ end and a quencher dye at the 3′ end. The close proximity of the two dyes in the intact probe quenches the fluorescent signal. However, during primer extension, Taq polymerase specifically displaces the annealed probe and allows the 5′-nuclease activity to cleave the probe and release the fluorescent dye. Light is then emitted. Although developed for quantitative real-time PCR, TaqMan technique is suitable for high-throughput SNP analysis. For the biallelic HPA system, two allele-specific probes with two reporter labels, which can be differentiated spectrally, are added into the same PCR mix, allowing single-tube genotyping. References for HPA-1–3 typing are listed in Ref. [8].

CONCLUSION

Once the genetic basis of HPAs had been resolved, a number of techniques for HPA typing were developed. Initially, conventional PCR techniques with elaborate post-PCR steps were introduced. Now, techniques with fluorescent probes have been developed which do not require extra post-PCR steps. These one-tube assays make HPA typing rapid and reliable and less prone to contamination. This makes high-throughput screening an achievable goal. Other techniques, such as minisequencing combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)[12] or pyrosequencing (http://www.pyrosequencing.com), might become more and more important for large-scale HPA genotyping in the future.

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PML/RARA and RARA FISH in Follow-up of Acute Promyelocytic Leukemia

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Dong Soon Lee
Seoul National University College of Medicine, Seoul, South Korea

INTRODUCTION

Retinoic acid receptor alpha-fluorescent in situ hybridization (RARA FISH) overcomes the drawback of promyelocytic leukemia (PML)/RARA FISH in the follow-up of acute promyelocytic leukemia. This is an analysis on the effectiveness of RARA FISH in acute promyelocytic leukemia (APL) patients during follow-up.

OVERVIEW

Detection of genetic change for t(15;17) is important for the diagnosis of atypical variants of acute promyelocytic leukemia (APL) and for predicting the response to ATRA treatment.[1–4] For this purpose, morphological diagnosis in conjunction with cytogenetics and reverse transcription-polymerase chain reaction (RT-PCR) for promyelocytic leukemia (PML)/retinoic acid receptor alpha (RARA) is generally used. At follow-up after treatment of APL, it is not easy to decide upon remission by morphology. With respect to the discrimination between leukemic promyelocytes and regenerating promyelocytes, many points of discrimination have been suggested;[5] however, the situation is sometimes perplexing. A positive result for RT-PCR in patients with PML after chemotherapy does not mean a persistence of malignant cells, because differentiated leukemic cells by ATRA treatment still harbor the PML/RARA gene rearrangement. Actually, although the majority of APL patients achieve morphological remission after complete remission induced by ATRA, PML/RARA transcripts remain detectable in all cases.[6–8] Hence serial quantitation of leukemic cells harboring the PML/RARA rearrangement is essential in the assessment of complete remission after consolidation.[9]

For the accurate and quantitative detection of minimal residual disease (MRD), real-time RT- PCR is available but is not yet used widely. Furthermore, persistence of PCR positivity in long-term remission without recurrence has been reported,[10] which means those clones with PCR^+ do not always have leukemogenic potential. Therefore the detection of PML/RARA fusion by FISH in the initial follow-up after treatment is very helpful. In addition, there are many factors that can influence the results of PCR, such as the quality of the mRNA, the timing of sampling, and the sensitivity of the RT-PCR assay. Although the sensitivity of FISH (10^{-2}–10^{-3}) is lower than that of PCR (10^{-5}–10^{-6}), the results of FISH are more precise, highly concordant between laboratories, and not influenced by other factors.[11] The only factor that can influence the interpretation of results is the different cutoff value used by laboratories. The scoring of fusion and split signals in FISH may vary by individual, resulting in different cutoff values among laboratories.

Single FISH probes for the PML/RARA fusion gene are commercially available, and the scheme of the PML/RARA probe involves the use of two probes: one from each of the fusion genes, differentially labeled. However, chance of colocalization of differently colored signals in normal specimens can produce some levels of false-positive nuclei with some traditional, two-color, come-together FISH translocation probes. The high normal range of probes with design of the PML/RARA probes is inherent in that design, but extra signal or dual-fusion PML/RARA probes are not available at the present time. This false-positive fusion encountered in normal cells makes it difficult to detect minimal residual disease (MRD) quantitatively, especially during follow-up. If we use RARA probes that are located in the proximal and distal sides of the breakpoint of the RARA gene, the signals would be split, resulting in one green signal, one orange signal, and one fusion signal (1G1O1F), and these probes would enable us to detect the translocations of the RARA gene with any partner chromosomes. The LSI RARA Rearrangement Probe (Vysis, Downers Grove, IL, USA) was originally designed to detect nuclei that have variant rearrangements involving the RARA gene and genes other than PML. However, this probe may also provide better information in the detection of nuclei that have t(15;17), because of its break-apart design.

We performed PML/RARA FISH and RARA break-apart FISH on bone marrow specimen of patients to investigate whether the RARA break-apart probe can overcome the disadvantage of the false-positive fusion signal of the single-FISH (S-FISH) for PML/RARA, and to evaluate the efficiency of RARA break-apart FISH in the diagnosis and monitoring of APL.

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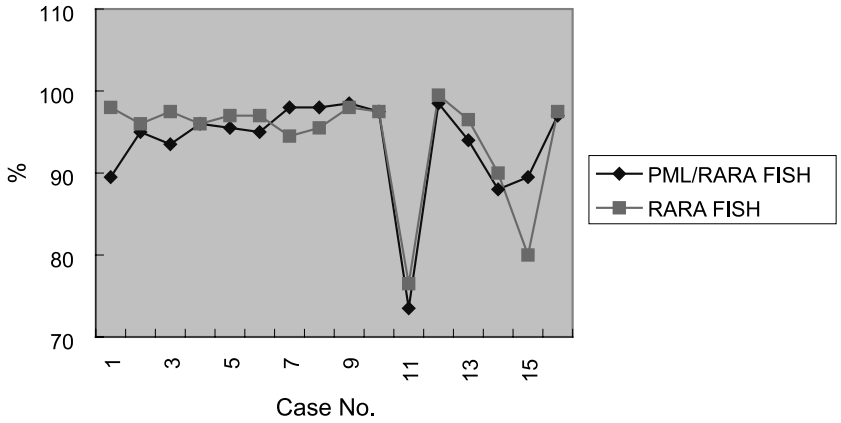

Fig. 1 Quantitative results of PML/RARA FISH and RARA FISH in 16 patients with acute myeloid leukemia (APL) at initial diagnosis (r=0.83). (*View this art in color at www.dekker.com.*)

MATERIALS AND METHODS

Patient Selection

A total of 78 cases tested for the PML/RARA rearrangement by FISH were retested with a RARA break-apart probe. Of these 78 cases, 27 were examined for the PML/RARA rearrangement by FISH to rule out M3 variant (M3v) at initial diagnosis, 16 cases were typical APL cases, which were positive for the PML/RARA rearrangement by RT-PCR at the time of initial diagnosis, and 21 cases were APL cases for follow-up after induction or consolidation therapy. Fourteen cases with hematological malignancies (non-APL) during the follow-up were included to determine whether there were any cutoff value differences when patients are under chemotherapy vs. the normal controls. To establish the reference range, PML/RARA and RARA FISH were conducted on specimens obtained from bone marrow examinations conducted in 20 normal controls without hematological malignancies (donors for bone marrow transplantation) and from the peripheral blood of 30 healthy persons. We scored the signals in 200 nuclei in each of the samples. Bone marrow mononuclear cells stored in methanol at $-20^{\circ}C$ were used for interphase FISH staining.

Conventional Cytogenetics Study and FISH Study

Bone marrow and peripheral blood samples were processed by conventional cytogenetic procedures with GTG (G bands by trypsin using Giemsa)-banding. Fluorescent in situ hybridization was performed according to the manufacturer's instructions, using an LSI RARA Rearrangement Probe (Vysis Inc.).

Probes of FISH for RARA

The Vysis LSI RARA Dual-Color Rearrangement Probe consists of a SpectrumOrange-labeled probe that

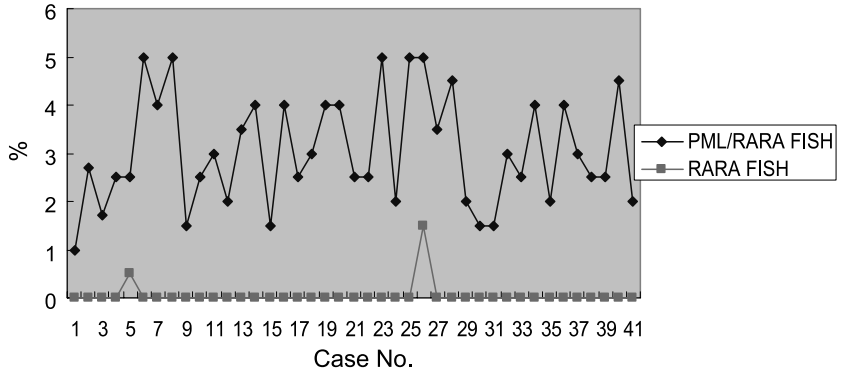

Fig. 2 The quantitative results of PML/RARA FISH and RARA FISH in 21 patients with acute promyelocytic leukemia at follow-up (r=0.75). (*View this art in color at www.dekker.com.*)

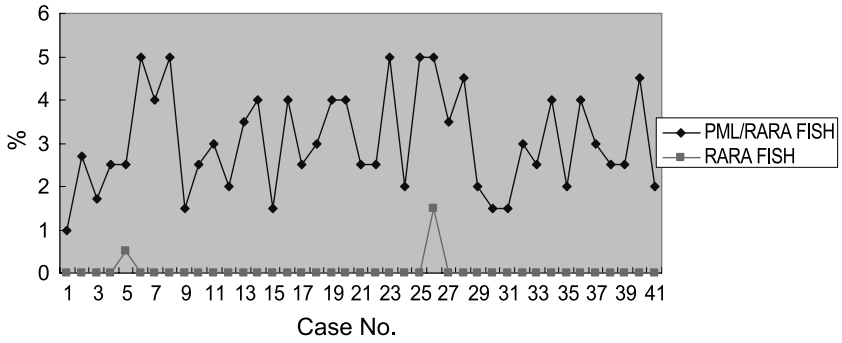

Fig. 3 Comparison of results of PML/RARA FISH and RARA FISH in 41 patients with nonacute promyelocytic leukemia (r=0.233). (*View this art in color at www.dekker.com.*)

hybridizes to a 130-kb region from the breakpoint cluster region (bcr) toward the centromere, and a SpectrumGreen-labeled probe that hybridizes to a 400-kb region. The signal pattern indicative of the RARA gene rearrangement is observed as a separation of the orange and green signals from the normal fusion signal, which indicates that the RARA gene has split.

Probes of FISH for PML/RARA

The Vysis LSI PML/RARA Translocation Probe is a mixture of a PML probe directly labeled with the SpectrumOrange fluorophore and a RARA probe directly labeled with the SpectrumGreen fluorophore.

PML/RARA RT-PCR

Mononuclear cells from the bone marrow or the peripheral blood were separated by dextran centrifugation, and cellular RNA was extracted with RNAsol according to the modified acid guanidium thiocyanate method, and reverse transcripted with avian myelomatous virus reverse transcriptase (Boehringer-Mannheim, Germany) with random primer according to the method of Borrow.[13]

RESULTS

Normal Range of PML/RARA and RARA Rearrangement by FISH

In PML/RARA FISH, the mean signal of 101G1F was 3.12% with a standard deviation of 1.02%, and therefore 6.2% was set as the normal reference range (mean ±3 S.D.). In RARA FISH, the mean signal of 1O1G1F was 0.05% with a standard deviation of 0.35%, and 1.2% was set as the reference range (mean±3 S.D.).

Comparison of PML/RARA, RARA FISH, and Cytogenetic Study in APL Patients at Initial Diagnosis and During Follow-up

The quantitative results of PML/RARA FISH and RARA FISH in 16 patients with acute myeloid leukemia (APL) at initial diagnosis showed good correlation (r=0.83): mean±S.D. was 95.3±6.5% for PML/RARA and 97.0±7.0% for RARA. The quantitative results of RARA FISH were rather higher than those of PML/RARA (Fig. 1). The quantitative results of PML/RARA FISH and RARA FISH in 21 patients with APL at follow-up were well correlated (r=0.75): mean±S.D. was 2.81±3.07%

Table 1 Analysis of the positive cases of RARA rearrangement FISH in comparison with results of PML/RARA translocation FISH

	PML/RARA		Positive RARA
Total (n=62)	≥5%	12 Cases	5 Cases
	< 5%	50 Cases	0 Cases
Initial diagnosis (n=27)	≥5%	3 Cases	1 Cases
	< 5%	24 Cases	0 Cases
Follow-up (n=35)	≥5%	9 Cases	4 Cases
	< 5%	26 Cases	0 Cases

PML/RARA, promyelocytic leukemia/retinoic acid receptor alpha.

for PML/RARA and 0.90±2.07% for RARA. In contrast to the situation at initial diagnosis, the quantitative results of RARA FISH were rather lower than those of PML/RARA at follow-up (Fig. 2). Of 62 cases with the results of PML/RARA fusion (1.0% to 11.6%), 46 cases (74.2%) showed absolute negative results (0%) with RARA split FISH. Interestingly, when we analyzed the results of non-APL patients, the results of PML/RARA and RARA were not correlated well (r=0.23), in contrast to those of APL patients (Fig. 3).

In 21 patients with APL during follow-up after chemotherapy, four cases showed positive signals for PML/RARA FISH over 6.2%, and three of these four cases showed a positive signal for RARA FISH over 1.5% (Table 1). These three cases (9.5%, 2%, and 2.5% positive signal for RARA FISH) showed continued remission by bone marrow examination and normal karyotypes by cytogenetic study after 6 months.

DISCUSSION

The present study differs from previous studies in that the normal control, for setting the reference range for the PML/RARA and RARA signals, included non-APL patients (14 cases) with hematological malignancies at the time of follow-up after chemotherapy, rather than normal individuals. We attempted to determine whether the alignment of chromosomes in the interphase after chemotherapy could affect the interpretation of FISH. Almost no difference was found in the reference ranges for PML/RARA between 20 specimens of normal bone marrow and 14 specimens of the bone marrow of non-APL patients at follow-up (p value=0.17, Student's t test). However, a difference was found in the cutoff values between 30 specimens of peripheral blood (5.47%) and 20 specimens of normal bone marrow (6.21%). The results of normal bone marrow were higher than those of peripheral blood, although the difference was not significant statistically (p value=0.59, Student's t test).

If a normal cutoff of 5% for PML/RARA FISH is applied, which is the same as the remission criterion (5%) by morphology, conducting FISH will not give any additional information. From the aspect of minimal residual disease detection, RARA FISH with a lower reference range of 1.5% would be more informative. Although the reference range of FISH does not usually matter in making a decision about APL at initial diagnosis, this range could significantly affect the clinical determination of complete remission during the treatment of APL, as positive RT-PCR results are frequently obtained after induction therapy, irrespective of the achievement of remission.

According to the results of the present study, the advantages of performing RARA FISH are that 1) false positivity due to the design problem of PML/RARA FISH can be reduced; 2) accurate quantitative results can be obtained; and 3) the interpretation difficulty experienced when the result is around the normal reference range with PML/RARA FISH can be resolved by RARA FISH. Thus at the time of follow-up of APL, employing RARA FISH would be more effective. Nevertheless, as it is important to determine the presence or absence of t(15;17) in the diagnosis of APL, PML/RARA FISH or PML/RARA RT-PCR needs to be considered at the time of initial diagnosis, and when their result is positive, we recommend performing RARA FISH, not PML/RARA FISH, during follow-up. On the other hand, in those cases that are difficult to interpret, because the PML/RARA FISH results are around the reference range, we believe that RARA FISH helps in the interpretation. We also recommend examining quantitative changes through repeated follow-up tests in each case during follow-up, rather than giving significance to only one test.

CONCLUSION

The cutoff value for RARA rearrangement must be changed by using the RARA split probe. In this study, using the RARA split probe, we were able to lower the cutoff value for the RARA rearrangement down to 1.05%, which is significantly lower than the 5–10% of PML/RARA. Actually, 82.7% of cases with PML/RARA positivity by FISH showed absolutely negative results with the RARA split probe.

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P

PNA–FISH—Application for Chromosomal Analysis and Other Forms of Genetic Testing

Franck Pellestor
Institute of Human Genetics, Montpellier, France

INTRODUCTION

Both the study and the development of modified oligonucleotides have known a significant upsurge of interest during the last decade and have become a central feature of biotechnology because of the tremendous potential of modified oligonucleotides as therapeutic agents and tools in molecular biology. Among all the synthetic nucleic acid analogs designed, the peptide nucleic acids (PNAs) constitute a remarkable class of nucleic acid mimics with important properties, which have been incorporated into an expanding variety of hybridization-based applications, including genome mapping, antigene therapy, mutation detection, and chromosome analysis.

PNA CHEMISTRY AND PROPERTIES

The first report concerning the design and properties of PNAs was published in 1991 by Nielsen et al.[1] PNAs are synthetic DNA analogs in which the phosphodiester backbone is replaced by repetitive units of *N*-(2-aminoethyl) glycine to which the purine and pyrimidine bases are attached via a methyl carbonyl linker (Fig. 1). The PNA molecules can routinely be labeled with biotin or fluorophores such as fluorescein or rhodamine.

A subsequent generation of PNAs could involve modification of the *N*-(2-aminoethyl) glycine backbone (PNA analogs) or chimeric architecture, such as PNA–peptide chimeras or PNA–DNA chimeras developed to improve the solubility and the cellular uptake of PNAs or to exhibit new biological properties. The synthetic backbone provides PNA with unique hybridization characteristics. Unlike DNA and RNA, the PNA backbone is not charged. Consequently, there is no electrostatic repulsion when PNA hybridizes to its target nucleic acid sequence, giving a higher stability to the PNA–DNA or PNA–RNA duplexes than the natural homo- or heteroduplexes. This greater stability is reflected by a higher thermal melting temperature (T_m) as compared to the corresponding DNA–DNA or DNA–RNA duplexes.

An additional consequence of the polyamide backbone is that PNAs hybridize virtually independently of the salt concentration. This property can be exploited when targeting DNA or RNA sequences involved in secondary structures, which are destabilized by low ionic strength. This facilitates the hybridization with the PNAs. The unnatural backbone of PNAs also means that PNAs are not degraded by nucleases or proteases. Because of this resistance to the enzyme degradation, the lifetime of PNAs is extended both in vivo and in vitro. Also, PNAs are not recognized by polymerases and therefore cannot be directly used as primers or be copied.

Peptide nucleic acids hybridize to complementary DNA or RNA in a sequence-dependent manner, according to the Watson–Crick hydrogen bonding scheme. In contrast to DNA, PNA can bind in either parallel or antiparallel fashion. However, the antiparallel binding is favored over the parallel one. PNA is able to adopt both A- and B-type structures when associating with RNA and DNA, respectively, and PNA–PNA duplexes formed an unusual helix conformation, called P type, and characterized by a large pitch of 18 base pairs.

Peptide nucleic acid probes can bind to either single-stranded DNA or RNA, or to double-stranded DNA (dsDNA). Homopyrimidine PNAs, as well as PNAs containing a high pyrimidine/purine ratio, bind to complementary DNA sequences to form highly stable $(PNA)_2$–DNA triplex helices displaying T_m over 72°C. In these triplexes, one PNA strand hybridizes to DNA through standard Watson–Crick base pairing rules, whereas the other PNA strand binds to DNA through Hoogsteen hydrogen bonds. The resulting structure is called P loops. The stability of these triple helixes is so high that homopyrimidine PNA targeted to purine tracts of dsDNA invades the duplex by displacing one of the DNA strands.[2]

Finally, PNA–DNA hybridization is significantly more affected by base mismatches than DNA–DNA hybridization. A single mismatch in a mixed PNA–DNA 15-mer duplex decreases the T_m by up to 15°C, whereas in the corresponding DNA–DNA complex, a single mismatch decreases the T_m by only 11°C. This high level of discrimination at single-base level has indicated that short PNA probes could offer high specificity and has thus allowed the further development of several PNA-based strategies for molecular investigations and diagnosis.

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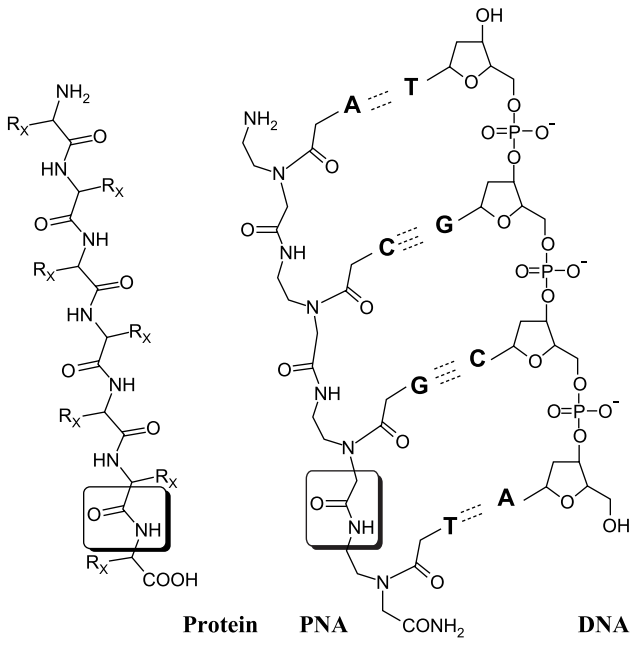

Fig. 1 Chemical structures of PNA as compared to DNA and protein. The amide bond characteristic for both PNA and protein is boxed in.

APPLICATIONS

Since its introduction, an increasing number of applications of PNA technology have been described, confirming the high potential of PNAs as a tool for molecular biology techniques.

Antisense and Antigene Applications

Originally conceived as agents for double-stranded DNA binding, the unique properties of PNAs as a DNA mimics were first exploited for gene therapy drug design. PNAs can inhibit transcription (antigene) and transcription (antisense) of genes by tight binding to DNA or mRNA.

Peptide nucleic acid-mediated inhibition of gene transcription is mainly due to the formation of invaded strand complexes or strand displacement in a DNA target. Several in vitro studies have shown that the binding of PNA or bis-PNA to dsDNA can efficiently block transcriptional elongation and inhibit the binding of transcriptional factors and helicases.[3] PNA targeted against the promoter region of a gene can form stable PNA–DNA complexes that restrict the DNA access of the polymerase, whereas PNA complexes located far from the promoter can block the polymerase progression and lead to the production of truncated RNA transcripts. Nielsen et al.[4] have demonstrated that even an 8-mer PNA can efficiently block transcriptional elongation by $(PNA)_2$–DNA triplex formation.

Peptide nucleic acids are able to interact with mRNA independently of the RNA secondary structure. However, unlike other antisense agents, PNA–RNA duplexes are not recognized by RNase-H. Studies on the mechanisms of antisense activity have demonstrated that PNA inhibits expression differently than antisense oligonucleotides acting through RNase-H-mediated degradation of the mRNA–oligonucleotide hybrid. Because PNAs are not substrates for RNAse, their antisense effect acts through steric interference of either RNA processing, transport into cytoplasm, or translation, caused by binding to the mRNA.

Application of PNAs as antisense reagents was first demonstrated in 1992. The nuclear microinjection of a 15-mer PNA targeting the translation start region of SV40 large T antigen mRNA inhibited transcription in cell extracts.[5] This inhibition was both sequence specific and dose dependent. More recently, Mologni et al.[6] reported the effect of three different types of antisense PNAs on the in vitro expression of PML/RARalpha gene. It was also reported that intron–exon splice junctions are very sensitive targets for PNA antisense probes because correct mRNA splicing can be altered by PNA binding.[7]

Although these in vitro results strongly emphasized the potential of PNAs for antigene and antisense applications, the limiting factors for in vivo use of PNAs are the weak uptake of PNAs by living cells. Several modifications of PNAs have led to significant improvements. Cellular uptake can be speeded up by coupling PNA to DNA oligomers, to receptor ligands, or more efficiently, to peptides such as cell-penetrating peptides that are rapidly internalized by mammalian cells. Reports have demonstrated that PNAs conjugated to such peptides are efficiently taken up by eukaryotic cells.[8,9] Another strategy adapted to improve the in vivo delivery of PNA can be their incorporation into liposomes.

PCR and Q-PNA PCR

Peptide nucleic acid probes have no direct interaction with DNA polymerase but PNAs can terminate the elongation of oligonucleotide primers by binding to the template or competing with the primers.[10] Moreover, PNA–DNA chimeras can be recognized by the DNA polymerase and can thus be used as primers for PCR reactions.[11] The high-affinity binding of PNAs has also been used for detecting single base pair mutations by polymerase chain reaction (PCR). This strategy, named PNA-directed PCR clamping, uses PNAs to inhibit the amplification of a specific target by direct competition of the PNA targeted against one of the PCR primer sites and the conventional

PCR primer.[12] More recently, novel automated real-time PCR has been developed using PNAs. In this method, named Q-PNA PCR, a generic quencher labeled PNA (Q-PNA) is hybridized to the 5′ tag sequence of a fluorescent-dye-labeled DNA primer to quench the fluorescence of the primer. During PCR, the Q-PNA is displaced by incorporation of the primer into amplicons and the fluorescence of the dye label is liberated.[13]

Nuclear Acid Capture

Peptide nucleic acids can be used for sequence-specific capture of single-stranded nucleic acids, taking advantage of the tight complex formation at low ionic strength, which destabilizes nucleic acid secondary structure. Several studies have reported such applications of PNAs. Short PNA probes can then be used as generic capture probes for purification of nucleic acids.[14]

Solid-Phase Hybridization Techniques

Peptide nucleic acids can be used in many of the same hybridization applications as natural or synthetic DNA probes but with the added advantages of tighter binding and higher specificity. This leads to faster and easier procedures in most standard hybridization techniques, such as Southern and Northern blotting. An alternative to Southern analysis is also the PNA pregel hybridization process, which significantly simplifies the procedure of Southern hybridization.[15] Labeled PNAs are then used as probes, allowing hybridization to a denatured dsDNA sample at low ionic strength before loading on the gel. The method is sensitive enough to detect a single mismatch in a DNA sample. Another gel-based strategy, known as affinity electrophoresis can be exploited with PNA probes. In this case, the slowdown of DNA during electrophoretic passage is related to its hybridization with a complementary PNA probe entrapped in the gel matrix. Likewise, hybridization PNA-based biosensor procedures have been developed in which a single-stranded PNA probe is immobilized onto optical or mass-sensitive transducers to detect the complementary strand or corresponding mismatch in a DNA sample solution. The hybridization events are converted into electric signals by the transducers.[16]

Fluorescence In Situ Hybridization (PNA–FISH)

The efficiency of PNAs as hybridization probes has also been demonstrated in fluorescence in situ hybridization (FISH) applications. The properties of PNAs have allowed the development of fast, simple, and robust in situ assays. Because of their neutral backbone, PNA probes present in situ high specificity and require low concentrations and short hybridization time. Additional in situ benefits of using PNA probes are reduced background binding, low photobleaching, and mild washing procedure. In situ labeling can efficiently be obtained with a single 15-mer PNA oligomer carrying a single label.

The PNA–FISH technique was first developed for quantitative telomere analysis. Using a unique fluorescein-labeled PNA probe, Lansdorp et al.[17] performed the in situ labeling of human telomeric repeat sequences and the data obtained allowed accurate estimates of telomere lengths. Subsequently, telomeric PNA probes were used in several in situ studies of cancer and aging.[18] Further developments were focused on the in situ specific identification of human chromosomes on both metaphases and interphase nuclei, using PNA probes specific for satellite repeat sequences of various chromosomes. Multicolor PNA experiments were thus reported on lymphocytes (Fig. 2), amniocytes, and fibroblasts from normal subjects and patients with numerical abnormalities.[19,20] These experiments demonstrated the superiority of PNA probes over satellite DNA probes in both intensity and sequence discrimination. Chen et al.[21] reported that PNA probes could discriminate in situ between two centromeric DNA repeats that differ by only a single base pair. The discriminating power of PNA could be very useful for the study of chromosomal variations and

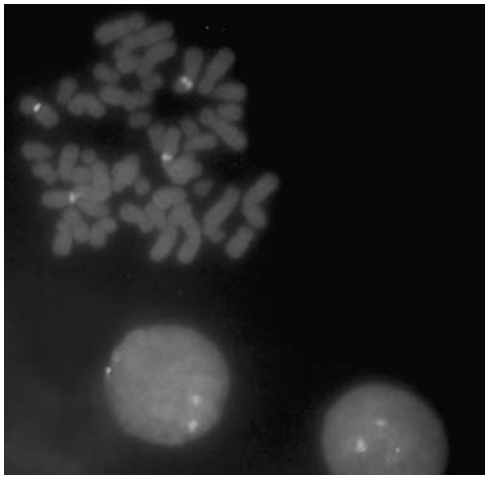

Fig. 2 In situ identification of chromosomes 1, X, and Y on human metaphase and interphase nuclei using multicolor PNA–FISH procedure with satellite-specific PNA probes. Chromosome 1 is labeled in blue, chromosome X in red, and chromosome Y in green. (*View this art in color at www.dekker.com.*)

polymorphisms. Recently, PNA–FISH has been adapted to in situ chromosomal analysis of the human sperm.[22] This adaptation constituted an interesting challenge because of the particularities of human spermatozoa nuclei in terms of genomic compaction and accessibility of DNA sequences. Comparative estimates of disomies X, Y, and 1 were performed in sperm from healthy subjects using FISH, primed in situ labeling (PRINS), and PNA procedures in parallel. Equivalent quality of in situ nuclear labeling and similar results was obtained with the three methods. However, the hybridization timing of PNA probes (i.e., 45 min) appeared to be significantly shortened in comparison with FISH reaction on sperm. The fast hybridization kinetics of PNA was similar to the kinetics of PRINS reaction.[23] The similarity between PNA and PRINS might be essentially due to the small size of both PNA probes and PRINS primers, which do not exceed 30 bases in length. This finding points out the importance of the probe size for in situ assays and, consequently, the great potentiality of the PNA approach, because high specific binding can be obtained with a short, unique PNA oligomer. This study also demonstrates the efficiency of multicolor PNA procedure on male gametes and shows that PNA could be a powerful alternative to FISH and PRINS for in situ chromosomal investigations.

CONCLUSION

Since their invention, PNAs have quickly evolved from basic research to the application of PNA technology to diagnosis procedures. PNA-based applications benefit from the unique physicochemical properties of PNA molecules, enabling development of simple and robust assays. The data presented here illustrate the large number of PNA applications in genetics and cytogenetic testing. Very powerful applications of PNAs have also emerged in microbiology, virology, and parasitology. New chemical modifications of the original PNA backbone may contribute to increasing the potentialities of PNAs and lead to the development of novel applications and PNA-dependent projects in many areas of biology and biotechnology.

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PNA–FISH—Applications for Rapid Diagnosis of Bloodstream Infections

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Henrik Stender
AdvanDx, Inc., Woburn, Massachusetts, U.S.A.

INTRODUCTION

Bloodstream infections have become an important ''disease'' in the industrialized world and are associated with significant morbidity and mortality. Each year, an estimated quarter million hospital-acquired bloodstream infections and a similar number of community-acquired bloodstream infections occur in the United States alone. The ''disease'' has further intensified due to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and an increasing number of fungemia caused by non-*Candida albicans* yeasts—all associated with increased costs and mortality.

Today, the diagnosis of bloodstream infections is based on Gram staining of positive blood culture bottles; however, the final identification and susceptibility results are not available until 1–3 days later. Therefore, meanwhile, patients are often treated empirically, and delayed laboratory results have little impact on patient therapy, leading to an inappropriate use of broad-spectrum antibiotics.

Peptide nucleic acid fluorescence in situ hybridization (PNA–FISH) is a novel in vitro diagnostic platform, which has recently been applied for rapid and accurate diagnosis of bloodstream infections. Benefiting from the unique features of PNA probes, PNA–FISH assays enable clinical microbiology laboratories to report important information for patient therapy within a time frame not possible using current methods.

THE PNA–FISH PLATFORM

PNA Probes—A Superior Probe Molecule For Diagnostics

PNA probes hybridize in a sequence-specific manner to DNA and RNA by obeying Watson–Crick base-paring rules.[1] However, because of their structure, PNAs have significant advantages compared with DNA probes, as outlined below:

- *Higher sensitivity*: PNA binds stronger and faster to complementary RNA or DNA, thereby facilitating the development of more rapid and sensitive diagnostic tests. Where DNA probes often require overnight incubation, reactions with PNA are completed within a few hours.[2]
- *Higher specificity*: PNA probes are particularly well suited for discriminating closely related sequences, even single nucleotide differences, thus making PNA-based assays 100% definitive.[3]
- *Robust assays*: PNA is a synthetic molecule resistant to nucleases and proteases. It is extremely stable in prepackaged kit formats, as well as during the actual assay when in contact with the sample.[4]
- *Novel assay formats*: The unique properties of PNA enable the development of assay formats that go above and beyond the possibilities of DNA probes, thereby reducing the complexity related to the performance of molecular diagnostic tests.[5]
- *No target limitation*: The noncharged backbone allows PNA probes to hybridize under conditions that are destabilizing to DNA and RNA. Attributes that enable PNA probes to access targets, such as highly structured rRNA and double-stranded DNA, are known to be inaccessible to DNA probes.[6]
- *In situ hybridization*: The hydrophobic nature of PNA relative to DNA makes PNA probes superior for in situ hybridization assays where the probes must penetrate the hydrophobic cell wall prior to hybridization.[7]

rRNA—A Superior Phylogenetic Marker

Comparative ribosomal RNA (rRNA) sequence analysis has become a widely accepted method for establishing phylogenetic relationships between bacterial species, and rRNA sequence information has become a well-established method for microbial identification.[8]

rRNA sequence differences between closely related species enable the design of species-specific probes targeting almost any microorganism. Such probes are obvious reagents for microbial identification and enable diagnostic microbiology to be based on a single genetic marker rather than a series of biochemical tests.

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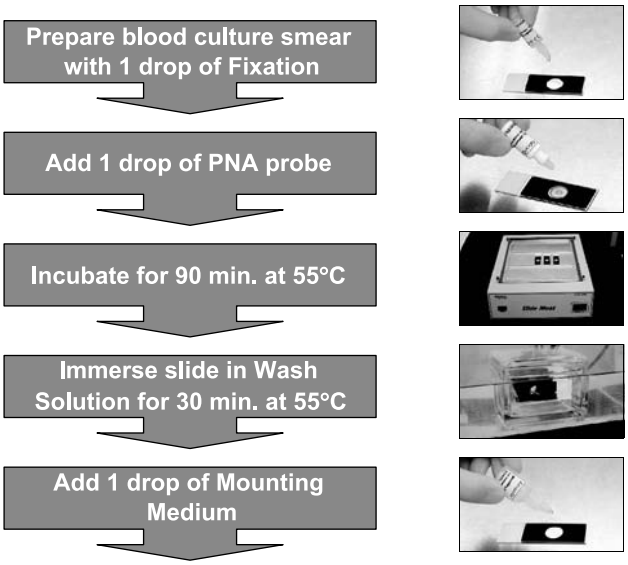

Fig. 1 PNA–FISH assay procedure. (*View this art in color at www.dekker.com.*)

PNA–FISH—An Intelligent Stain

PNA–FISH is a novel technique that combines the simplicity of current staining methods such as Gram staining with the specificity of molecular techniques (Fig. 1).[9] In this way, presumptive detection of the causative agent routinely performed by microscopical examination can be improved to provide final identification. Importantly, PNA–FISH is performed directly on smears fixed onto microscope slides by standard methods, such as flame fixation, methanol fixation, or heat fixation, without any other pretreatment or permeabilization steps known from DNA-based tests.

PNA–FISH uses rRNA as target not only because of its phylogenetic superiority as described above, but also because each microorganism harbors several thousand rRNA molecules. This abundance of rRNA target molecules allows individual cells to be detected and identified directly by fluorescent-labeled probes, also called ''phylogenetic stains''[10]—attributes that make the PNA–FISH technology extremely simple and user-friendly compared with polymerase chain reaction (PCR) and other molecular technologies.

Moreover, the PNA–FISH concept is directly compatible with a variety of sample types.[9] Blood cultures, as will be reviewed later, are one example where the complex medium contains well-known inhibitors and interference substances, such as sodium polyanetholesulfonate, charcoal particle, or resins, for diagnostic procedures. Numerous attempts to perform blood culture identification directly from the positive blood culture bottles have been reported in the past, but with variable sensitivities and specificities, and have often only been evaluated on a single blood culture medium and rarely on highly complex media with charcoal or resins. In contrast, PNA–FISH is compatible with all major blood culture media[11]—a key feature that is ascribed to the inert and synthetic PNA probe molecule.

IN VITRO DIAGNOSTIC APPLICATIONS

The generic PNA–FISH assay procedure allows PNA–FISH to be applied for various blood pathogens without procedural changes, such that separate tests can be performed in parallel. Using the results from Gram staining, appropriate PNA–FISH tests can be selected to identify or rule out relevant pathogens (Fig. 2).

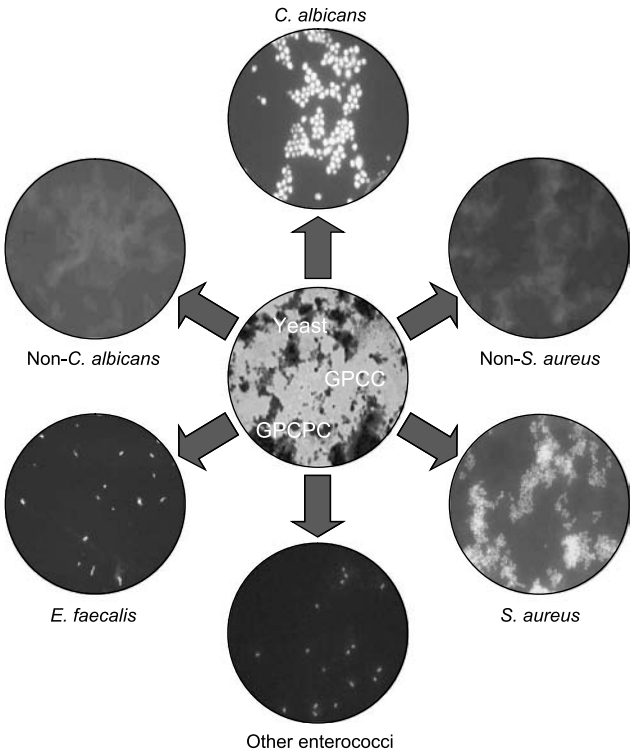

Fig. 2 Application of PNA–FISH tests for direct identification of positive blood culture bottles. The result of the Gram stain provides a basis for the selection of appropriate PNA–FISH test. GPCC: *S. aureus* PNA–FISH is used to identify *S. aureus*. Negative test results are indicative of coagulase-negative staphylococci. GPCPC: *Enterococcus faecalis* PNA–FISH is used to distinguish *E. faecalis* from other enterococci. Negative test results (not shown) are indicative of streptococci. Yeast: *C. albicans* PNA–FISH is used to identify *C. albicans*. Negative test results are indicative of non-*C. albicans* species. (*View this art in color at www.dekker.com.*)

Identification of *S. aureus* in GPCC-Positive Blood Culture Bottles

S. aureus bacteremia is both a serious and frequent nosocomial and community-acquired infection that is further complicated by the emergence of methicillin-resistant *S. aureus* and, recently, vancomycin-resistant *S. aureus*[12] which are ascribed to the widespread use of these antibiotics in healthcare settings. *S. aureus* is preliminary identified as gram-positive cocci in clusters (GPCC) along with other staphylococci; however, whereas *S. aureus* is often clinically significant, other staphylococci, such as *Staphylococcus epidermidis*, are often contaminants and not clinically significant. Therefore, rapid identification of *S. aureus* and differentiation from other staphylococci directly from positive blood culture bottles are important for patient diagnosis and selection of appropriate therapy to reduce empirical therapy with broad-spectrum antibiotics, such as ampicillin and vancomycin.

S. aureus PNA–FISH is based on a fluorescein-labeled PNA probe targeting *S. aureus* rRNA for both rapid and accurate identification of *S. aureus*. The performance of *S. aureus* PNA–FISH has been reviewed elsewhere[9] and showed 97.3–100% sensitivity and 98.9–100% specificity in four separate studies.[11,13–15]

Identification of *E. faecalis* in GPCPC-Positive Blood Culture Bottles

VRE have become a serious nosocomial pathogen because of the widespread use of vancomycin and require aggressive therapy with novel antibiotics, such as linezolid and quinupristin/dalfopristin. Resistance to vancomycin and also ampicillin among enterococci is primarily associated with *Enterococcus faecium* and a few other rare-occurring *Enterococcus* species, whereas *E. faecalis*, the most frequent species accounting for approximately two thirds of enterococcal isolates, is rarely resistant to these antibiotics. Therefore rapid identification of *E. faecalis* and differentiation from other enterococci directly from positive blood culture with gram-positive cocci in pairs or chains (GPCPC) are important to institute appropriate antibiotic therapy and to limit aggressive therapy to *E. faecium* and others with increased resistance to ampicillin and vancomycin.

E. faecalis PNA–FISH is a novel dual-color approach using a fluorescein-labeled PNA probe targeting *E. faecalis* and a rhodamine-labeled PNA probe targeting other enterococci, including *E. faecium*. Negative results are indicative of streptococci. The performance of *E. faecalis* PNA–FISH has been reviewed elsewhere[9] and showed 92.8–100% sensitivity and 88.9–100% specificity in two separate studies.[16,17]

Identification of *C. albicans* in Yeast-Positive Blood Culture Bottles

The number of candidemia cases has increased during the past decade because of the increasing population of immunocompromised patients, organ transplantations, and the common use of intravascular devices. Furthermore, species distribution is changing such that the prevalence of *C. albicans* is decreasing because of the emergence of other *Candida* species, in particular *Candida glabrata* and *Candida parapsilosis*.[18] Whereas *C. albicans* is often susceptible to fluconazole, *C. glabrata* and other emerging species, such as *Candida krusei*, have a different resistance pattern. Therefore rapid and accurate methods for species identification of yeast in blood cultures are important for the selection of appropriate antifungal therapy. Rapid identification of *C. albicans* and differentiation from other yeast species are particularly important as they allow the majority of cases caused by *C. albicans* to be treated safely and cost-effectively with fluconazole, such that only the remaining cases caused by other *Candida* species require ''empirical'' treatment with amphotericin B, a broad-spectrum but nephrotoxic drug, until further identification and susceptibility results become available.

C. albicans PNA–FISH contains a fluorescein-labeled PNA probe targeting *C. albicans* rRNA that can distinguish *C. albicans* from the closely related *Candida dubliniensis* often identified as *C. albicans* by standard methods. The performance of *C. albicans* PNA–FISH has been reviewed elsewhere[9] and showed 100% sensitivity and 100% specificity in two separate studies.[19,20]

UNLIMITED OPPORTUNITIES

The applications reviewed previously were all related to rapid and accurate identification of blood culture bottles with yeast or gram-positive bacteria; however, PNA probes for important gram-negative bloodstream pathogens, such as *Escherichia coli* and *Pseudomonas aeruginosa*, have also been described[21] and are obvious candidates for an almost complete test panel for blood culture identification. The PNA–FISH platform has also been studied in several other applications, such as *Mycobacterium tuberculosis* in acid-fast bacilli (AFB)-positive sputum samples,[7] cultures,[22] and biopsies,[23] and *Trypanosome brucei* in blood smears,[24] with the latter being the first PNA–FISH application on parasites and yet another example of the broad applicability of the PNA–FISH platform.

Therefore, PNA–FISH offers almost unlimited opportunities for novel diagnostic applications to not just

replace existing tests, but also to offer attractive solutions to unmet diagnostic needs that potentially lead to better patient therapy and management.

CONCLUSION

Rapid and accurate diagnosis of bloodstream infections by PNA–FISH is an important application utilizing the unique attributes of PNA probes to provide important diagnostic information in time to support the selection of appropriate antibiotic therapy. Furthermore, PNA–FISH has several similarities to standard staining technique, making it easily adaptable for routine testing without the need for specialized equipment, laboratory facilities, or personnel, as often required for molecular techniques.

It is well accepted that empirical use of antibiotics plays an important role in the spread and emergence of antibiotic resistance, and that a more appropriate use of antibiotics requires new and rapid diagnostic tests to change from empirical treatment practices based on clinical symptoms and worst-case scenarios to more personalized therapies based on individual diagnostic information. The speed and accuracy, combined with the simple and universal assay procedure, make PNA–FISH a promising tool for the development of therapy-directing diagnostics—the new generation of diagnostics.

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Pneumocystis jiroveci

Hans Henrik Larsen
Jannik Helweg-Larsen
Bettina Lundgren
Hvidovre University Hospital, Hvidovre, Denmark

INTRODUCTION

Pneumocystis is an unusual opportunistic fungus which causes a severe and—if untreated—lethal pneumonia (*Pneumocystis* pneumonia, or PCP) in immunocompromised humans and mammals.

Two major difficulties have hampered the study of PCP. First, no reliable in vitro system for the cultivation of *Pneumocystis* exists, and although animal models are widely used, considerable genetic difference between human *Pneumocystis* (*Pneumocystis jiroveci*) and *Pneumocystis* spp. infecting other mammalian species exists. Second, live organisms have never been detected outside its hosts, and clinical samples containing large quantities of *P. jiroveci* organisms are not easily obtained. As a consequence, the study of the organism and the infection has relied greatly on molecular and immunological approaches. These have led to the identification of clinical relevant antigens and enzymes, and have enabled typing. The latter is of outmost importance in elucidating the epidemiology of human pneumocystosis.

The standard method for diagnosis of PCP is microscopical examination of invasive lower respiratory tract specimens. Molecular detection systems have the potential to provide a higher degree of sensitivity than microscopy. Therefore PCR methods have been applied to clinical specimens for diagnosing PCP, and oral wash examination by PCR may be an alternative as a noninvasive diagnostic test.

PNEUMOCYSTIS—THE ORGANISM

Pneumocystis is a eukaryotic extracellular lung pathogen that has been detected in almost every mammalian species evaluated for its presence.

Pneumocystis was long considered as a protozoan based on morphological features and its resistance to classical antifungal agents. However, in the late 1980s, *Pneumocystis* was included in the fungal kingdom based on phylogenetic analysis of ribosomal RNA (rRNA) sequences and observations of genome size. This position was substantiated by functional and phylogenetic comparisons of several other genes.[1]

Pneumocystis organisms were first identified and named *Pneumocystis carinii* in the beginning of the last century. Studies including chromosomal karyotyping, nucleotide sequence analysis, antigenic characterization, and experimental transmission of infection have shown that *Pneumocystis* has considerable genetic diversity and host specificity. Thus an interim trinomial name change was adopted to reflect the diversity (e.g., *P. carinii* f. sp. *hominis* for *Pneumocystis* infecting humans). Finally, in recognition of the special forms' genetic and functional distinctness as separate species, human *Pneumocystis* was renamed *P. jiroveci*.[2]

In the lungs, two major forms of the organism can be identified by light microscopy: the cystic form (cysts) and the trophic form (trophozoites). The cystic form (sporangium) is thick-walled, oval, approximately 5–8 μm in diameter, and contains up to eight intracystic bodies (endospores), which will become trophic forms after excystation. The trophic form (yeast) is small (2–5 μm), thin-walled, pleomorphic, and often has an eccentric nucleus. The trophozoites are often seen in clusters. Not much is known about the lifecycle, and the mode of replication has not been definitely established, but both asexual and sexual life cycles have been proposed.

CLINICAL MANIFESTATIONS

Pneumocystis is primarily an opportunistic pulmonary pathogen, causing PCP. Extrapulmonary pneumocystosis is very rare.

PCP was an uncommon disease occurring in immunocompromised patient populations until the beginning of the 1980s, when it emerged as a significant cause of morbidity and mortality among human immunodeficiency virus (HIV)-infected patients.[3] PCP remains one of the most common acquired immunodeficiency syndrome (AIDS)-defining events, despite the use of prophylaxis and highly active antiretroviral therapy (HAART).[4]

The major presenting symptoms of PCP are shortness of breath, fever, and nonproductive cough. Immunosuppressed, HIV-uninfected patients are typically sick for days to 2 weeks before seeking medical attention, whereas PCP in HIV-infected patients typically has a more

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insidious onset, with symptoms lasting several weeks. The organism burden is higher, but the lung damage less severe in HIV-infected patients.

On physical examination, tachypnea and tachycardia are found in acutely ill patients. Although lung auscultation is usually not helpful, the chest radiograph classically exhibits diffuse bilateral interstitial infiltrates. Atypical findings range from normal examination to unilateral localized findings.

The magnitude of impaired arterial oxygenation is used to evaluate the severity of the disease, with a p_aO_2 of 70 mmHg discriminating between mild to moderate disease and severe disease. In addition, serum lactic dehydrogenase levels can be of some help in evaluating the severity of lung injury, but the analysis is nonspecific for PCP and lung injury.

TREATMENT

Management of PCP has improved over the past decades: chemoprophylaxis is used routinely during periods of immunological susceptibility in various patient populations, and if an episode of PCP does occur, the prognosis is improved because of earlier diagnosis and earlier institution of therapy. The use of adjunctive corticosteroid treatment for moderate to severe PCP has also led to a decrease in mortality.

Few advances in drug treatment have occurred during the past 20 years. The major chemotherapeutic agents available for treatment of PCP in the pre-HIV era were parenteral pentamidine and trimethoprim sulfamethoxazole (TMP-SMX). To date, TMP-SMX remains the drug of choice for both treatment and prophylaxis of PCP because of its well-established safety and efficacy, as well as its low cost. Alternatives, if the patient is intolerant or fails TMP-SMX, include dapsone-containing regimens, pentamidine (intravenous form for treatment and aerosolized form for prophylaxis), atovaquone, and clindamycin/primaquine (treatment only).

The oral route can be used to treat mild to moderate cases of PCP when no obvious gastrointestinal dysfunction is apparent. All patients with severe disease should be placed on an intravenous regimen and should receive adjunctive corticosteroid therapy.

MOLECULAR CHARACTERIZATION OF *PNEUMOCYSTIS*

The genomes of *Pneumocystis* species infecting mice, humans, rats, and ferrets have been estimated to be 6.5, 7.0, 7.7, and 11 Mb of DNA, respectively, which are relatively small genome sizes for a fungus when compared with, for example, the 12-Mb *Saccharomyces cerevisiae* genome. Chromosome organization has not been definitely determined because extensive chromosome polymorphism has been characterized among different strains of rat *P. carinii*. By pulsed field gel electrophoresis (PFGE), the estimation is that the genome of rat *P. carinii* is likely divided into 15 chromosomes of 0.3–0.7 Mb size.[5] There is an ongoing sequencing project with a goal to provide physical maps and gene sequences for the entire genomes of rat *P. carinii* and human *P. jiroveci* (http://biology.uky.edu/Pc/).

Phylogenetic analysis based on nuclear 16S-like RNA has not identified any close relatives, but fungal organisms on neighboring branches include the fission yeast, *Schizosaccharomyces pombe*. However, in contrast to most other fungi, *Pneumocystis* has a fragile cell wall and contains little or no ergosterol.

The typical *Pneumocystis* gene is rich in A and T (60–65%), and contains numerous (up to nine) introns of less than 50 bp length.[5]

To date, the major surface glycoprotein (MSG; also called glycoprotein A) is the only antigen of human *P. jiroveci* that has been well characterized.[6] MSG is the most abundant surface protein present in all *Pneumocystis* strains examined to date. The 95- to 140-kDA MSG is encoded by a multicopy gene family, distributed among all chromosomes. This protein has a crucial role in interactions with the host, possibly by acting as an attachment ligand to lung cells, and in elicitation of an immune response. MSG shows a high level of antigenic variation by switching the expression of MSG genes using a single expression site termed the upstream conserved sequence (UCS).[7] It is likely that it serves for evasion of the host immune response by use of antigenic variability.

Recombinant MSG fragments have been generated, which could be used in serological studies investigating the epidemiology of human pneumocystosis.[8,9]

A number of genes encoding enzymes have been described. Of particular potential clinical interest is the dihydropteroate synthase (*DHPS*) gene encoding the target enzyme of sulfa drugs. Nonsynonymous mutations occurring at six nucleotide positions, resulting in amino acid changes, have been reported.[10] Two of these mutations, encoding amino acids at positions 55 and 57, are located in the active site of the enzyme, and mutations at homologous positions have been shown to cause sulfa resistance in other organisms. Most studies have found a correlation of these two *DHPS* mutations with prior sulfa exposure, suggesting evolutionary selective pressure of sulfa drugs and hence a pharmacologically significant effect. The clinical significance of *DHPS* mutations still has to be elucidated.

Because *P. jiroveci* cannot be readily cultured,[11] one can speculate that molecular methods could eventually

provide the means for assessing the antibiotic susceptibility of a particular *Pneumocystis* ''isolate.''

MOLECULAR TYPING OF *PNEUMOCYSTIS*

Pneumocystis is likely transmitted through the respiratory route. The reservoir for *P. jiroveci* is unknown, but could include other human or environmental sources, whereas animal reservoirs are unlikely due to host specificity.[12] Serological studies suggest that a high proportion of healthy individuals has had a primary infection during childhood.[13] It is still debated whether PCP is caused by reinfection or reactivation of latent infection.

Development of a typing system for *P. jiroveci* is critical for investigating the transmission and epidemiology of PCP. Clinical samples contain relatively small amounts of organisms mixed with host DNA, and pure extractions of the organism are difficult or impossible to obtain. Therefore, standard genetic methods such as karyotyping or random amplification of polymorphic DNA cannot be performed. Thus, investigation of the epidemiology has relied on PCR-based DNA sequence analysis.

Different sequence types of *P. jiroveci* have been identified in a number of genetic loci (Table 1). Because of the relatively low discriminatory power of several of these loci, alternatives were sought. The internal transcribed spacer (ITS) regions, ITS1 and ITS2, of the rRNA locus have been central to the study of genomic variations and taxonomic relationships in fungi and other species. In contrast to other fungi, *Pneumocystis* possesses only one

Table 1 Genes used in the typing of *P. jiroveci*

Gene	Size (bp)	Number of different sequences types	Polymorphic nucleotide position	Nucleotide change
mt LSU rRNA	356	5	81	C/T
			85	T/C/A
			248	C/T
mt SSU rRNA	300	2	160	C/A
			196	T/G
5S rRNA	120	6	23	T/G
			63	A/T
			79	T/C
			82	A/G
			88	T/C
Arom	237	4	121	T/C
			208	A/G/C
ITS1	161	21	Refs. [15] and [16]	
ITS2	192	29	Refs. [15] and [16]	
ITS1+2 combinations		65		
DHPS	834	5	Wild type	
			Codon 55	
			Codon 57	
			Codon 60	
Cytochrome *b*	1038	7	279	C/T
			362	C/T
			369	G/T
			516	C/T
			1032	T/A
SSCP		35	Not applicable	Number of patterns
ITS1	204			3
26S	426			6
mt26S	340			4
β-Tubulin	309			3
Tandem repeats in the *MSG* gene	123	5	Six tandem 10-nucleotide repeats	Five patterns: number of repeats from 2 to 6

Source: Refs. [10,14–20].

copy of this locus. Studies have revealed that several ITS types exist (Table 1).

Single-strand conformation polymorphism (SSCP) analysis and restriction fragment length polymorphism (RFLP) assays have been investigated as alternatives to DNA sequencing.[14,21] These methods require prior characterization of the polymorphisms to be analyzed.

Although application of these typing methods has not yet provided unequivocal data on the mode of transmission and epidemiology of human pneumocystosis, studies continue to address these questions. At this point, molecular approaches seem to be the most promising way to investigate the epidemiology of *Pneumocystis*.

MOLECULAR TESTING FOR *PNEUMOCYSTIS*

The standard method for diagnosis of PCP is direct microscopical visualization of *Pneumocystis* organisms in invasive lower respiratory tract specimens (e.g., bronchoalveolar lavage, or BAL).

Upper respiratory specimens usually present insufficient numbers of organisms to enable diagnosis by microscopy, but PCR increases the sensitivity.[22] Even oral washes, which are easily obtained by gargling the mouth with sterile saline, are suitable for PCR diagnosis.[23] Various PCR assays targeting different genes and the use of nested PCR, in which a second round of PCR reamplifies the primary PCR product, have been investigated.[24] However, some of these PCR techniques employed have been too complicated for routine use in a clinical laboratory. Single-round PCR is to be preferred for diagnostic purposes because nested PCR offers a limited increase in diagnostic sensitivity with an increased risk of biological false-positive results (carryover contamination). Choosing a multicopy gene target provides increased sensitivity compared with a single-copy target. Furthermore, employment of touchdown PCR may increase the sensitivity. An internal inhibitor control is recommended to be included in a diagnostic assay. Oligonucleotide sequences and PCR conditions for three different PCR assays are presented in Table 2.

Although PCR methods have the potential to be very sensitive, specimens have been reported to be positive by PCR in patients without clinical PCP, who are presumed to be colonized with *Pneumocystis*. Recent research suggests that organism burden, as assessed by quantitative PCR, is higher in cases of PCP compared with cases of colonization.[25] If these findings are supported by further investigation, quantitative real-time PCR could improve the specificity by distinguishing between infection and colonization.

In addition, the viability of *P. jiroveci* organism may be assessed by the study of *Pneumocystis* mRNA, which could offer a potential method for monitoring treatment.[26]

CONCLUSION

Pneumocystis is an unusual organism, which has been determined to be a fungus by phylogenetic analysis of DNA sequences.

Because of our inability to culture the organism in vitro, molecular and immunological techniques have had

Table 2 Selected PCR assays, targeting three different multicopy genes using touchdown PCR

Gene target	Primers/probes	PCR conditions
mtLSU rRNA	pAZ102-E 5′-GATGGCTGTTTCCAAGCCCA-3′ pAZ102-H 5′-GTGTACGTTGCAAAGTACTC-3′ pAZ102-L2 5′-ATAAGGTAGATAGTCGAAAG-3′	95°C/10 min [94°C/15 sec, 72–62°C/30 sec (−1°C /cycle)] × 10 (92°C/15 sec, 62°C/30 sec, 72°C/15 sec) × 40, 72°C/5 min
mtSSU rRNA	pAZ112-10F 5′-GGGAATTCTAGACGGTCACAGAGATCAG-3′ pAZ112-10R 5′-GGGAATTCGAACGATTACTAGCAATTCC-3′	95°C/10 min [94°C/15 sec, 65–55°C/30 sec (−1°C /cycle), 72°C/60 sec] × 10 (92°C/15 sec, 55°C/30 sec, 72°C/60 sec) × 40, 72°C/5 min
MSG	JKK14/15 5′-GAA TGC AAA TCY TTA CAG ACA ACA G-3′ JKK17 5′-AAA TCA TGA ACG AAA TAA CCA TTG C-3′ PCMSGFRET1U 5′-CAA AAA TAA CAY TSA CAT CAA CRA GGC G-3′ PCMSGFRET1D 5′-TGC AAA CCA ACC AAG TGT ACG ACA GG-3′	95°C/10 min [95°C/5 sec, 65–60°C/10 sec (−1°C /cycle), 72°C/20 sec] × 6 [95°C/5 sec, 58–50°C/10 sec (−2°C /cycle), 72°C/20 sec] × 5 (95°C/5 sec, 50°C/10 sec, 72°C/20 sec) × 35

Source: Refs. [16,22,23,25].

great impact on our understanding of the organism itself and of PCP.

The main fields of research that have benefited from the development of these techniques include characterization of the basic biology of the organism, biology and epidemiology of PCP, and diagnosis of PCP. Although much is still to be learned about this organism and PCP, it is likely that molecular approaches will continue to be among the most important tools available. The ongoing project to sequence the genome will, when finished, provide important information for investigators conducting research on *Pneumocystis*.

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Point-of-Care Testing (POCT)—Nucleic Acid-Based Testing in a Point-of-Care Setting

Jürgen Fuchs
Maurizio Podda
J.W. Goethe University, Frankfurt, Germany

INTRODUCTION

The latest achievements in molecular biology and medicine and the evolution of new diagnostic microdevices are driving the implementation of nucleic acid-based technologies (NABT) in the medical laboratory and are contributing to the emerging utilization of point-of-care testing (POCT). These developments will be changing the medical laboratory and medicine in general. With the increasing recognition of new infectious diseases, the potential threat of global bioterrorism, the evolving identification of genetic markers for disease risk and drug side reactions, and new technology available, there is an increased interest in the potential applications of nucleic acid-based tests in a point-of-care setting. Several years ago nucleic acid-based technologies (NABT) seemed to move only slowly into the medical laboratory,[1] but currently several nucleic acid-based tests have already replaced traditional assays and turned into gold standard, in particular in the field of diagnostic microbiology.[2] Differentiating POCT from regular laboratory testing is somewhat artificial, but is a useful simplification from the health-care management perspective to describe testing by nonlaboratoty staff using specialized diagnostic equipment adapted for near-patient applications. Point-of-care testing has a number of names and definitions: ancillary testing, bedside testing, alternate site testing, and decentralized testing all mean POCT. Point-of-care testing includes simple self-care tests that patients can perform by themselves at home, tests that are performed in the doctor's office, and tests that are performed by personnel, e.g., in the intensive care unit, the emergency room, the ward, or in the battlefield area.

HISTORIC DEVELOPMENTS

Before clinical laboratories evolved in the late 19th and 20th century, simple laboratory testing was performed next to the patient, such as organoleptic analysis (perceived by a sense organ) of body fluids and excrements. An archaic example of POCT from ancient times is tasting the patients' urine to analyze the presence of glucose indicative of diabetes. With the beginning of modern clinical chemistry centrally located laboratories emerged, and later laboratory testing transformed medicine fundamentally when it became available on a broad scale and even more so as it became automated. The trend toward miniaturization, portability, and simplicity of use in combination with the advance of a black-box technology is again changing medicine fundamentally,[3] allowing personnel who do not have intensive training in laboratory medicine or molecular biology to run moderately or highly complex analytical tests. This will result in some reembodiment of laboratory testing in the primary health-care provider's hand, but in contrast to prelaboratorian times, the personnel will be equipped with the finest technology on the market.

QUALITY ISSUES

Point-of-care testing has many advantages and disadvantages, and numerous arguments have been raised in support of and against it.[4–6] A discussion of this topic is out of the scope of this contribution, but data indicate that POCT has (may have) a positive benefit on morbidity and mortality.[7–9] The cost of POCT is usually greater than traditional laboratory testing, but the increased cost may be offset by improvements in the management of patient care, improvements in patient outcomes, and decreased utilization of the health-care system.[7,9] An underestimated aspect of POCT is the importance of established quality-management procedures. These procedures have to ensure that POCT results are accurate, reliable, and performed by competent personnel, and that regulatory compliance is achieved.[10,11] Concerns over the quality of POCT have resulted in a hierarchy of laboratory regulations, and POCT guidelines are appearing in a number of countries worldwide.[12,13] In the United States, all laboratories are required to be CLIA (Clinical Laboratory Improvement Amendments) certified (http://www.cms.gov/clia), and several physician's office laboratories have been forced to close their doors in

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response to regulatory restrictions.[14] The continuing integration of nucleic acid-based POCT in the health-care system is considerably affected by quality-regulations matters, and these measures serve as an excellent controlling instrument.

BLACK-BOX TECHNOLOGY

The development of microfluidic systems,[15] nanotechnology-based lab-on-a-chip devices,[16] micro-total analysis systems (uTAS),[17] and microelectromechanical systems (MEMS)[18] is one of the driving forces for the implementation of nucleic acid-based testing in the point-of-care setting. For example, microelectromechanical systems (MEMS) can revolutionize the commercialization of diagnostic tests by development of compact, potentially disposable, automated diagnostic systems that could be used to perform molecular diagnosis in a point-of-care setting. These systems can be used for automated sample preparation and analysis, such as cell separation, nucleic acid purification, nucleic acid amplification and detection.[18] It was predicted that the health-care implications of successfully developed MEMS are enormous, including rapid identification of disease and risk condition, as well as more accessible health-care delivery at a lower total cost.[19] Recently, a biochip device was developed that integrates sample preparation with PCR and DNA microarray. The on-chip analysis starts with the preparation process of a whole-blood sample, which includes magnetic bead-based target cell capture, cell preconcentration and purification, and cell lysis, followed by PCR amplification and electrochemical microarray-based detection. This biochip performs pathogenic bacteria detection or hemochromatosis genotyping from a whole-blood sample within about 4 and 3 hr, respectively.[20] Another example is the Verigene™ device from Nanosphere, Northbrook, USA. The Verigene™ is a handheld nucleic acid analyzer, which performs clinical sample processing and real-time PCR detection in one instrument, and is scheduled to be available for use at the end of 2004.

ROLE OF NUCLEIC ACID-BASED TECHNOLOGY IN POCT

Nucleic acid-based technology is considered to be of critical importance to public health worldwide and is transforming clinical microbiology, pathology, genetics, pharmacogenetics, prenatal, and preimplantation medicine.[21] Nucleic acid-based technology will also have a significant impact on forensic diagnostics, veterinary medicine, food hygiene and technology, and monitoring of genetically modified agricultural products.

Genetics

Primary-care physicians are playing an increasing role in selecting and counseling patients, and in interpreting genetic test results, which are usually performed in specialized genetic laboratories. Currently, the number of laboratory tests to diagnose monogenic diseases is slowly increasing, and DNA testing is now available for more than 1000 inherited diseases (694 clinical testing and 345 research-only testing) (http://www.geneclinics.org). Mass screening has already commenced for a few of these diseases such as cystic fibrosis, sickle cell disease, hereditary hemochromatosis, and thrombophilias, which have a high prevalence and penetrance. The real challenge lies in the molecular diagnosis and prognosis of complex, multifactorial diseases, but testing for most of these disorders is still experimental. Because of the highly sensitive nature of genetic testing, professional practice guidelines have been developed, and, in addition to these general guidelines, special laboratory guidelines have been developed. It was proposed that testing for disease-causing mutations will become routine, and in time will be used massively in both hospital and community medicine,[22] but currently the implementation of POCT for hereditary diseases is impeded by strict regulations.

Pharmacogenetics

Much of the individual variation in drug response is due to genetic polymorphisms of drug-metabolizing enzymes, and genomic testing for drug-metabolizing enzymes has significant potential for improving the efficiency of drug treatment and reducing adverse drug reactions and thus promises to improve future health care in a number of ways. It is very likely that these tests will make up a significant proportion of total nucleic acid-based testing in the coming years.[23] Currently, however, only a few pharmacogenetic tests and associated products are commercially available, and most are for research use only. Pharmacogenetic tests pose fewer risks for the patient than genetic tests for disease mutations; however, these tests still raise several ethical, economical, and regulatory issues that need to be addressed before they can be integrated into medicine in a large scale.[24]

Microbiology

Molecular theranostics is an emerging concept in clinical microbiology in which molecular biology tools are used to provide rapid and accurate diagnostic assays to enable better initial management of patients and more efficient use of antimicrobials.[25] These applications will probably evolve into standard laboratory and POCT protocols.

Especially in virology, the application of both qualitative and quantitative nucleic acid detection techniques has had a major impact on diagnostics.[26] Point-of-care testing for biological warfare agents by ''nontrained'' personnel using devices such as the RAPID™ (Idaho Technology, Salt Lake City, USA), and handheld instruments such as the RAZOR™ (Idaho Technology), or the BioSeeg™ (Smiths Detection, Edgewood, USA) is already the reality.[27] It seems to be just a matter of time when this kind of technology will become available for primary-care providers for routine microbiological testing in the point-of-care setting.

Pathology

Nucleic acid-based tests have found major applications in pathology, particularly in oncology, and the number of clinically useful molecular assays in the routine clinical pathology laboratory is continuously expanding.[28,29] One example is human papillomavirus (HPV) testing by nucleic acid-based methods, which is becoming an integral part of cervical cancer screening.[30,31] However, several issues still need to be clarified before HPV screening can be recommended unequivocally.[32,33] Another example is evaluation of molecular alterations in fecal DNA, which is a potential, noninvasive, alternative tool for the detection of colorectal cancer and may be suitable for POCT. Stool testing by a multitargeted DNA-based assay has the potential to improve the effectiveness and efficiency for colorectal cancer screening, but the clinical value of this nucleic acid-based test still needs to be assessed by further studies.[34]

POCT MARKET ANALYSIS

The POCT market is expected to be a rapidly increasing and very lucrative market. The spectrum and the total volume of POCT are continuously expanding, and the market of POCT devices is growing at a 5% rate per year. It was estimated that in the developed world just 25% of laboratory testing took place outside the central laboratory in 1998, but this figure is estimated to grow to 45% by 2008.[35] This increase will be most prominent in the United States, but similar trends exist in Europe, Australia, and Asia.[36] In addition, there is a tendency to move laboratory testing away from the clinical chemical laboratory to other diagnostic laboratories, e.g., to pathology, hematology, or oncology. It was estimated that the diagnostic molecular pathology laboratory of the early 21st century will perform 5–10% of the volume of all laboratory testing.[35] Aging of the population in the industrialized Western world will cause a substantial increase in home health care, and in association with telemedicine, POCT is believed to have a significant impact on the management of these patients.[37,38] Many drug stores and supermarkets sell over-the-counter POCT kits intended for home use. These developments will move away test volume from the central laboratory to the point-of-care setting and thus shifting financial volumes away from the laboratory to POCT providers, triggering a distribution conflict. ''POCT is a game of tug-of-war, it's pulled and pushed and shoven between laboratorians, physicians, nurses and other health care providers'' (Karen Titus, contributing editor and comanaging editor of *CAP TODAY*, 2004). Various government guidelines and legislation impact the use of POCT and some of these regulations can be considered strategies in a turf war between the traditional provider of laboratory tests and the new incoming POCT providers.

CONCLUSION

Point-of-care testing is a fact of life in nearly every health-care institution in the Western world. Point-of-care testing is definitely not a replacement for conventional laboratory service but a supplement. It seems realistic that some parts of diagnostics will inevitably leave the central clinical laboratory to be performed instead at the bedside, in the doctor's office, or even at the patient's home. Miniaturization of in vitro diagnostics and NABT will play a significant role in this scenario. Many clinical disciplines deal with a wide spectrum of nucleic acid-based diagnostic assays, including tests for infectious diseases, neoplasias, and inherited diseases, which have the potential to be used at the point-of-care setting. Examples are pathology,[28,29] hematology,[39] oncology,[40] urology,[41] dermatology,[42] obstetrics and gynecology,[43,44] pediatrics,[45] and STD specialty.[46] With the development of prenatal and preimplantation diagnostics, ''obstetrics has been claimed to have assumed a natural leadership position in molecular diagnostics, not only in terms of applied technology, but also in the social, clinical, economical, and political ramifications of these technologies.''[47] The integration of POCT in the health-care system, particularly the implementation of NABT in the point-of-care setting, and finally patient care, will profit more from an inter- and multidisciplinary management approach between equals than by the ''natural leadership position'' of an individual discipline. It remains to be seen what the health-care system will look like when all the critical issues of POCT and NABT have

been resolved, and what impact these changes will have on medical practice in general, laboratory medicine, and on the individual patient in particular.

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Polymer Nonviral Delivery Vehicles

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Ernst Wagner
Ludwig-Maximilians-Universität, Munich, Germany

INTRODUCTION

Polyplexes are based on the condensation of negatively charged DNA by electrostatic attraction with polycationic condensing compounds. The resulting compact particles protect the nucleic acid and also improve the uptake into the cells. Numerous polycations have been used for formulating DNA into complexes. Ideally, the cationic polymer will carry out multiple tasks which include compacting DNA into particles that can migrate to the target tissue, shielding the particles against degradation and undesired interactions, and enhancing cell binding and intracellular delivery into cytoplasm and the nucleus. In practical terms, the polymer is unable to carry out all these tasks. Additional functional domains have to be integrated into the formulation. Advantageously, polymers can be chemically linked to molecules such as cell-targeting ligands, including proteins (antibodies, growth factors) and small molecules (carbohydrates, peptides, vitamins). Various polymer–ligand gene delivery systems have been demonstrated to facilitate receptor-cell-mediated delivery into cultured cells. Targeted delivery to the lung, the liver, or tumors has been achieved in experimental animals, either by localized or systemic application.

TARGETED POLYPLEXES FOR GENE THERAPY

Polycations for DNA Complex Formation

DNA-binding polycations include synthetic polymers such as polylysine, polyethylenimine, cationic dendrimers, carbohydrate-based polymers such as modified chitosan or dextran, and natural DNA-binding proteins such as histones or protamines. The characteristics of these polymers and their use in transfections have been reported extensively.[1–3] Of the ''first-generation'' cationic polymeric carriers evaluated, polyethylenimine,[4] also termed PEI, has the highest transfection efficiency. This can be explained by its intrinsic ability to facilitate endosomal release. The polymer acts as a ''proton sponge,'' containing protonable amines which after endocytosis slow down endosomal acidification, triggering enhanced endosomal chloride accumulation followed by osmotic swelling and breaking up of endosomes.[5] Biocompatible and biodegradable polymers with further enhanced efficiency would be advantageous. Approaches currently under evaluation include the use of low-molecular weight polymers oligomerized into larger polymeric structures by biodegradable, connecting disulfide linkages[6] or esters.[7,8]

In Vivo Applications of Polyplexes

For targeted delivery into a distant organ, the following factors have to be taken into account (Fig. 1): ideally 1) DNA polyplexes are stable and inert in blood; 2) they must be able to reach their target tissue and therefore to cross different biologicals barriers, including vascular endothelium, extracellular matrix, and others; 3) once reaching the target cell they should internalize; 4) should disassemble at the right moment, but still protect the DNA against intracellular degradation; 5) release the DNA into the nuclear compartment; 6) they must elicit as low an inflammatory or immune response as possible. Although no polyplex or other nonviral gene transfer system exists which would fulfill all these requirements, targeted delivery to the lung, the liver, or tumors has already been achieved in experimental animals (Table 1).

Several strategies have been evaluated for gene transfer to the lung. Systemic application of PEI polyplexes resulted in very high gene transfer to the lung.[9] In this application, the linear polymer form of PEI mediates a much higher transfection activity than branched PEI of similar molecular weight.[10] However, a positive charge ratio (cation of polymer to DNA phosphate anion ratio) is required, with a narrow window between efficiency and severe toxicity, as PEI/DNA activates the lung endothelium and forms small aggregates.

As an alternative approach, Ferkol and colleagues generated polylysine polyplexes for targeting the polymeric immunoglobulin receptor by using an antibody Fab fragment as ligand conjugated with polylysine. Systemic delivery of these polyplexes in rats resulted in reporter gene expression in cells of the airway epithelium and submucosal glands.[11] Also, different lung-targeted ligand–polylysine polyplexes were evaluated, using a

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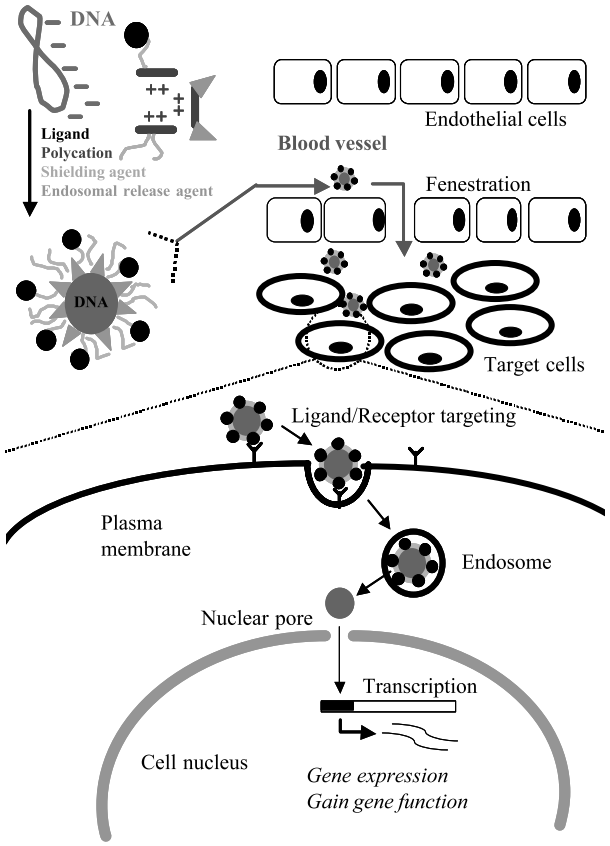

Fig. 1 Targeting opportunities for DNA polyplexes. Polyplex formation with polymer compacting DNA into particles, shielding the polyplex against degradation and undesired interactions, enabling migration to the target tissue, enhancing cell binding and intracellular delivery into cytoplasm and the nucleus. (*View this art in color at www.dekker.com.*)

synthetic peptide ligand for the serpin–enzyme complex receptor. These polyplexes upon nasal administration were able to transiently correct the chloride transport defect in the nasal epithelium of CF mice.[12]

Targeted gene transfer to the liver was reported by Wu and Wu.[13] DNA/asialoorosomucoid–polylysine complexes were administered for targeting to the hepatocyte-specific asialoglycoprotein receptor, and intravenous (i.v.) injection in rats resulted in marker gene expression in rat livers. This work was the very first successful in vivo application of a targeted polyplex system for gene transfer. Using the same type of formulation, Nagase analbuminemic rats were injected with a human albumin expression plasmid, followed by partial hepatectomy.[14] Circulating human albumin increased in concentration to a maximum by 2 weeks postinjection and remained stable for further 2 weeks.

A synthetic hepatocyte-directed, multifunctional polyplex system was applied by the group of Nishikawa et al.,[15] consisting of DNA complexed with polyornithine which was modified with galactose (to serve as asialoglycoprotein receptor ligand) and a fusogenic peptide derived from the influenza virus hemagglutinin subunit HA2 (to serve as endosomal release domain). Upon intravenous injection in mice, a large amount of a marker gene product was detected in the liver, with the hepatocytes contributing more than 95% of the total activity in all tissues. In another approach, a conjugate of low-molecular weight PEI with Pluronic 123 (a block copolymer of polyethylene oxide and polypropylene oxide) was synthesized by the group of Nguyen et al.[16] In combination with unmodified Pluronic 123 and DNA, the conjugate forms small and stable complexes which after i.v. injection into mice exhibit highest gene expression in liver.

Targeting tumors might present a unique opportunity to reach and attack multiple-spread metastases. Direct intratumoral delivery of PEI polyplexes has been investigated. Expression levels were low, and a special form of administration, the local infusion of PEI polyplexes into the tumor mass by a micropump,[17] had to be applied to obtain satisfactory results. As an alternative for lung tumors, Gautam and colleagues successfully delivered PEI polyplexes to lung metastases of melanoma as aerosol through the airways.[18]

A series of targeted DNA polyplex formulations with the potential of systemically targeting tumors have been established. A charge-neutral surface of the DNA particles is essential to minimize nonspecific interactions with blood components, allowing greater intravenous circulation time for the vector to reach its target, and also reducing vector toxicity.[4] The hydrophilic shielding agents investigated include the serum protein transferrin and hydrophilic polymers such as hydroxypropyl methacrylate or polyethylene glycol (PEG). For example, transferrin-shielded polyplexes[19] or PEG-shielded polyplexes[20,21] demonstrated potential for systemic in vivo targeting of tumors. Intravenous injection resulted in gene transfer into distant subcutaneous neuroblastoma tumors of syngeneic mice[19–21] with luciferase marker gene expression levels in tumor tissues approximately 100-fold higher than in other organ tissues. Specificity was confirmed by luciferase imaging in living mice.[22] In analogous manner, EGF–PEG-coated polyplexes were successfully applied for systemic targeting of human hepatocellular carcinoma xenografts in SCID mice.[23] Similar observations of in vivo hepatoma targeting were made with polylysine polyplexes linked with EGF-derived peptides and an endosomally active peptide.[24]

Table 1 In vivo delivery of polyplexes

Polyplex system	Delivery mode	Target organ	Results	Reference
PEI	Intravenous	Lung	High lung gene expression, toxicity	[9]
Anti pIgR–polylysine	Intravenous	Lung	Highest expression in lung; also in liver	[11]
SECR ligand–polylysine	Intranasal	Nasal epithelium	Functional CFTR gene transfer	[12]
ASOM–polylysine	Intravenous	Liver	First demonstration of targeted gene transfer	[13]
Galactose–polyornithine–HA2 peptide	Intravenous	Liver	Hepatocyte-targeted gene expression	[15]
Pluronic–PEI	Intravenous	Liver	Highest gene expression in liver	[16]
PEI	Infusion into tumor	Tumor	Micropump required	[17]
TfR-targeted, shielded PEI	Intravenous	Tumor	Up to 100-fold higher expression in tumor	[19,21]
EGF–PEI, PEG shielded	Intravenous	Hepatoma	Up to 100-fold higher expression in tumor	[23]

ASOM, asialoorosomucoid; EGF, epidermal growth factor; PEG, polyethylene glycol; PEI, polyethylenimine; SECR, serpine–enzyme complex receptor; TfR, transferrin receptor.

Therapeutic Approaches Using Polyplexes

Table 2 lists an example of therapeutic concepts evaluated in animal models. Using the first targeted in vivo gene delivery system developed,[13] Wu and colleagues demonstrated hepatocyte-specific gene transfer of the LDL receptor in a rabbit animal model for familial hypercholesterolemia. This resulted in a temporary amelioration of the disease phenotype.[25] In analogous manner, the albumin serum levels of Nagase analbuminemic rats were transiently raised by human albumin gene delivery.[14]

For cancer, Gautam and colleagues[18] successfully delivered PEI polyplexes expressing the p53 gene as aerosol through the airways to established melanoma lung metastases, which, especially in combination with 9-nitrocamptothecin chemotherapy, resulted in tumor growth inhibition.

Polyplexes with appropriate surface shielding can target the tumor tissue and exploit the unique neovasculature for extravasation from the blood stream (compare Fig. 1) and delivery of therapeutic genes into the tumor tissue. Repeated systemic application of transferrin- and/or PEG-shielded polyplexes encoding tumor necrosis factor alpha (TNF-alpha) into tumor-bearing mice induced expression in tumor cells close to the feeding blood vessels, which triggers TNF-mediated destruction of the tumor vasculature, tumor necrosis, and inhibition of tumor growth as demonstrated in several

Table 2 Examples for therapeutic strategies using polyplexes

Polyplex system	Delivery mode	Gene (target organ)	Therapeutic result	Reference
ASOM–polylysine	Intravenous (rat)	Human albumin (liver)	Human albumin in serum of Nagase rat	[14]
ASOM–polylysine	Intravenous (rabbit)	LDL receptor (liver)	Reduced hypercholesterolemia	[25]
PEI	Aerosol (mice)	p53 (lung metastases)	Growth inhibition of established lung metastases	[18]
TfR-targeted, shielded PEI	Intravenous (mice)	TNF-alpha (tumors)	Tumor necrosis, inhibition of tumor growth	[21]
EGFR-targeted polylysine	Intravenous (mice)	p21WAF-1, GM-CSF (hepatoma)	Inhibition of hepatoma growth	[24]

ASOM, asialoorosomucoid; EGFR, epidermal growth factor receptor; GM-CSF, granulocyte macrophage-colony stimulating factor; LDL, low density lipoprotein; PEI, polyethylenimine; TfR, transferrin receptor; TNF, tumor necrosis factor.

murine tumor models.[21] The gene expression of TNF-alpha was localized within the tumor; no significant systemic TNF-related toxicities were observed. Systemic administration of combined p21(WAF-1) and GM-CSF genes formulated into EGF receptor-targeted polyplexes inhibited the growth of subcutaneous hepatoma cells and increased the survival rate of tumor-bearing mice.[24] It remains to be demonstrated whether encouraging findings such as those obtained in mice can be transferred to studies in larger animals and humans.

CONCLUSION

Targeted delivery to the lung, the liver, or tumors has been achieved in experimental animals, either by localized or systemic application. Therapeutic effects have been demonstrated, although efficiencies currently appear to be still too low to justify clinical use. The limitations of first-generation polymeric carriers (modest activity and significant toxicity) have to be overcome by developments of new biodegradable polycationic polymers, incorporation of targeting and intracellular transport functions, and polyplex formulations that avoid unspecific adverse interactions with the host. A key future step will be the further development of polyplexes into ''artificial viruses,'' i.e., polyplexes possessing viruslike entry functions which are presented by smart polymers and conjugates. These ''smart'' polymers in contrast to conventional polymers have to respond in a more dynamic and controlled manner to alterations in their biological microenvironment such as pH or redox environment, and have to undergo programmed structural changes, to more accurately switch on the individual delivery functions only when required in the individual steps (Fig. 1) of the delivery process.

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Polymerase Chain Reaction (PCR)

Guido Krupp
artus GmbH, Hamburg, Germany

Ulrich Spengler
artus Biotech USA, Inc., San Francisco, California, U.S.A.

INTRODUCTION

This brief presentation cannot replace in-depth textbooks (e.g., Gelfand et al.).[1] The principle of the polymerase chain reaction (PCR) method is outlined in Fig. 1. Although it looks simple, a genius like Kary Mullis was needed for its invention, initially using the heat-labile Klenow enzyme as DNA polymerase, which had to be replenished in each amplification cycle.[2] Mullis, at that time employed by Cetus Corporation in California, was awarded the Nobel Prize in Chemistry in 1993. Since then, PCR technology has radically changed the face of medicine and biology. The basics of the method and some special applications will be outlined.

BASICS OF PCR TECHNOLOGY

The original goal of the PCR inventors was to develop a new diagnostic technology for the detection of an otherwise hardly traceable virus. The intended amplification procedure should work with tiny amounts of DNA, generating millions of amplicons from a single copy of the virus genome. Optimization of basic PCR protocols[1] for the amplification of specific targets involves several steps:

1) *Considerations for the amount of input PCR template*: In theory, a single target copy can be amplified (30 PCR cycles will yield up to a billion-fold amplification or femtomole amounts of amplified PCR product). But statistical variations will always result in undesired blank samples, and a threshold of 3–10 copies is a more practical lower limit. But there is also a maximum of input target, which should not exceed 10^5–10^6 copies. Apart from rapid consumption of all PCR components, rapid reannealing of both DNA strands leads to kinetic interference with primer hybridization or elongation, and PCR products can act as primers. As a drastic consequence, only a smear of PCR products with divergent sizes will be observed due to premature polymerase termination and staggered priming of PCR products.
2) *Primer design*: Length should be between 18 and 30 nucleotides with a GC content in the range of 40–60%. The following features should be avoided: complementarity of two or more nucleotides at the 3′ ends of primer pairs (to reduce formation of primer dimers); T at the 3′ end (3′-terminal T increases mismatch tolerance); complementarity within the primers and between primer pairs (detailed help is available from numerous software programs, e.g., the free *primer3* at www-genome.wi.mit.edu/genome_software/other/primer3.html or the commercial PrimerExpress® from Applied Biosystems).
3) *Adjustment of primer annealing temperatures*: The simplified formula of T_m [°C] = 2*x*(A:T) + 4*x*(G:C) can be used as a guideline. Whenever possible, use primer pairs with similar T_m values and optimize the PCR protocol by a stepwise increase of the annealing temperature, starting about 5°C below the calculated T_m value.
4) *Variety of cycling conditions*: In general, three-step amplification cycles are used (Table 1). If high annealing temperatures can be used, two-step amplification cycles are possible (Table 2), and some reports[3,4] suggest that this can result in less premature termination and the formation of more defined PCR products.
5) *Optimized concentrations of dNTPs and buffers (especially the divalent cation Mg^{2+}), as well as the use of reaction additives*: Some commercial suppliers (e.g., Q-solution from Qiagen with a mixture of monovalent K^+ and NH_4^+ ions) offer solutions with a wider window for optimal annealing temperatures, as well as Mg^{2+} and dNTP concentrations (Table 3).
6) *Choice of commercial thermocyclers*: A wide range is available, including instruments with several independently operated heating/cooling blocks or with gradient blocks that permit analysis of a temperature series in a single experiment. Blocks are available for your preferred PCR reaction tubes and sample numbers (brief overview in Table 4).
7) *''Hot-start'' PCR or heat-activated DNA polymerase*: If highly stringent PCR conditions are desired, consider using ''hot-start'' PCR or heat-activated

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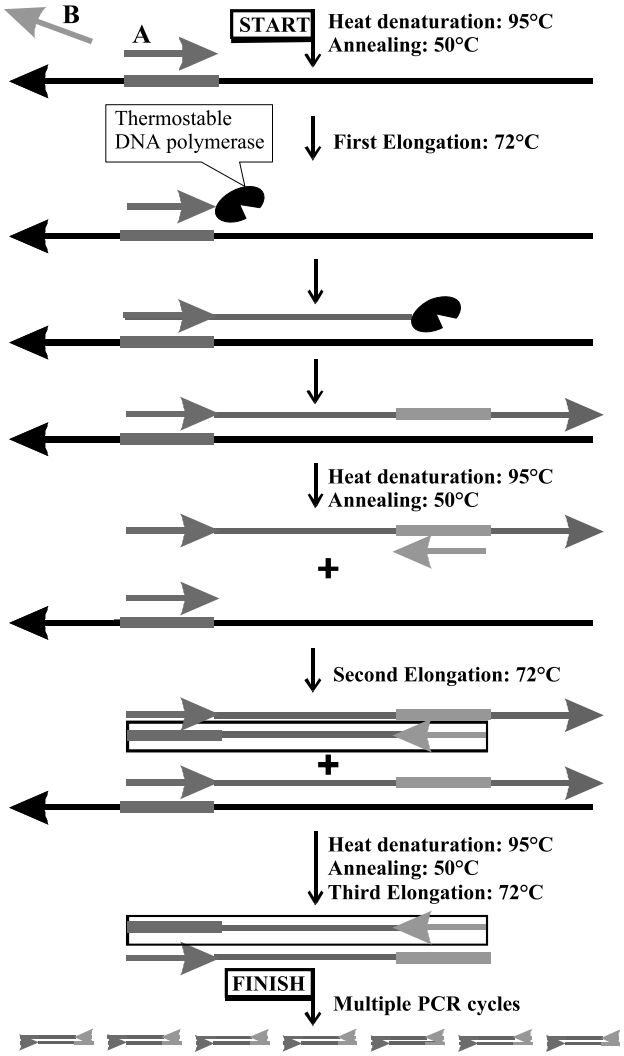

Fig. 1 Principle of PCR technology. Technology for the amplification of a single-stranded DNA target is outlined. *Start*: After heat denaturation (95°C), primers are annealed at a lower temperature (e.g., 50°C). Initially, only primer A can anneal to its complementary sequence (thick red line) in the target DNA. *First elongation*: The optimal reaction temperature for the thermostable Taq DNA polymerase is 72°C, and the annealed primer A is elongated, in principle, all the way to the end of the target DNA, including the sequence (thick green line) complementary to primer B. *Second elongation*: After heat denaturation and annealing, this step generates the first *specific PCR product* with a defined length, containing only the sequence stretch flanked by the two primer sequences (boxed product with green arrow, blue stretch, and thick red line). *Finish*: Multiple copies of the *specific PCR product* are generated (shown as smaller schemes). Primer A (red arrow), primer B (green arrow), target DNA (black), and PCR-generated DNA (blue). (*View this art in color at www.dekker.com.*)

DNA polymerase. The thermostable DNA polymerases have a high temperature optimum but they are not completely inactive at room temperature, and this can lead to elongation of misannealed primers or excessive primer dimer formation. These low-temperature products can be avoided by hot-start PCR: An essential component such as polymerase or magnesium solution is added only after the ''initial template denaturation'' step, during the ''primer annealing'' step (Tables 2 and 3). Another, more convenient option is the use of inactivated DNA polymerase enzyme (commercially available, using heat-labile chemical modification, e.g., from Qiagen; or by antibodies, e.g., from Roche Diagnostics). The enzyme activation requires an extended heat treatment and, for this purpose, the ''initial template denaturation'' at 95°C can be extended to 10 min.

After solving the basic problems of the PCR procedure, one serious practical problem is the occurrence of PCR inhibition, leading to false-negative results. This can be avoided by the inclusion of a separately amplified PCR target as internal control (Fig. 2).[5] A further complication derives from the required high precision in diagnostics. This means that the occurrence of an amplicon with the expected size is not sufficient proof for the positive detection of the targeted pathogen, considering the far-reaching consequences. Therefore in all diagnostic procedures, a sequence-specific identification was and still is a mandatory additional requirement to confirm the amplification of the desired target. Two basic procedures can be applied:[6] 1) Southern hybridization involves transfer (blotting) of the PCR products (with or without prior gel electrophoresis) to nitrocellulose or nylon membranes, followed by hybridization with a labeled probe. Stringent washes remove all nonspecifically bound probes. Finally, the specifically bound, labeled probe is detected. Different types of labels can be used: direct labels are radioactive isotopes (detected with X-ray film, or phosphoimager) or fluorescent dyes (detected with flourimager). Indirect labels are biotin or digoxigenin, combined with enzymes (phosphatase or peroxidase), which carry the corresponding haptens—streptavidin for biotin or antidigoxigenin antibodies for digoxigenin. A wide variety of enzyme substrates are available, which can be converted to fluorescent or nonfluorescent dyes, or produce a chemiluminescent reaction. The multiple turnover of substrates results in much higher sensitivity of these enzyme-coupled detections. Products and detailed protocols are available from many commercial suppliers (e.g., Amersham and Roche Diagnostics). 2) Higher throughput and automation are possible by a combination of capture and detection probes. Both probe sequences must be present in the PCR amplicons. First, amplicons

Table 1 General PCR conditions

Useful range of template copies	Primers	dNTP/$MgCl_2$	Thermostable DNA polymerase	Additives
Theoretical lower limit: one single copy (30 cycles can generate up to 1 billion copies)	5–50 pmol	20–300 μM/1.5 mM[a]	0.2–5 U *Taq* DNA polymerase (from *Thermus aquaticus*; no proofreading)	Increased annealing stringency: 2–5% DMSO, 5% formamide
Upper limit: 100,000 to 1 million copies	0.1–1 μM	20–400 μM/2–4 mM[a]	Pfu DNA polymerase (from *Pyrococcus furiosus*; with proofreading)	Increased efficiency and specificity: 1–10% glycerol, or nonionic detergents such as Tween, Nonidet, CHAPS, Triton

[a]A surplus of free Mg^{2+} is required. Therefore higher dNTP concentrations require higher Mg^{2+} concentrations.

are hybridized to an immobilized capture probe (on membrane or paramagnetic beads), followed by hybridization with a labeled detector probe, stringent washes, and detection, as described above.

This means that the nowadays fully automated and straightforward PCR is complicated by these extra steps after PCR completion, which include the risk of spreading the multimillion-fold amplified DNA segment. If high numbers of samples are analyzed in parallel, laboratory contamination can lead to many false-positive results. Therefore in routine diagnostics, conventional PCR is now widely replaced by sequence-specific real-time PCR techniques (see ''Real-Time PCR Technology'').

Exciting applications in molecular biology derive from the use of composite primers (principle in Fig. 3), combining a 3′-terminal segment with the usual target-specific primer sequence and an unrelated 5′ track that can introduce functional DNA sequence recognition sites. Examples are cleavage sites for restriction enzymes in cloning applications, or a promoter sequence of an RNA polymerase for the production of large amounts of PCR-designed RNA transcripts,[7] useful for in vitro translation, RNA processing, tRNA charging, ribozyme studies, etc. For cloning applications, it is important to keep in mind that the most widely used thermostable *Taq* DNA polymerase has an error rate of about 1:1000.[1] If the PCR-generated mixture of millions of amplicon molecules is used directly (e.g., in sequencing, or for the generation of RNA transcripts), the mutated molecules can be ignored. However, if PCR products are cloned, these clones are derived from individual amplicon molecules. This means that, if only one clone is analyzed, the derived sequence can have one or several mutations. Therefore multiple clones have to be sequenced, and a functional PCR construct (e.g., for overexpression of a protein) with the correct sequence has to be selected.

The aim to perform quantitative PCR analysis is a major challenge in conventional PCR. In principle, the amount of PCR product (*P*) is directly related to the

Table 2 General thermoprofile for three-step PCR

Number of cycles	Temperature [°C]	Time range	Description
1	94	2 min	Initial template denaturation
25–35	45–65	5–60 sec	Primer annealing
	72 (68 for very long PCR products)	20 sec–10 min	Extension/ elongation
	94	5–20 sec	Template denaturation
1	72	2–10 min	Final elongation for complete dsDNA PCR product

Table 3 General thermoprofile for two-step PCR

Number of cycles	Temperature [°C]	Time range	Description
1	94	2 min	Initial template denaturation
25–35	60–69	60 sec–10 min	Primer annealing and extension/ elongation
	94	5–20 sec	Template denaturation
1	72	2–10 min	Final elongation for complete dsDNA PCR product

Table 4 Some commercially available thermocyclers—a brief overview

Supplier	Instrument name	Number of heating blocks (change possible)	Block options	Heating (cooling) rate [°C/sec]	Temperature uniformity [± °C]	Gradient temperature range [°C]	Storage capacity: programs (steps per program)
Applied Biosystems	GeneAmp 9700	1 (Yes)	96 × 0.2 mL, 60 × 0.5 mL of MTP	Up to 5	0.5	No	100 (99)
	Dual GeneAmp 9700	2 (Yes)	2 × 96 × 0.2 mL of MTP	1 (1.5)	0.5	No	100 (99)
Biometra	Tgradient	1 (Yes)	96 × 0.2 mL, 48 × 0.5 mL of 384-well MTP slide	4 (3)	0.3	Yes, 40	>100 (99)
	T3 thermocycler	3 (No)	3 × 48 × 0.2 mL, 3 × 20 × 0.5 mL of MTP	1.5 (1.3)	0.3	No	100 (99)
	Tpersonal	1 (No)	48 × 0.2 mL, 20 × 0.5 mL of MTP	3 (3)	0.5	No	>100 (99)
BioRad	iCycler[a]	1 (Yes)	96 × 0.2 mL, 60 × 0.5 mL of MTP	3.3 (2)	0.3	Yes, 25	255 (99)
Eppendorf	Mastercycler	1 (No, universal)	96 × 0.2 mL, 77 × 0.5 mL	3 (2)	0.4	No	100 (99)
	Mastercycler Gradient	1 (No, universal)	96 × 0.2 mL, 77 × 0.5 mL	3 (2)	0.4	20	100 (99)
	Mastercycler ep Gradient	1 (Yes)	96 × 0.2 mL of MTP slide	6 (4.5)	0.4	Yes, 20	>700 (99)
MJ Research	Opticon[a]	1 (Yes)	96 × 0.2 mL of MTP	3 (3)	0.4	Yes, 24	External PC control
	PTC 150	1 (Yes)	25 × 0.2 mL, 16 × 0.5 mL	2.4 (2.4)	0.4	No	80 (99)
	PTC 225 Tetrad	4 (Yes)	96 × 0.2 mL, 60 × 0.5 mL of MTP slides	3 (3)	0.4	Yes, 24	400 (99)
Labtech	Techgene	2 (Yes)	25 × 0.2 mL, 20 × 0.5 mL	3 (2)	0.5	No	80 (99)
	Touchgene Gradient	4 (Yes)	96 × 0.2 mL, 40 × 0.5 mL of MTP slides	3 (1.8)	0.4	Yes, 20	50 (99)
	Genius	7 (Yes)	96 × 0.2 mL, 40 × 0.5 mL of MTP slides	2.8 (1.8)	0.5	No	99 (99)
MWG-Biotech	Primus 25	1 (Yes)	25 × 0.2 mL, 13 × 0.5 mL	4 (3)	0.5	No	90 (99)
	Primus Multiblock	4 (Yes)	96 × 0.2 mL of MTP slide	4 (3)	0.5	No	External PC control
PEQLAB	Cyclone 25	1 (Yes)	25 × 0.2 mL, 20 × 0.5 mL	3 (2)	0.5	No	80 (99)
	Cyclone Gradient	1 (Yes)	96 × 0.2 mL, 40 × 0.5 mL of MTP slide	3 (2)	0.5	No	External PC control
Thermo Hybaid	PCR Sprint	1 (Yes)	24 × 0.2 mL, 20 × 0.5 mL	3 (2)	0.5	No	60 (5)
	Px2	1 (Yes)	96 × 0.2 mL, 48 × 0.5 mL of MTP slide	3 (2)	0.4	Yes	99 (10)

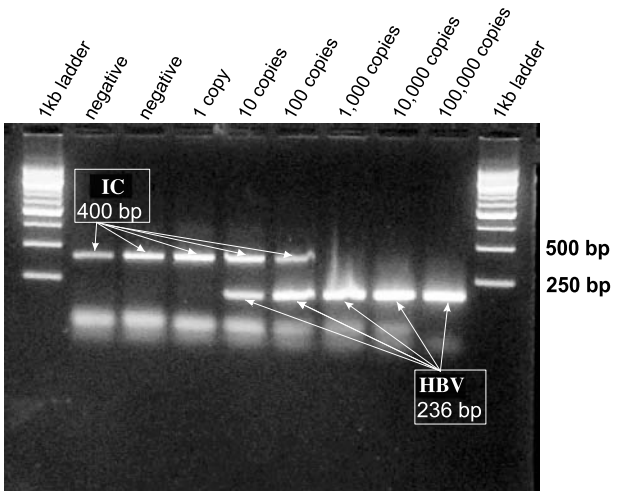

Fig. 2 Coamplification of internal PCR control. All PCR reactions contain primers for the specific pathogen (here, a 236-bp segment of hepatitis B virus, or HBV, is amplified) and for the internal PCR control template IC (here leading to a 400-bp amplicon). The IC sequence and primers are unrelated to the target pathogen and all PCR reactions are spiked with a defined amount of IC. In the absence of the pathogen (negative controls and negative samples), amplification of IC indicates proper PCR conditions and the absence of PCR inhibitors. These inhibitors frequently occur in biological samples and have to be removed during isolation of the DNA, prior to PCR analysis. Theoretical dilution down to one HBV copy per reaction frequently leads to no copy and thus to negative results. At low HBV titers (up to 100 copies per PCR reaction), amplicons from IC and HBV are observed. At higher HBV titers (1000 copies and more), only the HBV-specific amplicon is generated due to competition for reaction compounds. (*View this art in color at www.dekker.com.*)

number of input target copies (T): $P=TE^n$ (E=PCR cycle efficiency, ideally 2.0; n=number of PCR cycles).[8] However, this calculation is based on a constant value for E, and this requirement is met only for a limited number of cycles, the logarithmic or exponential phase. With increasing product concentration and stepwise consumption of primers and other reaction components, E will decrease and, finally, a plateau phase is reached with $E=1$ (Fig. 4). In consequence, product quantities reflect the initial target amounts only until certain cycle numbers are reached, whereas this relation is lost at higher cycle numbers (Fig. 5). In practice, unknown target amounts require the comparative quantitative analysis of PCR products after the completion of several different PCR cycle numbers. Again, real-time PCR techniques are preferred in routine applications. However, the previously mentioned sequence-specific detection is not mandatory and a simplified and much cheaper detection of amplicons by means of intercalating dyes such as ethidium bromide or SYBR Green is sufficient (see ''Real-Time PCR Technology'').

TROUBLESHOOTING

Problem: No PCR Product

Check the quality of primers and template.
Increase the number of cycles (by steps of five).
Increase template DNA by 50-ng increments.
Increase enzyme concentration (up to 5 U/50 μL).
Check if the PCR tubes fit the thermocycler used. Poor thermal contact prevents effective temperature transfer.
Check for air bubbles trapped after mixing the PCR components. Air bubbles prevent homogenous temperature distribution throughout the reaction volume.

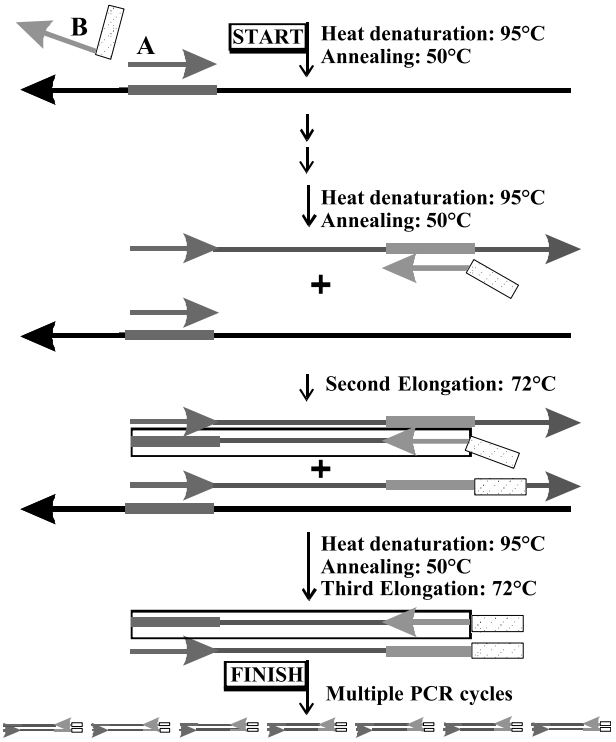

Fig. 3 PCR with functional primer(s). The procedure is as outlined in Fig. 1, but one or both primers contain an extra 5′-terminal functional segment. Here, it is shown only for primer B, indicated by a striped box and shown as tilted element for easier perception. During annealing of primer B, this box does not find a complementary target and sticks out, essentially without affecting PCR performance. After the third elongation step, this box is fully integrated in the *specific PCR product* and further amplified as shown before. (*View this art in color at www.dekker.com.*)

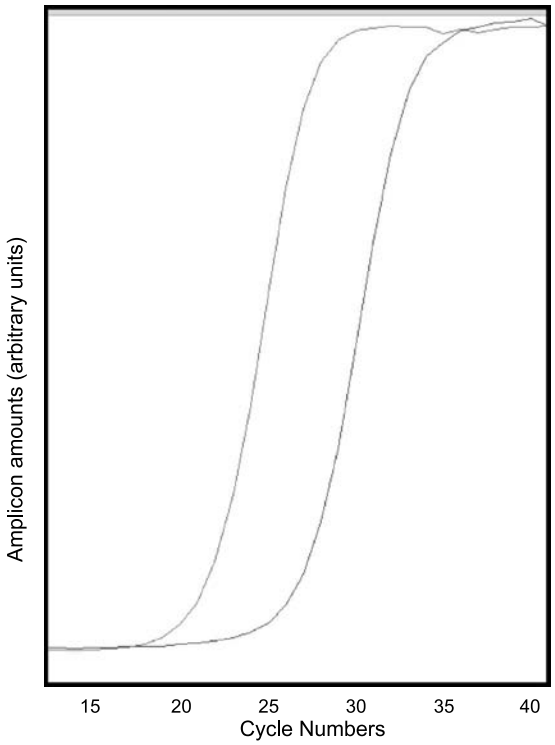

Fig. 4 Quantitative dynamics in PCR amplicon generation. After a certain number of PCR cycles, detectable amplicon amounts are generated (with the target amounts used here, after about 18 and 24 cycles, respectively). These amounts are amplified logarithmically for only about five subsequent PCR cycles, and, finally, a static plateau phase is reached (at about 26 and 32 cycles, respectively). (*View this art in color at www.dekker.com.*)

Problem: Low Amount of Defined PCR Product on High Background Smear

Reduce the number of cycles by steps of two.
Reduce template DNA by decrements of 50 ng.
Increase the time of the elongation step.
Check the ratio of magnesium ions/dNTP. A surplus of free Mg^{2+} is required; therefore a final dNTP concentration at 500 μM requires 2.5 mM Mg^{2+}.
Reduce the amount of enzyme in decrements of 0.2 U.
Increase the concentration of magnesium ions up to 3.5 mM in 0.25 increments if more than 500 ng of template DNA, or more than 500 μM dNTPs is used.

Problem: Low Yield of PCR Product

Increase the final buffer concentration in the reaction assay to 1.6×.
Increase the number of cycles (especially for complex genomic targets and GC-rich targets).
For GC-rich targets, consider replacing dGTP by base analogs with reduced T_m, such as deaza-dGTP or dITP.
Increase the amount of template DNA per reaction by increments of 50 ng.
Increase the amount of enzyme (up to 5 U/50 μL).
Increase the magnesium ions (up to 3.5 mM).

Problem: Nonspecific Products

Try higher annealing temperatures in increments of 5°C.
Reduce the annealing time down to 8 sec in 2-sec decrements.
Reduce time of the elongation step.
Decrease the enzyme amount per reaction in decrements of 0.2 U.
Reduce the amount of template DNA.
Reduce the number of cycles by steps of two.
Add 2–5% dimethyl sulfoxide (DMSO) to the PCR reaction.
Design new (maybe longer) primers.

CONCLUSION

Although conventional PCR has lost ground in the detection of pathogens for diagnostic applications, it remains the workhorse, with many applications in molecular biology. Future developments might reintroduce large-scale diagnostic applications of conventional PCR where cross-contamination and false positives are much less important, such as in tests for mutation(s) in one specific gene involved in a genetic disease and for single nucleotide polymorphism (SNP) analyses. Here, the target of interest is always present and amplified; only the subsequent detection of sequence variations is important. Beyond the scope of this entry are emerging combinations with advanced detection technologies suitable for automation and high throughput, such as pyrosequencing[9] and the rather complex mass spectrometry.[10]

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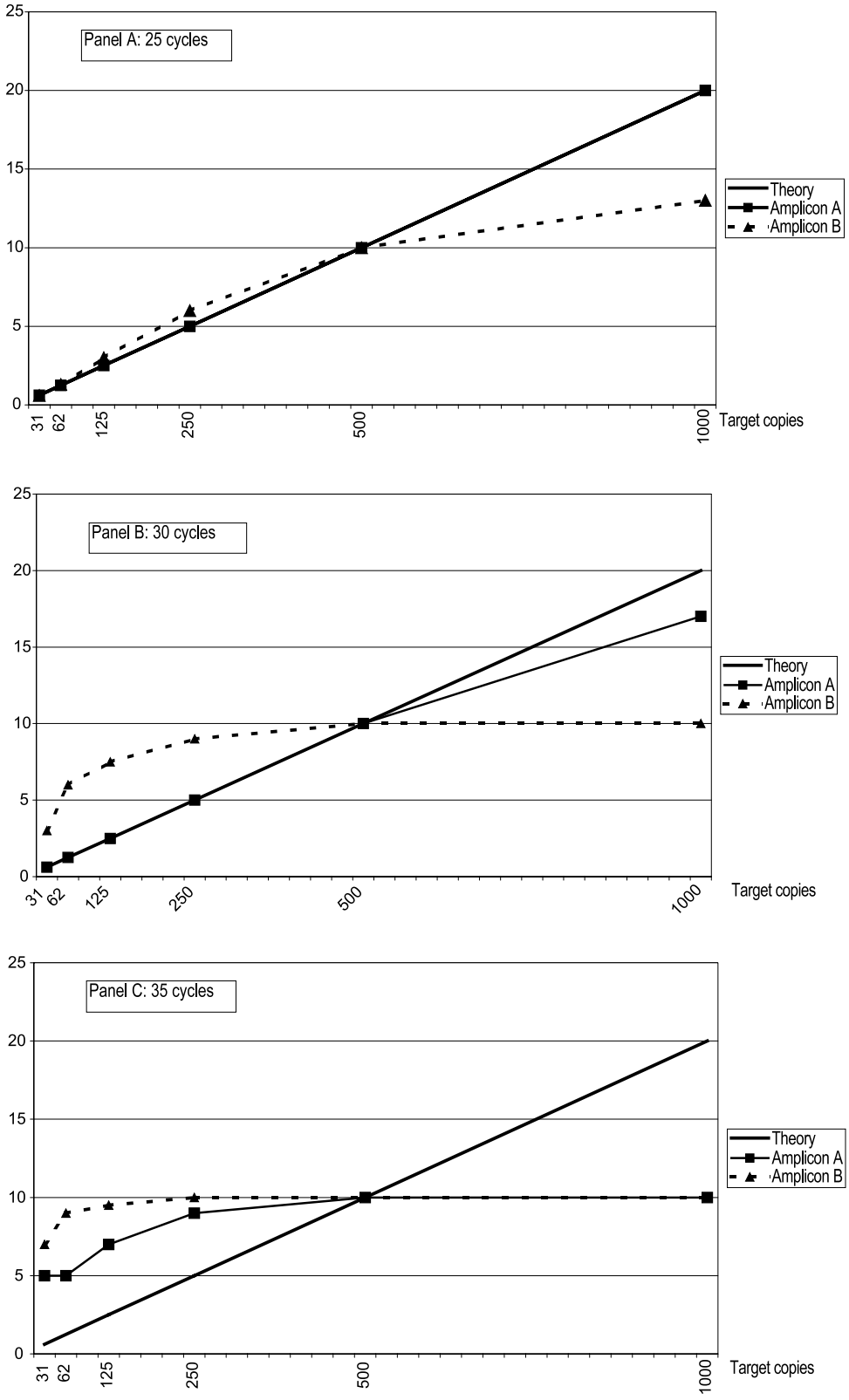

Fig. 5 Quantitative PCR by comparing several results after the completion of different PCR cycle numbers. As an example, dilution curves for two different targets are compared. (From Ref. [8].) Panel A: Data after 25 cycles. For all amounts of target A, the observed amplicon amounts follow the theoretical prediction; with target B, the highest amount levels off. Panel B: Data after 30 cycles. With target A, the highest amount is off, whereas essentially all values for target B are off from the theoretical prediction. Panel C: Data after 35 cycles. Plateau phase is reached for all amounts of targets A and B.

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Prader–Willi Syndrome

Milen Velinov
Edmund C. Jenkins
W. Ted Brown
New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, U.S.A.

INTRODUCTION

Prader–Willi syndrome (PWS), a condition initially described by Prader, Labhard, and Willi in 1956,[1] is a neurodevelopmental disorder with a clinical presentation that includes neonatal hypotonia, obesity, mental retardation, and behavioral abnormalities. After introduction of high-resolution chromosome analysis, PWS was the first abnormal phenotype found to be associated with a submicroscopic chromosomal deletion.[2] The PWS-associated microdeletion spans about 4 Mbp in the chromosomal region 15q11q13 and always affects the paternally derived chromosome. In this chromosomal region several genes are imprinted—only their paternally derived alleles are functional. Among them, candidates for major gene contributors for the PWS phenotype are the unusual polycistronic gene *SNURF-SNRPN*, the gene *NDN*, and others.[3] Four main molecular mechanisms may lead to deficiencies of genes located in the PWS area. In all PWS cases, with the exception of the rare cases of chromosomal balanced translocations, DNA methylation abnormalities can be detected and are used for diagnostic evaluations. Although in most cases the recurrence risk in the family is lower than 1%, complete diagnostic evaluation and determination of the particular PWS type is always appropriate because in some rare instances the recurrence risk is increased. The management of PWS is generally supportive. One useful recent development in PWS therapy is the use of growth hormone (GH) to counteract obesity and short stature.

PREVALENCE

Most frequently reported prevalence of PWS is in the range of 1:10,000–20,000.[4,5]

CLINICAL FEATURES

Patients with PWS have central muscle hypotonia that is manifested prenatally as decreased fetal movements, abnormal fetal position, and difficult delivery. The hypotonia is evident in the newborn period, accompanied by poor sucking and failure to thrive. Hypogonadism resulting in cryptorchidism and scrotal hypoplasia in males and labial hypoplasia in females are also seen in the newborn period.

Hyperphagia and food-seeking behavior start at about 1 year of age and lead to obesity by 1–6 years. The obesity is central and tends to spare the distal extremities. There is mild facial dysmorphism consisting of almond-shaped palpebral fissures and thin upper lip with downturned mouth. Most patients with PWS have small hands and feet, as well as strabismus. About one third have hypopigmentation.[6]

In addition to the prominent motor delay, there is cognitive developmental delay and, ultimately, mild mental retardation in most cases. The verbal skills are relatively spared.

Characteristic behavioral abnormalities with tantrums, stubbornness, and obsessive–compulsive characteristics are seen in the early childhood. True psychoses later develop in up to 10% of patients.

Clinical diagnostic criteria have been established for PWS to assist clinicians with making clinical diagnosis[4] (Table 1). Laboratory confirmation (see Laboratory Testing) is necessary for all suspected cases and will identify all true PWS cases.

MOLECULAR BASIS AND GENETIC COUNSELING

PWS is the result of deficiency of one or more genes that map on the proximal long arm of chromosome 15 (Table 2). These genes are imprinted. Only their paternally derived alleles are functional.[3] The genomic imprinting on chromosome 15 is reflected in the patterns of cytosine methylation in the CpG islands of this region. These cytosines are methylated only in the maternally derived sequences in the imprinted area. These detectable differences can be used for the diagnostic workup of individuals with suspected PWS. Four mechanisms

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Table 1 Consensus clinical diagnostic criteria for PWS

Major criteria (one point each)
Neonatal/infantile central hypotonia
Feeding problems, failure to thrive
Rapid weight gain between 1 and 6 years
Characteristic facial features
Hypogonadism
Developmental delay/mental retardation
Minor criteria (1/2 point each)
Decreased fetal movements
Abnormal behavior
Sleep disturbances/sleep apnea
Short stature
Hypopigmentation
Small hands/feet
Narrow hands with straight ulnar border
Esotropia, myopia
Thick viscous saliva
Speech articulation defects
Skin picking
Supportive findings
High pain threshold
Decreased vomiting
Temperature instability
Scoliosis/kyphosis
Early adrenarche
Osteoporosis
Unusual skill with jigsaw puzzles
Normal neuromuscular studies

The diagnosis of PWS is strongly suspected in children under 3 years of age with five points (three from major criteria) and above 3 years of age with eight points (four from major criteria).
Source: Adopted from Refs. [4] and [15].

can lead to PWS. (All but Type IIb among those listed below have DNA methylation abnormalities and can be diagnosed using DNA methylation analysis; see Diagnostic Methods.)

Type I: Chromosome 15q Deletion

About 70% of all PWS cases are the result of a de novo interstitial deletion involving the 15q11q13 region.[2] Most cases have similar/identical common deletion of about 4 Mbp with the same distal breakpoint and two alternative proximal breakpoints.[3] The deletion always involves the paternally derived chromosome 15. The same deletion is observed in patients with Angelman syndrome (AS) (see Angelman Syndrome), but in those cases the deleted chromosome is maternally derived.[7] The 15q deletion cases typically occur de novo and the recurrence risk for subsequent pregnancies is less than 1%.

Type II

Chromosomal rearrangements are seen in less than 1% of the PWS cases.

Type IIa: Unbalanced translocation affecting chromosome 15.[8,12] In these cases, if the transmitting parent has a balanced translocation, the recurrence risk for PWS in the family can be as high as 50%.

Type IIb: De novo balanced translocations affecting the paternally derived chromosome 15. Five such cases that resulted in PWS were reported.[3] In all cases of balanced translocations the translocation breakpoint lies within the locus of the gene *SNURF-SNRPN*. The balanced translocation cases do not have imprinting/DNA methylation abnormalities (see Diagnostic Methods). The mechanism here appears to be the disruption of sequences in the *SNURF-SNRPN* locus and subsequent deficiency of one or more of the multiple transcripts that originate from this locus (see The PWS/AS Chromosomal Region).[3] For these de novo translocations the recurrence risk is less then 1%.

Type III: Maternal Uniparental Disomy 15

Most of the deletion-negative PWS cases (about 25% of all cases) are due to maternal uniparental disomy 15 (UPD 15) inheritance of both chromosome 15s from the mother with resulting deficiency of paternally derived alleles in the imprinted 15q11q13 region.[9,10] Paternal UPD 15 is also observed and leads to Angelman syndrome (see Angelman Syndrome). However, the paternal UPD is not as frequent as the maternal (only 2–3% of the AS cases are the result of UPD). It appears that the mechanism of UPD occurrence differs in PWS, and AS maternal UPD in PWS occurred in most cases in meiosis I, whereas most paternal UPD cases that resulted in AS occurred postzygotically.[11] Although the recurrence risk for PWS in cases of UPD (Type IIIa) is generally low, chromosomal rearrangements in the parents involving chromosome 15 (Type IIIb) may increase the risk for maternal UPD 15 and PWS in the offspring.[12]

Type IV: Imprinting Defect

About 1% of individuals with PWS have neither a FISH identifiable 15q deletion nor UPD 15, but have DNA methylation abnormalities as if they had only maternally derived chromosome 15s. Up to 20% of these individuals (Type IVa) have a small deletion at the 5′ end of the *SNURF-SNRPN* locus, involving its first exon.[13] This region is referred to as imprinting center (IC) because it seems that this locus takes part in the regulation of the imprinting in a large (about 2 Mbp) imprinted region on the proximal 15q. A small proportion of the patients with

Table 2 Molecular types of PWS

Type	Mechanism	Laboratory testing	Proportion of all (%)	Recurrence risk
I	De novo Del 15q11q13	MT+[a] FISH+	70	<1%
IIa	Chromosomal unbalanced translocations	Chromosome analysis—abnormal MT+	<1	Up to 50%
IIb	Chromosomal balanced translocations	MT−, FISH− Chromosome analysis—abnormal	<1	<1% (de novo occurrence)
IIIa	Maternal UPD	MT+, UPD analysis+	25	<1%
IIIb	Uniparental disomy and chromosomal aberration	Chromosome analysis—abnormal MT+ UPD study+	<1	? Increased, but limited data
IVa	Imprinting defect with IC deletion	MT+, FISH− UPD−, IC del+	<1	Up to 50%
IVb	Imprinting defect without IC deletion	MT+, FISH− UPD−, IC del−	1	<1%

[a]MT = DNA methylation testing.

AS also have deletions in this region. However, the analysis of patients with imprinting mutations resulting in PWS or AS showed that the smallest regions of overlap for the imprinting mutations are not the same for the two groups of individuals. The IC is thus a bipartite structure with one proximal region of about 880 bp that regulates the paternal-to-maternal imprinting switch, and when disrupted may lead to AS, and another, distal 4.3-kb region that when disrupted results in PWS[14] (see Angelman Syndrome). The imprinting center deletions are also identified in the fathers of the affected individuals, and are associated with a 50% recurrence risk for subsequent pregnancies. On the other hand, the imprinting defects without identifiable IC deletions (Type IVb) are considered sporadic, and are not associated with greater recurrence risk.[13] A summary of Types I–IV is given in Table 1.

THE PWS/AS CHROMOSOMAL REGION

The common deletion in PWS spans about 4 Mbp on the proximal long arm of chromosome 15 (for a review, see also Ref. [3]). The imprinted region consists in about 2 Mbp within the commonly deleted region. One gene in the region, *UBE3A*, functions only with its maternally derived allele in the brain, and its deficit leads to AS (see Angelman Syndrome). A *UBE3A* mutation alone can produce the whole AS phenotype. On the other hand, there are at least five genes that are expressed only by their paternal alleles. Thus, in PWS the relative contributions of different gene deficiencies for the phenotype is unclear. Among the imprinted genes that are deficient in PWS one important candidate for a major phenotype contributor is *SNURF-SNRPN*, which is a very complex, polycistronic gene coding for two independent proteins. One of them, the protein SmN, translates off exons 4–10, and is a core spliceosomal protein that participates in the process of mRNA splicing in the brain. The five reported balanced translocations (see Type IIb PWS) that resulted in PWS had their breakpoint within the *SNURF-SNRPN* locus, thus suggesting the gene's major role in the PWS phenotype. However, mouse models with altered *SNURF-SNRPN* sequence do not seem to show the PWS-like phenotype. SmN's function in the brain overlaps with the SmB/B proteins. The SmB/B proteins have elevated levels in the mouse model with an ablated SmN sequence, thus supporting the idea that the SmB/B proteins can compensate for the SmN deficiency in the mouse model. SNRPN upstream reading frame (SNURF) (exons 1–3) encodes another protein that may be involved in the regulation of SmN or in the process of imprinting because its locus overlaps with the IC. In addition, there are genes for many small nucleolar RNAs located within the *SNURF-SNRPN* introns. The impaired transcription of these RNA molecules may also be an important contributor for the PWS phenotype.[15]

Finally, another major candidate contributor for the PWS phenotype is the gene *NDN* whose products are predominantly expressed in the brain.[3]

PHENOTYPE–GENOTYPE CORRELATIONS

The different PWS molecular subtypes have very similar phenotypes with only minor differences noted by some

studies. Patients with UPD tend to lack facial dysmorphisms, and African–American PWS patients often are of normal height and do not have small hands.[16] One recently reported important phenotype–genotype correlation is the higher prevalence of psychotic disorders among the patients with UPD compared to the other PWS types.[17] The molecular basis of this finding is currently unclear.

DIAGNOSTIC EVALUATION

The diagnostic tests bellow are listed in order of their application for diagnostic evaluation:

G-banded chromosome analysis is undertaken in every individual with suspected PWS to rule out chromosomal abnormalities leading to PWS or producing similar phenotypes.

DNA methylation testing is done as an initial evaluation for all patients with suspected PWS and will identify all patients except the ones with balanced chromosomal translocations. (For more detailed explanation of DNA methylation testing, see Angelman Syndrome).

Fluorescence in situ hybridization (FISH) analysis using commercially available probes specific for the SNRPN locus is done to look for 15q11q13 deletions after positive DNA methylation testing,[18]

UPD analysis is done in cases of abnormal DNA methylation and normal FISH analysis. This testing protocol uses DNA polymorphisms to trace the inheritance of chromosome 15. DNA specimens from both parents are necessary.

IC mutation screening. In methylation-positive and FISH- and UPD-negative cases a search for an IC deletion can be done, but is not currently available for routine use.

For patients with negative DNA methylation analysis and normal chromosome analysis the diagnosis of PWS is ruled out.

MANAGEMENT

The management of PWS is supportive and problem oriented.[16] The following more important disease aspects are usually addressed:

Developmental delay/mental retardation is managed by early intervention, special education, and occupational therapy.

Obesity is an important morbidity and mortality contributor in PWS. Its management includes low calorie diet, exercise, and limiting the access to food.

GH deficiency. GH therapy was recently recommended for PWS because GH deficiency is well documented in patients with PWS.[19] The favorable effect of GH therapy on linear growth and body composition was later reported, although issues of the priority of such therapy given the presence of more important PWS-associated problems was also discussed by Paterson and Donaldson.[20] Finally, several fatalities of children with PWS after initiation of growth hormone therapy were reported by Pfizer Inc. (the manufacturer of recombinant growth hormone; see www.pharmacia.com). The fatalities had one of the following complications: severe obesity, history of respiratory impairment/sleep apnea, or unidentified respiratory infection. These risk factors are now considered contraindications for growth hormone therapy in PWS.

Support and information for families with affected members can be obtained from the website of the PWS Association (USA) at www.pwsausa.org.

CONCLUSION

PWS is the result of the deficiency of imprinted gene(s) in the chromosomal region 15q11q13. Although no single gene was shown to be a major contributor for the phenotype, *SNURF-SNRPN* and *NDN* are strong candidates. The typical PWS phenotype is most likely the result of deficiency of more than one gene, and thus fits the definition for continuous gene syndrome. DNA methylation testing in combination with chromosome analysis will identify all PWS cases, but further testing may be required to determine the patient subtype. Future research on this condition may address the higher prevalence of psychotic manifestations in PWS patients with UPD. In addition, the long-term effects of growth hormone therapy on PWS need to be evaluated.

ACKNOWLEDGMENTS

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Preimplantation Genetic Diagnosis—An Overview

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The-Hung Bui
Erik Iwarsson
Helena Malmgren
Karolinska Institutet/Karolinska Hospital, Stockholm, Sweden

INTRODUCTION

Couples known to be at increased risk of transmitting a specific monogenic disorder or chromosomal imbalance to their offspring will find increasing use of preimplantation genetic diagnosis (PGD) as an alternative to conventional prenatal diagnosis by invasive procedures during pregnancy. Routine assisted reproductive technologies [in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI)] are needed for PGD. Genetic tests are performed before embryo transfer, thus allowing the selection of unaffected embryos before a pregnancy has been established. This approach has the advantage that the couple avoids having to consider termination of pregnancy because of an affected fetus. More recently, PGD for aneuploidy screening (PGD-AS) has been used in an attempt to improve the pregnancy rates in infertile couples treated by IVF.

This chapter focuses on the current status of clinical PGD, the technical limitations, and further prospects of this technology.

EMBRYO AND POLAR BODY BIOPSY

Currently, most centers performing PGD use cells obtained from the six- to eight-cell cleavage stage (day 3 of embryonic development).[1] Embryo biopsy is a two-step procedure in which a hole is first drilled in the zona pellucida surrounding the embryo at this stage, and next, one or two cells are aspirated through it (Fig. 1). The hole made in the zona pellucida is usually 30–50 μm, and it is believed that a larger hole may be detrimental to embryo development and viability.[2] Until recently, most centers used acidified Tyrode's solution for zona drilling, but a noncontact laser method has become very popular and is also widely used for assisted hatching (references in Ref. [1]). No controlled randomized studies have yet been conducted to compare the effects of acid Tyrode's solution or the laser on embryo viability. However, comparative data from the ESHRE PGD Consortium and others have shown no difference in pregnancy rates between these two methods.[1,3] Another controversy is whether taking two cells rather than one is detrimental to embryonic development.

Sequential or simultaneous biopsies of the first and second polar body have also been performed for PGD (Fig. 2). The main application has been for age-related aneuploidy screening (PGD-AS), but this strategy has also been used in some cases of PGD for single-gene defects and female carriers of chromosomal abnormalities.[1] Genetic analysis of the polar body is restricted to maternally derived diseases, as information of the paternal genetic contribution cannot be obtained by this approach.

CHROMOSOME ANALYSIS OF PREIMPLANTATION EMBRYOS

Karyotype analysis of early embryos is difficult because of limitations in culturing and obtaining metaphase spreads from biopsied embryo cells. This is circumvented by the use of fluorescent in situ hybridization (FISH) with locus-specific DNA probes on interphase nuclei.

FISH for Sexing and Inherited Chromosomal Abnormalities

Any X-linked disease, for which no specific single-cell polymerase chain reaction (PCR) test is available, may be considered for PGD sex selection. By using DNA probes for the sex chromosomes and an autosome as a hybridization control, the sex of embryos can be determined and only female embryos are transferred to the woman. For this particular application FISH is more advantageous and robust than PCR; it can additionally detect potential aneuploidy for the analyzed chromosomes while the problem with allele dropout (ADO) associated with PCR is entirely avoided. The first application of FISH in PGD was for sex selection and it remains the method of choice for this indication. Nevertheless, sexing and transfer of only female embryos cannot be regarded as the optimal treatment for carriers of an X-linked disorder as theoretically half of the discarded male embryos are unaffected. On the other hand, the workup of a new diagnostic test for every X-linked disease is very

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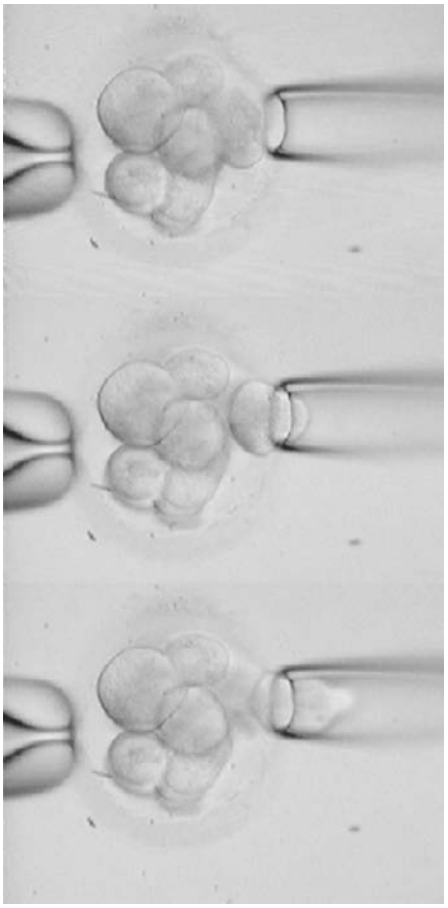

Fig. 1 Embryo biopsy. Embryo biopsy with removal of one blastomere at cleavage stage. (Courtesy of Dr. José Inzunza, Karolinska Institutet.) (*View this art in color at www.dekker.com.*)

expensive and time-consuming, thus sexing is still considered a reasonable option for these patients.

Fluorescent in situ hybridization is also used for PGD of inherited structural chromosomal abnormalities. Previously, the limiting factor was the need to design unique probes for every case as most structural chromosome abnormalities are ''private'' with patient-specific breakpoints. However, this has to a large extent been solved by the recent publication and subsequent commercial availability of subtelomeric probes from all chromosomes. The majority of reported cases of PGD for structural chromosomal abnormalities concern carriers of balanced translocations.[3] Two types of translocations are known: Robertsonian translocations, the centric fusion of two acrocentric chromosomes, and reciprocal translocations, an exchange of segments from at least two chromosomes. To discriminate between embryos showing balanced and unbalanced chromosome content, a minimum dual-color FISH assay with one DNA probe from each q-arm of the chromosomes involved is required for Robertsonian translocations (Fig. 3). Generally, a three-color FISH assay is used for reciprocal translocations with one probe from each side of the breakpoint on either of the chromosomes involved and one probe located anywhere on the other chromosome. However, this diagnostic approach does not allow distinguishing between normal and balanced embryos. To be able to discriminate between them, the DNA probes used need to span or flank the translocation breakpoints. Such a strategy will be restricted by the time required to develop specific probes for each translocation carrier and the additional costs. Also, as both normal and balanced embryos will result in normal offspring it is ethically questionable whether only normal embryos should be transferred and not balanced translocation carriers. Preimplantation genetic diagnosis has also been successfully applied for other structural chromosomal abnormalities including inversions and deletions.[4]

FISH for Aneuploidy Screening

Cytogenetic studies have shown that up to 60% of the early spontaneous abortions in humans are caused by numerical chromosomal abnormalities and that as many as 70% of all human preimplantation embryos may contain aneuploid cells.[5] This prompted the development of PGD for aneuploidy screening of oocytes and preimplantation embryos (PGD-AS). Fluorescent in situ hybridization is

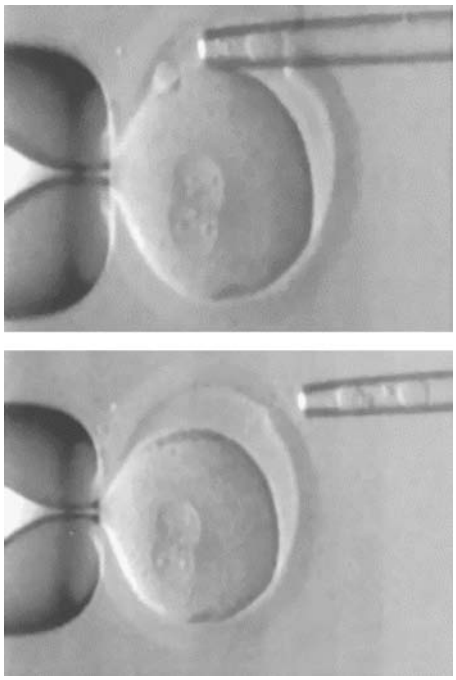

Fig. 2 Polar body biopsy. Removal of the first and second polar body.

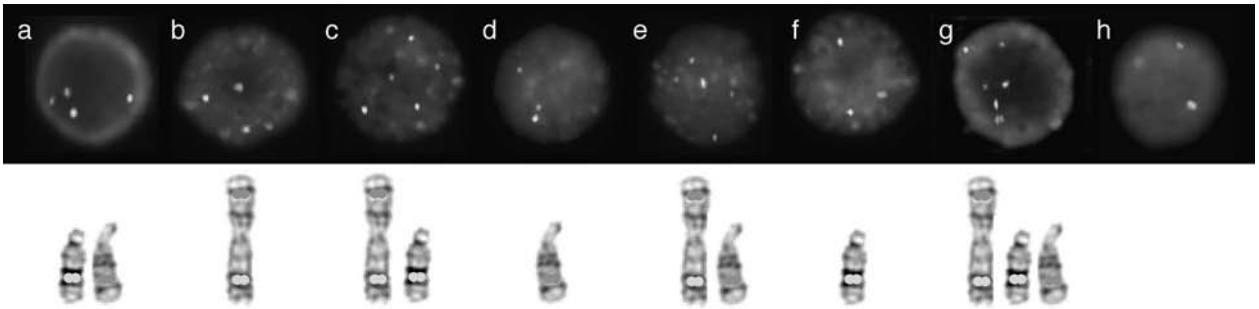

Fig. 3 Preimplantation genetic diagnosis for a Robertsonian translocation. Upper row: Blastomeres, obtained by biopsy of eight different embryos, analyzed by FISH using locus-specific probes (green for chromosome 13 and red for chromosome 14). The embryos were the result of fertilization of gametes from a carrier of a Robertsonian translocation, der(13;14)(q10;q10). Bottom row: Corresponding, and also all possible gametes in a der(13;14)(q10;q10) carrier, only chromosomes 13, 14, and der(13;14)(q10;q10) being depicted. Only embryos whose blastomere demonstrates two signals of each probe will result in an unaffected child. These embryos will be either noncarrier (a) or balanced carrier (b); the remaining embryos are unbalanced (c–h). (*View this art in color at www.dekker.com.*)

performed with enumerating probes for an increasing number of different chromosomes to select embryos that are more likely to implant and thereby enhance IVF success. However, there are limits to the number of probes used simultaneously in interphase FISH as this may result in signal overlapping. New technologies, such as comparative genomic hybridization (CGH), which enable full karyotyping of single cells, may solve these problems.[6]

Several reports have suggested an increased pregnancy rate using PGD-AS for patient groups with poor prognosis including those with increased maternal age, recurrent pregnancy losses, or IVF failures.[5] For this reason, PGD-AS is already practiced and is the most common reason for embryo diagnosis.[7] However, the first randomized, prospective study evaluating PGD-AS in these three high-risk groups could not find proof for an added benefit for any of them.[8] Thus there is an urgent need for further evaluation of this technology with additional large, controlled, randomized studies.

The presence of mosaicism shown in human preimplantation embryos can result in diagnostic difficulties as the biopsied blastomere may not be representative of the embryo. The risk of misdiagnosis could be assessed by reanalysis of nontransferred embryos. Data from PGD-AS indicate a misdiagnosis rate of 7.2%, of which 5.6% was attributable to mosaicism.[9] The estimated risk for false negative diagnosis was 4.3%. However, this may be reduced if two cells are analyzed.

PGD FOR SOCIAL SEXING AND HLA TYPING

Preimplantation genetic diagnosis for family balancing purpose, also known as social sexing (PGD-SS), is currently offered by a couple of PGD centers worldwide and 78 cycles were reported by May 2001.[3] This is a highly controversial application and a matter of contention. The vast majority of PGD centers registered with the ESHRE PGD Consortium does not offer PGD-SS.[3]

For a number of hematological diseases, transplantation of hematopoietic stem cells is the only curative treatment. Some parents with an affected child may contemplate PGD in the hope of conceiving an HLA-identical donor sibling. This issue is also much debated. However, if the child to-be is at risk of having the same disease and a specific PGD analysis is performed along with the HLA analysis, the added benefits of possibly saving the life of the sibling have been generally considered to outweigh the disadvantages.[10] A detailed account of the ethical issues of PGD is outside the scope of this chapter and these are discussed elsewhere.[10]

PGD OF SINGLE-GENE DISORDERS USING PCR

Preimplantation genetic diagnosis has been developed for an increasing number of single-gene disorders and in most cases a PCR-based method is used.[3,11,12] The sample is lysed in order to release the nuclear DNA and the 5–10 pg of DNA contained in a single cell needs to be amplified before mutation analysis can be performed using restriction enzyme digestion, single-strand conformational polymorphism (SSCP), sequencing, etc.[12] Nested PCR, a two-step PCR procedure, was introduced to achieve specific PCR products in large enough amounts. The more recent introduction of fluorescent PCR allowed for a more sensitive detection of PCR products (1000-fold more sensitive) and thus facilitates single rounds of PCR.[13] In

addition, primers can be tagged with different fluorescent molecules that facilitate amplification and simultaneous detection of several different DNA sequences.

Problems with Single-Cell PCR

Single-cell PCR is technically demanding, with high risks of contamination and ADO. Contamination can occur from the operator's DNA or carryover of previous PCR products. This type of contamination can be avoided by working in sterile manners, using different pre- and post-PCR rooms and materials dedicated only for the setup of single-cell PCR. Another source of contamination is genomic DNA from the patients, e.g., maternal cumulus cells or sperms sticking to the zona pellucida accidentally sampled during biopsy procedures. To avoid the risk of paternal contamination, ICSI should be used to achieve fertilization in all cases of PCR-based PGD. Allele dropout is a phenomenon observed when one of the examined alleles fails to amplify during the PCR reaction. This can result in diagnostic errors, especially for autosomal dominant disorders, as ADO of the mutated allele would wrongly diagnose an affected embryo as healthy.

Strategies for Avoiding Misdiagnosis

There are different ways to optimize the PCR to avoid or detect contamination and ADO in PGD.[14] The use of fluorescent PCR has reduced the observed frequency of ADO due to the increased sensitivity. In addition, most recent protocols use multiplex PCR with fluorescent tagged primers. Contamination can be detected by the simultaneous amplification of the DNA fragment encompassing the mutation and any informative polymorphic marker. The biopsied blastomere should show one marker allele from each parent; any other pattern will be indicative of contamination. Multiplex PCR of the DNA fragment encompassing the mutation and a linked polymorphic marker makes it possible to detect ADO by comparing the results of the two loci. This strategy doubles the chance of identifying the mutation, as one of the marker alleles will be linked to it, and thus decreases the risk for misdiagnosis due to ADO by half. Another advantage of multiplex PCR is that a combination of linked markers can be used for diagnosis. Linkage analysis could be an alternative strategy for PGD especially for diseases exhibiting wide mutation spectra such as cystic fibrosis.

Clinical Applications

The list of monogenic disorders for which single-cell PCR has been developed is continuously extending. So far, the largest number of PGD cycles has been performed for cystic fibrosis, myotonic dystrophy, Huntington's disease, beta-thalassemia, spinal muscular atrophy, the fragile X syndrome, Charcot–Marie–Tooth type 1A or 2A, and Duchenne's muscular atrophy.[3]

OUTCOME OF PGD

Preimplantation genetic diagnosis has been used clinically for more than 10 years and is presently offered by more than 50 centers worldwide. Although more than 5000 cycles have been performed and over 1000 babies born,[11] the impact of PGD is still low in comparison to conventional invasive prenatal diagnosis. This is due to the complexity of the procedure and the relatively low pregnancy rate following IVF. Two forums have been established to evaluate the clinical outcome of PGD, the ESHRE PGD Consortium and the International Working Group, IWGPG (Yury Verlinsky, Reproductive Genetics Institute, Chicago, U.S.A.). The most recent data collection from the ESHRE PGD Consortium included 1670 embryo transfer cycles from 25 different centers.[3] These data show a pregnancy rate of 17% and 25% per oocyte retrieval for PGD and PGD-AS, respectively, with 279 babies born. Children born after PGD have not been found to have malformations or complications to a higher extent than children born after IVF with ICSI.[15]

A few misdiagnoses are unfortunately still being made. Until now eight cases out of 451 pregnancies have been reported to the ESHRE PGD Consortium.[3] The total rate of misdiagnosis is 1.8%, with a higher incidence for PCR (3.4%) than for FISH (0.9%).

FUTURE PROSPECTS

For PGD-AS, CGH is an attractive method as it displays all the chromosomes at the same time. Although single-cell CGH has been applied on early embryos for research purposes,[16,17] its clinical application is not straightforward. All present CGH protocols have a time requirement of 4 to 5 days that is impossible to fit into the PGD situation with biopsies at day 3 and embryo transfer on day 4 or 5. However, one group has reported the use of CGH in PGD-AS, resulting in the birth of a healthy baby.[6] The obstacle was overcome by cryopreservation of the embryos after biopsy while awaiting the results. Alternatively, an accelerated protocol with polar body biopsy at day 1 could be used.[18]

The main disadvantage of CGH is that it is labor intensive and demands the knowledge of karyotyping. In microarray-CGH the target for hybridization are arrays of genomic clones spotted to glass slides. This setting allows

for automation as well as improved resolution, down to 100–200 kb depending on the number and density of clones present on the slide, compared to 2–20 Mb for conventional CGH. The use of microarrays in PGD for monogenic disorders will probably have a limited value because in most cases only one or two mutations have to be identified.

CONCLUSION

Preimplantation genetic diagnosis is a developing technology introduced in 1990 as an alternative to invasive prenatal diagnosis. The issue of pregnancy termination is avoided as genetic tests are performed before embryo transfer. Many would consider PGD ethically acceptable for cases where prenatal diagnosis would be offered. However, the clinical indications for PGD will widen and those performed for family balancing (sex selection) or other nondisease conditions (e.g., HLA-typing) are the most controversial. Whether PGD for aneuploidy screening will increase the success rate for IVF treatment is still being investigated.

Development in the field of PGD has been focusing on methods to eliminate any risks of misdiagnosis. For chromosome analysis, mosaicism is problematic and analysis of two blastomeres may increase the chance of detecting it. Ideally, a full chromosome analysis should be performed and techniques such as CGH are being adapted for PGD. In PCR, diagnostic errors can be reduced by using fluorescent PCR and informative markers in a multiplex reaction.

The overall impact of PGD is still low because of its complexity and relative costs. However, for couples at high risk of having an affected child, PGD may be an attractive alternative to prenatal diagnosis. For some couples PGD could even be the only available method that will enable them to have unaffected children.

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Preimplantation Genetic Diagnosis—Single-Cell DNA and FISH Analysis

Anver Kuliev
Yury Verlinsky
Reproductive Genetics Institute, Chicago, Illinois, U.S.A.

INTRODUCTION

Preimplantation genetic diagnosis (PGD) is a principally new approach for genetic testing before pregnancy is established. In contrast to prenatal diagnosis, performed already after the pregnancy, PGD avoids the need for pregnancy termination, which is the major limitation of prenatal diagnosis. Preimplantation genetic diagnosis has become realistic only after introduction of polymerase chain reaction (PCR), which made it feasible to perform genetic analysis in single cells biopsied from preimplantation embryo. This was first performed 13 years ago by testing single blastomeres, removed from the cleaving embryos, or the first polar bodies removed from the mature oocyte.

Since then, more than 7000 PGD cases have been performed in the framework of assisted reproduction and genetics practices, demonstrating an increasing acceptance of PGD in different populations around the world. A wider use of PGD has also been due to offering it to IVF patients of advanced reproductive age and application of FISH technique for a possible preselection and avoidance from transfer of oocytes and embryos with common aneuploidies and translocations.

SINGLE-CELL DNA ANALYSIS

Because PGD for single gene disorders is based on single-cell genetic analysis, its accuracy depends largely on the limitations of single-cell DNA analysis, which may potentially cause misdiagnosis. One of the key contributors to misdiagnosis is the phenomenon of preferential amplification, also known as allele-specific amplification failure [allele dropout (ADO)], requiring the application of special protocols to ensure the highest ADO detection rate.[1,2] A few previously reported misdiagnoses, involving PGD for cystic fibrosis (CF), myotonic dystrophy (DM), and fragile-X syndrome (XMR1), might have been due to this phenomenon, which has not initially been fully realized.[3,4]

It has been demonstrated that ADO rates in single cells might be different for different types of heterozygous cells.[5] The ADO rate may exceed 20% in blastomeres, compared with ADO rate in single fibroblasts and PB1, which was shown to be under 10%. A high rate of ADO in blastomeres may lead to an obvious misdiagnosis, especially in compound heterozygous embryos. As mentioned, most misdiagnoses, especially those at the initial stage of application of PGD for single gene disorders, were in the cases of blastomere biopsy from apparently compound heterozygous embryos. To avoid a misdiagnosis because of preferential amplification, a simultaneous detection of the mutant gene together with up to three highly polymorphic markers, closely linked to the gene tested, was introduced.[1,2] Each additional linked marker reduces the risk for misdiagnosis significantly, which may practically be eliminated with the application of three markers. So a multiplex nested PCR analysis is performed, with the initial PCR reaction containing all the pairs of outside primers, so that following the first-round PCR, separate aliquots of the resulting PCR product may be amplified using the inside primers specific for each site. Only when the polymorphic sites and the mutation agree are embryos transferred. So the multiplex amplification allows detecting ADO and preventing the transfer of misdiagnosed affected embryos.

Another efficient approach for avoiding misdiagnosis is a sequential genetic analysis of the PB1 and PB2 in PGD for maternally derived mutations.[1] Detection of both mutant and normal alleles in the heterozygous PB1, together with the mutant allele in the corresponding PB2, leaves no doubt that the resulting maternal contribution to the embryo is normal, even without testing for the linked markers as a control. However, it is ideal to test simultaneously at least for one linked marker to confirm the diagnosis. Alternatively, the mutation-free oocytes may also be predicted when corresponding PB1 is homozygous mutant, in which case the corresponding PB2 should be hemizygous normal, similar to the resulting maternal pronucleus. However, the genotype of the resulting maternal contribution may be quite opposite, i.e., mutant, if the corresponding PB1 is in fact

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heterozygous but not detected because of ADO of the normal allele.

The other method with the proved potential for detecting and avoiding misdiagnosis because of preferential amplification is fluorescence PCR (F-PCR), which may allow detecting of some of the heterozygous PB1 or blastomeres misdiagnosed as homozygous in conventional PCR.[2] In addition, the method also allows a simultaneous gender determination, DNA fingerprinting, and detection of common aneuploidies.

Finally, because of high rate of chromosomal mosaicism at the cleavage stage, testing for the chromosome, in which the gene in question is mapped, is of an obvious value to exclude the lack of mutant allele because of monosomy of this chromosome in the biopsied blastomere. As mentioned, aneuploidy testing is technically feasible and may be performed by adding primers for chromosome-specific microsatellite markers to the multiplex PCR protocols worked out for specific genetic disorder.[6]

Because of need for the development of a custom-made PGD design for each mutation and each couple, a preparatory work has become an integral part of PGD for single gene disorders to ensure avoiding the potential misdiagnosis. For example, in some cases, a particular set of outside primers has to be designed to eliminate false priming to the pseudogene, as described in PGD for long-chain 3-hydroxyacyl-Coa dehydrogenase deficiency.[7] In addition, the preparatory work may frequently involve a single sperm typing needed for establishing paternal haplotypes, so that linked marker analysis could be performed in addition to mutation analysis, especially in cases of paternally derived dominant conditions or PGD combined with preimplantation HLA matching.[8]

SINGLE-CELL FISH ANALYSIS

Preimplantation genetic diagnosis for the age-related aneuploidies is currently performed by FISH analysis using commercially available chromosome-specific probes (Abbott, Downers Groves, IL). It was first applied in 1991 for gender determination using DNA probes specific either for the X or Y chromosome.[9] Because testing for only one of the sex chromosomes could lead to misdiagnosis of gender because of a possible failure of hybridization, a dual FISH was introduced, involving the simultaneous detection of X and Y, each in different color.[10] Furthermore, the dual FISH analysis was combined with a ploidy assessment by adding a centromeric probe specific for chromosome-18.[11] Testing was then extended to up to 5 autosomes, including chromosomes 13, 16, 21, and 22, and then to up to 12 chromosomes, using additional rounds of rehybridization.[12]

The reliability of the FISH technique for aneuploidy detection in blastomeres has been extensively studied. However, a high rate of mosaicism was observed at the cleavage stage, which was particularly high in slow embryos exhibiting an arrested development.[13–15] According to the present data, approximately half of all preimplantation embryos are mosaic, suggesting that the biopsied blastomere might not represent the genotype of the embryo. As the origin of mosaicism in preimplantation embryos is still not well understood, it may be useful to test the chromosomal status of both oocyte and the resulting embryo, at least to exclude the majority of chromosomal aneuploidies originating from female meiosis.[16]

The overall experience of preimplantation FISH analysis currently involves more than 5000 clinical cycles, approximately half performed by FISH analysis of blastomeres and half by FISH analysis of PB1 and PB2, which resulted in hundreds of unaffected pregnancies and healthy children born at the present time. The follow-up confirmation studies of the preselected abnormal embryos, and the babies born following the procedure, demonstrated an acceptable accuracy of the FISH analysis.[3,17]

Preimplantation genetic diagnosis for aneuploidies was also achieved by comparative genome hybridization (CGH), which is a PCR-based method for testing of chromosomal abnormalities in a single cell. The method was designed for the detection and exclusion from the embryo transfer of the aneuploid embryos of which a sizable proportion could be misdiagnosed as normal by the commercially available five-color probe. The standard CGH protocol, which takes 5 days to complete, has presently been applied in 20 frozen cycles for poor prognosis IVF patients, resulting in a few unaffected clinical pregnancies.[18] The major limitation of CGH protocol involves a 3-day duration of the procedure incompatible with the current laboratory framework for PGD, which has presently been overcome either by performing the polar body CGH or by accelerating the procedure to be completed in 38 hr.[17,19] However, the accuracy and practical usefulness of CGH for PGD need to be further evaluated.[20]

PREIMPLANTATION GENETIC DIAGNOSIS APPLICATIONS

Preimplantation genetic diagnosis has presently been applied for more than 50 different conditions, among which the most frequent ones were cystic fibrosis and hemoglobin disorders.[3,4,17] In contrast to prenatal diagnosis, the

indications for PGD include inherited predisposition to late-onset disorders, such as inherited cancer predisposition and Alzheimer disease.[21,22] Most recently, PGD was applied for congenital malformations, including Sonic Hedgehog mutation and Crouzon syndrome.[8,23] The most controversial application of PGD has been HLA matching as a tool for the preselection of a potential donor progeny for bone marrow transplantation.[24] Previously, this was applied to the Fanconi anemia (FA) case, in which the preselection and transfer of unaffected embryos with HLA matched for the affected sibling yielded a clinical pregnancy and birth of a healthy carrier of FA gene, whose cord blood was transplanted to the affected sibling, resulting in a successful hematopoietic reconstitution. The method has currently been applied for the HLA genotyping for thalassemia, Wiskott–Aldrich syndrome, hyperimmunoglobulin M syndrome, X-linked adrenoleukodystrophy, and for leukemia, which resulted in the preselection and transfer of 19% of the HLA-matched embryos, demonstrating the practical usefulness of PGD for HLA typing.[25,26]

Finally, the most practical application of PGD is currently the preselection of aneuploidy-free embryos for IVF patients of advanced reproductive age and also detection of normal or balanced embryos in carriers of chromosomal translocations.[3,17] The usefulness of PGD in assisted reproduction is obvious from the data on the prevalence of chromosomal abnormalities in the oocytes of women of 35 years and older, which is over 50% even by testing for only 5 chromosomes (chromosomes 13, 16, 18, 21, and 22).[16] This is comparable to those detected in preimplantation embryos in PGD for aneuploidies at the cleavage stage, taking into consideration additional fertilization-related abnormalities and paternally derived meiotic errors, which was shown to be as high as 60%. As mentioned, the origin of high frequency of mosaicism, comprising approximately half of the chromosomal abnormalities at the cleavage stage, is still unclear. A significant proportion of mosaic embryos may originate from the oocytes that are aneuploid from the onset through a process similar to that known as a phenomenon of aneuploidy ''rescue.''

The overall experience of PGD for chromosomal disorders indicates a positive impact of the procedure on the clinical outcome in terms of the improved pregnancy and implantation rates, which may be almost doubled, as well the improved outcome of pregnancies through the statistically significant reduction of spontaneous abortions in IVF patients of advanced reproductive age, and other poor prognosis patients, including those with repeated IVF failures and repeated spontaneous abortions.[27,28] The clinical impact of PGD is even more significant for the carriers of balanced translocations who have an extremely poor chance of having an unaffected pregnancy. The data suggest a more than threefold reduction of spontaneous abortion rate in PGD patients with balanced translocations.[29,30]

The data show that single-cell DNA and FISH analysis has presently become an important tool for application of PGD in assisted reproduction and genetic practices, providing an important option for couples at the genetic risk to avoid the birth of an affected child and have a healthy child. In addition, the application of the technique has been extended to nondisease testing, such as the preselection of an HLA-compatible donor progeny for treatment of affected siblings requiring stem cell transplantation.

CONCLUSION

Preimplantation genetic diagnosis has been expanded to a variety of conditions, which have never been considered as an indication for prenatal diagnosis, including the late-onset disorders with genetic predisposition and preimplantation nondisease testing. Preimplantation genetic diagnosis has become a useful tool for the improvement of the effectiveness of in vitro fertilization (IVF), through avoiding the transfer of chromosomally abnormal embryos, representing more than half of the embryos routinely transferred in IVF patients of advanced maternal age and other poor prognosis patients. Preimplantation genetic diagnosis is of particular hope for the carriers of balanced chromosomal translocations, allowing accurately preselecting a few balanced or normal embryos, resulting from an extremely poor meiotic outcome. With the current progress in PCR-based detection of chromosomal abnormalities in oocytes and embryos, PGD may soon be performed for both chromosomal and single gene disorders using the same single cell, which is frequently required with the current expansion of PGD application. More than 1000 children have already been born following PGD, further confirming the accuracy and reliability of DNA and FISH analysis in single cells.

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Prenatal Diagnosis of Fetal RhD Status by Molecular Analysis of Maternal Blood

Jean-Marc Costa
American Hospital of Paris, Neuilly, France

Alexandra Benachi
Hôpital Necker Enfants Malades, Paris, France

INTRODUCTION

Hemolytic disease of the newborn (HDN) affects the fetus or neonate and results from the transplacental passage of maternal alloantibodies directed against fetal red cell antigens inherited from the father. Over 90% of all cases of clinically significant HDN affect rhesus D (RhD)-positive infants born to RhD-negative mothers. When fetomaternal hemorrhage occurs antenatally or at delivery, maternal B lymphocyte clones that recognize the RhD antigen are established. In a subsequent pregnancy, if stimulated by the RhD antigen on fetal red cells, they proliferate and produce immunoglobulin (Ig) G antibodies that cross the placenta and destroy any RhD-positive red cell, resulting in fetal anemia.[1] The incidence of HDN has dropped dramatically (1–6/1000 live births) since 1968, when effective prophylaxis by anti-D Ig injection to the mother become available. In the United States, anti-D Ig is systematically administrated at 28 weeks' gestation and at delivery, or if an identifiable risk event occurs to RhD-negative mothers. Administration of blood derivative is not devoid of risk, and every effort should be made to improve techniques that reduce the number of injections.[1] In 1991, the RhD locus was localized to the short arm of chromosome 1. The elucidation of the molecular basis of the various blood group system alloantigens allowed the development of polymerase chain reaction (PCR)-based assay for blood group typing. These assays can be performed with fetal DNA obtained from amniocentesis or chorionic villus sampling.[2] In 1998, Lo et al.[3] and Faas et al.[4] discovered that fetal DNA was present in maternal blood and that fetal RhD status could be determined by a noninvasive method.

For clinical purposes, fetal RhD genotyping using PCR is a significant advancement. In case of a heterozygous father, fetal RhD genotype determination early in pregnancy is useful in the management of RhD-negative sensitized women. Amniocentesis is now accepted as the primary modality that is used to test fetal blood type.[5] Chorionic villous sampling can also be used, but it should be discouraged in patients who wish to continue the pregnancy if the fetus is found to be RhD-positive. Disruption of the chorionic villi during the procedure can result in fetomaternal hemorrhage and an anamnestic response in maternal titer, thereby worsening the fetal disease. This phenomenon can also occur during amniocentesis, and all attempts should be made to avoid transplacental passage.[1] Although the quantity and quality of DNA obtained from fetal sampling are less than those of leukocytes, many groups showed concordant results with serology, although the importance of testing more than one region of the gene was established.[2] Moreover, these sampling procedures are invasive, resulting in an increase risk of fetal loss (2%). Fetal RhD genotyping is also useful in RhD-negative pregnant women at risk for RhD immunization [American College of Obstetrics and Gynecology (ACOG) practice bulletin, 1999] to adapt prophylactic anti-D Ig infusion, to avoid unnecessary administration in case of an RhD-negative fetus.

Many groups have worked on a reliable noninvasive approach to determine fetal RhD genotype. Recovery of fetal cells from maternal blood or cervical mucus has been reported, but techniques require isolation and enrichment of fetal cells, and questions regarding sensitivity and cost-effectiveness are still unresolved for routine use.[6,7] An RNA-based assay on fetal erythroblasts isolated from maternal blood was also evaluated, but results were not conclusive.[8] Moreover, the persistence of fetal cells from previous pregnancies renders this approach susceptible to false-positive results. Despite the fact that many studies have shown that it is theoretically possible to isolate fetal cellular DNA from maternal blood, none of the techniques used meets the accuracy needed for clinical usage.[2]

DISCUSSION

Fetal DNA has been known to be present in maternal blood since the work of Lo et al.[9] in 1997. They first

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showed that Y-chromosomal sequences could be amplified from DNA isolated from the plasma of pregnant women carrying a male fetus. Fetal DNA is present in maternal serum at 5 weeks' gestation[10] and the concentration of fetal DNA increases with gestational age. Fetal DNA represents 3.4% (0.4–12%) and 6.2% (2.3–11.5%) of total plasma DNA in the first and third trimesters of pregnancy, respectively.[11] The origin of fetal DNA is still unclear. One or more mechanisms might be involved in this process: Fetal DNA might be released from apoptotic fetal cells circulating in maternal blood, or by trophoblasts (by lysis of the cells at the fetal–maternal interphase, or after entrapment in the maternal lung); it could also passively cross the placenta. Regardless of its origin, fetal DNA is rapidly cleared (mean half-life, 16 min)[12] and it has been shown not to persist in maternal blood after pregnancy.[13]

Although anecdotal diagnosis has been made using circulating fetal DNA (myotonic dystrophy;[14] achondroplasia.[15]), most of the studies on the accuracy of PCRs based on fetal circulating DNA have focused on Y-chromosome and RhD. In the past few years, studies have reported a 100% accuracy in fetal sex detection using a PCR technique in the first trimester.[16,17] This technique has now modified the prenatal diagnosis of X-linked disorders,[18] as well as the management of pregnancies at risk for congenital adrenal hyperplasia.[19] The use of cell-free DNA in maternal plasma and serum for noninvasive fetal RhD genotyping has been achieved independently by five groups.[3,4,20–22] Faas et al.[4] reported a highly sensitive but conventional PCR specific for *RHD* exon 7. The RhD genotype was correctly predicted in 31 second-trimester pregnancies. Lo et al. used a real-time *RHD* PCR technique on exon 10 in 57 RhD-negative women at different gestational ages. Two false-negative results were obtained in the first trimester. Results of Bischoff et al.[20] were less accurate, with 30% false negatives. In their PCR, input DNA was isolated from only 10 μL of serum, and this could explain the low sensitivity observed in this study.[2] Zhong et al.[21] analyzed 22 pregnancies and obtained one false-positive result, but they used a multiplex nested PCR using Y-chromosome-specific and RhD-specific primers. Our team studied 106 sera from RhD-negative pregnant women in the first trimester, and maternal serum results were in complete concordance with those obtained on fetal cells isolated from amniotic fluid and with the RhD serotype of the newborn.[22]

The assay was based on real-time PCR targeted at the 3′ end (exon 10) region of the *RHD* gene. Being a closed-tube system, real-time PCR offers, to date, the highest level of safety and represents the most secure amplification procedure. Total DNA was extracted

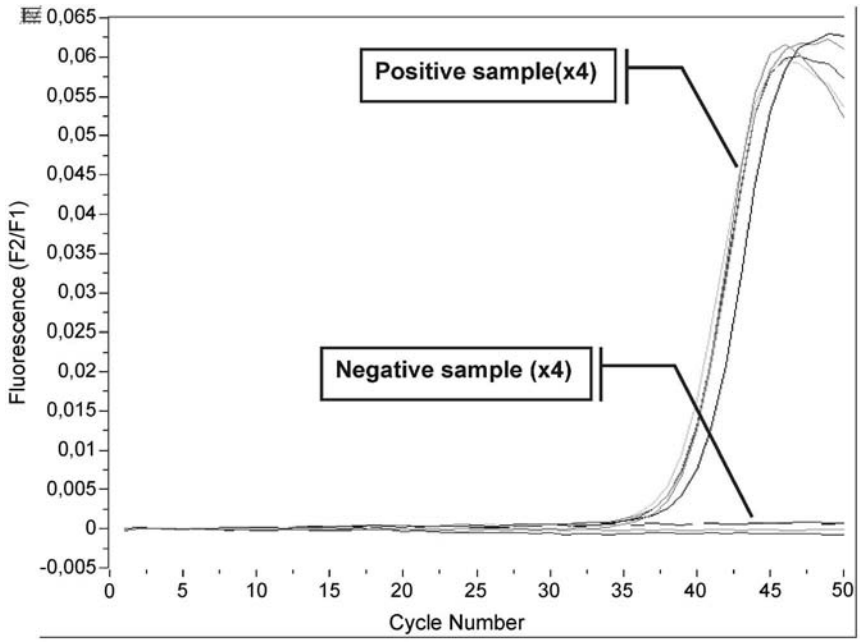

Fig. 1 Detection of the *RHD* gene in maternal serum using real-time PCR. Two patients' samples are tested in quadruplicate—one giving a positive result, and the second giving a negative one. (*View this art in color at www.dekker.com.*)

from 400 μL of serum using a silica-based commercial procedure and was then eluted with 50 μL of an elution buffer, of which 10 μL was used for the assay. Amplification as carried out in a LightCycler® instrument for 50 cycles. Each sample was analyzed, in duplicate, by different DNA extraction methods. Figure 1 illustrates typical results.

Van Der Schoot et al.[2] report a 100% concordance over 100 plasma samples (details on gestational age are lacking). The discrepancy on first-trimester determination of fetal RhD status might be a result of poor amplification efficiency. The real-time PCR assay (our team uses FRET instead of TaqMan technology) achieves a high level of sensitivity associated with a high level of safety because it is a closed-tube system; no false-positive results related to PCR product carryover are observed.[22] Poor efficiency of DNA recovery, or an inhibitor effect of DNA extract may also explain low sensitivity. As a control for amplifiability, the use of an endogenous gene (such as β-globin gene) seems inappropriate[3] because it evaluates mainly maternal DNA, which is present in large amounts in the plasma. For this reason, our group has proposed the addition of a low amount of a heterologous DNA as an internal control. Finally, the use of maternal serum instead of plasma used by Lo et al. may also explain these discrepancies.

One of the concerns for this technique is the internal control for the presence of fetal DNA. We postulate that fetal DNA was always present in maternal serum and that this control was not necessary. Van Der Schoot et al. consider that when no RhD-specific signal is obtained, the presence of fetal DNA in the plasma has to be confirmed by another fetus-specific DNA sequence from a highly polymorphic paternal antigen (STR markers) or from the Y-chromosome (e.g., *SRY*), which could be used only in women bearing a male fetus. However, most of those assays that have already been described are multiplex PCRs in which sensitivity is low because paternal alleles are difficult to detect within the high background of maternal alleles and because of poorer amplification.[21] Notwithstanding this, Pertl et al.[23] successfully applied multiplex fluorescent PCR of STR on fetal DNA in maternal plasma, but gestational age in this study was 34 weeks and over—a time when fetal DNA is particularly high in maternal serum, which is not the case in the first trimester.[2] To Van Der Schoot et al.'s point of view, only positive results are meaningful until allele-specific amplification strategies allow more sensitive systems. We do agree in theory with this statement, but our personal experience (over 250 cases with 100% accuracy) has shown that if internal control of the amplification is used, negative result is still of clinical value.

Another issue is genotyping errors caused by rare variants of the *RHD* gene. These variants could lead to false-positive or false-negative results. A fetus carrying an RhD-negative allele might be typed RhD-positive when the RhD-specific PCR assays are based on polymorphic sites still present in the silent allele. In already sensitized RhD-negative pregnant women, a false-positive result will not have much consequences as maternal IgG titer will be followed as usual and will not lead to any invasive intervention. In case of unsensitized women, unnecessary Ig injection will be performed if needed, which is acceptable. In the Caucasian population, RhD negativity is usually caused by deletion of the *RHD* gene, but negativity could also result from aberrant *RHD* alleles that do not lead to RhD expression. In the African black population, the variant *RHD* genes r′s (=Cde^S) and *RHDψ* can underlie serologic RhD negativity.[2] In the Caucasian population, 14 different RhD-negative *RHD* alleles have been described,[24] but the frequency of these alleles is extremely low (1/1500); therefore they do not influence the accuracy of *RHD*-specific PCR assays. It has been suggested that different regions of the *RHD* gene should be examined to increase the accuracy of RhD genotyping. However, the assay described by our group was only targeted at the 3′ untranslated region specific to the *RHD* gene (exon 10) and no false positive was detected. Lo et al.[3] and Zhang et al.[25] also used single *RHD* gene region assay on exons 10 and 7, respectively. The population of those studies is mainly Caucasian, which could explain the absence of false positives. Van Der Schoot et al.[2] have designed primers and probes located in exon 7. This exon is present in almost all RhD-positive *RHD* alleles (except some rare variants). False-positive results will be obtained, such as with the primers used by our team located in exon 10, in fetuses carrying only the silent *RHDψ* gene.

RhD positivity might be missed when the fetus is carrying an aberrant allele, which still leads to RhD positivity, although not all RhD-specific polymorphic nucleotides are present.[2] This kind of variant might lead to alloimmunization in an RhD-negative mother, but HDN in a fetus whose red blood cells have a partial D antigen is rare. Van de Schoot et al. suggest that for immunized mothers, no aberrant RhD alleles should be missed; therefore a multiplex PCR approach on more than one region of the *RHD* gene has to be taken and the *RHDψ* gene has to be recognized. From our point of view, the sensitivity of a multiplex PCR will be less than the actually reported protocols, and the frequency of these aberrant alleles is so small that sensitivity of the test should be preferred. However, in a non-Caucasian population, the existence of those variants should be kept in mind.

CONCLUSION

The high level of accuracy of fetal RhD genotyping obtained by different groups could enable this technique to be offered on a routine basis for the management of RhD-negative patients during the first trimester of pregnancy. This strategy offers many advantages. The potential risk for maternal infection from contaminated anti-D Ig has to be taken into consideration, and women should be informed of the risk involved.[22] Even if anti-D product was safe with regards viral transmission, the risk could not be formally excluded. Moreover, as unnecessary infusion of anti-D Ig would be avoided, this strategy could contribute to saving this limited product until clonal anti-D becomes available. First-trimester determination of RhD fetal status is of interest because several clinical events may be responsible for RhD alloimmunization very early in pregnancy, such as therapeutic or spontaneous abortion and bleeding. In case of an RhD-negative fetus, no further biological and clinical investigations should be performed during the rest of the pregnancy.

First-trimester pregnancy is a critical period for prenatal diagnosis using noninvasive and invasive procedures. The PCR assays actually used need to be evaluated on a large-scale population,[2,22] and could lead to a combined systematic analysis of fetal RhD genotyping in RhD-negative women in the first trimester, in conjunction with fetal nuchal translucency and Down's syndrome screening test by serum biochemical markers.

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Prenatal Diagnosis Using Fetal Cells and Cell-Free Fetal DNA in Maternal Blood

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Sinuhe Hahn
Wolfgang Holzgreve
University Women's Hospital, Basel, Switzerland

INTRODUCTION

The development of safe and efficacious methods for prenatal diagnosis has been the subject of intense research in the past decade. This has, in part, been spurred by a dramatic shift in population demographics in developed nations, typified by a significant increase in maternal age (>30 years) and a concomitant reduction in parity, with most couples opting only for one child. Because an increased maternal age is associated with an elevated risk for bearing a fetus with a chromosomal anomaly, this demographic change has led to an increased need for prenatal diagnoses, which can currently only be reliably performed using invasive practices, such as amniocentesis or chorionic villus sampling. As these procedures are associated with a significant risk for fetal loss (on the order of 0.1%), the need for safe alternatives has been voiced, as many couples were reluctant to expose their long sought-after unborn child to this risk.

FETAL CELLS: BRIEF HISTORICAL OVERVIEW

Even though the first description concerning the deportation of trophoblast cells into the maternal periphery was made by Schmorl at the turn of the 19th century, it was only in the late 20th century that the tools were developed, which permitted the enrichment of rare circulatory fetal cells (frequencies as low as 1×10^7 nucleated maternal cells). The main proponents were fluorescence-activated cell sorting (FACS) and magnetic cell sorting (MACS). Common consensus indicated that the target fetal cell of choice was the fetal erythroblast, also termed nucleated red blood cell (NRBC) because of the high prevalence of this cell in the fetal circulation (up to 50% of all nucleated fetal cells), its expression of potentially fetal-specific molecules (fetal and embryonic hemoglobin chains), and its short life span, which excluded cells from previous pregnancies from being analyzed.[1,2]

Further innovations that accelerated the pace of research in this field were the developments of fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR), both of which permit genetic analysis of individual cells. By use of these technologies, proof-of-principle studies demonstrated that fetal aneuploidies as well as single-gene Mendelian disorders, such as hemoglobinopathies, could be determined from the analysis of enriched circulatory fetal erythroblasts.[1,2]

FETAL CELLS: CLINICAL STUDIES

These promising results encouraged the instigation of at least two large-scale studies, the largest of which was conducted under the auspices of the National Institutes of Health (NIH)—the so-called NIFTY Study.[3] In this study, close to 3000 samples were recruited from pregnant women about to undergo invasive prenatal diagnostic testing because of an elevated risk for bearing a fetus with a chromosomal anomaly. These samples were then either processed by MACS or FACS and analyzed by multicolor FISH. All experimental results were compared to karyotypes obtained from the invasive procedure—the current gold standard. The results from this study, which were published in 2002, were in stark contrast to the euphoria that reigned a few years previously, in that fetal cells could, at best, be detected with a sensitivity of 48% using MACS and below 15% using FACS.[3] Consequently, the NIFTY Study reiterated the importance of large-scale well-designed and conducted studies in order not to be misled by short case reports such as high-profile publications.

An important aspect of the NIFTY Study that is frequently disregarded in other clinical studies is that it specifically addressed psycho-social issues concerning the introduction of a new noninvasive method for prenatal diagnosis, especially the potential problem of coercion. This part of the NIFTY Study clearly showed that coercion would not be an issue, and the introduction of such a noninvasive test would be especially welcomed by couples involved in assisted reproduction (ART) programs.[4,5]

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FETAL CELLS: DIAGNOSIS OR SCREENING?

The scarcity of fetal cells in the maternal circulation and the failure to reliably retrieve these cells from maternal blood samples are amply underscored by the sobering results of the NIFTY Study. As even more disappointing results were obtained in the study initiated by Applied Imaging, which used state-of-the-art technology for the automated detection of putative fetal erythroblasts,[5] questions have been raised as to whether the pursuit of fetal cells can have any future diagnostic value.[1] Although it is clear that current technologies are not suited to clinical applications, there are other facets worthy of future exploration. Among these is the observation that the trafficking of fetal cells into the maternal periphery appears to be greater in pregnancies with aneuploid fetuses, particularly in cases with trisomy 21.[6] Furthermore, reports indicating that trophoblast cells may be more prevalent in the maternal circulation than previously thought[7] suggest that unnecessary constraints may have been made in restricting previous studies solely to fetal erythroblasts. Therefore it is possible that a greater degree of success may be attained by casting the net out to catch a wider variety of fetal cells. Furthermore, a sensible strategy may be to move away from a diagnostic setting to a screening one. In such a scenario, the focus would be shifted to the detection of fetal cells only in those instances where suspicion of a fetal aneuploidy exists. These results are used in combination with those obtained from ultrasound and/or serum analyte examination to reduce the relatively high false-positive rate of other methods, or, at best, to obviate the need for any further confirmatory invasive procedures.

EXTRACELLULAR CIRCULATORY FETAL DNA

Possibly the observation that has changed future prospects for the noninvasive diagnosis of fetal genetic traits the most was that describing the presence of extracellular fetal DNA in maternal plasma and serum.[8] This analysis was prompted by reports indicating that cell-free tumor-derived DNA was readily detectable in the plasma of cancer patients.[9] By arguing that the placenta shares many similarities with tumors (high rate of cell turnover, tissue invasion), it was hypothesized that placenta-derived cell-free fetal DNA may be present in the maternal circulation. This indeed turned out to be the instance, with cell-free fetal DNA being readily detectable by conventional PCR assays. However, a caveat of this fetal genetic material is that, because cell-free maternal DNA is also present in the maternal circulation, only paternally inherited loci absent from the maternal genome can be reliably detected. Consequently, most studies have focussed on the detection of such facile fetal genetic loci such as Y chromosome-specific sequences.[10]

An advantage of cell-free fetal DNA is that the concentration of this material in maternal samples can be reliably quantified by the use of real-time PCR. Studies employing this technology indicated that this analyte had a very short half-life (on the order of 15 min) and rapidly disappeared from the maternal periphery postdelivery.[11] It was also shown that cell-free fetal DNA constitutes approximately 5% of the total circulatory cell-free DNA, and that the number of copies of this fetal material was approximately 100-fold higher than the estimated number of circulatory fetal cells. Current consensus is that cell-free fetal DNA is almost exclusively derived from the placenta.

Furthermore, by the use of such quantitative PCR technology, it was clearly demonstrated that the concentrations of this fetal analyte were elevated in pregnancies with aneuploid fetuses (again most notably with trisomy 21),[12,13] or those affected by preterm labor and preeclampsia.[14–16] A valuable aspect of these studies is that, by being performed independently, they verified the soundness of the initial observation and, for the first time, introduced a high degree of reproducibility into this research arena.

EXTRACELLULAR FETAL DNA: CLINICAL APPLICATIONS

The discovery of cell-free fetal DNA quickly led to a clinical application, namely the determination of the fetal RhD genotype in pregnancies at risk for hemolytic disease of the fetus and newborn (HDN).[10] This is largely because of the fortunate constellation existing in Caucasians where the *RhD* gene is generally absent in RhD individuals. Therefore the analysis of the fetal RhD genotype in pregnancies with an RhD constellation (father RhD, mother RhD) is similar to the facile detection of Y chromosome-specific sequences in a maternal background. Several large-scale studies have indicated that fetal loci such as the SRY locus on the Y chromosome and the *RhD* gene could be detected with sensitivities approaching 95%, coupled with 100% specificity.[17] Consequently, a few European centers have started offering this service in a clinical setting (i.e., either the determination of the fetal RhD status in pregnancies at risk for HDN, or fetal sex in pregnancies at risk for an X-linked disorder). The significance of this development truly becomes apparent when comparing the speed with which this transition from bench to bedside has occurred to more than a decade invested into circulatory fetal cells, where no similar test is yet in sight.

Despite this premise, other tests have been slow to follow, especially those for the detection of paternally inherited mutant alleles for heterozygous compound Mendelian disorders (e.g., hemoglobinopathies, cystic fibrosis). Such tests would be of importance in cases where the mother and father do not share the same disease allele. In this manner, the detection of the paternal mutant allele can serve to indicate that the fetus may be affected, whereas its absence would indicate that it is not affected, and thereby obviate the need for an invasive prenatal diagnostic procedure. It appears that the major factor hindering these assays is the predominance of maternal cell-free DNA over that of the fetus (at least 25:1), in that many of the allele-specific PCR assays are no longer capable of reliably determining single base changes under such conditions. It is clear that alternative strategies will have to be sought to overcome this problem.

EXTRACELLULAR FETAL RNA

A recent discovery, once again inspired by oncological observations concerning tumor-derived mRNA transcripts, is that placenta-derived mRNA species are present in maternal plasma samples.[18] Unlike their cell-free fetal DNA counterparts, which are truly acellular, these mRNA molecules are contained in small membrane particles. This difference becomes apparent when filtering the plasma sample, in that the mRNA containing particles are retained by the filter, whereas the cell-free DNA passes through. Disruption of the protective membrane particles renders the mRNA highly labile, and it is rapidly degraded by nucleases in the plasma sample. Consequently, stored or frozen samples are not suitable for these studies. Current analyses have focused on mRNA transcripts expressed in the placenta such as human placental lactogen (hPL), human chorionic gonadotropin (βhCG) or corticotropin-releasing hormone (CRH). In the latter instance, quantitative PCR analyses have indicated that the level of CRH mRNA species is elevated in pregnancies with manifest preeclampsia.[19] The immediate advantage of studying mRNA species is that it permits fetal sex-independent analysis in that the study is not restricted to the detection of Y chromosome-specific DNA sequences.

CIRCULATORY NUCLEIC ACIDS: ROLE IN DETECTING HIGH-RISK PREGNANCIES?

The observation that cell-free fetal DNA concentrations are elevated in pregnancies with certain fetal aneuploidies or pregnancy-related disorders, such as preeclampsia, have led to the suggestion that this may serve as a marker for such pregnancies. This is further supported by two independent studies, which have indicated that the levels of circulatory fetal DNA are elevated early (second trimester) in those pregnancies that later develop preeclampsia.[20,21] Unfortunately, these studies are too small to provide any indication of the possible predictive value. An examination of second-trimester fetal DNA levels has indicated a minimal increase in sensitivity for the detection of trisomy 21 fetuses when combined with the analysis of other serum analytes. The proposal that the analysis of circulatory fetal DNA could aid in the distinction between true and false preterm labor (i.e., those that do not respond to tocolytic treatment and still deliver prematurely, and those that respond and deliver at term) could, unfortunately, not be confirmed in a larger study.

It is perhaps in this area that the analysis of circulatory mRNA species will come to the fore, as apart from not being restricted by fetal sex, the description of mRNA species overtly expressed in the placenta of trisomy 21 placentae[22] may aid in the development of aneuploidy-specific screening tests. It is also possible that preeclampsia may be associated with the upregulation of different mRNA species when compared to other pregnancy-related disorders, such as preterm labor. This distinction may lead to the development of specific diagnostic or screening markers.

CONCLUSIONS

The conundrum facing research into the potential use of circulatory fetal cells for prenatal diagnostic purposes is whether it will be possible to develop a test that is rapid, reliable, and cheap enough to compete with the current high levels of sensitivity (>90%) and very low false-positive rate (<1%) for the detection of trisomy 21 fetuses, obtained by a combination of different ultrasound markers (nuchal translucency and nasal bone) and serum analytes.[23]

However, it is likely that once the hurdles regarding the detection of subtle fetal genetic alterations in a high background of maternal circulatory DNA have been overcome, the analysis of cell-free fetal DNA will further establish itself in day-to-day clinical practice. The usefulness of circulatory RNA species remains to be determined, but it is highly probable that by the inclusion of genomic methodologies, new markers may be identified for the screening of a number of high-risk pregnancies. In either instance, it appears that current challenge can only be met by an integrated approach that incorporates a wide range of disciplines and expertise.

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Protein Microarrays

Lance A. Liotta
Virginia Espina
National Cancer Institute, Bethesda, Maryland, U.S.A.

INTRODUCTION

Protein microarrays, in the simplest sense, are immobilized protein spots on a substratum. Individual protein spots may be heterogeneous or homogeneous, and may consist of cell or phage lysates, an antibody, a nucleic acid, drug, or recombinant protein. These immobilized bait molecules are detected by probing the microarray with a signal-generating molecule such as a tagged antibody, ligand, serum, or cell lysate. The tagging molecule generates a pattern of positive and negative spots. The signal intensity of each spot is proportional to the quantity of applied tagged molecules bound to the bait molecule. The spot pattern image is captured, analyzed, and correlated with biological information.

Protein microarrays have broad applications for discovery and quantitative analysis. Protein microarray technology may be applied to drug discovery, biomarker identification, and molecular profiling of cellular material. It is expected that the clinical utility of protein microarrays will depend on their ability to provide a map of known cell signaling proteins and to provide crucial information about protein posttranslational modifications. Protein microarrays help to provide a view of the disrupted cellular machinery governing disease. Identification of critical nodes, or interactions, within these networks is a potential starting point for drug development and/or design of individual therapy regimens.

CELLULAR SIGNALING PATHWAYS

Information Flow Through a Cell

Proteomics is considered the characterization of the entire cellular protein content. This broad categorization of cellular proteins may not yield immediate, clinically useful information because protein function is closely related to specific cell–cell interactions and the cellular microenvironment. Proteins relay information from the extracellular environment through a series of complex protein–protein interactions, allowing protein complexes to coalesce, then dissolve after the signal is gone, or by changing the shape of a receptor protein. As an example, some types of signaling mechanisms are mediated by phosphorylation events, cleavage events, or alteration of a protein's conformation. Characterization of these events may allow the elucidation of the information flow through a cell, which in turn can point to critical nodes or pathways required for cell survival.

Examples of functional proteomic information can be found in the phosphorylation states of signaling proteins and the flow of information within a cell and the organism. The ability to discern these specific interactions, or pathways, is a goal of clinical proteomics. The utility of protein microarrays is expected to be their ability to recapitulate the in vivo cellular signaling network, based on specific phosphorylation events, at any given point in time for a cell population.[1–13] Specific protein phosphorylation events are just one example of many types of posttranslational modifications that may affect information flow through a cell. Information concerning protein posttranslational modifications is not reflected in gene microarrays. Completion of a detailed protein network map, even for a subset of cellular processes or critical nodes comprising a network, coupled with detailed genomic and biologic insights, is expected to lead to the design of treatment targeted toward a specific cellular pathway or pathways.[14,15]

Tissue Microenvironment

The tissue microenvironment comprises multiple cell types, in a miniature ecosystem, permitting numerous interactions between diseased and disease-free cells. Host interactions, nutritional status of the individual, and immune system function influence the cell's microenvironment. Examples of molecules mediating cell–cell interactions include secreted factors, cell surface receptors, and adhesion molecules.[16–18] Cells carrying a constitutive genetic defect may express a variety of proteins depending on the context of the microenvironment. Thus cells, specifically diseased cells, are a product of their microenvironment as well as their genetic constitution.

A comprehensive analysis of the molecular basis of cancer and other disease states requires integration of the distinct, but complementary knowledge, garnered from the fields of genomics and proteomics. Genetic

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defects ultimately lead to altered functional proteins that confer survival advantages for tumor cells or disrupt normal cellular function.[19,20] Genomics and DNA microarrays point toward potential genetic defects that may cause disruptions in cell signaling pathways. On the other hand, protein microarrays help to provide information detailing the specific phosphorylation states of proteins. Changes in phosphorylation states may have inhibitory, activating, or neutral effects on a protein depending on the specific site of the phosphorylation event. Protein posttranslational modifications are not solely limited to phosphorylation events, but rather represent one molecule capable of indicating changes in signaling proteins that may reflect changes in the activation state of the protein.

Redundancy in Signaling Pathways

Cancer is an example of a genetically based disease process, but on a functional level, it is a proteomic disease. This genetic alteration is translated into a defective or altered protein that guides the survival of the diseased cell population. The genetic defect of the cancer cell selectively offers a survival advantage for the cell by altering cellular signaling pathways, possibly driving tumor invasion and metastasis.[17,19,21] As expected, these pathways are fluid and dynamic, adapting to the changing microenvironment.[15,22–26] Cellular survival mechanisms include redundancy and collateral signaling pathways. Recurrent tumors are prime examples of a completely natural and normal function of the cell's capacity to adjust to its microenvironment. The need for individual molecular profiling quickly becomes apparent in this context of redundancy and dynamic signaling pathways.

PROTEIN MICROARRAY TECHNOLOGY

Technology Overview

One means of profiling a protein network via protein microarrays is accomplished by comparing the proportion of total protein (phosphorylated and nonphosphorylated) to the phosphorylated protein. Comparison of a selected set of protein phosphorylation events, coupled with knowledge of the biology of the phosphorylation event, may allow us to infer information concerning the activity level of the protein(s). Monitoring the total and phosphorylated proteins before, during, or after treatment, or periodically over time, or between disease and disease-free states, may allow us to infer information concerning the activity levels of the proteins in real time for a

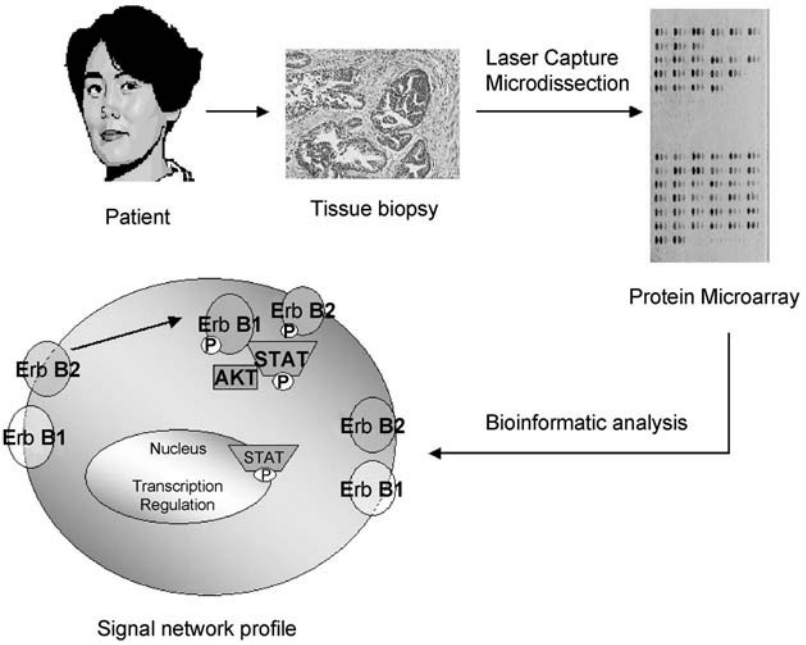

Fig. 1 Protein molecular profiling. Patient biopsy samples are applicable for molecular profiling via laser capture microdissection (LCM), protein microarray, and bioinformatic analysis. A heterogeneous biopsy is the starting point for cell procurement. LCM enhances the procurement of pure cell populations. Whole-cell protein lysates are prepared from the microdissected cells and printed on a microarray. Bioinformatic analysis reveals the activation state of key signaling proteins. The pattern of activated or phosphorylated proteins is developed into a signaling network profile for that particular patient sample.

particular protein pathway.[14,15] It is conceivable that this technology concept could be applied to other posttranslational modification events such as glycosylation, lipidation, or myristalization.

Microarray Formats

Forward phase microarray

There are currently two approaches to producing arrays capable of generating this type of network information. The first format is the forward phase array, in which a labeled bait molecule, typically an antibody, is immobilized on the substratum.[1–6] Each spot represents only one type of bait molecule or antibody. The array is incubated with only one test sample containing several different analytes of interest. The captured analytes are detected with a second tagged molecule that recognized the captured analyte or by labeling the analyte directly.

Reverse phase microarray

Reverse phase microarrays consist of immobilized analyte molecules, with multiple analytes in each spot (Fig. 1). Each spot represents an individual test sample, allowing an array to be comprised of multiple, different samples, such as clinical biopsy samples. The reverse phase array is probed with a single detection molecule, and a single analyte is measured for each spot on the array, across multiple samples. This format allows multiple samples to be analyzed under the same experimental conditions for any given analyte. The arrays are probed separately with two different classes of antibodies to specifically detect the total and phosphorylated forms of the protein of interest. Comparison of the proportion of phosphorylated proteins across patients on the same array provides insights into the cellular signaling network for individual patients. Each slide microarray is probed with a different antibody, generating a set of microarray slides for each set of probe antibodies. Construction of reverse phase

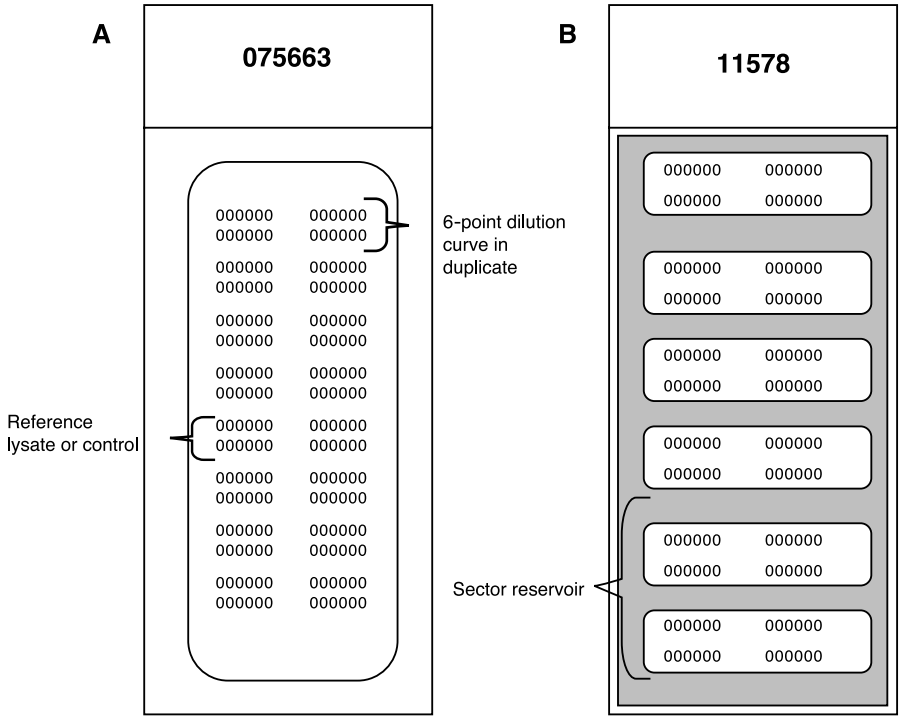

Fig. 2 Reverse phase protein microarray design. Protein microarrays may be designed as a single pad (A) or as sectors (B). The single pad design represents multiple samples, probed with a single antibody, on one microarray slide. Each sample is arrayed in duplicate in a 6-point dilution curve representing undiluted, 1:2, 1;4, 1:8, 1:16 dilutions, and a blank or negative control spot. Inclusion of a reference or control lysate permits process quality control within each slide. The single pad array design (A) allows multiple patient samples to be compared across arrays. Sector microarrays (B) represent one individual sample printed in multiple sectors and probed with a different antibody in each sector. The sample is prepared in a dilution curve in the same manner as for a single pad microarray. The removable reservoir effectively miniaturizes the assay, thus decreasing the volume of reagents for any particular assay. The sector array design allows analysis of multiple proteins for an individual patient on one microarray.

microarrays follows these general protocols: sample collection, sample preservation, preparation of frozen tissue sections, isolation of pure cell populations, protein extraction, microarray printing, and protein detection.

Reverse Phase Microarray Design

A typical array layout consists of a series of 4–6 horizontal spots, representing a mini dilution curve of each sample (Fig. 2). Multiple samples may be printed on each slide, representing before- and after-treatment samples or normal and malignant tissue lysates. The use of multiple sample dilutions ensures the protein of interest is within the linear dynamic range of the assay, based on the antibody sensitivity and affinity.[6]

Sector microarrays

Protein arrays may also be printed in sector formats. A sector array consists of multiple small pads of substratum on a slide. A reservoir placed around each sector permits a different antibody to be used for probing the samples. The sector format miniaturizes the array, providing an increased signal/noise ratio, and is well suited to individual patient assessments. Through the sector array, multiple antibody probes can be multiplexed on a single slide. Thus on one slide, a complete set of analytes can be characterized and used to support a therapy decision for a single patient. For example, a sector array can be probed with antibodies spanning a key cell growth or apoptosis pathway targeted for therapy.

Sensitivity Requirements and Clinical Samples

Clinical applications of protein microarray technology require systems that: 1) are capable of detecting a broad dynamic range of analyte concentrations; 2) exhibit adequate sensitivity (in the femtomolar range) and specificity for detecting low abundance proteins; and 3) are capable of limiting interference from contaminating biologically active molecules such as peroxidases, biotin, avidin, and immunoglobulins.

Protein microarray technology may be directly applied to biopsies, tissue cell aspirates, or body fluid samples.[1,27,28] The total number of cells required for microarray construction is dependent on the number of analyte molecules, the sensitivity of the detection system, and the number of analyte molecules/mole.[6]

Laser Capture Microdissection

Accurate interpretation of signaling pathways is dependent on the proportion of cells contributing to the total signal for a given cell population. Laser capture microdissection (LCM) is an established technique for procurement of pure cell populations from heterogeneous tissue sections.[29,30] Microdissection permits procurement of cells from any designated area of a tissue biopsy, such that cells representing normal, diseased, or stromal tissue may be obtained in relation to their microenvironment. Proteins from neighboring, contaminating cells will not be microdissected, thus limiting their contribution to the overall repertoire of proteins in the population of cells to be studied. Protein microarray construction may be performed with as few as 5000 microdissected cells/15 μL of extraction buffer.

Antibody Validation

A limiting factor for the successful application of this reverse phase protein microarray technology for monitoring phosphorylation events is the availability of specific, high-affinity antibodies.[6,12] DNA probes have been manufactured with known specific affinity constants. On the other hand, antibodies, aptamers, and ligands utilized for protein microarrays cannot be manufactured with predictable affinity or specificity. Reproducible protein microarray analysis relies on antibodies that are specific for the protein of interest. Validation of antibodies via Western blotting is typically performed with a complex biologic sample similar to the sample that will be used on the microarray. A validated antibody should reveal a single, specific band at the expected molecular weight, thus indicating its specificity.

Table 1 Comparison of contact and noncontact microarray printing technologies

	Technology	Types	Determination of spot size	Sample delivery volume
Contact printing	Direct contact of print head with substratum	Ink jet, pin and ring, quill	Pin diameter	0.3–2.0 nL
Noncontact printing	Fluid contact only	Piezoelectric, solenoid	Droplet volume	0.1–0.3 nL piezoelectric; 4.0–8.0 nL solenoid

Protein Extraction

Molecular profiling for elucidation of cell signaling networks requires liberation of the entire cellular proteome. Previously unidentified cell signaling partners could potentially be overlooked if a subcellular compartment was excluded from the extraction protocol. Thus extraction buffers with ionic and nonionic detergents will solubilize cells, generating proteins that are acceptable for use with microarray techniques.

Substratum and Arraying Devices for Reverse Phase Protein Microarrays

Protein microarray substrata may be nylon, nitrocellulose, or silanized silica.[31–33] The substratum requirements for protein arrays are as follows: 1) high binding capacity; 2) should not alter the protein structure; and 3) low background signal. Nitrocellulose-coated glass slides are a common substratum for protein arrays. Proteins bind to nitrocellulose via electrostatic interactions in an irreversible manner, limiting the number of probes that can be used with any one set of immobilized proteins.[32,33] The nitrocellulose slide format allows multiple slides to be printed for each set of samples, thus permitting multiplex analysis with a set of antibody probes (Fig. 1). Chromogenic, fluorometric, and luminescent detection methods may be used with an adequate signal/noise ratio.[33,35–44]

Microarray printing technology currently exists in two forms: contact and noncontact devices. Contact printing is accomplished by direct contact between the print head and the substratum. Noncontact printing dispenses a minute volume of sample above the substratum.[34] A comparison of printing devices is shown in Table 1. Selection of a printing device depends on the viscosity of the material to be printed, the throughput required, the space, and the ability to print replicate samples.

CONCLUSION

Protein microarray applications are being applied to address the challenges of translational medicine. Mapping pathophysiological defects, via protein microarrays, is enhancing our ability to discern treatment effectiveness at an early time point during treatment, thus hopefully leading to early intervention and positive patient outcomes. The need for discovery and quantitative analysis of protein–protein interactions and posttranslational modifications will undoubtedly benefit from this technology. Protein microarrays are proving useful for penetrating into phosphorylation events that are not related to gene expression. The ability to draw a functional map of the state of key protein pathways within a patient's tumor cells will hopefully become the starting point for individualized therapy. Under this scenario, therapy may be tailored to the individual tumor's molecular defect, making it feasible to administer combination therapy targeting multiple interdependent points along a pathogenic pathway or targeting separate pathways.

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Protein Truncation Test (PTT)

Sadanand Gite
Alex Garvin
Kenneth Rothschild
AmberGen Inc., Boston, Massachusetts, U.S.A.

INTRODUCTION

The protein truncation test (PTT) is a mutation detection technique that specifically detects mutations leading to the premature termination of protein translation. An increasing number of genes implicated in disease processes (primarily cancers) have been identified where the majority of the mutations result in premature termination of translation leading to an incomplete and nonfunctional protein product. These include the *APC* gene,[1,2] *BRCA*1 and 2,[3] *PKD*1,[4] *NF*1,[5] *TSC*1 and 2,[6] and *DMD*.[7] Protein truncation test was first reported in 1993[1,8] and has been used mainly in clinical research settings (http://www.genetest.org). However, until recently the PTT had not significantly advanced beyond its original form and has not gained wider acceptance because of several limitations that include 1) slow readout because of the use of electrophoresis followed by radioactive detection; 2) errors in the visual detection of mobility shifts on a gel (which depend on the level of the training of the technician); 3) safety issues involved with the use of radioactivity; and 4) the difficulty in automating SDS-PAGE. We describe here recent advances in the PTT including the introduction of an ELISA-based PTT (ELISA-PTT), and how these advances might lead to wider acceptance of this promising yet underutilized technique.

TECHNICAL DESCRIPTION OF PTT

PTT Protocol

The workflow for the standard-PTT and ELISA-PTT[9] is outlined in Fig. 1. The first step involves the isolation of genomic DNA/mRNA and amplification of specific regions of coding sequences from the target gene using PCR/RT-PCR. These PCR products are then used as template for in vitro (cell-free) translation and the synthesized proteins are traditionally analyzed by SDS-PAGE/autoradiography. Shorter protein products of mutated alleles are distinguished from the full-length protein products of normal alleles due to mobility differences. In the case of ELISA-PTT (Fig. 2A), a reduced C- to N-terminal ratio (C/N) indicates the presence of mutations.

Designing Primers for PTT

Several factors are important when designing PCR primer pairs for PTT. Proper design of the forward primer is the most critical factor. This primer contains four specific regions that are essential for efficient in vitro translation of the PCR amplicon. The 5′-primer must have a promoter sequence at the 5′-end (generally for T7 polymerase) followed by a 5–7-bp spacer sequence, a eukaryotic translation initiation sequence (Kozak sequence) including the ATG start codon, and at the 3′-end, a region of the target gene sequence ($\sim$17–24 bp) in-frame with the ATG codon. In addition, for ELISA-PTT, the 5′-primer also contains sequences coding for the N-terminal detection tag (epitope) and/or binding tags. The 3′-primer must be complementary to the 3′-end of target sequence and for ELISA-PTT must also contain a sequence encoding the C-terminal detection tag.[9] To ensure that truncation mutations near the beginning or at the end of a fragment (i.e., at the 5′- or 3′-end) are not missed, flanking segments for a large region of coding sequence should have an overlap of 350–500 bp. When RNA-based PTT is used, primers from overlapping segments should be located in different exons to reduce the possibility that a mutant allele having a deletion or splicing defect does not amplify with any of the primer sets.

Template Considerations for PCR Amplification

Although transcription/translation of PCR products up to 3 kb is not difficult, best results are obtained using fragments of 1.3–2.0 kb size. If a gene contains several kilobases of coding sequence, it is necessary to divide it into multiple segments. Most published PTT reports use genomic DNA as the template to analyze large exons, e.g., exon 11 of *BRCA*1 and exon 15 of the *APC* genes.[1,3]

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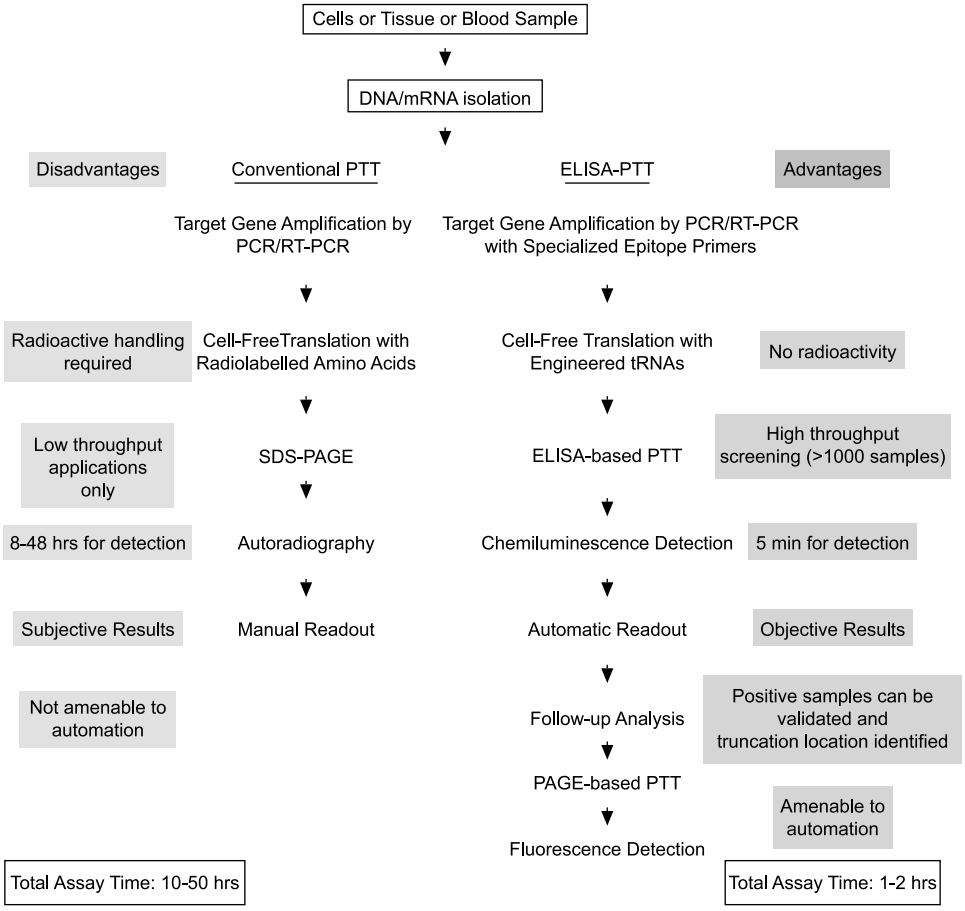

Fig. 1 Various steps involved in typical protein truncation test. (*View this art in color at www.dekker.com.*)

In addition, several attempts have been made to scan entire genes for truncating mutations using a combination of genomic DNA and mRNA (Table 1). In the case of RNA-based PTT, mRNA is first reverse transcribed to cDNA, which then functions as a template for amplification. The best source of RNA is cells in which the target gene is abundantly expressed. However, for practical reasons RNA is generally isolated from freshly drawn peripheral blood lymphocytes.

PCR Amplification

High-fidelity polymerase such as PFU (Stratagene) or Phusion (MJ research) should be used to reduce the possibility of PCR errors. In addition, PCR products should be analyzed by agarose gel electrophoresis prior to the transcription/translation. Direct analysis of the PCR products can indicate the presence of an abnormally sized amplicon suggesting a splicing error or genetic rearrangements such as deletions or duplications. However, purification of the PCR amplicon of the correct size is not necessary and should be avoided, because the aberrant-sized PCR products are often derived from mutant alleles.

In Vitro Transcription/Translation and Analysis of Translated Protein

The TNT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) is convenient for PTT as both transcription and translation are performed simultaneously in a single tube using the PCR product as a template. There are several ways to detect the newly synthesized proteins. The most common method is incorporation of radiolabeled amino acids (e.g., ^{35}S-methionine or ^{13}C-leucine). For separation of the translation products, appropriate SDS-PAGE conditions must be chosen for simultaneous detection of both full-length and smaller products. After electrophoresis, gels are dried and protein bands are detected using either X-ray film or a phosphorImager screen. In general, a wild-type sample will have a strong band at the expected size

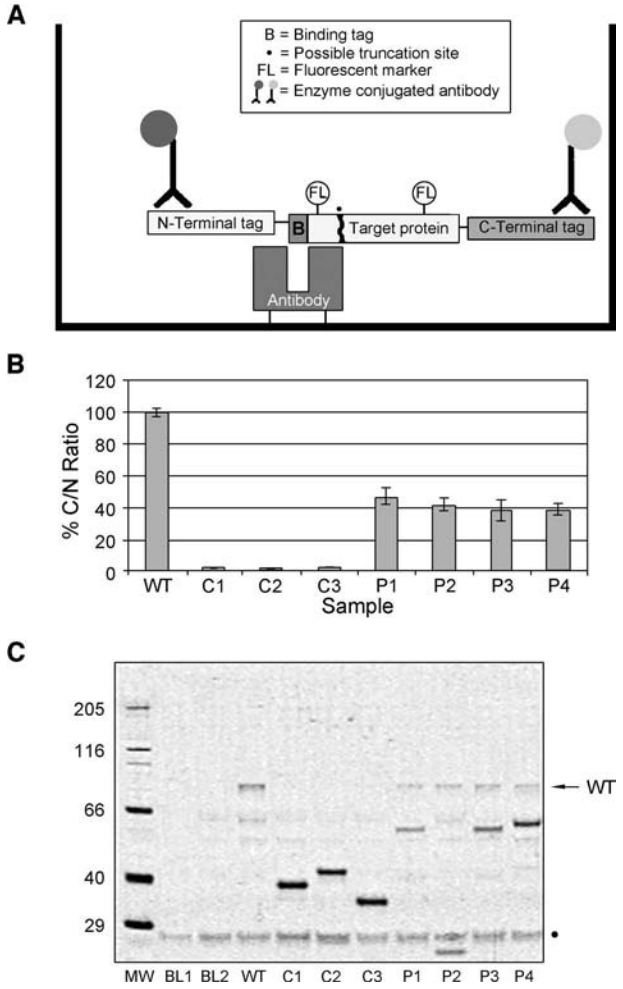

Fig. 2 ELISA-PTT. Panel A: Schematic representation of an ELISA-based Protein Truncation Test (ELISA-PTT). Unlike conventional PTT, the test uses a solid-phase ELISA format. B, Binding tag (either biotin which is incorporated randomly along the sequence using misaminoacylated tRNAs or epitope tag incorporated at N-terminal) acts to immobilize the cell-free synthesized fragments on the well surface. N- and C-terminal epitopes are detected by corresponding antibodies allowing for an estimate of the relative amount of truncated protein while simultaneously controlling for the level of translation. FluoroTags (FL) provide an independent confirmation of truncation and estimate of the fragment size by using fluorescence readout of gels. Panel B: Detection of various truncation mutations in the APC gene using ELISA-PTT. Panel C: Validation of ELISA-PTT result by gel-based fluorescence PTT. WT is wild-type, C1–C3 are mutant homozygous DNA samples from cell lines, and P1–P4 are the heterozygous DNA samples from patients prediagnosed with FAP. BL1 corresponds to a cell-free translation performed lacking both the added tRNAs and DNA. BL2 corresponds to a cell-free translation performed lacking only the added DNA. MW is molecular weight markers. The asterisk indicates the position of an autofluorescent protein band present in the cell-free translation extract. (*View this art in color at www.dekker.com.*)

of the full-length translation product compared to bands corresponding to smaller proteins. Ideally, these weaker bands correspond to truncated protein fragments derived from mutant template. However, weaker bands can also originate from internal weak translation initiation sites (AUG) and proteolytic degradation products. In some cases these bands can obscure the analysis and/or detection of the truncated fragments derived from a mutant template.

In addition to radiolabeling, methods have been reported which use nonisotopic detection. In one approach, biotin was incorporated during translation using biotin–lysine–tRNA and was detected by Western blotting.[10] Similarly, Kahmann and coworkers[11] used Western blotting to detect engineered epitope tags. Radioisotope labeling can also be eliminated by incorporating fluorescent labels using fluorescently labeled tRNAs.[9,12,13] Although these nonisotope-based methods have clear advantages compared to radioactive detection, they still suffer from the intrinsic throughput problems associated with electrophoresis. In order to overcome these limitations, ELISA-PTT has been developed which provides higher throughput and lower cost because it circumvents electrophoresis.[9]

The basic ELISA-PTT approach is illustrated in Fig. 2A. Specially designed primers, which incorporate N- and C-terminal epitopes, are used for amplification of target sequences. In addition to these epitopes, additional tags can be incorporated randomly along the protein chain for detection and capture purposes. This is accomplished using mis-aminoacylated tRNAs (e.g., biotin–lysine–tRNA or/and BODIPY–lysine–tRNA) which are added to the reaction mixture. A capture tag can also be incorporated into the protein by using a specially designed primer. After translation, the test proteins are captured in a single-well of a microtiter plate, and the N- and C-terminal epitope tags are detected using appropriate antibodies. The signals obtained are used to compare the total amount of target protein captured (N-terminal signal) vs. the fraction that is full length (i.e., has a C-terminus).

The results of ELISA-PTT for the *APC* gene are shown in Fig. 2B. In this experiment, DNA derived from normal controls, familial adenomatous polyposis (FAP) patients as well as cell lines with known mutations in the *APC* gene was analyzed. The C/N terminal ratio of wild type was normalized to 100% and the values obtained for all other samples are expressed as a fraction of the wild type. For cell line DNA (homozygous APC mutant), the C/N terminal ratio was close to 0%, as expected. The C/N terminal ratio for heterozygote samples derived from individuals with FAP ranged from 37% to 47% relative to the wild type (Fig. 2B). It is possible to validate the ELISA-PTT results and localize the mutation within the protein using the fluorescent labels incorporated into

Table 1 Top ten diseases where PTT is applicable

No.	Disease	Gene	% Truncated mutations	Ref.
1	Breast cancer	BRCA1/2	90	[3]
2	Colorectal cancer	APC	95	[21]
3	Familial adenomatous polyposis	APC	97	[1]
4	Hereditary nonpolyposis colon cancer	MSH2/ MLH1	70–80	[22]
5	Neurofibromatosis	NF1/2	60–75	[5]
6	Polycystic kidney disease	PKD1/2	95	[4]
7	Duchenne muscular dystrophy	DMD	95	[7]
8	Cystic fibrosis	CFTR	50	[23]
9	Ataxia telangiectasia	ATM	90	[24]
10	Tuberous sclerosis	TSC1/2	75–98	[6]

the in vitro translated proteins. Figure 2C shows results obtained from fluorescent imaging after SDS-PAGE using aliquots of the same translation mixtures used in Fig. 2B. The wild-type sample produces a band of the expected molecular mass (~70 kDa), whereas the homozygous mutant cell line samples exhibit single bands at approximately 35, 40, and 32 kDa (C1–C3, respectively). Samples derived from FAP individuals heterozygous for a mutation exhibited two bands corresponding to both wild-type and mutant alleles (lanes P1–P4).

ELISA-PTT does not require electrophoresis for the primary screen, but does require electrophoresis for sizing the proteins in order to locate and confirm the mutation in the amplicon. In contrast, Garvin et al.[14] reported a method to size in vitro synthesized proteins by mass spectrometry, thereby eliminating the need for electrophoresis. Experiments were carried out using a short sequence (21 bases) of the *BRCA*1 gene. After in vitro translation, the test peptide was purified using a FLAG-epitope and subjected to MALDI-TOF mass spectroscopy. Truncating mutations in heterozygotes are easily detected, and importantly, single amino acid substitutions are also detectable because of the high resolution of mass spectroscopy. Although this process can be multiplexed and is amenable to automation, the small test sequence size reduces its effective throughput compared to gel-PTT or ELISA-PTT.

GENERAL CONSIDERATIONS OF PTT

Sensitivity of PTT

The PTT is most often used to detect truncating mutations in tumor suppressor genes from patients that are heterozygous for the mutation, using genomic DNA as starting material. The mutant allele represents 50% of the alleles present in the sample, and the PTT, like all other scanning methodologies, can easily detect mutations at the 50% level. There are other applications, however, where the mutant allele is present at much less than 50% (e.g., in a tumor biopsy or fecal material from a colorectal cancer patients) and in these cases sensitivity becomes an issue. In comparison with other mutation detection techniques, the sensitivity of gel-PTT is high and is capable of detecting mutant alleles at one part in five. Recently, Traverso et al. have demonstrated detection efficiencies of chain truncation mutations as low as 0.4% relative to WT.[15] This was achieved by first diluting genomic DNA samples so that no more than two to four DNA templates are present in each sample prior to PCR amplification. This is followed by translation of the amplified DNA for approximately 144 samples and detection using radioactive gel-based PTT. As discussed previously,[9] ELISA-PTT is ideal for such an application as radioactive gel-based detection is not suitable for automation.

Advantages and Disadvantages of PTT

Despite the fact that chain-truncating mutations can be detected by conventional scanning methods such as DNA sequencing or single-strand conformation polymorphism analysis (SSCP), PTT remains the method of choice for many laboratories. This is primarily due to its ability to accurately scan a large sequence (as large as 3 kb) in a single reaction. Once a chain truncation is identified and its position on a gel is known, a much smaller region of DNA can be scanned by sequencing to confirm and characterize the mutation. Although PTT detects only chain truncating mutations, these mutations are invariably associated with disease, especially in the case of tumor suppressor genes because truncated proteins are almost always nonfunctional. In contrast, gene variants that result only in an amino acid substitution are often difficult for the clinician to interpret without additional information.

However, there are a variety of limitations for the PTT approach. First, PTT is usually only appropriate for those genes that contain a high proportion of protein truncating

mutations. In addition, it is only effective for large exons as protein fragments encoded by smaller exons are not easily analyzed by SDS-PAGE. This limitation can be overcome by using mRNA as the starting material. However, there are handling and storage concerns with the use of mRNA due to nonsense-mediated mRNA decay[16] and the lack of mRNA expression in accessible tissue (peripheral blood). In addition, PTT cannot detect mutations occurring outside the coding region, such as those that affect control of gene expression.

A number of problems can arise during the PCR amplification step used in PTT. For example, if the mutated allele has a large insertion in the amplicon or if the mutation is a deletion that includes the primer binding site, the mutant allele will not amplify. Also, polymerase error in the first few cycles can lead to an artifact that can be mistaken for an authentic mutation. Artifacts can also be produced from in vitro translation and include false-initiation from internal ATG codons (which is more severe in *Escherichia coli*-derived reaction mixtures) and proteolytic degradation of the full-length protein by endogenous proteases present in the in vitro synthesis lysate. These and other factors can give rise to background bands in the wild type control samples which can interfere with detection of an overlapping mutant band.

ALTERNATIVE METHODS FOR DETECTING CHAIN TRUNCATIONS

Many genotyping methods exist which can detect chain truncating mutations as well as other types of mutations. DNA sequencing is considered the gold standard for genotyping as it can detect any mutation that can be amplified by PCR including those that have never been previously described. However, it is expensive and time consuming to carry out complete gene sequencing even with high-throughput automated capillary sequencers. Furthermore, this method is not able to detect a mutant sequence that represents less than 25% of the sample because of the presence of the wild-type signal at every position.[17] Hybridization-based approaches for detecting mutations such as TaqMan® (Applied Biosystems), Invader (Third Wave Technology), and high-density chip arrays (Affymetrix) are high-throughput methods that are only capable of detecting previously identified mutations and will miss de novo mutations.[18] In one study,[19] five different mutation detection methods to detect truncation mutations in *BRCA*1 were compared. These included four DNA-based methods: two-dimensional gene scanning (TDGS), denaturing high-performance liquid chromatography (DHPLC), enzymatic mutation detection (EMD), SSCP, and PTT. Analysis of 21 samples showed that PTT correctly identified all 15 deleterious mutations. Not surprisingly, the DNA-based techniques did not detect a deletion of exon 22, and five truncating mutations were missed by SSCP. In another study,[20] researchers compared the effectiveness of PTT and SSCP for detecting *BRCA*1 mutations and concluded that PTT is a superior screening test as the specificity of PTT was 100% and sensitivity was 82.6%, whereas for SSCP, the specificity was 99% but the sensitivity was only 60.9%.

CONCLUSION

The protein truncation test is currently the fastest method in general use for detecting previously unidentified truncating mutations in tumor suppressor genes. In addition, by only identifying disease-causing truncation mutations, PTT analysis is not hampered by false-positive signals derived from phenotypically silent variants such as polymorphisms. Finally, the size of the truncated product localizes the DNA mutation and thus enhances sequence analysis for confirmation. In recent years, the identification of truncating mutations in tumor suppressor genes has greatly benefited from PTT. Improvements in the PTT, such as ELISA-PTT,[9] multicolor-PTT,[12] and mass spectrometry-based PTT,[14] should allow this method to gain wider acceptance.

ACKNOWLEDGMENTS

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Pseudoxanthoma Elasticum

P

Franziska Ringpfeil
Jouni Uitto
Jefferson Medical College, Philadelphia, Pennsylvania, U.S.A.

INTRODUCTION

Pseudoxanthoma elasticum (PXE) has long been considered a prototype of connective tissue diseases as it visibly alters the elastic fiber network of skin. Two French physicians, Felix Balzer in 1884 and Marie-Anatole Chauffard just five years later, first described the characteristic skin changes. The first of them also noted ''broken elastic fibers'' on histological specimen of the skin and heart. In his monograph about PXE, Neldner summarizes in 1988 that ''in both cases, efforts to indict the liver, often the target of blame for poor health amongst the French, were unsuccessful, although it was still a suspect.'' With the advent of molecular genetic research in general and the recent detection of the hepatic transmembrane transporter protein MRP6 as the affected gene product in particular, the suspicion of the French physicians was proven correct about 100 years later.

EPIDEMIOLOGY

Pseudoxanthoma elasticum is a rare heritable disorder of connective tissue. Its estimated prevalence varies between 1 in 25,000 and 1 in a million. Women seem to be slightly more often affected than men (3:2), although some investigators have attributed this to cosmetic bias. Age of onset of clinical symptoms may vary from infancy to over 50 years of age, and severity differs not only between families but also among members of the same family.[1]

MODE OF INHERITANCE

Pseudoxanthoma elasticum is caused by mutations in the *ABCC6* gene.[2–4] It is inherited in an autosomal recessive fashion, but is most frequently encountered sporadically.[1] However, affected individuals in two generations, suggesting autosomal dominant inheritance, have been frequently reported. In addition, a limited phenotype, often reflected only by positive histopathology at predilection sites of skin and by asymptomatic eye findings, has been reported in presumed obligate heterozygous carriers within PXE families. Molecular evaluation of some of these families revealed the presence of at least two different compound heterozygotes with segregation of a total of three different mutations[5] (Fig. 1). The disparities in phenotype within those families may well be explained by the difference in the set of mutations inherited. The mild phenotype was often associated with at least one missense mutation in the *ABCC6* gene, which would predict in some cases residual activity of the gene product. In other multigenerational families, consanguinity accounted for the pseudodominant inheritance of PXE. Refined mutation screening techniques led to the detection of two mutated alleles in almost all affected individuals, thus contradicting the existence of autosomal dominant forms of PXE. In fact, the mode of transmission is autosomal recessive in all affected individuals elucidated thus far at the molecular level.[5]

CLINICAL FEATURES

Pseudoxanthoma elasticum clinically affects three different organs of the body, namely, the skin, the eye, and the cardiovascular system. Initially, manifestations on the skin include yellowish papules, which tend to coalesce into larger plaques in the predilection sites, such as the lateral neck, the axillae, groin, antecubital, and popliteal fossae, and which typically are present at puberty. In long-standing lesions, sagging of the skin may be observed, which presents a major cosmetic problem. Involvement of the eyes can be severe as newly formed, fragile blood vessels along fractures (angioid streaks) in the elastic fiber-rich Bruch's membrane behind the retina often rupture and bleed, which leads to loss of central vision around the age of 40. Calcification in the cardiovascular system causes atherosclerotic disease often with morbid consequences beginning in young adulthood. While age of onset is variable and clinical manifestations are protean and differ significantly even within families, no racial or ethnic predilection has been observed.[1,6]

GENOTYPE–PHENOTYPE CORRELATION

To date, over 100 loss-of-function and missense mutations in *ABCC6* have been reported. Currently, it is unclear how consequences of mutations at the level of the

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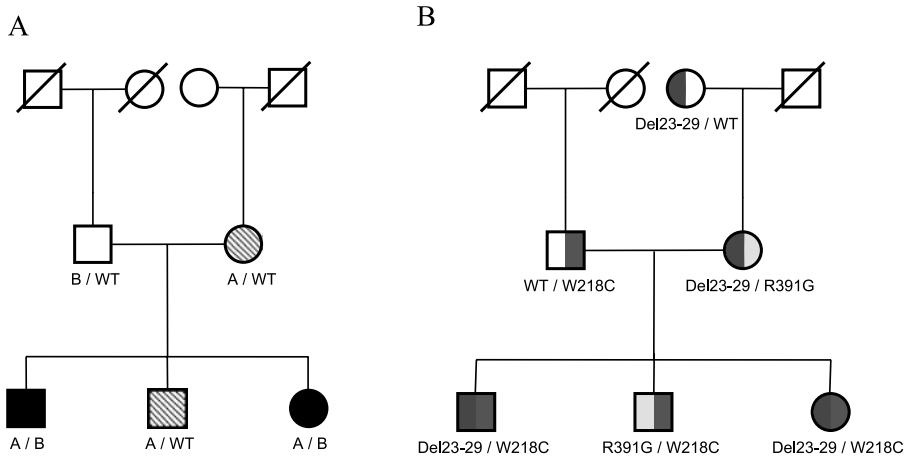

Fig. 1 A. Pseudodominant pedigree with clinically affected individuals (blackened) and presumed obligate heterozygous carriers with only histopathological evidence of PXE (shaded). Letters A and B designate the assumed mutations and their distribution prior to molecular analysis. B. Mutation analysis in the same pedigree reveals a total of three different mutations on three different alleles (colored symbols). Both affected and minimally affected obligate heterozygous carriers are compound heterozygous for mutations in *ABCC6*, WT: wild type. (*View this art in color at www.dekker.com.*)

encoded protein, MRP6, determine a particular phenotype ranging from mild to severely debilitating disease with a broad spectrum of cardiovascular involvement. The high prevalence of cardiac abnormalities in otherwise unaffected members of families or individuals with PXE also suggests the possibility of a dominant effect of specific mutations or polymorphisms on the cardiovascular system.

PATHOPHYSIOLOGY

Mutations in the *ABCC6* gene have recently been found to underlie PXE in nearly all affected individuals.[2–4,7–9] *ABCC6* is located on chromosome 16p13.1, and its 31 exons are dispersed over 73 kb of genomic sequence.[2] Its translated region spans 4.5 kb and encodes multidrug resistance-associated protein 6, MRP6,[10,11] an ATP-dependent efflux pump that is expressed at the basolateral membranes of hepatocytes and the proximal tubules in the kidneys.[12] The protein is composed of three transmembrane domains and two nucleotide-binding folds with ABC motifs located in the intracellular space. Mutations in *ABCC6* are mostly private with the exception of two frequently recurring mutations, R1141X and del exons 23–29, in patients of Northern European descent.[2,8] They are dispersed over the entire gene but the majority of mutations are predicted to interfere with proper function of the second nucleotide-binding fold, the site of substrate binding in ABC transporters. To date, no physiological substrate for MRP6 has been identified.[13–15] MRP6 is 45% homologous to the prototype within this family of proteins, MRP1.[3,10,11] The latter is instrumental to cellular detoxification, a property apparently not shared by MRP6, and has a capacity to confer chemotherapy resistance to the cells. While the exact pathomechanisms leading to abnormalities in the elastic fiber network of the target organs are poorly understood, deposition of calcium is considered merely a secondary change that can occur after trauma to elastic fibers. As a result of calcification, fragmentation or disorganization of elastic fibers in the midreticular dermis and the medium-sized arteries and veins as well as the fractures in Bruch's membrane behind the retina occurs. In the skin, this leads to inelasticity, loss of recoil, and subsequent sagging of the skin. In the eye, neovascularization occurs at sites of fracture due to calcium deposits. These newly formed vessels are fragile and often rupture with trauma. In the arteries, alteration of elastic fibers leads to calcification of the elastic media and later the intima, which facilitates atherosclerotic peripheral vascular disease.

DIAGNOSIS—CLINICAL AND LABORATORY FINDINGS

A trained dermatologist can often diagnose PXE clinically without difficulty, but skin biopsy confirms the clinical suspicion, especially in less obvious clinical scenarios. A punch biopsy should be obtained from the lateral neck or axillae even in the absence of obvious skin lesions. When lesions are clinically obvious, hematoxylin and eosin (H&E) stain usually demonstrates calcium deposits in the midreticular dermis. Otherwise, silver stains for elastin (Verhoeff–van Giesson) or calcium (von Kossa) may be necessary for visualization of microscopic anomalies in

Table 1 *ABCC6*-specific primers

Forward primer (5′–3′)		Reverse primer (5′–3′)		Size in bp	Annealing temperature in °C
1F	TGCTGGGTCCAAAGTGTTTA	1R	CAGCCCGAGAGATCTGCAGC	469	55
2F	GATCCAAAAAGTTGCCTGGC	2R	TGTCCCCTGCCTCCCCCGA**A**	328	60
3/4F	TCCCAGTTGGACATGGGGC**C**	3/4R	TATAAGTGTGTGCATCGTG*T*	736	60
5F	CCTCTGTCTCCATTCCTTA*T*	5R	AGACTGAGACCTCAAAGTGG	219	55
6F	CACAGTTCGTCCTGTCTTCC	6R	GGCCCTGGAGAAGCAGCTG*T*	624	57
7F	GATCCTGCAGGGGTGAATGG	7R	ATGATGAGCTTTTCTGAAG*T*	242	50
8F	CCCCCAACTCCCATGATTG*C*	8R	AAGGATGCCACTAAGAGACC	450	55
9F	AGGCACCTCCTCTCACCAGC	9R	GGTGACAGAGCAAGACTCC*A*	423	60

Underlined nucleotides eliminate the amplification of both pseudogenes, underlined nucleotides in bold eliminate ABCC6-ψ 2, underlined nucleotides in italics eliminate ABCC6-ψ1.

the dermis. Diagnosis has been challenging in presumed obligate heterozygous carriers, which have been found to display minimal clinical manifestations and have positive skin biopsies.[16–18] The recent discovery of *ABCC6* as the gene harboring causative mutations for PXE and the ability to perform mutation analysis have significantly improved our ability to render a definite diagnosis.

MOLECULAR GENETICS

Mutation detection strategies were initially complicated by the presence of two partial pseudogenes, *ABCC6-Ψ1* and *ABCC6-Ψ2*, in the human genome. They are both products of large genomic duplications containing 5′ portions of *ABCC6* as well as part of the neighboring *pM5* gene, located upstream of *ABCC6*. *ABCC6-Ψ1* contains the promoter region as well as the first nine exons of *ABCC6*, whereas *ABCC6-Ψ2* is nearly identical to the 5′ untranslated region and exons 1–4. They are located in close proximity to *ABCC6* on the short arm of chromosome 16. Both are highly homologous to their origin and differ only in a few nucleotide positions.[19] Current mutation detection methods employ gene-specific primers (Table 1) which allow detection of over 70% of mutations using denaturing high-performance liquid chromatography (dHPLC) through WAVE technology (Transgenomic™) as a screening technique[20] and over 80% with direct sequencing of the entire *ABCC6* coding region. Mutation detection has not only solved a historical debate about the mode of inheritance in PXE but allows timely and presymptomatic detection of affected individuals who may be counseled to avoid risk factors that would exacerbate their eye or cardiovascular disease. In addition, mutation detection has significantly improved genetic counseling and offers the possibility for prenatal diagnosis.

MANAGEMENT

The most important measure is prevention of severe cardiovascular or ophthalmologic disease. Biannual or annual evaluation by a cardiologist is recommended. Smoking should be avoided at all costs and regular low-impact exercise, e.g., running, biking, and swimming, is encouraged. The use of acetylsalicylic acid is controversial as it lowers the risk of myocardial infarction yet may facilitate serious complications in case of gastric or intestinal hemorrhage. Calcium intake should be appropriate for development and age but excess should be avoided as it may worsen the clinical symptoms. Claudication may be treated with pentoxyfylline (Trental) 400 mg tid or cilostazol (Pletal) 50 or 100 mg bid. Biannual ophthalmologic examinations, avoidance of head trauma, and regular use of an Amsler grid are crucial. When angioid streaks become symptomatic, laser surgery may improve symptoms in about 50% of patients treated. Photodynamic therapy and macular translocation are fairly novel techniques that should be reserved for the most seriously affected. Skin changes are disfiguring and may occasionally respond to plastic reconstruction. While wound healing is not impaired, scars may spread as a result of the loss of elastic properties of the skin. The use of oral phosphate binders is currently under investigation.

CONCLUSION

About 100 years after the first formal description of PXE, a major milestone has been reached with the discovery of the causative mutations in the *ABCC6* gene. However, the unsuspected role of a transmembrane transporter protein in liver and kidneys, far away from the affected organs, has raised many questions that remain to be answered.

Current hypothesis favors a metabolic pathway of disease development. Impaired excretion of a yet unidentified substance via urine or bile may lead to its resorption into the systemic circulation with adherence and subsequent trauma to the elastic fiber network and secondary deposition of calcium. Alternatively, a nonfunctional MRP6 pump may switch on an alternative mechanism of excretion in liver and kidneys that indirectly causes trauma to the elastic fiber network by removal of essential factors.

In addition, there is currently little evidence to suggest that certain mutations or combinations thereof elicit a specific phenotype. These observations emphasize the role of possible environmental factors as determinants of disease extent and severity, a question that may be resolved once adequate animal models for PXE become available.

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Pulsed Field Gel Electrophoresis (PFGE)

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Fann Wu
Preeti Pancholi
Phyllis Della-Latta
Columbia University Medical Center, New York, New York, U.S.A.

INTRODUCTION

Pulsed field gel electrophoresis (PFGE) was first described in the early 1980s and has proven to be one of the most powerful techniques in modern molecular epidemiology for genotyping microorganisms. The applications of this technology include determining genetic relatedness among microbial strains in industrial, agricultural, and hospital settings, as well as understanding the dynamics of antimicrobial resistance and epidemiological investigations. This chapter will focus on the clinical applications of PFGE, assessing its strengths and limitations.

THE HISTORY OF PULSED FIELD GEL ELECTROPHORESIS TECHNOLOGY

The Electrophoretic Separation of DNA Molecules

Conventional gel electrophoresis is based on the principle that negatively charged DNA fragments below 50 kb can be separated when subjected to a unidirectional electric field. The DNA samples are added to wells in an agarose matrix and migrate in the direction of the field at a rate that is inversely proportional to size, i.e., the larger the DNA fragments, the slower their rate of migration in the electric field. However, when DNA molecules are larger than the threshold size of 50 kb, all fragments exhibit size-independent mobilities.[1] This significant practical problem limited the use of electrophoresis for the study of megabase-sized molecules, such as the bacterial chromosomal DNA.

In 1984, Schwartz and Cantor introduced the concept of pulsed field gel electrophoresis by using two alternating electric fields, to separate large DNA fragments (50 to 2000 kb) within agarose gels.[2] Subsequently, a variety of alternative electrophoretic configurations, using currents ''pulsed'' in different directions over controlled time intervals, have been developed. These included orthogonal field alternation gel electrophoresis,[3] vertical alternating field gradient gel electrophoresis,[4] periodic field inversion gel electrophoresis,[5] and contour-clamped homogeneous electric field electrophoresis (CHEF).[6]

In PFGE, chromosomal DNA can be treated with a restriction endonuclease, resulting in DNA fragments that vary in size from 50 to 2000 kb. It is a powerful molecular typing technique for comparing and analyzing bacterial chromosomal DNA fragments. The two most common pulsed field methods used in DNA fingerprinting laboratories are the CHEF and the programmable autonomously controlled electrode gel electrophoresis (PACE).[7] Both systems contain three major components: a power module to generate the electrode voltages and store switching function parameters, a cooling module to keep the temperature at 14°C, and an electrophoresis chamber. The chamber contains 24 horizontal electrodes, some of which are clamped to eliminate DNA lane distortion. The electrodes are arranged in a hexagon, thus providing reorientation angles of 60° or 120° in contrast to traditional orthogonal field alternation gel systems with two perpendicular electrodes. The resolution of PFGE is dramatically affected by the number and configuration of the electrodes used, because these alter the shape of the applied electrical field. For high-resolution separation, the most effective electrode configurations yield angles of more than 110°.[8] In PACE, each electrode's voltage is independently controlled and can generate an unlimited number of electric fields of different voltage gradients, orientations, and intervals sequentially in time. However, the traditional CHEF systems are limited to two alternating electric fields at a fixed reorientation angle. The two leading PFGE technologies, CHEF and PACE, have been combined into a CHEF Mapper system (BioRad Inc.) which is widely used by molecular epidemiologists. It has the advantage of containing protocols embedded on a microchip, thus eliminating trial and error in setting parameters for obtaining good resolution of band patterns.

Sample Preparation for PFGE

Standard sample preparation procedures for DNA isolation involving mechanical forces are inappropriate for the

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analysis of fragile, high-molecular weight DNA molecules that easily break into small pieces. To prevent DNA damage, intact cells mixed with warmed, liquid agarose are pipetted into plug molds, $10 \times 5 \times 1.5$ mm in size. Cells embedded in the agarose plugs are lysed in situ by detergents and enzymes. The agarose matrix keeps the large DNA molecules intact, while permitting diffusion of lysed cellular components from the plug. Infrequent-cutting restriction endonucleases are used to digest the DNA. The choice of restriction enzyme is critical because it should generate between 10 and 30 numbers of DNA fragments necessary for strain discrimination. A list of endonucleases for typing specific microorganisms have been published.[9] After restriction enzyme digestion, the plugs are cut into appropriate sizes based on the DNA concentration and loaded into the wells of an agarose gel. The gel is then placed in the electrophoresis chamber, and pulsed field conditions are chosen based on the expected size range of the DNA. To separate 40–2000-kb-sized DNA fragments, PFGE is usually run for 18–48 hr. After staining with ethidium bromide, bands are visualized and photographed. Several commercially available software packages are available that provide computerized gel scanning and data analysis capabilities. They enable the clustering pattern of each gel to be represented as a dendrogram that shows the percent similarity obtained through Dice coefficient and the unweighted pair group method with arithmetic average. Pulsed field gel electrophoresis patterns can also be stored for future strain comparisons.

Performance Characteristics

The performance requirements for a good molecular typing system include a high index of strain discrimination (>0.95) and assay reproducibility, and the ability to provide results for a diverse group of microorganisms.[10] Pulsed field gel electrophoresis technology satisfies these criteria and therefore is the most frequently used molecular typing method in molecular epidemiology. The PFGE band patterns represent the entire microbial chromosome profile (>90%).[11] All manipulations for DNA extraction and restriction digestion are performed inside the agarose plug, which protects DNA from shearing forces during sample preparation. Pulsed field gel electrophoresis typically yields 10 to 30 well-separated, easy-to-read chromosomal DNA bands after digestion with infrequent-cutting restriction endonucleases. It has significant advantages compared with conventional electrophoresis techniques for analysis of restriction endonuclease digested chromosomal DNA. The latter when applied to conventional electrophoresis cannot separate large fragments, and treatment with frequent-cutting restriction endonucleases will generate hundreds of uninterpretable bands. Therefore conventional electrophoresis is best suited to the analysis of relatively small-molecular weight (<50 kb) DNA fragments of plasmids. The comparison of the procedural features of conventional electrophoresis and pulsed field gel electrophoresis is summarized in Table 1.

The major disadvantages of PFGE compared to other DNA-based typing methods include the relatively high cost and the labor-intensive techniques that can take 4 to 5 days to complete.[12] DNA fragments smaller than 50 kb cannot be reliably separated by PFGE, because the system is not able to switch the field orientation quickly enough to separate these smaller molecules. Furthermore, certain organisms such as *Clostridium difficile* and *Aspergillus* spp. may not be typeable by PFGE as their DNA cannot be isolated intact. Strain differentiation may not be possible for some bacterial species because of inherent nuclease contamination that can degrade DNA.[13]

Many different protocols have been developed in laboratories using PFGE, leading to variability in assay design and reproducibility. It is important to standardize the PFGE protocols, especially, some critical elements, such as the concentration and the integrity of the DNA in the plug, the effectiveness of specific restriction enzymes for digestion, and the electrophoresis conditions including gel volume and agarose concentration, ionic strength and volume of the buffer, and running conditions including voltage, switching times, reorientation angle, and total run times of electrophoresis.[14,15] To insure high-quality gels and consistent reproducibility, we recommend including a quality-control strain with each run for gel comparison.

Table 1 Comparison of the procedural features of conventional electrophoresis and PFGE

Procedural characteristics	Conventional gel electrophoresis	PFGE
Sample preparation	No specific requirement	Intact cells embedded in agarose
Restriction endonuclease	Frequent-cutting enzyme	Infrequent-cutting enzyme
Electric field	Single homogeneous	Pulsed homogeneous
Resolution in DNA size	<50 kb	50–2,000 kb
Typical run times	1–4 hr	18–48 hr
Instrument cost	<$2,000	>$15,000

Interpretation of strain relatedness can be performed by visual inspection of the PFGE bands. Criteria for PFGE interpretation have been proposed for epidemiological investigations[16] in the following manner: bacterial isolates yielding the same PFGE pattern are considered ''indistinguishable;'' isolates differing by one to three bands (a single genetic change) are considered ''closely related;'' isolates differing by four to six bands (two independent genetic changes) are considered ''possibly related.'' Six or more band differences represent three or more genetic changes and such isolates are considered ''unrelated.'' More recently, various computer-assisted programs have become available to enhance the capability of comparing DNA fragment patterns present on multiple gel sets. Using this approach, investigators can create a searchable database of PFGE fragment patterns and easily perform cluster analyses.[17]

APPLICATIONS

Pulsed field gel electrophoresis has a wide range of applications in the analysis of microorganisms and human or other mammalian DNA. Because of its high reproducibility and discriminatory power, PFGE has been utilized in genetic and epidemiological analyses of at least 98 different pathogens, including gram-negative and gram-positive bacteria and fungi.[18] Here we highlight some clinical applications of PFGE genotyping of bacterial pathogens to study hospital-related epidemiological investigations and international surveillance efforts to understand and contain the spread of antimicrobial resistant microorganisms.

Nosocomial Infections

An important application of PFGE is the comparative analysis of nosocomial pathogens. It is clear that specific etiological agents can be transferred between patients in hospital settings and cause nosocomial infections. Rapidly assessing the clonal relatedness of these isolates is critical in determining the extent of outbreak spread and the strategies for containment. Pulsed field gel electrophoresis is most often considered the typing technique of choice to examine the true genetic identity of the bacterial isolates in an outbreak investigation.[11] Figure 1 shows a variety of PFGE patterns that were generated when methicillin-resistant *S. aureus* (MRSA) chromosomal DNA was digested with *Sma*I restriction enzyme. These results were obtained from a published study describing an outbreak investigation in our hospital, which involved four cases of postpartum mastitis caused by nosocomial spread of a virulent strain of community-acquired MRSA.[19] The outbreak strains were found to be indistinguishable from each other by PFGE (lanes 1 to 4), and were distinct from other groups of hospital-acquired MRSA strains that were collected from different hospital units (lanes 5 to 9).

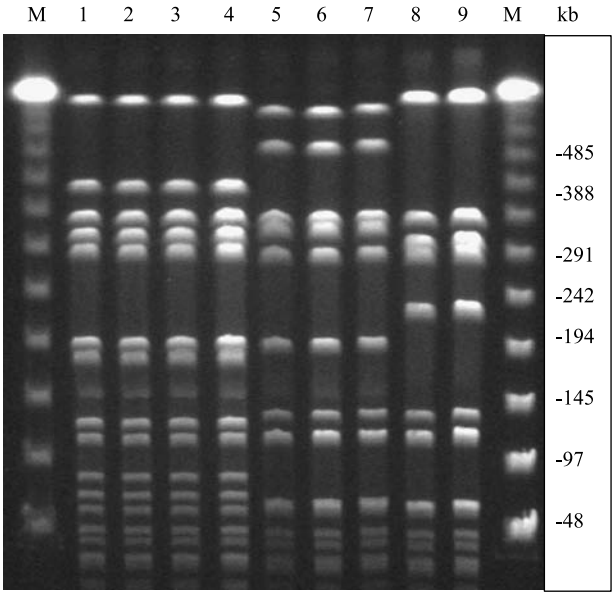

Fig. 1 PFGE of *Staphylococus. aureus* isolates. Genetic profiles were obtained by digestion of *S. aureus* chromosomal DNA with *Sma*I restriction endonuclease. Lanes 1 to 4 show chromosomal DNA from *S. aureus* isolates obtained during the outbreak. Lanes 5 to 9 show chromosomal DNA from unrelated *S. aureus* isolates obtained from other hospital units during the course of the outbreak. Lanes marked M show the lambda molecular weight standard with corresponding molecular weights in kilobase indicated on the right side of the gel.

Community-Acquired Infections: Colonization vs. Infection

Pulsed field gel electrophoresis has been successfully used as a tool to investigate community-acquired infections or colonizations and the epidemiological relatedness of strains within the community, school, or day-care centers.[20,21] We conducted an epidemiological study of multidrug-resistant *Acinetobacter baumannii* in our community.[22] A total of 103 isolates from patients in two Manhattan hospitals were compared with 23 isolates from the hands of community residents by analysis of antimicrobial resistance patterns and PFGE genotyping. The antibiogram results showed that 36.6% of hospital isolates were multidrug resistant, whereas those from the community were not ($p<0.005$). Pulsed field gel electrophoresis genotyping indicated that more than half of the isolates from both hospitals were either indistinguishable or closely related. However, hospital strains were found to be unrelated to most of the community isolates ($p<0.001$).

The results showed that *Acinetobacter* strains recovered from patients in the hospitals were not acquired from the community and suggested that the hospital environment harbored a reservoir for epidemic *A. baumannii* strains.

Public Health Surveillance

Pulsed field gel electrophoresis is the most commonly used molecular typing method for epidemiological investigations by national and international surveillance networks. Pathogens, such as *Salmonella*, *E. coli* O157, *Shigella*, and *Legionella*, often cause food or water-associated multicommunity outbreaks that require comprehensive public health measures.[23–25] Appropriate surveillance and timely detection of the outbreak sources are necessary for interruption of pathogen transmission. The strategy is that selected isolates from ongoing outbreak sources are submitted to a designated laboratory where PFGE genotyping is routinely performed. When a cluster of identical genomic profiles of the isolates are determined by PFGE, the local health authorities are immediately notified and an investigation is conducted. The implementation of PFGE typing technology enables reliable tracking of epidemic clones and determines the extent of the outbreak.

CONCLUSION

Pulsed field gel electrophoresis is widely used for genotypic characterization. It has been considered the method of choice for the analysis of most bacterial pathogens because of its high reproducibility and discriminatory power. The limitations include its labor-intensive nature, delayed turn-around time to results, and the inability to genotype some microorganisms. For the latter, a combination of PFGE and amplification-based technique may provide the best discriminatory power. Efforts are underway to simplify and standardize the methodology for broad applicability and interlaboratory comparisons.

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Pyrosequencing

Dong-chuan Guo
Dianna M. Milewicz
University of Texas-Houston Medical School, Houston, Texas, U.S.A.

INTRODUCTION

Pyrosequencing is a high-throughput technique for single nucleotide polymorphism (SNP) genotyping and DNA sequencing that is inexpensive, accurate, and easy to optimize. The pyrosequencing technique uses a non-electrophoresis-based bioluminometric DNA sequencing method that employs a cascade of luciferase-based enzymatic system that monitors DNA synthesis in real time. In the pyrosequencing assay, a sequencing primer is hybridized to a single-stranded, PCR-amplified DNA template and is then incubated with enzymes and their substrates. During the DNA sequencing, one of the four deoxyribonucleotide triphosphates (dNTPs) is added to the reaction system in an order that is preprogrammed. If the nucleotide is complementary to the nucleotide in the template, DNA polymerase catalyzes the incorporation into the DNA strand. When an incorporation event occurs, it is accompanied by the release of an inorganic pyrophosphate (PPi) in an equimolar amount to the incorporated nucleotide. The inorganic pyrophosphate produced is then converted to ATP by sulfurylase and is immediately utilized in a subsequent luciferase-based light-producing reaction. The pyrosequencer detects the visible light generated by luciferase during DNA synthesis, which is proportional to the number of nucleotides incorporated. The DNA sequence is then determined by monitoring the visible light produced from the reaction as each dNTP is added. The unincorporated nucleotides are degraded by apyrase, which allows the iterative addition of nucleotides in the following steps.

$$(\mathrm{DNA})_n + \mathrm{dNTP} \xrightarrow{\text{DNA polymerase}} (\mathrm{DNA})_{n+1} + \mathrm{PPi}$$

$$\mathrm{PPi} + \text{adenosine } 5' \text{ phosphosulfate} \xrightarrow{\text{ATP sulfurylase}} \mathrm{ATP}$$

$$\mathrm{ATP} + \text{luciferin} \xrightarrow{\text{luciferase}} \mathrm{AMP} + \text{oxyluciferin} + \boldsymbol{hv}$$

$$\mathrm{ATP} + \mathrm{dNTP} \xrightarrow{\text{apyrase}} \mathrm{ADP} + \mathrm{dNDP} + \mathrm{dNMP} + \mathrm{Pi}$$

SNP GENOTYPING

Single nucleotide polymorphisms are alterations in the DNA sequence at a single nucleotide and can serve as genetic markers. These genetic markers have been used extensively for population genetics, medical genetics, pharmacogenomics, and forensic DNA analysis. The technologies for analyzing SNPs have focused on rapid and efficient detection of these alterations. Pyrosequencing is one of the newer technologies that have allowed for a high-throughput SNP genotyping technique that performs DNA sequencing in real time. This methodology also creates an internal control for monitoring the specificity of SNP assays by reading a few base pairs of DNA sequence flanking the SNP.

Pyrosequencing begins by purifying PCR-generated single-stranded DNA as a sequencing template followed by annealing with a sequencing primer to perform real-time DNA sequencing. A biotin-labeled DNA fragment is generated by PCR amplification using a biotin-labeled PCR primer. The biotin-labeled strand is immobilized to streptavidin-coated magnetic beads to isolate the single-stranded DNA after denaturation of the double-stranded PCR product. The pyrosequencing primers are designed using the SNP Primer Design Software (http://www.biotage.com). The real-time sequencing reactions are performed and monitored on a Pyrosequencer^{TM} with the SNP Reagent Kit (Biotage AB and Biosystems, Sweden). A methodology of universal biotinylated primers has recently been developed to generate biotin-labeled sequencing templates, which tremendously reduces the cost of synthesizing biotin-labeled-specific PCR primers.[1]

Figure 1 depicts an example of SNP genotyping using the pyrosequencing assay. The C-to-G SNP and the flanking DNA sequence are C/G-A-A-C-G. Figure 1A shows the pyrosequencing result of the homozygous C/C genotype. The first position (T) and the position following SNP (the second T) are automatically assigned by the pyrosequencing as blank controls. The full peak height of the first C position followed by no signal in the G position indicates that it is a homozygous C/C genotype. The third peak in the A position indicates that the adenines follow the C/G SNP and the height of this peak indicates that there are two adenines in this position because the light signal is proportional to the number of nucleotides incorporated. The fourth and fifth peaks indicate that a cytosine and a guanine follow the adenines. Similarly, the half height of the peak in the first C and second G position in Fig. 1B and no signal in the first C and full height of

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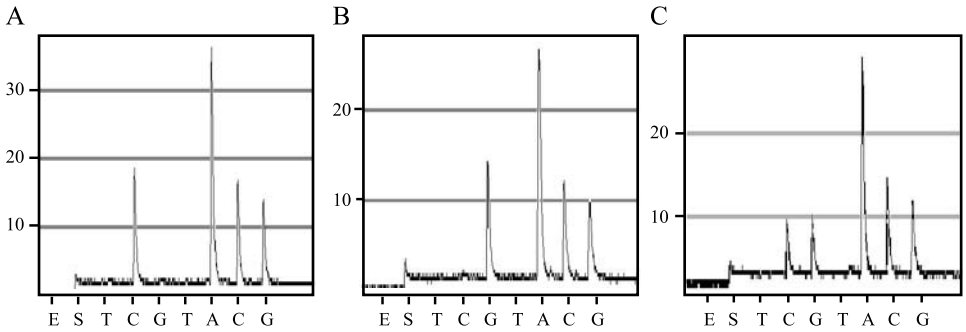

Fig. 1 Results of the pyrosequencing SNP genotyping. The C-to-G SNP and the flanking sequence are C/G-A-A-C-G. The SNP genotyping results show different peak patterns among DNA homozygous C (A), a homozygous G (B), and a homozygous C/G (C).

peak in the second G position in Fig. 1C indicate that they are the heterozygous C/G genotype and homozygous G/G genotype, respectively.

High-throughput detection of small genomic insertions and deletions can be achieved by the pyrosequencing SNP assay program. Small insertions and deletions of nucleotides are common causes of polymorphic variations and can result in a predisposition to particular diseases. Although conventional DNA sequencing techniques can analyze these variations, it is costly and time consuming. The pyrosequencing technique can detect these variations without sequencing an entire region of chromosome DNA. The most critical factor for detecting these variants is to preprogram the appropriate sequences for the pyrosequencing assay. The order of nucleotide preprogrammed should depend on the normal allele and the boundaries of the deleted nucleotide sequences, which maximizes the differentiated patterns of the pyrosequencing results among the different genotypes. It has been demonstrated that this technique can analyze up to 100 bp nucleotide insertions and deletions along with complicated genomic rearrangements.[2] The pyrosequencing SNP assay program greatly reduce the timing and cost for detecting small insertion and deletion of nucleotides while increasing the throughput for the assay.

To further reduce the cost and time and increase the throughput of this analysis, the pyrosequencing assays can be multiplexed. This can be performed on a single template which contains multiple SNPs or on multiple templates.[3,4] To ensure the accuracy of SNP genotyping, the assays require carefully designing sequencing primers and preprogramming the sequences. Interpretation of peak intensities and proper base assignment may be difficult when multiplexing assays on multiple templates because PCR amplification efficiencies may be different for different amplicons. Multiplexing pyrosequencing can yield better results when the multiple SNPs exist within a few hundred base pairs in the genome.[3,4]

Multiplexed pyrosequencing assays on a single template have been applied to SNP haplotyping. Traditionally, construction of SNP haplotypes is performed by computational analysis or cloning techniques. Pyrosequencing SNP haplotyping is based on the combination of allele-specific PCR with pyrosequencing SNP assays.[5] Utilizing an existing SNP, allele-specific PCR primers are designed to specifically amplify genomic DNA flanking each allele of the SNP. The allele-specific PCR products serve as templates for the pyrosequencing assays to detect other SNPs located within the PCR fragments. This method has been proven to be highly reliable for detecting SNP haplotypes. However, the pyrosequencing-based SNP haplotyping can only detect the haplotypes of SNPs located within a few kilobase pairs on a chromosome because amplification of large PCR fragments tends to reduce the specificity of allele-specific PCR and increase the background noise of the pyrosequencing assay. To reduce the background noise of the assay, linked SNPs can be amplified using allele-specific PCR followed by a nested PCR of each associated SNPs for pyrosequencing assays.[6]

DNA SEQUENCING

Pyrosequencing can be used as an alternative to the traditional Sanger dideoxy sequencing method. The sequencing assay is performed by a reiteration of steps in which one of the four nucleotides is added and tested sequentially for incorporation into the nascent nucleotide chain. When the nucleotide added is complementary to the DNA template, it incorporates into the DNA strand and a light signal is generated. The intensity of the light signal reflects the number of incorporated nucleotides in a single nucleotide repeat region. Compared to the Sanger sequencing method, the pyrosequencing technique is

limited to read up to 100 nucleotides.[7] However, the pyrosequencing technique has several unique advantages over Sanger sequencing. For example, the results of heterozygous genotypes or multiplexing assays by Sanger dideoxy sequencing show overlapping signal peaks in a single position, which may result in sequence misreading. Conversely, pyrosequencing obtains sequencing data from each allele independently, which allows it to simultaneously sequence multiplex loci of a gene family.[8] Additionally, pyrosequencing is able to capture the first base following the sequencing primer.

Pyrosequencing DNA sequencing is being used for a wide variety of disciplines.[9,10] Pyrosequencing has been employed to obtain 50–100-bp sequence data in the variable region of the 16S rRNA using PCR primers based on the conservative region for amplification. This sequencing assay allows for the rapid identification of bacteria.[11] Similarly, the pyrosequencing technique has been applied for yeast and virus typing, and mutation testing.[12,13]

The pyrosequencing assay is also used for human leukocyte antigen (HLA) genotyping, which is essential for matching the HLAs of a recipient and an unrelated donor to decrease graft rejection and transplant-related mortality. The genetic complexity of HLA genes results in extreme difficulty in genotyping the donor and recipient HLA haplotypes by traditional methods. Sequence-based genotyping is necessary because it provides critical information for HLA matches considering that histocompatibility between recipient and donor is inversely correlated with the number of mismatches. The high-throughput pyrosequencing technique can be used to obtain high-resolution sequencing information of HLA genotyping that overcomes these problems.[8,14]

ALLELE QUANTITATION

Pyrosequencing is a quantitative DNA sequencing technique because the intensity of a light signal is accurately correlated to the amount of nucleotide incorporated. This fact enables pyrosequencing to be applied to quantifying allele frequencies of SNPs,[15] estimating global DNA methylation,[16] and detecting variations in the copy numbers of duplicated genes.[17] The pyrosequencing SNP software automatically analyzes SNP genotyping data and quantifies the allele frequencies of SNPs.

Linkage disequilibria of SNPs have been employed to identify susceptibility genes for complex diseases. Although high-throughput techniques have been applied for accuracy and large-scale SNP genotyping, using individual genotyping is expensive and time-consuming for assays with large sample sizes and that also scan for numerous alleles. Directly assaying SNP allele frequencies in pooled DNA samples provides the advantages of higher throughput and lower cost. An accurate and reproducible assay is critical for determining SNP frequencies using DNA pools. The ability to quantify the amount of incorporated nucleotide enables pyrosequencing to accurately estimate the SNP allele frequencies in DNA pools. The correlation coefficients between estimated SNP allele frequencies in DNA pools and that of true allele frequencies were demonstrated to be between 0.979 and 0.996.[15,18] Scanning SNP allele frequencies in DNA pools allows the rapid estimation of the linkage disequilibria of SNPs in susceptibility genes in the genetic study of complex diseases.

DNA methylation is an epigenetic modification of DNA that plays an important role in controlling gene expression, regulating X-chromosome inactivation, DNA repair, and tumorigenesis.[19,20] In mammals, DNA methylation occurs at the 5-position of the pyrimidine ring of cytosine in the context of dinucleotide CpG. Quantifying global DNA methylation or DNA methylation in a specific locus is important for understanding the regulation of gene expression and inactivation of genes throughout the genome. The pyrosequencing technique has been demonstrated to be an accurate and high-throughput method for quantitating DNA methylation.[16,20] This method is based on bisulfite treatment of genomic DNA, which converts unmethylated cytosines to uracils, such that the ratio of methylated cytosines to unmethylated ones can be quantified as C/T SNP using the pyrosequencing allele frequency analysis program.

TECHNICAL CHALLENGES

A technical challenge for pyrosequencing is the limited annealing specificity for the sequencing primers to the templates as the pyrosequencing assay is performed at 28°C because of the low thermostability of firefly luciferase. Measures have been applied to minimize the background noise due to the nonspecific binding of the sequencing primers and the formation of 3′-end loops. Glycine betaine has been used to stabilize the luciferase so that the pyrosequencing assay can be performed at 37°C.[21] Single-stranded DNA-binding protein has also been tested to enhance specific hybridization between sequencing primers and templates, therefore reducing background noise and increasing the accuracy and intensity of the assays.[22] Increasing the annealing specificity would allow the pyrosequencing assay to be directly performed on double-stranded DNA and longer templates, therefore reducing the cost and time of assay.

Another disadvantage of pyrosequencing is the cost and time associated with purifying single-stranded DNA templates. A methodology has been developed to reduce the cost of generating biotin-labeled pyrosequencing templates.[5] This methodology uses a universal

biotinylated primer and two sequence-specific primers. One of the sequence-specific primers contains a 5′ tail that is complementary to the universal primer. During PCR amplification, a sequence-specific primer pair initiates PCR amplification of the genomic DNA. The resulting amplified fragments serve as templates for the universal biotinylated primer and a sequence-specific primer in subsequent amplifications, thus producing labeled amplicons. The cost savings result from ordering large amounts of the biotin-labeled universal primer rather than the biotin-labeled sequence-specific primers.

CONCLUSION

Pyrosequencing is a new DNA sequencing methodology and has been applied to SNP genotyping, DNA sequencing, and allele-specific DNA quantitation. This technique has the potential advantages of high-throughput, accuracy, flexibility, and can easily be automated. Future improvements in both chemical and instrumentation of pyrosequencing will reduce cost and improve specificity of primer hybridization, and will allow its widespread use in genomic and genetic studies. Developing a microarray-based pyrosequencing technique will enable performing genomewide SNP genotyping and DNA sequencing.

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Quality of In-House Made High-Density Microarrays

Paul van den IJssel
Paul Eijk
Erik Hopmans
Soemini Kasanmoentalib
Bauke Ylstra
Vrije Universiteit Medical Centre, Amsterdam, The Netherlands

INTRODUCTION

A modern-day technique has enabled tens of thousands of genes to be printed on a single microscope slide—high-density microarrays. The development of these arrays has boosted genomics research, exploiting the huge amount of data made available by the human genome project because all known genes could now be included. Complete RNA expression profiles can be acquired from tissue samples as small as those obtained by laser dissection microscopy using RNA amplification. Expression profiles can be clustered and related to disease status. From these clusters, a molecular profile or ''signature'' of the disease can be extracted. Thus research can take full advantage of developments in microarray technique to find diagnostic and prognostic profiles for various diseases. A major challenge is to implement these signatures as diagnostic or prognostic tools in the clinic.

Despite the availability of a number of commercial platforms, many institutes have raised core facilities that provide them with homemade microarrays to minimize cost and to maximize flexibility. Because all these facilities use their own combination of probes, slides, printing and scanning equipment, hybridization conditions, and sample preparation, this gives rise to a huge variability in quality. Because regulatory federal and state agencies will allow only excellent quality microarrays to be used for diagnostics, many of today's platforms will not pass.

Many statistical ''tricks'' are being applied to correct for artifacts in the results of microarray experiments. In this review, we will indicate ways to solve these artifacts by techniques rather than by statistics. We will pinpoint essential steps to raise the quality of microarrays produced in core facilities to the perfection required for diagnostics or prognostics.

MICROARRAYS FOR DIAGNOSTIC PURPOSES

The development of high-density microarrays offers an enormous potential for diagnostics. Where standard pathological and molecular techniques cannot distinguish between tumor subclasses, molecular profiles are now able to predict clinical behavior.[1–3] The Dutch Cancer Institute has developed a signature set of 70 genes that can predict the 5-year survival chance of breast cancer patients and is planning to use this information to decide whether or not to start chemotherapy treatment.[2] A set of 37 genes can discriminate between two lymphoma types of diffused large B-cell lymphomas.[4] Furthermore, the clinical outcome of children with medulloblastomas is highly predictable based on the gene expression profiles of their tumors at diagnosis.[5]

It goes without saying that the quality of microarrays used in diagnostics should be nothing less than excellent. Regulatory federal and state agencies require absolute confidence about all key issues in a microarray experiment, such as gene identity, sample preparation, hybridization procedures, and array analysis. Tests used for diagnosis must also be highly standardized, reproducible, and reliable. Here we present some ideas to raise the quality of microarrays provided by in-house facilities to a level essential for diagnostic tests.

FROM ARRAY TO HIGH DENSITY: LET ROBOTS DO THE WORK

Low-density arrays in the past used to be produced by simple methods such as slot or spot blotting on filters used with radioactively labeled probes. Nowadays, robotics has taken over, spotting tens of thousands of genes on a single microscope slide with high accuracy. The first robotic arrayers used solid titanium pins that spotted relatively slow and with medium density (spot–spot distance of 300 μM or more). Currently, most people use quill pins, which provide fast printing with high density (spot–spot distance of 200 μM or less). Nevertheless, quill pins have a number of disadvantages that prevent the microarray technique from developing to perfection.[6] As the pins spot the DNA solution, they touch the surface of the slide, which may damage the pins and the slide surface coating where the DNA probe is to be immobilized. As most

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arrays will be printed using more than one pin, there will be slight differences in spot morphology caused by pin-to-pin variation. Moreover, the number of slides that can be printed with one dip of a pin in the DNA solution is limited and dependent on the nature of the spotting buffer. Pins may fail during a run by picking up a dust particle or obstruction in the print head causing the whole—or part of the—subarray to drop out. Finally, it appears that thorough washing of the pins between dips is not invariably perfect, causing small amounts of carryover from one spot into the next one.[6]

It is important to notice that when an experimental set of arrays is analyzed, the variation in expression of a single gene across all arrays is analyzed, but not the variation of expression within an array. This implies that localization, pin-to-pin variation, or dye effects do not necessarily need to be corrected for as long as they are reproducible across an experimental set of arrays.

Most of these disadvantages have been overcome by noncontact printers, which are in development. Such printers use ''ink jet'' technology to deposit microdrops on a slide without touching it. Its tips can aspirate enough DNA solution to print thousands of spots, so all arrays within one print run will have identical quality. As the tips do not touch the glass surface, pin-to-pin variation is mostly eliminated and spot morphology is very even. In addition, ink jet technology allows detection of malfunctioning tips using a camera. Afterward, missing spots can be inserted, resulting in 100% complete arrays (Fig. 1).

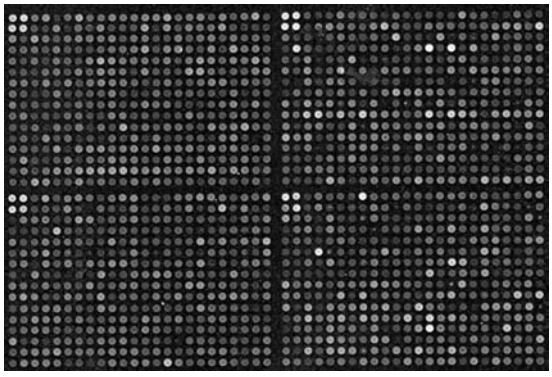

Fig. 1 Part of a microarray printed using the SpotArray™ Enterprise piezoelectric noncontact printer (PerkinElmer) showing complete arrays with very even spot morphology. The array has been constructed using the rat 5 K oligolibrary from Sigma/Compugen (www.cgen.com) on CodeLink slides. (From Ref. [7].) Target labeling, automated hybridization (Hybstation12; Perkin Elmer), and scanning were performed according to the protocols described on our website (www.vumc.nl/microarrays). Samples were from rat brain cerebellum (Cy3) and rat brain cortex (Cy5). (*View this art in color at www.dekker.com.*)

Finally, noncontact printing provides much more flexibility in designing arrays. It is much easier, for instance, to create replicates by set, rather than consecutively on the array, which introduces more statistical power to the array result as localization effects can be ruled out. In addition, the possibility to print a series of small arrays on one slide aids in the cost-effectiveness needed for diagnostic tests.

MICROARRAYS BY DESIGN: FROM cDNA TO OLIGONUCLEOTIDES

High-density microarrays were developed using spotted cDNA. At present, cDNA microarrays are still a major source for the production of valuable data, and they have the advantage that sequence analysis of the clones is not essential.[1] If one would like to perform an array on some exotic creature, a cDNA library can be printed and significant clones can be sequenced afterward. However, cDNA microarrays have a number of disadvantages that prevent the development of high-density microarrays to perfection. First and most important, cDNA clones used for microarrays are not all verified by sequence analysis, resulting in up to 25% of clones not representing what they should.[8] Such uncertainties are unacceptable for diagnostic microarrays. Second, cDNA clones have different lengths and basepair compositions resulting in different melting temperatures. Therefore it is impossible to achieve optimal hybridization conditions for all clones during a single hybridization experiment. Third, because most cDNA clones will have products of several hundreds of basepairs long, there is a major risk of cross-hybridization.

The best answer to these problems has proven to be the use of oligonucleotides to replace cDNA clones.[6,9,10] Oligonucleotides 50–70 nucleotides long have the advantage of being synthetically synthesized, thus they can be designed and customized for optimal performance (Compugen; www.cgen.com). They do not need to be sequence-verified and can be designed to minimize cross-hybridization and at similar melting temperatures. Furthermore, they can be designed to avoid known single nucleotide polymorphisms. Nowadays, most, if not all, of the commercial companies that offer high-density microarrays have platforms based on oligonucleotide technology.

SMALL SAMPLES: RNA AMPLIFICATION OR NOT?

The use of microarrays in diagnostic research requires that small samples give high-quality reproducible results. The size of a tumor sample or fine-needle biopsy can be limited, and researchers wish to perform microarray

analysis on microdissected material. One way to deal with this is to amplify the RNA of the sample, either using a homemade RNA amplification method[11] or one of the commercially available kits (i.e., www.agilent.com). The most challenging, yet difficult, part of amplifying an RNA sample is to do it linearly—meaning that the relative amount of all different RNA in the amplified sample is the same as in the original sample. Although many RNA amplification kits claim to perform linear amplification, in reality, when an unamplified sample is compared with its amplified RNA for many genes, a ratio other than 1 is obtained. As long as the nonlinearity is reproducible among all amplified samples that are compared in an array experiment, such an experiment will produce valid results. However, one should realize that all manipulations performed on the RNA samples will introduce extra noise in the results due to the limited stability of the RNA molecule. Moreover, the oligonucleotide libraries offered by various companies have been designed using the sense coding strand of the RNA. Using amplified RNA on these oligonucleotides enforces hybridization with unstable RNA, instead of cDNA. Although methods have been published to amplify even degraded RNA,[12] we would argue to avoid RNA amplification, if at all possible.

Instead, another approach used to deal with small size samples could be optimizing the yield of the cDNA labeling reaction and lowering the threshold for the amount of input RNA needed to achieve reasonable results in a microarray experiment. By optimizing column purification steps and hybridization conditions, we have scaled down the amount of input RNA by more than 10-fold—to as low as 4 μg of total RNA with very limited loss of signal intensity and reproducibility of the data. Whereas many researchers are using cover slips for their hybridization or hand-made hybridization chambers, automated hybridization, which enables moving targets in low volumes and very efficient washing, has been developed. All these measures increase the sensitivity of

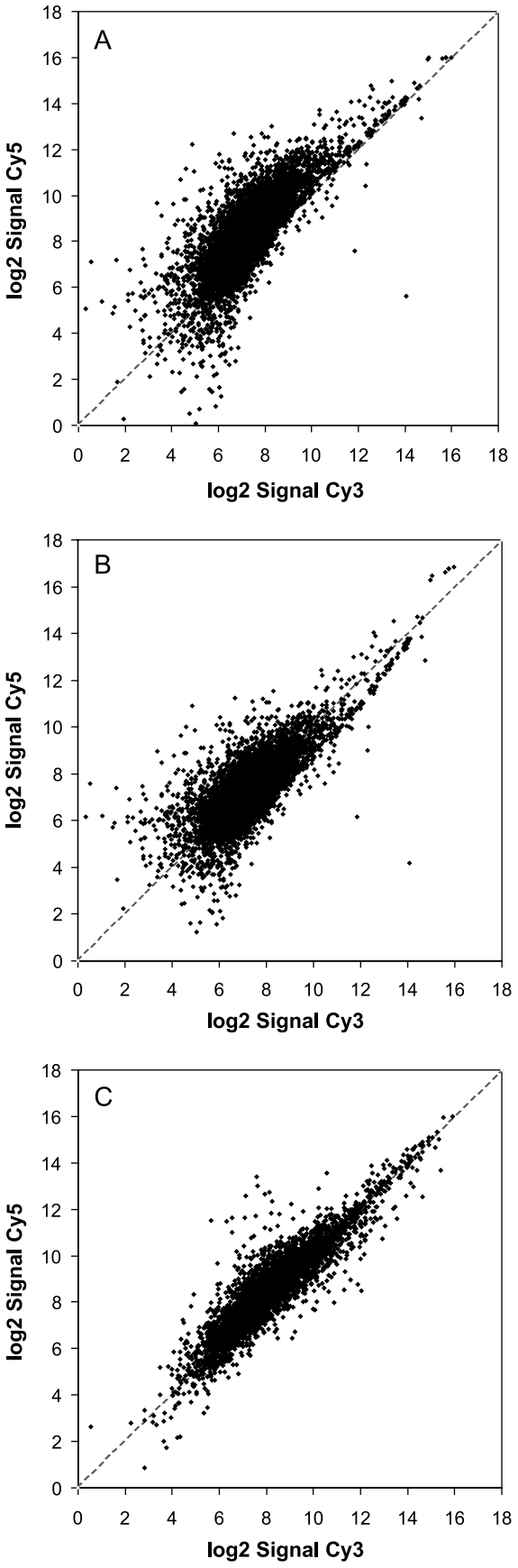

Fig. 2 Channel balancing omits the need for Lowess correction. Scatter plots showing the results of two microarray experiments using a human tumor cell line (Cy3 channel) and Human Universal Reference RNA (Stratagene) (Cy5 channel). The first experiment was performed without channel balancing (A) and results were corrected by Lowess smoothing (B). (From Ref. [15].) The second experiment was performed with channel balancing (C) showing that Lowess correction is not necessary. Arrays were performed by manual hybridization on 19 K human oligo arrays (Sigma/Compugen) as described in the legend of Fig. 1. (From Ref. [16].) Spots were measured using ImaGene software (BioDiscovery, USA). All plots have been constructed using signal mean values and all spots including flagged ones are included on the plots. (*View this art in color at www.dekker.com.*)

microarray experiments, thus omitting the need for RNA amplification. Currently, amplification is necessary for RNA samples smaller than 4 μg.

Another way of dealing with a limited size of samples is downscaling the size of the microarrays. Subsequent to the initial diagnostic research that has been performed on large, whole genome arrays, often a ''signature'' set of a limited number of genes that is specific for predicting a disease condition can be identified.[1,2,4] So if the size of the array can be downscaled from thousands to several hundred of genes, an enormous reduction of sample size can be obtained. In addition, there is technical room to decrease spot size by optimizing spotting solution in combination with the slide surface, or by other printing methods using capillary tips as currently being developed at the UCSF Cancer Center (cc.ucsf.edu). As a welcome advantage, this would also reduce the cost of a microarray experiment, thus rendering microarrays suitable for diagnostic purposes. The breast cancer study by Van't Veer et al.[2] has now led to the foundation of the first company that offers commercial microarray-based testing for diagnostics (Ref. [13]; www.agendia.com).

Investigations in which the RNA quality or quantity is not sufficient for expression profiling may be approached by microarray comparative genome hybridization (CGH)[3,14] because the more stable DNA is used as target instead of RNA.

FROM HIGH QUALITY TO PERFECTION

Many researchers perform all kinds of statistical ''tricks,'' such as Lowess correction,[15] to obtain valid data. It is our opinion that by using such tricks, valuable information is lost and that one should strive to solve array artifacts by the technique, rather than by statistics. As an example of this, Fig. 2 shows that ''channel balancing,'' a well-estimated chemical balance between the signal strength in both channels on the array, omits the need for high normalization factors and appears to have an enormous impact on data quality. Such a ''technical'' normalization can be achieved by measuring the amount of label incorporation in both channels and adjusting that for the hybridization. Rather than a conventional spectrophotometer, a Nanodrop (www.nanodrop.com) is an excellent piece of equipment for such measurements because it is highly reproducible and needs only tiny amounts of materials. A scatter plot of an array performed without channel balancing shows some sort of ''banana'' shape (Fig. 2A) that could be corrected by Lowess smoothing (Fig. 2B). An array performed with channel balancing (Fig. 2C) does not need such correction.

A second example is the improvement of hybridization conditions by going from a hand-made hybridization chamber[16] to an automated hybridization station (Hybstation12; PerkinElmer, USA). It is our experience that this alleviates the need for localization corrections such as border effects seen in the results of manual hybridizations.

To obtain the best hybridization results, it is essential to estimate hybridization stringency (ΔT). This is measured by the difference between the hybridization temperature (T_{exp}) and the melting temperature (T_m): $\Delta T = T_m - T_{exp}$. According to the equation by which T_m can be calculated, the four variables that affect melting temperature are: sodium concentration, formamide concentration, oligonucleotide length, and GC content.[17] If ΔT is too large, the chance of cross-hybridization and background signal is increased. If ΔT is too small, when the hybridization temperature approaches the melting temperature, too much signal is lost. Thus using the right ΔT during hybridization and washing can enhance reliability. For our homemade oligo arrays, we use a ΔT of 15°C. Compare it with a ΔT of approximately 35–40°C used in Southern blotting to detect gene families and 15–20°C to detect single genes.[17] Thus a ΔT of 15°C should completely abolish cross-hybridization as long as the design of the oligonucleotides is correct. The design of the oligonucleotides will keep pace with the quality of sequence data in the human genome databases. One should realize that things such as time, area, and nature of the surface (i.e., glass or nitrocellulose) are not in the ''T_m equation'' and thus will not affect hybridization stringency.

Finally, RNA quality is of crucial importance to the outcome of an array experiment. An RNA sample that has an excellent quality as judged by the ratio of the 28S and 18S ribosomal RNA bands and, an A_{260}/A_{280} ratio of 2.0 at a pH 7 usually guarantees good-quality microarray data, provided no phenol traces are present in the RNA samples, which severely affect enzymatic reactions that follow.

DATA ANALYSIS: HOW WAS THE EXPERIMENT SET UP?

Maybe the most underestimated consequence of high-density microarrays is the fact that every single experiment will generate thousands of results.[6] So the question is how to approach the analysis of series of tens or hundreds of microarray data. Although a huge number of both free and commercial software packages are available, this is no guarantee that a researcher will select the right analysis decisions. For example, clustering will always result in a number of clusters, but there is no assurance that these have a significant biological meaning. In some cases, unsupervised clustering using just the raw data is the best choice for analysis. In other cases (i.e., when the number of variables is small and the experiment has been

well set up), supervised clustering could lead to the desired results.

The major determinant in the analysis strategy is the experimental design of the study. If the experiment has been set up well and the quality of the RNA and arrays is optimal, then the analysis is straightforward. Often, researchers only find out how the experiment should have been set up when they run into difficulties while analyzing their results.

CONCLUSION

The development of high-density microarrays is having a great impact on diagnostic research. An increasing number of investigations are being published, showing that microarrays can classify tumor types based on specific ''signature'' sets that can be used as diagnostic or prognostic tools. The time has now arrived to let patients benefit from these tests. We believe that in-house facilities can raise the quality of their microarrays to diagnostic standards by implementing the latest technological developments and optimizing their protocols. Researchers will benefit from this by getting more reliable and reproducible data out of their microarray experiments.

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Ramification Amplification

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David Y. Zhang
Josephine Wu
Wandi Zhang
Fei Ye
Mount Sinai School of Medicine, New York, New York, U.S.A.

INTRODUCTION

Ramification amplification (RAM), also termed hyperbranched rolling circle amplification or cascade rolling circle amplification, utilizes a closed circular probe (C-probe) or padlock probe and a DNA polymerase with a high processivity. The C-probe can be exponentially amplified under isothermal conditions, eliminating the need for a thermal cycler. This type of amplification offers superb detection sensitivity for target DNA, RNA, or proteins.

OVERVIEW

Technical Description

Ramification amplification (RAM),[1,2] also termed hyperbranched rolling circle amplification[3] or cascade rolling circle amplification,[4] utilizes a closed C-probe or padlock probe and a DNA polymerase under isothermal conditions. The choice of DNA polymerase is based on the following requirements: a good strand displacement activity in order to separate downstream DNA from the template strand and a high processivity (i.e., incorporating nucleotides continuously on a given primer without dissociating from the template), allowing the polymerase to synthesize several thousand nucleotide-long ssDNA from the ligated C-probe. In this type of amplification, two primers, one complementary to the C-probe (forward) and the other identical in sequence to a second binding site in the C-probe (reverse), are added to the reaction. An initial rolling circle primer extension process is initiated and a single-stranded DNA (ssDNA) molecule is generated (rolling circle amplification). As the ssDNA molecule grows, multiple reverse primers are able to bind to the ssDNA and initiate a second ''round'' of primer extension templated by the initial ''rolling circle'' products. Once an upstream primer-extension strand ''bumps into'' a bound downstream primer, the polymerase displaces the downstream bound primer along with any extended sequence that is attached to it. The displaced dsDNAs serve as templates for further primer extension and amplification (Fig. 1). Like the constant unfurling of streamers, multiple primer extensions are simultaneously in progress, resulting in a large ramified complex. The reaction end-products are multimeric dsDNAs of various lengths, including smaller units such as monomers, dimers, trimers, and so on. Because the displaced DNAs are single-stranded, primers can bind to them at a constant temperature, eliminating the need for thermal cycling to generate ssDNA (as in the case of polymerase chain reaction, PCR).

The power of the exponential amplification of RAM can be expressed using the following formula: $x(2^U)$ where U is the number of repeats generated from the initial closed C-probe and where x is the number of the primer pair used.[1] For example, if one primer pair is used (one forward and one reverse), the formula is 2^U, indicating that RAM is an exponential amplification. Compared to PCR that can be expressed as 2^N, where N is the number of temperature cycles, RAM has an equivalent power of amplification. However, adding additional primer pairs can further enhance amplification. With three primer pairs (three forward and three reverse), the formula is $3(2^U)$, indicating that RAM can result in a far greater magnitude of amplification.[5] When using the RAM assay, the limiting factor is the length of the initial ssDNA generated from the closed C-probe; at least 20 repeats of ssDNA from the closed C-probe are required to achieve 1 million-fold amplification.

The two most important factors that affect amplification rate are stability of the DNA–polymerase complex and accessibility of the primer to its binding sites as soon as they are available. It is possible that formation of the large ramifying DNA–polymerase complex may interfere with the interaction of primers to their binding sites and disrupt the DNA–polymerase complex. Therefore the organic solvent dimethyl sulfoxide (DMSO, which stabilizes the DNA polymerase)[6] and a protein co-factor T4 gene 32 protein (which stabilizes single-stranded regions of DNA)[7] can be added to enhance primer binding to the growing ssDNA and stabilize the DNA–polymerase complex. The addition of these agents

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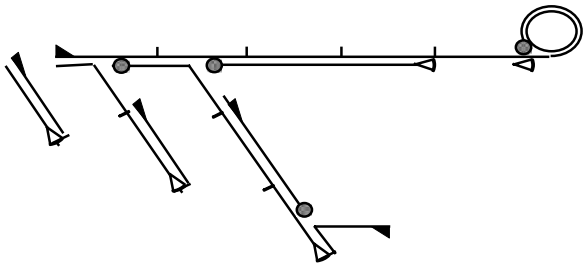

Fig. 1 Schematic representation of RAM. A forward primer (▶) bound to a C-probe is extended by a DNA polymerase (●), generating a long ssDNA. Multiple reverse primers (◁) bind to the nascent ssDNA as their binding sites become available. Each bound reverse primer extends and displaces the downstream primers and their extended products. The forward primer binding sites of the displaced ssDNA are then available for the forward primers to bind and extend similarly, thus forming a large ramifying DNA complex (RAM).

significantly stimulates DNA synthesis of many DNA polymerases such as 29 DNA polymerase[5] and Bst DNA polymerase.[2] Other components that can also stimulate DNA synthesis of DNA polymerases in RAM include Triton X-100 and 5% tetramethyl ammonium oxalate.[8]

The RAM assay offers many advantages over other amplification techniques. Primers can readily bind to ssDNAs displaced by DNA polymerase, enabling the reaction to be carried out under isothermal conditions, obviating the need for a thermal cycler. A multiplex assay can be designed using generic primers to amplify different probes with equal efficiency, resulting in a better multiplex capability than conventional PCR.[1,9] The RAM assay offers a uniform platform for both RNA and DNA detection with the ability to ligate both ends of the padlock probe regardless of the nature of target (DNA or RNA) eliminating the need for reverse transcription for detecting RNA.[9] Finally, ligation requires that both probe termini perfectly match in order

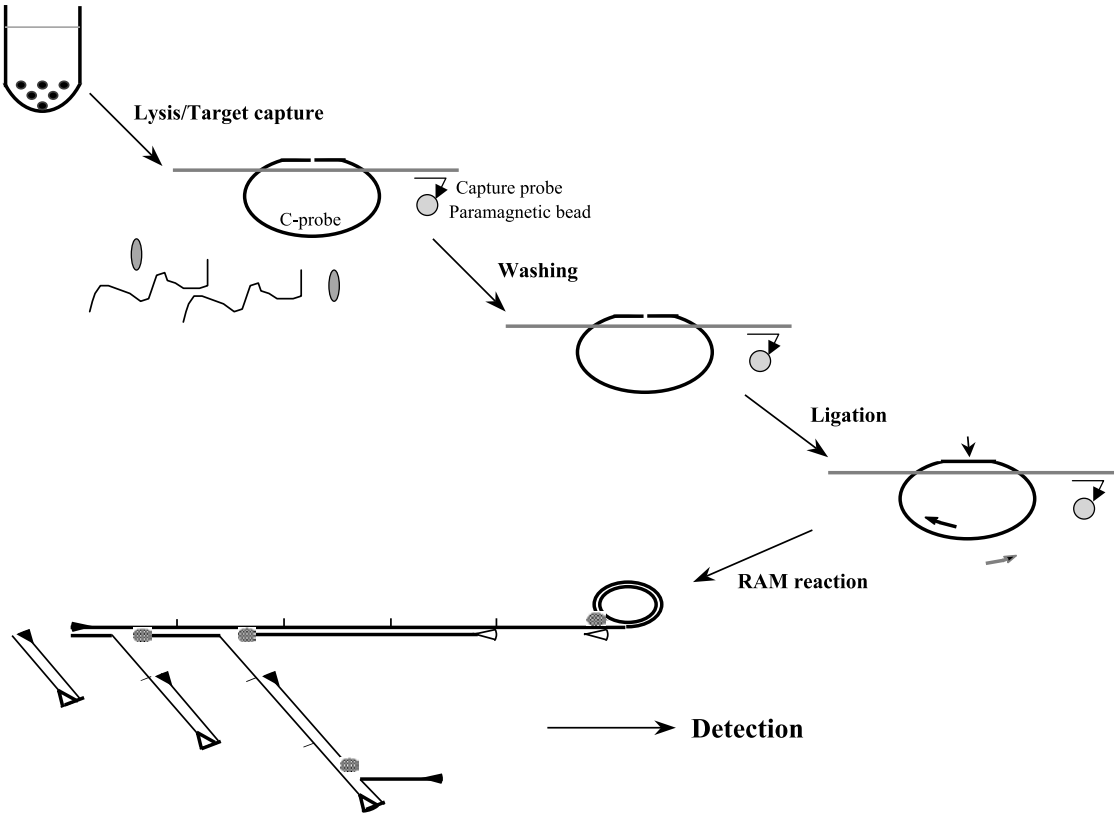

Fig. 2 Ramification amplification assay procedure. A clinical sample is lysed in a lysis buffer in the presence of capture probe, C-probe, and paramagnetic bead, and target DNA together with the bound C-probe are captured on a paramagnetic bead, allowing extensive washing to remove unbound C-probe and other components in the sample. The C-probe aligned on the target is linked together by a DNA ligase. RAM amplification is then carried out by the addition of forward and reverse primers and DNA polymerase. (*View this art in color at www.dekker.com.*)

for hybridization to occur, permitting the detection of a single nucleotide polymorphism.

Application

The practical uses of RAM assay to detect target nucleic acids in clinical samples have been demonstrated in several studies (Fig. 2). Zhang et al. have applied the RAM assay to detect *Chlamydia trachomatis*, a leading cause of sexually transmitted disease (STD) in the United States, in cervical specimens collected in PreservCyt cytological solution.[10] They demonstrated the RAM assay can detect as few as 10 *C. trachomatis* elementary bodies in less than 2 hr, comparable with those of Amplicor PCR and ligase chain reaction (LCR), and they further tested 30 clinical specimens and detected all positive samples confirmed by PCR and LCR. The RAM assay can be an alternative to PCR and LCR to detect sexually transmitted agents because of its simplicity and isothermal amplification nature. Furthermore, it is possible to screen simultaneously cervical intraepithelial lesions and to detect STD agents in a single collection vial. Zhang et al. have also successfully applied the RAM assay for detection of Epstein–Barr virus in human lymphoma specimens.[2] They demonstrated the feasibility of RAM to detect a rare DNA target in clinical specimens and achieved an analytical sensitivity of as low as 10 molecules, comparable to that of PCR.

Fluorescent-based RAM assays have been developed to monitor and quantify the RAM products in a real-time fashion in a homogeneous reaction. Zhang et al. have demonstrated that RAM products can be monitored in the presence of SYBR green, and SYBR green can be incorporated into RAM reaction for the real-time detection of RAM products. The major limitations of intercalating dyes are that it can only detect one target per reaction, and that the signal can also be generated from nonspecific polymerization products. Fluorescent molecular beacons can also be used for real-time RAM assay.[8,11] Amplifluor is used as a primer and the reverse sequence displaces the stem structure, resulting in the release of fluorescence.

The RAM mechanism has been applied to amplify large, circular DNA such as the M13 phage (30,000 bp) or plasmid DNA from single colonies or plaques.[8] Dean et al.[12] showed that using random primers and 29 DNA polymerase, circular DNA templates can be amplified up to 10,000-fold in a few hours. This procedure removes the need for lengthy growth periods for phage and plasmid as well as traditional DNA isolation methods. The amplified products can be used directly for DNA sequencing, in vitro cloning, library construction, and other molecular biology applications.

The RAM mechanism can also be used to amplify linear DNA, such as genomic DNA. Dean et al.[12] recently demonstrated that whole genomic DNA can be amplified with 3′ thiophosphate-modified random hexamer and 29 DNA polymerase. About 20–30 μg DNA can be generated from as few as 10 copies of genomic DNA in about 6 hr of incubation at 30°C; the average product length was >10 kb. The products can be used directly for sequence analysis, SNP detection, comparative genome hybridization, or loss of heterozygosity analysis.

CONCLUSION

In summary, RAM represents a novel, isothermal, exponential amplification method of nucleic acid and protein based on primer extension, displacement, and ramification. The inherent simplicity will allow RAM to be used in a wide variety of clinical and research applications.

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Real-Time PCR

Stephen A. Bustin
University of London, London, U.K.

INTRODUCTION

The real-time polymerase chain reaction (PCR) uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. This combines the DNA amplification and detection steps into one homogeneous assay and obviates the need for gel electrophoresis to detect amplification products. Appropriate data analysis and/or use of apposite chemistries also eliminates the need for Southern blotting or DNA sequencing for amplicon identification. Its simplicity, specificity, and sensitivity, together with its potential for high throughput and the ongoing introduction of new chemistries, more reliable instrumentation, and improved protocols, has made real-time PCR the benchmark technology for the detection of DNA.

THE ASSAY

The concepts underlying fluorescence-based real-time PCR are straightforward and are described in detail in the accompanying review on real-time reverse transcription-PCR. Real-time PCR technology is based on the detection of a fluorescent signal produced proportionally during the amplification of a DNA target (Fig. 1). Rather than having to look at the amount of DNA target accumulated after a fixed number of cycles, real-time assays determine the point in time during cycling when amplification of a PCR product is first detected. This is determined by identifying the cycle number at which the reporter dye emission intensity rises above background noise. That cycle number is referred to as the threshold cycle (C_t). The C_t is determined at the exponential phase of the PCR reaction and is inversely proportional to the copy number of the target. Therefore the higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed, and the lower the C_t. Real-time PCR assays are highly reproducible (Fig. 2A) and can easily discriminate between twofold differences in target numbers (Fig. 2B).

At its simplest, real-time PCR can be used as a qualitative assay. However, as fluorescence output is linear to sample concentration over a very broad range, this linear correlation between PCR product and fluorescence intensity can be used to calculate the amount of template present at the beginning of the reaction.

There has been an explosion of protocols, instruments, and chemistries, which on the one hand is evidence for the popularity and ubiquity of the assay, but also highlights the need to be aware of problems associated with the use of nonstandardized assays for diagnostic assays.[1]

CHEMISTRIES

Real-time PCR can utilize general nonspecific DNA-binding fluorophores (e.g., SYBER Green I), fluorophore-labeled primers (e.g., LUX™), or sequence-specific probes (e.g., Scorpions™).

Nonspecific Chemistries

An important advantage of nonspecific chemistries is that the design and setup of the assays is straightforward and, except for the cost of the real-time PCR apparatus, material costs are low. This makes them particularly attractive for the analysis of single nucleotide polymorphisms (SNP), which have become the markers of choice for identifying the multiple genes associated with complex diseases such as cancer or diabetes. Biallelic polymorphisms can be detected by combining allele-specific amplification with the detection of SYBR Green I. Allele-specific amplification takes advantage of the relative inability of *Taq* polymerase to extend primers that are mismatched to their targets at the 3′ end. The assay is carried out in two separate tubes, each of which contains a primer pair specific to one or the other allelic SNP variant.[2] Although there will be amplification of the mismatched allele, this occurs much less efficiently than that of the matching allele, delaying amplification and resulting in a much higher C_t being recorded. The specificity of the assay can be improved by using hairpin primers for the allele-specific PCR, as they are better than linear ones at discriminating between closely related sequences.[3]

The use of dissociation curve analysis to identify different amplicons obviates the requirement for two

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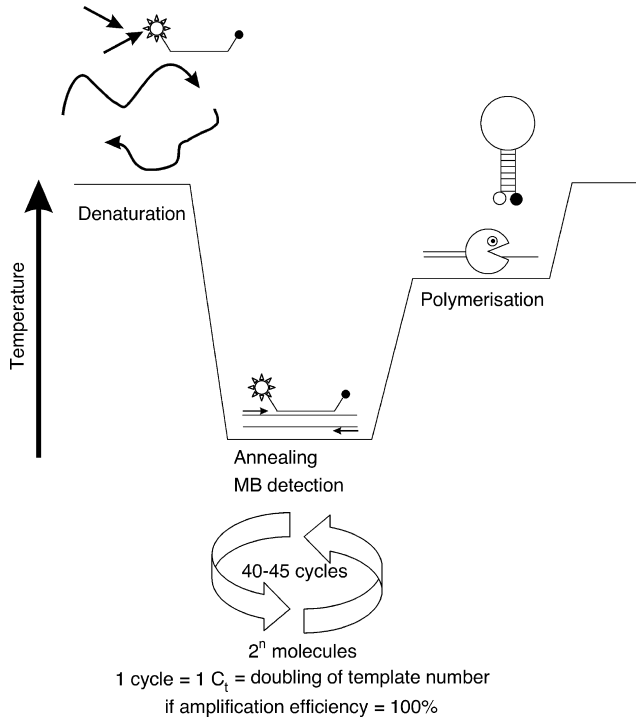

Fig. 1 Schematic representation of a real-time PCR assay using MB probes. At the annealing temperature, MB bind to their complementary target and fluorescence readings are taken. At the polymerization temperature MB dissociate from their target allowing *Taq* polymerase to read through and replicate the amplicon. A well-designed assay has an amplification efficiency of near 100% at the time fluorescence is first detected. Hence, any twofold difference in initial template concentration is reflected in a ΔC_t of 2.

separate amplification reactions. Following the PCR assay, double-stranded (ds) DNA product is melted into single-stranded (ss) DNA by a stepwise increase in temperature, with fluorescence data being collected at each temperature step. The magnitude of the reduction in fluorescence intensity of the SYBR Green dye due to its release from dsDNA provides an indicator of the amount of dsDNA dissociated at each step in the dissociation curve (Fig. 3). Furthermore, as different amplicons will melt at different temperatures, SYBR Green I can be used to distinguish different alleles through their melting temperatures (T_m). For example, Huntington's disease is caused by an expanded number of CAG repeats in the Huntington gene and the dissociation curve of a normal subject shows a single melting peak, whereas that of an affected individual has two.[4] Several closed-tube systems have been developed that can be used in combination with melting analysis of PCR products to identify both heterozygous and homozygous sequence variants,[5] and it has even been possible to develop triplex assays that use SYBR Green I and dissociation curves to identify different gene targets in the same tube.[6]

Fluorophore-Labeled Primers

Despite their attraction, there are several problems associated with the use of nonspecific dyes. In particular, there is a broadening of the melting transition and T_m compression among genotypes, which can lead to ambiguous results. Furthermore, dsDNA-specific dyes may redistribute during melting causing the release of dye from low-melting heteroduplexes and redistribution to higher melting heteroduplexes. The use of labeled primers for melting analysis avoids these problems while retaining the advantage of not having to use specific probes for each assay. The use of one fluorophore-labeled and one unlabeled primer enables melting profiles of the amplicon to be obtained immediately after completion of the PCR reaction and results in distinct melting curve shapes for different alleles, including those that differ by only a single base.[7]

Another method makes use of the principle of fluorescence resonance energy transfer (FRET). This is a distance-dependent interaction between the excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule at distances up to 70–100 Å without emission of a photon. As a result, the emission of the reporter fluorophore is quenched. In this approach, one of the amplification primers has a reporter and a quencher moiety attached to a hairpin structure at its 5′ end (Fig. 4A). When in solution, fluorescence emission from the reporter is quenched. A fluorescent signal is generated only when the labeled oligonucleotides are incorporated into the ds amplification product.[8] Labeled primer synthesis has been simplified recently by using the same type of hairpin primer, but only a single reporter fluorophore whose fluorescence emission is self-quenched based on sequence context.[9] These primers, also known as LUX™, quench when free in solution, fluoresce weakly when denatured, and emit light strongly when incorporated into DNA (Fig. 4B). Differential fluorescence labeling of primers allows the use of allele-specific PCR in a single tube.

Target-Specific Probes

Despite the evident usefulness of these chemistries, the specificity of these assays remains dependent on the specificity of the primers. Therefore the use of chemistries employing hybridization probes remains the reference method for genotyping. Two fluorogenic probes, labeled with two spectrally distinct dyes, are used to discriminate between the wild-type and mutant alleles. If amplification

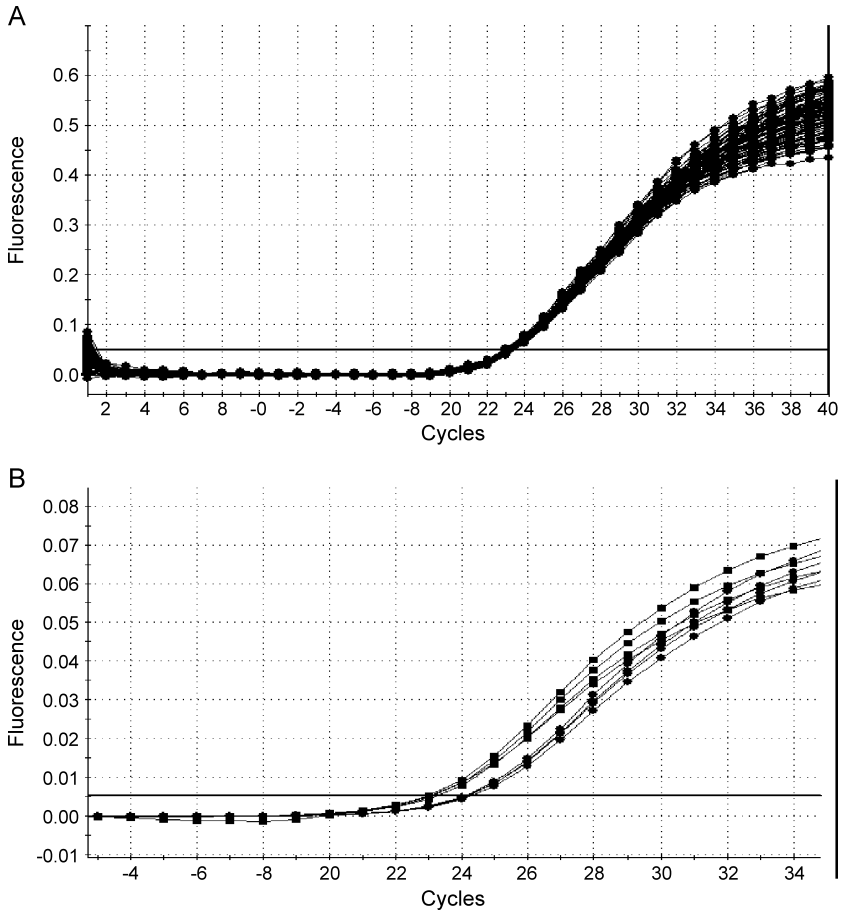

Fig. 2 Reproducibility and accuracy of real-time PCR. (A) Analysis of a 96-well plate containing replicates of a single template/mastermix. The assay was carried out on a Stratagene MX 4000 instrument using TaqMan™ chemistry. The average C_t for all 96 reactions is 23 ± 0.3. (B) Two reaction mixes were set up in triplicate. One contained 1×10^3 copies of template DNA and recorded a C_t of 23.1 ± 0.15. The other contained 2×10^3 copies and recorded a C_t of 24.1 ± 0.1. This corresponds exactly to the expected ΔC_t of 1.

in an unknown DNA sample is detected for the fluorophore identifying the wild-type allele but not for the one identifying the mutant allele, the sample is designated as wild-type homozygous. If amplification in an unknown DNA sample is detected for the fluorophore identifying the mutant allele but not for the dye identifying the wild-type allele, the sample can be designated as mutant homozygous. If the sample generates intermediate values for both dyes, it is designated as heterozygous for the two alleles (Fig. 5).

Assays based on the 5′-nuclease (''TaqMan'') use two allele-specific oligonucleotides that are labeled with different fluorophores at their 5′-ends. During PCR, fluorescence is generated after cleavage of the annealed probes by the 5′ nuclease activity of the Taq polymerase. Different sequences can de distinguished from one another by the differential fluorescence emission of the two reported dyes.[10]

Another assay, most commonly used on Roche's LightCycler™, uses two sequence-specific probes that bind adjacent to each other on the amplicon in a head-to-tail arrangement. One has a donor dye at its 3′-end, and the other has an acceptor dye on its 5′-end and is blocked at its 3′-end to prevent its extension during the annealing step. In solution, the two dyes are apart and only background fluorescence is emitted by the donor. Following the denaturation step, both probes hybridize to their target sequence in a head-to-tail arrangement during the annealing step. The reporter is excited and passes its energy to the acceptor dye through FRET. For SNP/mutation detection one probe is positioned over the polymorphic site and the mismatch causes the probe to dissociate at a different temperature to the fully complementary amplicons. Melt curve analysis after the PCR reveals which alleles are present as one probe dissociating from the amplicon causes a decrease in fluorescence as FRET can no longer occur.[11]

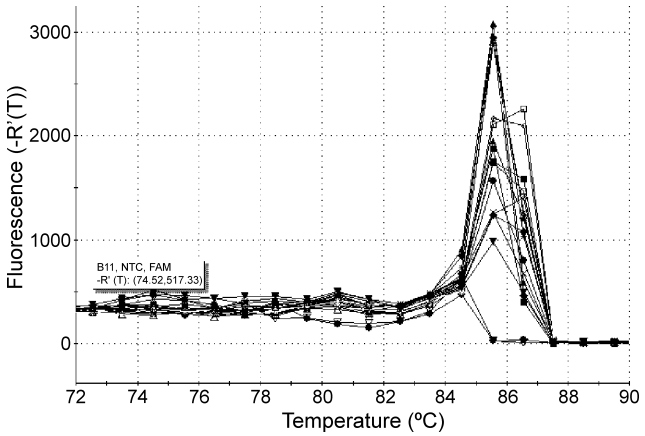

Fig. 3 Analysis of SYBR Green 1 dissociation curve. The first derivative view with respect to temperature provides a clear view of the rate of SYBR Green 1 loss and the temperature range over which this occurs. The example shows a view of data between 72°C and 90°C. The small peak at 74.5°C is probably due to primer dimer product formation as this is the only peak to occur in the NTC sample. The main peaks occur around 85.55°C although there are some with a distinctly different profile and a peak at 86.55°C. These distinct profiles represent different products in the final PCR product.

Molecular beacons (MB) and Scorpions are based on stem-loop hairpin structures, and MB in particular have found wide-ranging application as diagnostic tools.[12] Molecular beacons consist of a hairpin loop structure, with the loop complementary to a target nucleic acid and the stem formed by the annealing of complementary termini (Fig. 6A). One end of the stem has a reporter fluorophore attached and the other a quencher. In solution, free MB adopt a hairpin structure and the stem keeps the arms in close proximity, resulting in efficient proximal quenching of the fluorophore. During the denaturation step, the MB assume a random-coil configuration and fluoresce. At the annealing temperature, MB bind to any target amplicons as the probe/target duplex is designed to be thermodynamically more stable than the hairpin structure at that temperature. Once the probe binds to its target the hairpin is opened out and the fluorophore and quencher are separated, resulting in fluorescence. A major strength of this technology is the high specificity of the MB in recognizing nucleotide sequence mismatches in DNA and RNA. The hairpin shape of the MB causes mismatched probe/target hybrids to easily dissociate at significantly lower temperatures than exactly complementary hybrids. This is because the thermodynamic properties of the MB favor the formation of a hairpin form rather than continued hybridization to a less than perfectly matched target sequence. When the temperature is raised to allow primer extension, the MB dissociate from their targets and do not interfere with polymerization. A new hybridization takes place in the annealing step of every cycle, and the intensity of the resulting fluorescence indicates the amount of accumulated amplicon. Again, as this is a reversible process, melt curves can be used to analyze the dynamics of the reaction and determine the best temperature for fluorescent acquisition (Fig. 6B). There is some evidence to suggest that the measured signal ratios

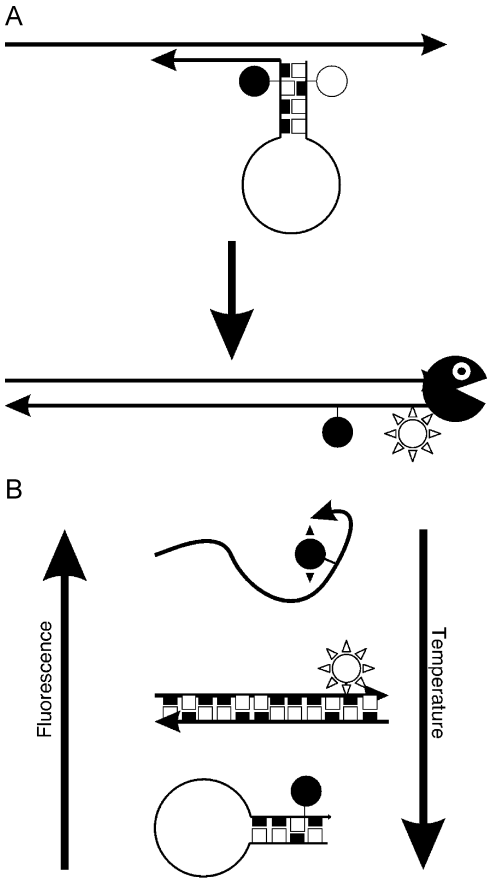

Fig. 4 Fluorophore-labeled probes. (A) The original design used a primer with a hairpin structure at its 5′-end that contained a fluorophore and quencher at opposite ends of the hairpin. During the first cycle of PCR the primers are extended and become templates during each subsequent cycle. This linearizes the hairpin, separates the donor and acceptor moieties, and results in fluorescence emission from the fluorophore. (B) LUX™ primers. One primer contains a fluorophore, the other one is unlabeled. The fluorogenic primer has a short sequence tail of 4–6 nucleotides on the 5′-end that is complementary to the 3′-end of the primer. The resulting hairpin secondary structure provides optimal quenching of the attached fluorophore. When the primer is incorporated into the double-stranded PCR product, the fluorophore is dequenched and a signal is reported.

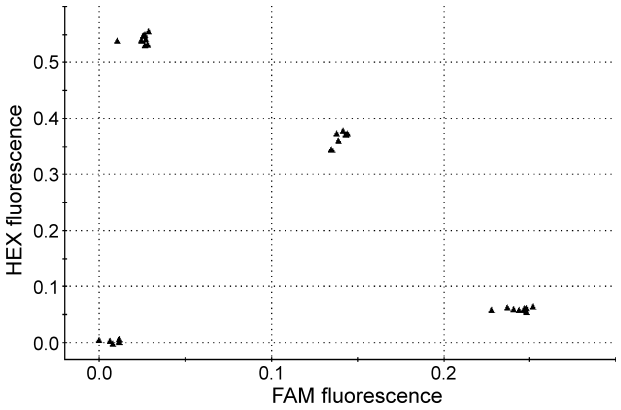

Fig. 5 Scatter plot for real-time SNP analysis using MB. Fluorescence is reported during each annealing step when the MB is bound to its complementary target and the C_t value for each dye in the sample is used to determine the genotype of the samples. A C_t value equal to the final cycle of the PCR reaction (typically 40 or 45) indicates the absence of a specific allele. Each plotted point represents the coordinates of the C_t values for the two dyes in a single well. Here the x axis corresponds to FAM C_t whereas the y axis corresponds to HEX C_t and the plotted points (x,y) correspond to the coordinates describing the two C_t values determined for a given well. The position of the data point for a given well on the scatter plot indicates the presence or absence of each allele, providing a rapid method for clustering the samples that are homozygous for either of the two alleles or are heterozygous. If a highly quantitative measurement of copy numbers is not necessary, it is also possible to measure fluorescence when cycling is complete.

with MB assays are proportional to the amount of the minor allele over a wider range than with the TaqMan assay.[13] Multiplexing of MB is enhanced by the use of wavelength-shifting MB (Fig. 6C).[14] These extend the range of fluorophore/quencher pairs that will function at a given wavelength and contain a generic harvester fluorophore, a probe-specific emitter fluorophore, and a quencher. Scorpions were originally made up of a single oligonucleotide containing a 5′ fluorophore, a stem-loop structure containing the probe, a quencher, and a PCR blocker to prevent read-through by DNA polymerase and primer. In the presence of a target, the specific probe sequence folds back on itself to bind its complement within the same DNA strand, opening up the hairpin loop and separating the fluorophore and quencher. This unwieldy structure was replaced by the second-generation Scorpions that are made up of two oligonucleotides. One contains the fluorophore-coupled probe, the other a quencher-coupled complementary sequence. For SNP analysis, the fluorescence is monitored above the T_m of the mismatch probe/target duplex and below the T_m of the fully complementary probe/target duplex. Under these conditions the mismatched probe reassociates with the quencher element to become nonfluorescent, whereas the hybridized wild-type probe is separated from the quencher element and is fluorescent. Because the hybridization of probe sequence to amplicon is intramolecular, Scorpion probes are more efficient than binary systems such as MB and as a result generate significantly greater signal intensities.[15]

There are many more probe chemistries available, all with their own advantages and disadvantages. These include Hybeacons, which require only a single fluorophore and make use of the quenching properties of DNA. This makes them easy to design and synthesize.[16] Light-up probes are composed of thiazole orange conjugated to peptide nucleic acid (PNA) (see below) and combine the excellent hybridization properties of PNA, which allows the use of shorter probes, with the extraordinary fluorescence enhancement of asymmetric cyanine dyes upon binding to nucleic acids.[17] Eclipse™ probes are linear probes that have a minor groove binder (MGB) and quencher on the 5′-end and the fluorophore on the 3′-end.[18] This is the other way round compared with hydrolysis probes, and the presence of the MGB at the 5′-end prevents cleavage of the Eclipse probe by *Taq* polymerase. Other chemistries are described in detail elsewhere.[19]

Real-time PCR assays generally use symmetric primers. However, this results in the reactions typically slowing down and entering the plateau phase in a stochastic manner, because reannealing of the template strands gradually outcompetes primer and probe binding to the template strands. This is a particular problem when the aim is to detect specific DNA targets down to alleles of single-copy genes in single cells. Asymmetric PCR potentially circumvents the problem of amplicon strand reannealing by using unequal primer concentrations. However, asymmetric amplification is much less efficient and requires extensive optimization to identify the proper primer ratios, the amounts of starting material, and the number of amplification cycles that can generate reasonable amounts of product for individual template/target combinations. A recent innovation termed linear-after-the-exponential-PCR (LATE-PCR) uses unequal primer concentrations but takes into account the effect of the actual primer concentrations on primer T_m.[20] It corrects for the fact that the T_m of the limiting primer is often several degrees below the T_m of excess primer and allows the asymmetric PCR to proceed as efficiently as symmetric PCR. Furthermore, ss amplicons are generated with predictable kinetics for many cycles beyond the exponential phase. This permits uncoupling of primer annealing from product detection. As a result, the T_m of the probe no

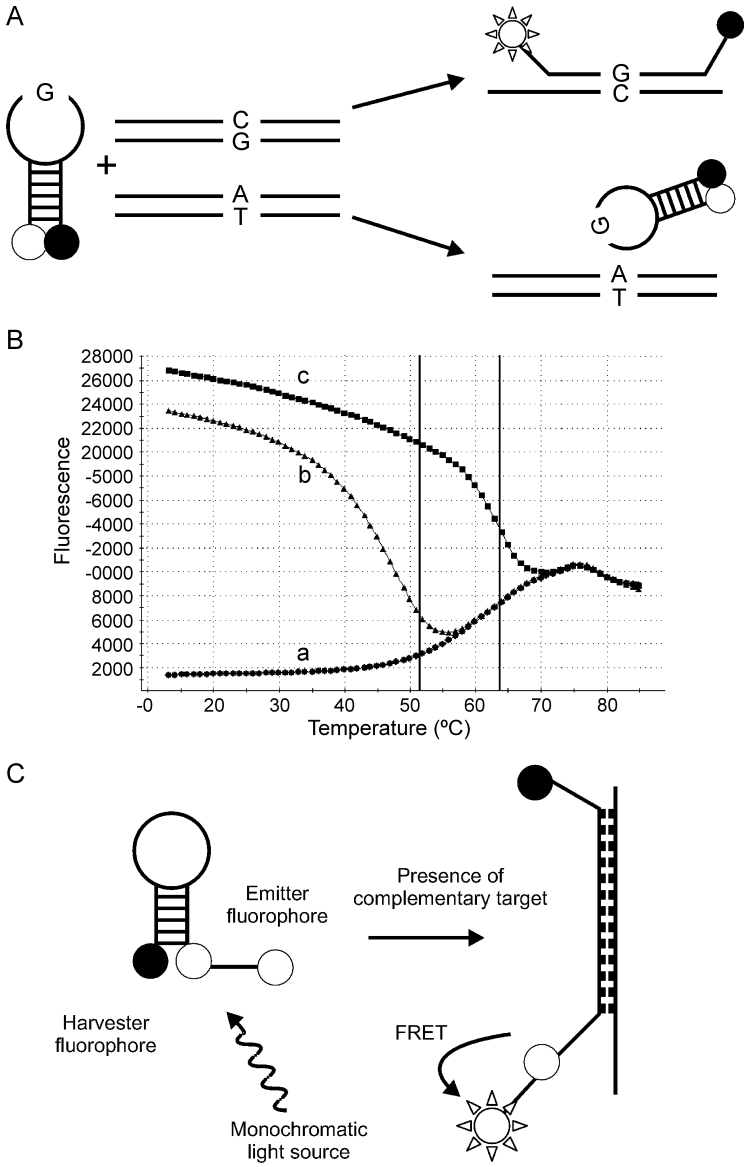

Fig. 6 Molecular beacons and melting curves. (A) Structured probes are better at discriminating single base-pair mismatches than linear ones such as TaqMan™. Only one MB is shown, which hybridizes to its perfect complement and emits fluorescence but remains closed and unbound to a target with a single nucleotide mismatch. (B) Molecular beacon melting profile for allelic discrimination. Three melting curves are visible. One for MB alone (a), a second one for MB and its perfectly complementary single-stranded oligonucleotide target (b), and a third one for MB plus a single-strand target that produces a probe/target hybrid containing a single mismatched base pair (c). The two vertical bars indicate the optimal annealing temperature range in which the perfectly matched MB will have greater fluorescence than the mismatched MB, with background fluorescence still low. (C) Wavelength shifting MB. The MB has two fluorophores on one end, a ''harvester'' and an ''emitter,'' and a quencher on the other end. In the hairpin loop structure, the quencher forms a nonfluorescent complex with the harvester. Upon hybridization of the MB to a complementary sequence, quenching of the harvester fluorophore is relieved, and it transfers energy via FRET to the emitter, which emits fluorescence.

longer needs to be higher than the T_m of either primer. This permits the use of low-T_m probes, which are inherently more allele-discriminating, generate lower background, and can be used at saturating concentrations without interfering with the efficiency of amplification.

Another important advantage of using probe-based chemistries is that it is possible to multiplex, i.e., amplify multiple targets in a single tube, as fluorescent dyes with different emission spectra may be attached to the different probes (Fig. 7). Probes afford a level of discrimination

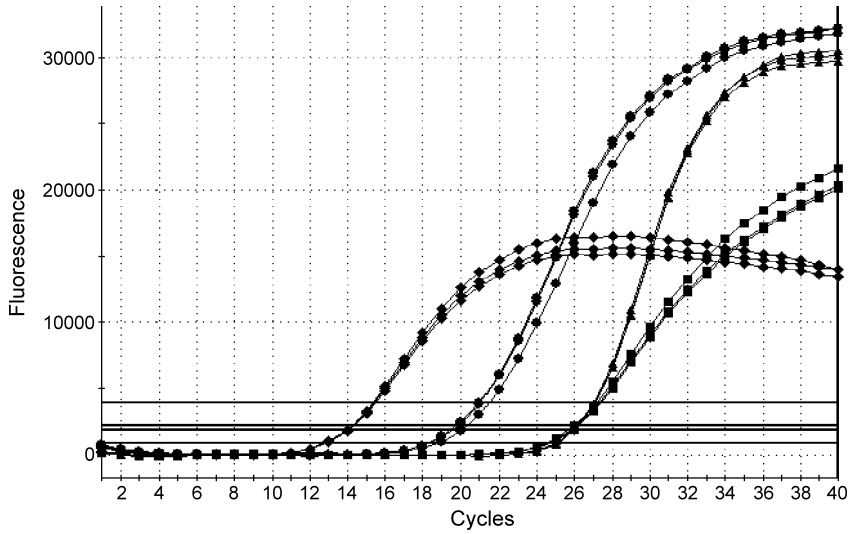

Fig. 7 Multiplex PCR. Four TaqMan™ probes were labeled with FAM, HEX, ROX, or Cy5, and a PCR assay was performed using the Stratagene ''Brilliant® Multiplex QPCR'' master mix on the MX4000 real-time PCR system. Each sample was analyzed in triplicate. The four horizontal lines indicate the four thresholds calculated for the individual fluorophores. The four targets differ by 14 C_ts, which translates into a 1.6×10^4-fold difference in target abundance.

impossible to obtain with SYBR Green, as they will only hybridize to true targets in a PCR and not to primer–dimers or other spurious products.

DNA ANALOGUES

A recent development has been the use of probes incorporating peptide nucleic acid (PNA) or locked nucleic acid (LNA™). In PNA the entire deoxyribose phosphate backbone is replaced by a structurally homomorphous backbone consisting of repeating *N*-(2-aminoethyl)-glycine units linked by peptide bonds. As the PNA backbone is not charged, hybridization is not affected by intrastrand repulsion, and no salt is necessary to facilitate and stabilize the formation of PNA and DNA or RNA duplexes. Furthermore, the ΔT_m of a single PNA/DNA mismatch is significantly higher than that of a DNA/DNA mismatch, making PNA probes useful for mismatch detection. PNA technology has been used in an ingenious approach utilizing quencher-labeled primers. This system uses two labeled molecules: 1) a quencher-labeled PNA probe with a C-terminal DABCYL group; and 2) a primer with a target-specific sequence at its 3′-end and a fluorophore and a PNA-probe-complementary sequence tag at its 5′-end.[21] The PNA/primer duplex has a T_m higher than the primer annealing temperature, but lower than the T_m of the primer/amplicon duplex. This ensures that excess primer is quenched at the annealing temperature and the fluorescence measured during the annealing step indicates the amount of primer hybridized to amplicon, plus any full-length ds amplicon as the end result is a fluorescently labeled ds amplicon. The main disadvantage with PNA probes is that they can aggregate and precipitate.

In LNA the furanose ring conformation is restricted by a methylene bridge that connects the 2′-oxygen position of ribose to the 4′-carbon. This bridge reduces the conformational flexibility of the ribose, which imparts superior affinity and specificity in binding complementary sequences of DNA or RNA. In addition, the change in T_m caused by a mismatch is significantly greater with a LNA/DNA duplex than with of a DNA/DNA duplex, resulting in enhanced specificity for SNP/mutation analyses.[22] Using appropriate dyes, it is possible to use probes as short as 7 nucleotides long for mismatch discrimination, making it possible to generate universal genotyping reagents. Unlike PNA, there is no problem with solubility of LNA molecules.

OTHER APPLICATIONS

The real-time PCR-based focus on pathogen genotype is having a significant impact on the detection of viruses, bacteria, and parasites in diagnostic microbiology.[23] The biggest application of real-time technology is probably in virology, where these assays have been used to investigate the role of viruses in a range of human diseases. The ability to multiplex has allowed the reliable measurement

of different viral nucleic acid targets within a single sample, and the discrimination of multiple viral genotypes within a single reaction tube. A major application is in the detection of viral load, and real-time assays have become very useful as indicators of the extent of active infection, host/virus interaction, and the efficacy of antiviral treatment. Other uses include the assessment of viral gene therapy vectors before their use in clinical trials and the study of new and emerging viruses and clinical symptoms experienced by patients.[24]

A major benefit lies in the rapidity with which results can be obtained. This is of major importance when detecting bacterial pathogens, as it allows a specific and timely application of antibiotics. Real-time assays are ideal for distinguishing between different serotypes of a single bacterial species,[25] for detecting and monitoring drug resistance among clinical isolates,[26] for detecting pathogens in food,[27] and not least for identifying microbes used as agents of biological warfare.[28]

The worldwide approval of a large number of genetically modified organisms (GMOs) among countries, and the associated labeling requirements, has resulted in the development of real-time PCR-based methods for the detection of the presence of GMO in food or food additives.[29] Genetically modified organism detection through PCR relies on parallel amplification of the transgene and of an endogenous reference gene that provides a control both for the lack of inhibition and for the ability to amplify the target DNA in the sample. Additionally, for quantitative analyses, amplification of the reference gene provides an estimation of the total amount of target DNA present in the sample. Targeting the DNA is particularly appropriate, because of the high stability of this molecule under the extreme conditions used during processing of some food products.

CONCLUSION

These examples are but a few of the huge number of applications that have benefited from the introduction of real-time fluorescence-based PCR assays and that have contributed to the transformation of this technique from an experimental tool into the scientific mainstream.[30] The advances in robotic nucleic acid extraction and liquid-handling systems, together with the continuous introduction of less expensive, yet more capable thermal cyclers make real-time PCR an attractive and essential technology for routine diagnostics. Recent developments in multiplexing make it possible to envisage easy identification, genotyping, and quantification of DNA targets in single, rapid reactions. However, the technology is only as reliable as the accompanying controls and associated quality-assurance programs. This includes the quality of standards, the use of suitably controlled standard curves, and the need to fully optimize, validate, and evaluate each new assay against previously standardized assays. Nevertheless, it is clear that real-time PCR is a technique whose time has come.[31]

ACKNOWLEDGMENTS

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Real-Time PCR Platforms

Mikael Kubista
Neven Zoric
TATAA Biocenter AB, Göteborg, Sweden

INTRODUCTION

During the last decade, the scientific community has undergone a technology shift in studying nucleic acids in biological samples. With the development of real-time polymerase chain reaction (PCR), it became possible to quantify the amount of specific nucleic acids target in virtually any biological sample with unprecedented accuracy, sensitivity, and specificity. Real-time PCR combines the exponential amplification of the PCR with optical detection using fluorescent dyes or fluorescent probes, which makes it possible to measure the amount of product after each cycle of the reaction. From this information, the initial amount of template can be determined. During the last years, real-time PCR has been used in a variety of applications such as human[1] and veterinary[2] diagnostics; detection of pathogens in food,[3] water,[4] and soil;[5] and gene expression studies.[6] To meet the various demands for real-time PCR instruments and the large variety of applications, several instrument platforms have been developed, many with specific properties. A real-time PCR instrument must have certain components to perform its intended function. The sample must be contained in a container that can be heated and cooled rather rapidly. An optical unit that illuminates the sample and records the emitted fluorescence is also required. In this article, we will briefly describe the variety of different components used in the most common real-time PCR machines.

HEATING AND COOLING MECHANISMS

To perform PCR, the unit must thermocycle the sample and heat it to temperatures close to 100°C. This is typically done using either Peltier elements, resistive heating, and/or airflow.

Peltier Elements

Peltier elements are solid-state heat pumps that make use of the Peltier effect to create a hot side and a cool side of the element. Peltier elements are common in electronic components in laboratory equipment. They are relatively small, silent, and have no moving parts. This makes them reliable. Peltier elements are operated by a direct current (d.c.) and, by reversing the polarity of the current, one can alternate heating and cooling. The amount of heat transferred is proportional to the current that flows through the element. The block temperature may not be uniform throughout the element, particularly the corners may deviate, which might affect reaction efficiency. Temperature variations within the block are usually within ±0.5°C after holding at constant temperature for a certain amount of time. Peltier elements can create temperature gradients, giving extra flexibility when, for example, optimizing annealing temperatures in PCR. Instruments that use Peltier elements usually have rather slow heating and cooling ramp rates. They are typically of 1–5°C/sec because of large thermal inertia, which cause a regular PCR run of 40 cycles in approximately 90 min. Real-time PCR instruments that use Peltier elements include the Bio-Rad iCycler and MyCycler, ABI, real-time PCR instruments, MJ Research DNA Engine Opticon, Techne Quantica and Stratagene Mx4000/Mx3000p.

Air

Using air as media for transfer of heat requires mechanisms to maintain sample temperature uniformity. This usually entails either the samples being spinned, or the air being thoroughly mixed using fans. Air has low heat capacity and, in direct contact with the reaction vessel, ensures efficient thermal transfer. Ambient air is heated with a resistive coil and is transferred to the reaction chamber using fans. Therefore for cooling, ambient air is used directly and samples cannot be cooled below room temperature. Instruments that use air for thermocycling include Corbett Research Rotorgene 2000/3000, Roche LightCycler, and also in the fast real-time PCR instrument under development by AlphaHelix.

Conductive Polymers

Recently, conductive polymers have been introduced for thermocycling to further reduce the thermal barrier

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between the heating/cooling material and the reaction media. Using electrically conductive polymers as reaction vessel, each reaction can be temperature-controlled independently using infrared temperature sensors. This results in high-temperature uniformity between samples and increased flexibility by allowing individual temperature cycling for different tubes in the same plate. Direct heating of the reaction tubes results in temperature transfer rates up to 15°C/sec,[7] enabling shorter cycling times. The BioGene InSyte uses conductive polymers for thermocycling.

EXCITATION SOURCES

Lasers

Laser excitation results in a highly intense and monochromatic signal. Therefore lasers are reliable, but they are expensive to manufacture and usually included in the more expensive range of real-time PCR instruments. Conventional lasers are also bulkier than other illuminating sources and are found only in larger instruments.

Halogen Lamp

Halogen lamp as illumination source requires the use of filters to excite fluorophores at the appropriate wavelength. Halogen lamps emit light between 350 and 750 nm. Light intensity is lower than from lasers and may result in lower sensitivity. They are cheap but have a limited lifetime and must be replaced regularly.

Light-Emitting Diodes (LEDs)

LEDs are small and cheap and, in combination with filters, can deliver narrow bandwidth illumination source. LEDs consist of two electrodes surrounding semiconductor elements and convert electric energy into light energy.

DETECTORS

Photomultiplier Tubes (PMTs)

A typical PMT consists of a photocathode followed by focusing electrodes, electron multipliers, and an electron collector. When light enters the PMT, photons hitting the photocathode emit electrons into the tube and are directed toward the multiplier electrodes by the focusing electrodes' voltage. The electrons are multiplied at each electrode and finally detected as an electric signal from the anode. Because each photon gives rise to a cascade of electrons, PMTs are highly sensitive detectors that generate signals with very low noise. PMTs are not

Table 1 Summary of common real-time PCR instruments' reaction vessels and thermocycling mechanisms

Instrument	Company	Heating mechanism	Heating/cooling rate (°C/sec)	Reaction vessel	Number of samples	Volume (μL)
7000/7700/7900	ABI	Peltier	1.5/1.5	Tubes/plates	96 (394 for 7900)	Up to 100
iCycler/myCycler	Bio-Rad	Peltier	3.3/2.0	Tubes/plates	96	15–100
LightCycler	Roche	Air	20/20	Glass capillaries	32	10–20
LightCycler 2.0	Roche	Air	20/20	Glass capillaries	32	10–20 or <100
Rotorgene 3000	Corbett Research	Air	2.5/2.5	Tubes	72	10–100
SmartCycler	Cepheid	I-CORE™	10.0/2.5	Plastic capillaries	16	25–100
Mx4000/ Mx3000P	Stratagene	Peltier	2.2/2.2	Tubes/plates	96	10–50
Opticon 2	MJ Research, Inc.	Peltier	3.0/2.0	Tubes/plates	96	10–100
InSyte	BioGene	Conductive polymer	15/15	Conductive polymer plate	96	Up to 50
7300/7500	ABI	Peltier	1.5/1.5 (7500 block upgradable to faster ramping block)	Tubes/plates	96	25–100
Quantica	Techne	Peltier	2.6/2.6	Tubes/plates	96	15–50

Table 2 Summary of common real-time PCR instruments' optical properties

Instrument	Company	Illumination source	Excitation wavelengths	Detector	Detection wavelengths
7000/7700/7900	ABI	7000: Halogen lamp; 7700/7900: argon laser	7000: 350–750; 7700/7900: 488 and 545	CCD	7000: 520, 550, 580, and 602; 7700/7900: 500–650
iCycler/myCycler	Bio-Rad	Halogen lamp	400–700/400–585	CCD	Variable filters available
LightCycler	Roche	LED	470	Photodiodes	530, 640, 710
LightCycler 2.0	Roche	LED	470	Photodiodes	530, 560, 610, 640, 670, 710
Rotorgene 3000	Corbett Research	LED	470, 530, 585, 625	Photodiodes	510, 555, 610, 580 hp, 610 hp, 660 hp
SmartCycler	Cepheid	LED	450–495, 500–550, 565–590, 630–640	Photodiodes	510–527, 565–590, 606–650, 670–750
Mx4000/Mx3000P	Stratagene	Halogen lamp	350–750	PMT	350–850
Opticon 2	MJ Research	LED	470–505	PMT	523–543, 540–700
InSyte	BioGene	Blue laser	473	PMT	520–720
7300/7500	ABI	Halogen lamp	7300: Single broad excitation 7500:5 filters for FAM/SYBR/VIC/JOE, NED/TAMRA/Cy3, ROX/Texas Red and Cy5	CCD	7300: Filters for FAM/SYBR, VIC/JOE, TAMRA and ROX 7500: Filters for FAM/SYBR, VIC/JOE, NED/TAMRA/Cy3, ROX/Texas Red and Cy5
Quantica	Techne	Halogen lamp	470–650 (up to 4 user selected filters)	PMT	500–710 (up to 4 user selected filters)

Source: Refs. [7–16].

wavelength-discriminative and must be used in combination with filters. Therefore instruments equipped with PMTs have only one PMT alternatively, one PMT per detection wavelength, and can only measure one sample at a time. Reading out of many samples requires moving parts. It can be achieved by rotating the sample holder, or by moving an optical fiber across the different samples.

Photodiodes

Photodiodes are electrical components that convert incident light to electric current. The photodiode consists of a positive electrode, a P-layer, a neutral layer, and an N-layer followed by a negative electrode. When light hits the P-layer, electrons in its crystal structure are excited and, in an electric field, accelerate toward the N-layer, creating a current. Photodiodes are cheap; there may be several diodes in a single instrument. Photodiodes can also be wavelength-discriminative; different diodes are used for different wavelengths.

Charged Coupled Device (CCD) Cameras

CCD cameras are basically a large array of photodiodes containing many spots—pixels—which convert light to an electric signal. The higher is the amount of spots, the higher is the resolution of the camera. CCD cameras are, in general, not as sensitive to light as PMTs, and this may affect the sensitivity of the instrument. Instruments equipped with a CCD camera will measure all samples simultaneously for each wavelength and must be used together with filters. The excitation and detection wavelengths of some instruments are set for certain fluorescence detection chemistries, which reduce the flexibility of the instrument and may lead to higher running costs.

REACTION VESSELS

Capillaries

Glass capillaries have the best optical properties for fluorescence measurements. They also have a large surface-to-volume ratio, leading to fast temperature ramping rates as well as being highly suitable as fluorescence measurement vessels. The high surface-to-volume ratio also increases the adsorption of reaction components to the glass walls, and protocols intended for plastic containers may need reoptimization. LightCycler capillaries have an outer diameter of 1.5 mm and a length of 3 cm, and can hold sample volumes of 5–20 μL.[8] The new LightCycler 2.0 instrument can also use new 100-μL capillaries in a special sample carousel. The shape of the capillaries concentrates the fluorescent signal to the tip, where emitted light is collected. The capillary has three parts: the actual capillary, a sample deposit chamber from where the reaction mix is centrifuged into the capillary, and a plastic cover to seal the capillary. Glass capillaries are rather expensive and containers are a major cost component of each PCR reaction run. The capillaries are also fragile and may break during handling.

Tubes/Plates

Tubes and plates are the most common reaction containers and are usually sealed with optical caps or optical film for head on detection. It is important to have plasticware that is thermally stable to avoid evaporation. Optical-grade plastic tubes and plates are available from a number of manufacturers, which have pressed prices, making them the most cost-efficient reaction vessel for real-time PCR. The ABI, Stratagene, Bio-Rad, and MJ Research instruments recommend a reference dye not participating in the reaction to be present. The reference dye is usually ROX or fluorescein in the reaction mix and is used to compensate for detection differences across the plate.

Special Containers

Certain real-time PCR instruments require special containers. These include electrically conductive polymer tubes/plates of the BioGene InSyte and the special plastic small-volume containers of the Cepheid SmartCycler. The cost of these containers is significantly higher than that of regular tubes and plates. Both containers are designed to achieve fast cycling times.

CONCLUSION

Combining various technologies for heating/cooling and illumination/detection has given rise to a large selection of real-time PCR instruments that have unique advantages, making them the preferred choice for certain applications, but they also have limitations. Tables 1 and 2 summarize the most common real-time PCR instruments available today and their properties.

Development in real-time PCR instrumentation is branching into high-throughput instruments for routine diagnostics, research instruments with maximum flexibility, and battery-operated instruments for field use. The high-throughput instruments are developing into fully automated machines, with miniaturized reactions enabling many hundreds of samples to be run simultaneously. The instruments are also becoming increasingly integrated with sample preparation robots.

Although the main use of real-time PCR is in diagnostics, the technique is rapidly becoming popular in gene expression profiling. Today, real-time PCR is mainly used to validate results from microarray expression experiments. With the introduction of high-throughput automated instruments, the unsurpassed sensitivity, accuracy, and specificity of real-time PCR are likely to make real-time PCR the method of choice also for large-scale gene expression studies. We think that development will eventually lead to a merger of real-time PCR and microarray platforms, resulting in real-time PCR microarrays with the throughput of today's microarrays and the sensitivity, accuracy, and specificity of real-time PCR.

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Real-Time Reverse Transcription PCR

Stephen A. Bustin
University of London, London, U.K.

INTRODUCTION

Real-time, fluorescence-based reverse transcription polymerase chain reaction (RT-PCR)[1] has been transformed from an experimental technology into a mainstream scientific tool for the detection of RNA.[2] This is because of several factors: 1) it is a homogeneous assay, which eliminates the requirement for post-PCR processing; 2) it has a wide dynamic range; 3) there is little interassay variation; and 4) it realizes the inherent quantitative capacity[3] of PCR-based assays, making it a quantitative, rather than a qualitative, assay. These properties match the evident requirement in molecular medicine for quantitative data (e.g., for measuring viral load,[4] monitoring of occult disease in cancer,[5] or examining the genetic basis for individual variation in response to therapeutics through pharmacogenomics).[6]

THE ASSAY

The principle of fluorescence-based real-time RT-PCR assays is simple: reverse transcription of RNA is reverse-transcribed into cDNA; a suitable detection chemistry reports the presence of PCR products; an instrument monitors the amplification in real time; and an appropriate software analyzes the data.[7] Because the quality of the RNA template is the single most important determinant of the reproducibility of RT-PCR results,[8] it is essential to ensure that no inhibitors copurify during the RNA extraction process.[9]

Real-time RT-PCR can be either a one-tube assay using a single buffer, or a two-tube assay where both first-strand cDNA synthesis and the subsequent PCR step are performed separately under optimal conditions for the respective polymerases. The former is more convenient and reduces the risk of cross-contamination;[10,11] the latter may be more sensitive and more reproducible.[12]

The priming of the cDNA reaction from the RNA template is best performed using oligo-dT or target-specific primers. Although random primers yield the most cDNA, they initiate transcripts from multiple points along the RNA, including ribosomal RNA (rRNA), thus producing more than one cDNA per original target. Oligo-dT priming results in a faithful cDNA representation of the mRNA pool, but it is not a good choice for poor-quality RNA from formalin-fixed archival material. Target-specific primers synthesize the most specific cDNA and provide the most sensitive method of quantification,[13] but require separate priming reactions for each target.

Viral RTs, used mainly in two-step assays, have a relatively high error rate and a strong tendency to pause, hence producing truncated cDNA.[14] Avian Myoblastosis Virus-RT (AMV-RT) is more robust and processive than Moloney Murine Leukemia Virus-RT (MMLV-RT)[15] and retains significant polymerization activity up to 55°C,[16] whereas native MMLV-RT has significantly less RNaseH activity than native AMV-RT[17] but is less thermostable. Several DNA-dependent DNA polymerases exhibit both RNA- and DNA-dependent polymerization activities in the presence of Mn^{2+}.[18,19] It is also possible to use blends of reverse transcriptases in RT-PCR reactions, which can result in higher reverse transcription efficiencies than the individual component enzymes.

CHEMISTRIES

Detection chemistries fall into two groups:

1. Nonspecific chemistries usually involve the detection of an intercalating dye (e.g., SYBR green I) (Fig. 1A).[20] The PCR product can be verified by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon.[21] Because the melting temperature (T_m) of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product. A characteristic melting peak at the amplicon's T_m will distinguish it from amplification artefacts that melt at lower temperatures in broader peaks.
2. Specific chemistries make use of template-specific fluorescent probes for each PCR assay. These probes can be structured (e.g., Scorpions, Molecular Beacons) or linear (e.g., TaqMan, Light-Cycler probes). Probes may contain reporters and quenchers, or make use of the quenching properties of the DNA. All operate on the same principle: A fluorescent signal is only generated if the amplicon-specific probe hybridizes to its complementary target (Fig. 1B).

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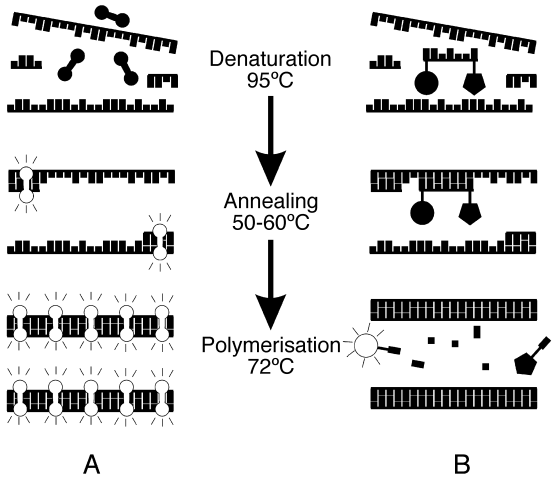

Fig. 1 Real-time detection chemistries. (A) Nonspecific (e.g., SYBR green I). In solution, the unbound dye exhibits little fluorescence; during the PCR assay, increasing amounts of dye bind to the nascent double-stranded DNA. When monitored in real time, this results in an increase in the fluorescence signal as the polymerization proceeds, and that falls off during the denaturation step. Consequently, the increasing amounts of amplified DNA can be monitored by measuring the fluorescence measurements at the end of each elongation step. (B) Specific (e.g., 5′-nuclease; TaqMan). Although the fluorophore (circle) and a quencher (pentagon) are bound to the same probe molecule, any light emitted by the fluorophore on excitation is quenched. When the polymerase displaces and cleaves the probe, the fluorophore and the quencher become physically separated, and emissions from the fluorophore can be detected.

INSTRUMENTATION

Instruments used for real-time PCR use: 1) an excitation light source, to excite the fluorophores; 2) a detector, to register photon emissions that are proportional to the concentration of the amplification product being measured; and 3) a software, which allows analyses of the data.

Fluorescence emission data are collected from each tube and the levels of background fluorescence detected by the fluorimeter are established. Platform-specific algorithms are used to define a fluorescence threshold. Finally, the algorithm searches the data from each sample for a point that exceeds the baseline. The cycle at which this point occurs is defined as C_t (Fig. 2) and is used to calculate the amount of template present at the beginning of the reaction.[22]

DATA ANALYSIS

Results obtained using real-time RT-PCR assays are significantly less variable than conventional RT-PCR protocols, which can be subject to significant error.[23] In principle, quantification by real-time assays is easy: the more copies of mRNA there are at the beginning of the assay, the fewer cycles of amplification are required to reach the C_t. In practice, there are some problems in converting a C_t value into a biologically meaningful copy number.[24]

Relative Quantification

Relative quantification expresses the changes in steady-state mRNA levels of a gene relative to the levels of a coamplified internal control mRNA.[25] Target C_t values are compared directly to an internal reference C_t and results are expressed as ratios of the target-specific signal to the internal reference. This produces a corrected relative value for the target-specific mRNA product, which can be compared between samples and allows an estimate of the relative expression of target mRNA in those samples. Amplification efficiencies of a target and a reference must be similar because they directly affect the accuracy of any calculated expression result and must be incorporated into copy number calculations.[26,27] However, because the expression of the internal control itself is often variable, relative quantification can be misleading.[28]

''Absolute'' Quantification

''Absolute'' quantification is not really absolute, but is relative to an external standard curve.[2] A standard dilution series with a known concentration of initial target

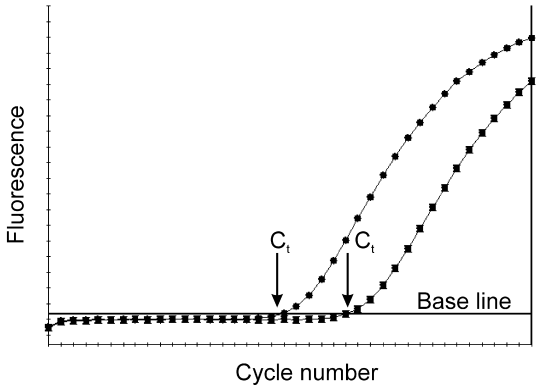

Fig. 2 Threshold cycle C_t. The threshold cycle is defined as the number of PCR cycles where the fluorescence generated from the amplification product first exceeds a baseline level. It depends on the sensitivity of the detection system and can vary significantly depending on assay-specific background levels. The two amplification plots have C_t values that differ by six cycles (i.e., represent an approximately 100-fold difference in template starting copy numbers).

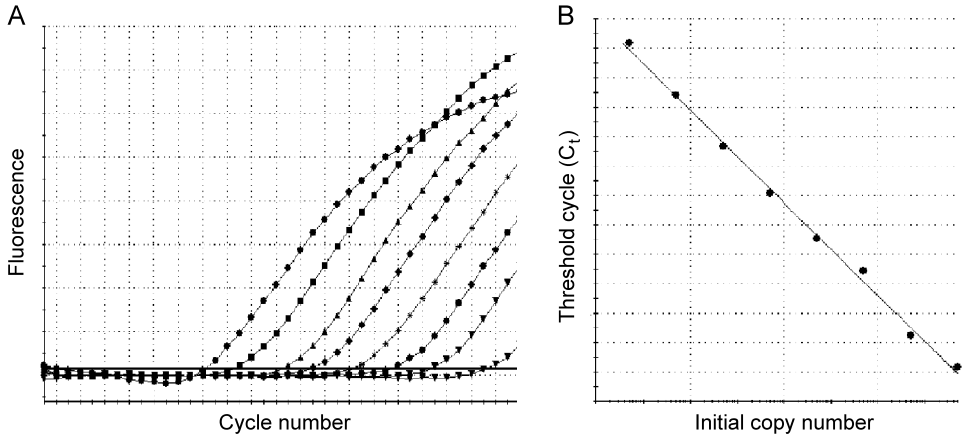

Fig. 3 Preparation of a standard curve. (A) Sense-strand amplicon-specific oligonucleotides are serially diluted from 1×10^8 to 10 copies, and their respective C_t values are recorded. (B) A plot of C_t against the log of the initial oligonucleotide copy number results in a straight line that is linear over at least seven orders of magnitude, and linear regression analysis permits the calculation of the ''absolute'' copy number of any unknown target relative to that standard curve.

copy number is used to generate a standard curve by plotting the C_t values against the logarithm of the initial copy numbers (Fig. 3).[29] Its dynamic range must include the C_t values expected for the experimental RNA samples. The copy numbers of unknown samples can be calculated from the linear regression of that standard curve, with the slope providing the amplification efficiency. Standard curves can be constructed from PCR fragments, in vitro T7-transcribed RNA, single-stranded sense-strand oligodeoxyribonucleotides, or commercially available universal reference RNAs.[30]

Absolute quantification is most obviously used for quantifying tumor cells or infectious particles such as viruses or bacteria in body fluids, but it is also usefully applied to quantitate changes in mRNA levels. The accuracy of absolute quantification depends entirely on the accuracy of the standards. However, external standards cannot detect or compensate for inhibitors that may be present in the samples.

Data Reporting

RT-PCR-specific errors in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples.[31] This is especially relevant when dealing with in vivo samples that have been obtained from different individuals, or when comparing samples from different tissues.

The most common method for minimizing these errors and correcting for sample-to-sample variation is to amplify a cellular RNA specified by a housekeeping gene that serves as an internal reference against which other RNA values can be normalized.[32] However, because there is no single mRNA with a constant expression level among different tissues of an organism,[33] its use as an internal calibrator is inappropriate.[34] rRNA has been proposed as an alternative normalizer,[35] but there are serious concerns regarding its expression levels, transcription by a different RNA polymerase, and possible imbalances in relative rRNA-to-mRNA content in different cell types that caution against its use as a normalizer.[36] Copy numbers can also be normalized to total cellular RNA and reported as copies per microgram of total RNA.[24] However, total RNA levels may be increased in highly proliferating cells, and this will affect the accuracy of any comparison of copy numbers between normal and tumor cells.

BIOLOGICAL RELEVANCE

Biopsies contain a range of different cell types—a problem exacerbated in heterogeneous tumor samples that include normal and inflammatory cells as well as diversely evolved cell populations. In addition, normal cells adjacent to a tumor may be phenotypically normal but genotypically abnormal, or exhibit altered gene expression profiles because of their proximity to the tumor.[37] Hence expression profiling of such biopsies provides a composite of the whole population, and this may result in the masking of the expression profile of a specific cell type, or it may be ascribed to and dismissed as illegitimate transcription.[38]

Laser capture microdissection (LCM) is useful for accurate expression profiling from such biopsies[39] and has become a powerful technique for extracting pure subpopulations of cells from heterogeneous in vivo cell samples for detailed molecular analysis.[40] Isolation of

RNA from such small samples is possible, and mRNA expression levels can be accurately and reproducibly quantified,[41] even from archival paraffin-embedded tissue specimens[42] and after immunohistochemical staining.[43]

APPLICATIONS

The increasing utility of real-time RT-PCR promises a paradigm shift in molecular clinical diagnostics. Its ability to detect the nucleic acid of a pathogen allows it to identify the actual causes of a disease, as opposed to merely detecting its symptoms. Alterations in mRNA expression profiles are associated with a tissue's reaction to pathological states or drug treatments, and are likely to prove useful for more accurate postoperative staging of cancer patients. Its speed, simplicity, specificity, and sensitivity make this technique ideally suited for this task, making it a cost-effective and time-efficient assay that could become part of a routine protocol of specimen processing.

However, it is important to be aware that issues such as sample processing, assay standardization, and reproducibility, as well as the use of appropriate diagnostic controls, remain to be resolved before real-time RT-PCR can become a realistic practical diagnostic assay. Furthermore, because data interpretation remains highly subjective, there is a need for strict quality control of the reported results to achieve a consistent, standard, and valid diagnosis based on real-time RT-PCR.

CONCLUSION

Real-time technology has revolutionized the use of, and applications for, RT-PCR assays. However, considerable doubts remain about the reproducibility of real-time RT-PCR data, and statistical analyses of the numerical data may obscure the actual results, leaving considerable scope for misinterpretation. Although there can be no doubt of its value as a research tool, its use as a routine clinical diagnostic tool remains unproven.

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Restriction Fragment Length Polymorphism (RFLP)—Application for Mycobacteria Typing

Karim Chemlal
Isdore Chola Shamputa
Nabil El AiLa
Leen Rigouts
Françoise Portaels
Institute of Tropical Medicine, Antwerp, Belgium

INTRODUCTION

Outbreaks of infectious diseases often result from exposure to a common source of the etiologic agent. Generally, the etiologic agent involved in an outbreak of disease is derived from a single cell whose progeny are genetically identical or closely related to the source organism. In epidemiological terms, the organisms involved in the outbreak are clonally related; that is, they have a common origin. Clonally related organisms are members of the same species that share virulence factors, biochemical properties, and genomic characteristics. However, there is sufficient diversity at the species level that organisms isolated at different times and in different geographical areas may be differentiated or classified into subtypes or strains.

OVERVIEW

The process of subtyping is epidemiologically important for recognizing outbreaks of diseases, detecting the cross-transmission of nosocomial pathogens, determining the source of infection, and in some cases recognizing particularly virulent strains of organisms.

Subtyping or strain classification has been accomplished by a number of different approaches. In recent years, the development and extensive use of high-resolution molecular typing systems based on direct analysis of genomic polymorphism have greatly improved the understanding of the epidemiology of infectious diseases.[1,2] These molecular typing methods can be applied to answer a number of different questions, such as:

In an outbreak, what is the extent and mode of transmission of the epidemic clone(s)?

In long-term surveillance, what is the prevalence over time and the geographic spread of epidemic and endemic clones in the population?

A large number of molecular typing methods are available for a wide range of microorganisms and can provide a good epidemiological tool. However, the rapid diversification and incomplete comparative evaluation of some of these methods leave the microbiologist and the epidemiologist faced with a number of questions dealing with selection of appropriate typing system(s), to address a particular problem, as well as a lack of consensus about interpretation and communication of results.

Several criteria have been proposed for evaluating the performance of typing systems.[1,2] These criteria include typeability, reproducibility, stability, and discriminatory power.

- *Typeability*. Typeability refers to the proportion of isolates that can be scored in the typing system and assigned to a type.
- *Reproducibility*. Reproducibility refers to the ability of the typing system to assign the same type on repeated testing of the same strain.
- *Stability*. Stability is based on the biological features of clonally derived isolates to express constant markers over time and generations.
- *Discriminatory power*. This is a key characteristic of typing systems, as it estimates the probability that isolates sharing identical or closely related types are truly clonal and part of the same chain of transmission.

Additional comparative studies are needed to establish the relative value of systems currently used for typing microbial pathogens. Moreover, there are important variations in the performance of any given method depending on the species and on modifications of the procedure as applied by different investigators.

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SOUTHERN BLOTTING AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) METHODS

Technical Description

Southern blotting has been used for many years to detect and locate genomic sequences from a variety of prokaryotic and eukaryotic organisms. For gene detection, whole chromosomal DNA is digested with a restriction enzyme, and the fragments are separated by electrophoresis through an agarose gel. The separated fragments are transferred from the agarose gel to either a nitrocellulose or a nylon membrane by Southern blotting (Fig. 1). The membrane-bound nucleic acid is then hybridized to one or more labeled probes homologous to the gene to be examined. Probes can be labeled with a number of detectable moieties, including colorimetric and chemiluminescent enzymes to bus with the appropriate substrates. This classical method has been adapted to differentiate bacterial strains on the basis of the observation that the location of various restriction enzyme recognition sites within a particular genetic locus of interest can be polymorphic from strain to strain, resulting in gel bands that differ in size between different strains. Thus the name restriction fragment length polymorphism (RFLP) refers to this polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. Only the genomic DNA fragments that hybridize to the probes are visible in RFLP analysis, which greatly simplifies the analysis. Different types of nucleic acid probes can be used for typing: 1) genes encoding metabolic, virulence or resistance functions; 2) multicopy elements, including insertion sequences (IS) and transposons. Insertion sequences typing technique using insertion sequences as a probe are in general very reproducible and can provide a highly discriminating typing tool. Discrimination is related to the presence of multiple copies of these elements at diverse locations in the chromosome. However, RFLP is a slow and labor-intensive technique requiring specialized equipment and expertise. Besides, careful selection and optimization of probe sequence, restriction endonucleases, electrophoresis, and hybridization conditions need to be developed for each species or pathovar to be typed. Furthermore, large-scale application of RFLP analysis requires international standardization of the technique, reagents, type strains, and nomenclature. In the field of *Mycobacterium tuberculosis* this was established by several public health reference laboratories for IS*6110* RFLP-fingerprinting, which integrates standard computer analysis of patterns and a common database, and it is now widely applied for large-scale surveillance of tuberculosis.[3]

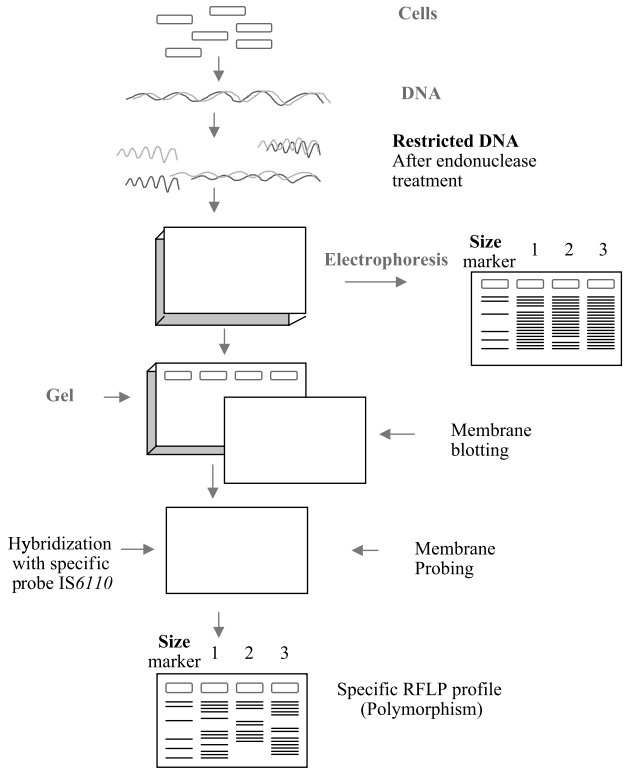

Fig. 1 Schematic representation of RFLP technique. (*View this art in color at www.dekker.com.*)

Example of Application of RFLP (*Mycobacterium tuberculosis*)

The most commonly used typing system for *M. tuberculosis* is IS*6110*-based RFLP,[4] so far considered the gold standard for TB typing. IS*6110* is an IS3-like element, also known as IS*986* or IS*987*. It occurs at various locations in the genome of *M. tuberculosis* in variable copy numbers.[5,6] As its sequence is apparently invariant,[7] it is an ideal target sequence. The copy number of the element ranges from 1 to 25 copies per genome, although rare *M. tuberculosis* cultures have been reported with no inserts. To visualize IS*6110* RFLP patterns, DNA is extracted and purified from bacterial culture. Thereafter, the DNA is digested with the restriction enzyme *Pvu*II, the restriction fragments are separated on an agarose gel and transferred to a DNA membrane. A peroxidase-labeled probe with a DNA sequence complementary to the IS*6110*-DNA sequence is added in order to visualize the IS*6110* containing restriction fragments, by chemiluminescence, which is initiated by adding two substrates. Restriction fragment length polymorphism

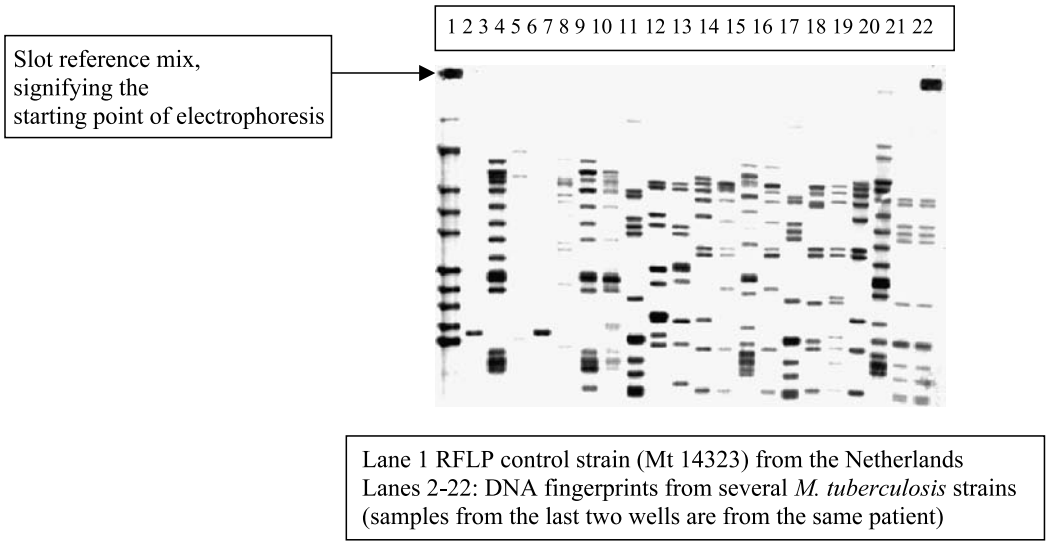

Fig. 2 Representative RFLP profiles of *M. tuberculosis* using IS*6110* as a probe.

patterns are detected by placing a light-sensitive film on the wrapped membrane in a light-blocked cassette[8] (Fig. 2). Restriction fragment length polymorphism has been used to confirm outbreaks of tuberculosis or incidents of suspected laboratory cross contamination,[9] to investigate the question of reinfection vs. reactivation, to examine the existence of multiple infections, and to track the spread of multidrug resistance.[10] DNA fingerprints of *M. tuberculosis* do not change during the development of resistance to various antituberculous drugs.[11] However, there are a number of notable limitations with standard IS*6110* typing. For example, it is labor intensive and thus costly. The typing patterns produced have varying numbers of bands which may also vary in position, so that sophisticated software is needed to analyze large numbers of patterns and to compare results between laboratories.[12] Furthermore, RFLP typing of strains with a low copy number of IS*6110* is not sufficiently discriminatory.[6,13,14] Additional genetic typing method in cases where *M. tuberculosis* isolates contain fewer than five IS*6110* copies is necessary.

In addition to IS*6110*, many other different DNA sequences that have been used as probes for typing *M. tuberculosis* complex strain by RFLP include the major polymorphic tandem repeat (MPTR), the polymorphic GC-rich sequence, the direct repeat region, $(GTG)_5$, repetitive DNA elements, and rDNA (ribotyping).[6,15,16]

Application for Other Organisms

Ribotyping is the most versatile and the most widely used strategy of Southern blot analysis of bacterial genome polymorphism. The evolutionary conservation of ribosomal RNA makes it applicable as a universal bacterial probe. Many important pathogens, including *Enterobacteriaceae*, *Listeria*, *Pseudomonas* spp., and staphylococci have more than five ribosomal operons and thus produce ribotype patterns of 5 to 15 bands.[17] Ribotyping is a robust method that exhibits excellent reproducibility and stability, during the course of the outbreaks. It is commercially available and fully automated and well standardized. However, its discriminatory power is only moderate, and this is related to the fact that ribosomal operons cover less than 0.1% of chromosomal length and tend to cluster in one particular region of the genome. Discrimination of ribotyping depends on the species and on the choice and number of restriction endonucleases used. No consensus has been achieved on optimal procedure and no general rules are available for interpretation of technically problematic results, such as weakly hybridizing fragments.

LOCUS SPECIFIC PCR-BASED RFLP

Technical Description

In recent years, a number of PCR-based strategies have been developed for strain discrimination of microbial pathogens. In PCR *gene* RFLP *typing*, a target sequence, 1 to 2 kb long and known to show polymorphism among strains of species of interest, is amplified at high stringency. The amplified product is cut with restriction endonucleases and isolates are compared by RFLP pattern on an agarose gel after staining with ethidium bromide avoiding the need for Southern blot.

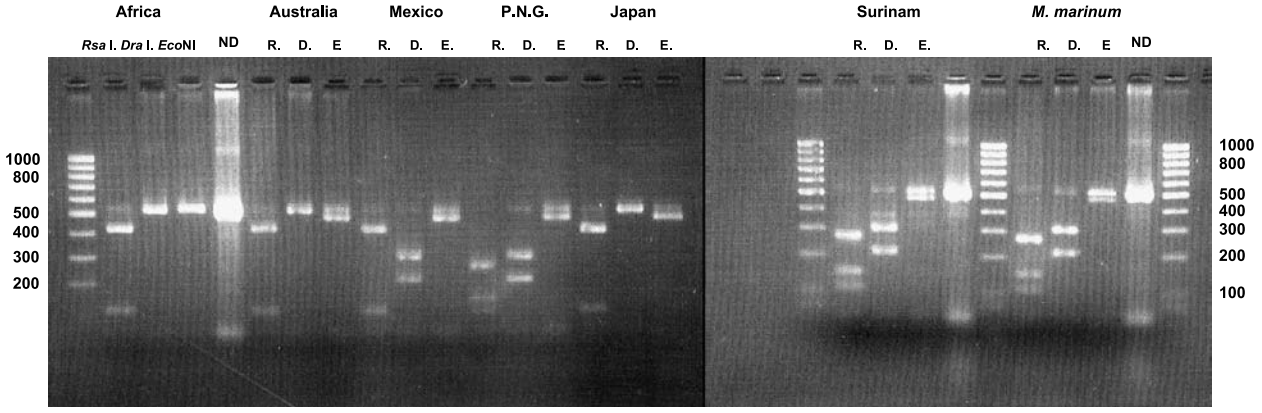

Fig. 3 Examples of PCR restriction profiles obtained from a representative set of *M. ulcerans* (from different geographic areas) and one *M. marinum* strain by using three restriction enzymes, *Rsa*I, *Dra*I, and *Eco*NI. The first and last lanes show the 100-bp ladder. ND, no digested PCR product; R, D, and E, *Rsa*I, *Dra*I, and *Eco*NI, respectively, P.N.G., Papua New Guinea.

Application for Mycobacteria (*M. tuberculosis*, *Mycobacterium ulcerans*, and *Mycobacterium marinum*)

In the field of *M. tuberculosis*, PCR-based RFLP is not so widely applied as the classical RFLP method. In a novel application of locus-specific RFLP, Cockerill et al.[18] have identified point mutations in the *katG* gene of *M. tuberculosis* that correspond to different levels of resistance to isoniazid by comparing the different RFLP banding pattern produced by the amplified gene. However, the discriminatory power is limited compared to other methods.

M. ulcerans and *M. marinum* are slow-growing mycobacterial species with optimal growth temperatures of 30°C to 33°C. These organisms are emerging necrotizing mycobacterial pathogens that reside in common reservoirs of infection and exhibit striking pathophysiological similarities. The interspecific taxonomic relationship between the two species is not clear as a result of the very high phylogenetic relatedness. To help understand the genotypic affiliation between these two closely related species, another approach of RFLP has been performed; namely, PCR restriction profile analysis (PRPA).

By targeting the 3′ end of 16S rRNA gene and by using three restriction enzymes, a set of geographically diverse *M. ulcerans* and *M. marinum* have been investigated.[19] The results (Fig. 3) showed that *M. ulcerans* can be typed at both intra- (three subtypes related to the geographical origin) and interspecific levels (differentiation between *M. marinum* and *M. ulcerans*). However, the major limitation of the technique is related to its discriminatory power as it cannot usually differentiate between these two species (Fig. 3); this may be related to the high degree of conservation of the mycobacterial 16SrRNA genes.

Application for Other Organisms

This new RFLP approach has been applied in a number of situations. Shortridge et al.[20] used the RFLP of the *ureC* gene to demonstrate the genetic diversity of *Helicobacter pylori* strains in the United States. The 16S, 23S, and 16S–23S spacer regions have also been used as targets for locus-specific RFLP.[21] In this variation of ribotyping, the ribosomal DNA is amplified and subjected to digestion with restriction enzyme, and the DNA fragments are visualized following separation by gel electrophoresis avoiding the need for Southern blotting.

Gene-specific probes have been used to subtype *Brucella* species,[22] *Legionella pneumophila*,[23] and *Pseudomonas aerugenosa*.[24] Furthermore, ribotyping has been applied successfully in many studies to differentiate bacterial strains.[25,26] Other applications of locus-specific RFLP found place in epidemiological studies of hepatitis C virus (HCV). By this technique, the virus can be subtyped into six major genetical groups.[27] The RFLP of the 5′ untranslated region has facilitated studies of the geographical distribution of viral genotypes and natural history of the disease.

CONCLUSION

Restriction fragment length polymorphism strategies are undergoing rapid technical improvements to circumvent

the different limitations (labor intensity, rapidity, and cost). Advances in the understanding of biological basis of microbial biodiversity at subspecies levels will improve the conceptual framework required for proper epidemiological interpretation of typing results. Wider application of these systems should shed more light on the epidemiology of hospital- and community-acquired infections, and therefore allow for more effective prevention and control strategies.

ACKNOWLEDGMENTS

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Retinoblastoma

Dietmar Lohmann
Universtiätsklinikum Essen, Essen, Germany

INTRODUCTION

Retinoblastoma is childhood cancer of the eye that originates from developing retina and affects one per 20,000 newborns. Familial aggregation of this retinoblastoma has been recognized in the early 19th century and, since then, this tumor has served as a model for familial cancer. For some time it was assumed all cases of retinoblastoma are hereditary and follow an autosomal dominant pattern of inheritance. Later it was recognized that most patients with tumors in one eye only, which account for about 60% of cases, have a nonhereditary form of this disease.[1] Knudson's seminal two-mutation model set the framework to explain the development of both hereditary and nonhereditary retinoblastoma.[2] His theory also guided the discovery of the causative gene, the RB1.[3] This tumor suppressor gene was the first of a now long list of genes that can cause hereditary predisposition to cancer.

CLINICAL ASPECTS

Diagnosis

Diagnosis of retinoblastoma is usually made in children under the age of 5 years. In most children, the first presenting sign is a white pupillary reflex (leukocoria) or strabismus. Diagnosis of retinoblastoma is usually established by examination of the fundus of the eye using indirect ophthalmoscopy. Most children have retinoblastoma in one eye only (unilateral retinoblastoma, 60% of cases).[1] Occasionally, multiple tumor foci are present in one eye (unilateral multifocal retinoblastoma). About 40% of children with retinoblastoma have tumors in both eyes (bilateral retinoblastoma). Most patients with bilateral retinoblastoma have more than one tumor focus per eye (bilateral multifocal retinoblastoma).

Family History

Patients with unilateral retinoblastoma most often have sporadic disease, i.e., no other case of retinoblastoma has been noted in their family. About 75% of patients with bilateral retinoblastoma are also sporadic; in the remainder (25%) there is a positive family history (familial retinoblastoma). Examination of the fundus of the eye in all first-degree relatives of children with retinoblastoma is required to identify retinal scars or quiescent tumors (retinomas).[4] The presence of such lesions in a relative indicates familial disease.

Therapy and Prognosis

Treatment of retinoblastoma depends on tumor stage, the number of tumor foci (unifocal, unilateral multifocal, or bilateral disease), localization and size of the tumor(s) within the eye, presence of vitreous seeding, and the age of the child. Treatment options include enucleation, external-beam radiation, systemic chemotherapy, cryotherapy, photocoagulation, brachytherapy with episcleral plaques. Following successful treatment, children require frequent follow-up examinations for early detection of new intraocular tumors. If the tumor(s) have not invaded extraocular tissues, treatment is favorable in most patients.

Second Tumors

Patients who are predisposed to retinoblastoma have an increased risk of specific neoplasms outside of the eye (second tumors). The spectrum of second tumors includes osteogenic sarcoma, soft-tissue sarcoma, and malignant melanoma.[5] Patients who have received external beam radiation for treatment of bilateral retinoblastoma have a higher risk of second tumors.

GENETICS OF RETINOBLASTOMA

Retinoblastoma Is Caused by Two Mutations

In 1971 Knudson hypothesized that two mutational events are sufficient for development of retinoblastoma (two-mutation model).[2] Later, molecular analysis showed that the two mutations alter both alleles of a single gene, the retinoblastoma gene (RB1).[6] This gene was cloned in

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1986[3] and the finding of inactivating mutations confirmed Knudson's two-mutation hypothesis:[7,8]

- Familial retinoblastoma is caused by germ-line mutations in the RB1 gene. Family members that have inherited a mutant RB1 allele from a parent are at a high risk because only one mutation that alters the remaining normal allele is sufficient for complete functional loss of the retinoblastoma gene. Second mutations can occur in several retinal precursor cells and each of these cells may be the beginning of an independent tumor focus.
- Most patients with sporadic bilateral retinoblastoma are heterozygous for a mutant RB1 allele. This mutation has either occurred de novo in germ-line cells of one of the parents (most often in the germ line of the father) or is inherited from a parent who is a carrier of the mutation but has not developed retinoblastoma (incomplete penetrance). Tumors arise from retinal precursor cells that have acquired mutational loss of the second RB1 allele.
- Mutational inactivation of the RB1 gene is also a prerequisite for tumor development in patients with sporadic unilateral retinoblastoma. In most of these patients, both RB1 gene mutations occur in somatic cells and none of the two somatic mutations is detectable in DNA from peripheral blood.[9]

In recent years, molecular analysis in patients with retinoblastoma has shown that Knudson's original model needs to be extended:

- In some patients with sporadic bilateral or unilateral retinoblastoma the predisposing RB1 gene mutation is present in a mosaic state.[9,10] Mutational mosaicism occurs when the first mutation has occurred de novo during embryonic development of the child. It has to be assumed that in some children with mutational mosaicism the mutation is present in only a sector of retinal precursor cells. Tumor development can only occur in cells that are part of the mutant sector. This may account for the observation that patients with mosaicism for a given mutation develop fewer tumors than patients who are heterozygous for the same mutation.

The Retinoblastoma Gene and Protein

The RB1 gene consists of 27 exons and occupies over 183 kb of genomic sequence on chromosome 13q14. A CpG-island, which is normally unmethylated, is located at its 5′-end. In diverse tissues, the gene is transcribed into a 4.7-kb mRNA, which contains a 2.7-kb open reading frame. Orthologs with a high level of sequence similarity in translated regions of the human RB1 gene have been identified in several vertebrate organisms.

The protein encoded by the RB1 gene, pRb, is a 928-amino acid nuclear phosphoprotein that migrates at 110 kDa in SDS-PAGE when hypophosphorylated. It belongs to a small family of nuclear proteins that includes p107 and p130. These proteins are termed pocket proteins because of significant sequence similarity in two discontinuous regions (pockets A and B). Conditional on the phosphorylation status at multiple serine and threonine residues, this pocket can bind to members of the E2F family of transcription factors as well as to endogenous nuclear proteins that contain the LxCxE peptide motif. The C-terminal region of pRB contains a nuclear localization signal and a cyclin–cdk interaction motif that enables it to be recognized and phosphorylated by cyclin–cdk complexes. In addition, C-terminal region can bind to the nuclear c-Abl tyrosine kinase and to MDM2. One role of pRb is its function as a gatekeeper that negatively regulates progression through the G1 phase of the cell cycle. During the G1 phase of the cell cycle pRb is hypophosphorylated. This form can bind E2F and causes a repression of E2F-mediated transcription. Beginning in late G1 and continuing to the M phase, pRB is phosphorylated by G1 cyclin-dependent kinases. Upon phosphorylation of pRb, E2F is released and promotes transcription of genes that are required for cell division. Consequently, pRB controls cell-cycle phase transition by transcriptional repression. In addition to phosphorylation, cell cycle-dependent acetylation has been found to control pRb function. Acetylation hinders phosphorylation of pRb and enhances binding to the MDM2 oncoprotein. However, besides cell-cycle regulation, pRb has several other roles including control of apoptosis and stimulation of differentiation (for review of pRB functions, see Refs. [11–13]).

SPECTRUM OF RB1 GENE MUTATIONS

Spectrum Mutations That Cause Hereditary Predisposition to Retinoblastoma

Mutational analysis in DNA from peripheral blood identifies predisposing RB1 gene mutations in almost all patients with familial retinoblastoma (>95%) and in most patients with sporadic bilateral retinoblastoma (>85%) (Refs. [14,15] and unpublished results).

- *Large deletions*. About 5–10% of patients with bilateral and almost 5% of patients with sporadic unilateral retinoblastoma have interstitial cytogenetic deletions involving 13q14.

- *Gross deletions*. This class of mutations includes deletions of one or more exons up to the loss of the whole RB1 gene. Recent studies indicate that gross deletions account for 10–20% of predisposing RB1 gene mutations. The location and size of these mutations are heterogeneous.[15]
- *Point mutations*. More than 70% of mutations that predispose to retinoblastoma are single-base substitutions and small-length mutations (database of RB1 gene mutations: http://www.d-lohmann.de/Rb/mutations.html and Refs. [15,16]). Most of them are nonsense or frameshift alterations. Recurrent nonsense mutations are observed at 12 of the 15 CGA codons within the open reading frame. Missense mutations and small in-frame length alterations are infrequent (less than 10% of point mutations) and located in the regions that code for the pocket domains A and B of pRB.

Spectrum Somatic Mutations in Retinoblastomas and Other Tumors

It is possible that all retinoblastomas have mutations in both alleles of the RB1 gene. RB1 gene mutations have also been identified in several other tumor entities including osteogenic sarcoma and small cell lung cancer. The spectrum of somatic mutations comprises that of germ-line mutations with two important additions:

- More than 60% of retinoblastoma show loss of constitutional heterozygosity (LOH) at polymorphic loci located on chromosome 13. In these tumors, one RB1 allele is lost in consequence of deletions or one of several chromosomal mechanisms such as mitotic recombination and nondisjunction.[17]
- Another class of somatic mutation is hypermethylation of the CpG-rich island at the 5′-end of the RB1 gene. Hypermethylation is observed in about 10% of retinoblastomas and causes silencing of the transcription of this gene.[18]

GENOTYPE–PHENOTYPE ASSOCIATIONS

Tumor formation in carriers of predisposing RB1 gene mutations depends on the chance occurrence of second mutations. Because of the influence of stochastic events, a given predisposing RB1 gene mutation is associated with variable expressivity. Statistical analysis of phenotypical variation shows that the distribution of phenotypical expression within most families complies with a Poisson distribution (Böhringer und Lohmann, unpublished). However, there are a few notable exceptions.[19] In patients with sporadic retinoblastoma, the delineation of genotype–phenotype associations is further complicated by mosaicism. As noted above, phenotypical expression in patients who show mosaicism for a given mutation is usually milder compared to patients who are heterozygous for a mutation of the same kind. Analysis of families with retinoblastoma offers the best opportunity to determine differences in phenotypical expression between different classes of predisposing mutations. The number of tumor foci per mutation carriers varies between families. Some families show a high proportion of heterozygous carriers that stay free of retinoblastoma (incomplete penetrance). Moreover, affected members of these ''low-penetrance'' families develop fewer tumor foci when compared to mutation carriers in families with complete penetrance. Most of the variance of penetrance and expressivity between families is due to allelic heterogeneity.

Families with Complete Penetrance

Most families with retinoblastoma show complete penetrance, and, with few exceptions, mutation carriers have bilateral retinoblastoma. Almost invariably, predisposing RB1 gene mutations identified in these families cause premature termination codons.[14] Analysis of RNA from the blood of carriers of such mutations has shown that transcripts from alleles with premature termination codons are less abundant than transcripts from the normal allele. This indicates that these mutant mRNAs can be subject to nonsense-mediated decay. This may explain why oncogenic alleles with premature termination codons in any of exons 2 to 25 show a similar phenotypical expression.[14]

Families with Incomplete Penetrance and Milder Expressivity

In about 10% of families with retinoblastoma, penetrance is incomplete (''low-penetrance retinoblastoma''). In some of these families, less than one in five mutation carriers is affected. Usually, incomplete penetrance is accompanied by milder expressivity, i.e., affected mutation carriers have retinoblastoma in one eye only. The spectrum of mutations in families with incomplete penetrance and mild expressivity is distinct from that of families with complete penetrance and includes:

- *Promoter mutations*. Predisposing RB1 gene mutations have been identified in motifs that bind transcriptions factors. The most likely result of such mutations is a reduced level of structurally normal transcript.[20]

- *Missense and small in-frame mutations*. These mutations result in substitution, deletion, or insertion of one or few amino acids that are part of the A/B pocket of the pRB. Functional studies have shown that mutant pRB expressed from these alleles shows only a partial loss of normal function.[21]
- *Gross in-frame deletions*. Deletions of exon 4[22] and of exons 24–25[23] have been found in extended pedigrees. These mutations result in an in-frame loss of 40 and 58 amino acids that are not part of the A/B pocket.
- *Mutations that result in leaky splice defects*. Splice mutations that affect splice signals in exons or less conserved intronic splice signals can be associated with milder expressivity and incomplete penetrance. One reasonable explanation is that the effect of these mutations on splicing is ''leaky,'' and therefore a part of the mutant transcript is processed into a normally spliced mRNA.[24] This would result in higher levels of normal transcript compared to cells heterozygous for null mutations.

Second Tumors

Carriers of oncogenic RB1 gene mutations also show variable phenotypical expression with regard to the development of second tumors. A higher incidence of second tumors in patients who were exposed to external beam radiation for treatment of retinoblastoma indicates that environmental factors are important.[5] The finding of interfamilial variation of the occurrence of second tumors suggests risk modification by genetic factors.[25]

CONCLUSION

Retinoblastoma, a rare childhood tumor of the eye, has served as the prototype of hereditary cancer predisposition. As predicted from Knudson's two-mutation hypothesis, mutational change in both alleles of the RB1 gene is a prerequisite for tumor formation. Genetic analysis has shown that in most patients with sporadic unilateral retinoblastoma these two mutations have occurred in somatic cells. Most patients with sporadic bilateral retinoblastoma and patients with familial disease are heterozygous for a predisposing RB1 gene mutation that can be transmitted via the germ line. The spectrum of RB1 mutations that can cause retinoblastoma is heterogeneous and includes structural and epigenetic alterations. Mutations that leave intact some of the functions of the encoded protein can be associated with a milder phenotypical expression (''low penetrance retinoblastoma''). It is a challenge for future research to identify genetic factors that modify the risk of retinoblastoma and second cancers in individual mutation carriers.

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Retinoschisis, Juvenile (X-Linked)

R

Maria A. Musarella
SUNY Downstate Medical Center, Brooklyn, New York, U.S.A.

Ian M. MacDonald
University of Alberta, Alberta, Canada

Paul A. Sieving
National Institute of Health, Rockville, Maryland, U.S.A.

INTRODUCTION

X-linked juvenile retinoschisis (RS, OMIM# 312700) is a vitreoretinal degeneration and is a common heritable early onset macular degeneration affecting males. A characteristic cystic spoke-wheel maculopathy is seen in virtually all affected males and results from foveal schisis or splitting within the inner retinal layers. Peripheral retinal schisis, predominantly in the inferotemporal quadrant of the fundus, is present in approximately 50% of cases. Most affected males are discovered with mild visual impairment early in their schooling, but some are identified in infancy with strabismus or nystagmus. The electroretinogram (ERG) is a key diagnostic test for RS. Typically, the ERG of affected males exhibits a selective reduction in the amplitude of the b-wave. The visual prognosis is good in most affected males as long as retinal detachment or vitreous hemorrhage does not occur. Carrier females remain asymptomatic and have no clinical signs.

OVERVIEW

RS is an inherited early onset macular degeneration affecting males.[1,2] *RS1*, the gene that is mutated in RS, encodes a 224-amino acid protein called retinoschisin. As a member of a family of cell adhesion proteins, retinoschisin may maintain cellular organization and synaptic structure of the retina. Retinoschisin contains a highly conserved carboxy-terminal discoidin domain in which mutations cluster, suggesting that the domain is essential for normal function. A number of missense and protein-truncating mutations of *RS1* have been identified; truncation or nonexpression of retinoschisin appears to have greater clinical severity.[3]

Molecular genetic analysis of *RS1* can complement fundus examination and clinical diagnostic testing of at-risk individuals. Mutation analysis may provide a definitive diagnosis where clinical tests and fundus examination are not sufficient to make a diagnosis. Carrier testing can be accomplished by either mutation analysis or linkage analysis with closely linked markers. This can be practically accomplished when sequence analysis has not yet identified a mutation in a particular family. Patients and families should be offered the opportunity of discussing these issues of testing with a genetic counselor.

CLINICAL FEATURES

Haas[4] provided the first clinical description of the disease in 1898. The phenotype of RS and its variability have been described by several authors.[5–8] The predominant phenotype includes foveal and peripheral retinoschisis and vitreous veils of partial retinal layers in the vitreous. Fundus examination may show the Mizuo phenomenon, an inner retinal sheen that occurs with the onset of light exposure after a period of dark adaptation.[9] An area of schisis may leave a retinal vessel unsupported in the vitreous cavity, called a ''congenital vascular veil.'' Affected males are identified between 5 and 10 years of age with poor visual acuity, typically 20/60–20/120.[10] A small number of patients present in infancy with bilateral bullous retinal detachment.[11] Visual impairment may be stable until age 50–60 when macular atrophy results in further central vision loss.[5,10] Acute sight threatening complications such as retinal detachment and vitreous hemorrhage occur in 10% and 5% of patients, respectively. Variation in disease presentation and disease progression is observed among affected members of the same family. Carrier females remain asymptomatic and generally have no signs of the condition.[10] Rarely, examination of the peripheral retina of a female carrier may show white flecks or areas of schisis.[12] The ERG is not able to detect carriers.

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PREVALENCE

The prevalence of RS ranges from 1:5000 to 1:25,000 depending on the population in which one ascertains cases. RS has a worldwide distribution across all races.

CLINICAL DIAGNOSTIC TESTING

The full field ERG of a male affected by RS shows a selective reduction of the amplitude of the dark-adapted b-wave and a normal amplitude and latency of the a-wave, leading to a reduction of the b/a ratio.[13,14] Recently, the multifocal ERG (mfERG) has been introduced as a clinical test of central retina function. The mfERG can be helpful as a diagnostic test for RS in patients where a significant reduction of the b-wave amplitude cannot be demonstrated with a full field ERG. In one such case, the waveforms of the mfERG showed a reduction in amplitudes of the central waveforms.[14–16] Optical coherence tomography (OCT) is a new technique that allows one to view a two-dimensional profile of the macula. Areas of retinal schisis can be identified in multiple retinal layers by OCT in eyes with RS.[14,15,17,18] These OCT findings are in contrast to the previous clinical understanding that the schisis separation occurred only in the innermost retinal layers.

MOLECULAR GENETICS OF RETINOSCHISIS

Retinoschisis was first mapped to Xp22 and then the *RS1* gene was identified by positional cloning.[19] *RS1* contains six exons and encodes a 224-amino acid protein called retinoschisin. Studies indicate that the protein is secreted by retinal cells as a disulfide-linked oligomeric protein complex, and that it is associated with the cell surface of all retinal neurons except adult horizontal cells.[20–22] Retinoschisin may function as a cell adhesion protein to maintain the integrity of the central and peripheral retina. The polypeptide consists of a leader sequence with a putative signal peptidase cleavage site and a highly conserved region in exons 4–6 known as a discoidin domain.[19] Discoidin domains are present in a family of extracellular or transmembrane proteins implicated in cell–cell adhesion or cell–matrix interactions—functions which correlate well with the observed splitting of the retina in RS.[23,24] Mutations cluster within the discoidin domain, exons 4–6, suggesting that this domain is essential for the normal function of retinoschisin.[25,26] Retinoschisin contains a high number of cysteine residues, 10 in total. Most mutations are missense mutations located in the discoidin domain of retinoschisin, with over 25% involving the loss or gain of a cysteine residue. Over 125 different missense, nonsense, insertions, deletions, and splice-site mutations in the RS1 gene have been associated with RS. An up-to-date listing of these mutations is maintained in RetinoschisisDB of The Retinoschisis Consortium (http://www.dmd.nl/rs/index).

MOLECULAR GENETIC TESTING

RS1 is the only gene known to be associated with RS. Molecular genetic analysis is an effective approach for the early detection of at-risk males, carrier detection, and prenatal diagnosis.[27] The phenotype of RS is sufficiently variable that Eksandh et al.[28] emphasized the importance of supplementary molecular genetic testing in boys with visual failure of unknown etiology. Three mutations within the *RS1* gene, E72K (214G>A), G74V (221G>T), and G109R (325G>C), are associated commonly with RS in individuals of Finnish descent. Approximately 95% of patients of Finnish heritage have one of these three founder mutations.[29] Mutation analysis for these mutations is available on a clinical basis. Sequence analysis of the six exons of the *RS1* gene identifies nearly 90% of males with a clinical diagnosis of RS, and mutational analyses are available at various clinical centers (http://www.geneclinics.org).[27]

HISTOPATHOLOGY

Historically, the histopathological findings in RS were considered to be primarily within the nerve fiber layer with degeneration of photoreceptors, thinning of the ganglion cell layer, and a focally absent or proliferative retinal pigment epithelium.[30–32] Newer clinical analysis based on noninvasive OCT shows lamellar dissections through retinal layers at multiple levels.[17]

MOLECULAR GENETIC PATHOGENESIS

For many years, RS was thought to be a result of a defect in the Muller cell that acted as a cellular scaffold within the retinal architecture. Recent studies on gene expression and immunolocalization of retinoschisin indicate that it is expressed in all major classes of adult retinal neural cells, with the possible exception of horizontal cells, and that there is no retinoschisin detected in Muller cells or processes,[22] although this is reported by others.[33,34] *RS1* is abundantly expressed in the inner segments of photoreceptors in human and mouse eye sections.[21,22,32] The exact cause of the retinal separation in RS is not known. To address this and understand the pathological basis of RS, Wang et al.[35] conducted in vitro experiments and

reported that the cause of RS is due to intracellular retention of the majority of mutant proteins. The phenotype may depend on the secretory capacity of the cells and the degree to which they may induce the unfolded protein response. Another study showed that misfolding of the discoidin domain, defective disulfide-linked subunit assembly, and inability of retinoschisin to insert into the endoplasmic reticulum membrane as part of the protein secretion process are all responsible for the loss in function of retinoschisin as a cell adhesion protein and lead to the development of RS.[36] Other investigators suggest that the mutant retinoschisin may be functionally defective leading to schisis formation.[32,37] A study of retinoschisin during development of the mouse retina showed that retinal ganglion cells are the first to express RS protein, by postnatal day P1; RS expression subsequently occurs in posterior retinal layers over a 14-day developmental period, indicating the presumptively important role of this protein in early eye development.[22]

MOUSE MODEL

To gain further insight into the function of the *RS1* gene and its role in the cellular pathology of RS, Weber et al.[38] generated knockout mice deficient in the mouse gene, *RS1h*. The $RS1h^{-/Y}$ male mouse has a retinal phenotype which closely resembles the human disease. These mice show generalized disruption of retinal cell layer architecture, splitting of the inner retinal layer with gaps between bipolar cells, and disruption of the synapses between photoreceptors and bipolar cells. These findings suggest that retinoschisin may be directly or indirectly involved in retinal cell layer architecture and structural properties of the retinal synapse.

CONCLUSION

Retinoschisin is a member of the discoidin domain family of cell adhesion proteins. Therapeutic intervention in RS may target restoring the function of retinoschisin in the retina or providing a substitute adhesive that prevents the separation of the retinal layers. Gene therapy is being attempted in the mouse model. Molecular genetic analysis of the *RS1* gene will be a necessary step before initiating clinical trials with or without gene therapy to treat RS patients.

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Retroviral Vectors

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Erin L. Weber
Paula M. Cannon
Childrens Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, California, U.S.A.

INTRODUCTION

The goal of gene therapy is to treat disease by the introduction of new genetic material into affected or susceptible cells of the body. Gene therapy has been proposed as a treatment for a variety of disorders. Monogenic diseases may be treated simply by replacement of the defective gene with a functional copy. Alternatively, the new genetic material may selectively eliminate tumor cells, provide protection against viral infection, or stimulate the immune system against a specific antigen.

Initiated in 1990, the first gene therapy clinical trial aimed to introduce functional copies of the adenosine deaminase gene into deficient T cells using a retroviral vector.[1] Today, more than 600 clinical trials have been approved, a third of which employ retroviral vectors. The website for the *Journal of Gene Medicine* (http://www.wiley.co.uk/genetherapy/clinical/) provides helpful statistical summaries of approved gene therapy clinical trials to date. The majority of proposed gene therapy treatments are still being evaluated as phase I trials; only one phase III clinical trial has been conducted using retroviral vectors.[2] This trial involved injection of glioblastoma multiforme tumors with murine producer cells to yield in situ production of retroviral vectors expressing the thymidine kinase suicide gene. Unfortunately, therapeutic effectiveness was not improved over standard treatments of surgical resection and radiotherapy.

In general, despite major improvements in vectors since their first clinical use, the therapeutic effectiveness of retroviral vectors remains limited. Advantages and limitations of retroviral vectors, as well as current areas of research, will be discussed in the following review.

OVERVIEW

Retroviral Life Cycle

Retroviruses are RNA viruses containing two copies of a single-stranded RNA genome encapsidated by a protein core and outer lipid layer or envelope. The retroviral genome codes for three basic polyproteins. The *gag* gene codes for structural proteins of the viral core, whereas the *pol* gene generates viral protease, reverse transcriptase, and integrase. The third gene, *env*, codes for a glycoprotein which, following incorporation into the outer lipid layer, directs the retrovirus to bind to receptors on target cells, triggering fusion of the viral lipid envelope with the cell membrane. Following entry of the retroviral core into the cell, the viral RNA genome is copied into a double-stranded DNA provirus by reverse transcriptase. The proviral DNA is then inserted into the host genome by the integrase enzyme, and viral proteins are synthesized from the integrated provirus using cellular machinery (Fig. 1).

Retroviral Vectors

Stable integration of the viral genome into host chromosomes allows the possibility of long-term gene expression, making retrovirus-based vectors prime candidates for correction of a variety of deficiencies. The most common retroviral vectors used for gene therapy clinical trials are based on the murine leukemia virus (MLV). The most current vector system is composed of three components: a vector genome which contains only those viral sequences necessary for packaging, reverse transcription, and integration, and which can accept approximately 6–8 kb of exogenous DNA; a packaging construct which provides Gag and Pol proteins in trans but is not packaged due to a deletion in the packaging signal (ψ); and an envelope construct which codes for the Env protein (Fig. 2).[3] Removal of viral genes from the vector genome and their provision in trans generates replication-defective viral particles, capable of delivering genes to a cell but incapable of subsequent rounds of replication.

LIMITATIONS OF RETROVIRAL VECTORS

For retroviral vectors to be maximally effective, several limitations need to be addressed. First, there is a limited vector size which can be efficiently packaged within the viral core. Additionally, the presence of two copies of the

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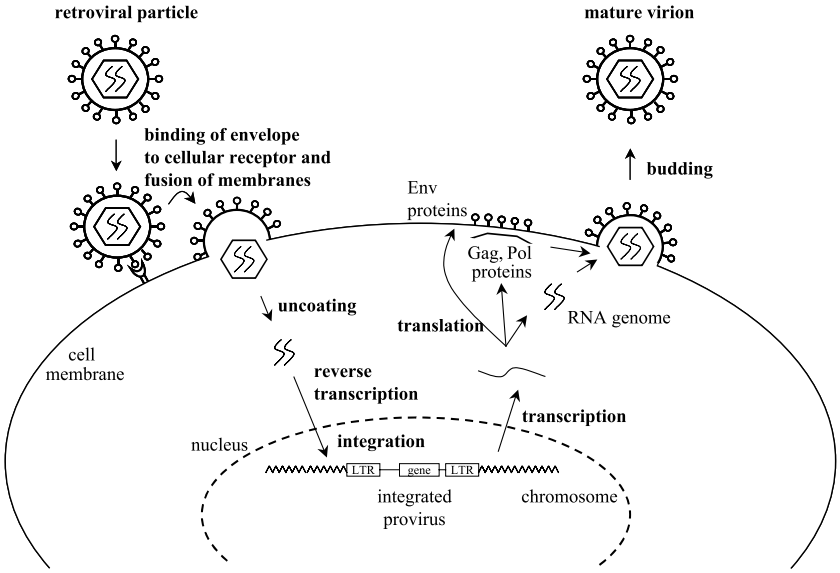

Fig. 1 The retroviral life cycle begins with interaction of the viral envelope with its host receptor, followed by fusion of viral and cellular membranes and entry of the viral core. The viral core disassembles (uncoating) and the viral genome is reverse transcribed into DNA, enters the nucleus, and is integrated into the host genome. The integrated provirus is transcribed by cellular transcriptional machinery, and Gag, Pol, and Env proteins are synthesized. Viral proteins and the newly transcribed viral genome colocalize at the cellular membrane to form viral particles which bud into the extracellular space.

vector genome and the nature of the inserted sequences themselves may contribute to vector instability and rearrangements during reverse transcription. Beyond these intrinsic limitations of viral structure, improvements are required in three major areas: transduction efficiency, gene expression, and safety.

IMPROVING TRANSDUCTION EFFICIENCIES

The number of cells transduced, or infected, by retroviral vectors is often insufficient to produce a therapeutic effect. Murine leukemia virus vectors require breakdown of the nuclear membrane during cell division for entry

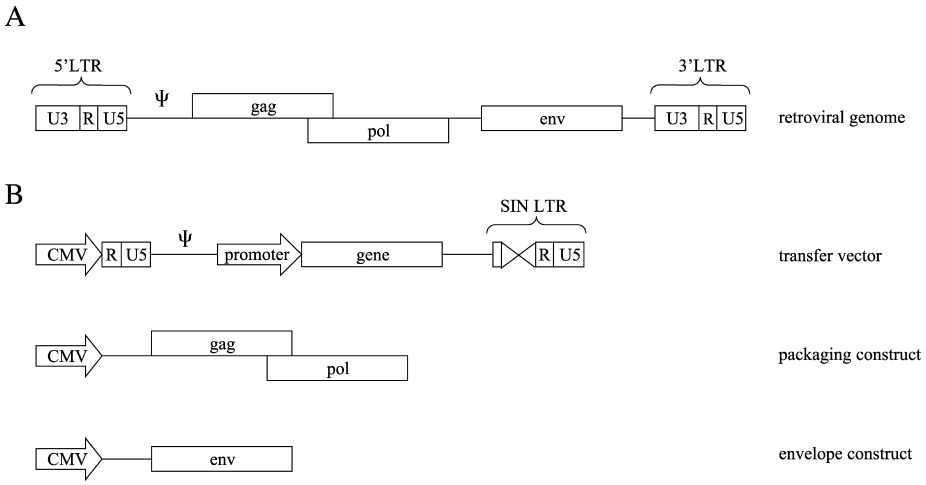

Fig. 2 A. The retroviral genome is composed of three genes, *gag*, *pol*, and *env*, flanked by long terminal repeats (LTR) which contain sequences necessary for initiation and termination of transcription as well as signals for integration into chromosomal DNA. The packing signal, ψ, is required for packing of the retroviral genome into virions. B. Production of retroviral vector particles is achieved by cotransfection of three plasmids and expression of the viral proteins in trans. The transfer vector is the only sequence packaged into the vector particle as the packaging signal has been removed from the other constructs. Safety of the transfer vector has been improved by deletion of viral enhancer and promoter sequences from the 3′LTR, creating a self-inactivating (SIN) vector with no active viral promoters.

into the nucleus and integration into the genome, thus limiting the number of cells that can be transduced. The traditional approach for enhancement of MLV vector transduction efficiencies has been stimulation of cell proliferation by the addition of various growth factors ex vivo.[4] Alternatively, lentiviruses, a subclass of retroviruses, possess nucleophilic signals which permit entry into the nuclei of nondividing cells. However, lentiviral-based vectors are not yet widely used in clinical trials.

The entry of a retrovirus into a cell is also largely determined by binding of the envelope glycoprotein to its cellular receptor, and the distribution of the receptor on various cell types will influence the vector's host range. Envelope proteins from heterologous retroviruses can be used to create pseudotyped vectors with broadened host ranges. For example, pseudotyping with the VSV-G rhabdovirus envelope protein is a common technique used to improve stability and extend the host range of retroviral vectors.[5]

Although pseudotyping and stimulation of cell proliferation have been successful in improving transduction efficiencies of particular cell types ex vivo, such expansion of viral host range may not provide the cell specificity required for gene delivery in vivo. To improve transduction efficiencies in vivo, several methods involving modification of natural retroviral envelopes have been explored to target vectors to a specific cell type (Fig. 3). The retroviral Env protein is composed of two subunits, the surface (SU) protein containing the receptor recognition domain and the transmembrane (TM) protein which anchors the complex within the viral lipid envelope. Binding of SU to its receptor is thought to trigger conformational changes which result in exposure of a fusion peptide on the TM subunit and subsequent fusion of viral and cellular membranes. Most attempts to reengineer the receptor binding site of the SU subunit have involved replacement of the natural receptor-binding domain with a ligand or single-chain antibody with tropism for an alternative cell surface molecule and have resulted in a lack of virus–cell fusion.[6] Some strategies have resulted in limited success, such as the replacement of the MLV Env receptor-binding surface of SU with the peptide ligand, SDF-1α, which resulted in transduction of human cells via the SDF-1α receptor, CXCR4.[7] However, transduction efficiencies were low.

A second approach to Env targeting, called tethering, concentrates vectors on the surface of specific cell types by the addition of a second binding moiety to the natural Env protein. The native receptor binding domain remains functional and mediates virus–cell fusion. The insertion of a von Willebrand factor-derived collagen-binding sequence at the N-terminus of the MLV envelope has resulted in improved gene delivery in vivo to sites of exposed extracellular matrix within tumor vasculature following systemic administration in mice.[8]

An alternate approach, the use of adaptor proteins which function as a bridge between the native MLV Env and cell surface receptors, has generally failed due to a lack of virus–cell fusion. However, greater success has been observed with the avian leukosis virus (ALV) Env protein. Use of an adaptor protein consisting of the single-chain antibody, MR1, which binds to a tumor-specific form of the EGF receptor, joined to the extracellular domain of the natural ALV receptor resulted in successful

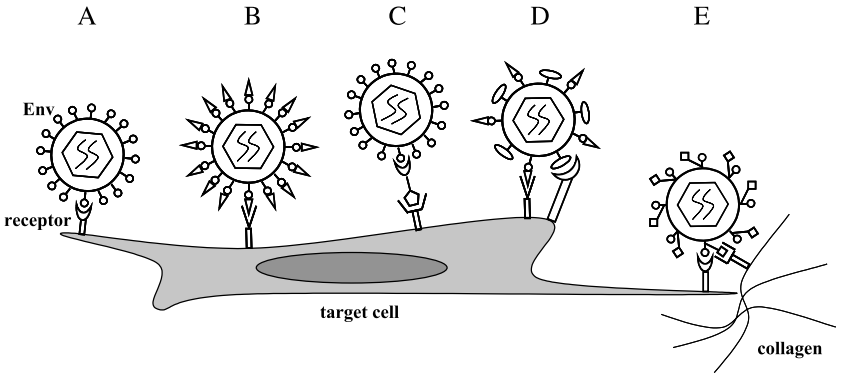

Fig. 3 Efforts to improve transduction of distinct cell populations have involved modification of viral envelope proteins. A. Retroviral infection begins with interaction of the native retroviral Env with a susceptible cellular receptor. B. Replacement of the native Env receptor binding domain with a peptide ligand may direct retroviral binding to a specific receptor. C. Adaptor proteins contain ligand binding sites from natural Env receptors linked to alternative ligands in an effort to direct retroviral binding to specific receptors. D. Trans-complementation of binding-defective influenza hemagglutinin proteins and fusion-defective retroviral Env proteins expands the retroviral host range while circumventing fusion defects often seen with retroviral envelopes possessing an alternative ligand at the receptor binding site. E. Attachment of a peptide ligand to the retroviral envelope protein outside of the receptor binding domain allows concentration of retroviral particles in the vicinity of target cells by interaction with receptors on nearby structures, e.g., collagen.

targeting of ALV-pseudotyped MLV vectors to cells expressing the tumor-specific EGF receptor.[9]

Finally, trans-complementation represents a strategy for circumventing the fusion defect. Orthomyxoviruses and paramyxoviruses possess two distinct envelope proteins which mediate binding and fusion functions separately, suggesting that segregation of the two functions may be possible for retroviral vectors as well. Lin et al. have demonstrated improved transduction efficiency into cells expressing Flt-3 by coexpression of a binding-defective influenza hemagglutinin protein and a fusion-defective MLV Env protein possessing the Flt-3 ligand at the receptor binding domain on an MLV vector.[10]

As an alternative method to improve transduction of tumors in vivo, the use of replication-competent retroviral (RCR) vectors has recently become of interest. As the majority of cells in the body are quiescent, transduction by MLV-based retroviral vectors should be selective for tumor cells. Following the initial transduction event, RCR vectors would replicate, essentially turning each transduced cell into a virus producer cell, and perpetuating gene delivery throughout the tumor. Importantly, RCR vectors typically contain a suicide gene which is toxic to the transduced cell following prodrug administration, thus eliminating tumor cells and minimizing the spread of virus to noncancerous cells. Injection of human glioma cell tumors in rats with MLV-based RCR vectors delivering the cytosine deaminase suicide gene showed 70–90% transduction rates, compared to 1% transduction with replication-defective vectors, and 100% survival of the rats for at least 60 days.[11] The RCR vectors were not detected in other tissues.

Improving Gene Expression

Variable levels of gene expression are observed following integration of current retroviral vectors into target cells. These can be attributed to chromosomal position effects at the site of integration, as well as limitations of vector function. Depending on the site of integration, vector genes may be silenced by a closed chromatin environment or subject to inappropriate regulation by host elements. Expression of retroviral vectors is restricted in several cell types, including embryonic stem cells and murine fibroblasts, as a result of the association of repressive factors with sequences in or near the 5′LTR and methylation of viral promoter elements. Additionally, host cells have a tendency to recognize foreign promoters, especially strong viral promoters such as SV40 and CMV typically used to drive expression of the gene of interest, and inactivate them by various mechanisms, including methylation. Even if gene expression remains active, transduced cells often lose viability over time because of an immune response elicited by the product of the delivered gene.

Replacement of repressive viral sequences with similar sequences from heterologous retroviruses and the minimization of CpG dinucleotides within viral promoters has lessened, but not eliminated, silencing of transgene expression.[12] Silencing of viral promoters has sparked an interest in the use of gene-specific regulatory sequences to direct transgene expression. Logg et al. have reported prostate-specific gene expression which persists over time by incorporation of the prostate-specific probasin promoter into RCR vectors, demonstrating the benefits of improved gene expression and tissue-specificity achieved with an authentic human promoter.[13] Incorporation of gene-specific locus control regions (LCR) into retroviral vectors confers integration site-independent and tissue-specific vector gene expression.[14] Additionally, the use of authentic genomic elements such as scaffold or matrix attachment regions and insulator elements has been reported to improve transgene expression when included in a retroviral vector.[15]

Improving Vector Safety

As retroviral transduction efficiencies and gene expression levels improve, so will the potential for serious adverse effects including retroviremia, generation of replication-competent revertants, germline transmission, and insertional mutagenesis. The recent demonstration that retroviral vectors favor integration sites within transcriptionally active loci has increased the probability of insertional mutagenesis caused by vector integration.[16] Additionally, the development of leukemia-like disease in two French SCID-X1 gene therapy patients as a result of retroviral vector integration in the vicinity of a known oncogene, LMO2, has underscored the necessity for improved safety mechanisms within gene therapy vectors.[17]

Many of the vector modifications described above to improve transduction efficiency and gene expression may also improve vector safety. Elimination of dispensable viral sequences from the vector, separation of necessary viral genes (*gag*, *pol*, *env*) onto individual packaging vectors, and replacement of viral sequences with heterologous retroviral components or exogenous *cis*-acting regulatory elements all combine to reduce the risk of homologous recombination between vectors and endogenous viruses and the development of chimeric replication-competent vectors. Additionally, transcriptional targeting, or the use of gene-specific regulatory elements to limit gene expression to desired cell types, will help prevent expression in inappropriate cell types. Targeting of vectors to specific cell types or tissues by reengineering

of envelope proteins will also limit gene expression to appropriate tissues. The use of insulator elements and matrix attachment regions within vectors may prevent inappropriate activation of cellular oncogenes by confining the action of vector regulatory elements to vector transgenes. Finally, attempts to target integration to specific genomic locations by modification of the integrase enzyme may eventually direct vector integration to benign sites within the genome. Tan et al. have shown that fusion of the synthetic polydactyl zinc finger protein, E2C, to the HIV-1 integrase results in preferential vector integration near the E2C binding site in vitro.[18] The E2C binding sequence is a unique site within the human genome; however, preferential integration has not yet been demonstrated in vivo.

RETROVIRAL VECTORS AS PHARMACEUTICALS

The development of gene therapy vectors for large-scale therapeutic use will also require improvements in several areas. As viral vectors can only be made by living cells, large-scale manufacturing of gene therapy vectors will require the establishment of producer cell lines which consistently yield stable, unrearranged viral vectors. Additionally, purification of vectors from producer cell supernatant is a cumbersome procedure and all processes must be carried out using Good Manufacturing Practices and the highest levels of quality control. Safety of viral vectors, namely, the potential to generate replication-competent vectors by recombination between vector components and endogenous retroviruses and insertional mutagenesis, is of significant concern in the development of vector therapy for use in humans. Finally, with in vivo vector therapy as a future goal, innovations are required which will minimize innate and humoral immune responses to systemically delivered vectors, especially inactivation of vectors by human complement and the possible development of antivector antibodies.

CONCLUSION

The therapeutic potential of retroviral vectors was first recognized over a decade ago. Due mainly to their ability to integrate into the host genome and the potential for long-term gene expression, retroviral vectors have now become one of the most widely used vehicles for gene therapy. However, despite many beneficial modifications, retroviral vectors are limited by the inability to transduce nondividing cells and inconsistent levels of gene expression. Additionally, with the recent development of leukemia-like disease due to insertional mutagenesis in two patients enrolled in a retroviral vector clinical trial, the safety of retroviral vectors is now a significant concern.

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Reversed Line Blot Hybridization

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Bert Gold
National Cancer Institute, Frederick, Maryland, U.S.A.

INTRODUCTION

Reversed line blot (RLB) hybridization is a specialized form of allele-specific oligonucleotide assay that provides a robust method for the molecular characterization of genes with numerous nonsynonymous variants. In most highly refined form, these assays rely upon highly multiplexed PCR reactions containing biotinylated primers to provide a substrate for nonradioactive detection systems. Reversed line blot technology involves mechanized covalent attachment of activated primary amine-conjugated oligonucleotides to carboxylated nylon membranes or BSA. Printed membranes are stored or sold dry in preparation for hybridization. Lines are visualized colorimetrically after hybridization using streptavidin horseradish peroxidase incubation then developed using a tetramethylbenzidine derivative and hydrogen peroxide. For rapid prenatal diagnosis of cystic fibrosis (CF), β-thalassemia, and other hemoglobinopathies, this technology is especially applicable. Commercial instruments are available to provide greater uniformity for the hybridization and color development.

TECHNICAL DESCRIPTION

Reversed line blot-polymerase chain reaction requires a moderate number of biotin tagged primers in robust multiplex PCR in order to provide a driver for solid support-bound probes. Optimal multiplex PCR reactions directed at a single locus rarely contain more than 20 separate amplicons. In single nucleotide polymorphism (SNP) typing, multiplex PCR can contain up to 100 amplicons; however, these experiments are not limited to a single genetic locus, thus the sequence optima are not subject to the same constraints as those used in genetic testing.

After denaturation and hybridization of tagged amplified sequences to probes attached to solid supports, filter-bound hybridization products are washed under conditions of increasing stringency in order to remove nonspecifically bound amplicon, followed by the use of a blocking buffer to further reduce background. Streptavidin conjugate is used to provide a chemical bridge between the solid support covered with a tagged, hybridized PCR product and an enzyme substrate complex susceptible to visualization using a colorimetric substrate. Detection of the labeled oligonucleotide probes is accomplished through incubation with substrate in a developing buffer. Peroxidase conjugate-mediated color development occurs during a 10-min incubation, in the dark, in a buffer containing low-concentration hydrogen peroxide. Filters are then washed in distilled water. Photography is best accomplished while the filters are still wet.

Molecular genetic tests have two different classes of validity: The first class is analytical validity, including analytical specificity and sensitivity, which provide a basis for judgment as to how a test performs in the laboratory. Another class of validation is clinical validity, which informs clinical decision making.

SPECIFICITY

Validation of RLB hybridization tests involves retrospective scoring of samples that have been reliably characterized by some other method, most often polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). Polymerase chain reaction–restriction fragment length polymorphism takes advantage of base-pair changes that create or interrupt palindromic restriction-enzyme recognition sites. Although this is a simple approach, restriction-enzyme cleavage is limited to linkage analysis of polymorphic restriction sites and to situations in which the mutation serendipitously creates or disrupts a restriction site. It also requires that the amplification reaction produce a unique product fragment. For historical reasons, PCR-RFLP remains the gold standard for clinical diagnosis of point mutations. New technologies for mutation detection are often compared against it. In 1999, polymorphisms discovered in the binding site of PCR primers within the hemochromatosis locus changed clinical practice by exposing the myth that PCR-RFLP is the most reliable genotyping technique (see Ref. [1] for details).

A statistical rating of analytical specificity provides a measure of how often a test is negative when a mutation is not present. This amounts to measuring the number of false negatives, or mutations missed. This statistic is expressed as a percentage of mutations missed over all other classes of test outcome. The analytical specificity of

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RLBs is extremely high. Palomaki et al.[2] found the analytical specificity of cystic fibrosis tests conducted in the United States to be 99.4% (95% CI 98.7–99.9%) after removing challenges involving ΔI507. The few exceptions to the rule of high specificity are discussed in ''Limits and Clinical Applications'' below. Clinical specificity is the quotient of normals (or noncarriers) with negative results divided by all normals.

SENSITIVITY

Generically, analytical sensitivity is defined as the proportion of positive test results, when a detectable mutation is present. Analytical sensitivity is equivalent to the analytical detection rate. Between 1996 and 2001, when RLBs were widely used, Palomaki et al.[2] found an analytical sensitivity of 97.9%,[2] in U.S. CF molecular testing. They added that analytical sensitivity was consistent over the 6 years.

Two genetic tests, one for the hemoglobinopathies and β-thalassemia that would have broad (95% clinical sensitivity) worldwide coverage, and the other for a series of relatively common mutations in the human CFTR, were the first application of RDBs. The clinical sensitivity of the 25 mutation panels for CF carrier testing recommended by the ACMG in 2001 varies by ethnic group.

REPRODUCIBILITY (PRECISION)

Zhang and colleagues,[3] working at Cetus, reasoned that probes bound by UV or heat, were attached in random fashion to the filter and sometimes to each other, making it difficult to determine consistent or optimal hybridization conditions. Until then, Saiki and his collaborators[4–9] had been using nucleotidyl transferase-tailed polyT oligonucleotides under UV radiation to conjugate amino-derivatized membranes (Biodyne B); but Zhang et al. showed that oligonucleotides could be amino-functionalized by introducing these nucleophiles at 5′ oligomer ends during synthesis, and that these would be reactive with charged membranes. After attachment, reactivity of activated carboxyl groups on the membrane remains high; therefore blocking previously activated sites through charge neutralization is necessary before using the membrane in a hybridization reaction, as nucleic acids can otherwise attach to the sites at a low, but significant level, even without an amino-linker. Although charge neutralization and blocking were initially achieved by the Cetus group using hydroxylamine, the UCSF group later found NaOH just as effective.

A Belgian group[10] found that empirical adjustment of the hybridization conditions was a requirement in developing appropriate molecular interrogators for the common Belgian mutations, ΔF508, G542X, and N1303K. Cuppens et al. also showed that a multiplex PCR was a requirement for detecting the desired variety of mutations at the CF locus. Both of these important observations, the requirement for multiplex PCR and that for reproducible, empirically derived hybridization probes and conditions, have become understood as necessary prerequisites for a successful RDB.

Zhang et al.[3] also emphasized the importance of the physical relationship between the interrogated oligonucleotide and the solid support surface through the use of spacer linkers. In controlled experiments, they found that steric spacers aided hybridization and that oligonucleotides without linkers were up to fourfold less efficient in hybridization (see Ref. [1] for details).

Despite the development of covalent attachment by the predecessor Cetus group, the Roche Molecular Systems progeny group developed a proprietary process for the manufacture of BSA-conjugated oligonucleotides. The process involves the synthesis of 5′-amino link oligonucleotides followed by incubation with BSA and coating onto nylon membranes.[1,11] This proprietary process is used by Roche for the manufacture of their Linear Array Panel (LAp).

Commercialization of RLB hybridization strips required significant effort in ensuring their reproducibility and uniformity. During the period of scale-up at Roche Molecular Systems, it was found that dot blots themselves often gave equivocal results in the form of a halo around the dots. This had more to do with surface tension considerations when applying the amino-conjugated or BSA-conjugated oligos (''printing'') than it had to do with any other consideration. As a consequence, a decision was made to convert the dots to lines of uniform width. Roche developed its own in-house technology for printing line probes, although off-the-shelf commercial technology is available.[1] Extensive improvements in reproducibility and manufacture accompanied the commercialization process. Incubation temperature, temperature equilibration, and uniformity in conjugate and substrate distribution are known to be critical variables for color development. Optimum conditions for nucleotide hybridization and development of the RDB strips were devised and automated by Roche and Innogenetics, Inc., through the use of Tecan's Profiblot and Dynal's AutoReli. These shaking baths give uniformity to the strip development that is not easily achieved in any other fashion.

ROBUSTNESS

Because of speed, simplicity, high sensitivity and specificity, large numbers of individuals can be rapidly

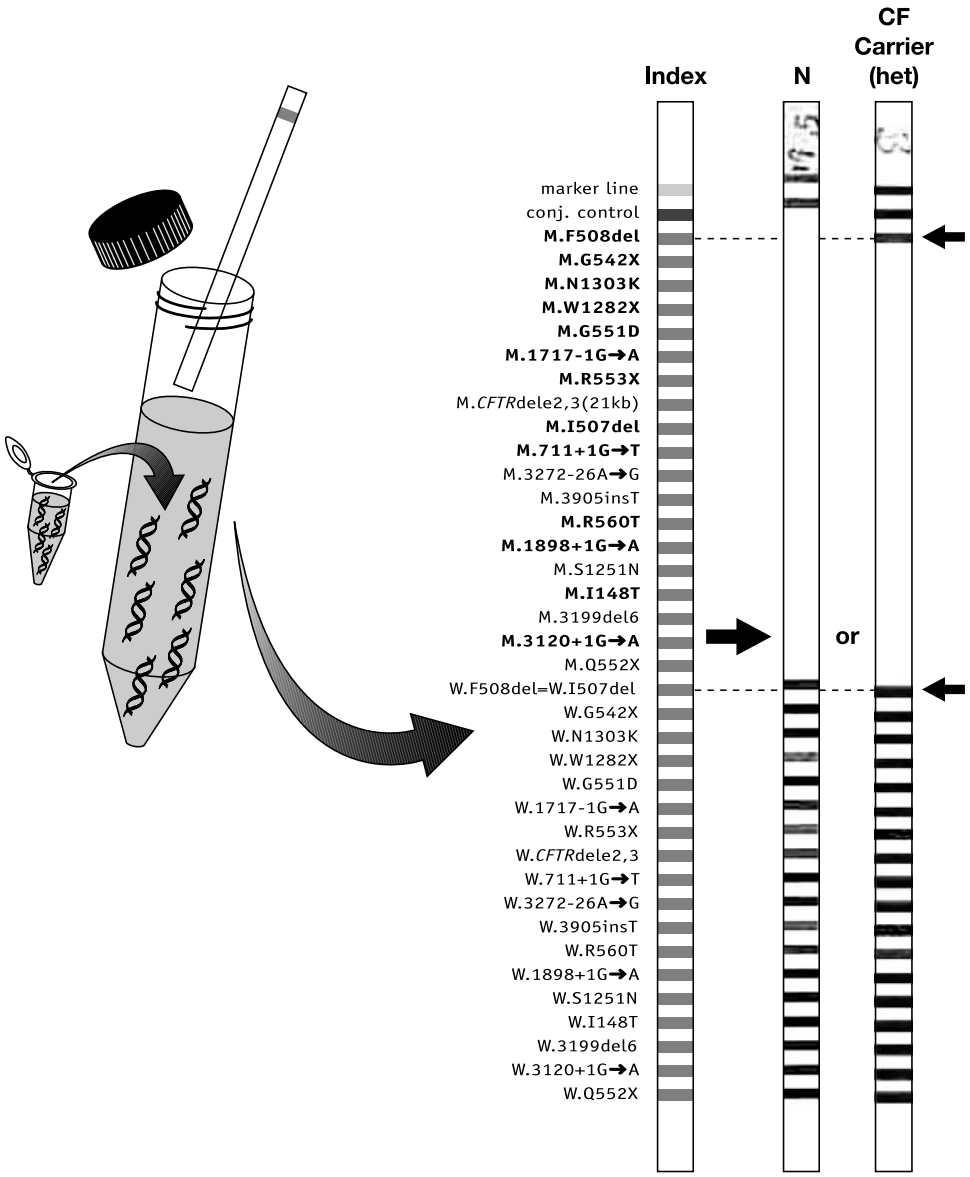

Fig. 1 Schematic of multiplexed reversed line blot assay for cystic fibrosis. In an RLB, primers for amplification are synthesized using a 5′-biotin. These are incorporated in a multiplex PCR for most CFTR exons. Amplified exons are represented by double helices in the smaller test tube on the left. These are hybridized against short variant and normal oligonucleotide probes arrayed on solid supports, represented by the larger screw cap tube. The index for the Innogenetics CFTR19 LiPA RLB provides a guide to printed oligonucleotide probes. Subsequent to hybridization, the filters are extensively washed, blocked, and incubated with a horseradish peroxidase-conjugated streptavidin. For visualization, a synthetic substrate is added and the reaction is permitted to proceed for a short time. The resultant arrays shown here and labeled as a normal strip (N) and CF Carrier (heterozygous individual) are photographed and subjected to interpretation. Note that the normal strip evidences colorimetric indicators corresponding only to normal oligonucleotide probes, whereas the carrier displays a colorimetric reaction when probed with the M.F508del oligonucleotide (upper arrow on right). (RLB photos courtesy Donna Adams of Innogenetics and Dr. Amy E. Krafft of the Armed Forces Institute of Pathology.)

tested using this technology, often in less than 1 day. One prerequisite is that binding of oligonucleotides must be sequence-specific under uniform hybridization conditions. In most cases, the destabilizing effect of a single base-pair mismatch is sufficient to disrupt the formation of stable probe–target duplex. This specificity requirement can often be met either by adjusting the length, position, and strand specificity of the probe, or by varying the amount of probe applied to the membrane. Most probes are as short as is required to achieve specific hybridization, usually 15–23 base pairs in length, with a destabilizing mismatch toward the middle, but at least 3 base pairs from

either end. The classic formula for predicting the dissociation temperature T_d of an oligonucleotide, 2°C for each A/T and 4°C for each G/C, is simple but not necessarily accurate. In recent years, many algorithms have been developed that appear to give much better estimates and can assist in the design of probes for particular hybridization conditions. Some mismatches are less destabilizing than others. G/T mismatches are less destabilizing than C/A and these can be avoided by using probe designs on the opposite strand. Increased length provides for efficient competition with the secondary structure of the amplicon. Destabilizing mismatches are favored in RLB probe design. Trimethylammonium chloride (TMAC) is a quaternary ammonium salt that is used as an adjunct in hybridization reactions. Trimethylammonium chloride eliminates the dependence of melting temperature on G–C content by reducing hydrogen bond energy between G–C base pairs. Additionally, TMAC binds specifically to A–T base pairs and increases their thermal stability. Thus the presence of TMAC at 3 M concentration in hybridization buffers reduces the melt temperature of an oligo with its complement to a function of length alone.[12]

Reversed line blots are often arrayed to permit rapid interpretation of heterozygotes where one normal and one variant allele are indicated (Fig. 1). However, where two mutations are close to each other and fall within the sequence of the oligonucleotide probe (a situation often observed with respect to HbA, HbS, and HbC), a different pattern is obtained. DNA from individuals with HbS/HbS (sickle cell disease) will not hybridize to the normal probe at either the S or the C position. Instead, such DNA hybridizes solely to the HbS probe.[13] This individual is distinguishable from HbS/HbC compound heterozygotes because the latter DNA will hybridize to both the mutant S and the mutant C probes. Similar RDB results are observed in the case of variants neighboring the ΔF508 CF mutation and those neighboring the IVS 1-1 and IVS 1-6 mutations, five nucleotides apart, that cause β-thalassemia.

In the past, RDBs have permitted the production of screening strips for aldolase B mutations (causing hereditary fructose intolerance),[14] nondeletion α-thalassemia,[15] or adult onset mitochondrial disorders such as Leber hereditary optic neuropathy[16] or hepatitis A contamination in food.[17] A recently published reverse dot blot assay for congenital adrenal hyperplasia has high clinical utility in identifying sexually ambiguous newborns.[18] These assays are flexible, inexpensive to implement, and use off-the-shelf commercially available hardware, reagents, and software. Both Tecan and Dynal manufacture specially engineered incubators and chemical dispenser devices that automate part of the RDB hybridization and developing process. While RDB assays are generally limited by an inability to detect large or quantitative deletions and an inability to characterize all but modestly expanded repeat sequences (ascertainment of the exact size of an expansion is often required for accurate molecular diagnosis), these strips can provide a means of accurate and reproducible genotype assignments. Automated spotting or line blotting of RLB strips allows the printing of large numbers of these with a minimum of operator intervention. Automation also permits higher density.

LIMITS AND CLINICAL APPLICATIONS

In molecular diagnosis there are two types of variant characterization: one for diseases with a major etiologic mutation, as in medium chain Acyl CoA dehydrogenase deficiency where almost 90% of affected pediatric patients have the same mutation (985A > G). Thus it is a simple matter to design a high-throughput test by either forward dot blot or PCR-RFLP. This, however, is not the case for the second type, CF or β-thalassemia. Hundreds of mutations are known that cause β-thalassemia or hemoglobinopathies, and nearly all are recessive, some causing milder and some more extreme phenotypes. And, even today, after a massive screening effort in the Mediterranean and Italy, where β-thalassemia is endemic, most patients are born into families with no history of the disease. Another example is CF in which one major mutation accounts for 70% of observed carriers and is present in homozygous form in less than 50% of affected individuals. Thus the clinical sensitivity, which is ethnicity specific, is much lower than most diagnostic tests found in a pathology laboratory. Like β-thalassemia, the vast majority of individuals who have CF are born into families with no history of the disease. Each of these diseases has a relatively wide mutation spectrum; that is, many different alleles are present that in combination can give rise to the disease. As a consequence, there is great motivation for carrier screening for each of these diseases, to provide early warning to at-risk families and to ascertain genetic carrier status far back in families presenting for screening. Chehab and his collaborators designed a series of very successful reverse dot blots aimed at these applications.[19–21] As the CF LAp offered by Roche Diagnostics and Innogenetics' Line Probe Assay (LiPA) have received the broad endorsement of U.S. molecular diagnostics laboratories, the RLB has come of age. In addition to these commercial offerings, there remains a large market of home-brew testing via reverse allele-specific oligonucleotides, for a variety of hereditary illness and infectious-disease typing. With the recent publication and evaluation of a 58-allele line probe assay by a Johns Hopkins and Roche Molecular Systems collaborative group,[11] the RLB has proved its analytical

utility in a variety of diagnostic situations. Limitations, such as the individuals who are compound heterozygote at closely spaced loci, occasionally fail to signal the presence of one or the other allele. This is because of interference. It is a characteristic of all sequence-specific assays that nucleotide variants within the probed region (usually $\sim$17 nucleotides) affect test accuracy. One example of this kind of interaction is detection of a S549N/R553X heterozygote in Wang et al.;[11] this failed to hybridize with the G551D wild-type probe which is encompassed by the probe for R553X. Another example is a ΔF508 mutation/I506V polymorphism heterozygote which failed to hybridize against the normal sequence in the region. These confounding results should notify the alert clinician of a test uncertainty that bears further investigation. This type of observation is one of the motives for the American College of Medical Genetics (ACMG) guideline of a two-tiered testing strategy for population-based CF screening.[22]

CONCLUSION

Polymerase chain reaction–restriction fragment length polymorphism has been the standard for molecular mutation screening; however, this method is not practical for high mutation spectrum disorders. Rapid reliable molecular diagnosis of these disorders requires a unique approach. Because the majority of these hereditary illnesses occur in families with no prior history, RLBs provide a high-sensitivity screening test to identify at-risk carriers. Direct DNA sequencing would be the most thorough mutation-detection approach, but it is arduous and capital intensive. Molecular carrier screening tests must be cost-effective to be practical. Commercialization of molecular diagnostics for high mutation spectrum disorders involves special considerations. Among these are low cost, high throughput, and rapid interpretation. Genotyping assay efficiency and throughput has considerably improved as a consequence of automation. Automation of many of the subtasks of nucleic acid hybridization including amplification, electrophoresis, and homogeneous signal detection has enabled the establishment of higher throughput molecular assays and diagnostics. Technical factors, such as methods of attachment of oligonucleotide probes to solid supports, the nature of the solid supports themselves, and PCR product visualization techniques, play an important role in formatting commercial diagnostics. Process automation has been inspired in part by the successful effort to map and sequence the human genome. Commercial tests have been developed for HLA class I and class II regions of human chromosome 6, mutations and polymorphisms in the cystic fibrosis transmembrane conductance regulator (*CFTR*) at 7q31, and for genotyping strains of human hepatitis B and C virus (HBV and HCV). Several diagnostics manufacturers have participated in the development of RLBs. There will undoubtedly be many improvements in automation and detection of DNA sequences using this technology in the short-term future.

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ARTICLES OF FURTHER INTEREST

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Rh Genotyping—Clinical Aspects

R

Carlos M. Cotorruelo
Claudia Biondi
Amelia Racca
Universidad Nacional de Rosario, Rosario, Argentina

INTRODUCTION

The Rhesus blood group system is of clinical interest because it is involved in the pathogenesis of hemolytic disease of the newborn, hemolytic transfusion reactions, and some autoimmune hemolytic anemias. More than 48 different antigens have been serologically defined, making the Rh system the most polymorphic of all the erythrocyte blood group systems. There are five most frequently typed Rh antigens: C/c, E/e, and the D antigen, which is the most immunogenic, defining an individual as RhD-positive or RhD-negative. Classical hemagglutination used for Rh typing is still a powerful and practical technique, with specificity and sensitivity suitable for clinical applications, but has limitations. Molecular genotyping techniques such as polymerase chain reaction (PCR) can be used to overcome some of these limitations and, in addition, to improve the current level of resolution.

GENETIC BASIS OF THE Rh SYSTEM

The discovery of the Rh blood group system was made just over 60 years ago. A three-gene theory and a single-gene theory had been proposed to explain the inheritance of Rh antigens, but it was in 1991 when Colin et al.[1] demonstrated that the *RH* locus was composed of two genes and established the genetic basis for RhD-positive and RhD-negative polymorphism, as follows:

The *RH* locus is composed of two homologous genes denoted *RHD* and *RHCE*.
Caucasian RhD-positive individuals have either one or two *RHD* genes per cell, whereas the RhD-negative phenotype is caused by the absence of the entire—or at least part of the—*RHD* gene (Fig. 1).
The *RHD* gene encodes the RhD protein that expresses the epitopes of the D antigen.
The *RHCE* gene has four prevalent allelic forms: *RHCe*, *Rhce*, *RhcE*, and *RHCE*, and each allele determines the expression of two antigens in Ce, ce, cE, or CE combination carried by the RhCcEe protein (*RHCE* is the collective name of the four alleles).

Rh PROTEINS

The Rh proteins consist of 417 amino acids, have 12 transmembrane spans with six extracellular loops, and are involved in the export of ammonium from red blood cells (RBCs).[2,3]

D is the major Rh antigen detected on the surface of RBCs obtained from individuals with presumed genotypes *RHD*/*RHD* or *RHD*/−, commonly referred to as ''RhD-positive.'' It consists of a collection of at least 24 different epitopes expressed on the RhD polypeptide. Whether from RhD-positive or RhD-negative individuals, virtually all normal RBCs bear the antithetical antigens C and/or c (involving the second extracellular loop of the RhCcEe protein), in addition to E and/or e (involving the fourth extracellular loop on the same protein).[2,4,5]

Rh antigens may have a depressed, partial, or ablated expression on the RBC membrane because of allelic variants of the *RH* genes. A serological weakly reacting form of D was described as weak D because these red cells possess a relatively small number of D antigen sites. Another phenomenon related to D antigen expression is the partial D status associated with both qualitative (lack of one or more D epitopes) and quantitative polymorphism,[4] which can be identified because of anti-D production and/or lack of reactivity with some monoclonal anti-D. However, molecular analyses have shown that ''weak D'' possesses slightly modified D antigens,[6] suggesting that the terms ''weak D'' and ''partial D'' should be replaced by ''aberrant D'' because each case of altered D expression investigated to date demonstrates a unique D phenotype.[3,5]

RH LOCUS

The *RH* locus is localized on the short arm of chromosome 1p34–p36. Each *RHD* and *RHCE* gene encompasses approximately 57 kb of DNA and consists of 10 exons (Fig. 1). There is about 90% coding sequence homology between the *RHD* and *RHCE* genes. The 3′ untranslated region of *RHD* exon 10 covers more than 1500 bp, whereas a shorter stretch is known for *RHCE*. Deletions

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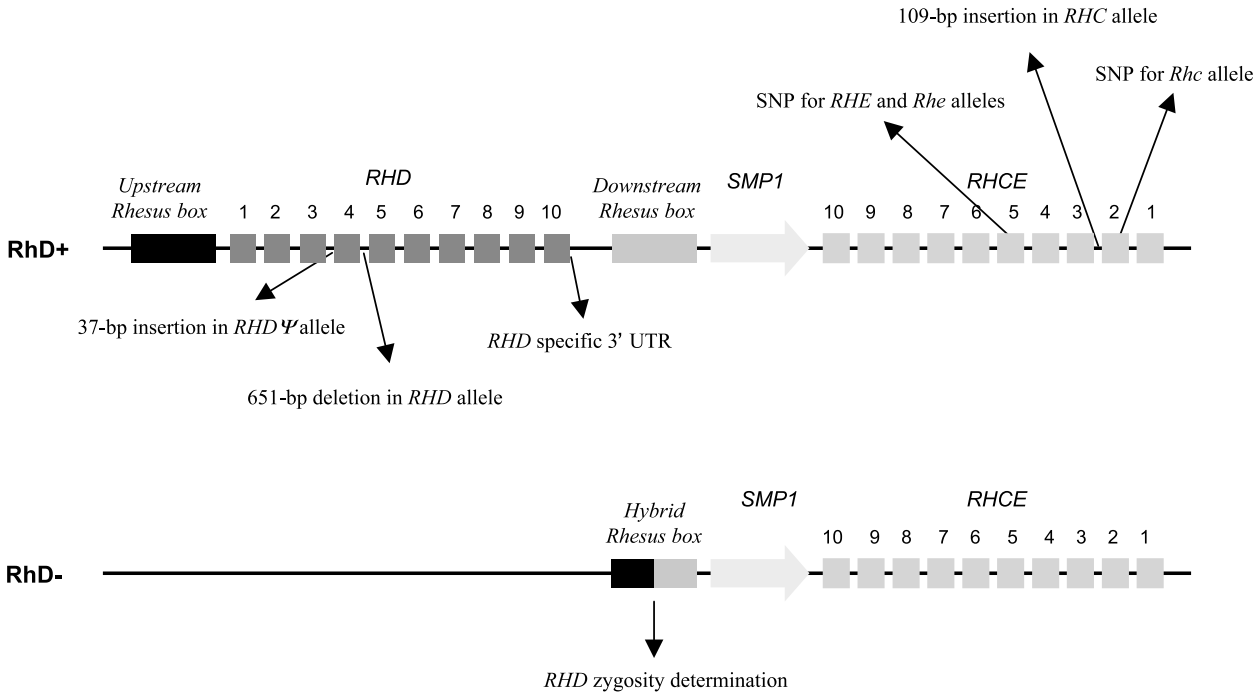

Fig. 1 Genomic organization of the *RH* locus in RhD-positive and RhD-negative chromosomes. Arrows indicate polymorphic sites used for *RH* genotyping. SNPs in *RHD* exons 1–7 and 9 are also detected in some *RHD* genotyping strategies.

of 109 bp in intron 2, 288 bp in intron 3, and 651 bp in the very short intron 4 have been reported.[2,3,5,7]

The physical distance between *RHD* and *RHCE* is approximately 30,000 bp, and it is filled with a *Rhesus box* and the *SMP1* gene. The two *RH* genes have opposite orientations and face each other with their 3′ ends. Considering this chromosomal organization, the lack of the *RHD* gene in most RhD-negative Caucasians was probable because of an unequal crossing-over event between two highly homologous DNA segments termed *Rhesus boxes*, which flank either side of the *RHD* gene, resulting in the formation of a hybrid *Rhesus box*. The analysis of the hybrid *Rhesus box* would be a predictor of *RHD* zygosity.[8] One copy of the hybrid *Rhesus box* predicts a heterozygous *RHD*-positive genome (Fig. 1).

Rh PHENOTYPE AND *RH* ALLELES

Besides the most common *RHD* DNA sequence, several allelic *RHD* variants are responsible for partial D phenotypes, weak D phenotypes, or unexpressed RhD proteins.[9,10]

Molecular alterations in partial D phenotypes generally affect at least one extrafacial amino acid. On the contrary, molecular studies of weak D phenotypes have shown that weak D alleles lead to aberrant RhD proteins with amino acid substitutions in transmembranous and intracellular segments that may disrupt the secondary or tertiary structure, or may alter the optimal membrane integration.[2,3,5,6]

It is important to note that there also exist unexpressed *RHD* alleles in RhD-negative phenotypes. These *RHD*-positive antigen D-negative alleles are most frequently found in non-Caucasian populations and are rare in whites. In Africans, the major *RHD*-negative allele is a *RHD* pseudogene designated *RHDΨ*.[11]

The *RHCE* gene also bears its own heterogeneity, which is responsible for C, c, E, and e polymorphisms. *RHC* and *RHc* alleles differ in one nucleotide in exon 1 and in five nucleotides in exon 2, whereas *RHE* and *RHe* have a difference of a single nucleotide in exon 5.[2,3,5,7] Different *RHCE* rare alleles are responsible for depressed, partial, or ablated expression of C/c and/or E/e antigens.[9,10]

RH GENOTYPING

Different PCR amplification protocols have been applied to determine the Rh phenotype in DNA obtained from normal donor peripheral blood, amniocytes, and trophoblastic cells.[12]

Analyses of *RHCE* single nucleotide polymorphism (SNP) of exons 5 and 2 have been used for E/e and c genotyping, respectively. A 109-bp insert in intron 2 of

the *RHCE* gene, found only in C-positive individuals, was the target for the *RHC* allele typing[3] (Fig. 1).

RHD genotyping was more complex because of the large number of different *RHD* alleles described. Developed strategies varied from *RHD* single-region PCR tests (involving exon 10, intron 4, or exon 7) to multiplex PCR tests for scanning all *RHD*-specific exons. However, *RHD* genotyping by probing the presence of *RHD*-specific polymorphisms was not suitable for certain population groups, such as Africans, where the presence of *RHDΨ* can reach 40%. Generally, checking two sufficiently distant regions of the *RHD* gene together with the *RHDΨ* has proven to be safe for determination of the RhD phenotype[3,12] (Fig. 1).

CLINICAL ASPECTS OF Rh GENOTYPING

Routinely, Rh typing is performed by agglutination with polyclonal and immunoglobulin (Ig) M monoclonal antibodies, or blends of IgM and IgG monoclonal antibodies. However, there are some clinical situations in which serological techniques cannot determine the RBC phenotype accurately:[13]

In fetuses at risk for hemolytic disease of the newborn because periumbilical blood sampling must be performed to obtain fetal erythrocytes.
In cases of autoimmune hemolytic anemia because autoantibody coating of RBCs may render serological typing impossible.
After a massive transfusion, when agglutination methods would detect antigens on both the patient's and the donor's RBCs.
In patients with altered expression of the D antigen and where serology is inconclusive for deciding transfusion therapy or anti-D prophylaxis.

RH genotyping with specificity and sensitivity comparable to serologic methods is of practical importance to overcome the limitations of classical hemagglutination and, in addition, to improve the currently possible resolution. Moreover, the application of molecular techniques to the identification of rare alleles is increasingly important, as many typing reagents on which serology is reliant are now either no longer available or in short supply.

Hemolytic Disease of the Newborn

The RhD hemolytic disease of the newborn continues to affect at least 1:1000 live births despite the use of prophylactic anti-D Ig. Current management strategies for RhD alloimmunization include invasive procedures such as serial amniocentesis (to predict fetal anemia) and cordocentesis (to directly determine fetal blood type serologically), but both alternatives involve a high risk of fetal loss and may result in an important increase in anti-D titers.[4]

Since the molecular cloning of the Rh system, efforts have been directed toward the development of methods for DNA-based Rh typing particularly for the prenatal assessment of the fetal RhD status. The extreme sensitivity of assays based on PCR made it possible to determine the presence of the *RHD* gene and also *RHCE* alleles in fetuses using DNA derived from a single amniocentesis.[3,11,12,14] The high level of accuracy reached in *RHD* genotyping strategies allowed the incorporation of this prenatal diagnosis test into the management scheme applied to erythrocyte sensitization. If the fetus proves to be RhD-negative, the need for subsequent invasive procedures is obviated. If testing predicts that the unborn baby is RhD-positive, treatment can be planned with sufficient time.

However, to circumvent this risk associated with amniocentesis, several groups have investigated the possibility of determining the fetal RhD status through the use of fetal cells isolated from maternal blood.[15] The main problem is that the procedures needed to isolate sufficient numbers of fetal cells from maternal blood are time-consuming and technically demanding. Moreover, a cell subpopulation has been shown to persist for up to decades after delivery; thus it could interfere with fetal cell analysis in women who have had multiple pregnancies. An alternative approach based on the detection of *RHD* messenger RNA in fetal nucleated red cells has also been described, but the small number of subjects analyzed precludes any firm conclusion as to the reliability of this method.[16]

Recently, it has been found that during pregnancy, cell-free fetal DNA can be found in maternal plasma and may be utilized to determine fetal blood group status without invading the fetomaternal circulation. Lo[17] was able to demonstrate that fetal DNA is present at very high fractional concentrations in maternal plasma, constituting approximately 3% of total maternal plasma DNA during the second trimester of pregnancy and that fetal DNA is cleared very rapidly from the maternal plasma after delivery, with a half-life on the order of minutes. This high relative concentration suggests that fetal DNA could be robustly detected in maternal plasma using modern molecular technology and, unlike fetal cells in maternal blood, plasma DNA analysis is not complicated by the effect of persistence from prior pregnancies.

Both real-time PCR and conventional PCR technology have been applied to successfully determine the RhD status of unborn infants by analysis of plasma samples obtained from RhD-negative pregnant women. However,

it is very likely that for future large-scale clinical usage, protocols based on real-time PCR may be preferred because of the high sensitivity and homogenous nature of the assays. In addition, genotyping of fetal DNA extracted from maternal plasma can potentially be used for the diagnosis of many disorders involving single genes.[17]

One of the latest developments in hemolytic disease of the newborn is the discrimination of *RHD* heterozygosity from homozygosity in RhD-positive fathers by the specific detection of *RHD* deletion through PCR amplification of the hybrid *Rhesus box*.[8] This determination is of considerable value in the prenatal assessment of the fetal RhD status.

Autoimmune Hemolytic Anemia

Rh polypeptides are the most common targets for pathogenic anti-RBC autoantibodies in patients with autoimmune hemolytic anemia.[4] In patients whose RBCs are heavily coated with IgG, testing with antiglobulin-reactive sera is difficult, whereas tests with high-protein agglutinin reagents are impractical. For RhD phenotyping of these patients, it is necessary to dissociate antibodies from erythrocytes by elution, without damaging RBC membrane integrity or altering antigen expression. Yet, when the affinity of the antibody is high, the dissociation procedure may be incomplete and lead to wrong results.[13] In some cases, the removal of autoantibodies coating the erythrocyte membrane is reduced after the elution procedure, but the direct antiglobulin test remains positive. If the sample is serologically typed as RhD-negative with a direct agglutination technique, doubts may arise when performing the indirect antiglobulin test. In these cases, DNA analysis can confirm the RhD-negative phenotype and discard a probable weak expression of D. When serological typing is inconclusive and cannot be accomplished with its usual ease, DNA-based phenotype prediction is superior to serotyping.

Transfusion

Patients requiring blood transfusion who present with panreactive antibodies pose great difficulty to blood banks. Appropriate antisera in sufficient volume are necessary to screen for compatible antigen-negative blood donors. Moreover, in polytransfused patients, persisting transfused red cells hamper a definitive RBC antigen profile and hence antibody identification.[13] Genotyping is important in determining the true blood group of many polytransfused patients, can assist in the identification of suspected alloantibodies, and can help perform a more accurate selection of antigen-negative RBCs for transfusion.

Altered Expression of the D Antigen

Rh phenotyping may be inconclusive when erythrocytes possess a reduced expression of the D antigen. Limiting antibody sensitivity often hampers serological discrimination of such RhD phenotypes. Moreover, the use of monoclonal antibodies may fail to detect some weak D antigens.[13] In these cases with reduced antigen density, DNA-based analyses may be better than serological typing in inferring the true phenotype. Discrimination of *RHD* variants, such us partial D and weak D alleles, from the prevalent *RHD* allele would be advantageous to guide optimal RhD transfusion strategies or anti-D prophylaxis, considering the possibility of anti-D immunizations in these phenotypes that express aberrant RhD proteins.[6]

CONCLUSION

Molecular methods can be applied to overcome the limitations of classical hemagglutination for blood grouping. Both methodologies, in conjunction, are beneficial when attempting to identify antigen incompatibilities between a pregnant woman and the fetus. They are undoubtedly useful not only for diagnosing an Rh incompatibility but also for guiding therapeutic options to prevent Rh immunization. Molecular genotyping constitutes a supplemental technique to hemagglutination tests for the determination of the true blood group antigens in cases of warm-type autoimmune hemolytic anemia and polytransfused patients. It is also a useful tool for the selection of compatible blood units for alloimmunized patients. *RHD* genotyping in individuals with altered expression of the D antigen may be better than agglutination assays in inferring the appropriate phenotype when serology is inconclusive for deciding transfusion therapy or anti-D prophylaxis.

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Ribotyping

Tim J. Inglis
PathCentre, Perth, Western Australia, Australia

INTRODUCTION

Ribotyping describes a series of methods used to characterize the ribosomal DNA operon in bacteria and other more complex organisms. In bacteria, rDNA is organized into the 16s, 23s, 5s, and spacer sequences. As ribosomal DNA is present in all bacteria and corresponds to genus and species, it has proved useful for identification and taxonomic and subtyping applications. The importance of rDNA to bacterial survival is reflected in the multiple copies present in most species.

TECHNICAL DESCRIPTION

Early ribotyping methods used digestion of extracted rDNA with restriction endonucleases, separation of rDNA fragments by electrophoresis, and hybridization with a cDNA probe in a Southern blotting process.[1,2] Unlike conventional restriction fragment length polymorphism (RFLP) analysis, which resolves the difference between bacterial isolates by comparison of ethidium bromide-stained DNA bands, ribotyping uses a cDNA probe to resolve differences between isolates by generating a smaller number of bands. The number of bands will depend on how many fragments the cDNA can hybridize to and therefore the number of cutting sites for the restriction endonuclease.[3,4] REs employed for ribotyping are frequent cutters such as *Eco*R1, *Bam*H1, and *Pvu*2, but some species require less commonly used restriction endonucleases such as *Pvu*2.[5] DNA probes hybridize to either 16s or 23s rDNA or both. Ribotyping probes include an *Escherichia coli* DNA and other sequences.[6,7] The membrane used for blotting can be either nitrocellulose or nylon, and the labeling system can be a fluorophore, digoxigenin, or a radioisotope.

Several variations have been introduced to adapt ribotyping for wider use. A version of ribotyping based on digestion with *Eco*R1, an *E. coli* cDNA probe, and a fluorescent tracer has been automated to simplify and speed up the procedure[8](Fig. 1). In PCR ribotyping, PCR primers to highly conserved regions of the 16S–23S intergenic spacer region are used to amplify the product for subsequent length polymorphism analysis.[9]

In outline, the manual ribotyping method is as follows:

1. Bacterial DNA extraction (phenol-chloroform, guanidium, etc.).
2. DNA check (concentration and mini-gel).
3. Digestion with restriction endonuclease.
4. Preparatory gel for blotting.
5. Southern blot.
6. rDNA digoxigenin labeling.
7. DNA marker ladder digenin labeling.
8. Prehybridization.
9. Hybridization.
10. Wash and development.
11. Ribotype pattern recording and analysis.

SPECIFICITY

As all bacteria have rDNA, ribotyping is theoretically practicable for all bacterial species. In the Enterobacteriaceae, the presence of 5–7 rDNA operons results in 10–15 bands, with potential for a good level of discrimination between strains of a given species.[10] On the other hand, Mycobacteria have only 1 or 2 rDNA operons and produce few bands on ribotyping with a correspondingly lower level of discrimination.[11] Southern blotting with alternative probes specific to mycobacteria (e.g., IS 6110 for *Mycobacterium tuberculosis*) has been much more productive for these bacteria.[12] The limited genetic diversity of some important gram-negative pathovars, such as *E. coli* O157, renders ribotyping less suitable for molecular epidemiology of these.[13] On the other hand, bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Listeria monocytogenes* can be effectively subtyped by ribotyping.[14–16] A large proprietary database of *Eco*R1 ribotypes obtained with the automated system exists for these bacteria. The automated method has been adapted for use with other restriction enzymes (e.g., *Pvu*2 and *Pst*1) but is not easily adapted to the other probes used in manual ribotyping protocols. Clearly, most ribotyping methods have been developed for highly specialized purposes, the details of the method being specific to the application. Each method requires validation against a representative collection of the

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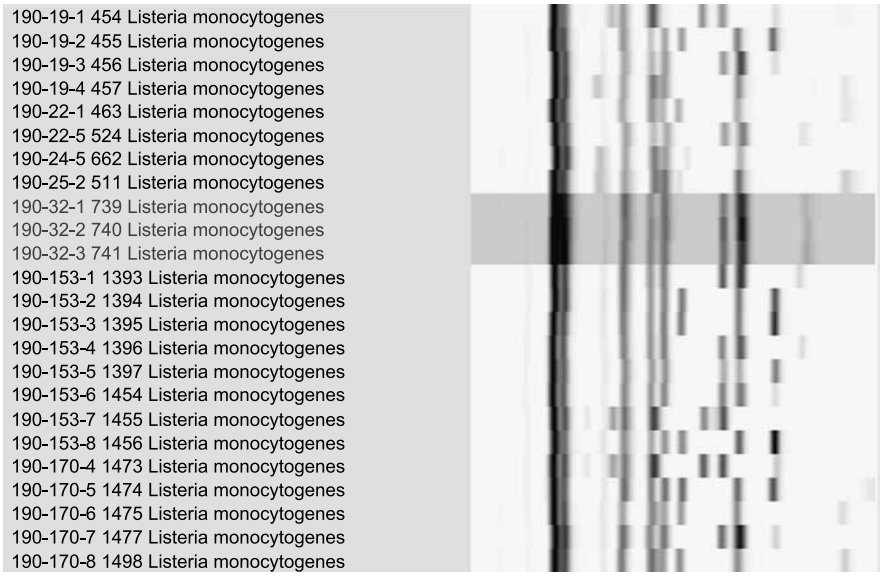

Fig. 1 *Eco*R1 ribotype patterns generated from *Listeria monocytogenes* analyses, showing highlighted cluster of indistinguishable isolates. (From L O'Reilly, PathCentre.)

species in question. The taxonomic value of the information generated by ribotyping therefore depends on the intraspecies variability of the rDNA operon, the number of copies present, the restriction endonuclease used, the cDNA probe, the range of strains already ribotyped, and the epidemiological setting from which the isolates were obtained. Ribosomal DNA has been used as an important arbiter of taxonomic status in determinative bacteriology.[17] Whereas this may be generally true down at species level, there have been reports of species that cannot be distinguished by 16s rRNA studies that have subsequently been resolved by DNA–DNA hybridization.[18] Ribotyping should thus be seen as less discriminating than DNA hybridization studies and therefore taxonomically subordinate. The automated ribotyping procedure is less suited to discriminating closely related fragments from some pathogens than traditional *Eco*R1 ribotyping, thus reducing its capacity to discriminate between strains.[19]

SENSITIVITY

Ribotyping requires only small amounts of high-quality bacterial DNA extract, particularly if the automated process is used. The use of a DNA hybridization probe reduces the sensitivity of band detection compared with conventional RFLP analysis, but, in doing so, improves the readability of the result.

REPRODUCIBILITY

Ribotyping is more reproducible than conventional RFLP analysis. As this is due in part to the specificity of the technique, the ribotype patterns obtained by a given method can give a higher reproducibility when a combination of several REs is used.[20] In the automated procedure, molecular markers and image optimization software are used to increase between-batch reproducibility so that band patterns can be compared with archived patterns using a computerized similarity index.

ROBUSTNESS

The various manual methods of ribotyping depend on a high level of technical skill, take a lot of dedicated laboratory time to complete, and are generally restricted to larger research laboratories.[21] Nevertheless, once established, the method is robust because a positive result depends on hybridization of digested bacterial rDNA with a specific probe. Automation of one of the more commonly used protocols has reduced the technical skill and dedicated time required without affecting the overall robustness of the method.

LIMITS

Ribotyping is limited mainly to analysis of bacterial species and has variable utility depending on the genera in

question. Ribotyping can be used to discriminate or identify as far as species level for most bacteria. Some species (e.g., Mycobacteria and some Enterobacteriaceae) cannot be reliably subtyped by this method without resorting to either multiple procedures (each with a different restriction endonuclease) or alternative probes (Southern blotting but not ribotyping,[12]). Ribotyping is not a satisfactory alternative to DNA hybridization studies as a final arbiter of taxonomic status[18] but can be used to gain an approximate assessment of taxonomic placing where the hybridization studies have already been performed.[17] At a practical level, the skills, time, and equipment required make manual ribotyping unsuitable for routine bacterial identification or molecular epidemiology service work.[21] Some of these shortcomings are met by the automated *Eco*R1 protocol.

CLINICAL APPLICATIONS

Ribotyping has seen wide application as a molecular epidemiology tool in clinical bacteriology.[3,8,10,13–16,19–21] It has often been used to complement other molecular typing methods such as DNA macrorestriction analysis when used in epidemiological investigations.[8,10,15,16,21,22] The most common of these applications are in hospital infection control and public health outbreak investigations where ribotyping is used to identify a cluster of isolates to confirm or refute preliminary epidemiological inferences.[23] In this setting, it has been argued that ribotyping is suitable for a wide range of typing applications.[22] More recently, the speed of automated ribotyping has made this version of the method more attractive to public health laboratories with a biosecurity role.[24] Larger reference laboratories have also found a use for ribotyping as one of a series of methods used to complete the identification and typing of species of uncertain status.

CONCLUSION

Ribotyping, or the characterization of the ribosomal DNA operon, has become an established analytical tool for bacteria and other more complex organisms. Automation of what was previously a lengthy experimental procedure now ensures that this technique can be used for a wide variety of molecular epidemiology tasks. The use of ribotyping, in connection with other genotyping procedures, is likely to play an increasing part in laboratory-based investigations of hospital- and community-acquired infections.

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RNA Storage

Martin Hofmann
Institute of Virology and Immunoprophylaxis, Mittelhaeusern, Switzerland

INTRODUCTION

RNA can be isolated from many different types of cells from eukaryotic and prokaryotic origin, including animals, plants, and bacteria. Depending on the source and the type of sample (organ tissue, cultured cells), appropriate procedures must be applied for RNA extraction to 1) maintain the integrity of the RNA, 2) inhibit RNA degradation because of RNases, and 3) obtain clean RNA preparations by removing protein and DNA contaminations.

Because RNA extraction can be quite a lengthy procedure and, furthermore, in many cases, numerous samples have to be analyzed in parallel, it is necessary to store the isolated RNA. Again, storage conditions should be selected to preserve the RNA in such a way that storage does not modify the RNA in any way. However, the intended use of the stored RNA also determines the way how the RNA is to be stored.

In this article, an overview of currently used techniques to obtain RNA from various sources and a description of the factors to consider for RNA storage are presented.

HANDLING OF RNA: THE PROBLEM OF RIBONUCLEASE CONTAMINATION

The isolation of intact, clean RNA is crucial for downstream experiments such as reverse transcription of RNA for cDNA cloning, RNA fingerprinting and secondary structure analysis, as well as for functional studies involving RNA metabolism. RNA stabilization is an absolute prerequisite for reliable gene-expression analysis. Stabilization of RNA in biological materials immediately after harvesting the sample is necessary because changes in the gene-expression pattern occur because of specific and nonspecific RNA degradation as well as to transcriptional induction.

Even a single nick can render the RNA molecule nonfunctional. This has to be considered when gene expression is studied by performing in vitro transcription and translation experiments, or transfection of infectious, self-replicating viral RNA.[1]

Whereas double-stranded RNA is relatively resistant against most RNases,[2] particular care must be used when handling single-stranded RNA.

The most important factor determining the quality of the RNA to be studied is the ubiquitous presence of RNases. This is a group of RNA-degrading enzymes, each acting in a sequence-specific way on double- or single-stranded RNA. Furthermore, some RNases such as RNase A are extremely resistant to inactivation by heat. For this reason, a suitable method for RNA extraction must be chosen which allows instant inactivation of RNases. This is achieved by dissolving proteins in denaturing agents such as guanidinium thiocyanate, phenol, or beta-mercaptoethanol.

To avoid or minimize ribonuclease contamination problems, all materials used for working with RNA should be RNase-free. Water should be treated with diethylpyrocarbonate (DEPC) that inactivates nucleases or should be obtained by ultrafiltration through a high-efficiency organics removal system. Glassware must be baked at least for 4 hr at 180°C because autoclaving does not inactivate ribonucleases completely. Plasticware straight out of the package can generally be considered free from nuclease contamination and can be used without any pretreatment. However, reused plasticware such as electrophoresis tanks should be filled with 3% H_2O_2 solution for 10 min at room temperature, followed by thorough rinsing with RNase-free water. Because hands are a major source of RNase contamination, gloves must be worn and changed frequently. It is also a good idea to keep separate stocks of chemicals that are exclusively used for RNA work and are removed from the bottle only with RNase-free tools (e.g., baked spatula).

Several types of specific RNase inhibitors can be used. 1) Protein inhibitors of RNases, either isolated from human placenta or produced synthetically as a recombinant protein, can be added to the extracted RNA. Furthermore, because these RNase inhibitors generally do not interfere with reverse transcriptases and polymerases, they are frequently used to prevent RNA degradation during subsequent reactions such as reverse transcription. 2) Vanadyl–ribonucleoside complexes (VRC) bind to many RNases thereby inhibiting their activity. VRC can

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be added to the intact cells or tissue before lysis and remain in the RNA-containing fraction during all stages of RNA extraction and purification. However, VRC can only be used for certain in vitro enzymatic reactions (e.g., reverse transcription), whereas they interfere with others such as cell-free in vitro translation of mRNA.

Numerous commercial products for RNA extraction are available today. Most of them are either based on a combination of guanidinium isothiocyanate and phenol based on a single-step RNA isolation method originally described by Chomczynski and Sacchi[3] followed by RNA precipitation, or by binding of RNA in guanidinium-lysed samples to a silica-gel membrane from which the RNA is subsequently eluted after washing. These methods allow disruption of the cells and simultaneously inactivate ribonucleases.

PRINCIPLES OF RNA STORAGE

Basically, RNA can be stored in purified form after its extraction or synthesis, or unprocessed samples containing the RNA of interest are stored. However, even in the latter case, proper care must be taken to avoid (re) contamination of RNA with RNases once it is extracted from the stored sample.

Little information is available on the long-term stability of isolated and purified RNA during storage. Most studies have been carried out focusing on various storage conditions of unprocessed samples before RNA extraction, or the material was stored at the stage of lysed cells or tissue but before RNA was isolated.

Storage of Extracted RNA

Depending on the extraction method used, purified RNA is either being eluted directly from a filter or column after ion exchange chromatography, or it is obtained as a pellet after precipitation with ethanol or isopropanol. Irrespective of the storage conditions, it is of great importance that all materials (glassware and plasticware, chemicals, water, etc.) are free from RNase contamination.

The most common way to store RNA is by dissolving it in RNase-free water and freezing at $-70°C$. This bears the advantage that the RNA can be used virtually for any subsequent experiments without having to worry about interference of the storage medium with the reaction conditions in the experiments. However, RNA stored in water is prone to degradation because of unfavorable pH, as well as traces of RNases. For this reason, RNA should be dissolved in or eluted with a low salt buffer such as 1 mM sodium citrate or TE (10 mM Tris, 1 mM EDTA) at pH<7, thereby preventing base hydrolysis by chelating free cations present in the RNA preparation.

If the higher-order structural integrity of the RNA is crucial, it should be stored in a buffer with an increased salt concentration to maintain hybridization of double-stranded RNA as well as self-complementary sequence elements (stems, pseudoknots) in single-stranded RNA. This is particularly important if such RNA is to be analyzed for secondary structure elements by targeted chemical/enzymatic degradation.[4,5]

Stabilized formamide (FORMAzol®, Molecular Research Center, Inc., Cincinnati, OH) can be used to dissolve precipitated RNA. It allows to store the RNA for at least 2 years at $-20°C$, whereas RNA dissolved in aqueous solutions should always be kept at $-70°C$. However, formamide must be replaced by water if the RNA is to be used in any in vitro enzymatic reaction.

To avoid RNA degradation as a result of multiple freeze thawing, it is recommended to store the RNA in small aliquots rather than in one single volume.

As an alternative to storing in solution, RNA can also be kept frozen or even at room temperature as a precipitate that is often obtained anyway at the end of the extraction procedure. Aliquots are removed from the precipitate after vortexing, and the RNA is pelleted by centrifugation and redissolved under RNase-free conditions immediately before use.

Storage of Samples Before RNA Extraction Under RNA Stabilizing Conditions

Samples containing the RNA of interest can also be stored prior to RNA extraction. Tissue or cells are allowed to be perfused by chemicals that inhibit RNase activity by denaturing all proteins. A rapid inactivation of enzymatic activity in the sample is required, in particular, in gene expression studies where any induction or repression of mRNA transcription because of changed environmental conditions must be prevented immediately after specimen collection to reflect the expression profile of the intact tissue.

It has repeatedly been shown that Trizol®, a monophasic solution of phenol and guanidinium isothiocyanate (Invitrogen Life Technologies, Carlsbad, CA), can be used to store samples for extended periods of time even at ambient temperatures, without a significant decrease of the quantity or quality of RNA.[6,7] Therefore, storage of samples in Trizol should be considered whenever samples cannot be kept frozen immediately after collection.

Recently, another commercially available product, RNAlater® (Ambion, Austin, TX), has been introduced on the market. RNAlater® is a patented aqueous, nontoxic tissue and cell storage reagent that stabilizes and protects

cellular RNA in intact, unfrozen tissue and cell samples by quickly permeating the tissue.[8] It allows to store collected tissue specimens and cells at 4°C or even at ambient temperature, eliminating the need for 1) immediate snap-freezing of tissue in liquid nitrogen after collection and 2) instant RNA extraction. Most of the commonly used techniques can be used to extract RNA from RNAlater®-treated samples. RNAlater® has been shown to be suitable for RNA stabilization in animal and plant tissue and cells as well as in bacteria. Further information is available from Ambion's Web site at http://www.ambion.com.

Storage of Unprocessed Samples

Tissue, cell, and serum samples can also be stored in a native, unprocessed form. However, to block RNA degradation as soon and as efficiently as possible, specimens should be frozen immediately after collection, and thawing and refreezing before RNA extraction must be avoided. It has been shown repeatedly that handling and storing conditions of diagnostic samples have a significant impact on the RNA levels detected by RT-PCR.[9–11] Tissue samples collected for gene expression studies should be snap-frozen in liquid nitrogen. All samples should be stored at −70C at all times.

CONCLUSION

There is no universally applicable ''ideal'' method for storing RNA. The procedure to be chosen is dependent on the source, the kind, and the intended use of the RNA. The most important factor that determines the quality of the stored RNA is the ability to protect the RNA from degradation by RNases. Therefore, if purified RNA is stored, RNase-free tubes and solutions are crucial, and the RNA should be stored frozen at temperatures as low as possible. Thawing and refreezing should be avoided.

If storage or shipment in a frozen state is not possible, samples from which RNA is to be extracted should be kept in a protein-denaturing agent to prevent RNase activity. For gene expression studies, any ongoing mRNA transcription activity should be suppressed as soon as possible after sample collection by snap-freezing the samples or by storage in an excess volume of RNA-stabilizing agent.

Detailed protocols for RNA extraction and storage can be found in molecular biology laboratory manuals.[12,13]

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Rolling-Circle Amplification (RCA)

R

Vadim V. Demidov
Boston University, Boston, Massachusetts, U.S.A.

INTRODUCTION

The rolling-circle amplification (RCA) reaction is an isothermal in vitro method for the hybridization-triggered enzymatic synthesis of hundreds to billions of linear copies of small, single-stranded, circular DNA probes. Thus, obtained long repeats of DNA sequences may serve as a signal amplifier for ultrasensitive detection of specific nucleic acids and other biologically important molecules in diagnostic genomics and proteomics. Depending on the goal and/or target molecule to be detected, RCA is performed using a variety of protocols and testing formats with either linear or exponentially branching kinetics. Because of their robustness and simplicity, the RCA-based assays hold a distinct position in the area of molecular diagnostics among other single-temperature amplification techniques.

RCA BASICS

RCA is based on the rolling replication of short, single-stranded DNA circles by certain DNA polymerases at constant temperature, the process discovered in the mid-1990s.[1,2] This reaction is initiated by the hybridization of a linear DNA single strand to a specific DNA minicircle, and it is widely used for diagnostic purposes in direct or indirect detection of different DNA/RNA, protein, and other biomarkers via a set of various biomolecular recognition events. A similar reaction was described for RNA polymerases as well,[3] but the RNA-generated process does not require any hybridization-dependent priming (or even promoter sequences). Therefore, the latter is only used to produce functional RNA sequences, such as RNA ladders and self-processing ribozymes.

Description of Method

Types and major features of RCA reactions

In its original formulation,[1,2] the RCA reaction involves numerous rounds of isothermal enzymatic synthesis in which DNA polymerase extends a circle-hybridized primer by continuously progressing around the circular DNA probe of several dozen nucleotides to replicate its sequence over and over again (Fig. 1A). This process is characterized by linear kinetics, easily yielding in one hour up to several thousands of sequence-complementary tandem repeats of an original DNA minicircle. These amplification products generally exhibit a wide, essentially continuous distribution over length and are normally seen in gel-electrophoretic images as a broad smear of high-molecular weight DNAs (Fig. 2, lane 2). The single-stranded nature of amplicons in case of linear RCA may be beneficial for subsequent manipulations with these DNAs towards their detection.[4]

A more complicated version, the double-primed RCA, called hyperbranched,[4] ramification[5] or cascade RCA,[6] operates with a pair of different primers. One primer is complementary, as in the linear RCA, to a DNA minicircle, whereas the other is targeted to the repeated, single-stranded DNA sequences of the primary RCA product.[4–6] Consequently, the double-primed RCA proceeds as a chain reaction with geometric kinetics featuring a ramifying cascade of multiple-hybridization, primer-extension, and strand-displacement events involving both primers (Fig. 1C). As a result, a discrete set of concatemeric double-stranded DNA (dsDNA) fragments is formed, yielding the distinct, ladder-type gel-electrophoretic bands (Fig. 2, lane 3).

Sometimes, this reaction produces amplicons so long that they cannot move through the gel during common gel electrophoresis (see lane 4 in Fig. 2). In terms of the degree of amplification, the geometric RCA is more potent, as compared with its linear alternative, yielding 10^9 or more copies of a circular sequence in about an hour.[4,6] Accordingly, it enables a bioanalyst to readily detect just a few probe molecules.[4–8] The double-primed RCA is usually performed at elevated temperatures by thermostable DNA polymerases, although in some cases it is performed by common polymerase enzymes at physiological or ambient temperatures.

Practical RCA formats

Generally, the RCA-based diagnostics can be classified into two groups: some of them operate with the preformed circular probes [Fig. 1D and E for the peptide nucleic acid (PNA)-assisted, nick-induced RCA and immuno-RCA,

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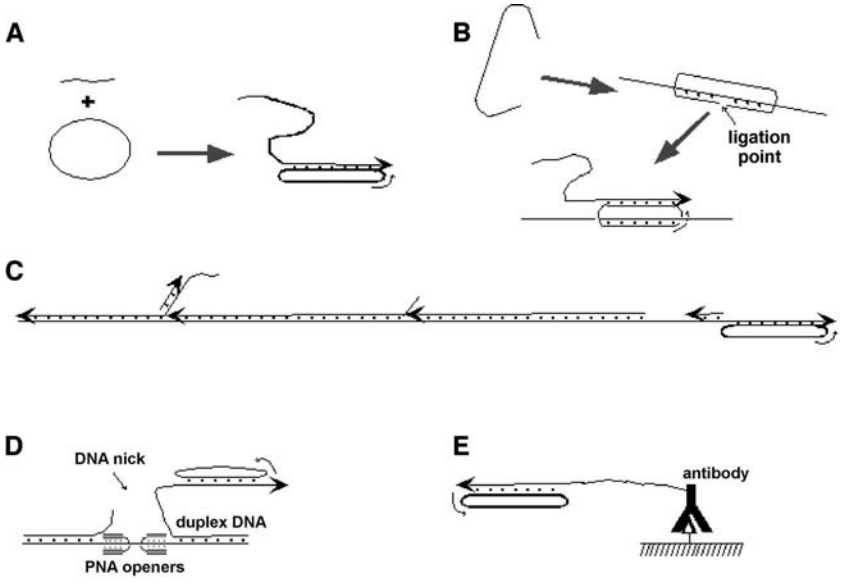

Fig. 1 Schematics of different RCA processes; the arrowhead symbolizes the DNA polymerase. Given a small, $\leq$100-nt size of DNA minicircles used in RCA and the strong rigidity of dsDNA fragments with these lengths, only part of the circular probe can be base-paired at any given time. Consequently, the geometry of RCA-generating complexes resembles a fiddlestick. (A) The RCA reaction carrying on a free DNA minicircle with the use of a single primer. If the DNA target is used to prime the RCA reaction, amplification products are fixedly linked to target molecules (see schematics D, for example). For surface-attached targets, these products will be immobilized on the solid phase. (B) RCA-based diagnostics of probe amplification with an in situ circularized linear oligonucleotide probe and a target-unrelated primer (ligation–RCA/L–RCA). In some cases, the topological linkages between a DNA minicircle and the marker/target DNA site may affect the rolling replication. A circular probe should then be released from the DNA target following the hybridization. (C) Initial stages of the double-primed RCA.[4–6] In these reactions, the second primer, which is complementary to the original RCA product, is used. Here, the DNA polymerases capable of strand-displacement synthesis are necessary. (D) The RCA reaction, which proceeds on dsDNA if assisted by PNA openers and DNA nicking.[14] (E) In immuno-RCA, the 5′ end of a primer is attached to a reporter antibody, which selectively binds to a test analyte immobilized on a solid surface.[7]

as examples], whereas others involve the circularization of hybridized linear probes by ligation followed by RCA (L-RCA; Fig. 1B). In the latter case, the in situ assembled DNA minicircles, called padlocks and earrings,[9–13] provide the corresponding DNA/RNA diagnostic assay with a higher sequence specificity, which is warranted by a multiple (at least dual) probe–target recognition and is also owing to the fact that mismatches close to the ligation point severely interfere with the ligation process. Importantly, padlocks and earrings ensure a higher stability of hybridization complex because of additional topological stabilization through the probe–target concatenation.[13] All this improves the hybridization stringency, allows one to more efficiently distinguish single-base sequence variants, and results in a highly localized hybridization/amplification signal retaining the positional information.

Further localization of the RCA-generated signal to essentially single visible points can be reached by condensation of amplication products after their hybridization to labeled oligonucleotides, known as RCA-CACHET.[4] In this way, the RCA amplicons are tagged with fluorescent labels at multiple sites in the tandem RCA-amplified DNA sequence. Thus ''decorated'' amplicons can be compacted into tiny objects by cross-linking with multivalent proteins, such as streptavidin and antibodies, which bind to amplicon-incorporated tags. If necessary, further increase of the RCA-generated signal to a superexponential level can be achieved by combining the RCA and PCR reactions.[5] Until recently, the RCA reactions have been run only on the single-stranded DNA and RNA targets, but, with the aid of PNA openers, these reactions can now be performed with dsDNA.[12,14] In immuno-RCA assays, the attachment of a reporter antibody to an RCA primer makes it possible to extend the RCA-based diagnostics on the non-nucleic-acid analytes, including proteins, which can be detected with superior sensitivities, compared to conventional enzyme immunoassays in ELISA and microparticle formats.[7]

The RCA-based analyses can be executed both as homogeneous assays in solution and as heterogeneous ''on surface'' assays, including the microtiter plate and microarray approaches for high-throughput genomics and

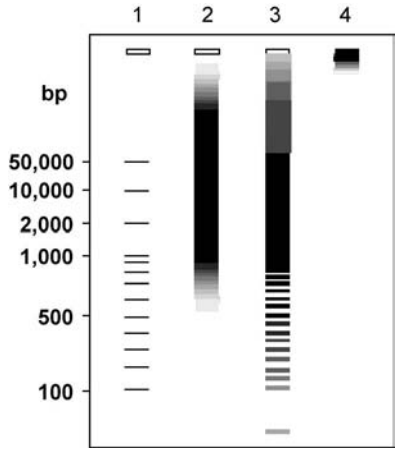

Fig. 2 Typical patterns of the RCA products generated with linear or geometric kinetics and resolved by gel electrophoresis (SYBR green or ethidium bromide staining). Usually, linear amplification is characterized by smearlike amplicons corresponding to essentially continuous distribution of single-stranded RCA products over length (lane 2). On the contrary, ladder-like amplicons are normally observed after geometric amplification representing linear concatemeric double-stranded copies of a circular template (lane 3). In some cases, most of the RCA products are so large that they cannot enter the gel (lane 4). Lane 1 corresponds to the size marker.

proteomics studies.[15,16] The RCA amplicons can be detected in several ways, such as fluorescence,[7] radiolabeling,[10] UV absorbance, and gel electrophoresis[4,12] by using either direct incorporation of various labels into the RCA products[10] or label-decorated amplicons.[4,7] Since it is not possible to even briefly describe this whole diverse range of RCA testing formats, the interested reader is referred to recent reviews[17,18] and corresponding original papers.

Specificity, sensitivity, and reproducibility

The RCA-based diagnostics exhibit an exceptional specificity for particular DNA/RNA sequences, as well as for marker molecules other than DNA/RNA, allowing the multiplex genotyping/detection of single-base mutations and specific antigens. Besides, RCA is an ultrasensitive method of detection: a variety of RCA formats permit essentially single-molecule counting of the DNA, RNA, or protein targets and some other analytes. Furthermore, RCA-based diagnostics are characterized by good reproducibility, with amplification errors being at a lower level compared to PCR. Consequently, such an exquisite sensitivity makes it possible to accurately and reliably quantify the gene copy number as well as to detect single-copy genes, discrete antigen–antibody complexes, and mRNA expression levels in individual cells.

Advantages and Limitations

RCA has several substantial advantages over other amplification techniques, which could be called ''the power of simplicity.''[17] Most importantly, RCA is an isothermal procedure and, therefore, has no need of special instrumentation to cycle the temperature, which is required with the widely used PCR-based diagnostics. This RCA feature significantly simplifies the automation and miniaturization of RCA-based diagnostics. In addition, RCA can be performed by a larger variety of DNA polymerases compared to PCR, which relies on only thermostable enzymes. Besides, RCA represents an inexpensive, more error-proof, and more sensitive (compared to PCR) analytical technology with a very wide dynamic range and higher multiplexity to serve as a potent alternative to the thermocycling diagnostic methods.

In comparison with other isothermal methods of signal, probe, or target amplification, such as transcription-based amplification, strand-displacement amplification, use of branched (or dendrimeric) probes, invasive signal amplification, or loop-mediated amplification, the RCA-based assays are less complicated and in many cases do not require any substantial preoptimization of an experimental protocol, thus being readily used by a beginner. RCA is the most flexible and adaptable amplification methodology featuring merely few drawbacks. The shortcoming of supersensitive RCA assays is that they require certain caution to avoid possible contamination/false positives. In some cases, release of probes from the hybridized targets and removal of the nontargeted DNA strands by additional treatment with endo- and exonucleases are also necessary to reach the requisite sensitivity of detection.[10,19]

CLINICALLY RELEVANT PILOT APPLICATIONS

The practical RCA potential to identify nucleic acid targets, antibodies, and antigens in clinical samples has recently been demonstrated in several feasibility studies. Specifically, the RCA-based protocol for an automated scoring of single nucleotide polymorphisms (SNPs) in a set of human genomic DNA samples with the nanogram sensitivity was developed.[15,20] Highly sensitive multiplex detection of hotspot somatic mutations present at very low abundance was also reported.[21] The RCA capability for pathogen diagnosis was convincingly proved by comparative study of RCA vs. PCR and ligase chain reaction (LCR) in detection of *Chlamydia trachomatis* in cervical specimens.[22] The workability of immuno-RCA for identification of allergen-specific immunoglobulins in samples from patients was shown in a microarray format.[23] In addition, RCA has been

adapted to immunohistochemistry, flow cytometry, and in situ hybridization to significantly improve their sensitivity without compromising cellular and tissue morphology.[24,25] RCA-mediated multiplex profiling of cytokines on microarrays with femtomolar sensitivity offers an advantageous approach for proteomic surveys.[16] All these pilot applications establish the firm basis for future clinical use of RCA methodology in a range of diagnostic applications, enabling the integration of genomic and proteomic information into cell- and tissue-based tests.

CONCLUSION

The RCA methodology represents a powerful and simple procedure for signal amplification that may serve as universal platform for in vitro diagnosis of a variety of biomarkers based on either nucleic acid sequence or antigenicity. Consequently, this technology should soon provide the laboratory researchers and clinical diagnosticians with highly sensitive and efficient customary diagnostic assays to expedite and to facilitate testing of miscellaneous analytes.

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ARTICLE OF FURTHER INTEREST

Padlock Probes, p. 962

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Rotavirus

C. Anthony Hart
N. A. Cunliffe
The University of Liverpool, Liverpool, U.K.

INTRODUCTION

Diarrheal disease is the fourth commonest cause of death worldwide and most of the deaths are in children in developing countries. Rotavirus is the major cause of diarrheal disease in children and is increasingly recognized as a problem in adults. The virus is unenveloped and has a characteristic double-shelled capsid (80 nm) with a double-stranded RNA genome. The genome comprises 11 linear segments and each segment encodes one or more virus proteins. The polypeptides are highly variable and thus genetic and antigenic changes occur by mutation (antigenic drift), genome rearrangement, genome reassortment (antigenic shift), and cross-species transmission of animal rotaviruses. Infection can be diagnosed by antigen detection (ELISA) or detection of the genome by reverse transcriptase-PCR (RT-PCR) or polyacrylamide gel electrophoresis (PAGE) of RNA extracted from feces.

THE VIRUS

Rotavirus is a medium-sized (70–80 nm) unenveloped round virus (Fig. 1), with a characteristic wheel-shaped morphology (rota is Latin for a wheel). *Rotavirus* is a genus within the family Reoviridae.[1,2] Its genome consists of 11 segments of linear double-stranded (ds) RNA which range in size from 0.6 to 3.3 kbp.[3,4] Each segment encodes one or more polypeptides (Table 1). The polypeptides expressed are either nonstructural (NSP1–5) or structural (VP, virus proteins) proteins. The nonstructural proteins are involved in virus replication and are found only in the infected cell. The mature virion has a trilayered structure (Fig. 2). The inner layer is composed of 11 dsRNA segments surrounded by VP1, VP2, and VP3. The middle layer or inner capsid is composed of VP6 and the outer layer is made up of two proteins VP4 and VP7. VP4 is cleaved by proteolysis to produce VP5* and VP8* and if this does not occur the virus is not fully infective. There are 60 spikes or knobs that extend 120 Å from the virion surface.[3] The virion has icosahedral symmetry with 132 surface capsomers and a triangulation number T13. There are 132 large channels that traverse both the inner and outer capsid layers.

REPLICATION AND PATHOGENESIS

Rotavirus was not maintained in artificial culture until it was realized that proteolytic cleavage of VP4 was required for full infectivity. Thus culture in a variety of cells including MA104 is achieved by including trypsin in the culture medium. Some rotavirus strains attach to their cellular receptor (acetylated sialic acid) via VP5*. However, recent work has shown that sulfated sialyl lipid inhibits rotavirus attachment to MA104 cells in vitro and prevents rotavirus infection in mice. Following attachment, some rotaviruses enter host cells by receptor-mediated endocytosis, but others, particularly following trypsin pretreatment, enter directly by membrane permeabilization. Uncoating of rotavirus to release dsRNA is facilitated by low intracytoplasmic [Ca^{2+}]. Virus replication takes place entirely in the cytoplasm. The majority of VP and NSP are synthesized on cytoplasmic ribosomes but the glycoproteins, VP7 and NSP4, are synthesized on the rough endoplasmic reticulum. Subviral particles assemble in cytoplasmic viroplasms and then, facilitated by NSP4, bud through the endoplasmic reticulum transiently acquiring a membrane. The lipid is gradually replaced by a thin protein layer which becomes the outer capsid as the virion matures. The progeny virions are then released by cell lysis.

During the peak of rotavirus replication, infants excrete up to 10^{11} virus particles per milliliter of stool. The infective dose is of the order of 10^2 virus particles. Rotavirus infects the mature villous enterocytes of the small intestine. It cannot infect immature crypt cells or colonic enterocytes. In addition to acetylated sialic acid and sulfated sialyl lipids, some human rotaviruses bind to integrins. Four mechanisms have been suggested for rotavirus-induced diarrhea.[3,4] First, within 12–24 hr following infection small intestinal brush border disaccharidase enzyme levels fall to less than one-third of normal. This is a result of rotaviral inhibition of transport of disaccharidases to the microvillar membranes. Dietary

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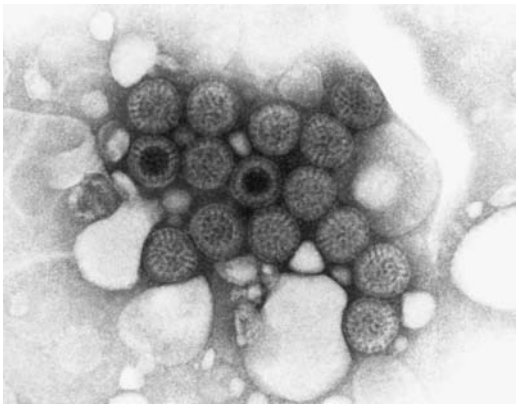

Fig. 1 Negative stain electron micrograph of rotavirus.

disaccharides must be broken down to monosaccharides as only monosaccharides can be absorbed. If they accumulate in the gut lumen then they hold water leading to an osmotic diarrhea. Recently, it has been found that NSP4 or even a 22-amino acid fragment from it can induce diarrhea in neonatal mice when administered orally or intraperitoneally.[5] It is thought that NSP4 acts as an exterotoxin causing diarrhea by opening a cellular cation channel which causes an increase in intracellular [Ca^{2+}], activation of adenyl cyclase, and cAMP-dependent Cl^- secretion. This causes a secretory diarrhea in a manner analogous to some bacterial enterotoxins.[6] Rotavirus is released from enterocytes by lysis and the increased cytoplasmic [Ca^{2+}] causes enterocyte death by oncosis. Thus the rate of dying of villous enterocytes exceeds the rate of production of new enterocytes in the crypts. This results in blunting of the villi and subsequent loss of surface area for absorption of fluid, electrolytes, and nutrients. Finally, there is some evidence that rotavirus affects the intestinal neuroendocrine axis which may also contribute to diarrhea. There are suggestions that NSP4 might be involved in each of the above mechanisms.[6]

IMMUNITY

Rotavirus diarrhea lasts on average 5–6 days and seems to resolve as the virus exhausts enterocytes to infect when it reaches the colon. In addition, it appears that T-cell-mediated immunity is important in resolution, at least in mice. Natural rotavirus infection protects against subsequent symptomatic infection but to a lesser extent against virological infection. It is likely that we will all experience a rotavirus infection, usually asymptomatic, every 2–3 years. However, immunity does appear to decrease with age and rotavirus outbreaks are increasingly reported in elderly patients. In a cohort study in Mexican infants the risks of rotavirus disease and rotavirus infection after one, two, or three prior episodes of infection were 0.23, 0.17, or 0.08, and 0.62, 0.40, or 0.34, respectively.[7] However, it is not clear which are the most important correlates of immunity. In several studies, serum IgG and IgA rotavirus antibodies correlate best with protection. Fecal antirotavirus IgA (which reflects duodenal IgA) also reflected protection against infection.

Table 1 Rotavirus genome and gene products

Genome segment	Molecular size (bp)	Gene product	Molecular weight (kDa)	Location in virion	Function
1	3,302	VP1	125	Core	RNA polymerase
2	2,690	VP2	94	Core	RNA binding
3	2,591	VP3	88	Core	Guanylytransferase
4	2,362	VP4 (VP5*+VP8*)	88	Outer capsid	Cell attachment, hemagglutinin, membrane permeabilization, neutralizing antigen (P-serotype)
5	1,581	NSP1	53	Nonstructural	RNA binding (zinc finger)
6	1,356	VP6	41	Inner capsid	Group and subgroup antigen, major capsid protein
7	1,104	NSP3	34	Nonstructural	RNA binding
8	1,059	NSP2	35	Nonstructural	RNA binding
9	1,062	VP7	38	Outer capsid	Glycoprotein, neutralizing antigen (G-serotype)
10	751	NSP4	28	Nonstructural	Glycoprotein, virus assembly, enterotoxin
11	667	NSP5	26	Nonstructural	RNA binding

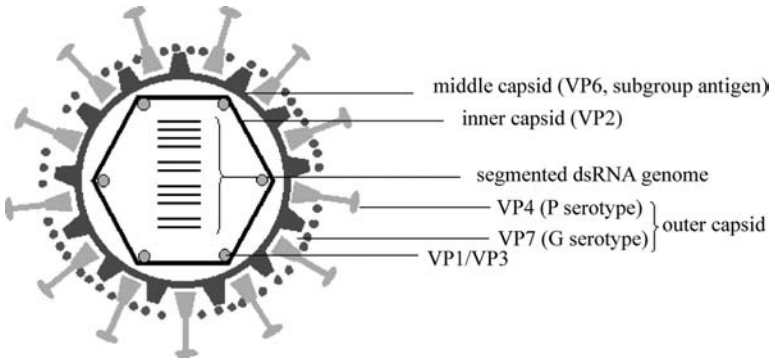

Fig. 2 Drawing showing the organization of the various rotavirus structural proteins. (*View this art in color at www.dekker.com.*)

TYPING, ANTIGENS AND ANTIGENIC VARIATION

Serogroups

Rotaviruses are subdivided into seven groups (Table 2). These serogroups are determined by epitopes on VP6 which forms the inner capsid. Of these, humans are infectable by groups A, B, and C, as are many other animal species. Most human infections are due to group A rotaviruses. Group B rotaviruses are associated with large epidemics of disease in adults and children alike.[8,9] Group C rotaviruses usually cause only mild disease and by adulthood 30% have antibodies. VP6 is the most abundant viral protein (780 molecules per virion) and the group-specific epitopes are localized between amino acids 48 and 75. Although antibodies to group epitopes are nonneutralizing in vitro they do give protection in mice.

Group A rotaviruses can be further divided into subgroups based on other, as yet undefined, epitopes on VP6. Four subgroups are possible: I, II, I and II, and neither.

Serotypes

There are two major serotype antigens: on VP4, the P (protease sensitive) type, and on VP7, the G (glycoproteins) type. These are expressed on the surface of the virus and antibodies to P and G epitopes are protective against infection.

VP4 forms the 60 knobs that protrude from the virion surface. It is nonglycosylated and makes up only 1.5% of the virion protein. Cleavage of VP4 (to VP5* and VP8*) stabilizes the knobs which become icosahedrally ordered. VP5* forms the body and base of the knob in β-pleated sheets and α-helices, respectively, VP8* forms the head in β-sheets, and both are involved in virus entry by permeabilization. Monoclonal anti-VP4 antibodies neutralize infectivity, and by use of such antibodies a number of different P-serotypes have been defined (Table 3).

Table 2 Reservoirs of different rotavirus groups

Group	Reservoirs in which infection has been detected
A	Man, primates, horse, sheep, pig, cattle, dog, cat, turkey, chicken, mice
B	Man, pigs, cattle, sheep, rats
C	Man, pigs, cattle, ferret
D	Chicken, turkey
E	Pigs
F	Chicken
G	Chicken

Table 3 Group A rotavirus P-serotypes and genotypes

Genotype	Serotype	Human pathogen	Other animals
[1]	6	–	Cattle, monkeys
[2]		–	Monkeys
[3]	5	+	Monkeys, dogs, cats
[4]	1B	+	
[5]	7	–	Cattle
[6]	2A, 2B, 2C	+	
[7]	9	–	Pigs
[8] and [8*]	1A	+	
[9]	3	+	Cats
[10]	4	+	
[11]	8	+	Cattle
[12]		–	Horses
[13]	3B	+	
[14]		+	Pigs
[15]		–	Sheep
[16]	10	–	Mice
[17]		–	Cattle, birds
[18]		–	Horses
[19]		–	Pigs
[20]		–	Mice

Table 4 Group A rotavirus G-types

Sero(geno)type	Host species
1 and 1*	Humans, pigs, cattle
2	Humans, pigs
3	Humans, monkeys, dogs, cats, horses, rabbits, mice, goats
4	Humans, pigs
5	Humans, pigs, horses
6	Humans, cattle
7	Birds, cattle
8	Humans, cattle
9	Humans, pigs
10	Humans, pigs, sheep
11	Pigs
12	Humans
13	Horses
14	Horses

Other P-types have been delineated by RT-PCR and sequencing (P-genotypes), and, unfortunately, P-genotypes do not correspond to serotype designations. VP8* carries the P-specific epitopes whereas VP5* epitopes are cross-reactive. There are 20 different P-genotypes and 12–15 P-serotypes (Table 3). Infection with one P-type does not necessarily protect against infection with the others. By convention P-genotypes are designated by a number in a squared bracket.

VP7 is the major neutralization antigen and comprises 30% of protein in the mature virion. It forms the smooth surface of the outer capsid through which the VP4 knobs protrude. It is a glycoprotein containing N-linked high-mannose oligosaccharides. It is encoded on the seventh, eight, or ninth segment depending upon the rotavirus strain. Its role in viral replication is unclear. Currently, 14 G-serotypes have been defined by use of neutralizing monoclonal antibodies and by RT-PCR. Fortunately, the G-serotypes and G-genotypes coincide (Table 4). Within a particular G-genotype the deduced amino acid sequences are 91–100% similar, but between genotypes the differences are significantly greater. Most of the divergence is represented in nine regions of VP7. Of the variable regions (VR), VR5 (αα 87–101), VR7 (αα 142–152), and VR8 (αα 208–221) are the major neutralization epitopes and escape mutants with point mutations in these regions occur easily.[10] Infection with one genotype does not necessarily impart protection against others.

Electropherotypes and Genogroups

Because there are so many virus particles excreted in the stool of an infected infant it is possible to extract rotavirus dsRNA in sufficient quantities for PAGE. The migration patterns of the 11 dsRNA segments allow rotaviruses to be typed. There are three major electropherotypes: long, short, and super-short. With the short electropherotype, the 11th genomic segment is larger than usual and thus migrates more slowly resolving between the 9th and 10th segments. With the super-short electropherotype, the 11th segment is even larger. By using radiolabeled, positive sense single-stranded RNA transcribed from viral cores in Northern blots at high stringency, it is possible to subdivide group A rotaviruses into two major genogroups. These are named for the prototype strains, namely, Wa (genogroup 1) and DS-1 (genogroup 2). The Wa genogroup viruses are generally long electropherotype, subgroup II with VP4 P[6] or P[8] and VP7 G1, G3, or G4 genotypes, whereas the DS-1 genogroup are usually subgroup I, short electropherotype, and P[4] and G2 genogroup. However, these broad subdivisions are being eroded by the tremendous variability of rotaviruses infecting man and other animals.[4]

Variability

Rotavirus populations can alter by point mutation (antigenic drift), genome rearrangement, genome reassortment (antigenic shift), and zoonotic introduction. The rotaviral genome is replicated by an error-prone RNA-dependent RNA polymerase and there is no proofreading capacity. This results in a mutation rate of one mutation per genome replication which is of the same order of magnitude as that of influenza A virus. Sequence variation has been most studied in the genes encoding VP7 and VP4, and there is more and more evidence of divergence from the original G-types.[4] Thus G1 and P[8], which globally represent some of the most common genotypes, have drifted sufficiently to require design of new primers for RT-PCR detection.[4] For example, P[8] rotaviruses can currently be divided into three lineages (Wa-like, F45-like, and OP354-like) on the basis of deduced amino acid substitutions at 11 sites on the VP4 protein. Similar variability in NSP4 even in the toxic peptide region is common.[4] Variability in non-group A rotaviruses is less well studied, but the nucleotide sequence of segment 8 (putative NSP2) of a group B adult diarrhea rotavirus from Calcutta showed only 77% homology to that of a murine strain and 93% similarity to other human isolates.[9]

Rotavirus genome rearrangements were detected during studies of chronic infection.[4] On PAGE of serial samples of feces from immunodeficient children, RNA segments were lost or diminished in concentration and new heavier bands emerged. Sequencing the new bands indicated that there were genome rearrangements but that these did not usually result in abnormal viral proteins.

Table 5 Primers used for VP4 (P) typing

Primer	Strain	Serotype ([GT])	NT	Sense	Sequence (5′ to 3′)	Primer type
Con3	Ku	1A[8]	11–32	+	TGG CTT CGC CAT TTT ATA GAC A	Consensus
Con2	Ku	1A[8]	868–887	–	ATT TCG GAC CAT TTA TAA CC	Consensus
1T-1	Ku	1A[8]	339–356	–	TCT ACT TGG ATA ACG TGC	Type specific
2T-1	RV5	1B[4]	474–494	–	CTA TTG TTA GAG GTT AGA GTC	Type specific
3T-1	1076	2A[6]	259–278	–	TGT TGA TTA GTT GGA TTC AA	Type specific
4T-1	K8	3[9]	385–402	–	TGA GAC ATG CAA TTG GAC	Type specific
5T-1	69M	4[10]	575–594	–	ATC ATA GTT AGT AGT CGG	Type specific

In contrast, genome reassortment is a major generator of diversity. This happens when two different rotaviruses infect the same cell at once. Mixed rotavirus infections have been detected in humans and a recent study of 3601 rotavirus strains from U.K. children with rotavirus diarrhea found approximately 2% to have novel [P]- and G-type combinations.[11]

Although it was considered that rotaviruses were species-specific it is becoming increasingly apparent that this is not so. The simian rotavirus SA11 can be used to infect mice, and some human rotavirus vaccines are reassortants of bovine or simian rotaviruses with human rotavirus VP7. How frequently this occurs naturally is unclear and most often it is inferred by detecting an animal-specific VP4 or VP7 in a human rotavirus strain. Examples of this include G5 (pigs), G6, and G8 (cattle) rotaviruses, and, for example, G8 rotavirus makes up over 50% of the G types in Malawi and Nigeria. Analysis of the Malawian rotavirus VP7 gene indicated that it is most closely related to bovine strains A5 (93.6% identical) and Cody (92.3%). In Nigeria a G8 cattle rotavirus isolate was found to be closely related to a human isolate in its VP7 (99.9%) and by Northern blot where all 11 segments cohybridized. Interspecies transmission combined with genomic reassortment appears to be a powerful generator of diversity.

THE DISEASE

Rotavirus diarrhea is as highly prevalent in developed as in developing countries, and all children have been infected by 2 years of age. It is responsible for from 20% to 60% of cases of infantile gastroenteritis that require hospitalization and for up to 40% in community-based studies.[2] In temperate countries it peaks in winter or early spring, whereas in sub-Saharan Africa peaks coincide with dry seasons.[1] Infection is transmitted feco-orally, and after a short incubation period (2–3 days) patients present with acute dehydrating watery diarrhea sometimes with vomiting. Illness lasts on average 5–6 days but prolonged rotavirus excretion can occur after severe disease occasionally with relapses of diarrhea. Extraintestinal disease including acute myositis, hemophagocytic lymphohistiocytosis, aseptic meningitis, and encephalitis has been described rarely.[1] Of particular interest has been the recent detection of rotavirus viremia which might be linked to such extraintestinal infection.[12]

Diagnosis

Laboratory diagnosis depends upon detection of the virus, its antigens, or genome in the feces of infected patients.

Table 6 Primers used for VP7 (G) typing

Primer	Strain	Serotype	NT	Sense	Sequence (5′ to 3′)	Primer type
9Con1	Wa	G1	37–56	+	TAG CTC CTT TTA ATG TAT GG	Consensus
9Con2	Wa	G1	922–941	–	GTA TAA AAT ACT TGC CAC CA	Consensus
9T-1	Wa	G1	176–195	–	TCT TGT CAA AGC AAA TAA TG	Type specific
9T-2	S2	G2	262–281	–	GTT AGA AAT GAT TCT CCA CT	Type specific
9T-3P	107E	G3	484–503	–	GTC CAG TTG CAG TGT TAG C	Type specific
9T-4	ST3	G4	423–440	–	GGG TCG ATG GAA AAT TCT	Type specific
9T6A	Hun4	G6	178–196	–	TAT CGG TGG TAA TGC TTA	Type specific
9T6B	Hun6	G6	178–196	–	CAC YGG TAG TAA CAC TTG	Type specific
9T6C	Hun5	G6	178–197	–	GAY TGG TGG CAY TGT TTAC	Type specific
106	HMG89	G8	681–697	–	TCT TCA AAA GTC GRA GTG	Type specific
9T-9B	116E	G9	131–147	–	TAT AAA GTC CAT TGC AC	Type specific

Where Y=thymine or cytosine.

Negative stain electron microscopy was the method first used to detect rotavirus. Its major advantages are that virus is detected by its distinctive morphology (Fig. 1), so the test is highly specific and the technology is ''catch-all'' in that other viral enteropathogens can also be detected. Its disadvantages are that it requires specialized expensive equipment with an experienced electron microscopist and that to be detectable there must be over 1 million viral particles per milliliter of sample.

A number of antigen detection formats have been designed including latex particle agglutination (LPA) and enzyme-linked immunosorbent assays (ELISA). In general, ELISA is more sensitive than LPA but with both there are problems of specificity. Latex particle agglutination has the advantage over ELISA of being in a format for single tests whereas ELISA is better for batch testing. Both are available as commercial kits.

Genome detection is either by PAGE of dsRNA extracted from stool or RT-PCR. Silver-stained PAGE gels are of equivalent sensitivity and specificity to electron microscopy. However, RT-PCR is the most sensitive technique but there are no commercial kits available. Using RT-PCR has the added benefit of allowing virus genotyping. Some of the PCR oligonucleotide primers available for detection and genotyping are shown in Tables 5 and 6.

TREATMENT AND PREVENTION

The mainstay of treatment is the assessment and degree of dehydration and appropriate rehydration as necessary. There are no antirotaviral drugs available but probiotics (*Lactobacillus* spp.) and oral pooled human immunoglobulin have been shown to decrease the frequency and duration of vomiting and diarrhea.[1] It is difficult to prevent transmission of rotavirus by simple hygienic measure, but in hospitals, children should be nursed in isolation or cohorted in rotavirus wards. Specific prevention by vaccine is possible as was demonstrated by the recently withdrawn live rhesus reassortant vaccine (containing G1–G4). It was one of the first rotavirus vaccines to be equally efficacious in infants in developing and developed countries. It was withdrawn from use because of concerns over its potential to cause intussusception. There are, however, a number of new candidate vaccines in production.

CONCLUSION

Rotavirus is the most important cause of infantile gastroenteritis both in terms of mortality and morbidity. It has a highly mutable genome changing by mutation, rearrangement, and reassortment, thus new strains are continually emerging. Continued vigilance is needed.

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Salmonella spp.

Kenji Hirose
National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan

Ken-ichiro Itoh
National Institute of Infectious Diseases, Musashi-Murayama City, Tokyo, Japan

Haruo Watanabe
National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan

INTRODUCTION

Salmonella species have been isolated from humans and most animals, including domestic and wild mammals, reptiles, and birds. Many *Salmonella* serovars cause salmonellosis in humans and animals. *Salmonella* infection of humans and animals is one of the major public health problems in the world. *Salmonella* is also one of the major pathogens of foodborne disease throughout the developed and developing countries. *Salmonella* is transmitted to humans by contaminated food or poor hygiene. Most *Salmonella* infections result in self-limited gastroenteritis, and less commonly cause bacteremia with focal infection. The bacteremia caused by *Salmonella enterica* serovar Typhi and Paratyphi A are called typhoid fever and paratyphoid fever, respectively.

TAXONOMY

The genus name ''*Salmonella*'' is derived from Dr. D. E. Salmone, who first isolated *Salmonella choleraesuis* from porcine intestine. The nomenclature and classification of *Salmonella* have changed many times over the past century and still are not entirely stabilized. The classification has divided *Salmonella* into two species, *S. enterica* and *S. bongori*.[1] The *S. enterica* can be classified into six subspecies by its biochemical characteristics and host range (Table 1). Almost all serotypes pathogenic to humans belong to the subspecies *enterica*. The genus *Salmonella* is extremely polymorphic. Members of the seven *Salmonella* subgroups have been classified into over 2300 serovars according to somatic O, capsular Vi, and flagellar H antigens.[2] Differences within *Salmonella* serovars are based on those of the surface antigen such as O and H antigens. The O antigens are derived from polysaccharide domains of lipopolysaccharides (LPS) in the cell wall and the H antigens are derived from flagellin proteins in the flagella. O and H antigens are used for identification of *Salmonella* serovars by agglutination tests with O and H antigen specific antisera, and *Salmonella* serovars are determined by the combination of O and phase 1 and 2 H antigen type. Different *S. enterica* serovars produce different disease syndromes and show host specificities according to their antigenic profiles (Table 2). Therefore, it is necessary and important to differentiate *Salmonella* serovars from one another to ensure that each pathogen and its epidemiology are correctly recognized.

Major human pathogenic serovars include serovars Typhi, Paratyphi A, Shottmuelleri (Paratyphi B), Hirschfeldii (Paratyphi C), and Sendai. These serovars are almost always pathogens only of humans. The clinically important serovars, serovar Typhi and Paratyphi A, which are pathogens only of humans, cause enteric fever. The remaining serotypes of salmonellae are broadly spread into the animal kingdom. *Salmonella* has been isolated from all species, including mammals, poultry, birds, reptiles, amphibians, and insects. Human salmonella infections are generally caused by ingestion of contaminated food that is made from animal products, such as eggs and meat. *S.* Typhimurium and *S.* Enteritidis are the most frequently isolated serovars from foodborne outbreaks worldwide.

ORGANISM

The genus *Salmonella* belongs to the family Enterobacteriaceae. *Salmonella* is a gram-negative, motile, facultative anaerobic, non-spore-forming bacillus, 2–3 μm by 0.4–0.6 μm in size. It is differentiated from other Enterobacteriaceae by biochemical tests. *Salmonella* spp. ferment glucose, maltose, and mannitol but not lactose or sucrose. Most *Salmonella* strains produce acid and gas by fermentation. However, some *Salmonella* strains have variable biochemical tests. The following exceptional characteristics are helpful in identification; *S.* Typhi

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Table 1 Classification of the genus *Salmonella*

Species	Subspecies
S. enterica	*S. enterica* subsp. *enterica*
	S. enterica subsp. *salamae*
	S. enterica subsp. *arizonae*
	S. enterica subsp. *diarizonae*
	S. enterica subsp. *houtense*
	S. enterica subsp. *indica*
S. bongori	

does not produce gas and is ornithine-negative, *S.* Choleraesuis is trehalose-negative, and *S.* Gallinarum–Pullorum is nonmotile.

IDENTIFICATION

Isolates with biochemical characteristics typical of *Salmonella* should be serogrouped by agglutination with polyvalent or monovalent antisera. Three kinds of surface antigens, namely, O and phase 1 and phase 2 H antigens are detected by agglutination by antigen-specific antisera in slide and tube agglutination tests. Although complete O-serotyping is used for identification, most laboratories examine only agglutination reactions to differentiate into groups O2(A), O4(B), O7(C), O9(D), O3,10(E_1), and several other groups. *S.* Enteritidis, which causes gastroenteritis, and *S.* Typhi, which causes typhoid fever, are both in the O9 group. Similarly, another cause of gastroenteritis, *S.* Typhimurium, and *S.* Paratyphi B, another cause of enteric fever, are both in the O4 group. Classical methods that rely on cultivation and biochemical tests for detecting *Salmonella* are time-consuming and often labor-intensive. In addition, for serotyping, antibodies must be produced for each serovar, which is extremely complex and time-consuming. Easier and more rapid methods are needed to identify *Salmonella* serovars and these are now available.

IDENTIFICATION OF MAJOR *SALMONELLA* SEROVARS BY PCR

Polymerase chain reaction (PCR) protocols have been developed to detect major *Salmonella* serovars, such as Typhi, Paratyphi A, Typhimurium, and Enteritidis. However, these methods are not yet widely used and are impractical in the areas where such salmonella infections are common. Detection of *Salmonella* species and major *Salmonella* serovars by PCR has been reported by many researchers, as shown in Table 3. *S.* Typhi and Paratyphi A are important in clinical laboratories, and their direct detection in blood or stool has been intensively studied.

Diagnosis of typhoid and paratyphoid fevers is performed on blood or stool by using culture methods and requires at least 4 or 5 working days. A rapid, alternative diagnostic method is required for the diagnosis of typhoid fever and paratyphoid fever. Targets for detection of *S.* Typhi by PCR include the *fliC-d* gene[3] Vi capsular antigen gene,[4] and 16S-23S rRNA spacer region gene.[5] If only one gene is targeted for the

Table 2 Major pathogenic *Salmonella* serovars

			H antigens		Pathogenicity to	
O serogroup	Serovar	O antigens	Phase 1	Phase 2	Humans	Animals
O2 (A)	Paratyphi A	1,2,12	a	[1,5]	Paratyphoid fever	
O4 (B)	Paratyphi B	1,4,[5],12	b	1,2	Gastroenteritis	
	Typhimurium	1,4,[5],12	i	1,2	Gastroenteritis	Mouse (typhoid fever)
O7 (C)	Paratyphi C	6,7,[vi]	c	1,5	Gastroenteritis	
	Choleraesuis	6,7	c	1,5	Gastroenteritis	Pig (typhoid fever)
O9 (D)	Dublin	1,9,12,[vi]	g,p	—		
	Enteritidis	1,9,12	[f],g,m,[p]	1,7	Gastroenteritis	Mouse, guinea pig (typhoid fever)
	Typhi	9,12,[vi]	d or j	z_{66}	Typhoid fever	
	Gallinarum	1,9,12	—	—		Chicken (diarrhea)
O3,10 (E1)	Give	3,10,[*15*],[*15*,*34*]	[d],l,v	—	Gastroenteritis	
	Anatum	3,10,[*15*],[*15*,*34*]	e,h	—	Gastroenteritis	
O1,3,19 (E4)	Senftenberg	1,3,19	q,[s],t	—	Gastroenteritis	

[ ], O or H antigens that may be present or absent without relation to phage conversion.
—, O antigens that are produced by phage conversion.

Table 3 Identification of major *Salmonella* serovars and detection of fluoroquinolone resistance gene by PCR

Serovar	Target genes	References
All (*Salmonella* specific)	*invA*	Rahn et al.[14]
	stn	Makino et al.[13]
	phoP, *Hin*, *fliC-i*	Way et al.[15]
	spaQ (real-time PCR)	Kurowski et al.[12]
Typhi	*rfbE*, *rfbS*, *viaB*, *fliC*	Hirose et al.[6]
	viaB	Hashimoto et al.[4]
	fliC	Song et al.[3]
	16S-23S rRNA spacer region	Zhu et al.[5]
	gyrA mutation (quinolone resistance gene)	Hirose et al.[22]
Paratyphi A	*rfbE*, *rfbS*, *viaB*, *fliC*	Hirose et al.[6]
	gyrA mutation (quinolone resistance gene)	Hirose et al.[22]
Typhimurium	*sefA*, *fliC*	Soumet et al.[8]
	rfbJ, *fliC*, *fljB*	Lim et al.[7]
	gyrA mutation (quinolone resistance gene)	Giraud et al.[21]
Enteritidis	*sefA*	Woodward et al.[9]
	spvA	Lampel et al.[10]
	rfbE, *fliC*	Itoh et al.[11]
	sefA, *fliC*	Soumet et al.[8]
Dublin	*rfbE*, *fliC*	Itoh et al.[11]
Galinarum–Pullorum	*rfbE*, *fliC*	Itoh et al.[11]

identification of *S.* Typhi in these methods, other *Salmonella* serotypes can also be amplified. Multiplex PCR detecting two or more genes is now thought to be a more specific method for PCR identification. Recently, specific identification of *S.* Typhi by multiplex PCR, which targets the *rfbE*, *viaB* and *fliC-d* genes, has been reported, and this method correctly identified *S.* Typhi and differentiated *S.* Typhi from other *S. enterica* serovars with similar antigenic formulae.[6] This system enabled us to identify and differentiate *S.* Typhi from *S.* Paratyphi A, both clinically important serovars in human infections, by only a single PCR on bacteria isolated from blood or stool culture. A similar method for specific detection of *S.* Typhimurium has also been developed.[7] The antigenic formula of *S.* Typhimurium is O antigen (O4), phase 1 (H1) antigen (H:i), and phase 2 (H2) antigen (H:1,2). Three primer sets were designed for three genes—*rfbJ*, *fliC-i*, and *fljB-1,2*—encoding O:4, H:i, and H:1,2 antigens, respectively, and these were used in a multiplex PCR to identify *S.* Typhimurium. In another PCR, *sefA* and *fliC* genes were used as specific targets for *S.* Typhimurium.[8] PCRs for identification of *S.* Enteritidis, *S.* Dublin and *S.* Galinarum–Pullorum have also been developed.[8–11] Similarly, specific detection of *Salmonella* spp. has also been reported, and *Salmonella* spp. is detected by *invA*, *stn*, *phoQ*, *hin*, *fliC-i*, and *spaQ* genes with multiplex PCR or real-time PCR.[12–15]

The whole genome sequences of *S.* serovar Typhimurium LT2 and *S.* serovar Typhi CT18 have been determined and have been published in 2001. The whole genomic sequence data will make it possible to develop better diagnosis methods that are based on DNA.[16,17]

ANTIMICROBIAL RESISTANCE

Typhoid fever and paratyphoid fevers are still serious public health problems in many developing countries and are endemic in many countries, especially in Southeast Asia and Africa. Typhoid and paratyphoid fever are sometimes fatal infections of adults and children, with bacteremia and inflammatory destruction of the intestine and other organs. They require urgent treatment by the administration of appropriate antibiotics. Recently, fluoroquinolone-resistant and nalidixic acid-resistant strains of *S.* Typhi and *S.* Paratyphi A have emerged in the Indian subcontinent, becoming problematic in many other countries.[18,19] Fluoroquinolone resistance has been associated with mutations in *gyrA* and *parC*, which encode DNA gyrase and topoisomerase IV, respectively. Particularly in Enterobacteriaceae, *gyrA* mutations contribute greatly to fluoroquinolone resistance.[20] The product of the *gyrA* gene, DNA gyrase, is thought to be a target of fluoroquinolones. *gyrA* mutations lead to

conformational changes in DNA gyrase and cause fluoroquinolone resistance. Mutations at codon 83 and/or 87 of *gyrA* have been associated with fluoroquinolone resistance in *Salmonella* spp. Screening methods for antimicrobial resistance genes are also being developed by PCR. Screening methods for *gyrA* mutations giving fluoroquinolone resistance by PCR-restriction fragment length polymorphism (RFLP) have been developed and have been reported for *S.* Typhimurium,[21] *S.* Typhi, and *S.* Paratyphi A.[22] PCR-RFLP seems to be one of the best methods for the rapid detection of *gyrA* mutations.

CONCLUSION

Most of these PCR methods described here cannot be used for direct detection from clinical samples such as blood or stool. One has to isolate the causative agent by ordinary culture methods using SS agar or Maconkey agar plates, then apply several colonies on selective media to the PCR identification system. Thus, it takes 2 or 3 working days to get a final result.

The PCR methods described here may make it possible to detect and/or identify clinically important *Salmonella* serovars, such as *S.* Typhi, *S.* Paratyphi A, *S.* Enteritidis, and *S.* Typhimurium, within a few working days of arrival in the diagnostic microbiology laboratory. Furthermore, if specific primers to amplify other flagellar antigen genes and other O antigen synthesis genes are designed, it might be possible to identify other human pathogenic *Salmonella* serovars by a combination of PCR primers responsible for O and H antigen genes using multiplex PCR.

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Serial Analysis of Gene Expression (SAGE) Technology

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Farid E. Ahmed
East Carolina University, Greenville, North Carolina, U.S.A.

INTRODUCTION

Serial analysis of gene expression (SAGE) technology has provided rapid and comprehensive approaches for elucidating quantitative gene expression patterns that do not depend on a priori knowledge of gene sequence or transcript information. The SAGE method is based on the isolation of unique short sequence tags (9–21 bp long) from individual transcripts and their serial concatenation (linking) into long deoxyribonucleic acid (DNA) molecules, thereby overcoming the problem of transcript redundancy and allowing the method to be performed without subtraction or normalization. Sequencing of concatemer clones in parallel reveals individual tags and allows for the identification of up to $\sim$30 tag sequences in each reaction. Matching tags to genome sequences identifies the gene corresponding to each tag, and reveals novel internal exons and uncharacterized genes. Furthermore, the number of times a particular tag is observed provides a quantitative measure of transcript abundance in the ribonucleic acid (RNA) population.

The SAGE protocol can be used for studying any kind of biological phenomenon resulting from changes in cellular transcription. The technology not only provides a global gene expression profile of a particular type of cell or tissue in any organism, but also identifies a set of specific genes responsible for function by comparing the profiles obtained from a pair of cells kept at different circumstances.[2]

PRINCIPLES OF SAGE

SAGE is an ingenious method that allows for determining the expression patterns of thousands of unknown genes simultaneously in parallel (i.e., an open system) and quantitatively.[1] It was invented in the early 1990s, and was published in 1995 by distinguished investigators from the Johns Hopkins University Oncology Center (Baltimore, MD).[2] The original SAGE is based on two principles: 1) a unique 9- to 10-bp oligonucleotide fragment, or SAGE tag, can unambiguously identify a specific transcript [i.e., because a 9-bp fragment has 4^9 (or 262,144) potential combinations], and estimates suggest that the human genome encodes about 80,000 transcripts. Thus, statistically speaking, a 9-bp sequence tag obtained from a defined position in the cDNA can theoretically identify all human transcripts; and 2) concatenation (linking) of these tags, followed by their cloning into a plasmid vector, allows for serial analysis of transcripts by the sequencing of multiple tags within a single clone. Figure 1 depicts the SAGE method.

Double-stranded (ds) copy (c) DNA is synthesized from poly $(A)^+$ messenger (m) RNA using biotinylated oligo deoxythymine (dT) primer. The cDNA is then cleaved with an anchoring enzyme (AE)—a restriction endonuclease with a 4-bp recognition site, which cleaves most transcripts, on the average, every 256 bp (4^4). *Nla*III is the most frequently used AE. The most 3′ position of the cleaved cDNA is isolated by binding to streptavidin beads, providing a unique site on each transcript that corresponds to the restriction site located close to the polyadenylated tail. The cDNA is then divided in half and ligated via the anchoring restriction site to one of two linkers containing type IIS restriction sites (tagging enzyme, or TE) such as *Bsm*II, which cleaves at a defined distance up to 20 bp away from their asymmetrical recognition site. The linkers are designed so that when they are cleaved with TE, they are released with a short piece of the cDNA—a 9-bp tag. The two pairs of released blunt-ended tags are ligated to each other in a tail-to-tail formation by T4 polymerase, thus serving as a template for a polymerase chain reaction (PCR) with primers specific to each linker. Because amino groups block the 5′ ends of the linkers, only the mRNA-derived termini can be ligated. Thus the resulting amplification products contain two tags linked tail-to-tail (DITAGS), flanked by sites for the AE.

Analysis of DITAGS before amplification allows checking for any distortions as a result of the PCR amplification because the probability of any two tags being coupled in the same DITAG is small. Repeated DITAGS produced by biased PCR could then be excluded from analysis without affecting final results. Cleavage of the PCR product with an AE allows release of DITAGS that could then be concatenated by ligation, recovered by polyacrylamide gel (PAG) electrophoresis, cloned into a plasmid vector, and sequenced. Theoretically, inefficient enzymatic reactions that occur during the generation of a SAGE library can lead to inaccurate data. However,

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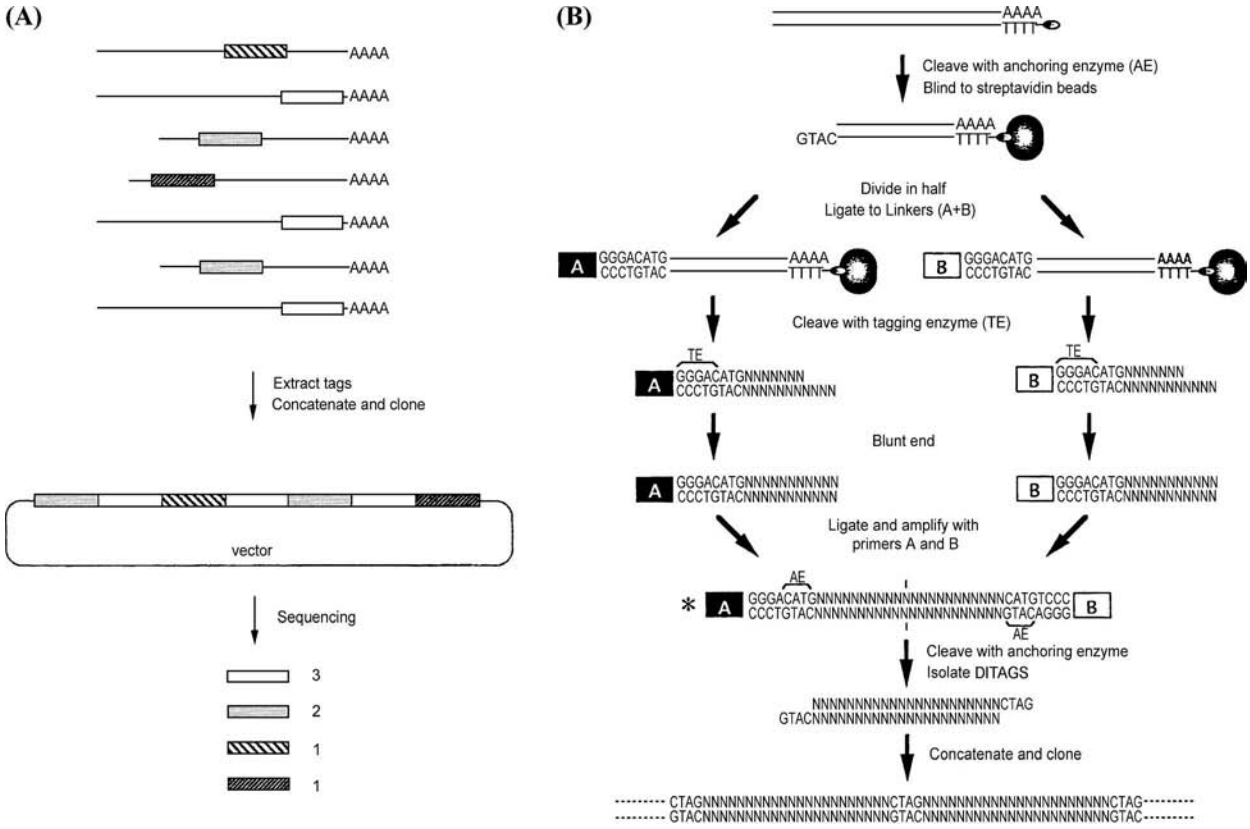

Fig. 1 Principles of SAGE. (A) A model showing the hypothetical eukaryotic cell that contains seven mRNA molecules composed of four species. Boxed are tags for the appropriate mRNA species. (B) A schematic of the procedure showing the AE, the TE, and linkers A and B, whose 3′ positions are designed to contain TE sequences. Transcript-derived tag sequences are denoted by Ns. *Blunt-end ligation step. (From Ref. [11].)

rigorous testing of the reagents and the use of appropriate controls can avoid this problem. The analysis of SAGE data is performed by statistical methods, which extracts the tags from the sequence and determines their abundance and identity. Informatics is also available for data presentation.[3]

Several technical and conceptual difficulties intrinsic for the original SAGE method have been raised, including: 1) the need for a relatively high quantity of mRNA; 2) problems of constructing tag libraries; 3) short length of tags (9 or 10 bp); 4) inefficiency of blunt-end ligation; 5) possible loss of a fraction of mRNA species because of anchoring and tagging errors; 6) low cloning efficiency of concatemers; 7) reliance on PCR to amplify ligated DITAGS that may compromise the quantitative aspect of the method by altering the representation of mRNA; 8) tag sequencing errors; 9) incorrect matching of SAGE tag to sequences available in databases; and 10) options in case of unavailability of matches. Solutions to these problems are addressed below.

MODIFICATION TO THE ORIGINAL SAGE PROTOCOL

Since the inception of the original protocol, several modifications have been instituted to increase its efficiency. To reduce the amount of RNA needed, a modification named microSAGE was made, which required 500- to 5000-fold less starting input RNA. The new method utilized a single-tube format for all steps from RNA isolation to tag release in which the RNA—and later the cDNA—remained immobilized to the wall of the tube by means of streptavidin–biotin binding. Furthermore, total RNA was used instead of poly $(A)^+$ mRNA, resulting in reduction of the number of manipulations and accompanying loss of materials. In addition, a limited number of additional PCR cycles were performed on DITAGS.[4]

Other attempts to lower the quantity of input materials relied on the inherent poly (C) terminal transferase activity of reverse transcriptase to switch templates during

DNA polymerization (Clontech SMART system), which allowed the generation of PCR-amplified cDNA prior to the SAGE procedure. In PCR-SAGE, 900 pg of poly $(A)^+$ RNA generated a library validated by the presence of transcripts expressed in human oocytes.[5] In SAGE-Lite, starting with 50 ng of total RNA derived from cerebrospinal tissues or HT1080 cells, first-strand cDNA was PCR-amplified to generate large quantities of ds-cDNA, which was utilized as a substrate for SAGE analysis.[6] MiniSAGE[7] and SADE[8] employed procedures to diminish the loss of materials throughout the various steps, thereby allowing the use of only 1 μg of total RNA and 10^5 cells, respectively. Using an approach similar to microSAGE, a two-round PCR amplification of the DITAGS, and other minor modifications, a SAGE library was generated from a single PC-3 cell (scSAGE), but without adequate validation of results.[9] Although the above approaches suggest that amplified transcripts represent the original DNA mixture, a general concern about using PCR for tag amplification is the potential tag bias during PCR resulting in distortion of the true transcript level, which is especially important for template transcripts present at low abundance, leading to them being underrepresented in the final cDNA population.[1]

Another modification, dubbed small amplified RNA (SAR) SAGE, employed linear amplification of small mRNA fragments containing the SAGE tags, which allowed for preparation of libraries of over 100,000 tags from as few as 2500 cells (or ~50 ng of total RNA).[10] The procedure is similar to classical microSAGE, but employs a T7 RNA polymerase-dependent transcription of the mRNA segment between the tag and the poly $(A)^+$ tail, which allowed for synthesis of small mRNA molecules harboring the SAGE tags. For correctly identifying unknown tags, which contain ~10 bases, and ascertaining that they are not due to sequencing errors, methods employing reverse transcription (RT) PCR and other techniques to recover longer cDNA fragments were also reported.[10] A technical difficulty encountered in the original SAGE is that major products of PCR amplification are often linker dimers. By employing biotinylated PCR primers, biotinylated DITAGS were produced early on in the method, allowing removal of unwanted linkers by binding them to streptavidin beads at a later stage.[11]

To eliminate a small average size of cloned concatemers, which decreases the efficiency of tag collection, a heating step was carried out at 65°C for 15 min, followed by a quick chill on ice for 10 min of ligated concatemers. This step resulted in cloned concatemers with an average size of 67 tags, compared with 22 tags obtained by the original protocol.[12]

Modified SAGE protocols that generate longer TAGS have been attempted. One method employed *Rsa*I instead of the *Nla*III as the AE, which resulted in a tag length of 14 bases. In addition, this modification resulted in cohesive termini generated on cDNA tags for the ligation of both 5′ and 3′ linkers, allowing for higher efficiency in tag linker ligation and a lower possibility for linker dimer production.[11] Another modification for increasing the tag length by 2–3 bp, which employed *Sau*3A as the AE, has also increased the potential for transcript detection.[13] A technique dubbed as the generation of longer cDNA fragments from SAGE tags for gene identification (GLGI) extended the 10 tags to hundreds of bases long until they reached the 3′ end of the cDNA. This method employed a primer containing the 10-base SAGE tag as the sense primer, and another single-base anchored oligo (dT) as an antisense primer in a PCR reaction, together with a *pfu* DNA polymerase.[14] A procedure on that line, which also generated a 21-bp tag derived from the 3′ end of the transcriptase called LongSAGE, utilized a different type IIS tagging endonuclease (*Mme*I).[15] The longer cDNA fragment promises to permit a wider application of SAGE for quantitative analysis of global gene expression to complete the catalogue of expressed genes in humans and other species, to allow the identification of the 3′ cDNA sequence from any exon within the gene, and to delineate the 3′ boundary of expressed genes.

The use of different reverse transcriptases could play an important role in generating more representative cDNA. Although initial SAGE cDNA libraries have been constructed with the Moloney murine leukemia virus (MMLV), reverse transcriptase RNase H^- reverse transcriptase (Superscript II; Invitrogen) was reported to generate as much as four times more cDNA from a similar starting material. Furthermore, improved reverse transcriptases such as Sensiscript and Omniscript (Qiagen), used for generating high-quality cDNA from a limited amount of RNA that have been more recently introduced, promise to increase the efficiency of cDNA production.[1]

To overcome intrinsic PCR amplification problems resulting in transcript misrepresentation, a method that does not employ any PCR amplification at any stage of the process and identified as TALEST (or tandem arrayed ligation of expressed sequence tags) was used. The method utilized a series of restriction (*Eco*RI, *Bsg*I, and *Not*I) and punctuating (*Msp*I) endonucleases, which acted on ds-cDNA, resulting in the production of an *Eco*RI/*Not*I-tailed DNA fragment containing a 12-bp cDNA tag flanked at both ends by GC-clamped *Msp*I punctuation sequence. These sequences were resistant to thermal denaturation, permitting their concatemeration into long arrays and their subsequent recognition and analysis by high-throughput DNA sequencing.[16]

Table 1 SAGE databases found on the Internet

Database site	Web address	SAGE data
NCBI CGAP SAGEmap	www.ncbi.nlm.nih.gov/SAGE	Brain, colon, ovary, pancreas, prostate, breast, fibroblasts, endothelial cells
Johns Hopkins Oncology Center	www.sagenet.org	Colon, pancreas, endothelium, p53, yeast, adenomatous polyposis coli (*APC*) gene
University of Rochester	www.urmc.rochester.edu/smd/crc/swindex.html	Human skeletal muscle
Stanford University *Saccharomyces* Genome Database	http://genome-www.stanford.edu/cgi-bin/SGA/SAGE/querySAGE	Yeast, *Saccharomyces cerevisiae*
Aldaz Lab, Global Gene Expression Group, University of Texas M.D. Anderson Cancer Center	http://sciencepark.mdanderson.org/ggeg/SAGE_proj_1.htm ggeg/p53/default.htm	Estrogen-treated MCF-7 breast cancer cell line, normal $p53^{null}$ mammary epithelium
Howard Florey Institute, Tan Laboratory, University of Melbourne	www.hfi.unimelb.edu.au/research/developmental_biology/sage/c6sage.html developmental_biology/sage/neocortexsage.html	Rat C6 gliosarcoma cell line, mouse neocortex
Commissariat a l'Enegie Atomique (CEA), Saclay, Elalouf Laboratory	www-dsv.cea.fr/thema/get/sade.html	Mouse kidney microdissected tubules
University of Tokyo School of Medicine	www.prevent.m.u.tokyo.ac.jp/SAGE.html	Immune cells, dendritic cells, liver cells, estrogen-treated MCF-7 breast cells
Genecarta by Compugen	www.LabOnWeb.com	Variety of human, mouse, rat, zebrafish, and *Arabidopsis* tissues

STATISTICAL AND BIOINFORMATICS CONSIDERATIONS

SAGE is a sampling method that determines the expression level of a gene (i.e., a resulting transcriptome) by measuring the frequency of unique short sequence tags derived from the corresponding mRNA transcript. Although the method is a very effective approach for determining the expression of mRNA populations, there can be significant biases in results caused by sampling errors, sequencing errors, nonuniqueness, and nonrandomness of tag sequences. Moreover, there are often transcripts that are present at a low copy number. To obtain a valid estimate of transcriptome size from SAGE libraries, one must sample a number of tags in a fashion inversely proportional to the lowest abundance level, which is not always known. Taking these mathematical considerations into account puts a strain on the design of a SAGE experiment.[17] Several statistical tests, all having their limitations, have been published for the pairwise comparison of SAGE libraries, and should be consulted before a SAGE experiment is designed. Computer programs, incorporating different statistical methods, are also available—free of charge—to facilitate data handling and analysis.[18]

To enhance the utility of SAGE data, the NCI's Cancer Genome Anatomy Project (CGAP) has created an informatics tool ''SAGE Genie,'' a web site for the analysis and presentation of SAGE data (http://cgap.nci.nih.gov/SAGE). SAGE Genie provides an automatic link between gene names and SAGE transcript levels, accounting for alternative transcription and many potential errors, and allowing for an invaluable means to archive and analyze the expression profiles for any given gene under any biological context.[19]

APPLICATIONS OF SAGE

More than 6.8 million SAGE transcript tags have been generated from 171 libraries and stored in CGAP database (http://cgap.nci.nih.gov/SAGE). Table 1 shows various SAGE databases available on the Internet. Many SAGE tags may have no match to known expressed sequences, and the majority of these unmatched novel SAGE tends to be low copies. Use of these novel tags and probes was proposed to allow identification of novel genes in human genomes that are difficult to identify by conventional methods.[20]

SAGE technology has been applied to the fields of cancer research, immunological research, cardiovascular research, brain research, pharmacological and drug discovery research, taxonomy, and transcriptome discovery in all phyla.[1–3,15,20,21]

CONCLUSION

Gene expression profiling is a powerful tool for discovering genes and their products, and for generating new mechanistic hypotheses at the core of understanding the function of the genome. Approaches using SAGE share a number of strengths and limitations. Considering SAGE limitations, first, a small number of transcripts would be expected to lack an AE site and should be missed in the analysis. Although it is possible to utilize a second AE to overcome this problem, this has rarely been attempted in practice because of the effort and expense involved. Second, the approach relies on DNA sequencing, with its inherent cost and throughput limitations. Third, it is a statistical sampling method, which depends on the appropriate understanding and design of the study before the rigorous experimental part is even attempted.

A notable strength of the SAGE method, like no other gene expression profiling, is that results from any experiment are directly comparable to existing SAGE gene expression databases. Significant differences among these comparisons can be spotted by using a variety of statistical methods and bioinformatics to identify new transcriptomes.[21] Contrary to the static genome, an approach represented by the transcriptome is an interactive module that allows the study of the influence of external and internal factors, thereby serving as a dynamic link between an organism's genome and its physical characteristics.

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Single Cell Gel Electrophoresis Assay (Comet Assay)

Lisa Giovannelli
Vanessa Pitozzi
University of Florence, Florence, Italy

INTRODUCTION

Single cell gel electrophoresis allows detection of DNA breaks based on the electrophoretic migration of DNA from single nuclei embedded in agarose. The assay is commonly known as the ''comet'' assay because of the typical shape of the DNA fragments migrated out of the nucleus, resembling the tail of a comet when they are viewed through a microscope. The idea of electrophoresing single nuclei by embedding cell suspensions in thin agarose layers on microscope slides was originally developed for detection of radiation-induced DNA damage. A few years later, a modification of the original version of the method was introduced, consisting in conducting the electrophoresis after a DNA unwinding step in alkaline conditions, which greatly improved detection of single-strand breaks (SSBs) and also allowed detection of alkali-labile sites (ALS). Since then, the comet assay has gained increasing popularity, and today it is one of the most widely used methods in genetic toxicology, particularly in the alkaline version, which encompasses detection of a broader spectrum of DNA lesions. In addition to strand breaks, the comet assay can also be used for cross-link detection. The reasons for its popularity are also the small amount of cells/tissue required, high sensitivity, low cost, relative rapidity, and simplicity.

PRINCIPLES OF DETECTION

The principle underlying the comet assay is the increased electrophoretic migration of DNA upon formation of strand breaks or nicks.[1,2] To allow DNA migration, the agarose-embedded cells undergo a lysis process that eliminates cytoplasm, cellular membranes, and DNA-associated proteins such as histones. The naked DNA left in the gel retains its original nuclear shape and supercoiled arrangement, and is referred to as ''nucleoid.'' The undamaged nucleoid cannot migrate in an electric field because of its high molecular weight and compact structure. However, if breaks are present in the DNA molecule, the supercoil relaxes to some extent, and loops or free ends can be pulled out of the nucleus. It has been proposed that in the neutral version mainly loop migration takes place, unless the frequency of double-strand breaks (DSBs) is high enough to generate fragments that can move out of the nucleus.[3] In the alkaline comet assay, both relaxed loops and fragment migration take place, with the fragments being completely or partially single stranded, depending on breakage frequency.[3,4]

OUTLINE OF THE METHOD

The comet assay can be conducted on virtually any nucleated cell that can be brought into suspension. Thus, both cultured or freshly isolated cells from any organ can be used. Isolating cells from solid tissues usually involves time-consuming procedures, often implying the use of extracellular matrix-digesting enzymes, which can damage DNA. Instead, for the study of endogenous levels of DNA damage, it is necessary to use rapid isolation techniques to reduce the onset of artefacts, and, for some purposes, intact nuclei isolation by simple homogenization is advisable.[5] International expert guidelines have been provided for using the assay in in vivo genotoxicity testing.[6] Care should also be taken to prevent DNA damage induction during the procedure. For this purpose, it is convenient to work in dim light to avoid UV-induced DNA damage, and all solutions used should contain EDTA to inhibit endonuclease activity.

However, prepared, isolated cells or nuclei have to be obtained in a small volume of medium (10–20 μL) or in a pellet, to be either mixed or resuspended in low-melting point (LMP) agarose (0.5–1% in phosphate-buffered saline) at 37°C. A drop of mixture is transferred onto a fully frosted or agarose-precoated slide, covered with a coverslip, and allowed to solidify at 4°C. To obtain a suitable cell density, an 18×18-mm gel can be made with 70 μL LMP containing 20–50,000 cells. Smaller gels can be prepared if needed, and two or more gels can be accommodated on one slide.

The slides with the gel-embedded cells are immersed in a lysis solution containing detergents and having a high salt content to eliminate cellular membranes, cytoplasm, and organelles. The composition of one commonly used lysis solution is as follows: 1% *N*-lauroyl-sarcosine, 2.5 M

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NaCl, 100 mM Na_2EDTA, 1% Triton X-100, and 10% dimethylsulfoxide. Proteinase K can be added to improve deproteinization of DNA. The pH of the lysis solution can be neutral or alkaline, depending on the type of assay. The incubation is usually conducted at 4°C for 1 h, although longer times have been used.

In the neutral assay, the detectable lesions are mainly DSBs. In the alkaline assay, additional lesions can be visualized owing to the alkaline unwinding step preceding electrophoresis. The slides are submerged in a solution containing NaOH (0.3 or 0.03 mM) and EDTA (1–2 mM). The pH of the unwinding solution can vary, depending on the types of lesion to be detected: 12.1 for SSB (including those produced by the excision repair systems), and >13 for additional detection of ALS, i.e., modified sites that are converted in breaks at high pH, such as DNA base adducts. Cross-links can also be detected by measuring the prevention of DNA migration induced by X-ray.[7] The unwinding time chosen by many laboratories is 20 min; however, longer times can be used to increase the amount of single-stranded DNA and thus the extent of migration.

Electrophoresis is conducted with the slides placed side by side on a platform in a standard electrophoresis tank. The electrophoresis solution is usually the same as the unwinding solution for the alkaline assay, whereas in the neutral assay tris–borate–EDTA (TBE) or tris–acetate–EDTA (TAE) buffer is used. The applied voltage varies, depending on the desired extent of migration, from 0.55 to 1.0 V/cm for the alkaline (with 0.8 V/cm being the most widely used) and from 0.55 to 9.0 V/cm for the neutral assay. The associated amperage is generally rather high and it is advisable to conduct the run at 4°C.

Neutralization is required in the alkaline assay before staining. In this case, the slides are washed with a Tris buffer solution at neutral pH for a few minutes.

DNA staining can be brought about with ethidium bromide, 4,6-diamidino-2-phenylindole (DAPI), propidium iodide, or other fluorescent dyes by applying about 20 μL of a diluted staining solution (1–20 μg/mL) onto each gel and sealing with a coverslip. The slides can be stored in a dark, moist chamber at 4°C until they are viewed (usually overnight). For long-term storage, gels can be dried by placing the slides in a warm oven for a few hours, and then stored indefinitely at room temperature. For reexamination, the gels can be rehydrated[8] and stained as above.

DATA ANALYSIS

Comets can be analyzed visually in a quantitative way. A scheme has been described[4] for visual scoring based on five recognizable classes of comet, from undamaged, having no discernible tail, to highly damaged, with almost all DNA in the tail. Examples of comets in different classes of damage are given in Fig. 1. Fifty to 100 comets are selected randomly from each slide, and each comet is given a value according to the class it is assigned to, so that an overall score can be derived for each gel from the sum of all the comets' values. The advantage of visual scoring is that it is very quick.

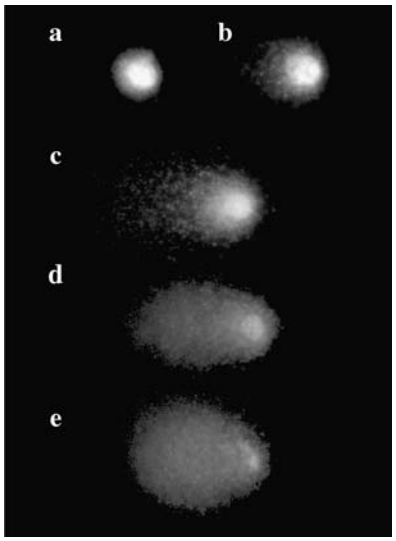

Fig. 1 Examples of comets in different classes of damage. Images of ethidium bromide-stained nuclei, showing increasing degrees of DNA damage (from image a to e) after electrophoretic migration.

Several software programs are also commercially available that automatically analyze individual comet images acquired in digital form with a CCD camera mounted on a microscope. These programs are designed to provide fluorescence profiles of comet head and tail and to measure various parameters such as percentage of total fluorescence in head and tail, tail length, and ''tail moment'' (conceptually the product of tail length and DNA fluorescence in tail).[6] The percent of DNA in the tail is a widely used parameter and is linearly related to DNA break frequency, up to about 80% in the tail.

MODIFICATIONS FOR SPECIFIC PURPOSES

Enzymatic Detection of Specific Classes of DNA Damage

An enzymatic modification of the alkaline comet assay to allow detection of oxidized bases was first described by Collins et al.[9] These authors suggested the use of lesion-specific DNA glycosylases/endonucleases involved in bacterial DNA repair to convert specific DNA base

modifications to strand breaks. The embedded nucleoids are incubated in the presence of the enzyme after the lysis step, so that additional breaks can be introduced at sites of damaged bases. The amount of enzyme-specific lesions can then be calculated by the difference between the breaks found in enzyme-treated and those in buffer-treated slides. Any base modification can be detected with this procedure, as long as the proper repair enzyme is available. Among the DNA repair enzymes used in the comet assay, formamidopyrimidine-DNA glycosylase (FPG) and endonuclease III (ENDO III) have been used for the detection of oxidized purines (8-OH-guanine, 7-methylguanine, 5-OH-cytosine, and 5-OH-uracil) and pyrimidines (thymine glycol, dihydrothymine, dihydroxydihydrothymine, and uracil glycol), respectively.

UV endonuclease for recognition of UV-damaged bases (6-4 photoproducts, pyrimidine dimers)[4] and uracil glycosylase (UDG) for detection of misincorporated uracil[10] have also been used.

Comet Assay—Fluorescence In Situ Hybridization

This procedure allows detection of DNA breaks within specific DNA sequences. It involves DNA unwinding of the embedded nucleoids followed by hybridization with a fluorescence in situ hybridization (FISH) DNA probe, based on the principle that DNA will be available for hybridization at single-stranded sites produced by alkaline unwinding in the presence of strand breaks.[11]

Treatment of Embedded Cells

The cells embedded in agarose can be kept viable and treated by incubating the slides in an appropriate medium containing the chemical to be tested for genotoxicity at the desired concentration. The radiation effect on DNA breakage and DNA repair can also be studied in embedded cells by irradiating the slides.[12] The advantage of this procedure is that the cells can be lysed immediately after treatment and run through the comet assay, avoiding further manipulation (trypsinization, centrifugation).

Measurement of DNA Content

When comet assay data are analyzed by means of a computer-assisted system, it is possible to evaluate the total DNA content from the value of the total fluorescence for each nucleoid. In this way, simultaneous measurement of the level of DNA breakage and content can be obtained in the same nucleus. This approach was first developed by Olive and Banath,[13] who showed dependence of the extent of induced DNA damage on the cell cycle point.

Identification of Subpopulations in the Sample

The comet assay can be used to differentially analyze cell subpopulations within a sample. A modification of the procedure can be used to label specific cell types that can be recognized along with the comets.[14] In some cases, one can take advantage of the fact that the nucleoids retain the morphological features of the original nucleus, so that, e.g., smaller or larger nuclei can be differentiated in a heterogeneous population of cultured cells, or polymorphonuclear blood cells can be separated from mononuclear cells.[15]

CALIBRATION AND STANDARDIZATION

Calibration of the comet assay is based on the fact that ionizing radiation produces strand breaks in DNA with known efficiency.[16] By using the comet assay to measure the breaks introduced into cells irradiated with different doses of X-rays, a standard curve can be obtained (Fig. 2), allowing conversion of the comet assay parameters (percent DNA in tail or tail moment) in break frequency. With this approach, the sensitivity of the method has been evaluated as less than 0.5 breaks/10^9 Da of DNA,[17] similar to other established methods for DNA damage measurement.[16] However, the comet assay saturates at lower levels of damage. A comparison between different methods for oxidative DNA damage detection was recently conducted by a European group to validate current protocols for the measurement of DNA oxidation.[18] This concerted effort showed that the values of

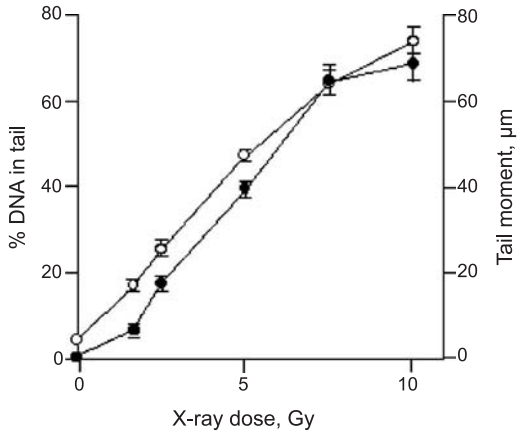

Fig. 2 Example of a calibration curve obtained by X-ray. Lymphocytes were embedded in agarose and irradiated with a range of doses before lysis and electrophoresis. The percent of DNA in the tail (open circles) and the tail moment (solid circles) are plotted. (From Ref. [17].)

DNA oxidation measured with the comet assay are significantly lower than those obtained in the same samples with chromatographic methods.

Protocol validation is an important issue, in view of the numerous variations of the method used in different laboratories, and guidelines have been provided by experts to improve intra- and interlaboratory comparisons.[19] To minimize variations due to visual scoring, each operator should undergo training involving ''calibration'' against a computer image-analysis system. Another potential source of variability is represented by reagents, gel preparation, lysis conditions, alkaline unwinding, and electrophoresis. To control these variations, standard samples should be included in every experiment. These should ideally be cells of the same type as those analyzed, prepared in aliquots to be frozen to $-80°C$ and stored until needed. Critical to the standardization is making sure that the storage process does not affect DNA integrity over time.[20]

CONCLUSION

Because of its unique design, the comet assay has proved useful in many fields. As a test for genetic toxicity, it has been widely used in human studies for biomonitoring environmental DNA-damaging substances.[21] A number of clinical applications have also been described, such as monitoring DNA damage in patients undergoing cancer chemo- or radiotherapy.[22] These studies, conducted in peripheral blood cells or in cancer biopsies, can provide information on both the sensitivity of tumor cells to the treatment and the toxic responses of normal tissues. Furthermore, measurement of the basal levels of DNA damage in cancer cells might have potential as a diagnostic and prognostic tool.[23] Other clinical conditions investigated with the comet assay include male infertility and oxidative stress-related pathologies such as diabetes.[22] Another important application is the measurement of oxidative damage in nutritional studies in humans.[24] Finally, in vivo and in vitro experimental studies with the comet assay have provided mechanistic information on the genotoxic effects of chemicals and radiation and on fundamental cellular processes such as DNA repair and apoptosis. Future developments and modifications will probably further enlarge the already wide scope of application of this assay.

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Single Nucleotide Polymorphism (SNP) Genotyping Techniques—An Overview

Richard M. Twyman
University of York, York, U.K.

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are individual base positions in the genome that show natural variation in a population. They represent the most abundant form of genetic variation in humans, accounting for more than 90% of all differences between unrelated individuals. SNP patterns are likely to influence many human phenotypes; therefore large-scale association studies based on SNP genotyping are expected to help identify genes affecting complex diseases and responses to drugs or environmental chemicals. SNPs play a major role in all stages of the drug development process—from target identification to clinical trials. The analysis of SNPs may also help to tailor drugs and drug regimes to particular genotypes—the underlying principle of pharmacogenomics. Given the potential impact of SNPs on healthcare, the biotechnology industry has focused urgently on the development of high-throughput methods for SNP genotyping. All genotyping methods are a combination of distinct methods for allele discrimination and signal detection, and these methods are the subject of this article.

SNP GENOTYPING

High-throughput SNP genotyping methods have been developed in order to exploit the many healthcare benefits which will arise from a detailed knowledge of genetic variation.[1–3] SNP genotyping technologies have two components—a method for determining the type of base present at a given SNP locus (allele discrimination), and a method for reporting the presence of the allele(s) (signal detection).[4–6] There are three general allele discrimination methods: hybridization/annealing (with or without a subsequent enzymatic discrimination step), primer extension, and enzyme cleavage. In each case, the technology platform may be homogeneous (in solution) or heterogeneous (involving both a liquid and a solid phase, such as a microarray). Some of the assays require prior amplification of the genomic target, whereas others are sensitive enough to work directly on genomic DNA or cDNA. There are many signal detection platforms, and most of these follow the fate of a label either in real time or at the assay end point. Uniquely, mass spectrometry can be used to detect the allele-specific product of a discrimination assay without the need for a label, by distinguishing the masses of DNA molecules containing alternative bases.

ALLELE DISCRIMINATION METHODS

Allele-Specific Hybridization

The simplest method for discriminating between alleles at an SNP locus is hybridization using allele-specific oligonucleotide (ASO) probes. Two probes are required—one specific for each allele—and stringency conditions are employed such that a single-base mismatch is sufficient to prevent hybridization of the nonmatching probe (Fig. 1a). The ASO probes can be used as labeled/unlabeled pairs, in which case two separate assays are required to genotype each SNP, one in which probe 1 is labeled and probe 2 is unlabeled, and one in which these roles are reversed. Alternatively and preferably for high-throughput assays, distinct labels can be used for each probe (e.g., different fluorophores or mass tags), so that each SNP can be genotyped in a single reaction. More sophisticated assays, such as TaqMan, use allele-specific hybridization as the primary discriminating reaction, but additional enzymatic steps are required to detect the signal.

Allele-specific polymerase chain reaction (PCR) is a modification of allele-specific hybridization in which discrimination is achieved by allele-specific primer annealing (followed by PCR amplification) rather than the direct detection of a hybridized people (Fig. 1b). Although it is possible to use a strategy in which the variable base position is in the middle of one of the primers, which is directly analogous to the ASO technique, this allows the extension of a mismatched primer/template if stringency conditions are not optimized. A more sensitive approach is to place the 3′ end of one of the primers at the variable base position because extension is dependent on perfect complementarity at the 3′ end of the primer.[7]

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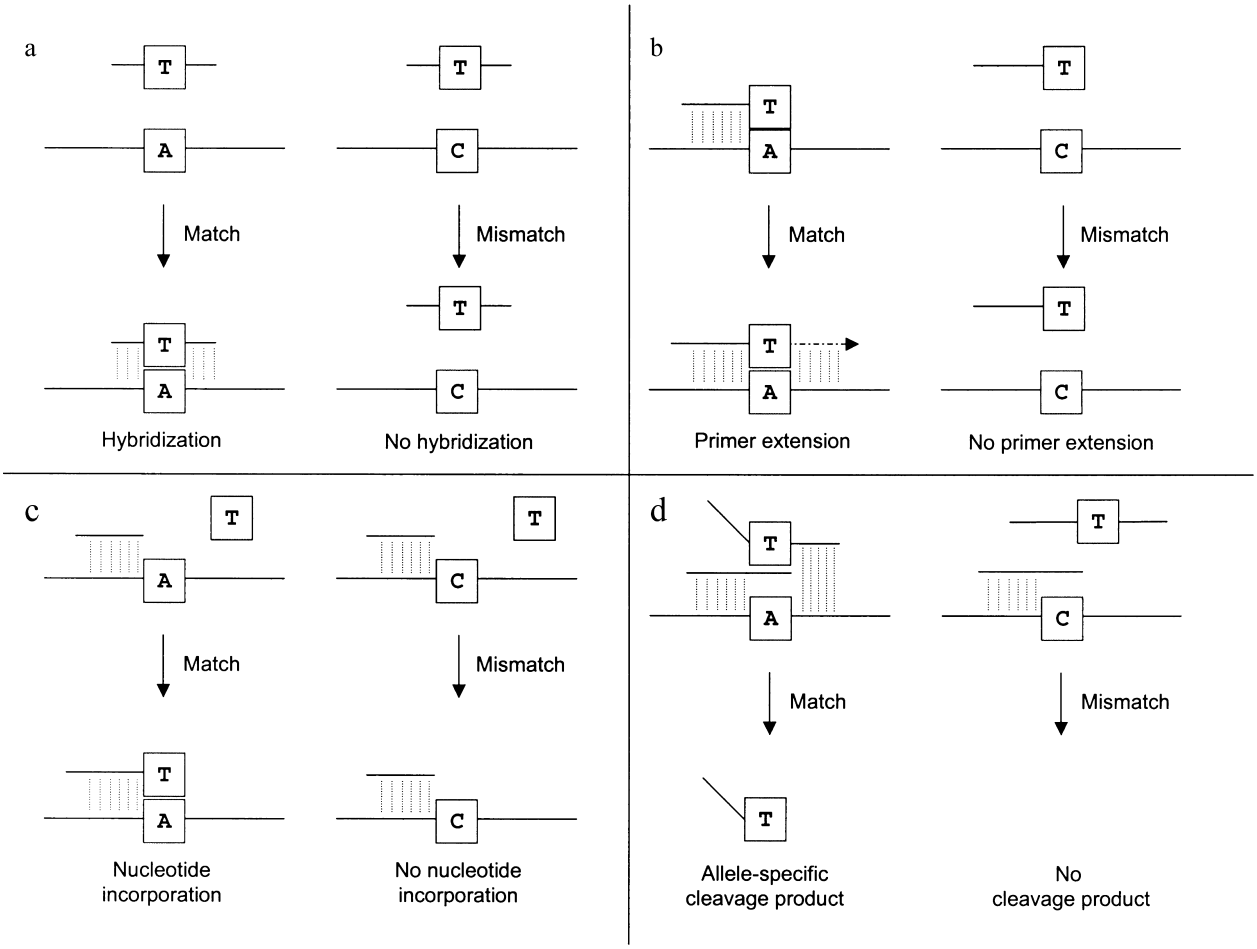

Fig. 1 Principles of allele discrimination methods. (a) Allele-specific hybridization. (b) Allele-specific PCR. (c) Allele-specific single-base primer extension. (d) Allele-specific invasive cleavage. In each case, the SNP position and the variable base position in the probe are shown. (*View this art in color at www.dekker.com.*)

Another modification of allele-specific hybridization is allele-specific ligation, in which two oligonucleotides are annealed at adjacent sites and DNA ligase is used to join them together. One of the oligonucleotides has two allele-specific variants, and the discriminatory base is at the most 3′ position. If there is perfect complementarity between the ASO and the template, it will be successfully ligated to the common oligonucleotide. If not, ligation will fail and the two oligonucleotides will remain separate. The assay in itself is not very sensitive, but sensitivity can be increased using additional steps such as the ligase chain reaction (in which the ligation reaction is reiterated with further oligonucleotides complementary to the first ligation product) or rolling circle amplification technology (RCAT; in which two ASOs about 80 nucleotides in length are designed to form a circle on the template, generating a closed loop or padlock probe when ligated). Primers annealing to this circle can be extended with a strand-displacing DNA polymerase, so that when the nascent strand completes the circle and encounters itself, it is continually displaced, generating a long concatemer that is easy to detect using fluorescence methods.[8]

Allele-Specific Single-Base Primer Extension

In allele-specific single-base extension (also called mini-sequencing), primers that anneal one nucleotide upstream of the polymorphic site are designed, and allele discrimination depends on the ability of this perfectly annealed primer to be extended (Fig. 1c).[9] This is distinct to allele-specific PCR where the discriminatory position lies within one of the primers and extension depends on the ability of the primer to anneal to its template. Allele-specific primer extension methods are more adaptable than hybridization/annealing assays because a much greater diversity of labeling strategies can be used. For example, the free nucleotides in solution can be labeled with four different

fluorescent tags, mass tags, or haptens, allowing the same mix to be used in the detection of multiple SNPs in parallel (e.g., on a microarray). Primer extension methods for genotyping have been extensively patented as Genetic Bit Analysis™ (GBA) technology (Orchid Biosciences, Inc.) and form the basis of many popular commercial genotyping systems (e.g., SnaPshot; Applied Biosystems).

Allele-Specific Enzymatic Cleavage

One of the earliest and most widely used genotyping methods, restriction fragment length polymorphism (RFLP) analysis, works on the principle of allele-specific enzymatic cleavage. An RFLP is generated when an SNP occurs at a restriction endonuclease recognition sequence, and one allele preserves the sequence while the other destroys it. If we consider any DNA fragment with three adjacent restriction sites, with the middle one containing an SNP, then digestion of amplified genomic DNA with the appropriate restriction endonuclease will produce either a single large fragment (if the central restriction site is absent) or two smaller fragments (if the central restriction site is present and cleavage occurs).

RFLP analysis is difficult to adapt for high-throughput genotyping, but this has been achieved for another cleavage-based assay known as Invader.[10] The Invader assay employs a unique method of allelic discrimination involving overlapping probes and an enzyme that specifically recognizes the resulting ''flap'' (Fig. 1d). An advantage of the Invader assay is that it requires no PCR amplification. Two signal probes are used, one recognizing each allele, plus a third invader probe. The signal and invader probes hybridize in tandem, and the signal probe overlaps the invader probe, generating a flap that is recognized by an enzyme called Cleavase, a modified form of the thermostable FEN-1 enzyme (flap endonuclease). A flap with the appropriate structure is generated only if the signal probe completely matches the template and there is a one-base invasion. The cleaved flap can then be used in a second round invader assay to generate a fluorescent signal, or the cleaved flap can be detected by mass spectrometry.

DETECTION OF ALLELE-SPECIFIC PRODUCTS

Methods Utilizing Fluorescent Labels

Direct fluorescence detection

The most straightforward way to detect an allele-specific product is to label it by incorporating one or more nucleotides conjugated to a fluorescent dye. Direct fluorescence detection is generally used with solid-phase assay formats (microarrays and bead arrays) and where allele-specific products are separated by gel electrophoresis or capillary electrophoresis.

A variation on this approach is to use an intercalating fluorescent dye such as ethidium bromide and to study the melting profile of DNA duplexes. This method for typing SNPs is known as dynamic allele-specific hybridization (DASH).[11] One (unlabeled) ASO is added to a PCR product and allowed to anneal in the presence of an intercalating dye, which fluoresces specifically in the presence of double-stranded DNA. The reaction is then heated, and fluorescence is measured in real time as the temperature rises. As the duplex denatures, fluorescence steadily decreases. When the melting temperature of the probe is reached, denaturation is completed and the fluorescent signal falls to background levels. The thermal melting profile of each allele is different because one is perfectly complementary to the probe and the other contains a mismatch.

Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) occurs where two fluorophores are in close proximity, and one of the fluorophores (the donor) has an emission spectrum that overlaps the excitation spectrum of the other fluorophore (the acceptor). When a lone donor fluorophore is excited, light is produced with a characteristic emission spectrum. However, when the donor fluorophore is excited in close proximity to the acceptor fluorophore, energy is transferred to the acceptor fluorophore with the result that the intensity of emission from the donor is reduced (quenched), whereas that of the acceptor is increased (enhanced).

Although FRET enhancement can be used for SNP genotyping, two of the most popular homogenous assays in current use employ FRET quenching. The TaqMan assay, mentioned earlier, uses the intrinsic 5′ nuclease activity of *Taq* DNA polymerase to generate a fluorescent signal from a short ASO probe.[12] Two ASO probes are required—one specific for each allele. Each probe contains a unique donor fluorophore and a common acceptor fluorophore, and is short enough for the donor to be quenched when the probe is intact (either hybridized or in solution). However, when the probe hybridizes to the PCR template, the 5′ exonuclease activity of *Taq* DNA polymerase digests it, thus releasing the two fluorophores into solution and eliminating the quenching effect (Fig. 2a). The Molecular Beacon assay[13] involves the use of longer probes that have self-complementary ends labeled with the donor and acceptor fluorophores. Non-hybridized probes will self-anneal, bringing the donor and

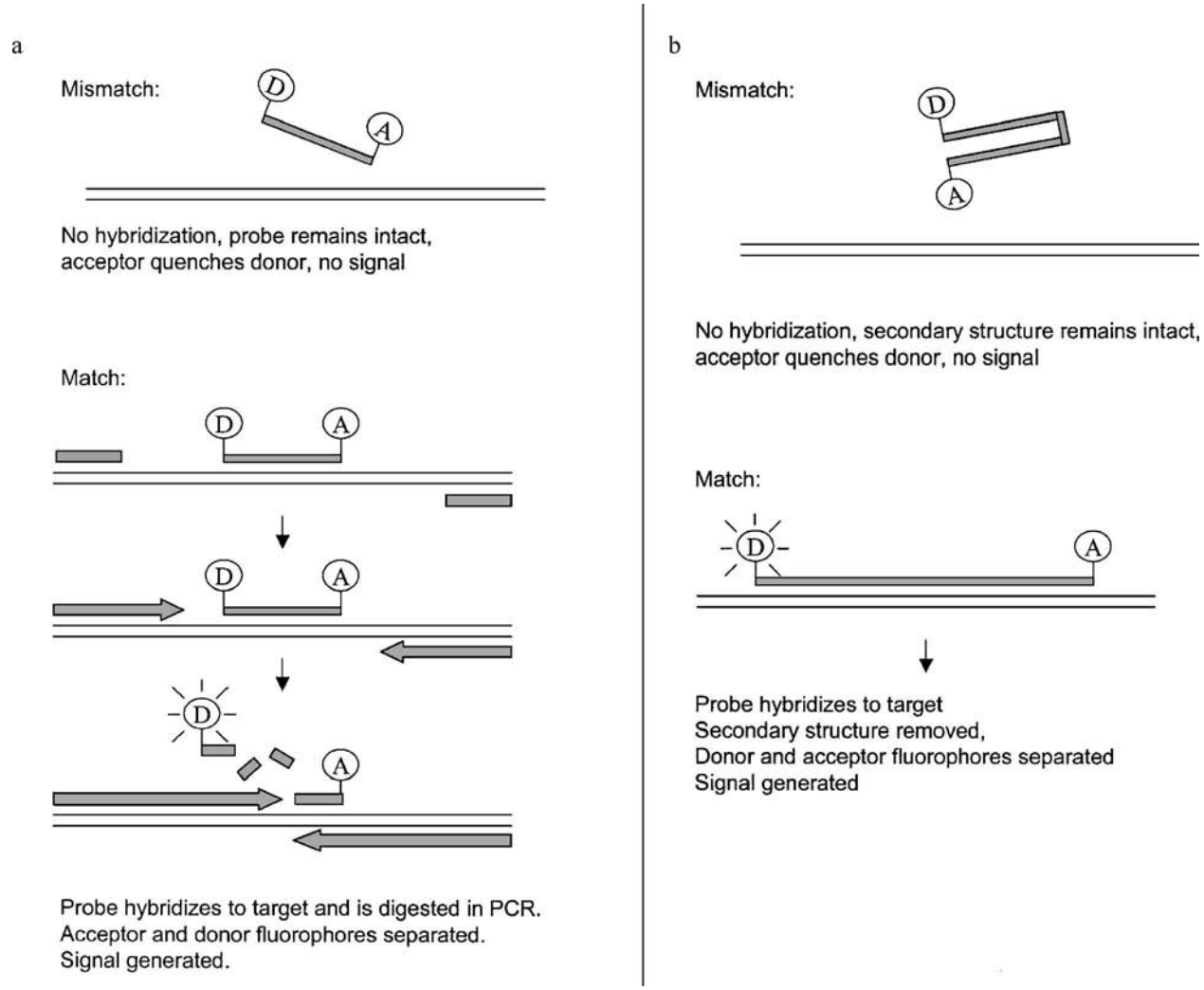

Fig. 2 Principles of the two most popular fluorescence-based homogenous genotyping methods: (a) TaqMan assay; (b) Molecular Beacon assay. (*View this art in color at www.dekker.com.*)

acceptor fluorophores together, resulting in quenching. Where hybridization occurs, the donor and acceptor fluorophores are separated such that the quenching effect is eliminated and a fluorescent signal is produced. As with the TaqMan assay, two probes labeled with different donor fluorophores are required—one specific for each allele (Fig. 2b).

Fluorescence polarization

When a fluorophore is excited by plane-polarized light, the fluorescence emitted by the dye is also polarized. This phenomenon is termed fluorescence polarization (FP). Complete FP occurs only when the dye molecule is stationary. Therefore the degree of observed FP is dependent on how fast a molecule tumbles in solution, and this is in turn dependent on the volume of the molecule, which is related to its molecular mass. Therefore changes in molecular mass (e.g., caused by primer extension, probe hydrolysis, or invasive cleavage) can be detected by changes in FP as long as all other conditions (temperature, viscosity, etc.) remain constant. FP is used as the detection method in the SnaPshot (Applied Biosciences) and Acycloprime (Perkin Elmer) commercial genotyping systems.

Mass Spectrometry

Genotyping assays involving mass spectrometry are distinct from those discussed above in that signal

detection is direct, based on differing molecular weights of small DNA fragments rather than the behavior of a label.[14] The analysis of DNA by mass spectrometry requires soft ionization (i.e., without fragmentation) and is usually achieved by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis. The MALDI procedure involves mixing the allele-specific products of the discrimination assay with a matrix compound on a metal plate. The mixture is then heated with a short laser pulse, causing it to expand into the gas phase where ionization is achieved by applying a strong potential difference. Ions are accelerated toward the detector and the time of flight (the time taken to reach the detector) is measured, allowing the mass/charge ratio to be calculated.

High-resolution mass spectrophotometers can accurately discriminate between alternative alleles in DNA fragments of 3–20 nucleotides in length. The smallest difference in mass between alternative allelic products is about 9 Da, representing an adenosine/thymidine polymorphism. Advantages of MS-based methods include its accuracy (as long as the sample is very pure) and the fact that each genotyping reaction takes a fraction of a second, allowing thousands of reactions to be carried out serially in a single day.

The majority of current MS genotyping platforms involve the use of DNA chips because the chip can be used directly as a MALDI plate. Thus far, the technique has been used to determine the masses of allele-specific hybridization probes, allele-specific primer extension products, and the products of invasive cleavage reactions. Commercial systems utilizing mass spectrometry include MassArray (Sequenom, Inc.) and PinPoint (PerSeptive Biosystems, Inc.).

Another promising MS genotyping technology involve the use of cleavable mass tags (chemical moieties of known molecular mass) as labels to replace fluorophores.[15] The advantage of mass tags is that, due to the accuracy of MS-based mass determination, the range of labels that can be used is virtually limitless, so ultra-high throughput genotyping is possible. The MassCode system (Qiagen Genomics) uses this technology.

Pyrosequencing

Pyrosequencing is a novel method for sequencing short stretches of DNA based on the detection of pyrophosphate, a normal by-product of DNA synthesis.[16] Although similar in principle to primer extension allele discrimination methods, pyrosequencing is suitable not only for typing SNPs but also for scoring entire haplotypes (groups of linked SNPs). Pyrosequencing works on the basis that pyrophosphate can be used to generate adenosine triphosphate (ATP), which then stimulates luciferase activity, causing the emission of a chemiluminescent signal. To achieve this, the primer extension reaction must include adenosine 5′ phosphosulfate (APS) and the enzyme ATP sulfurylase, which converts APS into ATP in the presence of pyrophosphate. Also present is luciferin (the substrate of luciferase) and the enzyme apyrase, which continuously degrades unincorporated dNTPs and excess ATP. The dNTPs are added to the reaction one by one. Only if the incoming dNTP is complementary to the template will it extend the primer and release pyrophosphate, resulting in the production of an equimolar amount of ATP. Visible light is generated in proportion to the amount of ATP and is detected as a peak on a pyrogram. When the degradation of the present dNTP is completed by apyrase, the next is added. A thioderivative of dATP must be used to avoid constant stimulation of luciferase activity.

CONCLUSION

Association studies aim to find patterns among 0.1% variation in the human genome (mostly represented by SNPs) corresponding to differences in complex phenotypes such as disease susceptibility and response to drugs. The success of such studies relies on accuracy, sensitivity, and high throughput in SNP genotyping. Many of the technologies described in this article are geared toward highly parallel analysis, although MS stands out as a platform where increased throughput is realized by ultra-rapid serial processing.

The sensitivity of most genotyping techniques reflects some form of amplification step either before, during, or after the actual allele discrimination assay. In the majority of cases, this amplification step relies on the PCR, but novel formats such as the Invader assay and RCAT are also becoming popular. Although homogenous assay formats are versatile, array-based technology platforms have the advantage of higher throughput. Recent developments[4] include the use of electrostringent hybridization arrays, where electric currents are used to increase the speed and sensitivity of hybridization, and the detection of allele-specific products based on their electrochemical properties when hybridized to oligonucleotide probes attached to minute electrodes. Another emerging technology, bead arrays, combines the flexibility of the homogenous assay format with the convenience and throughput of chip-based detection systems. Bead array systems that employ fluorescence-based detection and sorting are commercially available from Lynx Therapeutics and Illumina, Inc.

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Single-Tube Two-Round Real-Time PCR

Jörg Berg
General Hospital Linz, Linz, Austria

INTRODUCTION

The introduction of real-time PCR in routine diagnostic laboratories has markedly increased the convenience and speed of PCR-based diagnostic assays. As product detection and identification in real-time PCR is performed within closed PCR vessels, the necessity for cumbersome post-PCR analysis steps is omitted. When real-time PCR is carried out in glass capillaries, e.g., on the LightCycler instrument, the PCR cycling speed is markedly increased to approximately 30 sec per cycle only. This rapid-cycle real-time PCR permits product detection by the utilization of various formats of fluorescence dye technologies such as fluorescence resonance energy transfer (FRET) hybridization probes, TaqMan probes, molecular beacons, or SYBR Green I. With this technique numerous protocols have been developed for diagnostic purposes. The sensitivity of rapid-cycle real-time PCR has been demonstrated to the extent of detecting a single copy of DNA per reaction vessel. In order to achieve this high technical sensitivity, the amplification reaction is required to run highly efficiently. However, this may not always be obtained in diagnostic applications. Sequence constraints may prevent ideal primer and probe design, and the complexity of nucleic acids from a clinical sample may preclude ideal amplification conditions.

OVERVIEW

In many diagnostic applications the analytical sensitivity of PCR has been markedly increased, when two rounds of PCR were performed, one PCR after another. In the first round of PCR sample DNA is amplified below the detection limits. Thereafter, the products of the first round of PCR are further amplified to detectable levels in the second round of PCR. For the second round of PCR usually a second set of primers are used which anneal within the DNA sequence of the first-round products. This second set of primers is then termed nested primers or second-round primers. Thus the application of two-round PCR to diagnostic PCR assays has resulted in improved assay sensitivities. In addition, the use of second-round primers increased the specificity of the PCR analysis, because the second-round primers amplify only if the first-round PCR yielded a specific product.[1] So in virus diagnostic applications consensus or indiscriminate primers have been used in a first-round PCR to amplify target DNA, which in a second-round PCR was further amplified with primers that more specifically annealed to the selected viral target DNA. This has increased the sensitivity of PCR analysis to the extent that, for example, in the diagnosis of HIV infection a single copy of HIV-1 cDNA could be detected.[2,3] Furthermore, the two-round PCR technique has successfully been utilized with the simultaneous amplification of various viral genomes such as from herpes simplex virus-1, herpes simplex virus-2, and human cytomegalovirus. Furthermore, two-round PCR has been applied to the genotyping and subtyping of viral strains.[4] For PCR diagnosis and monitoring of hematological malignancies such as the chronic myelogenous leukemia, two-round PCR has successfully been employed for the assessment of the chromosomal translocation t(9;22). This has yielded in high sensitivity of detecting up to one malignant cell within 10^6 normal leukocytes.[5]

As a disadvantage, two-round PCR has suffered from the necessity to recover the products from the first PCR, in order to introduce them into the second-round PCR. During the recovery of first-round products carryover contaminations into samples assayed in parallel may sometimes occur and thus may cause false positive test results to occur. To avoid this, various techniques have been developed to perform two-round PCR in single closed reaction tubes for the conventional PCR technique.[6–8]

In real-time PCR using LightCycler capillaries, the disadvantage of two-round PCR also relates the necessity to recover first-round PCR products. As LightCycler capillaries are rather inaccessible for pipetting, PCR products are usually retrieved by a brief centrifugation step, which releases the capillaries' contents into small tubes. But this procedure is thought to be particularly prone to product carryover. In order to avoid the centrifugation procedure, a two-round real-time PCR technique has been developed for LightCycler capillaries that remain closed during both rounds of PCR.

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SINGLE-TUBE TWO-ROUND REAL-TIME PCR

Single-tube two-round real-time PCR was achieved in LightCycler capillaries by separating the first-round PCR mixture from the second-round PCR mixture by a layer of silicone oil placed in between for the duration of the first round of PCR.[9] Thus the first round of PCR was exclusively performed with the first-round primers and reagents. Then, after the completion of the first-round PCR a brief centrifugation step forced the second-round reagents including the second-round primers to move underneath the silicone oil. Thus second-round reagents were united with first-round products and the second-round PCR was performed (Fig. 1). As the capillaries remained closed during the entire procedure, product carryover into samples analyzed in parallel was avoided between both rounds of PCR.[9]

The feasibility of this method was initially demonstrated by using nested pairs of primers for the PCR-mediated detection of the MBR/JH chromosomal translocation t(14; 18) (q32;q21) in cells of the human B-lymphoma cell line DoHH2.[10] As those cells carry this translocation, they were used as source for MBR/JH-specific DNA. The first-round and second-round PCR mixtures were prepared with equal volumes of 15 μL that were separated in the LightCycler capillary by 5 μL of silicone oil. Both PCR mixtures contained SYBR Green I as detecting fluorochrome. First-round PCR primers were used at about half the concentrations more than were used for the second-round primers. The second-round mixture was void of Taq polymerase and contained only primers and buffer. Capillaries were loaded with the first-round PCR mixture followed by the silicone oil. A brief centrifugation ($735 \times g$ for 5 sec) established the two separate phases with the silicone oil on top, onto which the second-round PCR mixture was layered (Fig. 1). The capillaries were closed, and the first and second PCR cycling programs were performed interrupted only by the centrifugation step after the first round of PCR. Product generation was recorded during both rounds of PCR, and after the completion of the second round of PCR melting curve analysis was performed to discriminate MBR/JH-derived products from primer dimers (Fig. 2). The single-tube two-round PCR detected the MBR/JH fusion gene from as low as 50 pg DoHH2 cell-derived DNA (Fig. 2A–D). This detection limit corresponded to about two to three cells per PCR. For comparison, one-round PCR using the first-round primers only detected the MBR/JH fusion gene from 500 pg DoHH2 DNA. This corresponded to a detection limit of about 20 to 30 cells per PCR only. Thus, the two-round PCR was found to exhibit an approximately 10-fold increased sensitivity than the one-round PCR with this application.[9]

As mentioned above, the described principle of single-tube two-round real-time PCR relates to the physical separation of the first-round PCR mixture from the second-round reagents during the first round of PCR. To test whether the 5 μL of silicone oil sufficed for the effective separation of both reaction mixtures, mock single-tube two-round real-time PCRs were performed with first-round mixtures, prepared without primers. Instead, the primers were placed on top of the oil layer. After completion of the mock first round of temperature cycling, centrifugation was performed and the second round of temperature cycling was carried out. Thus, in repeated experiments amplification products were obtained only in the second round of temperature cycling, and not during the first round[11] (Fig. 3).

Furthermore, when first- and second-round reaction mixtures are unified after the completion of first-round PCR, both mixtures should mix up to homogeneity. In order to accomplish this, both mixtures were prepared to equal volumes. However, mixing experiments with bromphenol blue as indicator dye in the second-round mixture revealed that homogeneity of both mixtures was not obtained immediately after centrifugation. Instead, the centrifugation forced the upper liquid phase to move underneath the first-round mixture. When the temperature

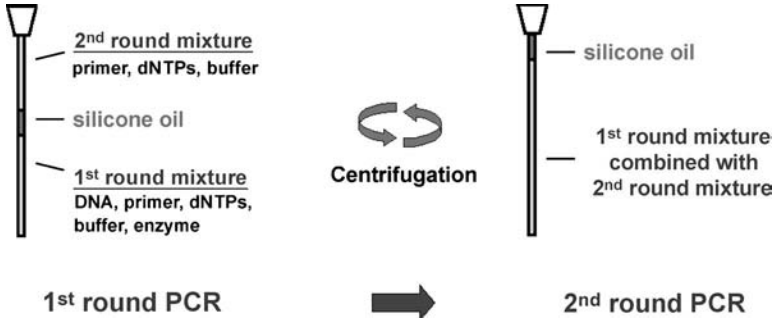

Fig. 1 Schematic sketch of single-tube two-round real-time PCR in glass capillaries on the LightCycler instrument. (*View this art in color at www.dekker.com.*)

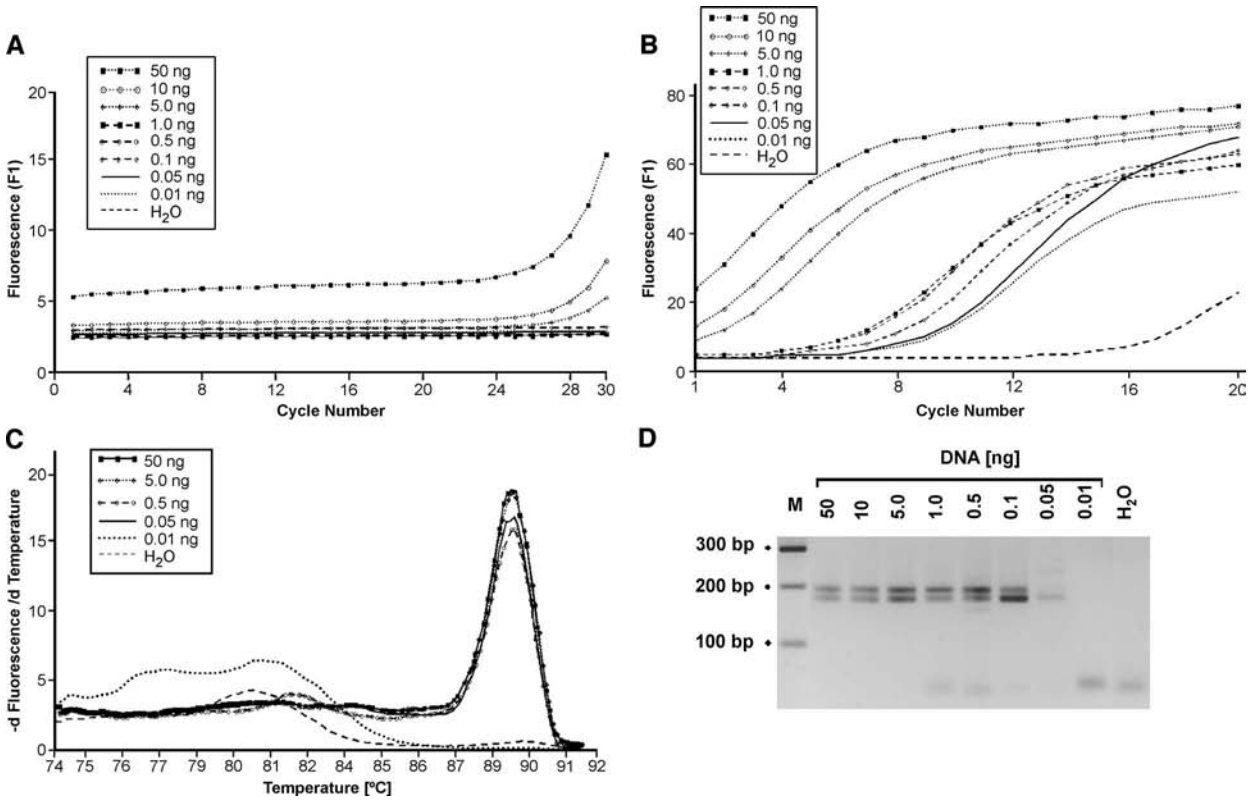

Fig. 2 Example of single-tube two-round real-time PCR for the detection of MBR/JH translocation. Real-time PCR on the LightCycler instrument was performed with graded amounts of genomic DNA (50 to 0.01 ng). Product generation was recorded in real-time fashion during the first round (A) and the second round (B) of PCR. Note that baseline normalization was switched off to show the increase of fluorescence during both rounds of the two-round PCR in comparable fashion. (C) After the amplification melting curve analysis was performed. The products specific for the MBR/JH fusion gene generated peaks corresponding to a melting temperature of about 89.5°C. As the melting peaks superimposed upon each other, only parts of the results are presented for clarity of the figure. (D) The amplification products after both rounds of PCR were resolved by standard agarose gel electrophoresis (3% agarose, 100 V for 1 hr, visualization by ethidium bromide staining and UV illumination). The first-round primers generated a 199-bp product whereas the second-round primers generated a 178-bp product. The nucleic acid molecular weight marker was resolved in lane M. (From Ref. [9].)

cycling was initiated, both mixtures were found mixed to homogeneity after about four to five temperature cycles. When much smaller volumes such as 2 or 5 μL for the second-round mixtures were used compared with the first-round mixtures, homogeneous mixing of both phases was not observed in experiments with bromphenol blue, and nested primer PCR did not occur either.[9]

In a further development of single-tube two-round real-time PCR reverse transcription (RT) was tried to perform within the same closed reaction tube used for the two-round PCR. As detection format, FRET hybridization probes were used instead of SYBR Green I. In this application a two-tube nested RT real-time PCR on the LightCycler instrument was merged into one capillary.[12] The first-round reaction mixture contained the reagents for the RT and for the first round of PCR. The primers for the first and second round of PCR were chosen to exhibit marked differences in their annealing temperatures (8–10°C). So the selected high annealing temperature of the first-round primers resulted in high specificity in the first round of PCR. As a lower annealing temperature was applied to the second-round PCR, potential spurious products of the first-round PCR were further amplified to arrive at a high sensitivity for the assay. In order to further increase sensitivity, the second-round PCR mixture was complemented with a hot start Taq polymerase. This enzyme needs extensive heating (about 7–10 min at 95°C) to be rendered active. As this was not reached with the temperature profile applied to the first round of PCR, the formation of primer dimers did not occur in the second-round mixture during the first round of PCR. As a result the developed HCV-specific assay exhibited an about 20-fold increased detection limit compared to a single-round HCV-specific real-time RT-PCR assay. With the

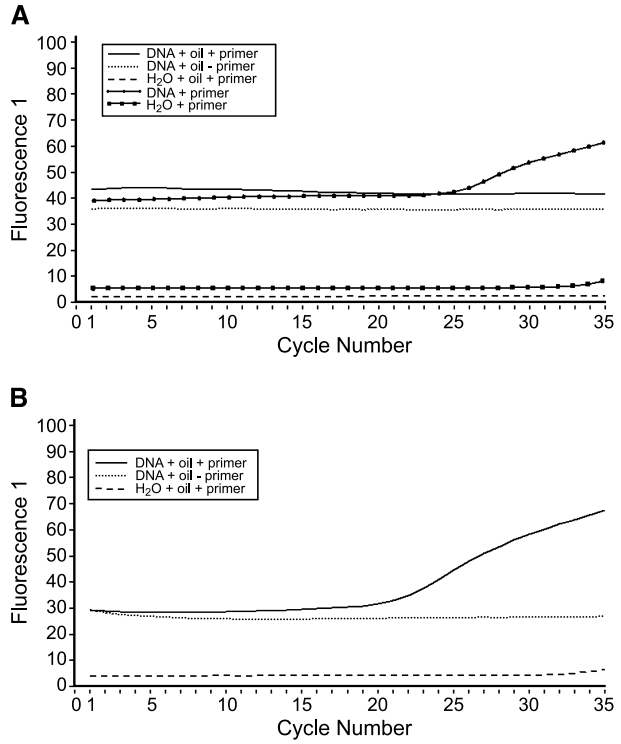

Fig. 3 Effectiveness of the oil layer for single tube two-round real-time PCR. (A) First-round PCRs in the presence of SYBR Green I were performed with primers (DNA+primer), with primers layered on top of the oil phase (DNA+oil+primer), and without primers (DNA+oil−primer) and with H_2O instead of DNA, with primers (H_2O+primers) and with primers layered on top of the oil phase (H_2O+oil+primers). Fluorescence intensities were recorded in real-time fashion. After the first-round PCR the capillaries were spun down and second-round PCR was performed with real-time recording of the fluorescence intensities. (From Ref. [11].)

detection limit of 100 IU/mL this single-tube two-round RT-PCR assay was found suitable for the routine testing of patient samples and for the routine screening of donor samples for the preparation of blood-derived products.[12]

The following limitations were, however, observed with this HCV-specific single-tube two-round real-time RT-PCR approach. The first-round PCR was performed in the absence of the FRET hybridization probes. When they were already added to the first round of PCR, the detection limit was found lower than without hybridization probes. As the probes were omitted in the first-round reaction mixture, quantification was not possible during the first round of PCR, only during the second round of PCR to which they were applied. Therefore this procedure caused a limited range of quantification from about 2500 IU/mL to about 100 IU/mL only. This is in marked contrast to the ranges of quantification with one-round real-time PCR, which usually covers several orders of magnitude.[13] Furthermore, real-time PCR assays have been demonstrated to exhibit very high precision with coefficients of variance usually in the range of a few percents. When this HCV-specific single-tube two-round real-time RT-PCR assay was analyzed for its precision, coefficients of variance of about 10% were obtained.[12] Nevertheless, the developed assay sufficed for the purpose of sensitive detection of HCV RNA in clinical samples, which was not achieved with one-round real-time RT-PCR.[14] As the reaction tubes remained closed during the whole procedure including RT, first- and second-round PCR, and product detection by melting curve analysis, carry-over contaminations into samples analyzed in parallel were precluded.

CONCLUSION

Various approaches have been applied to improve the sensitivity of diagnostic PCR assays. Greater amounts of sample have been used to extract nucleic acids from, in order to permit greater concentrations of sample nucleic acids applied to assays. Also, greater volumes of sample nucleic acids themselves have been applied to PCR assays. These approaches have usually resulted in about two- to fourfold increased sensitivities of PCR analysis. Markedly increased sensitivities have been observed when two rounds of PCR amplification, i.e., two-round PCR, were performed either with nested or with seminested primers. When two-round PCR was performed in single closed tubes with real-time PCR technology, an increase in sensitivity of about 10- to 20-fold was obtained compared to single-round real-time PCR. In various diagnostic applications real-time PCR per se has been shown to exhibit sensitivities comparable to those performed in two-round PCR by conventional thermocycler PCR.[15] However, when this sensitivity was not obtained, nested PCR has been developed with real-time PCR techniques and has yielded in improved sensitivity.[16–19]

In order to set up single-tube two-round real-time PCR for routine use in diagnostic laboratories, various parameters need to be considered and optimized. These include the volumes of the reaction mixtures for the first- and second-round PCR, the use of nested or seminested primers, the primer annealing temperatures, and primer concentrations for first- and second-round PCR. Furthermore, the choice of the detection format may influence the sensitivity and/or the quantification range of those assays, as described above. So the development of a single-tube two-round real-time PCR assay may be easy or laborious in some cases. However, assay development is facilitated by the opportunity to monitor amplifications during PCR in real-time fashion. The reward for the efforts will be a

robust single-tube two-round real-time PCR assay with improved sensitivities that also avoids carryover contamination of first-round amplification products into samples assayed in parallel.

ARTICLES OF FURTHER INTEREST

Real-Time PCR, p. 1117
Real-Time PCR Platforms, p. 1126

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SNP Genotyping Using Single Molecule Fluorescence

Rudolf Franz Zirwes
Stephanie Bousart
Evotec Technologies GmbH, Düsseldorf, Germany

INTRODUCTION

The differential susceptibility or resistance to many human diseases as well as the individual response to medications and environmental, nutritional, or pathogenic stimuli are based on the genetic diversity of the human population. Single nucleotide polymorphisms (SNPs) are single nucleotide positions in genomic DNA that show natural variation, occur on average once at every 500–1000 nucleotides in the human genome, and account for more than 90% of all human sequence variability. Although they have the potential to be tetra-allelic, almost all SNPs are bi-allelic. SNPs revolutionize human molecular genetics because they represent a dense panel of genetic markers distributed across the entire genome. Currently, SNP genotyping is employed in a variety of application areas including population dynamics and evolution, identification of disease-related genes, for the optimization of new drug development, as well as for forensic and diagnostic genetic testing. In addition, SNP genotyping holds great promise for the individualization of clinical diagnostics and therapeutics (i.e., the tailoring of drugs and dose regimens to individual patients) in the future.

This report gives an introduction into the GALIOS™ Genotyping System, a novel technology which provides homogeneous, rapid, and cost-efficient analysis of SNPs and mutations in genomic DNA. The accuracy, specificity, sensitivity, robustness, and reproducibility of GALIOS™ for diagnostic genotyping are illustrated and its potential for miniaturized high-throughput genotyping is discussed.

CHALLENGES AND CURRENT TECHNOLOGIES FOR SINGLE NUCLEOTIDE POLYMORPHISM GENOTYPING

The specific requirements for SNP genotyping technologies vary strongly with the application area. For example, large-scale genetic association studies performed for the identification of disease susceptibility genes and for the optimization of drug developments require the analysis of many thousands of identified SNP positions in hundreds to thousands of individuals to obtain statistically significant association data. Hence, they demand high-throughput SNP genotyping capable of providing up to 500,000 SNP scores (and more) per day. In addition to the evident need for very low running costs, such high-throughput SNP genotyping technologies must be highly automated and must feature economical, fast, and flexible assay development. In contrast, clinical SNP genotyping in forensic, diagnostic, or therapeutic applications usually does not require such high throughput but strongly demands the highest possible sensitivity, specificity, and reliability of the genotyping results. Moreover, prevention of cross-contamination and sample interchange is pivotal for the use of SNP genotyping results in clinical environments.

Currently applied methods for SNP genotyping are based on four general mechanisms for allele discrimination: primer (or probe) extension, allele-specific PCR, allele-specific hybridization, or structure-specific endonuclease cleavage. Although some current methods have considerable potential for high-throughput or clinical genotyping or both, they all have specific restrictions. Methods using primer extension (e.g., pyrosequencing[1] and template-directed dye-terminator incorporation[2]) and most chip-based methods require prior target amplification and purification. This causes many individual steps which complicate automation, rise costs, and provide uncontrolled error sources. Despite the fact that many assays based on allele-specific PCR [e.g., ''amplification refractory mutation system'' (ARMS) assay[3] or allele-specific hybridization (e.g., 5′-nuclease assays[4] and molecular beacon assays[5]) are homogeneous, they usually require expensive specialty probes. Moreover, most high-density chip-based methods do not allow for flexible, rapid, and inexpensive implementation of newly identified SNPs. Whereas the Invader assay[6] based on structure-specific enzymatic cleavage overcomes many of the described restrictions, it requires huge amounts of genomic DNA because its sensitivity is about 1 order of magnitude below PCR-based methods. Finally, all methods which employ traditional macroscopic fluorescence techniques—encompassing conventional fluorescence intensity, fluorescence polarization, and fluorescence resonance energy transfer—have only limited potential for

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miniaturization, an essential step toward cost-efficient high-throughput genotyping.

GALIOS™—HOMOGENEOUS HIGH-CONTENT SINGLE NUCLEOTIDE POLYMORPHISM GENOTYPING

A new technology has been developed for homogeneous, sensitive, rapid, and cost-efficient analysis of SNPs and mutations in genomic DNA. The GALIOS™ Genotyping System (Evotec Technologies GmbH, Düsseldorf, Germany) combines the high specificities of seminested PCR and allele-specific amplification, concurrently performs amplification and allele-specific labeling, and simultaneously interrogates both alleles of a given bi-allelic SNP in a ''one-tube'' reaction. Moreover, the GALIOS™ Genotyping System is fully generic and does not require the expensive and time-consuming synthesis of specialty primers or probes. Figure 1 illustrates the GALIOS™ principle of bi-allelic SNP scoring in a one-tube reaction. The reaction mix contains four target-specific and two universal primers; one pair of gene-specific amplification primers, two types of seminested, allele-specific diagnostic primers containing different universal sequence tails at their 5′-ends, and two types of fluorescent universal detection primers tagged with fluorescence dyes excitable at 543 nm (TAMRA) or 633 nm (EVOblue™), respectively. The tailed diagnostic primers have identical sequences, except for the 5′-tail portion and at least one internal nucleotide, which is complementary to the corresponding nucleotide of the respective allele. During a GALIOS™ reaction, the high-concentrated amplification primers dominate the early PCR cycles and amplify the genomic region of interest independent of the allele. In contrast, the low-concentrated diagnostic primers are extended predominantly in later PCR stages and lead to allele-specific generation of universally tailed PCR products. Upon progress of the

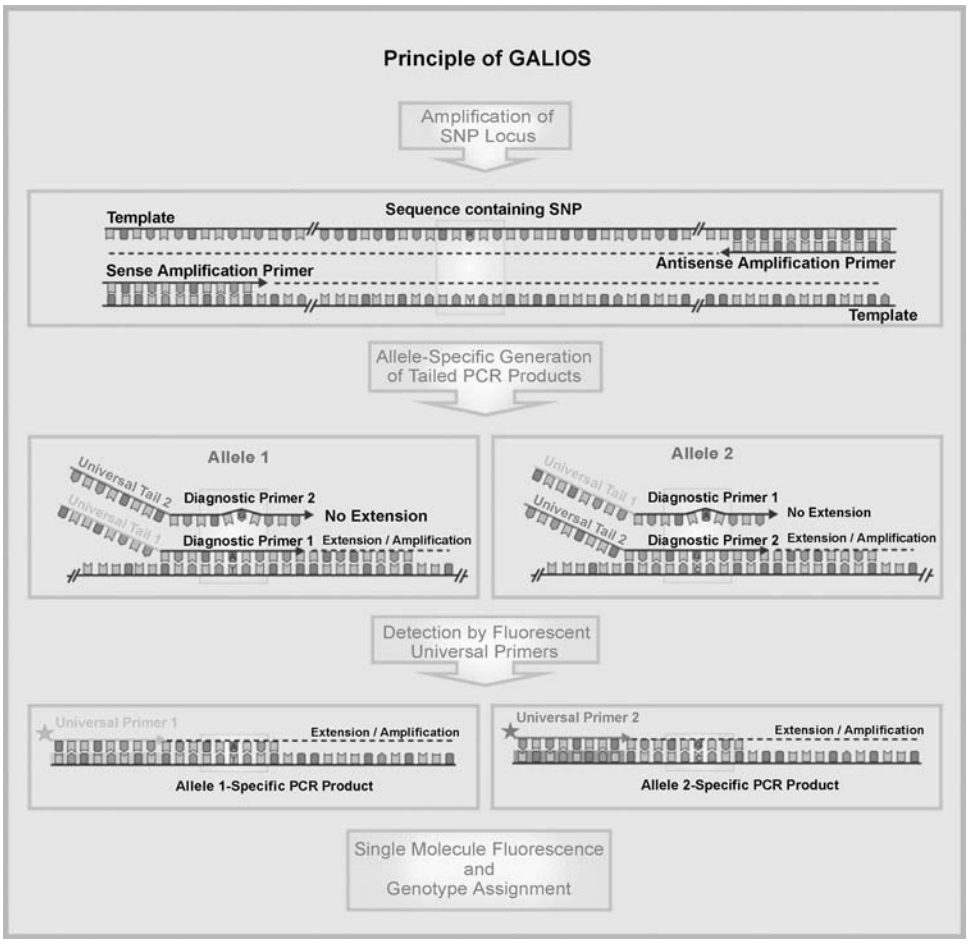


Fig. 1 The principle of biallelic SNP scoring with GALIOS™. (*View this art in color at www.dekker.com.*)

GALIOS™ reaction, these tailed PCR products serve as a template for the universal labeling primers, finally resulting in allele-specific accumulation of fluorescent amplification products.

After the GALIOS™ amplification, reaction mixes are analyzed directly, without any post-PCR processing or physical separation steps, by single molecule fluorescence (i.e., fluorescence correlation spectroscopy or FCS).[7–10]

Whereas traditional macroscopic fluorescence techniques monitor the average fluorescence output from the entire ensemble of emitting fluorophores in a given reaction, single molecule fluorescence employs confocal optics with a very tiny illumination volume of approximately 1 fL (10^{-15} L). Only fluorescence emitted within this confocal volume is observed and traced in a time-resolved manner. The resulting fluorescence raw data are amenable to different types of fluctuation analysis including FCS, which statistically samples the fluorescence fluctuations emitted from single molecules in discrete time intervals, with a temporal resolution down to microseconds and below. This process is termed autocorrelation and provides information on multiple fluorescence parameters including the average number, the molecular brightness, the triplet fraction, and the translational diffusion time of fluorescent molecules within the confocal volume. By fitting the autocorrelated fluorescence signal to a two-component model, the relative amounts of two species of fluorescent molecules differing in their translational diffusion time (e.g., the amounts of free universal primer and fluorescent PCR product in GALIOS™ reactions) are determined.

By combining confocal optics and autocorrelation, FCS virtually eliminates problems associated with conventional macroscopic fluorescence techniques, such as probe adsorption, inner filter effects, photobleaching of fluorophores, and particularly the decrease of signal with decreasing reaction volumes (i.e., with miniaturization). Thus, FCS provides a high signal-to-noise ratio (SNR) and a maximum sensitivity that is essentially independent of the assay volume. This allows ultimate assay miniaturization down to 1 μL or below sustaining a consistent signal quality—a favorable feature for economical high-throughput SNP genotyping. In addition, the obtained multiple fluorescence parameters serve as intrinsic quality controls within each reaction.

AUTOMATED GENOTYPING WITH FLUORESCENCE CORRELATION SPECTROSCOPY

Both the MF10S microplate reader [developed jointly by Olympus Optical Co., LTD (Tokyo, Japan) and Evotec Technologies GmbH] and the Analyzer ΔA2.0 (Evotec Technologies GmbH) are bench-top instruments for automated FCS analysis and perform automated adjustment, FCS measurements, autocorrelation, data fitting, and SNP genotype assignment. Reaction mixes are excited at 543 and 633 nm, and the quantities of allele-specific fluorescent amplification products (% amplimer) at each wavelength are determined. Genotypes are assigned by calculation of a normalized fluorescence signal (NFS), i.e., the difference of the quantities of fluorescent amplification products normalized by the sum of all amplimer-derived signal $[(543-633)/(543+633)]$. The software employs user-defined or default limit values for multiple fluorescence parameters and the quantities of fluorescent amplification products from negative and positive control reactions, thus providing multiple intrinsic quality controls for successful amplification and single molecule detection in each reaction. Additionally, user-defined limits for discrimination of the three possible genotypes allow for automated SNP genotype assignment.

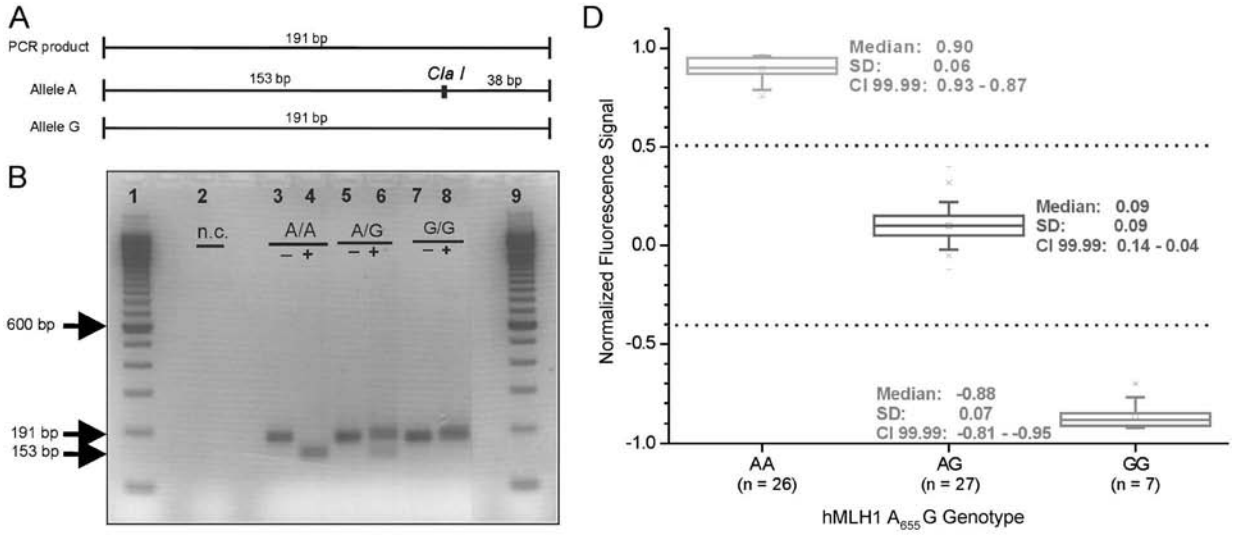
ACCURACY, REPRODUCIBILITY, AND PRECISION OF GALIOS™ GENOTYPING

The performance of GALIOS™ is demonstrated by genotyping the SNP at position 655 ($A_{655}G$) within the human MLH1 (mutL homolog 1) gene.[11] The hMLH1 protein functions as a mismatch repair protein and helps to maintain the fidelity of DNA during replication. The $A_{655}G$ transition within the *hMLH1* gene replaces isoleucine at position 219 by valine ($I_{219}V$) in the hMLH1 protein and is associated with the inherited familial cancer syndrome of hereditary nonpolyposis colorectal cancer (HNPCC).[12]

To compare the accuracy of the GALIOS™ genotyping technology to a conventional genotyping method, 60 genomic DNAs (gDNAs) were analyzed in parallel by 1) PCR amplification and subsequent restriction fragment length polymorphism analysis (PCR-RFLP) and by 2) GALIOS™.

For PCR-RFLP, a 191-bp PCR product encompassing the $A_{655}G$ polymorphism of the *hMLH1* gene was amplified from each gDNA. The antisense primer was designed such that a *Cla*I restriction site (AT/CGAT) was introduced into the 191-bp PCR product upon amplification from allele A but not from allele G. Consequently, restriction digests of the amplimers with *Cla*I analyzed by agarose gel electrophoresis revealed distinct restriction fragment patterns for the three hMLH1 genotypes (Fig. 2A and B).

For GALIOS™ genotyping, all 60 gDNAs were amplified in duplicate reactions, each at 20 μL final



C

Sample ID	SNP score	Signal 543 nm	Signal 633 nm	Allele Ratio	SNR	NFS
n.c	-	2.0	2.3	-	-	-
361	A/G	41.8	34.3	1.2	17.7	0.10
377	A/A	51.6	1.3	39.7	25.8	0.95
385	G/G	2.8	60.4	21.6	26.3	-0.91
386	A/G	39.3	32	1.23	16.6	0.10
397	G/G	2.8	45	16.1	19.6	-0.88
409	A/A	60.7	6.3	9.63	30.35	0.81

E

Genotype	Precision of NFS								
	Intra-Sample (n = 100)			Inter-Well (n = 10)			Inter-Sample (n = 10)		
	Mean	SD	CI 99.99	Mean	SD	CI 99.99	Mean	SD	CI 99.99
A/A	0.91	0.08	0.94 - 0.88	0.94	0.04	0.99 – 0.89	0.92	0.06	0.99 – 0.85
A/G	-0.03	0.09	-0.01 - -0.07	0.08	0.04	0.03 – 0.13	0.05	0.05	0.11 - -0.01
G/G	- 0.92	0.07	-0.89 - -0.95	-0.88	0.08	-0.79 - -0.97	-0.88	0.05	-0.81 - -0.95

Fig. 2 (A–D) Comparative analysis of the hMLH1 $A_{655}G$ SNP with PCR-RFLP and GALIOS™. (A) Scheme of the PCR-RFLP strategy. A 191-bp PCR product amplified from allele A, but not from allele B, is cut into a 38- and a 153-bp fragment by *Cla*I digestion. (B) Analysis of *Cla*I restriction digestion by agarose gel electrophoresis. Lanes 1 and 9: 100-bp DNA ladder. Lane 2: negative control (n.c.). Lanes 3–8: PCR products from gDNAs of hMLH1 genotype A/A (3 and 4), A/G (5 and 6), or G/G (7 and 8) before (''–'': 3, 5, and 7) and after (''+'': 4, 6, and 8) *Cla*I digestion. All samples show the expected restriction fragment pattern, i.e., 153 and 38 bp for A/A, 191, 153, and 38 bp for A/G, and 191 bp for G/G genotype. The 38-bp fragment is poorly visible on the gel. (C) Data generated by GALIOS™ and FCS analysis from reactions containing no DNA (n.c.) or gDNA samples of different hMLH1 $A_{655}G$ genotype (361–409). (D) Box plot illustrating the highly precise distribution of NFS values obtained from (*n*) gDNAs for each hMLH1 $A_{655}G$ genotype (A/A, A/G, and G/G). Median, S.D., and CI 99.99% values are denoted at the respective boxes. (E) Precision data (mean, S.D., and CI 99.99%) for intrasample, interwell, and intersample precision obtained from (*n*) determinations of each hMLH1 genotype. (*View this art in color at www.dekker.com.*)

volume containing 5 ng gDNA. GALIOS™ reactions consisted of $1\times EVO_{AMP}$ buffer (Evotec Technologies GmbH), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U Q-BioTaq (Q•BIOgene, Heidelberg, Germany). Amplification, diagnostic, and universal labeling primers were 300, 10, and 5 nM, respectively. Thermal cycling included an initial 2-min denaturation at 94°C, followed by 45 cycles of 94°C for 20 sec, 55°C for 40 sec, and 72°C for 45 sec. Samples were maintained at 72°C for 5 min before holding at 4°C prior to analysis. FCS measurements were performed for 3×3 sec in each reaction both at 543 and 633 nm. Mean values of the fractions of fluorescent PCR product (amplimer) from triplicate measurements were used for further genotype calculations. In all samples, amplimer values indicating allele-specific amplification signal were at least 8-fold higher than the corresponding background ''noise'' from no-DNA control reactions at the respective wavelength (SNR>8) (Fig. 2C). Bi-allelic SNP scores were made when the SNR was ≥ 8 and the ratio of the higher to the lower allele signal was ≥ 6 for homozygous and ≤ 2 for heterozygous scores. Calculation of the NFS revealed distinct values for each hMLH1 genotype (A/A, A/G, and G/G), and the resulting bi-allelic SNP scores were 100% identical for both GALIOS™ and PCR-RFLP, demonstrating the accuracy of GALIOS™ genotyping. The high confidence of the GALIOS™ genotyping results was also confirmed by the narrow intragenotype distribution of NFS values (Fig. 2D).

Reproducibility and precision of GALIOS™ genotyping were assessed by repetitive hMLH1 genotyping of subsets of gDNAs and evaluation of the distribution of the NFSs (Fig. 2E).

For intrasample precision, a single gDNA sample per hMLH1 genotype was amplified and measured by FCS for 10×3 sec at both 543 and 633 nm. All combinations of individual amplimer values from both wavelengths were used to calculate 100 individual NFSs per genotype. For interwell precision, a single gDNA per hMLH1 genotype was amplified in 10 individual GALIOS™ reactions. Each reaction was measured by FCS, each for 3×3 sec at both

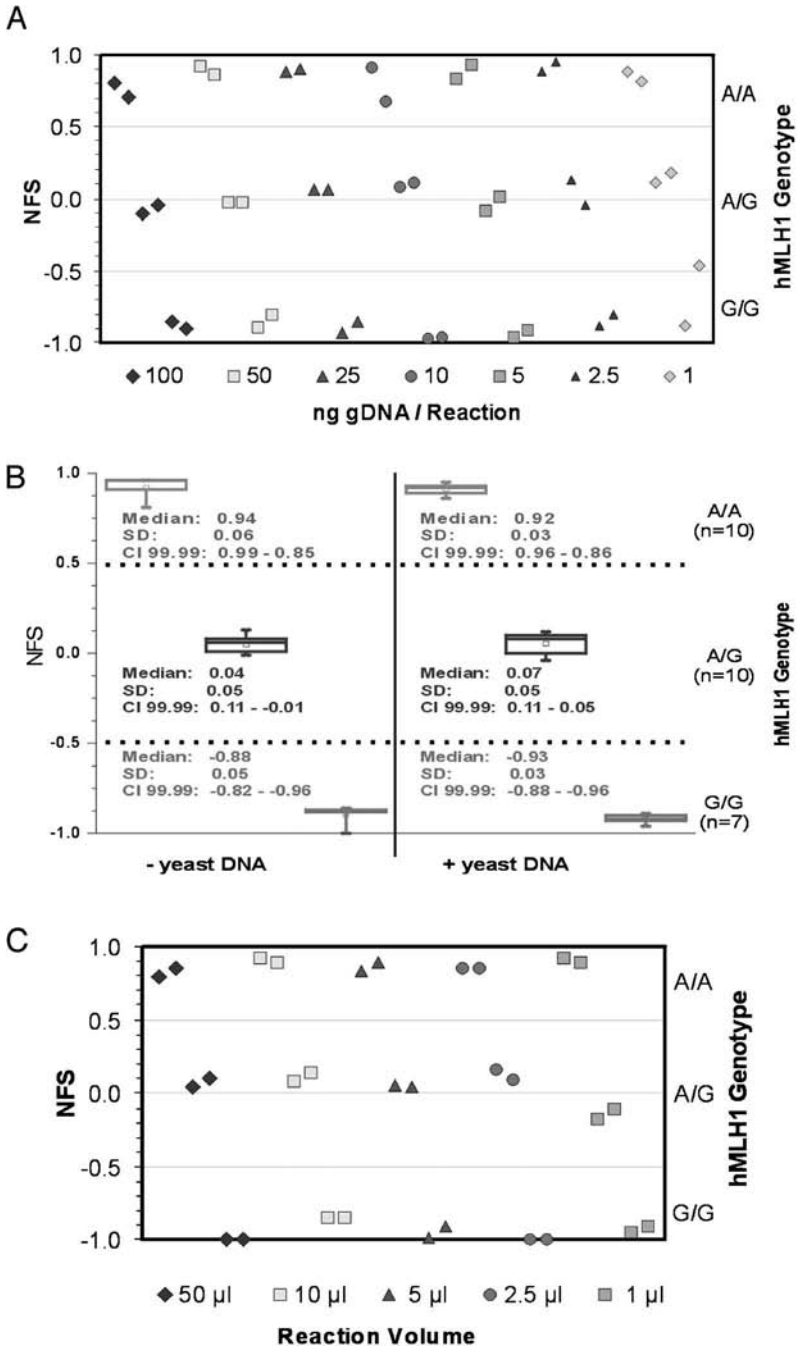


Fig. 3 Robustness, specificity, and miniaturization of GALIOS™ genotyping. (A) Plot of the NFS values obtained for hMLH1 $A_{655}G$ genotypes (A/A, A/G, and G/G) using 100–1 ng of gDNA per reaction. (B) Box plot illustrating the highly precise distribution of NFS values obtained for each hMLH1 $A_{655}G$ genotype from several ($n = 7$–10) gDNAs both in the absence (−yeast DNA) or presence (+ yeast DNA) of unspecific template. Median, S.D., and CI 99.99% values are denoted at the respective boxes. (C) Plot of the NFS values obtained for each hMLH1 $A_{655}G$ genotype obtained from standard and miniaturized reaction volumes (50–1 µL) containing a gDNA concentration of 1 ng/µL. (*View this art in color at www.dekker.com.*)

543 and 633 nm. Mean amplimer values from triplicate measurements were used to calculate 10 individual NFSs per genotype. For intersample precision, 10, 10, and 7 individual gDNAs for hMLH1 genotypes A/A, A/G, and G/G, respectively, were amplified and measured by FCS for 3×3 sec each at both 543 and 633 nm. Mean amplimer values from triplicate measurements were used to calculate individual NFSs.

All precision experiments yielded fully reproducible results and the NFS values showed a narrow distribution for each hMLH1 genotype (Fig. 2E), thus indicating the high reproducibility and precision of GALIOS™ genotyping.

Accuracy, reproducibility, and precision of GALIOS™ genotyping were highly identical when FCS measurement time was reduced to 1×2 sec at both 543 and 633 nm (data not shown).

ROBUSTNESS AND SPECIFICITY OF GALIOS™ GENOTYPING

Upon variation of the amount of genomic DNA from 100 to 1 ng per 20 μL reaction, unambiguous genotype discrimination was obtained (Fig. 3A), illustrating the robustness of GALIOS™ against variations in template concentration.

The specificity of GALIOS™ genotyping was examined by parallel analysis of 27 gDNAs (10, 10, and 7 for hMLH1 genotypes A/A, A/G, and G/G, respectively) with and without addition of 10 ng yeast gDNA representing a 500-fold molar excess of ''contaminating'' template. The results of the FCS analysis show that accuracy and precision of genotyping results are not affected by the presence of excess unspecific DNA (Fig. 3B), thus proving the very high specificity of the system.

MINIATURIZED SINGLE NUCLEOTIDE POLYMORPHISM GENOTYPING WITH GALIOS™

Duplicate GALIOS™ reactions of each hMLH1 genotype were performed in final reaction volumes ranging from 1 to 50 μL and a final gDNA concentration of 1 ng/μL (Fig. 3C). The three genotypes were clearly distinguished irrespective of the assay volume. Even 1 μL reactions containing 1 ng of genomic DNA perform genotype discrimination with the same confidence as 50 μL reactions containing 50 ng of genomic DNA.

CONCLUSION

The GALIOS™ SNP genotyping technology is ideally suited for diagnostic SNP genotyping and has potential for high-throughput genotyping as well. The homogeneous assay principle and direct detection by single molecule fluorescence facilitate automation and minimize cross-contamination risks. Universal labeling primers provide a generic system for flexible, fast, and cost-efficient assay development without a need for the synthesis of labeled primers or specialty probes, and small reaction volumes reduce costs per bi-allelic SNP score effectively. The system is robust to variations in template concentration and provides highly sensitive and confident genotyping with as little as 1 ng of gDNA. High-content data from single molecule fluorescence provide intrinsic quality controls for highly reliable SNP scoring without additional control reactions.

Currently, by using FCS measurements, about 15,000 bi-allelic SNP scores per day can be analyzed on a single MF10 S instrument. Using Evotec Technologies' readers for ultra-high throughput screening (μHTS),[13] this number can be increased to at least 10-fold to allow for high throughput genotyping.

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Spinal Muscular Atrophy

Shuji Ogino
Brigham and Women's Hospital, Dana-Farber Cancer Institute, and Harvard Medical School, Boston, Massachusetts, U.S.A.

INTRODUCTION

Spinal muscular atrophy (SMA) is the second most common severe autosomal recessive disease after cystic fibrosis. SMA is caused by degeneration of the anterior horn cells of the spinal cord. Despite the complexity of SMA genetics, considerable progress has been made in the diagnostics for patients and carriers since the identification of the disease gene, *SMN1*.

CLINICAL DESCRIPTION

SMA is characterized by symmetric proximal muscle weakness. Sensory neurons may have also been affected in SMA type I (see below) patients.[1] SMA is classically subdivided into three types according to age of onset and clinical severity, based on the diagnostic criteria.[2] Type I SMA (Werdnig–Hoffmann disease; OMIM#253300) is characterized by the onset of severe muscle weakness and hypotonia in the first few months of life, and the inability to sit or walk. Fatal respiratory failure usually occurs before the age of 2 years. Type II SMA (intermediate type; OMIM#253550) is characterized by the onset of proximal muscle weakness before 18 months of age, the ability to sit but not to walk unaided, and survival beyond 4 years of age. Type III SMA (Kugelberg–Welander disease; OMIM#253400) is characterized by the onset of proximal muscle weakness after the age of 2 years, the ability to walk independently until the disease progresses, and survival into adulthood.

INCIDENCE AND PREVALENCE

SMA affects $\sim$1/10,000 live births and with a disease allele frequency of $\sim$1/100 and a carrier frequency of $\sim$1/50.[3] Approximately 60% of SMA patients have type I, and the remaining 40% have type II or III.[3] Type II is more common than type III SMA.

MOLECULAR GENETICS

SMN1, the Disease Gene, and *SMN2*, the Disease Modifier Gene

SMN1 (OMIM#600354) and its homologue, *SMN2* (OMIM#601627), lie within the telomeric and centromeric halves, respectively, of a large inverted repeat in 5q13.[4] *SMN* consists of nine exons (exons 1, 2a, 2b, 3 through 8), with the stop codon present near the end of exon 7. The coding sequence of *SMN2* differs from that of *SMN1* by a single nucleotide (840C>T) in exon 7. In contrast to *SMN1*, at least one copy of which is indispensable for the survival of motor neurons, $\sim$5–10% of normal individuals lack both copies of *SMN2*. The failure of *SMN2* to compensate fully for the loss of *SMN1* is due to the sequence difference in exon 7. Although translationally silent, the 840C>T in *SMN2* decreases the activity of an exonic splicing enhancer so that less full-length transcript is generated. A recent study also suggests the presence of exonic splicing silencer around the position 840 that is active in *SMN2*, but not in *SMN1*.[5] *SMN* gene duplication occurred more than 5 million years ago, before the separation of human and chimpanzee lineages, but that the *SMN2* gene sequence per se appeared for the first time only in *Homo sapiens*.[6]

SMN1 Deletion Mutations and Conversion Mutations

Approximately 94% of individuals with clinically typical SMA lack both copies of *SMN1* exon 7.[7] Loss of *SMN1* exon 7 can occur by a large deletion, typically including the whole gene, or by conversion to *SMN2* exon 7. Absence of the *SMN1* exon 7 sequence indicates a functional or a de facto absence of the *SMN1* gene.

Most converted genes from *SMN1* to *SMN2* had *SMN2* sequences in all of the polymorphic nucleotides except for the *SMN1* sequence in exon 8.[8] A half of the hybrid *SMN1*/*SMN2* genes had a single Ag1-CA/C212 haplotype,

Databases: *SMN1*—OMIM: 600354, 253300 (SMA type I), 253550 (SMA type II), 253400 (SMA type III); Genbank: XM_017078; GDB: 120378. *SMN2*—OMIM: 601627.

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suggesting that these hybrid *SMN* alleles had a common origin.[8] Other variants of hybrid *SMN* genes are rarely present. Considering increased number of *SMN2* copies in mild SMA patients in the absence of *SMN1* exon 7, gene conversion from *SMN1* to *SMN2* is a more likely hypothesis than unequal recombination and intrachromosomal deletion.[8] There is also evidence of gene conversion from *SMN2* to *SMN1*.[9]

SMN1 Small Intragenic Mutations

In a subset of the SMA patients who do not lack both copies of *SMN1*, ~30 small intragenic mutations (''subtle'' or ''nondeletion'' mutations) in *SMN1* have been identified (reviewed in Ref. [10,11]), for which we proposed the use of standard nomenclature.[10] These intragenic mutations provide solid evidence that *SMN1* is indeed the SMA gene, because intragenic *SMN1* mutations are associated with the SMA phenotype regardless of the status of other candidate genes. The three most frequently reported small intragenic mutations are the Y272C missense mutation (815A>G), the 768_778dupTGCTGATGCTT frameshift mutation, and the 399_402delAGAG frameshift mutation.

SMN1 De Novo Mutations

SMN1 has a high rate of de novo deletion mutations. There is a high frequency of recombination and crossover events at 5q13, presumably secondary to the presence of the large inverted repeat as well as smaller repeats around the *SMN1* and *SMN2* loci. The high rate of de novo mutations in *SMN1* likely underlies the high carrier frequency for SMA in the general population despite the high genetic lethality of the disease. De novo mutations of paternal origin are much more frequent (with a mutation rate of $\sim 1.6 \times 10^{-4}$)[3] than those of maternal origin.[12]

GENOTYPE–PHENOTYPE CORRELATION

At least one of the factors underlying the variable phenotype of SMA is the *SMN2* copy number. Individuals with type III SMA have, on average, more copies of *SMN2* than individuals with type II or type I SMA,[13–16] and likely express more full-length SMN protein from *SMN2*. The number of *SMN2* copies correlates with longer survival.[15] Mouse models of SMA also show dosage effects of *SMN2* in rescuing SMA symptoms.[17] However, other modifying factors must exist as *SMN2* copy numbers of the three SMA types overlap.

Various small intragenic mutations have also different effects on SMA phenotype. In in vitro experiments, G279V (836G>T) and Y272C (815A>G) caused a severe impairment of oligomerization, whereas S262I (785G>T) and T274I (821C>T) caused a mild impairment, and the degree of impairment correlated with the severity of SMA associated with the mutations in vivo.[18]

Other candidate modifier genes include *NAIP*, *H4F5* (*SERF1*), and *Htra2-β1*. It remains a possibility that SMA phenotypes are modified by genes responsible for neuromuscular disorders other than *SMN1*-linked SMA, including *IGHMBP2*, *HEXA*, *SCO2*, and others (reviewed in Ref. [10]).

POTENTIAL THERAPEUTICS

Currently, there is no therapy that cures SMA. However, active investigations are underway, and new therapeutic approaches will likely emerge in the near future. Potential therapeutic approaches (reviewed in Refs. [11,19]) are: 1) to increase full-length mRNA from *SMN2* by an in vivo alteration in the splicing pattern of *SMN2* exon 7 through activation of *trans*-acting factors, or antisense RNA molecules; 2) to increase the overall transcription level of SMN mRNA by activating the *SMN* promoter; 3) to stabilize the SMN protein; 4) to repair degenerated motor neurons; 5) to replace degenerated motor neurons by stem cell therapy; and 6) to target modifying factors other than *SMN2*. A high throughput drug screening for the identification of compounds that up-regulate full-length SMN protein is underway.

MOLECULAR GENETIC TESTING

Linkage Analysis

Localization of the SMA critical region allowed linkage analysis as the first genetic test. Informative linkage markers have been listed.[20] Linkage analysis remains an important tool in certain clinical settings.[3,21]

PCR-Restriction Fragment Length Polymorphism (RFLP)

The most commonly used method to confirm genetically the diagnosis of SMA is a qualitative PCR-RFLP assay to detect the homozygous absence of *SMN1*.[22] The PCR-RFLP assay takes advantage of the base differences in exons 7 and 8 to distinguish *SMN1* from *SMN2*. The restriction endonuclease *Dra*I digests only the *SMN2* exon 7 PCR products because of a restriction site generated by a mismatched reverse primer.[22] Another method introduces *Hin*fI control restriction sites in both the *SMN1* and the *SMN2* exon 7 PCR products to ensure complete digestion, in addition to the *SMN1* unique restriction site.[16]

PCR-Denaturing High-Performance Liquid Chromatography (DHPLC)

A rapid PCR-DHPLC method to detect homozygous absence of *SMN1* exon 7 has been described.[23]

Prenatal and Preimplantation Genetic Testing

Prenatal testing to detect the homozygous absence of *SMN1* can be performed on chorionic villous sampling (CVS) specimens, amniotic fluids, or maternal peripheral blood.[24] Preimplantation genetic testing to detect the homozygous absence of *SMN1* exon 7 has been described (reviewed in Ref. [10]).

Quantitative *SMN* Gene Dosage Analysis

To identify SMA carriers necessitates a quantitative approach, i.e., *SMN* gene dosage analysis. The first highly precise assay to determine copy numbers of *SMN1* and *SMN2* utilizes quantitative competitive PCR-RFLP, with *CFTR* exon 4 as a two-copy genomic reference.[13] To correct for variation in amplification efficiency, *SMN* and *CFTR* internal standards are used. Each standard has the same primer binding sites as its genomic counterpart, and a small internal deletion, which allows the internal-standard PCR products and the genomic PCR products to be separated and quantified independently.[13] The rationale for this quantification method has been described.[25] Various dosage analysis methods have since been described,[14,16,26,27] including real-time quantitative PCR.[15,28] *SMN* gene dosage analysis can be used for the detection of heterozygous absence of *SMN1* in SMA patients who do not lack both copies of *SMN1*.[16]

Heteroduplex formation between *SMN1* and *SMN2* PCR products may affect quantitative PCR-RFLP.[25] In addition to heteroduplex formation, reproducible PCR bias between *SMN1* and *SMN2* sequences may be present in *SMN* gene dosage analysis.[29]

SMN1 Small Intragenic Mutation Analysis

Analysis for the detection of small intragenic mutations is typically performed by DNA sequencing. Although labor-intensive and not routinely offered, it can be performed.[20] A multiplex, allele-specific PCR assay to detect the seven relatively common small intragenic mutations has been described.[30]

Monosomal Analysis

A monosomal-analysis method utilizes the separation of single human chromosomes of homologous pairs by fusing human cells with mouse cells and culturing with selection. A monosomal-analysis method to identify an *SMN1* deletion/conversion carrier who had two copies of *SMN1* on the normal chromosome 5 (the ''2+0'' genotype) has been developed.[31] This promising technology obviates the need to perform extensive linkage and dosage analyses to distinguish carriers with two copies of *SMN1* from noncarriers (the ''1+1'' genotype) who passed a de novo deletion mutation to an affected child.[3]

GENETIC COUNSELING

SMA Genetic Risk Assessment

Methods of SMA genetic risk assessment for various clinical settings have been described in great depth.[3,10] Risk assessment is an integral part of genetic testing. The purpose of genetic risk assessment is to provide family members with risk estimates as accurately as possible for their decision making. Risk assessment for SMA is particularly important, because of the complexity of SMA genetics. The disease allele frequency is relatively high; small intragenic mutations are undetectable by the standard assays; the paternal de novo mutation rate is high; and the presence of two copies of *SMN1* on one chromosome 5 can mask a deletion/conversion mutation on the other chromosome 5 in *SMN* gene dosage analyses.

It should be noted that genetic risk assessments are estimates based on published data from a finite number of cases in specific populations. It should also be noted that additional family information, especially additional genetic information, often dramatically improves the accuracy of genetic risk assessments. Thus genetic risk assessment is often an ongoing process, and genetic testing on additional family members should be performed whenever indicated by the initial results.[10]

SMN1 Allele and Genotype Frequencies

For SMA risk assessment, normal and disease allele frequencies have been estimated.[10] Most disease alleles in SMA are deletions (or conversions) of at least exon 7 of *SMN1* (''0-copy'' alleles). A normal chromosome 5 usually has one copy of *SMN1* (''1-copy'' allele). However, two copies of *SMN1* are often present on the same chromosome 5, for which we use the term ''2-copy allele.'' We refer to disease alleles with small, intragenic mutations in *SMN1* as ''1^{D}'' (''1-copy-disease'') alleles. Updated frequency of each *SMN1* allele or genotype is listed in Table 1 (modified from Ref. [11]).

Table 1 Updated *SMN1* allele and genotype frequencies

Allele			Designation	Frequency
''0-copy''			**a**	9.88×10^{-3}
''1-copy''			**b**	9.50×10^{-1}
''2-copy''			**c**	4.04×10^{-2}
''1^{D}-copy''			**d**	1.81×10^{-4}
Status	**Genotype**[a]	**Gene dosage**	**Designation**	**Frequency**
Noncarrier	''2+2''	4	$\mathbf{c}^2$	1.63×10^{-3}
	''2+1''	3	2**bc**	7.67×10^{-2}
	''1+1''	2	$\mathbf{b}^2$	9.02×10^{-1}
Carrier	''2+1^{D}''	3	2**cd**	1.46×10^{-5}
	''2+0''	2	2**ac**	7.98×10^{-4}
	''1+1^{D}''	2	2**bd**	3.43×10^{-4}
	''1+0''	1	2**ab**	1.88×10^{-2}
Affected	''1^{D}+1^{D}''	2	$\mathbf{d}^2$	3.27×10^{-8}
	''1^{D}+0''	1	2**ad**	3.57×10^{-6}
	''0+0''	0	$\mathbf{a}^2$	9.77×10^{-5}

[a]Genotype is indicated by ''(*SMN1* allele on one chromosome 5)+(*SMN1* allele on the other chromosome 5).''
Source: Modified from Ref. [11].

CONCLUSION

Since the discovery of the disease gene, *SMN1*, there has been considerable progress in the understanding of molecular genetics of SMA. Comprehensive SMA genetic testing, comprising qualitative PCR-RFLP, *SMN* gene dosage analysis, and linkage analysis, in combination with appropriate genetic counseling and risk assessment, currently offers the most complete evaluation of SMA patients and their families. In addition, new technologies, such as monosomal analysis techniques, may be widely available in the near future. Understanding of molecular genetics will also facilitate the development of new therapy that may cure SMA in the future.

ARTICLES OF FURTHER INTEREST

Ethical Issues Related to Genetic Testing, p. 409
Prenatal Diagnosis Using Fetal Cells and Cell-Free Fetal DNA in Maternal Blood, p. 1079
Real-Time PCR, p. 1117

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Staphylococcus aureus—Detection of MRSA

Sameer Elsayed
University of Calgary, Calgary, Alberta, Canada

INTRODUCTION

Antimicrobial resistance is becoming an increasingly recognized global health problem. One particularly important worldwide threat is that of methicillin-resistant *Staphylococcus aureus* (MRSA), which, by virtue of its highly virulent nature similar to its methicillin-sensitive counterpart, is capable of causing a wide array of serious illnesses, although with limited options for therapy. Recent advances in molecular science have resulted in an improved understanding of the genetics of methicillin resistance in *S. aureus* and have fostered the development of new molecular techniques for the laboratory detection of this emerging pathogen. This article will provide the reader with a comprehensive overview of MRSA, focusing on the epidemiology, microbiology, clinical features, genetics, and molecular detection of this microorganism.

BACKGROUND

S. aureus is a major human pathogen, causing a wide spectrum of hospital-acquired and community-acquired infections worldwide.[1,2] The use of potent antimicrobials, such as the β-lactam class of antibiotics, for the treatment of severe *S. aureus* disease has contributed significantly to reduced morbidity and mortality rates.[3] However, of great global concern is the rapid emergence and spread of MRSA strains. Since their first description in 1961 in the UK shortly after the introduction of methicillin into clinical use,[4] MRSA strains have spread to all parts of the world.[1,5–7] Rates of methicillin resistance in *S. aureus* vary markedly by geographical region, ranging from $\leq 2\%$ in Switzerland and The Netherlands to over 70% in Hong Kong and Japan.[1] Although methicillin is no longer used clinically or for laboratory susceptibility testing (having been replaced by cloxacillin and oxacillin, respectively), strains classified as methicillin-resistant harbor the *mecA* gene, which, when expressed, renders isolates resistant to all β-lactam agents, including penicillinase-resistant penicillins (e.g., methicillin, oxacillin), cephalosporins, and carbapenems.[8,9] These strains also have a tendency to carry other antibiotic-resistant genes, resulting in multiple drug resistance.[1,10,11] Although glycopeptides, such as vancomycin, and newer antimicrobials with purported activity against multidrug-resistant MRSA are available for clinical use, patient outcomes have been less than favorable with these agents.[12] Furthermore, the increasing use of vancomycin has contributed to the emergence of vancomycin-resistant enterococci[13] and, more recently, vancomycin-resistant staphylococci.[14] The MRSA epidemic has, until lately, been largely confined to the hospital setting. However, the epidemiology has now expanded into the community, with a number of community-acquired infections caused by newly emerging virulent clones of MRSA being recently described.[7] Nevertheless, MRSA remains a serious hospital infection control problem, whereby strict measures and surveillance activities are required to prevent the intrainstitutional and interinstitutional spread of these strains from colonized or infected patients.[15] To facilitate the institution of appropriate antimicrobial chemotherapy and/or infection control measures for MRSA, rapid and accurate laboratory diagnostic methods are critical. Laboratories have traditionally relied on phenotypic methods, particularly the standard oxacillin salt agar screen recommended by the National Committee for Clinical Laboratory Standards (NCCLS)[8] and also *mecA* gene product latex agglutination tests,[9,16] for the detection of methicillin resistance in *S. aureus*. However, *mecA* expression—and hence methicillin resistance—is variable, being influenced by a complex interplay between *mecA* and a number of other genes as well as various environmental and in vitro factors.[10,16,17] Phenotypic detection may also be confounded by the common occurrence of heterogeneous MRSA strains composed of susceptible and resistant subclones.[10,18] Additionally, strains of *S. aureus* lacking the *mecA* gene may occasionally demonstrate low-level, nonclinically significant, phenotypic resistance to methicillin because of other mechanisms, but are not considered to be true MRSA.[9,10,16] Furthermore, some of these phenotypic tests may be labor-intensive and slow (turnaround time >24 hr). Hence an increasing number of laboratories are resorting to the use of more widely available molecular methods, either alone or in combination with these phenotypic assays, for detecting MRSA.

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Molecular detection of *mecA* is now considered the gold standard method for MRSA diagnosis,[16] and a number of genotypic assays based on polymerase chain reaction (PCR) and real-time PCR platforms have been recently described, which allow for easy, rapid (2–5 hr), and precise detection of the *mecA* gene in clinical isolates of *S. aureus*.[19–22]

MICROBIOLOGICAL DESCRIPTION

S. aureus and other members of the genus *Staphylococcus* are gram-positive, catalase-positive, predominantly facultatively anaerobic, nonmotile, nonspore-forming, spherical-shaped bacteria, approximately 0.5–1.0 μm in diameter, which typically form irregular ''grapelike'' clusters and/or occasional configurations of single cells, pairs, tetrads, and short chains, and are differentiated from other closely related genera by their cell wall teichoic acids, susceptibility to lysostaphin, intrinsic bacitracin resistance, lack of cytochrome *c* (as determined by the modified oxidase test), and relatively low guanine plus cytosine DNA base composition.[23] Approximately 35 species of staphylococci have been described to date. Based on the production of the enzyme and virulence factor coagulase (encoded by the *coa* gene), the staphylococci are classified as either coagulase-positive (*S. aureus*, and the veterinary pathogens *Staphylococcus schleiferi* subspecies *coagulans*, *Staphylococcus lutrae*, *Staphylococcus delphini*, *Staphylococcus intermedius*, and *Staphylococcus hyicus*) or coagulase-negative.[23] A positive tube (free) coagulase test serves as the basis for presumptive identification of *S. aureus* because the other coagulase-positive staphylococcal species are almost never encountered in human infections. However, rare strains of *S. aureus* may lack the *coa* gene and/or fail to produce coagulase.[23] Heat-stable nuclease (encoded by the *nuc* gene) is another virulence factor produced almost exclusively by the coagulase-positive staphylococci and serves as the basis for the DNase test.[23] The organism is also characterized by the presence of protein A on its cell surface, allowing for rapid, species-specific identification through the use of IgG-coated latex bead-based agglutination kits. *S. aureus* is a very hardy organism that grows well on most microbiological media and under a variety of incubation conditions. The morphology of *S. aureus* on solid agar media is somewhat distinctive; after 24 hr of growth, colonies are approximately 3–5 mm in diameter, creamy yellow to orange in color, smooth, and slightly raised. Strains frequently demonstrate beta-hemolysis on blood-containing agar media. Sequencing of the 16S rRNA gene can be used for definitive speciation of *S. aureus*. However, colonial features, biochemical tests, and 16S rRNA profiles do not differentiate MRSA from methicillin-sensitive *S. aureus* (MSSA).

CLINICAL SIGNIFICANCE

Natural Habitat and Reservoir

S. aureus is widely distributed in the environment, although it may also be found as part of the normal bacterial flora of humans or other mammals. Strains may colonize various anatomic sites including the skin, mucous membranes, and the upper respiratory, gastrointestinal, and genitourinary tracts.[24] Nevertheless, *S. aureus* has a particular predilection for colonizing the anterior nares of humans.[15,24] A constellation of host factors is believed to play a role in the ability of an individual to asymptomatically carry this organism. It is estimated that 20% of humans are persistent *S. aureus* carriers, 60% are intermittent carriers, whereas the remaining 20% are noncarriers.[24] Carriage of MRSA has similar anatomic predilections but occurs less commonly, being more prominent in hospitalized patients and health-care workers.[15,24] Some patients who become colonized with MRSA eventually develop an MRSA infection. MRSA are easily spread from a colonized patient or health-care worker to other hospitalized individuals, usually via unwashed hands.[15,24] Airborne transmission may be important in the development of nasal carriage.[24]

Spectrum of Infection

S. aureus is characterized by an ability to harbor and express an impressive armamentarium of virulence factors, leading to a wide spectrum of infectious and toxin-mediated illnesses. Diseases caused by MSSA and MRSA have ranged from relatively mild skin and soft-tissue infections (cellulitis, folliculitis, furunculosis, and impetigo) and urinary tract infections to more severe conditions such as pneumonia, empyema, intra-abdominal infection, deep tissue infection, osteomyelitis, septic arthritis, device-related infections, septicemia, endocarditis, meningitis, brain abscess, and toxigenic states such as toxic shock syndrome, scalded skin syndrome, and food poisoning, leading to significant morbidity and/or mortality.[2] However, most individuals with MSSA/MRSA are colonized asymptomatically.

Therapy of Infections

Vancomycin is the first-line antibiotic used for the treatment of infections caused by MRSA. Newer agents with in vitro and in vivo efficacy against MRSA include

Table 1 Oligonucleotides used for conventional multiplex PCR

Target gene (GenBank accession no.)	Nucleotide sequence (5′ to 3′) of primer (F=forward, R=reverse)	Amplicon size (bp)	Source
mecA (X52593)	F primer: TGG CTA TCG TGT CAC AAT CG R primer: CTG GAA CTT GTT GAG CAG AG	304	[19]
nuc (V01281)	F primer: GCG ATT GAT GGT GAT ACG GTT R primer: AGC CAA GCC TTG ACG AAC TAA AGC	279	[25]
femA (X17688)	F primer: CTT ACT TAC TGC TGT ACC TG R primer: ATC TCG CTT GTT ATG TGC	685	Modified from Ref. [19]

linezolid (an oxazolidinone), quinupristin-dalfopristin (a streptogramin A/B combination), daptomycin (a lipopeptide), and the topical antibiotics mupirocin and fusidic acid.[4,22] Some strains, particularly those acquired in the community, commonly demonstrate sensitivity to several other non-β-lactam antistaphylococcal agents, including clindamycin, trimethoprim–sulfamethoxazole, fluoroquinolones, macrolides, and aminoglycosides.[1,2,6,18]

MOLECULAR CHARACTERIZATION

Methicillin resistance in *S. aureus* and other staphylococci is caused by the production of penicillin-binding protein 2a (PBP2a, also known as PBP2′), a novel 78-kilo Dalton penicillin-binding protein encoded by the *mecA* gene.[10] PBP2a, along with PBPs normally produced by *S. aureus*, are enzymes that catalyze peptidoglycan cross-linking reactions in the bacterial cell wall, resulting in a rigid structure that protects the bacterium against osmotic cell lysis and death.[10] However, PBP2a is distinct from putative *S. aureus* PBPs in that it has a low binding affinity for β-lactam antibiotics, resulting in continued cell wall synthesis, albeit not as efficiently, at β-lactam concentrations that would otherwise be lethal.[10] Nevertheless, *mecA* expression—and hence PBP2a production—is variable, being regulated by at least 20 additional genes of known or unknown function.[10,17,18] Two particularly important regulatory genes are located adjacent to the *mecA* locus, namely, *mecR1* (encoding a membrane-bound signal transducer/inducer protein triggered by the presence of β-lactam antibiotic) and *mecI* (encoding a *mecA* transcription repressor protein), which make up the *mec* regulatory gene system, and, together with *mecA*, form the *mec* gene complex (of which there are two configurations in MRSA, designated type A or B).[18] These complexes, in turn, are located on a recently described large (21–67 kb) mobile genetic element, termed the staphylococcal cassette chromosome *mec* (SCC*mec*), which, via its cassette chromosome recombinase A and B (*ccrA* and *ccrB*) genes, integrates into the *S. aureus* chromosome at a specific location near the origin of replication.[7,18] These two *ccr* genes form part of a *ccr* gene complex, of which there are three types.[7,18] There are five types of SCC*mec* element, based on their overall genetic composition, including the type of *mec* and *ccr* gene complex they carry.[7,18] These elements function as antibiotic resistance islands and are responsible for the horizontal transmission of resistance to methicillin and other non-β-lactam anti-

Table 2 Oligonucleotides used for molecular beacon probe-based real-time multiplex PCR

Target gene (GenBank accession no.)	Nucleotide sequence (5′ to 3′) of primer or probe[a]	Amplicon size (bp)	Source
mecA (X52593)	F primer: GAT GCT AAA GTT CAA AAG AG R primer: AAG GTG TGC TTA CAA GTG Probe: [6-FAM] AGA TGC **CTA TCC ACC CTC AAA CAG** CAT CT (Dabcyl or Black Hole Quencher™-1)	109	Modified from Ref. [22]
nuc (V01281)	F primer: ATG CAA AGA AAA TTG AAG TC R primer: GTT TAC CAT TTT TCC ATC AG Probe: [Cy5] TGC TGC **TCA AAG AAC TGA TAA ATA TGG ACG** GCA GCA (Dabcyl or Black Hole Quencher™-2)	98	Modified from Ref. [22]

[a]Underlined nucleotides form part of molecular beacon probe stem; those in bold form the hybridizing probe sequence.

biotics.[7] Types IV and V SCC*mec* elements are the smallest and most transmissible types, being prominent in community-acquired infections and typified by the lack of resistance factors to non-β-lactam antibiotics.[18] Because SCC*mec* elements are also found in methicillin-resistant coagulase-negative staphylococci, molecular assays for the detection of *mecA* or SCC*mec* alone are not diagnostic for MRSA unless a *S. aureus*-specific gene marker is included. A number of published protocols for the molecular detection of MRSA have been based on PCR and, more recently, real-time PCR platforms involving the detection of *mecA* and a *S. aureus*-specific gene marker such as *femA*, *femB*, or *nuc*.[19–22] The *fem* factors are normal chromosomal genes found in all *S. aureus*, mediating critical steps in peptidoglycan synthesis and being necessary for the expression of *mecA* in MRSA.

MOLECULAR TESTING

Conventional or real-time multiplex PCR methods may be used to detect MRSA. In the case of real-time PCR, a number of fluorescent-based technologies such as FRET,[20] TaqMan®,[21] and molecular beacon[22] probes have been used in this regard. This section will briefly outline methods for conventional and molecular beacon probe-based real-time PCR assays for MRSA detection.

Template DNA Preparation

Laboratory cultures

Isolates should be pure and phenotypically identified as *S. aureus* prior to testing. Control strains of MRSA (ATCC 33591), MSSA (ATCC 25923), and *mecA*-negative *Staphylococcus epidermidis* (ATCC 14990), in addition to a no-template (water) control, should be included with each run. Prepare a 1.0 McFarland standard suspension of the organism in 0.85% NaCl. Extract DNA using either commercial nucleic acid extraction kits or Chelex-100 resin. For the latter, Chelex-100 is added to 800 μL of bacterial suspension to a 1.5-mL final volume, followed by vortexing, heating to 80°C for 10 min, and centrifugation for 15 min at $16{,}000 \times g$, resulting in a supernatant containing template DNA.

Clinical specimens

Preferred specimens include nasal swabs or blood from positive blood cultures. For the latter, a single morphotype of gram-positive cocci in clusters resembling staphylococci should be confirmed prior to testing. Obtain a 0.5-mL aliquot of blood and centrifuge for 2 min at $900 \times g$ to remove particulate matter (e.g., charcoal). Resuspend the pellet in 0.85% NaCl and repeat centrifugation. Perform another centrifugation step at $12{,}000 \times g$ for 15 min to pellet down the bacteria. Resuspend the pellet in 0.85% NaCl to make a 1.0 McFarland standard suspension, then follow the subsequent directions for template extraction as outlined in ''Laboratory Cultures'' above. For nasal swabs, the use of commercial DNA extraction kits is recommended. Control strains should be included as for laboratory cultures.

PCR Primers and Probes

Tables 1 and 2 outline sets of primers and probes that may be used for conventional and real-time multiplex PCR assays, respectively.

Preparation of PCR Mixture

Conventional PCR: Prepare 25-μL reactions similar to that described previously,[26] but use 2 μL of supernatant containing template DNA and include the *femA* primer set.

Real-time PCR: Prepare 50-μL reactions as per the method of Elsayed et al.[22]

Amplification and Detection of Target DNA

Conventional PCR: PCR products may be separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet (UV) light.

Real-time PCR: The protocol of Elsayed et al.[22] may be used. Collect fluorescence data during the annealing stage of amplification. The presence of a specific amplicon is indicated by a probe-generated fluorescence signal exceeding the instrument-calculated threshold level.

CONCLUSION

Molecular detection of MRSA has greatly improved our understanding of the epidemiology of this resistant microbe. Further developments in molecular methods, heralded by the recent sequencing of the entire genome of a strain of MRSA, in addition to the emergence of DNA microarray technology out of the research setting, may lead to new and exciting diagnostic and therapeutic advances.

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Staphylococcus aureus—Toxin Detection

Karsten Becker
Westfälische Wilhelms-Universität Münster, Münster, Germany

INTRODUCTION

Staphylococcus aureus is an extraordinarily versatile pathogen, colonizing distinct ecological niches and causing a wide spectrum of mild to severe, life-threatening infections in humans as well as economically important infections in animals. In addition to superficial lesions and systemic infections, *S. aureus* is responsible for toxin-mediated diseases, such as toxic shock syndrome (TSS), staphylococcal food poisoning (SFP), and staphylococcal scalded-skin syndrome (SSSS). The virulence factors causing toxicosis are members of the family of bacterial pyrogenic toxin superantigens (PTSAgs) comprising the TSS-causing toxic shock syndrome toxins (TSST) and the staphylococcal enterotoxins (SEs) producing the food-borne illness. In addition, PTSAgs have been strongly implicated in other acute (e.g., Kawasaki syndrome) and chronic diseases, such as atopic dermatitis. Furthermore, the exfoliative toxins (ETs)—also known as epidermolytic toxins—cause SSSS (also known as pemphigus neonatorum and ''Ritter's disease'').

THE ''SUPERFAMILY'' OF PYROGENIC TOXIN SUPERANTIGENS AND THE EXFOLIATIVE TOXINS

The PTSAgs of *S. aureus* belong to a ''superfamily'' of true exotoxins sharing common phylogenetic relationships, sequence homology, structure, and function.[5] Because they are bivalent molecules that bind two distinct molecules, they are able to stimulate T-lymphocyte proliferation in a non-antigen-specific manner by recognizing T-cell antigen receptor (TCR) Vβ and interacting with invariant regions on class II major histocompatibility complex (MHC) products on the surface of antigen-presenting cells (Fig. 1). Thus, by bypassing intracellular processing, they are the most potent activators of T lymphocytes stimulating, at nano- to picogram concentrations, up to 30% of T cells (only 1 in 10^5–10^6 T cells is activated upon conventional antigenic peptide presentation to the TCR); hence they are termed ''superantigens.'' This excessive polyclonal activation results in a massive release of proinflammatory cytokines (Fig. 1).[1,2] In addition, staphylococcal PTSAgs have several other important properties contributing to their role in disease. They have mitogenic activity and are able to induce high fevers, stimulate neutrophil recruitment to a site of infection resulting in local inflammation, and enhance host susceptibility to endotoxin shock 100,000- to 1 million-fold.

In contrast to the TSST-1 and SEs, the superantigenic activity of ETs has been debated for years. Whereas the mass of the ETs is similar to that of the staphylococcal PTSAgs, the degree of primary sequence and structural homology is poor.

Staphylococcal Enterotoxins

In addition to their nature as superantigens, SEs function as potent gastrointestinal toxins causing SFP, which has a major public health impact.[1,3–5] Superantigenicity and emesis activities of SEs have been shown to result from distinct regions of the toxin protein. SEs comprise a family of homologous but antigenically different exotoxins, which are heat stable and resistant to inactivation by gastrointestinal proteases. SEs have also been implicated in several allergic and autoimmune diseases. The repertoire of SEs includes the five classical enterotoxins SEA through SEE and the more recently identified enterotoxins starting with SEG (Table 1). To date, the ''alphabet'' of SEs and their coding genes has reached the letter ''Q.'' However, some of the recently described SEs were shown to be nonemetic, thus actually lacking the defining property of SEs. Nevertheless, the standard convention hitherto has been to refer to proteins exhibiting sequence similarity to SEs as enterotoxins.

For some SEs, slightly divergent sequences and minor epitope differences are known, resulting in their further subdivision.[5] SEC comprises several unique molecular variants named SEC_1, SEC_2, and SEC_3 as well as variants produced by animal-adapted strains of *S. aureus* and *S. intermedius*, such as ovine (SEC_{ovine}), bovine (SEC_{bovine}), and canine (SEC_{canine}) types. In addition, variants of SEG are known.

Different *S. aureus* strains harbor different combinations of PTSAgs.[6] The majority of the SE-encoding genes are located on mobile genetic elements [plasmids,

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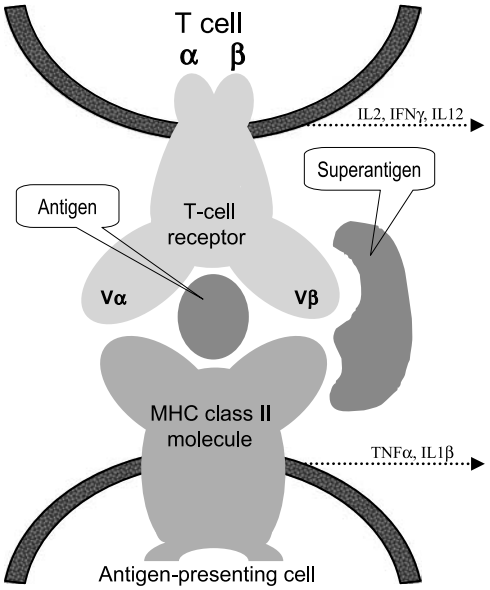

Fig. 1 Schematic representation of the different interactions of antigens and superantigens with the MHC class II molecule and the T-cell receptor.

prophages, and pathogenicity islands, e.g., SaPI1 (prototype), SaPI3, SaPI4, SaPIbov] except for the genes that encode SEG, SEH, and SEI, which are chromosomal. Analyzing the *seg–sei* intergenic and flanking regions, Jarraud et al.[7] found a highly prevalent operon encoding—in addition to SEG and SEI—further enterotoxins, designated SEK, SEL, and SEM. SEK and SEL were renamed subsequently as SEN and SEO, respectively. Phylogenetic analysis of all hitherto known SE genes indicates that they all potentially derived from this operon (named the enterotoxin gene cluster, *egc*), inferring that *egc* is in an SE gene nursery. Constructing phylogenetic trees from nucleic acid and amino acid sequences, respectively, three monophyletic groups were identified by Jarraud et al.: one composed of *sea*, *sed*, *see*, *seh*, *sej*, *sen*, and *seo*; another including *seb*, *sec*, and *seg*; and a third comprising *sei* and *sem*.[7]

Toxic Shock Syndrome Toxins

In 1981, Bergdoll et al. identified enterotoxin F, an exotoxin that caused TSS.[8] However, it was renamed toxic shock syndrome toxin-1 (TSST-1) because of its lack of emetic activity (Table 1). Despite its similar biological activities and three-dimensional structure, TSST-1 is generally considered to be different from other PTSAgs, forming its own group comprising—in addition to the classical TSST-1—a minor variant, $TSST_{ovine}$. Compared to other staphylococcal PTSAgs, TSST-1 is shorter and has no cysteines. Unlike the SEs, TSST-1 is able to cross the mucosa.

Exfoliative Toxins

Two classical, immunologically different ETs of *S. aureus* are recognized, ETA, which is chromosomally encoded and heat stable, and ETB, which is carried on a β-lactamase plasmid and is heat inactivated.[9] A serologically different heat-labile ET isolated from a horse strain of *S. aureus* was termed ETC. Recently, ETD, a novel ET integrated on a pathogenicity island, has been published.[10] Other SEs causing exfoliation in piglets were named SHETA and SHETB and have been purified from culture supernatants of *Staphylococcus hyicus* (Table 1).

Superantigenicity of ETs has been a subject of debate for many years. X-ray crystallographic structures of ETs suggest that they are members of the trypsinlike serine protease family. Although protease activity has not been demonstrated in vitro, ETA and ETB have intrinsic esterase activity, which is associated with serine proteases. Recently, desmoglein 1 has been identified as putative target protein for ETA.[11] Whereas some earlier studies describe ETs as mitogens (e.g., because of contamination of the toxin preparation with hitherto unknown superantigens), others fail to demonstrate superantigenic activity. A recent study with recombinant ETA and ETB demonstrates that these recombinant toxins do not act as superantigens.[12]

LABORATORY DETECTION OF EXOTOXINS

Phenotypic Detection Methods

Conventional methods for the detection of PTSAg-producing *S. aureus* strains are based on biological or immunological procedures detecting or measuring the toxin in culture supernatants of suspected *S. aureus* strains in contaminated food extracts or in patient specimens.

Biological methods using animals and human volunteers were the first approaches to determine the production of SEs. Monkeys have been considered to be the primary animal model to study the emetic activity of SEs. Investigating the emetic activity in biological assays is the most important basis to characterize a candidate exotoxin as yet unknown SE.

Since antigen–antibody reactions became available, the adaptation of immunodiffusion assays (e.g., Ouchterlony technique) for detection of toxin proteins have radically changed the diagnostics of PTSAgs. Further increase of sensitivity by about 100-fold because of the introduction of reversed passive latex agglutination, radioimmunoassay (RIA), and enzyme immunoassay (EIA) methods made it possible to detect as little as 1 ng/mL of toxin. Because biological assays are less specific and sensitive and restricted by high costs, availability of the experimental animals, and ethical considerations, immunological

Table 1 Staphylococcal pyrogenic toxin superantigens and exfoliative toxins

Toxin	Encoding toxin gene	Gene size (bp)	Gene localization	Molecular weight (Da)	Emetic activity
1. Enterotoxins					
SEA	*sea* [*entA*]	771	Prophage	27,078	Yes
SEB	*seb* [*entB*]	801	Pathogenicity island; plasmid (occasionally)	28,336	Yes
SEC family					
SEC_1	sec_1 [$entC_1$]	801	Pathogenicity island; plasmid (occasionally)	27,496	Yes
SEC_2	sec_2 [$entC_2$]	801	N.D.	27,531	Yes
SEC_3	sec_2 [$entC_3$]	801	Pathogenicity island	27,563	Yes
SEC_{bovine}	sec_{bovine}	816	Pathogenicity island	~27,000	N.D.
SEC_{canine}	sec_{canine}	720	N.D.	~27,600	Yes
SEC_{ovine}	sec_{ovine}	720	N.D.	~27,000	N.D.
SED	*sed* [*entD*]	774	27.6-kbp plasmid	26,360	Yes
SEE	*see* [*entE*]	771	Phage (?)	26,425	Yes
SEG family					
SEG	*seg*	777	Chromosomal (*egc* operon)	27,043	Yes
SEGv	seg_v	777	Chromosomal	26,985	N.D.
SEG_{L29P}	seg_{L29P}	777	N.D.	N.D.	N.D.
SEH	*seh*	654	Chromosomal	25,210	Yes
SEI	*sei*	729	Chromosomal (*egc* operon)	24,928	Reduced
SEJ	*sej*	804	27.6-kbp plasmid	28,460	N.D.
SEK	*sek*	776	Chromosomal on pathogenicity islands	~26,000	Lacking
SEL	*sel*	782	Chromosomal on pathogenicity islands	~26,000	Lacking
SEM	*sem*	719	Chromosomal (*egc* operon)	N.D.	N.D.
SEN	*sen*	777	Chromosomal (*egc* operon)	N.D.	N.D.
SEO	*seo*	786	Chromosomal (*egc* operon)	N.D.	N.D.
SEP	*sep*	783	N.D.	N.D.	N.D.
SEQ	*seq*	729	Pathogenicity island	26,000	Lacking
2. Toxic shock syndrome toxins					
TSST family					
TSST-1 [SEF]	*tstH* [*tst*]	705	Pathogenicity islands	~22,000	Lacking
$TSST_{ovine}$	tst_{ovine}	N.D.	Pathogenicity island (?)	~24,000	Lacking
3. Exfoliative toxins					
ETA	*eta*	840	Chromosomal	26,950	—
ETB	*etb*	831	Plasmid	27,274	—
ETC	*etc*	836	N.D.	~27,000	—
ETD	*etd*	845	Pathogenicity island	27,200	—
SHETA	*sheta*	920	Chromosomal	~27,000	—
SHETB	*shetb*	804	Plasmid	~27,000	—

N.D., no data.

methods, which are commercially available as diagnostic kits, are the basis for phenotypic detection of the classical PTSAgs.

However, conventional methods are relatively time- and labor-consuming. Nonspecific reactions due to cross-reactivity between PTSAgs and contaminations by nonstaphylococcal bacteria do occur. Furthermore, phenotypic assays depend on the concentration of PTSAgs expressed and thus can be negatively influenced by various factors (e.g., catabolite concentration, temperature, pH). Also, differences in the toxin production levels by strains grown on natural substrates and laboratory media have been described.

Regarding generalized SSSS, the superficial blisters are only very rarely culture-positive, but ET-positive strains are usually grown from specimens obtained from the suspected site of infection (e.g., umbilicus, breast, surgical wound). A number of different immunological

Table 2 Publications with description of specific sequences of synthetic oligonucleotides used in molecular methods to detect staphylococcal genes encoding TSST, enterotoxins, and exfoliative toxins

		Molecular target: staphylococcal genes encoding[a]				
Reference	**Detection method**	**TSST**	**Enterotoxins**	**Exfoliative toxins**	**Additional structures[b]**	**Applied for**
Rifai et al.[17]	Dot blot hybridization	N.D.	N.D.	*eta*, *etb*	N.D.	Culture
Neill et al.[18]	Colony blot hybridization	*tst*	*sea–sec*	N.D.	N.D.	Culture
Johnson et al.[15]	PCR	*tst*	*sea–see*	*eta*, *etb*	N.D.	Culture
Wilson et al.[19]	Nested PCR	N.D.	*sea*, *seb*	N.D.	*nuc*	Milk[c]
Sakurai et al.[20]	PCR	N.D.	N.D.	*eta*, *etb*	N.D.	Culture
Becker et al.[21]	Multiplex PCR with DNA-EIA	*tst*	*sea–see*	*eta*, *etb*	N.D.	Culture
Monday and Bohach[22]	Multiplex PCR	*tst*	*sea–see*, *seg–sej*	N.D.	16S rDNA	Culture
Gilligan et al.[23]	PCR-EIA	N.D.	*sea*, *seb*	N.D.	N.D.	Culture
McLauchlin et al.[24]	PCR	*tst*	*sea–see*, *seg–sei*	N.D.	N.D.	Culture, (spiked) food samples
Mehrotra et al.[25]	Multiplex PCR	*tst*	*sea–see*	*eta*, *etb*	*femA*, *mecA*[b]	Culture
Sharma et al.[26]	Single-reaction multiplex PCR	N.D.	*sea–see*	N.D.	N.D.	Culture
Omoe et al.[27]	Multiplex PCR	N.D.	*sea–see*, *seg–sei*	N.D.	N.D.	Culture
Rosec and Gigaud[28]	PCR with restriction analysis	N.D.	*sea–see*, *seg–sej*	N.D.	N.D.	Culture
Becker et al.[6]	Multiplex PCR with DNA-EIA	*tst*	*sea–see*, *seg–sej*	*eta*, *etb*	N.D.	Culture

[a]N.D., not done.
[b]Genes not noted elsewhere: *nuc*, thermonuclease (TNase) gene; *femA*, encoding factor A, which is essential for methicillin resistance; *mecA*, encoding penicillin-binding protein 2a.
[c]Dried skimmed milk artificially contaminated with enterotoxigenic *S. aureus* cells.

techniques have been developed to determine ET production by *S. aureus* recovered from patients with suspected SSSS. Although these methods have been reported to be specific and sensitive for detection of ETs, they rely on isolating putative toxinogenic *S. aureus* strains from the patient. A recently developed EIA based on $F(ab')_2$ fragments was able to detect ETA directly from serum with high sensitivity.[13]

Nucleic Acid-Based Detection Methods for PTSAgs

Hybridization methods

Since 1986, protein sequences have been established for PTSAgs and ETs as well as nucleotide sequences for the corresponding genes (Table 1). Early molecular approaches for the detection of these toxin genes have been developed that use DNA–DNA hybridization techniques based on gene fragments or oligonucleotide probes (enzyme-, hapten-, radiolabeled). For detection of toxigenic staphylococci, e.g., colony hybridization and single-phase liquid hybridization assays were used. The hybridization techniques have never gained much use for direct detection of PTSAg-bearing staphylococci in patient specimens or in food, because their sensitivity and specificity was too low.

Nucleic acid amplification methods

The advent of DNA amplification-based methods, in particular the development of the polymerase chain reaction (PCR), provides a new, highly specific and sensitive tool for the detection of the PTSAg-encoding genes.[14–16] In addition, the PCR technique allows the detection of toxin genes with little sample preparation in a relatively short period, most recently further shortened by the application of real-time PCR methods. The detection of SE- and TSST-encoding sequences by molecular methods has been reported by several investigators, mainly in DNA extracts derived from bacterial cultures and, to a lesser extent, directly in foods or patient specimens (Table 2). The development of multiplex PCR

systems allows the simultaneous detection of PTSAg genes for diagnostic and epidemiological purposes.

Nucleic acid-based methods are hampered by their inability to distinguish nonviable cells from viable ones because DNA from dead cells would also be amplified and detected. This may be of special importance for testing food samples, where enterotoxigenic staphylococci that have been killed by heat or other processing would yield a positive response. However, positive PCR results only indicate the presence of PTSAg genes in extracted DNA. They do not prove that production of toxin proteins occurs by a given isolate or indicate the presence of such proteins in samples. On the other hand, contamination of food products restricted on preformed SE proteins is not detectable by PCR but causative for SFP. In addition, false negative PCR results have been reported because of the presence of inhibitors of the DNA polymerases, which have been found to exist in food matrices.

Oligonucleotide arrays

Although oligonucleotide arrays (the so-called ''DNA chips'') have increasingly been used over the last few years to analyze DNA sequences as well as gene expression based on cDNA of mRNA for a number of microorganisms, applications to detect specifically staphylococcal toxins in medical specimens or food are still unavailable. Nevertheless, the potential is great because in contrast to traditional DNA detection methods restricted to target only a single or a few genes at a time at best, microarrays are able to integrate thousands of probes into the same chip-based hybridization procedure. This enables not only the detection of all staphylococcal toxins simultaneously, but also of other genes of specific interest, e.g., taxonomic marker genes, antibiotic resistance genes, and target structures for staphylococcal subtyping. However, this technique faces some of the same problems that have met traditional DNA hybridization, especially to get enough sensitivity to detect the target genes in specimens.

CONCLUSION

Detection of the genes involved in staphylococcal toxin-mediated diseases constitutes a challenge because the toxin-bearing bacteria are often present in low numbers and masked by other components of the specimen. Traditional phenotypic immunological and biological methods are time- and labor-consuming and often hampered by low sensitivity and specificity. DNA-based methods, especially amplification techniques, provide highly specific and sensitive techniques that allow the detection of staphylococcal target genes in a short period with little sample preparation. The ''all-in-one'' tool as offered by the microarray technology may further enhance our diagnostic options.

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Streptococcus pneumoniae

David R. Murdoch
Christchurch School of Medicine & Health Sciences, Christchurch, New Zealand

INTRODUCTION

Streptococcus pneumoniae is a gram-positive bacterium that typically grows as lancet-shaped pairs of diplococci. All pathogenic strains are surrounded by a polysaccharide capsule that allows the bacterium to evade phagocytosis, thereby delaying clearance.

There are more than 90 capsular serotypes of *S. pneumoniae* and the distribution of each serotype varies with age and geography. Nasopharyngeal carriage of *S. pneumoniae* is common, especially in young children where rates of 40–50% are frequently observed. Carriage occurs in approximately 10–30% of adults at any one time.

S. pneumoniae is the most common cause of community-acquired pneumonia, otitis media, and sinusitis. It is also an important cause of primary bacteremia and meningitis. Less common infections with *S. pneumoniae* include endocarditis, peritonitis, and osteomyelitis.

OVERVIEW

Laboratory diagnosis of *S. pneumoniae* infection relies on culture of the bacterium from relevant clinical samples.[1] Typically, *S. pneumoniae* colonies have an umbilicated appearance surrounded by a zone of α-hemolysis when grown on blood agar. Identification of *S. pneumoniae* isolates is usually based on the demonstration of capsule formation, bile solubility, and/or susceptibility to optochin. Blood and other samples from normally sterile sites are the samples of choice for culture. Sputum culture is complicated by contamination with nasopharyngeal colonizing bacteria. Recent developments in pneumococcal infection diagnostics include a new immunochromatographic urinary antigen test that is sensitive for diagnosing pneumococcal pneumonia in adults.[2]

β-Lactams are the antibiotics of choice for treating *S. pneumoniae* infections. However, the global emergence of penicillin-resistant strains over the past 20 years has restricted therapeutic options. Extended spectrum cephalosporins (e.g., ceftriaxone) have been successfully used to treat serious infections with penicillin-resistant pneumococci, but resistance to this class of antibiotic is also increasing. Resistance to other classes of antibiotics (macrolides, tetracycline, cotrimoxazole, fluoroquinolones, and chloramphenicol) has also emerged to significant levels, and many *S. pneumoniae* strains are now exhibiting resistance to multiple antibiotic classes.

BODY OF TEXT

There are several clinical applications of genomics-based technologies to *S. pneumoniae*. Genotypic tests may assist with the identification and typing of *S. pneumoniae* isolates cultured from clinical samples. Gene amplification methods have been developed to rapidly detect *S. pneumoniae* directly from clinical samples, thereby obviating the need to rely on culturing the organism. Detection of antimicrobial resistance genes can provide valuable clinical and epidemiological information. Recent publication of the complete genome sequence of *S. pneumoniae* has facilitated the use of genomics-based technology to identify important genes of this bacterium, including the identification of potential new targets for antimicrobial drugs. The clinical applications of proteomic technologies in medical microbiology are still being realized, although proteomics is likely to have a future role in the investigation of the epidemiology, taxonomy, and antimicrobial resistance of human pathogens.

Use of Genotypic Tests to Identify and Type *S. pneumoniae* Isolates

Optochin susceptibility, bile solubility, and encapsulation are the phenotypic characteristics most widely used to identify *S. pneumoniae* isolates. While most clinical isolates of *S. pneumoniae* are readily identified with these phenotypic tests, the occurrence of isolates with atypical characteristics has increasingly caused problems in diagnostic laboratories.

For these reasons, there has been increased interest in genotypic techniques, such as gene amplification and DNA hybridization, for identifying *S. pneumoniae* isolates, especially those strains exhibiting atypical characteristics. The most widely studied targets for DNA amplification are the genes encoding pneumolysin (*ply*), autolysin (*lytA*), and pneumococcal surface antigen A (*psaA*). Although once thought to be specific to

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S. pneumoniae, use of these targets has been complicated by the discovery that all three genes can occasionally be found in strains of other streptococci, especially *Streptococcus mitis*.[3,4] Modification of the polymerase chain reaction (PCR) primers used to amplify the *psaA* gene has enabled more specific identification of *S. pneumoniae*,[5] and positive results by this *psaA*-specific PCR are generally in agreement with the detection of capsule formation.[6] Initial reports of the results from partial sequence analysis of the gene encoding the manganese-dependent superoxide dismutase (*sodA*) indicate that this is a reliable method for species-level identification of streptococci from the mitis group (including *S. pneumoniae*).[7]

A commercial test using DNA probe complementary to the 16S rRNA of *S. pneumoniae* (AccuProbe®, GenProbe®, San Diego, CA) is being used by an increasing number of diagnostic laboratories. Evaluations of the AccuProbe® system have recorded positive results from streptococcal isolates with discordant combinations of phenotypic characteristics.[3,8] Without an absolute gold standard, it is difficult to be certain whether these atypical isolates are *S. pneumoniae* or not, although many of these isolates appear to be organisms that are genetically divergent from typical *S. pneumoniae* strains. Restriction digestion by *Rsa*I of the amplified 16S rRNA gene of *S. pneumoniae* produced identical results to the AccuProbe® system,[6] and may be a useful alternative to the AccuProbe® for identification of atypical strains of *S. pneumoniae*.

At present, the main role for genotypic tests is to aid the identification of atypical streptococcal isolates resembling *S. pneumoniae*, as optochin-sensitive, bile-soluble α-hemolytic streptococci are almost always *S. pneumoniae*. To date, no genotypic method, alone or in combination with phenotypic tests, has been shown to reliably identify all atypical *S. pneumoniae* isolates, although the *psaA*-specific PCR and partial sequence analysis of *sodA* are probably the most discriminatory of evaluated methods.

Typing of *S. pneumoniae* Isolates

Beyond the identification of *S. pneumoniae* strains, genotypic methods are increasingly being used for epidemiological typing purposes. Capsular serotyping by immunological means has been the standard typing method for *S. pneumoniae*, but requires technical expertise and is time-consuming; furthermore, not all strains are typeable. However, serotype data will continue to be required for historical comparisons and as part of pneumococcal vaccine studies. Serotype-specific multiplex PCR assays have been developed to provide a rapid and cost-effective method of providing serotype data,[9,10] obviating the need to perform immunological testing. When serotype data is unnecessary, the most commonly used genetic fingerprinting method for *S. pneumoniae* is restriction fragment-length polymorphism combined with pulsed-field gel electrophoresis.

Use of DNA Amplification Methods to Detect *S. pneumoniae* in Clinical Samples

PCR is the DNA amplification method almost exclusively evaluated in published studies for the detection of *S. pneumoniae*. A variety of PCR assays has been used, the most common targets being *ply* and *lytA*, although none has been sufficiently standardized for introduction into routine use.

Pneumonia

The use of PCR as a tool for diagnosing pneumococcal pneumonia has been evaluated in both adults and children. Most studies of *S. pneumoniae* PCR have focused on testing blood samples (whole blood, serum, or plasma) for the purpose of detecting occult pneumococcal bacteremia. Using positive blood culture as the comparison standard, the reported sensitivities for detecting *S. pneumoniae* in blood samples have ranged from 29% to 100% in adults, and from 57% to 100% in children.[11] When the definition of pneumococcal pneumonia also includes patients with positive sputum cultures, the reported sensitivities range from 26% to 88%.[11] The reason for this variation is unclear, as similar PCR protocols were used in most studies. Positive PCR results were also recorded from control subjects in many of these studies, and these findings are not readily explained.

For the few studies that have evaluated PCR for detecting *S. pneumoniae* in respiratory samples, sputum PCR positivity rates have been high (82–100%).[11,12] However, specificity of PCR testing of sputum samples is low because of its inability to distinguish colonization from infection.[12,13] It is possible that quantitative PCR may be useful in this setting, and that infection can be distinguished from colonization by a higher bacterial burden in the former,[14] but this is yet to be systematically evaluated. PCR has also been used successfully to detect *S. pneumoniae* in invasive respiratory samples.[15]

Meningitis

The diagnosis of pneumococcal meningitis traditionally relies on the appearance of gram-positive diplococci in cerebrospinal fluid (CSF) and/or the isolation of

S. pneumoniae from CSF. Both tests can be negative if antibiotics were given before CSF was obtained. A new immunochromatographic antigen detection test is a promising rapid method.[16] Several studies using a variety of assays have evaluated PCR as an alternative method for rapidly diagnosing pneumococcal meningitis,[17–22] including as part of assays designed to detect multiple bacterial pathogens.[17,20,21] These preliminary studies show that PCR is at least as sensitive as culture, and PCR may be particularly useful in Gram stain- and culture-negative cases. As yet, a standardized PCR assay has yet to be developed. Furthermore, no direct comparisons have been made between PCR and the immunochromatographic antigen detection assay for rapidly diagnosing pneumococcal meningitis.

One advantage PCR has over other rapid diagnostic techniques for meningitis is the potential to detect antimicrobial resistance as well as the pathogen itself. The rapid detection of penicillin resistance in *S. pneumoniae* is especially important for pneumococcal meningitis, as active antibiotics need to be commenced as soon as possible. Preliminary studies of seminested and real-time PCR systems targeting the pneumococcal penicillin-binding protein (PBP) 2B gene (*pbp2B*) enabled the rapid detection in the same assay of penicillin-resistant and penicillin-susceptible strains of *S. pneumoniae* in CSF.[18,22]

Otitis Media

PCR has been used to improve the sensitivity of detection of pneumococcal middle ear infections.[23] When testing middle ear fluid from patients with otitis media with effusion, almost all culture-positive samples are PCR-positive, and many other samples are culture-negative and PCR-positive. This almost certainly reflects the greater sensitivity of PCR over culture.

Detection of Antimicrobial Resistance Genes in *S. pneumoniae*

The genetic basis of resistance in *S. pneumoniae* to major antibiotics has been well documented. Resistance to β-lactams occurs when PBPs are altered through the acquisition of chromosomal DNA from other pneumococci or from other streptococcal species (especially *S. mitis*). Pneumococcal resistance to macrolides occurs by either target site modification through methylation or by active drug efflux. Tetracycline resistance is mainly attributable to the production of cytoplasmic proteins capable of interacting with the ribosome and making it insensitive to tetracycline inhibition. Resistance to fluoroquinolones is primarily mediated by point mutations in the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), and less commonly by a drug efflux mechanism.

Diagnostic laboratories rely on phenotypic tests to determine the antimicrobial susceptibility profile of bacteria isolated from clinical samples. Increasingly, genetic tests are being used to complement phenotypic tests in the identification of antimicrobial resistance genes or mutations associated with antimicrobial resistance. The main reasons for detecting these genes are for epidemiological purposes and for the rapid identification of resistant strains directly in clinical samples; the former is usually a function of regional reference laboratories, while the latter remains restricted mainly to research laboratories. The detection and monitoring of penicillin resistance in *S. pneumoniae* has been the principal focus, as this resistance phenotype is of the greatest clinical importance. Detection of alterations in the genes encoding PBPs by PCR can provide information on penicillin resistance at least one day earlier than conventional phenotypic antimicrobial susceptibility testing.[24–27] In addition, the pattern of changes can predict whether penicillin resistance is low-level or high-level as determined by minimal inhibitory concentrations (MICs).[26] As already noted, rapid PCR assays have also been applied to the detection of penicillin-resistant *S. pneumoniae* directly in CSF samples from a small number of patients with suspected meningitis.[18] A major problem with the development of these PCR assays is the relatively divergent nature of the alterations in the genes encoding PBPs that confer penicillin resistance, particularly in the transpeptidase-encoding region. However, within this divergent region, there are alterations that are common in many penicillin-resistant pneumococcal strains,[25] and these changes are seen in pneumococcal strains from different geographic locations. Further work is needed to determine the best targets for PCR-based diagnostic assays.

CONCLUSION

S. pneumoniae remains an important human pathogen throughout the world. The emergence of antibiotic resistance (especially to β-lactams) has fuelled efforts to develop improved diagnostic tests to identify *S. pneumoniae* in clinical samples and to rapidly detect antibiotic resistance. Genetic, particularly PCR-based, tests have many attributes that make them ideally suited for this purpose. To date, there are no standardized PCR protocols for routine use in diagnostic laboratories and there are few genomics-based commercial diagnostic kits.

Future research needs to determine ideal PCR targets and sample types to overcome specificity problems when diagnosing pneumococcal infection and to accurately and rapidly detect antibiotic resistance. Quantitative PCR assays may assist in distinguishing colonization from infection with *S. pneumoniae*, and may provide prognostic information. Efforts to create robust standardized protocols will greatly assist with the introduction of these assays into routine diagnostic laboratories.

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Streptococcus, Group A

Joseph E. Alouf
Heide Müller-Alouf
Institut Pasteur, Paris, France

INTRODUCTION

Streptococcus pyogenes (group A Streptococcus in the Lancefield classification scheme of serological typing) is a bacterial pathogen strictly associated with the human species (no animal reservoir known). This Gram-positive microorganism colonizes the throat or skin and is responsible for a wide spectrum of suppurative infections and nonsuppurative sequelae. The diseases caused by *S. pyogenes* range from simple skin infections (impetigo, erysipelas) and uncomplicated tonsillopharyngitis to more severe diseases such as scarlet fever, invasive deep tissue infections (cellulitis, myositis, necrotizing fasciitis), bacteremia, puerperal sepsis, streptococcal toxic shock syndrome (STSS), and very likely Kawasaki disease. Furthermore, group A streptococci (GAS) play a major role in the development of poststreptococcal infection sequelae including acute rheumatic fever (ARF), acute glomerulonephritis, reactive arthritis, Sydenham's chorea, and the recently investigated obsessive-compulsive disorders and Tourette's syndrome.

VIRULENCE AND PATHOGENICITY FACTORS

Great progress has been achieved over the past decade in our knowledge of these factors which comprise a great number of both cell surface (somatic)-associated molecules and extracellular products, among them the family of the streptococcal superantigens.[1,2] The interaction of these factors with host's tissues contributes to the virulence and pathogenicity of GAS and thereby initiates the pathophysiological processes leading to illness.[1–6] Moreover, the immunogenetics of the host influences the outcome of the streptococcal infection.[7]

SOMATIC VIRULENCE EFFECTORS

These factors expressed at the surface of the bacteria comprise the M proteins (124 serotypes), the hyaluronic acid capsule, and fibronectin-binding proteins, and other factors (lipoteichoic acid, various proteins) which allow *S. pyogenes* to bind to, colonize, and invade human mucus tissues and skin.[1,2] M proteins are the major virulence factors of the microorganism and act as antiphagocytic effectors by interfering with opsonization and phagocytosis by polymophonuclear leucocytes via the alternate complement pathway.[1] Strains of certain GAS M-protein types are more often associated with particular severe clinical syndromes. For example, during the past two decades, strains of M1 and M3 types have been frequently isolated from patients with severe invasive diseases associated with increased mortality in North America and Europe.[1,4,8] The resurgence of ARF in the 1980s in the United States was strongly associated with type M18 strains.[1]

THE STREPTOCOCCAL GENOME

The advent of recombinant DNA technology and recent deciphering of the genomes of *S. pyogenes* serotypes M1,[9] M3,[10] and M18[11] have taught much about the organism's virulence and pathogenicity mediated by both cell-associated and extracellular products. They have also allowed the discovery of a number of streptococcal superantigens.

The sizes of these three genomes are ca. 1.85, 1.90, 1.89 bp, respectively, and open reading frames (ORF) range between 1752 and 1895. Approximately, 1500 ORF are conserved between the three serotypes. Four to six bacteriophage genomes were identified per bacterial chromosome confirming that many cell-associated and extracellular factors are encoded by phage genome.

EXTRACELLULAR PRODUCTS

Group A streptococci release more than 25 extracellular proteins (not all of which have been well characterized) in culture media and in the infected host as reflected by immunoelectrophoretic analysis of concentrated culture supernates against concentrated human γ-globulins[12] and by assay of specific antibodies against streptococcal proteins in human sera.[4,8,10,16] The secreted proteins comprise the membrane-damaging (cytolytic) toxins

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streptolysin O and streptolysin S, NAD-glycohydrolase, cysteine proteinase, DNAses, other hydrolytic enzymes, and 11 exotoxins of the family of the bacterial superantigens (SAgs). The molecular and immunological aspects of these SAgs and their implications in the pathogenesis of severe streptococcal diseases, particularly toxic shock syndrome (STSS), are thereafter described.

WHAT ARE SUPERANTIGENS?

Superantigens include a class of certain bacterial and viral proteins exhibiting highly potent lymphocyte-transforming (mitogenic) activity toward human and or other mammalian T lymphocytes.[13,14] Unlike conventional antigens, SAgs bind to certain regions of major histocompatibility complex (MHC) class II molecules of antigen-presenting cells (APCs) outside the classical antigen-binding groove. They simultaneously bind under their native form to T lymphocytes bearing certain specific motifs in the variable region of the β chain (Vβ) of the T-cell receptor (TcR). The human T-cell repertoire comprises about 24 major types of Vβ elements.[15] Each SAg interacts specifically with a characteristic set of Vβ motifs. As a consequence, SAgs stimulate at nano- to picogram concentrations up to 30% of T cells, whereas only 1 in 10^5–10^6 T cells are activated upon conventional antigenic peptide presentation to TcR in the immune response.

REPERTOIRE AND BIOCHEMISTRY OF *S. PYOGENES* SUPERANTIGENS

The repertoire of *S. pyogenes* SAgs includes 11 structurally and functionally related, single-chain proteins with molecular weights ranging from ca. 23 to 27 kDa.[14] Most of these proteins have been purified to homogeneity. The repertoire comprises: 1) the classical erythrogenic (scarlatinal) toxins A and C (due to their likely association with scarlet fever), also designated streptococcal pyrogenic exotoxins A and C (SPE A, SPE C); 2) a series of recently discovered mitogenic exoproteins, called streptococcal superantigen (SSA), streptococcal mitogenic exotoxin (SMEZ), and the pyrogenic toxins SPE G, SPE H, SPE I, SPE J, SPE K, SPE L, and SPE M, respectively.[4,9–11,14,16] The occurrence of these exotoxins discovered over the past 12 years was inferred for some of them from genome mapping of serotypes M1, M3, and M18 GAS. The respective genes were further transcribed, and the superantigenicity and lethality of corresponding SAg proteins established.[16] Serological studies showed the presence of antibodies against these SAgs.[4]

GENETIC DETERMINANTS OF THE SUPERANTIGENS

All known streptococcal SAgs with the exception of SMEZ, SPE G, and SPE J are encoded by bacteriophage genes integrated in the bacterial chromosome.[4,9,14] Four naturally occurring *speA* alleles have been found in strains recovered from patients with severe invasive diseases. Three of these *speA*1, *speA*2, and *speA*3 encode toxins differing by a single amino acid. The toxin encoded by *speA*4 was 9% divergent from the other three ones with 26 amino acid changes. Strains expressing *speA*2 and *speA*3 have caused the majority of STSS episodes in the past 12 years, suggesting that the gene products, SPE A2 and SPE A3, may be the more bioactive toxic forms of the toxin. This may be due to higher affinity to HLA-DQ molecule. Four alleles of *speC* have been described and 24 for *smez* gene.[17] Detailed studies on SMEZ-2[19] and SMEZ-16[20] proteins showed that they are the most potent SAgs (in terms of mitogenic activity) discovered thus far.

Molecular Relatedness

The determination of the nucleotide sequences of the 11 streptococcal SAgs listed above allowed the establishment of their amino acid sequences which revealed various degrees of molecular and structural relatedness at both amino acid sequences and three-dimensional (3-D) levels. These SAgs were found to share important structural homologies or similarities with *S. aureus* enterotoxin SAgs. Interestingly, certain streptococcal SAgs are more similar to some staphylococcal SAgs than to other streptococcal SAgs.[14] The genomic and structural relatedness suggests that these SAgs share a common ancestor.[17,18]

Crystal Structure of the Superantigens

High-resolution X-ray crystallographic techniques have led to the elucidation of the 3-D structure of five *S. pyogenes* SAgs, namely, SPE C, SPE A, SSA, SPE H, and SMEZ-2.[17] All of these toxins as well as *S. aureus* SAgs investigated to date appear as compacted ellipsoidal proteins sharing a common characteristic two-domain folding pattern comprising a NH_2-terminal β barrel globular domain, a long α helix that diagonally spans the center of the molecule, and a COOH-terminal globular domain based on grasp motif.[18] Valuable information on the molecular architecture of MHC–SAg–TcR complexes was obtained by X-ray crystallography of the SAgs bound to human MHC class II molecules and (or) TcR.[14,18,19]

These studies revealed the essential role of Zn in this process for certain SAgs.

BIOLOGICAL AND MOLECULAR TEST METHODS FOR DETECTION OF SAGS AND SAG CARRYING *S. PYOGENES*

The detection and assay of SAgs in bacterial supernatants, purified preparations, or biological fluids (e.g., patient plasma) are based on the classical mitogen-induced lymphocyte proliferation test. Human peripheral blood lymphocytes, purified (Ficoll gradient) from the blood of healthy donors, are incubated in multiwell microtiter plates at 10^{-5} cells per well. After 48 hr, 0.1 μCi of tritiated thymidine is added for another 24 hr. The cells are harvested and thymidine incorporation is measured in a radioactivity scintillation counter.[4] This procedure is also used by testing the inhibition of SAg-induced lymphocyte proliferation by specific antibodies against the relevant SAg.[4]

The determination of SAg-specific TCR Vβ pattern is investigated by Vβ enrichment analysis using anchored multiprimer amplification.[15]

The identification of SAg genes in *S. pyogenes* isolates is undertaken by polymerase chain reaction (PCR). Bacterial isolates are grown overnight in brain–heart infusion medium. The bacterial cells are spun down and washed. Their DNA is purified and used for PCR with specific primers for the various *spe* genes. A primer pair specific to a DNA region encoding the 23S rRNA is used as a positive control.[4]

BIOLOGICAL AND PATHOPHYSIOLOGICAL PROPERTIES

Streptococcal as well as staphylococcal SAgs exhibits a remarkable spectrum of biological and pharmacological activities. These activities comprise among others 1) the pyrogenic effects resulting from the release of interleukin-1 and tumor necrosis factor-α;[20] 2) immunosuppression of humoral and cell-mediated responses, deletion of T-cell repertoire, anergy, and apoptosis of lymphocytes.[14]

Consequences of Immune System Cells Stimulation by Superantigens

The excessive activation of an unusual high proportion of T lymphocytes and APCs in vivo and in vitro upon binding the SAg molecules triggers massive production of a wide array of inflammatory and other Th1/Th2 and APC-derived cytokines, by the stimulated cells. This process takes place through a complex of upregulated and downregulated immunological networks, activation signals, and the cooperation of adhesion molecules on target cells leading to a cascade of events including further release (besides cytokines and chemokines) of other pharmacologically active products, particularly those of the arachidonic acid/lipid pathways.[4,14,21,22] This SAg-mediated hyperstimulation of the immune system of the host can overwhelm the host regulatory network and thereby assist pathogen evasion of the adaptive immune response.[18] Moreover, the excessive and aberrant activation of T cells causes damage to tissues and organs and cell apoptosis which may result in disease and even death.[13,22] Superantigen activity, particularly SPE A, has been found in acute-phase serum samples from streptococcal disease patients.[4]

CLINICAL ASPECTS OF SUPERANTIGEN-MEDIATED DISEASES

S. pyogenes represent a potent threat to both healthy and immunocompromised individuals. These bacteria release, besides the SAgs described here, a wide array of potentially harmful cytolytic toxins and exoenzymes.[1,2,4,12] As mentioned above, the production of these effectors during host infection by GAS contributes to a broad spectrum of diseases ranging from mild to severe cutaneous and other tissue infections, to life-threatening septicemia and toxic shock syndrome. A detailed description is beyond the scope of this review. We will summarize here the major aspects of STSS (reported death rate of 30% to 70%) mainly mediated by SAgs.[1,2,4,22] This illness is a significant portion of invasive *S. pyogenes* cases such as painful cellulitis, soft-tissue infections (necrotizing fasciitis, myonecrosis), bacteremia, deep-seated infections preceded by blunt trauma, muscle strain, hematoma, or joint effusion at the infection site. The early symptoms of STSS are often flu-like including myalgia, chills, and fever. Severe localized pain is a hallmark of the illness. These symptoms are followed by persistent fever, systolic hypotension, and shock. Multiorgan failure and tachycardia may occur as well fasciitis and myositis. Disseminated intravascular coagulation may lead to impaired circulation and subsequent gangrene of the limbs and ultimately death.[4,22]

Host Implication in Superantigen-Mediated Invasive Diseases

Several lines of evidence support the contention that certain individuals may be genetically more susceptible to the effects of individual SAgs in the pathogenesis of STSS and other severe streptococcal diseases in relation with the

excessive release of inflammatory cytokines triggered by the superantigens in host's organism.[7,13,23,24] Indeed, specific human leukocyte antigen class II haplotypes conferred strong protection from systemic diseases. Patients with the DRB1*1501/DQB1*0602 haplotype mounted significantly reduced responses and were less likely to develop severe systemic diseases.[7] This finding is supported by recent report showing that erythrogenic toxin SPE A binding to class II HLA is dependent on DQ α-chain polymorphisms such that HLA-DQA1*01 α-chains show greater binding than DQA1*03/05 α-chains.[24]

CONCLUSION

Streptococcal SAgs are very original and highly mitogenic bacterial protein toxins exhibiting particular mechanisms of molecular and functional action on immune system cells and indirectly on other cellular and tissue targets in the host. They play a major role in severe invasive streptococcal diseases, particularly in the pathogenesis of streptococcal toxic-shock syndrome. The in vivo SAg-mediated release of inflammatory cytokines has a pivotal role in these diseases. The immunogenetics of the host influences the outcome of invasive streptococcal infection.

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Subtelomeric Rearrangements in Unexplained Mental Retardation

Samantha J. L. Knight
University of Oxford, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Headington, U.K.

INTRODUCTION

Mental retardation (MR), also referred to as learning disability, learning difficulty, intellectual disability, developmental delay, impaired cognition, or mental handicap, is an extremely common condition, affecting about 3% of the population. Virtually all of those individuals with moderate to severe MR [intelligence quotient (IQ) under 50] need lifelong support and about half of those with mild MR (IQ 50–70) are significantly impaired throughout life. Although MR carries with it immense clinical, social, and psychological burdens, the origins remain poorly understood. Its causes tend to be thought of broadly, such as prenatal insult, social disadvantage, and inheritance. As a consequence, it has been impossible to provide effective therapeutic or preventive strategies.

One avenue that has been better pursued in recent years is the exploration of genetic causes. It has long been suspected that small chromosomal rearrangements, undetectable by conventional cytogenetic diagnostic methodologies, might be involved in the etiology of MR. However, it is only within the past decade that advances in molecular methodologies and technology have allowed this hypothesis to be tested. Initial studies focused on the ends of chromosomes (the telomeres and adjacent subtelomeric regions) and showed that small, previously undetected rearrangements are a significant cause of unexplained moderate to severe MR, occurring in 7.4% cases. These findings have been corroborated since through a vast number of independent patient studies, and the importance of subtelomeric anomalies in unexplained MR is now well recognized in clinical genetics.

This entry discusses the background to the subtelomeric studies, the methodologies available for testing subtelomeric regions, the MR subtelomeric studies to date, and how subtelomeric testing has become a widely used tool in clinical diagnostic laboratories, particularly in the diagnosis of unexplained MR. The conclusion addresses the overall impact that subtelomeric testing has had on the diagnosis of MR, the implications for patients and their families, and also future research avenues for exploring the genetic causes of MR and improving our overall understanding of neurocognitive development.

SUBTELOMERIC REARRANGEMENTS IN UNEXPLAINED MENTAL RETARDATION

Background

The standard test for suspected chromosomal rearrangements is cytogenetic analysis at a 400–550 band resolution. This has been available for many years, but cannot routinely detect rearrangements smaller than 5 megabases (Mb) and much larger abnormalities may be missed if they occur in regions where the banding pattern is not distinctive. The suggestion that a substantial proportion of MR might be a result of smaller chromosomal anomalies, undetectable using conventional cytogenetic analysis, gave the impetus to develop new molecular diagnostic assays which have a higher level of resolution and reliability.

A major advance came with the discovery that the very end portions of human chromosomes (the telomeres and adjacent subtelomeric regions) can be cloned by functional complementation in yeast. This paved the way for the identification of telomere-specific microsatellite markers and for generating telomere-specific cosmids, bacteriophage P1 artificial chromosomes (PACs), and bacterial artificial chromosomes (BACs).

Wilkie,[1] in 1993, first suggested that subtelomeric rearrangements might be responsible for MR. Screening telomeres for rearrangements held a number of attractions. First, the majority of translocations involve chromosome ends and therefore an assay that targeted telomeres would detect these with 100% sensitivity regardless of size. Second, the regions adjacent to telomeres are gene-rich so rearrangements involving these would be more likely to have phenotypic consequences than rearrangements in many other parts of the genome. Finally, rearrangements involving telomeres were emerging as an important cause of human genetic diseases. For example, Wolf–Hirschhorn, Cri du Chat, and Miller-Dieker syndromes and alpha-thalassemia with mental retardation of chromosome 16 were all known to be a result of the unbalanced products of subtelomeric translocations. In 1995, the results of a pilot study that used polymorphic loci to study the subtelomeric regions of 28 chromosomes in 99 mentally retarded individuals were reported by Flint

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et al.[2] The findings suggested that at least 6% of idiopathic MR might be explained by submicroscopic rearrangements involving telomeres. The first extended study was reported by Knight et al.[3] who used a novel multitelomere fluorescence in situ hybridization (FISH) approach.[4] The results confirmed those of the pilot study and found that subtelomeric rearrangements contributed to the MR in 7.4% cases with moderate to severe MR with dysmorphic features, in about half of those with a family history, and also in 0.5% cases with mild MR.[3]

Subtelomeric Testing Methods

The complex structure of subtelomeric regions and the practical difficulties of testing 41 telomeres in a single procedure proved particularly challenging when it came to developing a robust, cost-effective diagnostic technique with a high degree of sensitivity and specificity.[5] However, subtelomeric rearrangements have now been detected using at least nine different approaches: 1) multitelomere FISH;[3] 2) multiplex or multicolor FISH, including M-TEL[6,7] and combined binary ratio labeling fluorescence in situ hybridization (S-COBRA FISH);[8] 3) telomere spectral karyotyping (telomere SKY);[9,10] 4) primed in situ labeling (PRINS);[11] 5) high-resolution chromosome analysis;[12] 6) comparative genome hybridization (CGH) to chromosomes;[13,14] 7) scanning short tandem repeat polymorphisms;[15–18] 8) locus copy number measurement by hybridization with amplifiable probes (MAPH)[19–21] and multiplex ligation-dependent probe amplification (MLPA);[22] and 9) telomere array CGH.[23,24] Of all the available methods, the FISH-based approaches still provide the gold standard for the detection of copy number changes and for balanced rearrangements (Fig. 1). However, neither these nor any of the alternative methods can be singled out as ideal; concerns over cost or sensitivity and specificity or both are constantly recurring issues. The advantages and drawbacks of each method are summarized briefly in Table 1.

Subtelomeric Anomalies and Unexplained Mental Retardation

Since the initial findings, the results of testing subtelomeric regions of almost 3300 individuals with MR have been reported. These can be divided broadly into three categories: 1) case reports; 2) studies of few, although highly selected patient sets; and 3) studies of smaller or larger sample sets referred because the patients have unexplained MR (includes mild or moderate to severe MR, with or without dysmorphic features and normal karyotypes at a 550-band level). The latter allow an estimate of the overall frequency of subtelomeric anomalies associated with MR. Of the 2690 unselected unexplained MR cases reported, 136 have subtelomeric rearrangements, giving a frequency of $\sim$5% in this group. The results can be further subdivided, at least for some studies, according to the degree of MR. Combining these, subtelomeric anomalies are found in 0.3% of those with mild MR and 7.9% with moderate to severe MR. Overall, the results show that small, subtelomeric anomalies are more likely to be present in those with moderate to severe MR, those with dysmorphic features, and those with a positive family history, but as addressed in the following section, these features are present in a substantial number of MR referrals.

Who Should Be Tested?

Patients with unexplained MR account for approximately 15% of all referrals passing through genetics and

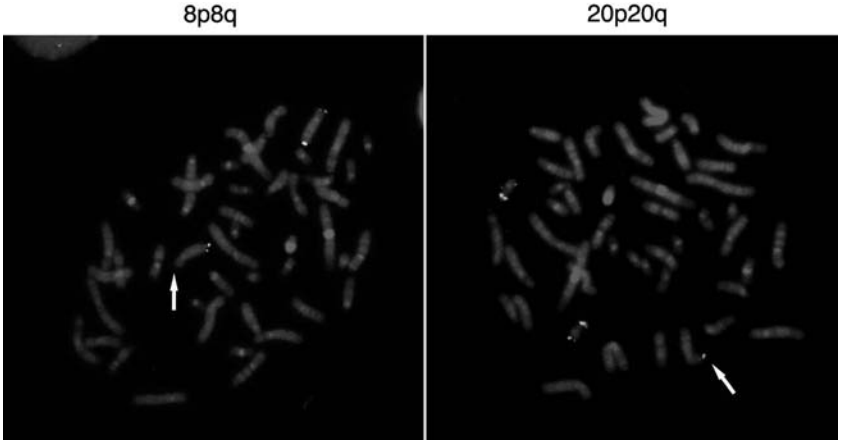

Fig. 1 Example of telomeric FISH result. The arrows highlight the unbalanced subtelomeric rearrangements in a patient monosomic for 8p (left metaphase) and trisomic for 20p (right metaphase). (*View this art in color at www.dekker.com.*)

Table 1 Subtelomeric testing methods

Method	Advantages and drawbacks
	Advantages
Multitelomere FISH	Interpretation is straightforward
	Tolerates poor chromosome resolution
SKY and M-FISH:	Tolerate chromosome preparations with low mitotic index
Telomere SKY, M-TEL, S-COBRA	Useful for samples where chromosomal material is limited
PRINS	Uses only a single probe for all telomeres
High-resolution chromosome banding	Extension of standard karyotypic analysis
	No additional expensive equipment required
CGH to chromosomes	Uses DNA sample
	Interrogates the whole genome as well as telomeres
Microsatellite genotyping	Uses DNA sample
	Can detect uniparental isodisomy as well as copy number changes
	Can be automated
MAPH and MLPA	Uses DNA sample
	FISH workload significantly reduced
Telomere array CGH	Uses patient's DNA sample
	Drawbacks
Multitelomere FISH	Labor-intensive
	Commercial kits expensive, but probes can be produced ''in house''
	Knowledge of polymorphisms and cross-hybridizations required
SKY and M-FISH:	Often labor-intensive
Telomere SKY, M-TEL, S-COBRA	Requires specialized, expensive imaging equipment and software
	Knowledge of polymorphisms and cross-hybridizations required
PRINS	Only detects monosomy
High-resolution chromosome banding	Labor-intensive. Requires highly experienced and diligent analyst
	Fully dependent on high-quality, high-resolution banding[a]
CGH to chromosomes	Resolution at telomeres only $\sim$4 Mb so smaller anomalies will be missed
	Efficacy yet to be proven for all telomeric regions
Microsatellite genotyping	Requires parental samples as well as probands
	Labor-intensive (can be many false positives to follow up)
	Requires highly informative markers (even with >85% heterozygosity, only 72% monosomies, 75% uniparental isodisomy, and 50% trisomies are detected)
MAPH and MLPA	False positive rate of $\sim$4%
	False negative rate of $\sim$2% (depending on the frequency of genuine copy number changes expected)
Telomere array CGH	Experimentally expensive and requires access to specialized arraying and scanning equipment
	False positive rate high and some probes give false negatives
	Rigorous evaluation and optimization pending

[a]In the United Kingdom, the recommended quality for microdeletion detection is quality score 7, as laid out by the National External Quality Assessment Scheme (NEQAS) (for information, see http://www.well.ox.ac.uk/~sknight/NEQAS/NEQASBandingCriteria.htm and http://www.ukneqas.org.uk/Directory/GENET/clincyto.htm). In practice, few laboratories achieve this standard routinely, although would endeavor to do so for selected cases, on request. According to the UK NEQAS for Clinical Cytogenetics 2000, sample slides submitted by the United Kingdom's 40 Clinical Cytogenetics laboratories ranged in quality from quality score 4 to quality score 8.

Source: Dr. Ros Hastings, organizer of UK NEQAS for Clinical Cytogenetics, personal communication.

pediatrics clinics, and so who then, among these vast numbers, should be investigated for subtle subtelomeric aberrations? Detection rates are higher among those with moderate to severe mental retardation, but screening all individuals in this group is usually considered impractical because of the cost. One argument is that the high rate of familial occurrence does justify blanket screening of all individuals with unexplained MR on the grounds that in familial cases, all results are informative.[3] However, a more cost-effective strategy would be to identify clinically a subgroup in whom small deletions occur at a much higher frequency. But is this possible? One approach that has been taken has been to investigate the clinical phenotypes of all of those MR individuals diagnosed

with a subtelomeric rearrangement and to identify features that occur more frequently in this group.[25] The results indicate that prenatal onset of growth retardation and a positive family history for developmental delay are good indicators for subtelomeric anomalies. These criteria, plus features suggestive of a chromosomal phenotype, have been included in a five-item clinical checklist, predicted to improve the diagnostic pickup rate of subtelomeric rearrangements among the developmentally delayed.[25] However, this checklist is not routinely adopted in clinical practice, presumably because diagnoses might be missed.

A more clinically useful approach is to determine whether anomalies of particular subtelomeric regions result in an identifiable and specific phenotype, thereby directing the clinician toward the diagnosis. This strategy is simplest when there is a de novo deletion or duplication affecting only a single subtelomeric region. The most striking example is the ''1p36 deletion syndrome.'' As shown in Fig. 2(a), cases of 1p monosomy account for

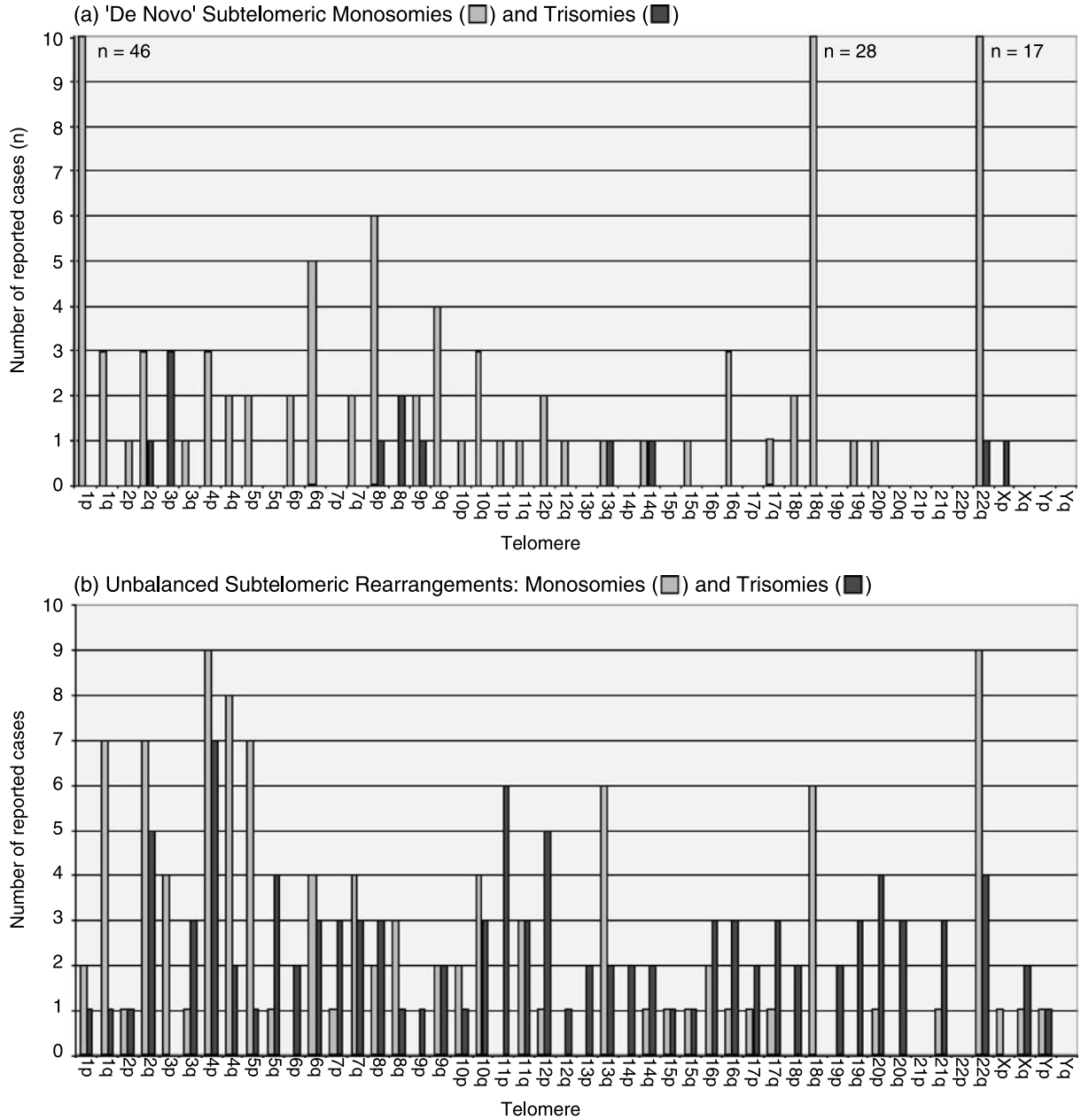

Fig. 2 Spectrum of telomeric involvement in reported MR studies (identical rearrangements within a family have been included only once). (*View this art in color at www.dekker.com.*)

one-third of all reported 'de novo' monosomies and trisomies, over twice that for any other telomere. From these cases, a specific 1p36 deletion phenotype has been defined and is already aiding clinicians. Monosomies for 18q and 22q also occur frequently [Fig. 2(a)]. Cytogenetically visible deletions both of the long arm of 18q and 22q13.3 are known to be associated with MR, although the overlapping phenotypes of patients with smaller subtelomeric deletions are likely to help refine and further delineate the chromosomal regions contributing to the phenotypes.

Figure 2(b) shows a summary of the anomalies reported in MR individuals with unbalanced subtelomeric translocations. The vast majority are familial originating from a phenotypically neutral, balanced translocation in one of the parents. Although some telomeres are more frequently involved than others, to date, there is no indication that specific translocation events between any two subtelomeric regions are favored over others. This makes it difficult to use unbalanced translocations to dissect out exactly which parts of a patient's phenotype are associated with the monosomy or trisomy because there may always be uncertainty regarding the phenotypic consequences of having both. For example, patients with unbalanced rearrangements involving monosomy 4p may have phenotypes atypical of Wolf–Hirschhorn Syndrome because the associated trisomy masks the features.[3] Furthermore, the actual combination of subtelomeric regions involved in an unbalanced rearrangement may determine the overall severity or even lethality of a phenotype. Nonetheless, some unbalanced translocations may prove informative, and studies have been undertaken to compare the anomalies found with their clinical phenotype.[26,27]

Spectrum of Telomeres Involved in Unexplained Mental Retardation

Of all of the subtelomeric anomalies noted in unexplained MR, there are only seven telomeres (including four acrocentric p-arms) for which no deletions have been reported and four telomeres (including two acrocentric p-arms) for which no duplications have been reported. For the acrocentric telomeres, this is most likely because chromosome-specific probes do not exist and, in most cases, available probes have not been tested. For the other telomeres, it may be because such anomalies are comparatively rare in MR. Alternatively, the subtelomeric deletions or duplications might be lethal, although this is unlikely to be the case for 18p, 19p, and Yq monosomies or for Yq and 18q trisomies because microscopically visible cases do exist. Another possibility is that some anomalies are being missed because they occur more distal to the current probe sets. Finally, some subtelomeric anomalies simply might not occur or might be phenotypically neutral or rare polymorphisms that will take more time to come to light. The latter do exist because a number of apparently normal individuals with subtelomeric anomalies have already been noted.[28]

CONCLUSION

The discovery that subtelomeric rearrangements are a significant cause of unexplained MR has led to a vast number of studies aimed at providing diagnoses in this group. Results from over 3000 patients show that rearrangements involving the ends of chromosomes can be detected in about 5% of unselected cases, that the prevalence of subtelomeric anomalies is higher in patients with moderate to severe MR (about 7%), and that in about half of those with subtelomeric anomalies, the rearrangements appear to have arisen de novo whereas in half they are familial, unbalanced rearrangements arising from balanced translocations in a parent. Efforts are in progress to refine our current knowledge and to identify more specific subsets of MR individuals by defining those phenotypes more likely to be associated with subtelomeric rearrangements. Some progress has been made based on reported cases, but many more cases need to be categorized before the phenotypes of particular deletions or duplications become clear.

Although originally developed for the study of idiopathic MR, subtelomeric testing is also proving useful for other clinical applications, including the full characterization of partially defined chromosomal abnormalities, the study of spontaneous recurrent miscarriages, infertility, and hematological malignancies, and for preimplantation diagnosis (in selected cases). Whatever the application, one diagnostically important issue will be to resolve how much monosomy or trisomy at each of the telomeric regions can be tolerated without phenotypic effect. This is being addressed by establishing a set of markers and clones to measure the size of monosomic/trisomic regions, thereby providing the foundations for future studies designed to determine whether a given subtelomeric rearrangement would be expected to be benign or pathogenic.[29]

The identification of subtelomeric rearrangements represents an important step toward understanding the genetic causes of unexplained MR. The findings thus far have been particularly relevant for providing clinical diagnoses and more accurate genetic counseling, but ultimately will be important for understanding cognitive development as a whole and for developing new therapeutic avenues. In this respect, subtelomeric studies have already opened important avenues for future research. For example, one crucial question is whether

small rearrangements occurring elsewhere in the genome might also be responsible for a significant proportion of unexplained MR cases. Indeed, there are already indications that whole-genome screening for copy number changes would be worthwhile.[15,30] One emerging technology that might prove suitable for this is CGH to microarrays as a single array-CGH experiment has the potential to identify copy number changes anywhere in the test genome at a resolution of 1 Mb or less. Using such approaches will undoubtedly lead to further advanced diagnostic, counseling, and therapeutic capabilities together with a greater scientific understanding of unexplained MR and will also find application in a wide range of other human conditions with genetic origins.

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TaqMan Oligoprobes

Dino A. De Angelis
Memorial Sloan-Kettering Cancer Center, New York, New York, U.S.A.

INTRODUCTION

The parallel development of fluorescent probes and of high-throughput thermal cyclers capable of continuously measuring fluorescence has fueled the emergence of real-time polymerase chain reaction (RT-PCR) as the diagnostic method of choice to accurately detect and quantify the occurrence of a specific DNA sequence in a given sample (see chapters on Real-Time PCR, Quantitative Real-Time PCR, Real-Time PCR platforms). The uses of RT-PCR range from gene copy number determination to RNA expression profiling to allelic discrimination, to name a few. Regardless of the chemistry used in RT-PCR, specific hybridization of the probe to the target DNA sequence ultimately results in the production of a specific fluorescent signal that can be measured in the reaction vessel directly, without any post-PCR processing of the samples (such as performing agarose gel electrophoresis of the reaction, or indeed even opening the reaction vessel). The major fluorescence detection technologies used in RT-PCR are described elsewhere in this encyclopedia, including DNA binding fluorophores, linear oligoprobes, hairpin oligoprobes (such as molecular beacons), scorpion probes, and hybeacons. The TaqMan assay has the privilege of being the most widely used fluorescence detection methodology for RT-PCR, and also one of the first ones to be developed. The purpose of this chapter is to introduce the principle behind the TaqMan assay, and to discuss different parameters in the design of TaqMan oligoprobes (also referred to as 5′ nuclease oligoprobes).

PRINCIPLE OF THE TAQMAN ASSAY

Like several eubacterial DNA polymerases, *Thermus aquaticus* (Taq) polymerase is a bifunctional enzyme consisting of a polymerization domain and a nuclease domain, and it is the latter that is crucial for the TaqMan assay.[1] In essence, the TaqMan assay is identical to a standard PCR reaction powered by Taq polymerase, except for the addition of the TaqMan probe, a nonextendable fluorescently labeled oligonucleotide hybridizing on the target DNA between the forward and reverse primers[2–3] (Fig. 1A). The TaqMan probe is modified with a fluorescent reporter dye (R) at its 5′ end and a fluorescence quencher dye (Q) at its 3′ end. Because of the proximity of the two dyes in the intact TaqMan probe, fluorescence resonance energy transfer (FRET) takes place from the reporter dye (FRET donor) to quencher dye (FRET acceptor) (De Angelis, FRET chapter, EDGP, and Ref. [4]). This proximity results in low fluorescence emission from the reporter dye because most of its excited state energy is transferred to the quencher dye. During extension from the forward primer, Taq polymerase encounters the TaqMan probe hybridized to the sequence being amplified. Taq then displaces the first few bases of the TaqMan probe off the template (Fig. 1B), and subsequently cleaves the TaqMan probe with its 5′ nuclease activity. While this was initially thought to be a 5′–3′ exonuclease, subsequent work showed that it is an endonuclease that cleaves at the juncture between the unhybridized and hybridized portions of the probe.[1,5] This cleavage results in an increased separation of the reporter dye on the 5′ end of the TaqMan probe from the acceptor (or quencher) on the 3′ end, yielding increased donor fluorescence emission (Fig. 1C). Therefore, when the TaqMan probe hybridizes perfectly to a DNA sample in the PCR reaction, an increase in fluorescence proportional to accumulation of the cleaved TaqMan probe follows every amplification cycle, and this signal is monitored in real time. On the other hand, in the absence of a perfect match, the probe remains intact and there is no increase of fluorescence above background.

The first use of the 5′ nuclease activity of Taq specifically designed to cleave a nonextendable, radioactively labeled oligonucleotide probe positioned between forward and reverse primers in a PCR reaction, was described in 1991.[6] The introduction of dual-labeled fluorescent probes[7] was a quantum leap that made the technology easier to use and safer; however, the analysis of fluorescence from accumulated cleaved probe in the sample still required some post-PCR reaction processing. The advent of the ABI PRISM™ 7700 system allowed continuous monitoring of fluorescence in real time.[8–10]

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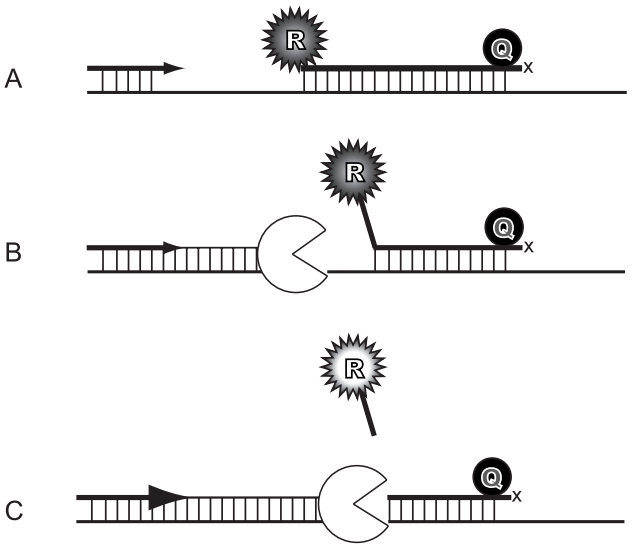

Fig. 1 Schematic depicting three stages of the TaqMan assay. (A) Hybridization: The forward primer (indicated by the arrow) and the TaqMan probe, labeled at the 5′ end with the reporter dye (R) and at the 3′ end with the quencher dye (Q) are shown annealing to the template DNA. The x on the TaqMan indicates that the probe is nonextendable. Proximity of R and Q yields low fluorescence from R through FRET. (B) Extension: Taq polymerase extends the forward primer and encounters the TaqMan probe. C) Cleavage: Taq polymerase cleaves the TaqMan probe, thus releasing the reporter from the quencher and causing an increase in the fluorescence of the reporter, which is monitored by the RT-PCR instrumentation.

PHYSICOCHEMICAL PROPERTIES OF TaqMan PROBE

TaqMan probes are covalently modified at the 5′ end with a reporter dye; commonly used dyes for this purpose are listed in Table 1. When excited by incident light, these dyes emit light at wavelengths ranging from 515 nm (FAM) to 565 nm (HEX, VIC). The first nucleotide of a TaqMan probe—the one covalently labeled with the reporter dye—can only be A, C, or T; a G residue is not allowed because this base partially quenches reporter fluorescence, whether the probe is cleaved or not.

The quencher dye can be positioned in the middle of the probe, but the 3′ end is preferred because the dyes can interact more effectively due to the greater flexibility of the probe, thereby increasing FRET efficiency and lowering reporter fluorescence before cleavage. Positioning the quencher at the 3′ end also ensures that the endonuclease activity of Taq can be activated at any point along the length of the probe, and still result in separation of the dyes. Tetramethyl rhodamine (TAMRA) is a commonly chosen dye because through FRET it can effectively quench the excited state energy of all the reporter dyes cited above (absorption range 500–570 nm) (Table 1). However, as it quenches the reporter, TAMRA also emits light of a different wavelength (~580 nm), which contributes to increasing the total fluorescence in the sample. Nonfluorescent quencher dyes such as DABCYL [4-(4′-dimethylaminophenylazo) benzoic acid] have been introduced. With an absorption range of 450 to 550 nm, DABCYL can effectively quench the fluorescence of the shorter wavelength emitting dyes (such as FAM, JOE, and TET) in the intact probe without contributing any fluorescent signal (compare the emission maxima of the reporter dyes in Table 1 with the absorption properties of the quencher dyes in Table 2). Proprietary dark quenchers with wider absorption ranges and evocative names are constantly being developed, such as Black Hole Quencher™ 1 and 2 (Biosearch Technologies), and Eclipse™ dark quencher (Epoch Biosciences). Nonfluorescent quenchers are particularly useful for multiplexing (chapter on Multiplexing, EDGP), where two (or more) TaqMan probes labeled with reporters with distinct emission maxima (such as FAM and HEX) are present in the same PCR reaction to simultaneously monitor the levels of different targets in the sample.[11]

In addition to being dual fluorescent labeled at the 5′ and 3′ end, TaqMan probes are also modified with a phosphate group at their 3′ end. This modification renders them nonextendable, preventing priming of DNA synthesis from the probe itself by Taq polymerase.

Table 1 Commonly used reporters for TaqMan probes

5′ Reporter	Full name	Absorbance (nm)	Emission (nm)
FAM	6-Carboxyfluorescein	492	515
TET	Tetrachloro-6-carboxyfluorescein	521	536
JOE	2,7-Dimethoxy-4,5-dichloro-6-carboxyfluorescein	520	548
HEX	Hexachloro-6-carboxyfluorescein	535	565
VIC™	A proprietary fluorescent dye (Applied biosystems)	535	565

Table 2 Commonly used quenchers for TaqMan probes

Quencher	Full name	Absorbance range (nm)	Emission
TAMRA	Tetramethyl rhodamine	500–575	580 nm
DABCYL	4-(4′-Dimethylaminophenylazo) benzoic acid	400–550	Dark
ECLIPSE™	A proprietary dark quencher (Epoch Biosciences)	390–625	Dark
BHQ-1™	A proprietary dark quencher (Biosearch Technologies)	480–580	Dark
BHQ-2™	A proprietary dark quencher (Biosearch Technologies)	550–650	Dark

SEQUENCE AND POSITIONING OF TaqMan PROBE

Many of the rules that apply to PCR primer design also apply to TaqMan probe design (see Primer/Probe Design chapter in EDGP). The sequence of the TaqMan probe should have GC contents within 40–60% preferably. Long sequences of identical nucleotides should be avoided in the probe. The sequence should be devoid of self-complementarity, or complementarity with the forward and reverse primers. The TaqMan probe should anneal as close as possible to the forward primer without overlapping. The size of the amplicon (dictated by the forward and reverse primers) should be quite short, never exceeding 300 bp.

The TaqMan probe should be 20–30 nucleotides in length, with a high melting temperature (65°C to 67°C). This is ~10°C higher than the forward and reverse primers of the PCR reaction, and ensures that the probe is bound more stably than the primers when there is a perfect match with the target DNA sequence (Fig. 1A). This is important, because hybridization of the probe with the template DNA activates the Taq 5′ nuclease activity, which in turn generates the fluorescence increase because of cleavage. In the case of a TaqMan probe designed to distinguish between two alleles of a specific marker gene, the polymorphic site should be positioned near the middle of the probe to cause as much destabilization as possible of the mismatched duplex (Fig. 2).

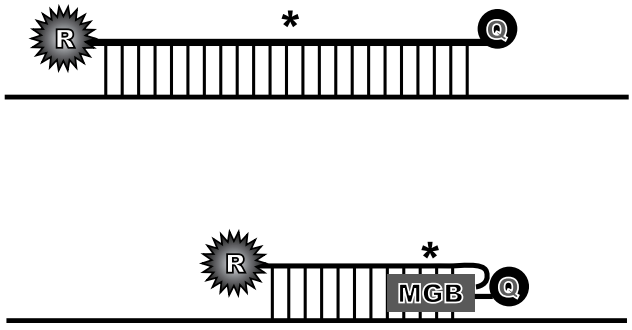

Fig. 2 Comparison of the classical TaqMan probe and the minor groove binder (MGB) TaqMan probe. Classical TaqMan probes (top) are longer than the MGB counterparts. Allelic discrimination is best achieved by positioning the polymorphic site (indicated by the asterisk) toward the center of the probe. The MGB probes (bottom) are shorter because of the additional duplex stabilization afforded by the MGB group over the span of approximately 5 base pairs. Allelic discrimination is best achieved by positioning the polymorphic site in the MGB portion of the TaqMan probe.

MINOR GROOVE BINDER PROBES

A recent improvement on classical TaqMan probes is the development of minor groove binders (MGB) chemistry.[12] Modification of an oligonucleotide with an MGB such as dihydroclyclopyrroloindole tripeptide (DPI_3) results in a substantial increase in the interaction of the oligonucleotide with a complementary DNA strand. The MGB effectively acts as a clamp that secures the base-pairing interaction between the oligonucleotide and the target DNA sequence (Fig. 2). The MGB interacts with the minor groove of the double-stranded DNA mainly through van der Waals forces, stretched over approximately 5 base pairs. The increase in stability is such that the binding energy of a 12-mer probe modified with an MGB group at either end is similar to the binding energy of a perfectly matching 27-mer, nonmodified oligonucleotide. For TaqMan probes, the MGB is added at the 3′end, which de facto makes the oligonucleotide nonextensible. The quencher dye can be conjugated directly to the MGB.

Unlike the TaqMan probe, where optimal discrimination of polymorphic sites is achieved by positioning the mismatch near the center of the probe, MGB-modified TaqMan probes discriminate between alleles better when the mismatch is in the 3′ end of the sequence (see asterisks in Fig. 2). Base mismatches in the MGB binding region of the TaqMan probe greatly affect the ability of the MGB to bind and stabilize the base-pairing interaction, resulting in destabilization of the DNA duplex. Whereas a mismatch in the non-MGB binding region results in a decrease of 3–9°C in the melting temperature of the duplex, a mismatch in the MGB portion of the probe can result in

decreases as much as 16°C.[12] Combined with the fact that MGB probes are much shorter than regular TaqMan probes (12–16 vs. 20–30 bp), this results in a superior mismatch discrimination overall when using MGB-modified probes.

CONCLUSION

Established more than a decade ago, the TaqMan assay is one of the most widely used several fluorescence-based detection methodologies for real-time PCR. Continuing improvements in the chemistry of reporter and quencher dyes have resulted in 1) lower background fluorescence and 2) higher discrimination potential of the probes for multiplexing purposes. These developments, along with the ease of designing the probes, ensure the TaqMan assay will remain very much in use for several years to come.

ARTICLES OF FURTHER INTEREST

DNA Binding Fluorophores, p. 351
Real-Time PCR, p. 1117
Real-Time PCR Platforms, p. 1126

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Temporal Temperature Gradient Gel Electrophoresis

Lee-Jun C. Wong
Özgül Alper
Hae-Young Kwon
Duan-Jun Tan
Georgetown University Medical Center, Washington, D.C., U.S.A.

Tian-Jian Chen
University of South Alabama, Mobile, Alabama, U.S.A.

INTRODUCTION

Methodologies for the detection of nucleotide substitutions as well as small insertions and deletions in clinical molecular diagnosis of human genetic diseases have been recently reviewed.[1] For unknown mutations, although the gold standard is direct DNA sequencing, it is usually not practical to routinely sequence the entire coding regions of candidate gene or genes in patients suspected of having the genetic disease. Very often, a high-throughput, low-cost mutation screening method is used to quickly scan the coding exons and intron/exon junctions for the presence of mutations, followed by sequencing of suspected regions prior to sequencing of the regions containing the mutations. Commonly used screening methods for unknown mutations include denaturing gradient gel electrophoresis (DGGE),[2] temperature gradient gel electrophoresis (TGGE),[3] single-strand conformation polymorphism (SSCP),[4] heteroduplex analysis (HA),[5,6] chemical mismatch cleavage (CMC),[7] enzyme mismatch cleavage (EMC),[8,9] protein truncation test (PTT),[10] mismatch binding protein (MutS),[11] and cleavase fragment length polymorphism (CFLP).[12] The drawbacks of these methods include low sensitivity (SSCP, HA), difficulty in casting denaturing gradient gel (DGGE), synthesis of long GC clamped primers (DGGE, TGGE), the inability to detect homozygous mutations (HA, CMC, EMC), high background (CMC, EMC), lack of documented reports (CFLP, EMC, MutS), and preferential elimination of unstable mutant transcripts (PTT).[1] Furthermore, most of these methods are used for the detection of mutations in nuclear genes. Application of these methods to the detection of mitochondrial DNA (mtDNA) mutations is limited because of the heteroplasmic feature of pathogenic mtDNA mutations and numerous benign, homoplasmic single nucleotide polymorphisms. Thus a unique requirement for the molecular analysis of mtDNA mutations is the ability to detect heteroplasmic mtDNA mutations and to distinguish them from homoplasmic sequence variations. In search of an effective and sensitive mutation detection method, we recently developed the temporal temperature gradient gel electrophoresis (TTGE) that not only resolves the wild-type and mutant homoduplexes and the wild-type/mutant heteroduplexes into four distinct bands, but also detects a heteroplasmic or heterozygous change in the background of homoplasmic or homozygous polymorphism. Here we describe the utility of TTGE in the detection of mutations in both nuclear and mitochondrial genomes.

PRINCIPLES OF TTGE

Temporal temperature gradient gel electrophoresis (TTGE) was first introduced by Yoshino et al.[13] in 1991. It is based on the difference in the sequence-specific melting behavior of the normal and mutant DNA in a temporal temperature gradient that gradually increases in a linear fashion over the duration of the electrophoresis (Fig. 1). In TTGE, the denaturing environment is formed by a constant concentration of urea in the polyacrylamide gel and a temporal temperature gradient (0.5–3°C/hr). During the course of electrophoresis, the gradual temperature increase causes the double-stranded DNA to become partially denatured, and the electrophoretic mobility of the partially denatured DNA decreases. As a result of the different melting behavior, mutant and wild-type molecules are separated on the gel as they begin to denature at different temperatures (Fig. 1). A homozygous mutation shows band shift and a heterozygous mutation shows multiple bands because of the presence of two homoduplexes and two heteroduplexes at optimal separation conditions. Usually, an AT base pair to GC base pair change results in band shift-down and the GC pair to AT pair change results in band shift-up. This is because three hydrogen bonds in GC pairs hold the double-stranded structure tighter than the two hydrogen bonds in AT pairs. TTGE differs from TGGE in that TGGE has a fixed

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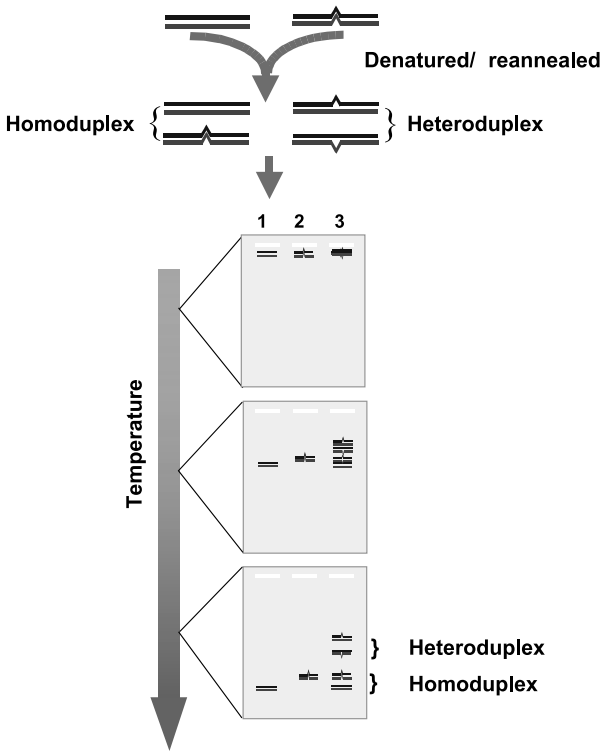

Fig. 1 Principles of TTGE. The PCR products are denatured and reannealed gradually to form homoduplexes, and heteroduplexes if there are mutations. During electrophoresis, the duplexes become partially denatured due to the temperature gradient. Based on the melting behavior of the duplexes, the partially denatured duplexes become separated while the temperature rises. (*View this art in color at www.dekker.com.*)

temperature gradient from top to bottom of the gel.[14] In TTGE, the temperature at any location of the entire gel is the same at any given time, but changes with respect to time (temporal temperature). Thus it is easier to modulate the temperature during electrophoresis and provide a broader separation range that leads to a much higher sensitivity of detection. Because the denaturant in TTGE is the temperature, there is no difficulty in the preparation of a chemical denaturant gradient gel. The size of the DNA fragments to be analyzed on TTGE can be as large as 1 kb. A GC clamped primer is not a requirement.

RUNNING CONDITIONS OF TTGE

DNA fragments of interest are amplified by polymerase chain reaction (PCR). The primers are usually about 18–22 nucleotides with T_m of 54–60°C, and the size of the PCR product is about 300–1000 bp. Prior to TTGE analysis, the PCR products are denatured at 95°C for 30 sec and slowly cooled to 45°C for a period of 45 min at a rate of 1°C/min. The samples containing reannealed homoduplexes and heteroduplexes are maintained at 4°C until loading onto the gel. TTGE is performed on a Bio-Rad D-Code apparatus. Two back-to-back 20 cm × 20 cm × 1 mm 4.5–6% polyacrylamide (acrylamide/Bis=37.5:1) gels are prepared in 1.25× TAE buffer containing 6 mol/L urea. The percentage of the gel depends on the size of the PCR product. Five microliters of denatured and reannealed PCR products are mixed with equal volume of 2× loading buffer (70% glycerol, 0.05% bromophenol blue, and 0.05% xylene cyanol) and loaded onto the gel. The electrophoresis is carried out at 110–145 V for 4–7 hr at a constant temperature increment of 1–2°C/hr. The temperature range for the electrophoresis depends on the melt curve of the DNA fragment determined by computer simulation using MacMelt (or WinMelt) software (Bio-Rad Laboratories). In addition, the melting temperature decreases by approximately 2°C for each molar of urea used. The gels are stained in 2 mg/L ethidium bromide for 5 min and imaged with a digital charge-coupled device (CCD) gel documentation system. DNA fragments showing alterations in banding patterns are sequenced to determine the nucleotide change. To confirm if a mtDNA mutation is homoplasmic or heteroplasmic, usually a second method, such as PCR/allele-specific oligonucleotide (ASO), dot blot, or PCR/restriction fragment length polymorphism (RFLP) analysis is used.[15,16]

APPLICATION OF TTGE ANALYSIS TO THE DETECTION OF NOVEL *CFTR* MUTATIONS

Cystic fibrosis (CF) is one of the most common and life-shortening autosomal recessive disorders, with an incidence of 1 in every 2000–3000 Caucasians. It occurs less frequently among African-Americans (1 in 15,300)[17] and Hispanics. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene.[18] Although it appears that the majority of CF mutant alleles carry the ΔF508 mutation, more than 900 *CFTR* mutations have been identified (Cystic Fibrosis Genetic Analysis Consortium, 2003, http://www.genet.sickkids.on.ca). The overall mutation spectrum and the common mutations found in specific geographic or ethnic background varied significantly among different populations. Current molecular diagnoses are designed to detect 80–97% of CF chromosomes in Caucasians and Ashkenazi Jews. However, by using the pan-ethnic mutation panel, the detection rate for Hispanic CF patients is only about 58%.[19] To improve the mutation detection rate and to provide a more accurate risk assessment for

Hispanic CF patients, TTGE was developed for comprehensive mutational analysis.

Primers were designed for all 27 exons, including at least 20 bp into the flanking intron regions.[20] The sizes of the PCR products range from 260 bp for exon 23 to 862 bp for exon 13. The PCR and TTGE conditions have been described above and also in a previous publication.[20] Figure 2 shows the detection of homozygous and/or heterozygous mutations and/or polymorphisms in *CFTR* gene. Panel A is a heterozygous G386A (G85E) mutation in exon 3 showing four bands. The two upper bands are heteroduplexes, and the lower two bands are homoduplexes, with the mutant homoduplex shift-up. Panel B is a heterozygous 875+40A>G polymorphism in intron 6A, showing similar four bands with the mutant homoduplex shift-down. Panel C is a heterozygous 124–146 del23 mutation showing two bands with the homoduplex deletion mutant shift-down. The mismatched heteroduplex bands are not formed, probably as a result of the large deletion. Panel D is a homozygous C2800T (Q890X) nonsense mutation in exon 5, showing single-band shift-up. Panel E shows a homozygous (lane 2) and a heterozygous (lane 3) 1285insTA mutation. The two homoduplex and the two heteroduplex bands are clearly separated.

These results indicate that TTGE banding patterns of homozygous and/or heterozygous mutations can be easily recognized. Using TTGE analysis, we have identified at least 22 novel mutations and 2 novel polymorphisms.[20,21] Sequencing of the 350 TTGE negative DNA fragments detected eight additional distinct mutations. These mutations are either located in the GC-rich region or close to the end of the DNA fragment, or nucleotide substitutions without the change in the number of hydrogen bondings. However, as soon as the TTGE condition is optimized for the melting domain in which the mutation located, the mutations were detected.

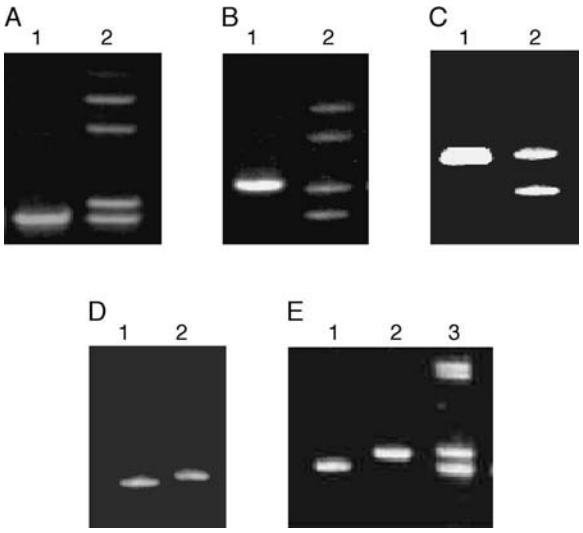

Fig. 2 Detection of mutations in *CFTR* gene by TTGE analysis. Lanes 1 are wild-type DNA and lanes 2 and 3 are mutant DNAs. A) Heterozygous G386A (G85E) mutation in exon 3. B) Heterozygous 875+40A>G polymorphism in intron 6a. C) heterozygous 124–146del23 mutation in exon 1. D) homozygous mutation C2800T (Q890X) in exon 15. E) Homozygous (lane 2) and heterozygous (lane 3) 1285insTA in exon 8.

DETECTION OF HOMOPLASMIC AND HETEROPLASMIC MUTATIONS IN MITOCHONDRIAL GENOME

Energy metabolism disorders caused by mutations in mtDNA represents a group of heterogeneous diseases. The most frequently found mtDNA point mutations are A3243G for mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), A8344G for myoclonus epilepsy, ragged red fibers (MERRF), T8993G/C for neuropathy, ataxia, retinitis pigmentosa (NARP), and Leigh syndrome, and G11778A for Leber's hereditary optic neuropathy (LHON).[16,22] Molecular analysis of the known mtDNA mutations is usually carried out by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) or multiplex PCR/ASO (allele-specific oligonucleotides) dot blot (cross reference) method. Recently, a capillary-based technique was also used for A3243G mutation analysis.[23] Using multiplex PCR/ASO method to screen 44 known point mutations in 2000 patients with mtDNA disorders, we have demonstrated that only about 6% of the patients had identifiable, disease-causing mutations.[16,22] These data suggest that the majority of the pathogenic mutations have yet to be identified.

One of the unique characteristics of mitochondrial genetics is heteroplasmy. Because there are hundreds to thousands of mitochondria per animal cell, the percentage of mutant mitochondria can vary from 0% to 100%, unlike the nuclear gene mutation that is 100% for homozygous and 50% for heterozygous. In addition, there are numerous homoplasmic mtDNA variations. Thus the requirement for an effective mutation detection method is the ability to distinguish between the heteroplasmic and homoplasmic mtDNA mutations.

Both psoralen clamp and GC clamp have been used for broad-range (0–80% denaturant) denaturing gradient gel electrophoresis (DGGE).[24,25] Michikawa et al.[25] developed the DGGE conditions for 18 psoralen-clamped DNA segments to analyze 20 different known mtDNA point mutations in tRNA genes. Sternberg et al.[24] used the traditional DGGE method with a GC clamp to investigate 35 unrelated patients with mitochondrial

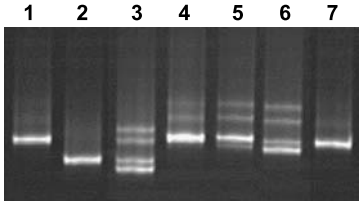

Fig. 3 Detection of heteroplasmic A3243G mutation in mitochondrial DNA. PCR products of 643 bp (nt3116 to nt3758) containing the $tRNA^{Leu(UUR)}$ and part of 16S rRNA and ND1 regions were analyzed with TTGE. Lanes 1 and 7: wild type; lane 2: homoplasmic T3197C polymorphism with band shift-down; lane 3: 47% heteroplasmic A3243G mutation in a homoplasmic T3197C polymorphism background; lanes 4–6: A3243G mutations with 7%, 24%, and 62% of mutant heteroplasmy.

encephalomyopathy. These studies were restricted to tRNA genes only. Recently, we have developed the temporal temperature gradient gel electrophoresis (TTGE) for the fast screening of the entire mitochondrial genome for mtDNA mutations.[26,27]

Thirty-two pairs of overlapping primers were designed to amplify the entire 16.6 kb of mitochondrial genome.[26,27] The primer sequences and detailed PCR and TTGE conditions have been previously described.[26,27] The sizes of the PCR products vary from 306 to 805 bp.[26] Figure 3 shows the ability of TTGE to detect various percentages of mutant A3243G heteroplasmy. The homoplasmic T3197C polymorphism shows a band shift down (lane 2) as a result of the TA pair to CG pair change. Under optimal conditions, a single heteroplasmic mutation produces four bands representing the wild-type and mutant homoduplex bands (lower two bands in lanes 3–6) and two wild-type/mutant heteroduplex bands (upper two bands in lanes 3–6). As can be seen in this figure, the intensities of the two heteroduplex and the mutant homoduplex bands increase as the percentage of the mutant A3243G mtDNA increases from 7% to 62% (lanes 4–6 of Fig. 3). The detection of the heteroplasmic A3243G mutation in the presence of a homoplasmic T3197C background is also illustrated, where the wild-type homoduplex and all other bands are down-shifted (Fig. 3, lane 3) because of the presence of T3197C polymorphism. These results clearly demonstrate the power of TTGE to detect and distinguish homoplasmic and heteroplasmic mutations, as well as to identify heteroplasmy in the presence of a homoplasmic polymorphism. If there is more than one heteroplasmic mutation within the same DNA fragment, TTGE will show more than four bands.[26,27]

TTGE analysis was performed on DNA specimens obtained from patients with mitochondrial respiratory disorders without identifiable common point mutations. DNA fragments showing abnormal banding patterns were sequenced to identify the exact mutations.[26] One of the difficulties is that direct DNA sequencing sometimes cannot unequivocally detect low levels of heteroplasmic mutations. Very often, TTGE detects heteroplasmic banding patterns, but sequencing does not detect the mutations although the reduced peak height of the wild-type nucleotide may suggest the presence of mutation. In this case, a second mutation detection method, such as ASO, should be designed to confirm the putative heteroplasmic mutation.[26]

SENSITIVITY, SPECIFICITY, FALSE POSITIVE, AND FALSE NEGATIVE RATE OF TTGE

From our experience in the study of *CFTR* nuclear gene mutations, the TTGE method has a sensitivity of detecting approximately 92% of all mutations. Its specificity is estimated to be about 99%, with a false negative rate of about 1%, without any false positives.

For mitochondrial genes, 109 samples with 15 different previously identified mutations in 6 different genes were analyzed. All the mutations and status of heteroplasmy/homoplasmy were correctly identified.[17] In the screening of unknown mtDNA mutations, some TTGE-positive DNA fragments did not show mutations upon direct DNA sequencing. This is probably a result of the limited sensitivity of DNA sequencing.

CONCLUSION

TTGE is a powerful method for mutation detection. It is simple, sensitive, high-throughput, and cost-effective. For nuclear gene mutations, the distinction between homozygous and heterozygous mutations is obvious. The identification of unknown heteroplasmic mtDNA mutations is more difficult. This is attributable to the limited sensitivity of direct DNA sequencing in the detection of low percentage of heteroplasmy. Very often, the TTGE showed the presence of heteroplasmic mutations but sequencing failed to score the mutation.

ABBREVIATIONS

ASO	allele-specific oligonucleotide
CFLP	cleavase fragment length polymorphism
CMC	chemical mismatch cleavage
DGGE	denaturing gradient gel electrophoresis
EMC	enzyme mismatch cleavage
HA	heteroduplex analysis

MutS	mismatch binding protein S mutation analysis
PTT	protein truncation test
SSCP	single-strand conformation polymorphism
TGGE	temperature gradient gel electrophoresis
TTGE	temporal temperature gradient gel electrophoresis

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Tetracycline Resistance

Marilyn C. Roberts
University of Washington, Seattle, Washington, U.S.A.

INTRODUCTION

Tetracyclines were discovered in the 1940s and consist of a group of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Tetracyclines are ''broad-spectrum'' agents with activity against a wide range of gram-positive and gram-negative bacteria, anthrax, chlamydiae, Lyme disease, traveler's diarrhea, brucellosis, *Helicobacter pylori* disease, *Francisella tularensis*, mycoplasmas and rickettsiae, and protozoan parasites.[1] These antibiotics are also used prophylactically for the prevention of malaria by mefloquine-resistant *Plasmodium falciparum*. Tetracyclines are also listed as alternative therapy for *Vibrio cholerae*, some atypical *Mycobacterium* diseases, *Burkholderia*, and *Donovana granulomatis*, and have potential use in the treatment of filarial nematodes and rheumatoid arthritis. In addition, a number of noninfectious conditions such as acne, blistering skin diseases, and rosacea use tetracyclines. Tetracycline was first available commercially in 1953, whereas the semisynthetic second-generation compound doxycycline became available in 1967. The favorable antimicrobial properties of these agents and the absence of major adverse side effects have led to their extensive use in the therapy of human and animal infections, and as food growth promoters in food animals.[1] In a span of 30 years, tetracyclines have accounted for almost one third of all antibiotics approved for human use. Although tetracyclines retain important roles in both human and veterinary medicine, the emergence of microbial resistance has limited their effectiveness. The increasing incidence of bacterial resistance to tetracyclines has in turn resulted in efforts to establish the mechanisms by which genetic determinants of resistance are transferred between bacteria and the molecular basis of the resistance mechanisms themselves.[1]

MECHANISMS OF TETRACYCLINE RESISTANCE

Resistance to tetracycline can be caused by mutations to host efflux pumps, their 16S rRNA sequences, or the altering permeability of the cell. This usually provides low-level stepwise resistance, which until recently has rarely been clinically important. The more common method is the acquisition of new genes conferring tetracycline resistance. It is believed that this second method occurred in the last hundred years, as illustrated by a study of Enterobacteriaceae collected between 1917 and 1954 where only 2% was resistant to tetracycline.[1,2]

Numerous studies have characterized acquired resistance genes. Presently, two genes are considered related and given the same gene designation if they share $\geq 80\%$ of amino acid sequences in common with each other. Two genes are considered different and given different designations if they share $\leq 79\%$ amino acid sequence identity.[3] This comparison can now be performed using GenBank sequence information. The number of *tet* genes has reached the end of the Roman alphabet and to accommodate new *tet* genes numbers are being assigned.[3] Levy et al.[3] have agreed to be the contact persons to confirm proposed new numbers for a new tetracycline resistance gene to prevent two distinct *tet* genes from being assigned the same numbers, or two related genes being assigned different numbers. There have been 33 different tetracycline-resistant *tet* genes characterized and three oxytetracycline-resistant (*otr*) genes characterized (Table 1).[4] This includes the *otr*(C) gene, which was recently sequenced in the laboratory and is another efflux protein. Nineteen of the *tet* genes and two of the *otr* genes code for efflux pumps, 11 tet genes and one *otr* gene, [*otr*(A)], code for ribosomal protection proteins, and three genes code for enzymatic alterations of the tetracycline molecules [*tet*(X), *tet*(36) and *tet*(37)]. (Table 1).[4] The *tet*(U) sequence is known but seems to be unrelated to other types of resistance genes (Table 1).[1]

In general, clinically important Tc^r pathogens rarely owe their resistance to mutations.[1] Some exceptions have been described including Tc^r cutaneous propionibacteria with mutations in the 16S rRNA.[1] Previously, little work has been performed specifically on the detection of tetracycline mutations, although more work is now being done.

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Table 1 Mechanism of resistance for characterized *tet* and *otr* genes

Efflux (21)	Ribosomal protection (11)	Enzymatic (3)	Unknown[a]
tet(A), *tet*(B), *tet*(C), *tet* (D), *tet*(E)	*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32),	*tet*(X)	*tet*(U)
tet(G), *tet*(H), *tet*(J), *tet*(V), *tet*(Y),	*tet*(Q), *tet*(T), *tet* (36)	*tet*(37)	
tet(Z), *tet*(30), *tet*(31), *tet*(33)	*tet*(A), *tetB*(P),[b] *tet*	*tet*(34)	
tet(35)			
tet(K), *tet*(L)			
tetA(P)			
otr(B), *otr*(C)			
tcr3			

[a]*tet* (U) has been sequenced but does not appear to be related to either efflux or ribosomal protection proteins.
[b]*tetB*(P) is not found alone and the *tetA*(P) and *tetB*(P) genes are counted as one operon.

DETECTION OF ACQUIRED TETRACYCLINE RESISTANCE GENES

DNA–DNA Hybridization

The use of molecular tests to determine the carriage of specific antibiotic resistance genes began over 20 years ago.[5] Initially, the entire plasmid, or a relatively large fragment ($\geq$600 bp) carrying the gene of interest, was radiolabeled and used as a DNA probe in DNA–DNA hybridization assays to distinguish different *tet* genes from each other.[6] Then smaller ($\leq$500 bp) intragenic fragments, representing internal segments of the structural gene, were used. These were made by cutting the desired band out of a plasmid after it had been cleaved with restriction enzyme(s) and then by separating the fragments on an agarose gel. Currently, small 20- to 30-bp probes are used for hybridization. The sequences are determined by the gene or genes of interest and are prepared de novo. The advantage is that cross-hybridization is minimized, hybridization time is short, and they are now cheap to purchase.

All the various probes need to be labeled (radiolabel or nonradiolabel) before use. A number of nonradiolabeled systems are currently available commercially, but we have found that using these systems requires that the test be purified DNA to reduce possible interactions with the antibody used for detection and the protein left in the test DNA. In contrast with ^{32}P-labeled probes, whole bacterial dot blots cannot be screened using nonradiolabeling systems because they give too many false-positive reactions. Hybridization conditions depend on the base composition (G+C content) of the probe and should be set so that different *tet* genes do not cross-hybridize with each other.[7] The hybridization conditions will vary depending on the type of probe used, the type of label, and the kit used for the detection. Positive and negative controls should be run with each set of experiments.

To prepare dot blots from whole bacteria, turbid bacterial suspension (3 McFarland standard) are spotted onto a support such as nylon and then lysed in situ. For gram-negative bacteria, the isolates are taken from grown plates or in broth, whereas for gram-positive isolates, they must grow in broth with reagents that weaken the cell wall (D-threonine and glucose). To increase the sensitivity of the dot blot assay, we have used purified DNA instead of lysed bacteria.

Each method (radiolabel vs. nonradiolabel) for labeling probes has advantages and disadvantages. In the author's laboratory, both nonradiolabeled and radiolabeled probes were used. The radiolabeled probes can be reused for at least five to seven times. A disadvantage of the nonradiolabeled method is that it is unsatisfactory when using anything other than purified DNA because of the high level of false positives generated. Thus, nonradiolabeled probes are not used in the author's laboratory for whole bacterial dot blots because of the high background and the nonspecificity of binding with this type of target sample. In contrast, ^{32}P-labeled probes have few false positives and are very specific when using whole bacteria dot blots. For verification of novel finding, a second method such as polymerase chain reaction (PCR) with hybridization of the PCR product should always be performed.

PCR Assay

Numerous PCR assays have now been developed in a variety of resistance genes discussed in this review.[6–10] Each PCR assay uses a pair of primers, which can be the same as those used for DNA–DNA hybridization. The template may be purified DNA, proteinase-K lysates of whole bacteria, and/or direct patient materials. The PCR assay can use direct patient material, if from a normally sterile site, as the source of the template to determine whether a particular antibiotic resistance gene is present in the sample without growing the bacteria. Using nonsterile sites as source of material is not desirable because most of

the genes discussed in this review are commonly found in commensal species[8] and will be positive.

Initially, the PCR assays detected single genes. We then modified our two primers to look for *tet*(M)/*tet*(O)/*tet*(S) and the *tet*(K)/*tet*(L) PCR assays.[6,11] Which gene was amplified was determined by hybridization with an internal probe specific for only one of the three genes. More recently, multiplex PCR has been developed where each *tet* gene has it own set of primers and four to six different genes are detected in one reaction.[9,10] Each gene could be determined by the size of the PCR fragment. In all PCR assays, it is important to verify that the fragment visualized carries the correct sequences. Using one of these two methods is the only way to verify that the PCR fragment is correct. The multiplex PCR could be performed using a real-time PCR system, or even an automated system if available. Manual PCR assays are not well suited for quickly screening a large number (>100) of isolates per run. Both PCR and DNA–DNA hybridization methods can give false-negative results. To reduce the false-positive PCR assay, the author's laboratory uses an internally labeled oligonucleotide probe that is hybridized to the PCR products. This allows one to verify that the PCR product visualized is the correct one. Positive and negative controls are run with each assay. Reduction in PCR conditions may be used to identify related unknown genes. This is how the *tet*(37) gene was identified.[12]

Microarray

Microarrays offer a new method for screening for the presence of *tet* genes. In this format, the specific gene probes are placed onto a solid substrate in a lattice pattern. The test DNA is then labeled and hybridized to the array, and specific target–probe duplexes are detected using a reporter molecule. Recently, we developed and tested a glass-based microarray using 17 *tet* genes.[13] We initially tried to use the short probes we had developed for PCR and DNA–DNA hybridization (see above), but these were too insensitive to low copy number genes, which are usually found in natural isolates. They were able to detect cloned genes where there are high copy numbers of the target *tet* gene. We found that 550-bp PCR products used with purified DNA that was biotinylated were able to detect low copy numbers of *tet* genes.[13] Each probe was printed as four replicated spots within each array. The target DNA was grown and DNA was extracted with a DNeasy Kit (Qiagen). Other DNA extraction methods were not adequate. One microgram of target DNA was biotinylated using nick translation and then used. Detection used a Tyramide Signal Amplification, Alexa fluor 546, and microarray scanner. The detection threshold of three times the standard deviation above the standard negative signal intensity was set so that any signal over this threshold limit was called positive. Positive detection was confirmed either by PCR or DNA–DNA hybridization.

The prototype assay demonstrates the feasibility of developing a high-throughput planar microarray for the detection of *tet* genes. However, the current process needs to be automated before microarrays can be widely adopted. One advantage is that a variety of different probes ($\leq$10,000) can be incorporated on a single slide.

Mutation

Mutations that upregulate innate efflux pumps can alter the host's susceptibility. The mutation in the *marR* region of the chromosome enhances intrinsic resistance to a large group of antibiotics including tetracycline.[15] The level of tetracycline resistance increases as the cells are grown continually in the presence of tetracycline. Another example is *Neisseria gonorrhoeae*, which have an *mtrCDE*-encoded efflux pump. When there is a 1-bp deletion of an A within the 13-bp inverted repeat sequence of the *mtrR* promoter region, the host cell shows a fourfold increased resistance to tetracycline, penicillin, and erythromycin.[16] In *N. gonorrhoeae*, the chromosomally mediated resistance is often more common than plasmid-mediated acquired antibiotic resistance. Mutations in efflux pumps have been documented in a variety of pathogens and opportunistic species including *Burkholderia cepacia*, *Campylobacter jejuni*, *Escherichia coli*, *Enterobacter* spp., *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Stenotrophomonas maltophilia*.[1,18] Recently, 15 Tc^r clinical isolates [minimum inhibitory concentrations (MICs): 2–64 μg/mL tetracycline and 1–32 μg/mL doxycycline] of cutaneous propionibacteria were found to have a cytosine instead of guanine at position 1058 in the 16S rRNA.[17] This change was associated with increased tetracycline resistance. Mutations, which alter the permeability of outer membrane porins and/or lipopolysaccharides, can also affect bacterial susceptibility to tetracycline and other agents.[18] How often these mutations occur and whether they are of clinical importance have not been established. At this time, mutations occur by sequencing the region thought to contain the base pair change(s).

CONCLUSION

The emergence of bacterial resistance to tetracyclines mirrors the situation with most antimicrobials currently in use. These drugs are relatively inexpensive, have a broad-spectrum of activity, and have been extensively used in the prophylaxis and therapy of human and animal

infections and as animal growth promoters. The selection pressures exerted by the use of tetracyclines in these various environments have resulted in the emergence of resistant organisms. The first tetracycline resistance *R* factors were identified over 40 years ago in Japan.[1] Since then, tetracycline resistance genes have spread in both gram-negative and gram-positive genera, primarily by conjugal transfer of plasmids and/or transposons. The dramatic increase in the number of species and genera that have acquired tetracycline resistance since the 1950s has led to a reduction in the efficacy and use of current tetracycline therapy for many diseases.

As PCR and microarray technology become less expensive and more automated, it will be easier for more laboratories to look at the genotypes of antibiotic-resistant bacteria. However, this information will need to be shown to be clinically relevant and cost-effective. This scenario assumes that clinical laboratories will continue to culture bacterial samples, although there are some suggestions that nonculture techniques such as PCR could replace standardized culture methods in the coming years because these methods can be less costly. Regardless of where culture methods go at this time, it is inadvisable to look for specific tetracycline resistance genes in direct samples other then those taken from normally sterile sites because many commensal bacteria carry the same resistance genes as do the pathogens.[1]

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Thyroid Cancer—Detection of Tumor Cells by Molecular Methods

Jörg Bojunga
Stefan Zeuzem
Saarland University Hospital, Homburg/Saar, Germany

INTRODUCTION

Over the last years, new PCR-based techniques have improved the sensitivity and specificity of detection of primary and recurrent cancer in different types of neoplasms. In the field of endocrinology, thyroid cancer makes up more than 90% of all endocrine cancers and accounts for 63% of deaths from endocrine cancer, although thyroid neoplasms are rare, constituting only 1% of all cancer in the United States. Because most of initial diseases as well as recurrences can successfully be managed with surgical and/or radioiodine treatment, effective methods for early detection of thyroid cancer and disease recurrence are crucial.

RATIONALE FOR MOLECULAR DIAGNOSTIC TESTING IN PATIENTS WITH SUSPECTED THYROID CANCER

The initial workup of a thyroid nodule consists of ultrasonography and—if indicated—a fine-needle aspiration biopsy (FNAB). Despite the fact that cytological examination of FNAB by an experienced pathologist provides the most accurate and cost-effective means of diagnosing thyroid neoplasms, in some situations, cytological examination may not provide a conclusive diagnosis. For example, biopsy samples are cytologically found to be ''intermediate'' or ''suspicious,'' but most of these lesions are benign. In addition, preoperative diagnosis of lesions with follicular histology is difficult, and a consistent number of not otherwise specified ''follicular nodules'' are surgically resected more for diagnosis than therapeutic purposes. Because operation could be recommended only to those patients with a high risk of malignancy, the use of a molecular-based approach with detection of tissue- or tumor-specific mRNA transcripts may provide a more objective method to assure reliable diagnosis. As a consequence, molecular techniques for the diagnosis of cancer in thyroid nodules are most needed for patients in whom conventional FNAB cytology yields an indeterminate ''follicular neoplasm,'' patients who have either adenomas or low-grade carcinomas.

Besides the restrictions of preoperative FNAB mentioned above, postoperative surveillance of patients with thyroid cancer includes several limitations as well. Since the 1970s, serum thyroglobulin, especially when performed with TSH stimulation, is an established tumor marker of residual and recurrent differentiated thyroid carcinoma. However, serum thyroglobulin immunoassay results may be unreliable or uninterpretable in patients with antithyroglobulin antibodies, which can be detected in up to 20% of patients. Therefore a molecular-based, sensitive, and convenient screening test to diagnose recurrent thyroid cancer, not requiring TSH stimulation or the absence of thyroglobulin antibodies, would be useful.

PAPILLARY AND FOLLICULAR THYROID CARCINOMA

Galectin-3

Galectin-3 (GAL-3) is a β-galactosil-binding protein, which is involved in regulating cell–cell and cell–matrix interactions. In addition, galectins have been implicated in the initiation and regulation of cell growth and malignant transformation. Galectin-3 has been widely used as a marker of thyroid cancer in immunohistochemistry. Hereby normal thyroid tissue and benign nodules have been considered not to express GAL-3, while its expression has been demonstrated in malignant transformation of follicular cells. Using GAL-3 as an additional marker, most studies evaluating it as a marker in immunohistochemistry reported high sensitivity and specificity in differentiating malignant from benign thyroid disease for both FNAB-derived cytology and conventional histology.[1] However, there is some concern about the specificity of GAL-3 expression, because GAL-3 expression has been found in Hashimoto's thyreoiditis (HT) using immunohistochemistry, and even a ubiquitous expression of galectin-3 mRNA in benign and malignant thyroid tumors using RT-PCR has been

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described. Taken together, recent data indicate that a search for GAL-3 protein overexpression by immunohistochemical methods, but not by RT-PCR, may yield an additional marker of malignant potential of thyroid nodular lesions, and may be a useful adjunct to the currently available diagnostic tools for the preoperative diagnosis of malignant thyroid tumors.

CD44v6

CD44 is a polymorphic family of immunologically related integral membrane glycoproteins associated with cell–matrix adhesion, lymphocyte activation and targeting, and tumor growth and metastasis. Some variant isoforms, especially those containing sequences encoded by v6–v10, are overexpressed in several human neoplasms, including thyroid cancer, particularly in the papillary variant. Reports of successful differentiation of thyroid papillary carcinoma from other lesions by CD44 immunostaining of thyroid fine-needle aspirates were followed by studies using RT-PCR to detect CD44 mRNA in leftover cells inside the needle used for FNAB.[2] These studies suggested that thyroid tumors may be more accurately diagnosed by a combination of cytological examination and detection of CD44 variants in RNA obtained by FNAB, in particular when coexpression of CD44v6 and galectin-3 was detectable. However, CD44 exons were also present in transcripts in a high proportion of benign samples. Taken together, in thyroid cancer the clinical applicability of CD44v detected by RT-PCR still has to be determined, in particular in the light of the excellent specificity and sensitivity of FNAB conventional cytology in papillary thyroid carcinoma (PTC).

Oncofetal Fibronectin

Oncofetal fibronectin has been proposed as a possible tool for the preoperative diagnosis of thyroid carcinomas, based on the finding that the expression of oncofetal fibronectin mRNA was significantly increased in papillary and anaplastic carcinomas, compared to normal thyroid tissues and follicular adenomas. Several reports using oncofetal fibronectin as a marker of malignancy showed that combined examination using both RT-PCR-based and cytological approaches may contribute to a more precise preoperative diagnosis of papillary and anaplastic carcinomas.[3] However, other groups found that oncofetal fibronectin overexpression detected by immunohistology, but not RT-PCR, may yield an additional marker of papillary and anaplastic carcinomas.[4] Taken together, this particular marker is unlikely to benefit patients with ''follicular neoplasms'' and most of papillary lesion, although it may be helpful in diagnosing patients with papillary lesion and limited cellularity.

RET/PTC Rearrangements

RET activation has only been found in PTC tumors and is therefore called RET/PTC. Activated forms of the RET proto-oncogene are the consequence of oncogenic rearrangements fusing the tyrosine kinase domain of the RET gene with the 5′ domain of different genes. Therefore several variants of RET/PTC have been observed, the three main types designated as RET/PTC1, 2, and 3. Two other types of RET rearrangements (RET/PTC4 and 5) have also been described recently. In most populations, generation of the fusion transcripts is identified in almost half of PTC lesions.

One study examined thyroid aspirates for the presence of RET/PTC gene rearrangements by RT-PCR for the three most common ret/PTC gene rearrangements (RET/PTC1, 2, and 3). In this study, no false-positive results were obtained. In addition, the identification of RET/PTC gene rearrangements refined the diagnosis that would otherwise have been considered indeterminate or insufficient for cytological diagnosis in several cases.[5] Another study reported RET/PTC activation, in particular the RET/PTC1 isoform, in a significant number of cases of Hurthle cell adenomas and carcinomas, but not in hyperplastic nodules.[6] However, RET/PTC rearrangements have been found in benign nodules as well. In addition, on the basis of a growing number of RET/PTC oncoproteins, evaluation of only one, two, or three isoforms appears to be incomplete in most cases.

MEDULLARY THYROID CARCINOMA

In medullary thyroid carcinoma (MTC), laboratory tests for preoperative diagnosis and postoperative follow-up are performed using basal as well as pentagastrin-stimulated serum calcitonin (CT), which is supplemented by estimation of serum levels of carcinoembryonic antigen (CEA).

In contrast to papillary and follicular thyroid carcinoma, to date only a few studies have been published concerning the preoperative diagnosis of MTC using PCR-based methods. The reason for that discrepancy may be that patients with MTC are usually readily diagnosed by conventional cytology and serum calcitonin measurements. One study reported the preoperative diagnosis of MTC by RT-PCR using RNA extracted from leftover cells of FNAB.[7] As transcripts specific for MTC, calcitonin, carcinoembryonic antigen, and RET proto-oncogene mRNA, respectively, were used. In another study using identical primer sequences, calcitonin mRNA was found in all samples of FNAB derived from patients with MTC, but not in samples from patients with follicular adenoma and follicular/papillary carcinoma, respectively.[8] On the other hand, all MTC patients in these studies presented

with ''high levels'' of serum calcitonin, whereas all of the non-MTC patients but one had serum calcitonin levels below the threshold. Therefore, RT-PCR-based assays did not add any additional information to the measurement of serum calcitonin alone.

MOLECULAR ANALYSES OF PERIPHERAL BLOOD AND POSTOPERATIVE SURVEILLANCE OF THYROID CARCINOMAS

Papillary and Follicular Thyroid Carcinoma

The application of RT-PCR to detect circulating thyroid-specific mRNA in recurrent thyroid cancer was first reported in a study in which all patients with thyroid carcinoma and metastases were tested positive for circulating thyroglobulin (TG) mRNA, whereas patients without metastases were tested negative.[9] In a subsequent study,[10] circulating mRNA transcripts of TG, thyroid peroxidase (TPO), and RET/PTC1 were used as tumor markers in patients with thyroid disease. Although there was a correlation between existence of these transcripts in peripheral blood and diagnosis of thyroid carcinoma, TG and TPO mRNA transcripts could be detected in the peripheral blood of all control subjects as well as in several human cell lines when PCR assay sensitivity was increased. Finally, an optimistic study was published,[11] which reported an RT-PCR assay to detect blood-borne TG mRNA that was more sensitive than the conventional TG serum assay. However, in the study of the authors,[12] only a weak correlation between the detection of circulating TG mRNA and the diagnosis of thyroid cancer was found with low assay sensitivities, whereas at high RT-PCR sensitivity, TG-mRNA expression was found not to be specific for thyroid tissue and was not correlated with a diagnosis of thyroid cancer in patients. The conclusion was that TG mRNA is not a tissue-specific marker, which could be confirmed by testing different human tissues for TG mRNA expression. At low assay sensitivities, thyroglobulin mRNA expression was exclusively found in thyroid tissue, whereas at high sensitivities, thyroglobulin mRNA expression was not only limited to thyroid tissue but was also found in lymphocytes as well as in several other tissues.

Our finding of missing tissue specificity of TG mRNA has been reproduced by many other groups (see below), and now there is a bright spot of evidence that detection of TG mRNA in peripheral blood might reflect detection of illegitimate transcription in cell types of nonthyroid origin—e.g., lymphocytes—as it has been described earlier, and circulating TG mRNA is not a reliable tumor marker for the follow-up of patients with thyroid cancer.[13–15]

Medullary Thyroid Carcinoma

Searching for local relapse or distant metastases in medullary thyroid carcinoma is often very difficult and includes laboratory tests as mentioned above as well as selective venous catheterization, ultrasound, octreotide scan, computer tomography, and magnetic resonance imaging. Therefore molecular detection of circulating tumor cells may again represent a new approach in order to improve early diagnosis of metastasized MTC.

One study has been published which has explored blood-borne cytokeratin 20 mRNA as a potential tumor marker in thyroid cancer, especially MTC.[16] However, the limitation of this study was that the tissue specificity of the transcripts was not evaluated, and, as others have shown, the detection of micrometastasis by cytokeratin 20 RT-PCR is limited because of stable background transcription in granulocytes. Another study[17] investigated whether circulating tumor cells can be detected in the peripheral blood of patients with MTC by RT-PCR targeted to CT mRNA and whether the results of this method are correlated with disease manifestation and metastatic potential. Although the authors concluded that the results of the RT-PCR-based procedure seem to reflect tumor spread and aggressiveness and thus may help with early identification of patients with disseminated and rapidly progressive disease, these results have not been confirmed yet. In the study of the authors,[18] RT-PCR on blood samples from patients diagnosed with MTC disease was performed using primers specific for CT and CEA, respectively. Because there was no significant correlation between CT/CEA mRNA status and clinical staging, and, furthermore, comparable limitations as mentioned in the chapter above have to be expected, we could not recommend circulating CT/CEA mRNA as a tumor marker in MTC.

FUTURE PERSPECTIVES

In the preoperative diagnosis of thyroid cancer, several tissue- and/or tumor-specific mRNAs have been described. Molecular techniques for the diagnosis of cancer in thyroid nodules are most needed for lesions with follicular histology on FNAB, lesions which are either adenomas or low-grade carcinomas. To prove significant clinical utility for new molecular diagnostic techniques, the sensitivity and positive predictive value of these methods must be demonstrated. In several cases published, a higher sensitivity and specificity could be achieved using molecular techniques compared to ''conventional''

methods. However, new immunohistochemical markers have markedly enhanced the differential diagnostic accuracy between benign and malignant thyroid neoplasms, in particular galectin-3, which has been shown to be an important preoperative marker of thyroid malignancy and, in addition, seems to be a reliable presurgical marker to differentiate between follicular adenomas and follicular carcinomas as well. Especially in medullary carcinoma, the existing serum protein markers—calcitonin and CEA—and conventional cytology are quite sensitive for establishing the diagnosis. Reverse transcription-polymerase chain reaction-based methods have to be significantly more sensitive and specific to rationalize their considerably higher costs and expenditure of energy and time compared to established methods. To date, this has not been proven.

In the postoperative follow-up of patients with thyroid cancer, conflicting data have been published and the use of PCR techniques revealed several problems of the molecular approach. These problems are based on some technical as well as biological limitations.

First, most of the technical problems of the initial studies derived from the nonquantitative, but only qualitative, approach. Using ''low'' assay sensitivities, a correlation between PCR result and metastases of thyroid cancer could be observed, whereas at ''high'' assay sensitivities, nonspecific results were obtained and illegitimate transcription in blood cells was suspected as a cause. Additional studies using quantitative assay format initially seemed to circumvent this problem of qualitative RT-PCR assays and optimistic reports were published. However, recent studies using a quantitative assay provide evidence for the existence of thyroid-specific mRNA in peripheral blood of all patients, irrespective of their thyroidal status. These results give rise to a question regarding the clinical applications of not only qualitative RT-PCR detection but also quantitative measurement of tumor-specific mRNA in peripheral blood. Furthermore, many TG mRNA RT-PCR assays had a poor specificity and low positive predictive value. To date, no standardized RT-PCR assay exists and many different results between the groups may therefore simply reflect technical differences between the assays. For medullary carcinoma, the existing serum protein markers are quite sensitive and the rationale for the use of RT-PCR-based methods is questionable. Now before considering the clinical application of these methods, an intensive and complete reevaluation is obligatory.

Second, biological limitations of molecular assays have to be considered. A prerequisition for a reliable assay detecting circulating tumor cells is that these cells indeed circulate continuously in peripheral blood. However, there is experimental evidence of intermittent release of tumor cells in the bloodstream, and analyses of different blood samples taken at different times indicate that tumor cells persist only transiently in peripheral blood. An additional limitation of the molecular approach is that the presence of circulating tumor cells does not necessarily lead to the development of clinically significant metastatic disease. Several studies have shown that the metastatic process in humans is inefficient on both a temporal and numeric basis, and this phenomenon has been termed ''metastatic inefficiency.'' The vast majority of tumor cells that enter the bloodstream are killed by immunological, mechanical, and unknown mechanisms, and the lungs as well as the liver are the major sites responsible for this ''metastatic inefficiency.'' As a consequence, the amount of tumor cells circulating in the peripheral blood is considerably altered by these mechanisms.

CONCLUSION

Polymerase chain reaction-based techniques may improve the preoperative diagnosis and staging of patients with thyroid malignancies and therefore help to avoid unnecessary radical procedures. However, there is a growing number of literature on the use of immunostaining markers for the preoperative diagnosis of thyroid cancer. To date, the studies published do not give good reasons for the use of technically more demanding and probably less specific RT-PCR assays. In the follow-up of patients with thyroid cancer, a reliable PCR-based assay that predicts relapse or metastases is to date—in view of the many conflicting data that have been published—not available. Nevertheless, molecular detection of tumor cells is likely to have an impact on the practice of thyroid malignancies in the future.[19]

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Tissue Microarrays in Pathology

Guido Sauter
Ronald Simon
Martina Mirlacher
University of Basel, Basel, Switzerland

INTRODUCTION

Advances in molecular medicine and biology have fundamentally changed biomedical research strategies. For example, the development of the DNA microarray technology, in conjunction with the completion of the human genome sequencing project, now enables researchers to analyze many human genes, in a highly parallel manner, on both the DNA and RNA levels. As a consequence, the rate of discovery of new genes involved in cancer and other diseases has increased significantly. However, a major drawback of the DNA array technology is the need for unfixed tissues and its enormous expense, which hampers comprehensive studies including statistically relevant sample sizes. In addition, such kinds of analyses require disintegrated tissues, obliterating information on the tissue distribution of marker molecules. Therefore, evaluation of the clinical importance of promising candidate genes is optimally performed using histological sections of diseased and nondiseased patient tissues. However, hundreds to thousands of tissue samples, optimally with attached follow-up data, must be analyzed to yield statistically sound results. This leads to a massive workload in involved pathology laboratories. Moreover, the analysis of multiple genes results in a critical loss of precious tissue material because the number of conventional tissue sections that can be taken from a tumor block usually does not exceed 200–300. To overcome the limitations of conventional tissue analysis, we developed the tissue microarray (TMA) technology, which significantly facilitates and accelerates in situ analyses. In this method, minute tissue cylinders (diameter: 0.6 mm) are removed from hundreds of different primary tumor blocks and subsequently brought into one empty ''recipient'' paraffin block. Sections from such array blocks can then be used for simultaneous in situ analysis of hundreds to thousands of primary tumors on DNA, RNA, and protein levels.

TECHNICAL DESCRIPTION

Preparatory Work

It is important to realize that most of the work (approximately 95%) devoted to TMA manufacturing is traditional pathology work that cannot be accelerated by machinery. This preparatory work is similar to what is needed for traditional studies involving ''large'' tissue sections, apart from the number of tissues involved, which can be an order-of-magnitude higher in TMA studies than in traditional projects. The preparatory work includes:

1. Identification of potentially suited tissues.
2. Review of all sections from all candidate specimens to select the optimal slide.
3. Definition of the structure (outline) of the TMA, including generation of a file that contains the identification numbers of the tissues together with their locations and real coordinates (Fig. 1 and Table 1).

Recipient Block Preparation

In contrast to normal paraffin blocks, tissue microarray blocks are cut at room temperature. Therefore a special type of paraffin is needed with a melting temperature between 55 and 58°C (''Peel-A-Way'' paraffin, Polysciences Inc., Pennsylvania, USA). The paraffin is melted at 60°C, filtered, and poured in a stainless steel mold (e.g., $30 \times 45 \times 10$ mm). A slotted plastic embedding cassette (as used in every histology laboratory) is then placed on the top of the warm paraffin.

TMA Manufacturing

TMA manufacturing is a simple three-step procedure that is repeated for each sample placed on the TMA:

1. Making a hole in an empty (recipient) paraffin block.

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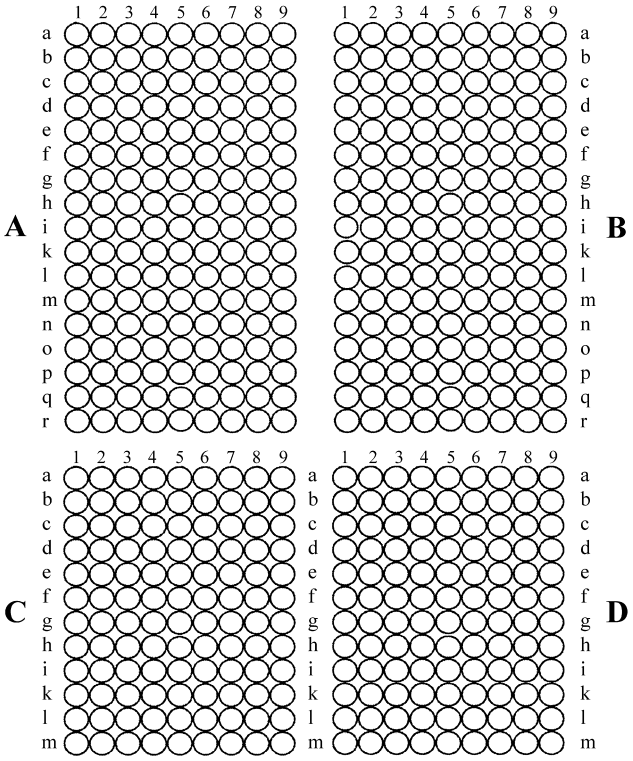

Fig. 1 TMA outline example. The TMA has been divided into four subsections to facilitate navigation during microscopy.

2. Taking a cylindrical sample from the tissue sample (donor) paraffin block.
3. Placing the cylindrical tissue sample in the premade hole in the recipient block.

Commercially available or various kinds of homemade tissue-arraying systems are used for TMA manufacturing. Exact positioning of the tip of the tissue cylinder at the level of the recipient block surface is crucial for the quality and the yield of the TMA block. Placing the tissue too deeply into the recipient block results in empty spots in the first sections taken from the TMA block. When the tissue cylinder is not positioned deep enough, empty spots are created in the last sections taken from this TMA. If tissue cylinders protrude, they may be gently pressed deeper into the prewarmed TMA block (40°C for 10 min) by using a glass slide.

Array Sectioning

TMAs can be sectioned like standard paraffin blocks by using regular microtomes. A special tape sectioning kit (Instrumedics Inc., NJ, USA) may be utilized to facilitate cutting, especially for large TMAs. The tape system leads to highly regular nondistorted sections (which are ideal for automated analysis) and helps to prevent arrayed samples from floating off the slide during incubation and washing steps.

TMAs from Frozen Tissues

TMAs may also be manufactured from frozen tissues.[1] Here recipient blocks are made from OCT compound (Sakura Finetek, the Netherlands) that is frozen down in a Tissue-Tek standard cryomold. The resulting OCT block is mounted on top of a plastic biopsy cassette. During the

Table 1 Example file for TMA construction

Location	Coordinates	Location	Coordinates	Location	Coordinates
A 1a	0/0	A 2a	0/800	A 3a	0/1600
A 1b	800/0	A 2b	800/800	A 3b	800/1600
A 1c	1600/0	A 2c	1600/800	A 3c	1600/1600
A 1d	2400/0	A 2d	2400/800	A 3d	2400/1600
A 1e	3200/0	A 2e	3200/800	A 3e	3200/1600
A 1f	4000/0	A 2f	4000/800	A 3f	4000/1600
A 1g	4800/0	A 2g	4800/800	A 3g	4800/1600
A 1h	5600/0	A 2h	5600/800	A 3h	5600/1600
A 1i	6400/0	A 2i	6400/800	A 3i	6400/1600
A 1k	7200/0	A 2k	7200/800	A 3k	7200/1600
A 1l	8000/0	A 2l	8000/800	A 3l	8000/1600
A 1m	8800/0	A 2m	8800/800	A 3m	8800/1600
A 1n	9600/0	A 2n	9600/800	A 3n	9600/1600
A 1o	10400/0	A 2o	10400/800	A 3o	10400/1600
A 1p	11200/0	A 2p	11200/800	A 3p	11200/1600
A 1q	12000/0	A 2q	12000/800	A 3q	12000/1600
A 1r	12800/0	A 2r	12800/800	A 3r	12800/1600

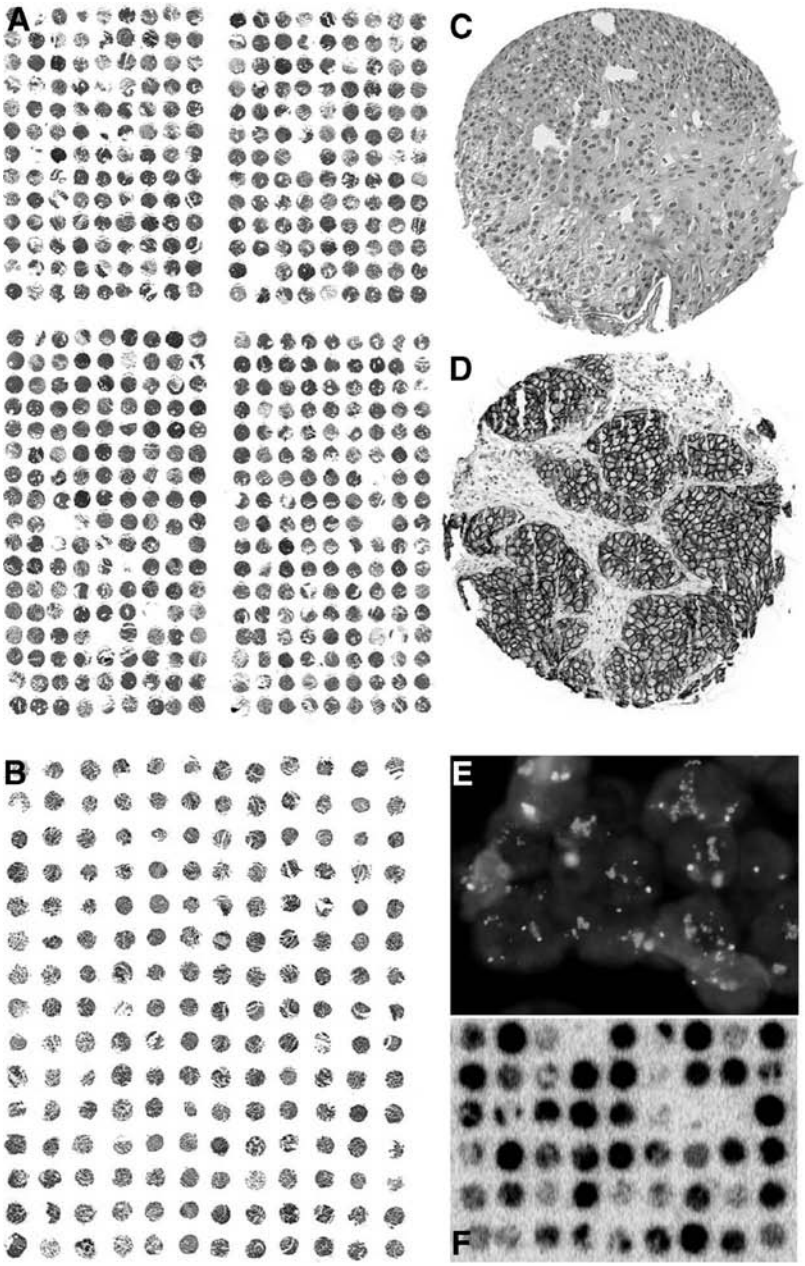

Fig. 2 Examples of stained tissue sections. Hematoxylin & eosin (H&E)-stained sections of (A) a TMA from formalin-fixed, paraffin-embedded tissues containing 540 tissue spots and (B) a TMA from frozen tissue containing 228 tissue spots. Each tissue spot measures 0.6 mm in diameter. Missing samples result from the sectioning/staining process or indicate samples that are already exhausted. Note that the distance between spots is larger on the frozen TMA compared to the paraffin TMA. (C) Magnified view of an H&E-stained 0.6-mm tissue spot of a bladder carcinoma. (D) Immunohistochemistry against the Her2/neu protein in a breast cancer sample using the DAKO HercepTest®. (E) FISH analysis of centromere 17 (green signals) and the *HER2* gene (red spots) in cell nuclei (blue staining) of a tissue spot (630×). The high number of HER2 signals indicates a gene amplification. (F) RNA in situ hybridization on a frozen TMA made from normal and malignant kidney tissues. A radioactively labeled oligonucleotide was used as a probe against vimentin mRNA. The black staining intensity indicates the level of mRNA in each tissue spot. (*View this art in color at www.dekker.com.*)

arraying process, it is important to keep the tissue in the needle frozen. This can be carried out by cooling the needle with a piece of dry ice before punching, and while dispensing the tissue core into the recipient block, 4- to 10-μm sections of the whole block are cut from the array block, using a cryostat microtome with or without a Tape Transfer System and slides.

REPRESENTATIVITY OF TMAs

An important concern connected to the TMA technology is the question whether or not ''tiny'' tissue cores measuring less than 0.6 mm in diameter are representative of an entire tumor.[2–6] To date, at least 20 studies have addressed this question, comparing immunohistochemistry (IHC) findings on TMAs with the corresponding traditional ''large'' sections (e.g., Refs. [2–5,7]). Most of these studies reported a high level of concordance of results, and concluded that the inclusion of more than one tissue core per donor block further increases the concordance. For example, Camp et al. studied the expression of ER, PR, and Her2 in 2–10 tissue cores obtained from the same donor blocks in a set of 38 invasive breast carcinomas. They found that analysis of two cores was sufficient to obtain identical results, compared to the corresponding whole tissue sections in 95% of cases. A concordance of 99% is reached if four cores were analyzed, and analysis of additional cores did not result in a significant further increase of concordance.[2]

However, it is important to note that both large sections and TMA tissue cores represent only a very small fraction of the tumor bulk from which they derive. Perhaps, even more important than comparing individual results obtained on large sections and on TMA sections, is the question of whether or not the whole tumor can be represented by any type of size-limited histological samples such as TMAs or large sections.

APPLICATIONS OF TMA TECHNOLOGY

In principle, all types of research requiring in situ tissue analysis can be performed in a TMA format. For example, TMA sections have been used for IHC, fluorescence in situ hybridization (FISH), or RNA in situ hybridization (Fig. 2).[8,9] To date, TMAs have mostly been used for cancer research, but there are also many applications in other research fields. Depending on the aim of a particular analysis, oncology TMAs may be divided into four groups: prevalence TMAs, progression TMAs, prognostic TMAs, and TMAs composed of experimental tissues.

1. **Prevalence TMAs** are assembled from tumor samples of one or several types without attached clinicopathological information. These TMAs are useful to determine the prevalence of a given alteration in tumor entities of interest. A typical example of a prevalence TMA has been reported by Schraml et al.[10] The TMA containing 4788 different samples from 130 different tumor types has been used for the analysis of multiple different markers on the DNA and protein level, including FISH and IHC analysis of cyclin E amplification and overexpression. Cyclin E amplification was detected in 15 different tumor types and Cyclin E protein accumulation in 48 tumor types.
2. **Progression TMAs** contain samples of different stages of one particular tumor type. They are instrumental in uncovering associations between tumor genotype and phenotype. For example, an ideal breast cancer progression TMA would contain samples of normal breast from patients with and without breast cancer history, several different nonneoplastic breast diseases, ductal and lobular carcinoma in situ, invasive cancers of all stages, grades and histologic subtypes, as well as metastases and recurrences after an initially successful treatment.
3. **Prognosis TMAs** contain samples from tumors with available clinical follow-up data. They represent a fast-and-reliable platform for the evaluation of the clinical importance of newly detected disease-related genes. Validation studies using prognosis TMAs readily reproduced all established associations between molecular findings and clinical outcome. For example, significant associations were found between estrogen or progesteron expression[11] or HER-2 alterations[12] and survival in breast cancer patients, between vimentin expression and prognosis in kidney cancer,[13] and between Ki67 labeling index and prognosis in urinary bladder cancer,[5] soft tissue sarcoma,[14] and in Hurthle cell carcinoma.[15]
4. **Experimental TMAs** may be constructed from tissues such as cell lines[16,17] or xenografts.[18] Cell line TMAs are optimally suited for screening purposes, e.g., the rapid identification of cell lines with a specific genotype. Selected cell lines can then be grown and, e.g., utilized for testing potentially inhibiting drug candidates.

AUTOMATION

TMAs are optimally suited for large-scale expression profiling projects. Automation of the interpretation is the absolute key issue for such projects. In principle, TMAs

are highly suited for automated analysis. The greatest difficulty for automated tissue analysis, the selection of an appropriate tissue area, has been solved to a large extent during TMA manufacturing. Several systems enabling automated TMA analysis have recently been introduced. Some of them utilize fluorescent dyes for immunostaining because of the better dynamic range compared to peroxidase-based systems and the potential for multicolor analyses. However, fluorescence-based immunostaining is limited by the complex staining protocols for multicolor applications and the difficult manual reevaluation of questionable staining results. Most commercial products for automated TMA analysis, therefore, concentrate on conventionally immunostained TMA sections. These are generally based on an automated microscope with a charge-coupled device (CCD) camera. An overview image is used to identify the localization of each tissue spot in the TMA for subsequent high-resolution scanning. Studies have shown, that such ''low-tech'' solutions quantifying the total signal intensity per TMA spot are easy to establish and surprisingly efficient for identification of clinico-pathological associations.

CONCLUSION

The TMA methodology is now an established and frequently used tool for tissue analysis. The equipment is affordable and easy to use in areas where basic skills in histology are available. Basically, all kinds of in situ analyses, such as IHC, in situ hybridization, and in situ PCR assays, may be adapted to TMAs with only slight (if any) modifications of the respective large section protocols.

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Treponema pallidum, T. pertenue, T. endemicum, and *T. carateum*

Hsi Liu
Centers for Disease Control and Prevention, Atlanta, Georgia, U.S.A.

INTRODUCTION

The genus *Treponema* includes pathogenic and saprophytic spirochetes. Four of the treponemes are human pathogens, and three of these are *Treponema pallidum* subspecies: subspecies *pallidum* (*T. pallidum*) causes venereal syphilis; subspecies *pertenue* (*Treponema pertenue*) causes yaws; and subspecies *endemicum* (*Treponema endemicum*) causes endemic syphilis, or bejel. The three subspecies of *T. pallidum*, with identical morphologies and similar antigenic properties and genomic structures, cannot be distinguished based on current serologic or genetic methods. The diseases are categorized according to their clinical presentations and the geographic distributions. *Treponema carateum*, the causative agent of pinta, remains classified as a separate species because of insufficient genetic information to determine whether it too is a subspecies of *T. pallidum*. Current efforts to control these diseases are focused on combining molecular and serologic methods with epidemiological surveillance and treatment.

T. PALLIDUM

T. pallidum causes venereal syphilis, also called ''the great imitator'' because the clinical features are similar to those of many other diseases. Untreated, syphilis progresses through several distinct stages. Following exposure and an incubation of 9 to 90 days, the primary stage becomes apparent and is characterized by a painless skin lesion called a chancre, and sometimes by regional lymphadenopathy. It is believed that *T. pallidum* is disseminated through the bloodstream within hours after initial contact with an infected person.[1–3] Chancres, which contain millions of organisms, usually heal spontaneously.[3] The secondary stage of the disease is characterized by generalized mucocutaneous lesions and lymphadenopathy. This stage is followed by a latent period, during which the infection can be detected only by serological tests. If the infection is left untreated, approximately one third of infected individuals will develop tertiary syphilis, characterized by gummatous lesions of any organ of the body including the cardiovascular or the nervous systems. Infants born to mothers infected with *T. pallidum* may contract congenital syphilis as a result of transplacental transmission. Congenital syphilis can be classified into distinct stages similar to those of acquired syphilis.

Epidemiology

In many industrialized countries, the number of syphilis infections has declined significantly. In the United States, the rate of infection so far in 2003 is fewer than four cases per 100,000 population; however, the number of cases is expected to fluctuate, and syphilis remains a significant health problem in many major U.S. cities. Meanwhile, the disease remains endemic in many other parts of the world. The World Health Organization in 1999 reported 12 million new cases worldwide, with some regions showing a significant increase in prevalence.[4] For example, the Russian Federation experienced a 26-fold increase of congenital syphilis from 1991 to 1999.[5] With current high rates of international travel, STD infections in one endemic region may easily become a significant problem in other parts of the world.[6] Concurrent HIV infection also alters the natural history of syphilis. Rapid progression from primary syphilis to neurosyphilis has been observed in many coinfected individuals. While no penicillin-resistant strains of *T. pallidum* have been reported, erythromycin-resistant *T. pallidum* has been described and results from a single base pair mutation in both copies of the 23S rRNA gene.[7]

Structure, Biochemistry, and Genomics

Treponemes are spiral in shape, 6 to 15 μm in length and 0.1 to 0.2 μm in diameter. They contain two membranes: the inner cytoplasmic membrane and an outer membrane, which is unique in its lack of membrane proteins and lipopolysaccharides. An endoflagella is located in the periplasmic space between the two membranes.

Use of trade names is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the Public health Services, and the Centers for Disease Control and Prevention.

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T. pallidum cannot be continuously grown in culture; however, up to a 2000-fold expansion has been achieved when treponemes are inoculated into eukaryotic cell cultures in the presence of serum, 1.5–5% O_2 and N_2.[8,9] The optimal temperature for in vitro growth is 33–35°C; this limited temperature range may result from the presence of heat-labile enzymes such as 3-phosphoglycerate mutase and may be regulated by minerals such as manganese.[10]

The complete genome of *T. pallidum* has 1.138 million base pairs, encoding an estimated 1041 open reading frames, and is relatively small compared to the genome of *Escherichia coli*.[9,11] The genome lacks almost all the pathways for fatty acid, amino acid, and nucleotide biosynthesis, but the genome sequence also reveals 18 ABC transporters, which may be important for acquiring these nutrients from the host environment. The genome lacks respiratory components or obvious pathogenic factors or plasmids and contains five possible hemolysins and many flagellar genes.

Analysis of the genome indicates a strong codon usage bias toward G and in the leading strand. Proteins made from the lagging strand are high in arginine but low in lysine content.[12] In addition, the DNA metabolism-related proteins contain twice the amount of cysteine as cell envelope proteins.[13]

Genetic Polymorphism and Molecular Typing

Despite the fact that pathogenic treponemes are closely related, a recent molecular system, based on two genes, *arp* (AF 411124) and the *T. pallidum* repeat gene (*tpr*; 8), has been used to type clinical isolates of *T. pallidum*.[14–16] The *arp* gene varies in the number of repeats of a 60-bp sequence, with clinical isolates demonstrating variations from 4 to 22 repeats. The *tpr* gene family consists of 12 genes (A to L). The typing system uses primers that amplify the *tpr* E, G, and J genes followed by *Mse I* endonuclease digestion and the RFLP patterns of the *tpr* genes. The system has been successfully used in several endemic regions and in outbreak situations in industrialized countries. However, this molecular typing method cannot distinguish between subspecies of *T. pallidum*. Other members of the *tpr* gene family (*tpr D* and *K*) have shown genetic polymorphism and multiple alleles, but may not be suitable for development into a typing system.[17,18]

Paster et al.[19] differentiated *T. pallidum* from oral and other spirochetes using a dendrogram generated from the 16S rRNA sequence, relying on a single base pair change. Several genes have been reported to have single base pair mutational differences when comparing *T. pallidum* with other nonvenereal treponemes.[20,21] However, unless the functions of the genes are known, a single base pair mutation alone may not be sufficient for typing purposes.

Recent Progress in Laboratory Tests for *T. pallidum*

An experienced clinician and a suitable laboratory test are essential for the diagnosis of syphilis. Two types of serologic tests are available: the nontreponemal tests, such as the VDRL and RPR tests, are used for screening, and the treponemal tests, such as TP-PA and FTA-ABS tests, are used as confirmation. Unfortunately, serological tests cannot distinguish active from previously treated or past infection. However, they remain the only means available to diagnose latent disease. One of the current goals is to develop treponemal tests utilizing cloned protein antigens.

Alternative diagnostic methods include direct detection of treponemes or specific nucleic acid sequences. The gold standard of direct detection of *T. pallidum* is the rabbit infectivity test (RIT). The RIT has a sensitivity of a single organism but is cumbersome, expensive, and time-consuming, requiring access to an animal facility, and takes 1 to 2 months to complete the test. Thus, it is not recommended for use as a routine laboratory test. Alternatively, treponemes can be visualized in lesion preparations by dark-field microscopy or direct fluorescent antibody tests (DFA-TP). Dark-field microscopy and DFA-TP tests have a sensitivity of approximately 10^5 organisms/mL in the lesion preparation.

Nucleic acid amplification tests (NAAT) such as the polymerase chain reaction (PCR), which amplifies and detects the presence of the nucleic acids of the organisms (dead or alive), are the newest laboratory tests developed in the past 15 years. Any segment of the genome of *T. pallidum* can be used to design primers for PCR. The primers, however, must be tested for specificity and sensitivity and validated using clinical specimens. Currently, six gene targets (*tpf*-1, *bmp*, *tmpA*, *tmpB*, 47-kDa protein gene, and *pol A*) and one RNA target (16s rRNA) have been reported as potential clinical tests. The analytic sensitivity of the targets varies from 10^{-3} of the 16S rRNA, because of multiple copies,[22] to one copy per reaction in the *pol A* PCR using the ABI 310 genetic analyzer.[23] The PCR primers designed from the 16S rRNA have the highest sensitivity; however, the method is complex, and it is difficult to control the specificity because primers' sequences resemble other species' 16S rRNA sequences. The primers for the 47-kDa protein gene (TP574) have been extensively tested. The *pol A* (TP105) PCR has been used in multiple samples, is relatively robust, and is sensitive to a single organism.[23] A combination of 47-kDa protein gene and *pol A* PCRs is recommended for use together as a diagnostic PCR test.

A newer generation of thermocyclers permit real-time PCR testing combined with microfluidic techniques, simplifying sample preparation to a single injection and making results available within 30 min. It is likely that a real-time PCR test will become the standard screening test for syphilis in the near future.

Nonvenereal Treponematoses

The three nonvenereal treponematoses have distinct clinical courses and can be separated into early and late stages similar to those of syphilis. Some excellent reviews on the characteristics of the diseases are available.[24–26]

T. PERTENUE

T. pertenue causes yaws (also known as frambesia, bouba, pian, parangi, or paru). The disease is transmitted nonsexually, by direct skin contact, with most patients infected in childhood. Yaws is characterized by skin ulceration and sometimes bone involvement. Yaws affects populations in humid tropical parts of West and Central Africa, in Southeast Asia, and in some Pacific Islands (Table 1). Sporadic infections are found in South America, primarily in Colombia, Venezuela, Haiti, and Brazil. However, the effects of intervention can be dramatic. For example, a 1988 survey performed in northern Ecuador found that 16.5% of the population had clinical symptoms of yaws and that 96.3% of the population was seropositive. As of 1998, after 10 years of community intervention, the TP-PA seropositivity rate had fallen to 3.5% in the same region, with no clinical yaws cases reported recently.[27] Although sporadic cases in rural areas or in the tropical rain forests still occur, this report illustrates that an intensive prevention and treatment program can eliminate yaws.

Table 1 Characteristics of treponemes and geographic distribution

Disease	***T. pallidum*** **syphilis**	***T. pertenue*** **yaws**	***T. endemicum*** **bejel**	***T. carateum*** **pinta**
Natural infection in host				
Systemic	+	±	–	–
Skin involvement	+	+	+	+
Bone involvement	+	+		
Sexually transmitted/congenital	+	–	–	–
Geographic distribution	Cosmopolitan	Warm humid tropics	Arid subtropical or temperate	Warm arid tropical Americas
South America				
Brazil, Venezuela, Colombia, Peru, and Ecuador		+		+
Mexico, Haiti		+		+
Africa				
Ghana, Benin, Togo, Central African Republic, Nigeria, Cote d'Ivoire, Botswana		+		
Sahel countries (Mali)			+	
Middle East				
Saudi Arabia, Syria, Iraq			+	
Pacific Island				
Papua New Guinea		+		
Southeast Asia				
Indonesia		+		
Europe		Bosnia and Herzegovina, Bulgaria, Botswana	Yugoslavia	
World Wide	+			
Laboratory animals				
Rabbits	+	+	+	–
Hamster	+	+	+	–
Guinea pigs	+	–	+	–
Primates	+	+	+	+

T. ENDEMICUM

T. endemicum causes endemic syphilis, or bejel [also known as Skrljevo disease, Rijeka (Fiume), or Grobnik disease]. Bejel is characterized by mucous, skin, or osseous lesions, and is found mainly in dry and arid or temperate areas of Africa and the Middle East (Table 1). However, it has been eradicated in many locations. Bejel is transmitted by direct contact or contaminated fomites, and it mainly affects children 5 to 12 years old. Like yaws and pinta, bejel affects rural populations with poor sanitation and hygiene.

T. CARATEUM

T. carateum causes pinta (also known as mal de pinto, azul, or carate). Pinta is characterized by primary skin lesions, which may last for years; a latent period may follow, or secondary, disseminated lesions, called pintides, may appear and may persist for the patient's life. Serologic tests do not become positive until after secondary lesions have appeared. Pinta was once endemic in much of Central and South America, and remains prevalent in remote rural populations (Table 1). *T. carateum* can be propagated only in primates; thus, no organisms are currently available for genetic studies.

CONCLUSION

Despite the fact that treponemal infections can be easily cured by penicillin treatment, the diseases caused by pathogenic treponemes remain a global problem. Prevention efforts must be focused on finding and treating active cases. A better understanding of the biology of these organisms and improved diagnostic methods are also important. By combining inexpensive diagnostic methods, including extensive screening and effective treatment, global elimination of treponemal infections is possible.

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Trichinella spp.

Edoardo Pozio
Istituto Superiore di Sanità, Rome, Italy

INTRODUCTION

Nematodes belonging to the genus *Trichinella* are the etiological agents of trichinellosis in humans, who acquire the infection by ingesting raw or undercooked meat and meat products from various types of animals. In animals, these parasites have a cosmopolitan distribution on all continents but Antarctica. According to the World Health Organization, the global prevalence of trichinellosis is around 10 million.

BIOLOGY

The adults of *Trichinella* parasites are small worms (1–2.5 mm in length and 0.025–0.038 mm in width), which live in the mucosa of the small intestine for 15–20 days and then in the striated muscles at the larval stage for years. The infecting stage is the larva (0.6–1.2 mm in length) present in muscles, which, after the ingestion by another host, develops to the adult stage in the small intestine in about 48 hr; male and female worms mate and females produce newborn larvae starting 5–6 days after infection. Newborn larvae, released in the lymphatic vessels by the female, migrate to the blood vessels and reach all body sites of the host. Only newborn larvae, which reach striated muscles, are able to develop to infective larvae. They penetrate the muscle cells and induce the host cell to change into a nurse cell, in which larvae can survive for years, waiting for a new host to ingest the infected muscles.[1] Some *Trichinella* species induce the formation of a collagen capsule around the larva in the nurse cell (encapsulated species), whereas other species do not induce capsule formation (nonencapsulated species).[2]

EPIDEMIOLOGY

The *Trichinella* genus includes eight species and three genotypes (Table 1),[2,3] which can only be distinguished through biochemical or molecular analysis.[4]

Both a sylvatic and a domestic cycle of *Trichinella* have been documented.[2,5,6] The sylvatic cycle has been observed for all *Trichinella* species, and it is maintained in nature by animals with cannibalistic and scavenger behavior (Table 1), although omnivores (e.g., wild boar) can also act as a reservoir. Human infections related to the sylvatic cycle are mainly caused by the consumption of game meat (e.g., bear, walrus, fox, cougar, wild boar, and warthog).[5] The domestic cycle is only established as a result of improper practices in animal rearing, mainly pig rearing[2] (e.g., the use of raw or uncontrolled pig scraps or the carcasses of sylvatic carnivores as food for pigs). In nearly all cases, the domestic cycle has involved *Trichinella spiralis*, although in particular epidemiological situations, *Trichinella britovi* and *Trichinella pseudospiralis* have also been transmitted between pigs and from pigs to humans.[2,6] A high prevalence of *Trichinella* infection in pigs has also been shown to be associated with the transmission to unusual hosts, such as herbivores. For example, in France and Italy, about 3500 human infections were caused by the consumption of raw horse meat imported from countries with a high prevalence of *T. spiralis* infection in pigs.[2] In China, where the prevalence of *Trichinella* infection in pigs is very high, hundreds of human infections were attributed to the consumption of mutton.[2] Whether or not rodents constitute a reservoir of *T. spiralis* in the domestic cycle has been a topic of debate, especially with regard to rats, although it is more likely that these animals act as a vector of *Trichinella* infection, as opposed to a reservoir.[2,6]

The countries reported to have the highest prevalence of trichinellosis in humans are Argentina, Byelorussia, Bulgaria, Chile, China, Croatia, Georgia, Latvia, Lithuania, Malaysia, Mexico, Moldova, Myanmar, Romania, Russia, Serbia, Thailand, and Ukraine. In the European Union, in the past 25 years, more than 6800 human infections, 5 of which resulted in death, have been documented, with the cause of infection reported to be the consumption of horse meat, pork, and game meat.[5] In the United States, in the past 10 years, about 40 human infections per year have been reported, including some deaths.[5] In Canada, about 20 persons per year acquire infection for the consumption of game meat, prevalently walrus meat consumed by Inuit populations in the Arctic.[5] A few human infections have also been documented in Africa (i.e., Ethiopia, Kenya, Senegal, and Tanzania) and in the Australian Region (New Zealand and Papua New Guinea).[2]

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Table 1 Geographical distribution, hosts, and main biological and clinical features of *Trichinella* species and genotypes

Species	Geographical distribution	Cycle	Hosts	Biological features	Clinical features
T. spiralis	Cosmopolitan	Domestic and sylvatic	Pig, wild boar, horse, bear, fox	Encapsulated; no resistance to freezing; high number of larvae per female	Highly pathogenic; short incubation period; lethality <0.2%
Trichinella nativa	Arctic and subarctic regions of Asia, Europe, and North America	Sylvatic	Bear, fox, wolf, walrus, raccoon, cougar, dog	Encapsulated; high resistance to freezing; low number of larvae per female	Moderately pathogenic; long incubation period; severe enteric symptoms; deaths have been reported
Trichinella T6	Rocky Mountains (USA) from Montana to Alaska	Sylvatic	Bear, fox, wolf, cougar	Encapsulated; high resistance to freezing; low number of larvae per female	Moderately pathogenic; long incubation period
T. britovi	Temperate areas of Europe and Asia, Northern and Western Africa	Sylvatic; seldom domestic	Fox, wolf, raccoon dog, wild boar, pig, horse, dog	Encapsulated; moderate resistance to freezing; low number of larvae per female	Moderately pathogenic; long incubation period; mild or no enteric symptoms; no deaths reported
Trichinella T8	Namibia, South Africa	Sylvatic	Lion, hyena	Encapsulated; low number of larvae per female	Unknown
Trichinella T9	Japan	Sylvatic	Fox	Encapsulated; low number of larvae per female	Unknown
T. pseudospiralis	Cosmopolitan in Asia, Europe, North America, and Tasmania	Sylvatic; seldom domestic	Pig, wild boar, fox, marsupials, carnivorous and omnivorous birds	Nonencapsulated; no resistance to freezing;	Highly pathogenic; short incubation period; deaths have been reported
Trichinella murrelli	United States	Sylvatic	Fox, bear, raccoon, horse, lynx	Encapsulated; no resistance to freezing	Moderately to highly pathogenic; long incubation period; mild or no enteric symptoms; deaths have been reported
Trichinella nelsoni	Kenya, Tanzania, South Africa	Sylvatic	Hyena, lion, warthog	Encapsulated; no resistance to freezing	Moderately to highly pathogenic; long incubation period; mild or no enteric symptoms; deaths have been reported
Trichinella papuae	Papua New Guinea	Sylvatic	Wild pig, crocodile	Nonencapsulated; no resistance to freezing;	Slightly to moderately pathogenic
Trichinella zimbabwensis	Zimbabwe	Sylvatic	Crocodile, varan	Nonencapsulated; no resistance to freezing;	Unknown, but pathogenic in experimentally infected monkeys

CLINICAL ASPECTS

The incubation period for trichinellosis lasts from 2 to 50 days, depending on the number of infective larvae ingested (a greater number of larvae correspond to a shorter incubation period).[7] The acute stage of trichinellosis corresponds to the phase in which the newborn larvae migrate from the lymphatic vessels and invade the muscle cells. The acute stage can be preceded by loose stools or diarrhea, with flatulence, moderately intense abdominal pain, loss of appetite, and vomiting.[7]

The principal manifestations of the acute stage are fever, eyelid and periocular edema, and muscle pain. The onset of the acute stage is sudden, beginning with general weakness, chills, headache, fever (up to 40°C), excessive sweating, and tachycardia. In nearly all cases, these symptoms are followed by symmetrical eyelid and periocular edema, and edema frequently affects the entire face.[7] In some persons, petechiae, intraconjunctival hemorrhages, and hemorrhages of nail beds occur. These symptoms are accompanied by eosinophilia (up to 19,000 cells/μL) and leukocytosis (up to 15,000–50,000 cells/μL). Although eosinophilia appears early (i.e., before the development of clinical signs and symptoms), it increases between 2 and 5 weeks after infection. Leukocytosis, although not as common as eosinophilia, is characteristic of the acute stage of trichinellosis, and it indicates the presence of inflammation. This symptomatology is followed by pain in various muscle groups, which may restrict motility. Both creatinophosphokinase and lactate dehydrogenase activities have been observed in 75–90% of infected persons, and they peak earlier with respect to anti-*Trichinella* antibodies.[7] The principal electrolyte disturbance is hypokalemia, which results in reduced muscle strength and cardiac disturbances, as revealed by ECG. Decreased levels of proteins and albumin result in a hydrostatic edema.[7] Trichinellosis can also cause miscarriage or premature delivery, although infected women have delivered healthy babies. When infection is severe, complications such as cardiovascular, neurological, respiratory, and digestive disturbances can occur and hospitalization is compulsory.[7] Twenty deaths have been documented worldwide out of a total of 10,030 cases.[8]

It has not been possible to determine whether or not individual *Trichinella* species have specific clinical or biological patterns; this is because the number of infective larvae ingested has remained unknown in most cases, making it impossible to establish whether the clinical patterns observed were related to the species or to the infective dose. Nonetheless, *T. spiralis* infection, which is the cause of most human infections,[2] is believed to be more severe than *T. britovi* infection, possibly because *T. britovi* females are less prolific. In fact, in *T. britovi*-infected persons, only asymptomatic, mild, and moderate infections have been reported. In *Trichinella nativa* infections, there is a greater involvement of the intestine, with severe diarrhea. *T. murrelli* seems to be more likely to provoke skin reactions and less likely to cause facial edema. *T. pseudospiralis* seems to cause signs and symptoms that last longer.

DIAGNOSIS

The diagnosis of trichinellosis should be based on three main criteria:

1. Anamnesis based on epidemiological data: the source of infection, the amount of infected meat ingested, the number of larvae present in the infected meat, and the number of cases involved in the epidemic focus.
2. Clinical evaluation, i.e., recognition of the signs and symptoms of trichinellosis and definition of the clinical manifestations, which significantly affect the choice of treatment.
3. Laboratory tests: immunodiagnosis and/or detection of larvae in a muscle biopsy. The most reliable test is an ELISA that uses an excretory/secretory antigen. Antibodies are detectable in serum samples from 2 to 8 weeks postinfection, depending on the infecting dose, the *Trichinella* species, and the host response.[7,9]

TREATMENT

The effectiveness of the most commonly used antihelminthics (i.e., albendazole and mebendazole) is strictly related to the amount of time that has elapsed since the individual acquired the infection: they are completely effective only when used in the early stages of infection. As a general rule, the later the treatment is prescribed, the higher the probability that the infected person will harbor viable larvae in their muscles for years, with possible persistent myalgia. Thus, as the time elapsed since infection increases, the doses of antihelminthics should be higher and they should be administered for longer periods of time.[7]

Mebendazole should be administered at a daily dose of 25 mg/kg/day, in 3–4 doses/day for 2 weeks. Albendazole should be administered at a daily dose of 20 mg/kg/day, in 3–4 doses/day for 2 weeks. For severe infections, treatment can be repeated after 5 days.[7]

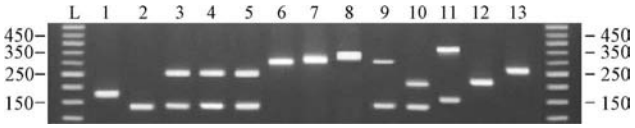

Fig. 1 Multiplex PCR amplification of single larvae of *Trichinella*. Photograph of an ethidium bromide-stained 2.5% agarose gel under ultraviolet light illumination. The samples are as follows: L (ladder 50), sizes are in base pairs; lane 1, *T. spiralis*; lane 2, *T. nativa*; lane 3, *T. britovi*; lane 4, *Trichinella* T8; lane 5, *Trichinella* T9; lane 6, *T. pseudospiralis* (Palearctic isolate); lane 7, *T. pseudospiralis* (Nearctic isolate); lane 8, *T. pseudospiralis* (Tasmanian isolate); lane 9, *T. murrelli*; lane 10, *Trichinella* T6; lane 11, *T. nelsoni*; lane 12, *T. papuae*; lane 13, *T. zimbabwensis*.

Glucocorticosteroids (e.g., prednisolone 30–60 mg/day in multiple doses) should be administered together with the benzimidazole and never alone because they could increase the larval burden by delaying the expulsion of adult worms from the intestine.

Pregnant women should only be treated if the infection is severe and under the strict supervision of a physician. Only antihelminthics (e.g., pyrantel) that are poorly absorbed by the intestinal lumen should be used. These antihelminthics are affective only against intestinal worms and not against migrating larvae or larvae in the muscles.[7] Prednisolone can be administered at a dose of 20–30 mg/day, tapering the dose, particularly for women in the third trimester.

MOLECULAR CHARACTERIZATION OF INDIVIDUAL LARVAE

The lack of morphological markers and the fact that the biological and biochemical markers do not allow the species to be easily or rapidly identified stress the importance of methods based on polymerase chain reaction (PCR) in identifying *Trichinella* parasites at the species level.[4] The use of PCR-derived methods also allows the species to be identified based on a single larva, which is important because, frequently, only one larva is detected in human biopsies and in muscle samples of animal hosts. Furthermore, the identification of single larva allows more than one species of *Trichinella* to be detected in the same host, i.e., mixed infections.

Although a variety of molecular methods have been developed in recent years,[4,10–13] the most reliable and simple-to-perform method for the molecular identification of single *Trichinella* larva is a multiplex-PCR analysis,[11,14] which is capable of identifying all species, one genotype, and three populations of *T. pseudospiralis* (Fig. 1).

PREVENTION AND CONTROL

Trichinellosis can be prevented through suitable food preparation. Meat and meat products should be cooked to an internal temperature of 70°C for at least 3 min. Meat and meat products can also be rendered innocuous by freezing them solid at −15°C for at least 3 weeks (for cuts up to 15 cm in thickness) or for at least 4 weeks (for cuts up to 60 cm in thickness),[15] although freezing-resistant *Trichinella* species do exist, and, in areas where these species are endemic, consumers should be informed that freezing is not recommended, especially for game meat. Those meat-preparation methods that are not considered as effective include microwave cooking, curing, drying, and smoking.[15] The consumption of raw meat products should be discouraged under all circumstances. An important means of preventing infection in humans is by performing veterinary controls (i.e., the artificial digestion of muscle samples for the detection of muscle larvae) at slaughterhouses for potentially infected animals, such as pigs, horses, and game animals.[15]

To prevent domestic animals from becoming infected, good animal-rearing practices are essential: avoiding the use of untested food, adopting microbiological barriers for preventing the entrance of rodents and other synanthropic animals into the pigsty and food storage areas, and the proper disposal of dead animals.[15] Although the infection cannot be feasibly controlled in wildlife, hunters should be discouraged from leaving animal carcasses in the field after skinning, a practice which favors the spread of the infection among sylvatic animals.

CONCLUSION

The risk of acquiring *Trichinella* infection through the consumption of raw or undercooked meat has, in recent years, increased as a result of the increased globalization of both the food industry and eating habits. Identifying the etiological agents of these infections at the species level is quite important in aiding physicians in diagnosis and treatment, in tracing the source of the infection, in determining the area of origin of the infected meat, and in developing the most appropriate measures for controlling infection at all phases of food production. With regard to diagnosis, although only molecular methods can be used to identify the parasites at the species or genotype level, they cannot replace the currently

available diagnostic techniques, such as ELISA for diagnosis in humans[9] and the digestion of muscle samples for diagnosis in animals,[15] given their high cost, lower sensitivity, and the need to perform a muscle biopsy.

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Trichomonas spp.

Holgar Mayta
Robert H. Gilman
Johns Hopkins School of Public Health, Baltimore, Maryland, U.S.A.

INTRODUCTION

Four species of the family Trichomonadidae are found in humans, *Trichomonas vaginalis* (Donne, 1836), *Trichomonas tenax* (O.F. Muller, 1773), *Pentatrichomonas hominis* (Davaine, 1860), and *Trichomitus fecalis* (Cleveland, 1928). Although there have been many nomenclature problems with *Pentatrichomonas hominis* (Davaine, 1860), it is clear that this parasite represents another genera of the family Trichomonadidae.[1,2]

The most widely and best studied parasite is *T. vaginalis*, the urogenital trichomonad, which occurs almost exclusively in the lower genital tract. *Trichomonas vaginalis* is considered the only typical trichomonad with definite pathogenicity for humans.

Trichomonas tenax is considered a normal commensal of the human oral cavity. It is found especially in the tartar around the teeth or in the defects of carious teeth; although there is no evidence of direct pathogenesis, it is frequently associated with phylogenic organisms in pus pockets or at the base of teeth and its incidence is higher among patients with periodontal disease.[3]

OVERVIEW

The present paper is a review on the current state in the diagnosis of parasites of the genera trichomonas; most information has been obtained from studies on *T. vaginalis*.

BIOLOGY AND LIFE CYCLE

Trichomonas are flagellated protozoan, which vary in size and shape. In axenic culture they are usually pear-shaped ranging in size from 4 to 30 μm, but when attached to epithelial cells the amoeboid forms are predominant.[4] Organisms of the genera Trichomonas are characterized by possessing a single nucleus and five flagella, four of which are free and located at the anterior portion, and the fifth is a recurrent anterior flagellum, which is attached to the body as an undulating membrane. The cell body is longitudinally bisected by a thin hyaline structure, the axostyle, which starts at the nucleus and protrudes from the posterior end of the parasite.[5] The nucleus located in the anterior portion has a porous double membrane characteristic of a eukaryotic cell; the Golgi complex is also highly developed. The cytoplasm is rich in glycogen granules and ribosomes, but lacks mitochondria, peroxisomes, or glycosomes.[4]

Trichomonas exist only as trophozoites; they lack a cystic stage and reproduce generally by longitudinal binary fission without disappearance of the nuclear envelope.[4] Mitosis in *T. vaginalis* has been well characterized and includes the four mitotic phases: prophase, metaphase, anaphase, and telophase;[6] the chromatin condenses to six chromosomes;[7] no recent data on the mitotic division exist for *T. tenax*.

The genome of *T. vaginalis* has been estimated to be approximately 2.5×10^7 bp with only 33% of unique sequences.[8] The overall content of G+C was estimated to be about 36%. *Trichomonas vaginalis* has six monocentric chromosomes, which seem to be haploid.[7,9] However, 0.1% of the population displays diploid metaphases with some cells exhibiting four nuclei suggesting a possible occurrence of meiotic division.[7]

No extra nuclear DNA is present in Trichomonas; detection of extra chromosomal DNA in the hydrogenosome was looked for but not found.[8,10]

EPIDEMIOLOGY AND TRANSMISSION

Although trichomoniasis (*T. vaginalis*) is the most common nonviral sexually transmitted disease, epidemiological data are limited because official reporting is not required. According to the World Health Organization 174 million new infections occurred in 1999[11] with 8 million occurring in North America. Prevalence varies in different populations; the prevalence of trichomoniasis among black American population is higher than any other population in the United States.[12]

Transmission of Trichomonas parasites is direct, person-to-person. *T. vaginalis* is almost exclusively transmitted directly by sexual intercourse. However, infection in newborns and infants is commonly a consequence of mother-to-child transmission during

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delivery.[13] Newborn girls can acquire infection from their infected mothers during passage through the birth canal and remain asymptomatic until puberty. Transmission of *T. vaginalis* in children before and after puberty may be indirect especially in an environment with infected family members.[13]

T. tenax is spread by direct as well as indirect mouth-to-mouth contact, sharing of oral hygiene utensils such as a toothbrush may be an important source of transmission.[1] A zoonotic transmission may also be possible, as a PCR-based molecular analysis of isolates obtained from dogs and cats confirmed the presence of *T. tenax* in these animals.[14]

CLINICAL MANIFESTATIONS

Clinical trichomoniasis in women varies from asymptomatic state in 10% to 50% of infected women to an obvious vaginitis,[15] which may be either acute or chronic in nature. Women with asymptomatic infection have a normal vaginal pH and flora[16] but during the following 6 months up to 50% of these patients will develop symptoms. Acute infection is characterized by diffuse vulvitis due to a copious leucorrhea; the discharge is usually frothy, yellow or green, and mucopurulent.[17] Small hemorrhagic spots may be seen in the vaginal and cervical mucosa, which is commonly referred as a ''strawberry appearance'' and is observed only in 2% of the patients. These signs and symptoms are cyclic and worsen around the time of menses.[4,17] In chronic infection symptoms are mild with pruritus and dyspareunia; vaginal secretion may be scanty and mixed with mucus.[15,20]

Although vaginitis is the most common manifestation of *T. vaginalis* infection the disease may also be associated with infertility, premature rupture of membranes, premature delivery, low birth weight, still birth, and neonatal death.[18] Other complications include an increased risk of pelvic inflammatory disease,[19] HIV infection,[20] and possibly cervical cancer.[21]

Trichomoniasis in men is largely asymptomatic; men are considered asymptomatic carriers of *T. vaginalis*. Disease in men may be categorized into three groups: an asymptomatic carrier who is identified by investigation of sexual contacts of infected women; acute trichomoniasis, characterized by profuse purulent urethritis; and mild symptomatic trichomoniasis, which is clinically indistinguishable from other causes of nongonococcal urethritis.[4] Complications in men include nongonococcal urethritis, prostatitis epydidymitis, urethral disease, and infertility.[22]

DIAGNOSIS

Diagnostic techniques have been developed primarily for the detection of *T. vaginalis* but are equally applicable to the detection of *T. tenax*. Diagnosis of Trichomonas traditionally depends on the microscopic observation of motile protozoa. Although this technique is rapid, specific, and cost effective, it has a low sensitivity detecting only half of the culture positive samples.[23] The low sensitivity is probably due to the loss of the distinctive motility after the parasite has been removed from body temperatures for any length of time. Sensitivity is increased if specimens are examined immediately after being taken.

The culture method is the gold standard for the detection of Trichomonas; however, culture requires an inoculum of 300 to 500 trichomonads/mL[24] and up to 7 days before a sample could be reported as negative. During this period positive patients may continue transmitting the disease. Moreover, inadequate transport or storage of samples may result in a negative culture. The InPouch TV system (BioMed Diagnostics, San Jose, CA) is considered the most advantageous culture systems. It consists of a two-chambered bag that allows performing an immediate wet-mount microscopic examination through the bag as well as a culture,[25] but as with other culture techniques sensitivity may vary especially according to rapid access to incubators.

Cell culture is more sensitive than broth culture and wet-mount preparation; however, cell culture is not routinely performed and is neither convenient nor used at present for rapid diagnosis.[24]

Papanicolaou (Pap) smear and other staining techniques have also been performed as a detection tool for trichomonas.[26,27] Papanicolaou smear is preferred as it is routinely performed in gynecological screening for cytological abnormalities. Staining techniques are not reliable, however, as Trichomonas does not always appear in its typical pear-shape form.

Detection of *T. vaginalis* based on antibody detection is not reliable because of serotype differences among different strains and because host response to the parasite is widely variable. In addition, infection of *T. vaginalis* by a double-stranded RNA virus (the *T. vaginalis* virus, TVV) may influence expression of antigenic proteins.[28]

Detection of the parasite in clinical samples using labeled monoclonal antibodies has shown similar sensitivity and specificity results to those obtained by culture.[29]

Techniques based on DNA analysis have been increasingly used in clinical laboratories to improve the specificity and sensitivity of *T. vaginalis* diagnosis. Hybridization techniques such as the Affirm VP system

(MicroProbe Corp., Bothwell, WA) or the dot blot and in situ hybridization demonstrated a better performance than the wet-mount preparation. However, the number of false-negative results was high when the Affirm VP system was tested.[30] The requirement for radioactive material for the dot blot and in situ hybridization is the main drawback of these techniques.

Polymerase chain reaction-based techniques have been widely explored for the detection of both *T. vaginalis* and *T. tenax*. Repetitive DNA sequences of *T. vaginalis* have been cloned, sequenced, and characterized for PCR amplification;[31,32] however, low sensitivity is observed because of the high variability of these sequences and the presence of insertion elements, causing more than a single product or absence of amplification. The beta tubulin gene has also been used as a target for PCR amplification, but this PCR shows a cross-reaction with some *T. tenax* isolates and fails to amplify some axenically cultivated *T. vaginalis* isolates.[33] Techniques based on the amplification of ribosomal coding DNA sequences have been described both for *T. tenax*[34] and *T. vaginalis*.[35] Ribosomal genes (rDNA) have been demonstrated to be highly conserved among different Trichomonas strains;[2] also, ribosomal genes are generally multicopy genes. The nature of the rDNA genes made them the most advantageous target for PCR-based detection. Both human Trichomonas are capable of invading organs and tissue that are not their natural environment making them difficult to identify using current techniques; rDNA-based PCR is especially useful when those cases occur.

Random amplified polymorphic DNA (RAPD) amplification may be useful to characterize *T. vaginalis* strains according to resistance to metronidazole, and infection with TVV or mycoplasmas; however, virulence of strains or geographic distribution has not been genotypically correlated.[36]

Antigenic differences between *T. tenax* isolates from respiratory passages and isolates from the oral cavity have suggested that the isolates from the respiratory passages may belong to a separate species.[3] However, no further studies have been conducted in this regard. The use of molecular biology techniques should be able to provide a definitive answer to whether there is a difference in the oral and respiratory *T. tenax* isolates.

TREATMENT

Metronidazole and other nitroimidazoles are effective against Trichomonas. The standard treatment for vaginal trichomoniasis is 250 mg of metronidazole, given orally, either three times a day for 7 days, or in a single 2-g dose.[37,38] Both the infected patient and sexual partner, whether symptomatic or asymptomatic, should be treated to prevent reinfection. Both regimens are equally effective.[37]

Although treatment is highly effective, resistance of *T. vaginalis* to metronidazole is now on the rise, and according to the Centers for Disease Control 5% of all *T. vaginalis* patient isolates have some level of resistance to metronidazole.[38]

The mechanism of metronidazole resistance may be a consequence of a reduced transcription of the ferredoxin gene and/or a consequence of abolished or decreased activities of the pyruvate–ferredoxin oxidoreductase and hydrogenase enzymes.[39]

CONCLUSION

Human parasitism by Trichomonas species has been demonstrated to cause serious health problems especially due to potential complications and increased susceptibility to other more serious transmitted disease agents. Although much research is still needed to determine the pathogenicity and virulence of Trichomonas it is clear that at least *T. vaginalis* infection causes several complications in pregnancy and increases the risk of transmission of other sexually transmitted diseases.

Although culture for Trichomonas has many drawbacks, it remains the gold standard for the identification and diagnosis of Trichomonas. Polymerase chain reaction-based amplification of DNA sequences is now both useful and accessible for the detection of these parasites and can be used both for patient diagnosis and epidemiological studies. Cost effectiveness does not constitute a barrier to the use of DNA amplification. Equipment and reagents are more accessible nowadays; prices are now comparable to those required for implementing an ELISA technique. Caution should be taken, however, when PCR techniques are applied in different settings. A great variety of Taq polymerases are now commercially available, most of them are genetically modified and perform differently. Standardization of the PCR condition under local settings should be performed before field application. We have seen a sensitive rDNA assay become insensitive because protocols were not followed and the type of Taq used was changed.[40]

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Tropheryma whipplei

R. Mahnel
T. Marth
Stiftung Deutsche Klinik für Diagnostik GmbH, Wiesbaden, Germany

F. Fenollar
D. Raoult
Unité des Rickettsies, Marseille, France

INTRODUCTION

Whipple's disease (WD) is a good example of progress resulting from the introduction and development of molecular genetic techniques. In 2003 Bentley et al. and Raoult et al. sequenced and published the full genome of the causative bacterium *Tropheryma whipplei* which allowed to ascertain specific targets for the detection of the bacterium in the host and the environment.

The first case report of a patient suffering from diarrhea, polyarthritis, and bronchitis was by G.H. Whipple in 1907. He described the disease as a new entity and it was known since as ''intestinal lipodystrophy,'' later on as Whipple's disease.[1] Although a systemic disease was considered, the underlying agent was unknown for many years. For a long time the diagnosis was based on clinical symptoms and later on histological investigations: periodic acid-Schiff (PAS) positivity in the affected tissue has been considered as a pathognomonic sign. In 1960, a bacterium was identified in the affected tissue using electron microscopy.[2] The start of genetic techniques in the disease was the sequencing of 16S rRNA in 1991, which allowed to determine the phylogenetic position as an actinomycete,[3] and the bacterium was named after its culture *T. whipplei*.[4]

TYPE OF PATHOGEN

T. whipplei is a short rod-shaped bacterium, approximately 0.3 μm in diameter and 1.5 μm long. Morphologically, the bacterium has a characteristically trilamellar ultrastructure including a plasmic membrane surrounded with a thin wall, itself surrounded with a structure like a plasmic membrane.[5] This morphology is not typical of Gram-positive or Gram-negative bacteria. However, the bacterium appears Gram negative at the Gram staining, even if the phylogenic analysis classified it among the Gram-positive bacterium. *T. whipplei* is also well stained by PAS staining.[6] The culture of *T. whipplei* has been an elusive goal for many generations of microbiologists. For the first time, in 1997, two strains of *T. whipplei* have been isolated from cardiac valves on a macrophage system in which microbicide functions have been inhibited.[7] Unfortunately, no isolate has been subcultured.[7] Two years later, the bacterium has not only been isolated but also established from a cardiac valve of a patient with WD endocarditis. More precisely, the bacterium has been propagated in human fibroblasts in minimum essential medium with 10% fetal calf serum and 2 mM glutamine.[6] Since this work has been reproduced, four other strains have been isolated and established. Two strains were obtained in the same laboratory where the first strain of *T. whipplei* was established. One strain was isolated from a blood culture of a patient with WD endocarditis, and another strain was isolated from a duodenal biopsy from a patient with digestive WD.[8] Two other strains obtained in another laboratory were from cerebro-spinal fluid samples.[9] Finally, the availability of the sequenced genome of *T. whipplei* has allowed the successful design of a cell-free culture medium for *T. whipplei*.[10] This medium provided missing amino acids (DMEM/F12 medium) and was supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% nonessential amino acids. The culture flask was incubated vertically at 37°C in a 5% carbon dioxide atmosphere. The doubling time of *T. whipplei* was 28 hr and was unchanged from passage three up to now.

CLINICAL FEATURES AND SPECTRUM

The clinical picture is often multifaceted. In the past, WD was often considered to be a gastrointestinal disease, but it is now established that nearly every organ system can be involved. The course varies in the same extent, so it was suggested that different clinical courses may result from *T. whipplei* subtypes, identified according to its 16S–23S rDNA.[11] Mostly affected are Caucasian middle-aged

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men (male/female ratio 8:1), but no valid estimates of the incidence are available.

The initial complaints are mostly arthralgia and swelling of the joints. This seronegative, nondestructive arthropathy in an approximate frequency of up to 85% can occur years before diagnosis and is often associated with HLA-B27. Affected joints are predominantly peripheral. Arthropathy in knees, wrists, and ankles occur in about 45% each.[12]

Diarrhea is the other major symptom. It is followed by a general malabsorption resulting in weight loss and anorexia. Weight loss can be found in about 90% of the patients. Gastrointestinal symptoms, which lead to the diagnosis, consist of episodic and watery diarrhea and steatorrhea accompanied by colicky abdominal pain and, in one-third, by occult blood in stool.[13] Endoscopic investigation reveals a pale yellow mucosa alternating with erythematous, erosive parts in the duodenum or jejunum, and duodenal biopsies are positive in the PAS stain.

A less frequent but severe course is the infection of the central nervous system (CNS). Dementia, personality changes, or memory disorders can become apparent as well as myoclonia, nystagmus, or ophtalmoplegia. In some cases CNS involvement is found without other affections.

PREVALENCE

The distribution of the bacterium in the environment seems to be ubiquitous. The DNA of the bacterium has been detected in excretions (feces) of patients suffering from WD and in sewage water.[14,15] The bacterial DNA was also found in saliva, gastric fluid of healthy humans, so that it seems possible that it can survive outside cells and body fluids are the way of distribution.[16,17] Normally, the bacterium can be found within membrane-bound vesicles in the cytoplasm of macrophages as well as in many other cell types such as plasma cells, granulocytes, epithel cells, endothelial cells, smooth muscle cells, and lymphocytes.

MANAGEMENT

Untreated disease may be fatal, but adequate and consequent antibiotic therapy often leads to complete remission. The systemic infection requires the use of antibiotic agents. However, treatment is based on empirical observations. A common antibiotic therapy starts with cephalosporine or meropenem (2 weeks intravenous), followed by cotrimoxazole orally for 1 year minimum. In the majority of cases this treatment results in a quick improvement of complaints. However, the disease may have a chronic relapsing course. In these cases changes in antibiotic treatment have to be considered. Second-line antibiotics include tetracycline, minocycline, or oral penicillin.[18] In therapy refractory cases the positive effects of immunosupportive treatment with interferon gamma have been described.[19] Additionally, general replacement therapy is necessary in many cases with malabsorption syndrome.

In patients responding to antibiotic treatment, control endoscopies and duodenal biopsies are recommended 6 and 12 months after diagnosis.[20] Treatment can be stopped after 1 year if the patients have no clinical signs and duodenal biopsy is no longer PAS positive.

MOLECULAR CHARACTERIZATION

In 1991, Wilson sequenced a fragment of 16S rRNA from a patient with WD.[3] This sequence was original and showed that, phylogenically, the bacterium was included to the *Actynomyces* clade. One year later, the sequencing of 1321 bp of the 16S rRNA from the biopsy of a patient confirmed these data;[21] the bacterium was named then *Tropheryma whippelii*. After the culture and the description of the bacterium, the bacterium was officially named *Tropheryma whipplei*.[4] At the time the genome sequencing of *T. whipplei* was initiated, only five genes were identified: 16S rRNA, 5S rRNA, 23S rRNA, *rpoB*, and *hsp65*.[22] Phylogenetic analysis classified *T. whipplei* within the high G+C content Gram-positive bacteria class (Actinobacteria, relatively near to two known species in human pathology, *Actinomyces pyogenes* and *Rothia dentocariosa*, and to bacteria principally found in the environment). The 16S–23S interregion of *T. whipplei* had been studied by PCR and sequencing from clinical samples from patients with WD.[11] The sequence analysis has revealed the existence of six different types of spacer.[11] The more frequent spacer types were types 1 and 2.[23]

Recently, the genome of two different strains of *T. whipplei* has been entirely sequenced.[22] Sequencing of *T. whipplei* Twist, propagated from the cardiac valve of a patient, revealed a 927,303-bp genome which encodes 808 predicted protein-coding genes and presents a GC% of 47%.[22] Sequencing of *T. whipplei* TW08/27, propagated from the cerebrospinal fluid of a patient, revealed a 925,938-bp genome which encodes 784 predicted protein-coding genes and presents a GC% of 46.3%.[24] *T. whipplei* presents a unique circular chromosome and is the only known reduced genome species (<1 Mb) within the Actinobacteria.[22,24] The two genomic sequences of the two strains are mostly (>99%) identical at the nucleotide sequence level and encode quasi-identical

gene complements. One of the specific genome features includes deficiencies in amino acid metabolisms. This observation points to a host-restricted lifestyle for *T. whipplei*. To compensate for the defective biosynthetic pathways, the missing amino acids must be obtained from the environment or the host. This information has allowed the design of a comprehensive medium, supplemented with amino acids, in which *T. whipplei* grows axenically.[10] Another specific characteristic of *T. whipplei* is the lack of clear thioredoxin and thioredoxin reductase genes. Thioredoxin reductase is a ubiquitous enzyme that reduces thioredoxin, which in turn acts as electron donor in various essential redox reactions in the cell.[22] A functional thioredoxin system has systematically been retrieved in all bacterial genomes sequenced so far. Thus *T. whipplei* might be the first example of a bacterium without the usually essential thioredoxin pathway, but this observation must be confirmed by further experiments. A mutation in DNA gyrase, which predicts a resistance to quinolone antibiotics, is also present. These data are consistent with the high minimal inhibitory concentration of ciprofloxacine observed in vitro for *T. whipplei*.[25] The genome of *T. whipplei* contains also a gene cluster that seems to direct the biosynthesis and export of extracellular polysaccharide. This observation could explain the fact that the *T.whipplei* cell wall includes, in addition to peptidoglycan, an unusual inner layer composed mainly of polysaccharides which accounts for the positive PAS staining reaction. The alignment of the two genome sequences revealed a large chromosomal inversion, the extremities of which are located within two paralogous genes.[24] These genes belong to a large cell-surface protein family defined by the presence of a common repeat highly conserved at the nucleotide level. The repeats appear to trigger fragment genome rearrangements in *T.whipplei*, potentially resulting in the expression of different subsets of cell-surface protein.[24] This phenomenon might represent a new mechanism developed to evade the host's immune response during the course of this chronic disease. It is also remarkable to note a high frequency of repeats in *T. whipplei* because usually repeated sequences are considered to be less frequent in the reduced genomes.[24] Besides, new primers were designed according to the sequence of this highly conserved domain, allowing the improvement of the current molecular diagnosis of WD.[26] The isolated lifestyle of parasitic bacteria has been proposed to induce ''ongoing genome degradation.'' However, *T. whipplei* genome exhibits a coding content (86%) comparable to the free-living bacteria and shows a few pseudogenes and a small sign of ''ongoing degradation.'' Finally, the important finding derived from *T. whipplei* genome sequences is the frequent genomic instability mediated by protein-coding repeats within genes of membrane proteins.

MOLECULAR TESTING

The histology analysis of biopsies using the PAS staining, which has been considered for a long time, as pathognomonic of WD, is not completely specific. Polymerase chain reaction assays could be performed on various biopsies (duodenum, adenopathy, cardiac valve, kidney, brain, synovial biopsy), liquid samples (cerebrospinal fluid, joint fluid, aqueous humor, blood), saliva, or stools.[27] The DNA extraction is a clue step and different protocols have been proposed.[27] These protocols could be applied in fresh sample or included in paraffin. Before the genome sequencing, all the five identified genes were used as targets for the molecular diagnosis of the WD.[28–31] For each PCR assay, it is necessary to include positive (DNA extracted from a suspension of *T. whipplei*) and negative controls. Indeed, samples must be separated every five samples by a negative control, including water and DNA extracted from control tissues (intestinal or other biopsies). The positive and negative controls must be correct to validate and interpret the results of each PCR assay. Recently, a PCR assay targeting the *rpoB* gene and using a real-time PCR apparatus has been successfully applied for the diagnosis of WD.[32] This technique offers the advantages of reducing PCR time and risk of contamination. The availability of the genome has offered the possibility to rationally choose DNA targets. Indeed, a real-time PCR assay targeting repeated sequences of *T. whipplei* performed in samples from patients with WD and in a control group has allowed the significant enhancing of PCR sensitivity without altering its specificity, when compared to regular PCR.[26] All the sequence primers available and published for the specific diagnosis of WD (except those targeting the 16S rDNA sequence), the target gene, and the molecular size expected of the amplified product are summarized in Table 1. When an amplification product is obtained by PCR, the identification of *T. whipplei* should be confirmed by sequencing or hybridization. Indeed, the main problem with PCR is the specificity, due notably to problems of contamination depending on the technique used and the studied samples. Besides, some divergences have been observed between laboratories. Positive results have been reported in saliva, gastric liquids, and duodenal biopsies in patients without WD.[33] However, these data have not been confirmed by other teams.[32,34] Presently, we recommend for the definite molecular diagnosis of WD, notably for atypical cases, to use at least two pairs of primers targeting two different sequences to avoid false-positive results.

Table 1 *Tropheryma whipplei*-specific amplification systems available for the diagnosis of Whipple's disease (except the 16 rRNA system)

Primers forward/reverse	Sequences forward/reverse	Gene target/product size	Reference
Tws3,f/	5′CCGGTGACTTAACCTTTTTGGAGA3′/	ITS	[17]
Tw1857r1	5′TCCCGAGCCTTATCCGAGA3′	498-bp	
Tw1662f/	5′ACTATTGGGTTTTGAGAGGC3′/	ITS	[17]
Tw1857r1	5′TCCCGAGCCTTATCCGAGA3′	214-bp	
Tws1,f/	5′ATCGCAAGGTGGAGCGAATCT3′/	ITS	[17]
tws2,r	5′CGCATTCTGGCGCCCCAC3′	728-bp	
tws3,f/	5′CCGGTGACTTAACCTTTTTGGAGA3′/	ITS	[17]
tw1857r1	5′TCCCGAGCCTTATCCGAGA3′	489-bp	
tws3,f/	5′CCGGTGACTTAACCTTTTTGGAGA3′/	ITS	[17]
tws4,r	5′CTCCCGTGAGCTTGTGCCCAAAC3′	489-bp	
TW-23InsF/	5′GGTTGATATTCCCGTACCGGCAAA G3′/	23S rDNA	[18]
TW-23InsR2	5′GCATAGGATCACCAATTTCGCGCC3′	250-bp	
Whipp-frw1/	5′TGACGGGACCACAACATCTG3′/	*hsp*65	[19]
Whipp-rev	5′ACATCTTCAGCAATGATAAGAAGTT3′	500-bp	
Whipp-frw2/	5′CGCGAAAGAGGTTGAGACTG3′/	*hsp*65	[19]
Whipp-rev	5′ACATCTTCAGCAATGATAAGAAGTT3′	357-bp	
TwrpoB.F/	5′TGAGCGCACGCCGGAAAAA3′/	*rpo*B	[20]
TwrpoB.R	5′GCACCGCAACCTCGGAGAAA3′	650-bp	
53.3F	5′AGAGAGATGGGGTGCAGGAC3′/	Repeat	[15]
53.3R	5′AGCCTTTGCCAGACAGACAC3′	164-bp	

Sequences of the primers, gene target, and expected product size are given.

CONCLUSION

Molecular techniques now play an important role in the diagnosis of Whipple's disease. The genome analysis allows new insights into the etiopathogenesis of the disease and potentially in therapeutic options. Improved assays for the detection of *Tropheryma whipplei* have increased the reliability in diagnosis and may offer the possibility to identify the natural reservoir of the bacterium.

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Trypanosoma cruzi

John M. Kelly
Martin C. Taylor
Shane R. Wilkinson
London School of Hygiene and Tropical Medicine, London, U.K.

INTRODUCTION

The insect-transmitted protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas' disease, a debilitating condition that has a considerable impact on public health in Latin America. Despite the importance of the infection, the mechanisms underlying the severe pathology associated with the disease are still poorly understood. In addition, the drugs in current use have limited efficacy and there is no immediate prospect of a vaccine. However, research on Chagas' disease is now receiving fresh impetus with the completion of the human and *T. cruzi* genome projects. Together with the application of postgenome technologies, this is providing new insights into the genetic and biochemical nature of the parasite and the mechanisms and consequences of host–parasite interactions. Rapid progress in these areas should facilitate the development of new chemotherapeutic approaches and provide the tools and reagents necessary to address the complex epidemiology of the disease.

THE NATURE OF CHAGAS' DISEASE

In Latin America, 18–20 million people are infected with *T. cruzi* resulting in up to 50,000 fatalities per year.[1] Chagas' disease is endemic in more than 20 counties with 100 million people at risk. It is by far the most serious parasitic infection in the region with an estimated economic burden of $8 billion per year. There is a close association between poverty and the disease because the reduviid bug that transmits the parasite is usually restricted to poor-quality housing. Most individuals are infected while sleeping, when parasites are deposited on their skin as the bug defecates during a bloodmeal. The parasites then penetrate the skin, typically via the bite wound or other lesion (Fig. 1). Transmission can also occur congenitally (5–10% of children born to infected mothers are themselves infected) or by blood transfusion. This latter route is increasingly recognized as a potential problem in the United States where there are estimated to be 100,000 infected individuals that have migrated from endemic areas.

In humans, Chagas' disease has three stages. The initial acute stage is characterized by a high parasitemia and a range of symptoms that can last for 4–8 weeks. Although the symptoms are usually mild, there can be a 5% fatality rate in children, often because of acute myocarditis or meningoencephalitis. The disease then subsides and most individuals are asymptomatic for the rest of their lives. However, they remain infectious and can serve as a reservoir. In approximately 30% of cases the disease develops into the chronic form, often many years after the initial infection. Premature and progressive heart disease is the most common clinical manifestation, and prognosis for these individuals is poor. Other serious symptoms that can be associated with chronic disease include swelling of the esophagus and colon (the so-called megasyndromes) and abnormalities of the central nervous system. Reactivation of *T. cruzi* infection in HIV carriers has also been recognized as an increasing problem. Most patients identified with dual infections have been found to display neurological dysfunction and/or heart lesions.

Pathology in Chagas' disease is mainly a consequence of interactions between the parasite and the host immune system. *T. cruzi* elicits polyclonal lymphocyte activation, a type of response common in many viral, bacterial, and parasitic diseases and which is thought to constitute a nonspecific immune evasion strategy. This has also been linked with the immunosuppression and pathology associated with the chronic form of Chagas' disease. Geographical variations in the percentage of infected people who go on to develop chronic disease has hinted that the spectrum of symptoms may reflect the genotypic diversity of the parasite (below) rather than differences in host genetic background, although this remains controversial.

There has been a major debate within the Chagas' disease research community about the factors responsible for triggering the development of the chronic pathology.[2] Some have argued that the continued presence of the parasite is required, whereas others consider that the disease is an autoimmune condition. There is now strong evidence, at least in a mouse model, that the continued presence of the parasite is both sufficient and necessary to promote chronic disease pathology.[3] This has important

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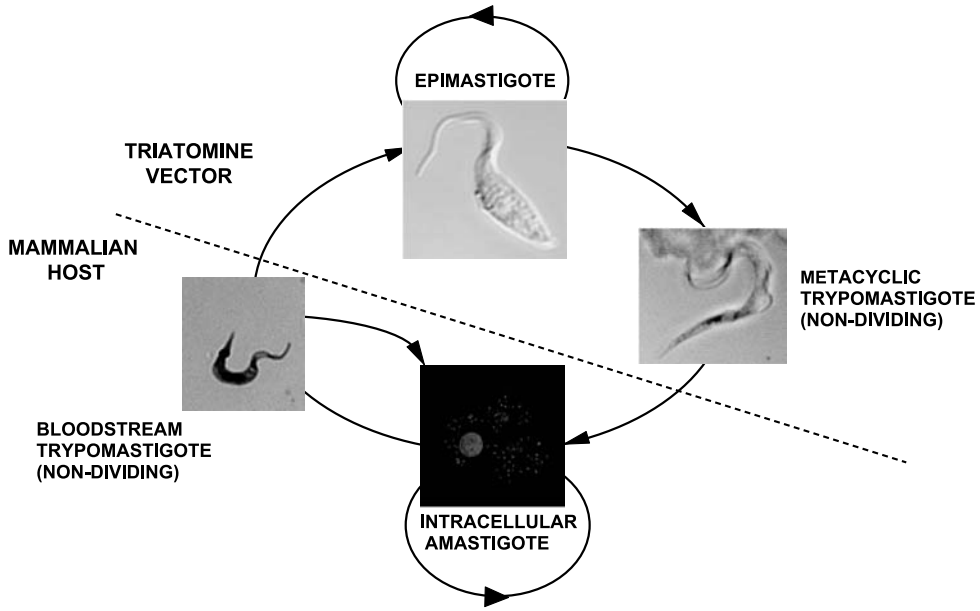

Fig. 1 Life cycle of *T. cruzi* showing the four major forms of the parasite. Epimasigotes and amastigotes divide by binary fission, whereas both of the trypomastigote forms are nonreplicative. The small rounded nonflagellated amastigotes can grow in almost any mammalian cell type. In the image shown intracellular amastigotes can be identified as small red dots following DNA staining. The large red dot is the host cell nucleus. Amastigotes differentiate into bloodstream trypomastigotes, which are released after host cell lysis. These can reinvade other cells or be taken by an insect vector during a bloodmeal. (*View this art in color at www.dekker.com.*)

implications because it provides a theoretical basis for the use of drugs targeted directly at *T. cruzi* as a means of reducing chronic disease severity and/or progression.

A wide range of techniques have been developed for the diagnosis of *T. cruzi* infection. Unfortunately, these can lack sensitivity and/or specificity and it is often necessary to use more than one approach. For example, the success of microscopic diagnosis is limited by the low level of parasitemia out with the acute phase and serological tests can suffer from cross-reactivity with antigens of *Leishmania* or the nonpathogenic trypanosome *Trypanosoma rangeli*. More recently, encouraging results have been achieved with PCR-based approaches that exploit the species specificity of high-copy-number satellite repeat sequences.[4] Only two drugs are currently available for the treatment of Chagas' disease, nifurtimox and benznidazole. They are partially effective against the acute stage but have several drawbacks. Both can produce toxic side effects, require long-term administration under medical supervision, and remain unproven as a treatment for chronic disease. Improved public health measures such as the ''Southern Cone Initiative'' have been successful in reducing infection rates in some countries where transmission is predominantly domestic.[5] However, even if all the routes of parasite transmission could be blocked throughout its entire geographic range, such is the nature of the infection, some individuals will still be suffering from chronic Chagas' disease in 40 years time.

CHARACTERISTICS OF *T. CRUZI*

T. cruzi is a member of the family Trypanosomatidae (order: Kinetoplastida), which also includes *Trypanosoma brucei*, the etiological agent of African sleeping sickness, and *Leishmania* species, which cause cutaneous/visceral leishmaniasis. Chagas' disease is a zoonosis and the parasite has been detected in more than 150 species of mammals. The opposum *Didelphis*, which is found throughout South America, plays a central role in the transmission between sylvatic (forest) and domestic habitats, where animals such as dogs, cats, and rodents serve as important reservoirs. *T. cruzi* is transmitted by blood-feeding reduviid bugs of the subfamily Triatominae. In the insect vector, *T. cruzi* undergoes differentiation from the noninfectious epimastigote form into the metacyclic trypomastigotes that are capable of initiating an infection in the host (Fig. 1). Trypomastigotes can invade a large number of mammalian cell types including macrophages, muscle cells, and nerve cells. Epimastigotes (Fig. 2) are the stage that is most easily cultured in the laboratory.

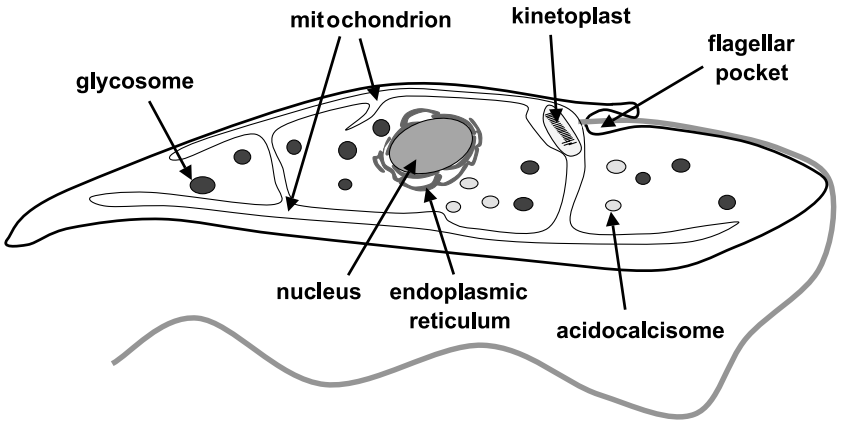

Fig. 2 Schematic representation of a *T. cruzi* epimastigotes. *T. cruzi* contains a large, single mitochondrion that is dispersed through much of the cell. The mitochondrial genome is organized as a defined structure, the kinetoplast (light blue), which is adjacent to the flagellar pocket and base of the flagellum. The parasite contains other subcellular structures typical of eukaryotes including the endoplasmic reticulum (red) and the nucleus (purple). It also possesses a number of unusual organelles[6] such as glycosomes (dark blue) and the acidic calcium storage compartments acidocalcisomes (yellow). (*View this art in color at www.dekker.com.*)

T. cruzi has a complex population structure that reflects at least 60 million years of divergence. On the basis of a large number of genotypic and phenotypic studies the species has now been divided into two major groups, *T. cruzi* I and II.[7] Group II parasites also display marked heterogeneity and have been separated further into five subdivisions (II a–e). It has been proposed, and there is some experimental evidence,[8] that *T. cruzi* II is more frequently associated with the development of chronic pathology, whereas *T. cruzi* I gives rise to more benign disease. Given the considerable genetic diversity of the parasite this is probably an oversimplification. Until recently, reproduction in *T. cruzi* was thought to be exclusively asexual with the parasite having a clonal population structure. However, new data from phylogenetic[9] and laboratory[10] studies have demonstrated that genetic exchange can occur and that it is initiated by parasite cell fusion within infected host cells. Although probably a rare event, the potential for genetic exchange could have major epidemiological consequences including the spread of undesirable phenotypes such as virulence and drug resistance.

Trypanosomatids diverged early from the main eukaryotic lineage and their study has led to the discovery of many novel genetic and biochemical phenomena. These include mitochondrial RNA editing, unusual secondary modifications to cell surface proteins, sequestration of glycolytic and fatty acid biosynthetic enzymes in organelles called glycosomes (Fig. 2), and an oxidative defense system based on the unique low-molecular weight thiol trypanothione. Parasite-specific features such as these have attracted considerable interest in the context of drug design.[11]

THE UNUSUAL NUCLEAR AND MITOCHONDRIAL GENOMES OF *T. CRUZI*

As with the other trypanosomatids, the chromosomes of *T. cruzi* do not condense during mitosis, and karyotype studies have had to rely on pulse field gel electrophoresis and related techniques. These indicate that the nuclear genome is organized into about 40 chromosomes ranging from 0.4 to 4.5 megabases. The genome size (haploid content) is 45 megabases and the total number of genes has been estimated to fall within the range of 8–10,000. There is significant variation in the size of chromosomes between different parasite strains and, although the genome is generally diploid, the sizes of homologous chromosomes within the same cell can often differ considerably. The genome contains an extensive range of repetitive DNA elements and several large surface glycoprotein gene families, some with over 500 members. *T. cruzi* has an extremely complex cell surface, and this large and diverse repertoire of genes, many of which are expressed in a stage-specific manner, is critical to several aspects of the parasite life cycle (Fig. 1). These include host cell invasion, protection from complement and disruption of the host immune response.[12] In the parasite genome there are no recognizable RNA polymerase II-specific promoters for protein-coding genes; transcription is polycistronic and each mRNA is modified posttranscriptionally by addition of spliced leader RNA to its 5′ end. In the case of surface antigen genes, evidence is now accumulating that their stage-specific expression is regulated predominantly at the level of RNA stability.[13]

T. cruzi contains a single mitochondrion that forms a complex structure that extends throughout much of the

cell (Fig. 2). The mitochondrial genome, which contains 20–25% of the total cellular DNA, is sublocalized at a specific site organized as a tight compact disc called the kinetoplast[14] (Fig. 2). It contains two classes of circular DNA, maxicircles and minicircles, which form a concatenated network. The maxicircle DNAs are between 20 and 30 kb and are present in 25–50 copies per cell. The minicircle DNAs, of which there are 5–10,000 copies per cell, have a size range from 0.5 to 2.5 kb. Maxicircle DNA is analogous to the mitochondrial genomes of other organisms and contains genes encoding ribosomal RNA and components of the mitochondrial respiratory system. Most unusually, many of the primary transcripts of the protein coding genes do not contain a complete open reading frame and need to undergo RNA editing to produce functional mRNA.[15] This requires posttranscriptional insertion or deletion of uridine residues, a process mediated by small transcripts called guide RNAs that are encoded by the minicircle DNAs. Minicircles have a conserved region, which encompasses the postulated origin of replication and a region of high variability that reflects the repertoire of expressed guide RNAs.

THE *T. CRUZI* GENOME PROJECT

The *T. cruzi* genome project is expected to be completed in 2004.[16] At the time of writing the ''shotgun-sequencing'' phase of the project has been finished to a 14× coverage. However, the recent finding that the genome sequencing strain CL Brener is a hybrid of two distantly related class II lineages[9] will make the final production of a contiguous and fully annotated sequence a complex procedure. The consequences of this hybridization event are that allelic copies of genes in CL Brener can vary in sequence by more than 2%, and that recombination has produced chromosomes that are mosaics of the parental genotypes. Despite this, the genome project is already having a major impact on research strategies with the focus changing from the characterization of individual genes to a more comprehensive high-throughput functional analysis. This is being further driven by improvements in the tools that are available for genetic modification of the parasite[17] and by the new microarray and proteomic techniques, which can provide a snapshot of the relative expression of thousands of genes simultaneously. Because the genome sequence is only now approaching completion, these types of techniques have yet to be applied to *T. cruzi* in a systematic manner. However, developmental regulation, responses to environmental stimuli and drug treatment, dissection of signal transduction pathways, and the identification of virulence factors are examples of questions that will soon be amenable to analysis on a genome-wide basis.

CONCLUSIONS

In terms of understanding why Chagas' disease is such a debilitating condition, the two most important questions are ''Does the development of chronic disease pathology require the continued presence of the parasite?'' and ''Do all *T. cruzi* parasites have the potential to induce chronic disease?'' Because of the long-term nature of the disease, the low level of parasitemia in chronic patients, and the possibility of mixed infections these are not trivial problems to address.

Results achieved with PCR-based diagnosis increasingly support the premise that all untreated (and many treated) patients remain infected with the parasite for life. In addition, most clinical trials have demonstrated a positive correlation between chemotherapy (despite its limitations) and some degree of prevention/alleviation of chronic disease pathology. These findings therefore argue strongly that the development of more effective and less toxic drugs should be a major goal of postgenome research. In this context it may ultimately be feasible to identify individuals in the indeterminate stage of the disease who are infected with parasites that predispose them to develop chronic pathology and who should be prioritized for chemotherapy. This will require exploitation of the new postgenome technologies to define genotypic or phenotypic markers diagnostic of parasites capable of inducing chronic disease.

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Tuberous Sclerosis

David J. Kwiatkowski
Brigham and Women's Hospital, Boston, Massachusetts, U.S.A.

INTRODUCTION

Tuberous sclerosis (TSC) is an autosomal dominant genetic disorder characterized by development of distinctive nonmalignant tumors termed hamartomas and hamartias, in several organ systems. Tuberous sclerosis hamartomas involve the skin, brain, and kidneys in most TSC patients. Tuberous sclerosis is due to inactivating mutations in either the *TSC1* gene, which encodes the protein hamartin, or the *TSC2* gene, which encodes the protein tuberin. Most TSC lesions follow the first germline hit-second somatic hit tumor suppressor gene paradigm, such that complete loss of either hamartin or tuberin leads to tumor development.

CLINICAL AND MOLECULAR GENETICS OF TSC

About two-thirds of TSC cases are sporadic, representing new mutations. Tuberous sclerosis occurs in up to 1 in 6000 live births without apparent ethnic clustering. Large families with the condition are rare due to reduced reproduction among those affected.[1–3]

The *TSC1* gene consists of 23 exons with an 8.6-kb mRNA encoding the 130-kDa hamartin, and occupies 53 kb on 9q34 (Fig. 1).[3] The *TSC2* gene consists of 42 exons with a 5.4-kb mRNA encoding the 200-kDa tuberin and resides in 40 kb on 16p13 (Fig. 1).[3]

More than 600 mutations in *TSC1* and *TSC2* have been identified.[3] Comprehensive mutational analyses indicate that about 85% of TSC patients will have a mutation found in either *TSC1* or *TSC2*.[4,5] *TSC2* mutations are about 4.2 times as common as *TSC1* mutations, reflecting a higher germline mutation rate.

In *TSC1*, about half of the mutations are single base substitutions, of which most are nonsense mutations (Fig. 1). Just over half of the nonsense mutations are recurrent C to T transitions at six of the seven CGA codons within the *TSC1* coding region. Missense mutations are rare in *TSC1*, but have been confirmed as disease causing in a few cases. In *TSC2* as well, about half of the mutations are single-base substitutions (Fig. 1).[3] Nonsense mutations comprise about a third of these and commonly occur at five of the seven CpG sites which can transition to nonsense codons in *TSC2*. In contrast to *TSC1*, missense mutations are relatively common in *TSC2* (Fig. 1). *TSC2* has two CpG sites that are mutational hotspots which lead to missense mutations—1831-2 (611R>W and 611R>Q) and 5024-5 (1675P>L). These two CpG sites account for nearly half of the missense mutations in *TSC2*. Another relatively common mutation in *TSC2* is an 18-bp in-frame deletion mutation near the C-terminus (Fig. 1). However, no mutation in either gene is seen in more than 2% of all TSC patients.

About a third of *TSC1* mutations are small deletions (<29 bp), while half that number are small insertions (<34 bp). Nearly all small insertions and most deletions arise as duplications/deletions of a tandem repeat of single or multiple bases. Similar observations apply to small deletions and insertions found in *TSC2*. Large deletions and rearrangements are relatively common in *TSC2*, accounting for 16% of all mutations, whereas they are rare in *TSC1*, accounting for only 2%. In *TSC2*, deletions show no consistency in either size or junction fragments involved, ranging in size from 1 kb to over 100 kb.[3]

Patients with *TSC1* mutations have symptoms and clinical features that are milder on average than patients with *TSC2* mutations, although there is considerable overlap in severity.[4] Linkage studies provide evidence against a third TSC gene. Patients in whom mutations cannot be found are likely partially explained by mosaicism, which is well documented in TSC, and hinders efforts at mutation detection.[3]

Tuberous sclerosis molecular genetic testing is available commercially in the United States through Athena Diagnostics, and through several academic laboratories in Europe. In most laboratories the overall mutation detection rate is less than 80%, and findings of sequence variation of uncertain significance are often made, including possible missense and splice site mutations. Mutation identification has benefit for genetic counseling and prenatal diagnosis, particularly for individuals who have TSC. For parents of sporadic cases of TSC, the benefit is less clear, as such parents have a very low recurrence risk (~2%) if they are thoroughly screened clinically (exam by an experienced clinician and imaging studies of brain and kidney) and have no evidence of TSC.

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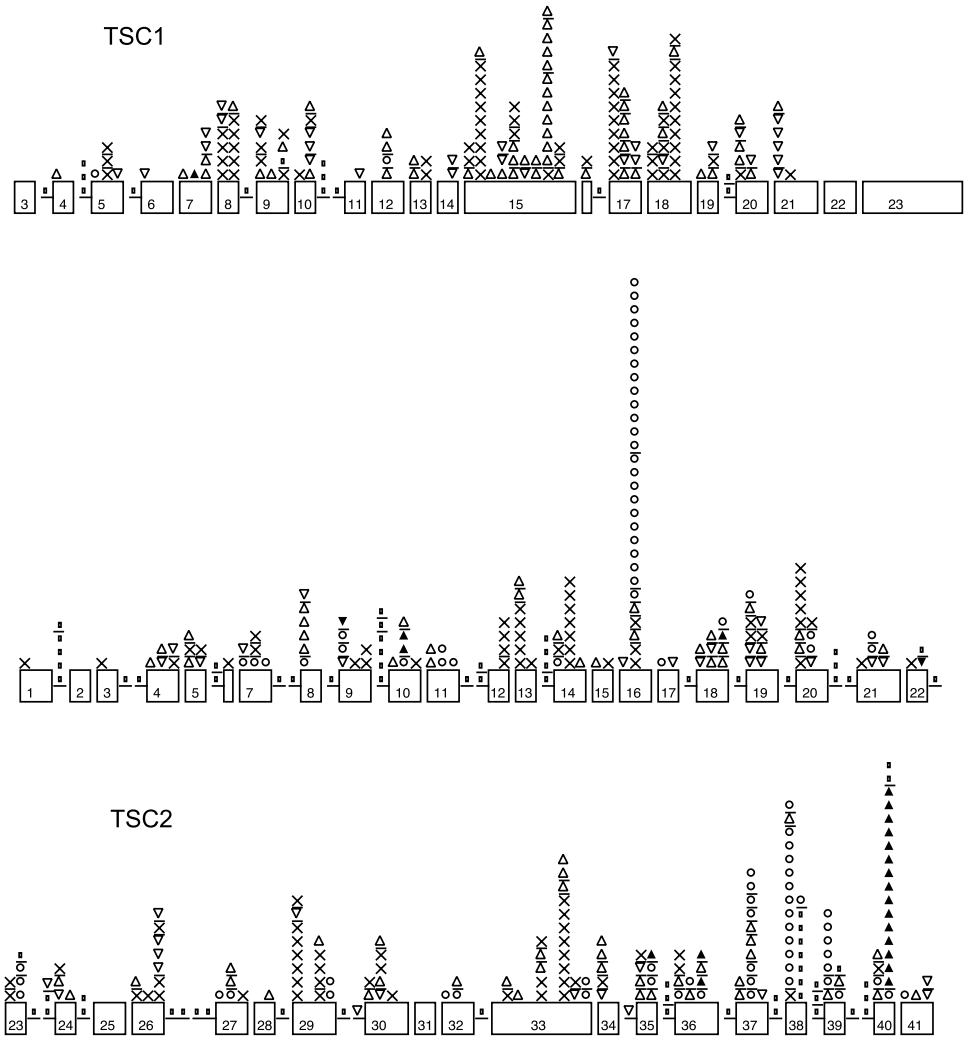

Fig. 1 Map of the sites of several hundred small mutations in TSC1 and TSC2. Proportional drawings (boxes) of all of the exons of TSC1 and TSC2 are shown. Intron regions are not drawn to scale and are expanded only when a mutation is present. Mutation symbols are: ×, nonsense; ■, splice site; △, deletion; ▽, insertion; ○, missense; □, in-frame deletion. A line separates mutations occurring at nearby but not identical nucleotide positions.

CLINICAL FEATURES OF TSC

Although hamartomas typically occur in several organ systems in TSC, the vast majority display limited growth potential and do not require intervention. A small fraction display persistent growth, necessitating surgical control. However, progression to malignancy is very rare in TSC and has been seen only in TSC renal hamartomas, termed angiomyolipoma (AML), at a frequency of about 2% of all TSC patients.[1,2] Another remarkable feature of TSC hamartomas is that they first appear at different ages during the patient's life and at times spontaneously resolve. For example, cardiac rhabdomyomas are often present at birth, but then usually disappear during childhood. The diversity and variability of clinical presentations and hamartomas in TSC have led to formal diagnostic criteria (Table 1).[6]

The cardinal feature of TSC is the cortical tuber, which is a distinctive form of brain hamartia.[1,2,7] Grossly, tubers are firm, smooth, somewhat raised, pale lesions of the cerebral cortex, and can number up to 40 in one brain, ranging in size from a few millimeters to several centimeters. The second common brain lesion seen in TSC is the subependymal nodule (SEN), typically found in the lateral ventricle. They occur as smooth nodules projecting into the ventricle and typically calcify in patients past the age of 10.

Histologically, cortical tubers are characterized by architectural disarray with disruption of cortical lamination.[1,2,7] Tubers contain a mixture of cells, including normal and dysplastic neurons, astrocytes, and the hallmark giant cell. Giant cells extend short aberrant processes and express both glial and neuronal proteins. Subependymal nodules consist of large cells

Table 1 Diagnostic criteria for TSC

Major features
1. Facial angiofibromas or forehead plaque
2. Nontraumatic ungual or periungual fibroma
3. Hypomelanotic macules (three or more)
4. Shagreen patch (connective tissue nevus)
5. Multiple retinal nodular hamartomas
6. Cortical tuber[a]
7. Subependymal nodule
8. Subependymal giant cell astrocytoma
9. Cardiac rhabdomyoma, single or multiple
10. Lymphangiomyomatosis[b]
11. Renal angiomyolipoma[b]

Minor features
1. Multiple, randomly distributed pits in dental enamel
2. Hamartomatous rectal polyps
3. Bone cysts
4. Cerebral white matter radial migration lines[a]
5. Gingival fibromas
6. Nonrenal hamartoma
7. Retinal achromic patch
8. ''Confetti'' skin lesions
9. Multiple renal cysts

Definite tuberous sclerosis complex: either two major features or one major feature plus two minor features.
Probable tuberous sclerosis complex: one major plus one minor feature.
Possible tuberous sclerosis complex: either one major feature or two or more minor features.
[a]When cerebral cortical dysplasia and cerebral white matter migration tracts occur together, they should be counted as one rather than two features of tuberous sclerosis.
[b]When both lymphangiomyomatosis and renal angiomyolipomas are present, other features of tuberous sclerosis should be present before a definite diagnosis is assigned.
Source: From Ref. [6].

in a vascular stroma, with occasional cells that attain giant cell proportions with aberrant and/or multiple nuclei. Subependymal giant cell astrocytomas (SEGA) develop from a SEN in 5–10% of TSC patients. Although there is growth to a size >1 cm, there is no histological progression. These lesions must be treated by surgical resection to prevent hydrocephalus and other adverse effects.

The major presenting symptoms and signs of cerebral TSC are a variety of epileptic seizures.[1,2,7] These seizures have their origin in the cortical tubers or in nearby transitional cortex. About two-thirds of TSC patients first present with infantile spasms usually between the ages of 3 and 9 months. There is a correlation between the number of cortical tubers in a TSC patient and both a younger age at onset and severity of seizures.

Mental retardation and developmental problems are serious consequences of brain involvement by cortical tubers.[1,2,7] Mental retardation is variable but can be profound, and rarely occurs in the absence of a generalized seizure disorder. Developmental problems are very common in TSC and include autistic behavior, hyperactivity, sleep disorders, and aggressive behavior.

Angiomyolipomas (AMLs) are the most common lesion in the TSC kidney, being seen in about 75% of children by adolescence using ultrasound imaging.[1,2] These lesions consist of a variable mixture of aberrant vessels, smooth muscle, and fat. Angiomyolipomas are typically bilateral and of variable size. Lesions >5 cm diameter are not rare, and large lesions are associated with a significant risk of bleeding as well as loss of nephrons due to compression.

Cutaneous findings are present in the great majority of TSC patients and are the most easily identified sign of the disease.[1,2] Most lesions are of minor clinical significance, but facial angiofibromas can be a significant cosmetic issue. Hypomelanotic macules or white spots typically have a lance-ovate shape (Ash-leaf) and are most common over the trunk and buttocks. Three or more of these lesions are very unusual in the general population and thus are considered a major diagnostic criterion (Table 1). The lesions are present at birth and do not change, although they are more easily observed following suntanning.

Facial angiofibromas are red to pink papules or nodules with a smooth surface that are found in a malar distribution and extending down to the chin.[1,2] They typically first appear between the ages of 2 and 6, and progress to a variable extent during puberty. Histological findings are dermal fibrosis and angiogenesis with occasional large glial appearing cells. Forehead fibrous plaque is a larger lesion related to facial angiofibroma, and Shagreen patch is also a related lesion found typically on the upper buttock. Ungual fibromas are another common cutaneous manifestation of TSC, being seen in most adults over the age of 30 years, but rarely prior to adolescence. These lesions are red- or flesh-colored papules or nodules occurring in the finger or toe nail regions.

Lymphangiomyomatosis (LAM) is a disorder seen almost exclusively in females which is characterized by smooth muscle cell proliferation and cystic changes in the lung parenchyma. Most LAM patients also have AMLs in the kidney or elsewhere in the abdomen. Lymphangiomyomatosis presents clinically as progressive dyspnea, or acute dyspnea due to pneumothorax, and can be progressive and fatal. However, 40% of adult TSC women have CT-scan evidence of LAM, and most of those are asymptomatic.

The retina is involved by hamartomas in about 50% of TSC patients. Cardiac rhabdomyomas are also found in most TSC infants when echocardiographic screening is done. These lesions can be up to several centimeters in diameter and consist of glycogen-filled myocytes. In most cases, there are no symptoms and the lesions spontaneously decrease in size or disappear over time. Less often, rhabdomyomas cause heart failure due to obstruction, affect myocardial function by replacement, or cause rhythm disturbances, and surgical resection is considered.

PATHOGENESIS OF TSC

Tuberous sclerosis hamartomas, particularly renal AMLs, usually display loss of heterozygosity (LOH) for the wild-type allele of either *TSC1* or *TSC2*, consistent with a two-hit mechanism for complete inactivation of either *TSC1* or *TSC2*.[3] This has not been seen as consistently for other types of TSC lesions, but microdissection has been effective in some cases (e.g., LAM) to demonstrate LOH events. Cortical tubers do not show evidence of LOH either, and cell admixture likely explains this finding.

Consistent with a relative lack of malignancy in TSC patients, limited surveys of human cancer specimens have failed to show evidence of either *TSC1* or *TSC2* mutation, with the possible exception of bladder carcinoma.

Seminal studies in Drosophila led to major insights into the cellular functions of TSC1 and TSC2.[8] Translation and extension of these studies in mammalian cells have led to a model of P13 K–Akt–TSC1/TSC2–Rheb-mTOR signaling that is shown in Fig. 2. In normal quiescent cells, P13 K and Akt are inactive, the TSC1/TSC2 complex is active as a GTPase activating protein (GAP) for Rheb, there are low levels of Rheb-GTP, and mTOR is inactive. In response to growth factor stimulation, P13 K and Akt become activated, TSC2 is phophorylated by Akt, and the TSC1/TSC2 complex becomes inactive as a GAP, so that Rheb-GTP levels rise, stimulating mTOR. In cells lacking TSC1 or TSC2, the residual TSC2 or TSC1 does not function as a GAP for Rheb, and Rheb-GTP levels are high, leading to constitutive activation of mTOR and phosphorylation of S6K1 and 4E-BP1. The model shown captures the main pathway involving TSC1 and TSC2, but omits many details and interconnections in the complex biochemical pathway that regulates cell growth. One

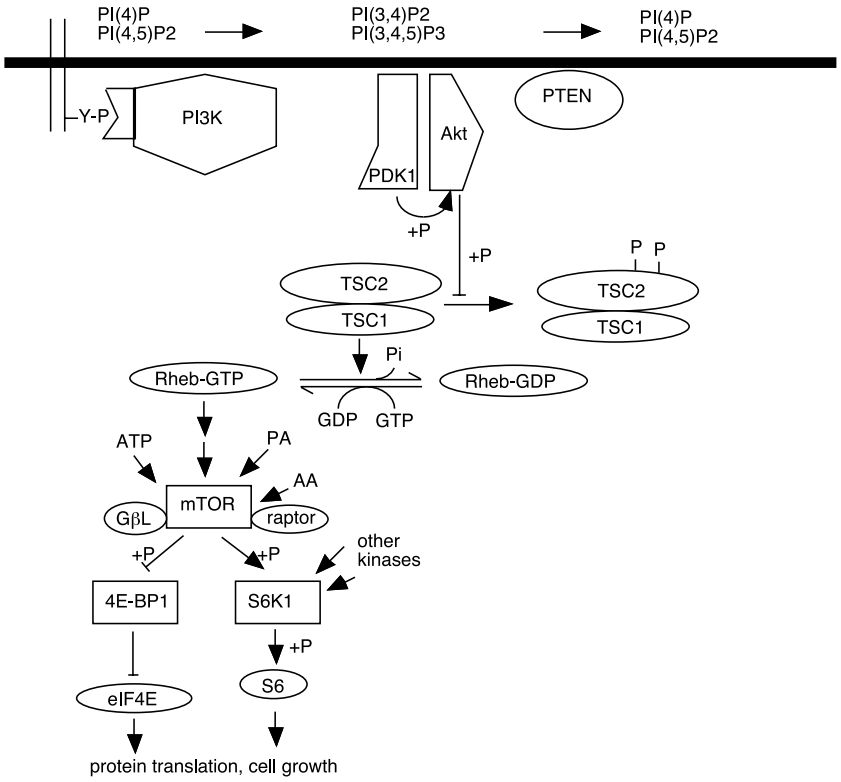

Fig. 2 Signaling pathway model for the function of TSC1 and TSC2 in mammalian cells. A phosphorylated growth factor receptor is shown at the upper left, to which a PI3 K molecule is binding. This leads to conversion of the indicated phosphoinositides to 3′-phosphoinositides, which leads to recruitment of Akt to the membrane in a position where it can be phosphorylated and activated by PDK1 and a second kinase. PTEN functions to terminate this signaling pathway by acting as a 3′ phosphatase on these phosphoinositides. Activated pAkt phosphorylates TSC2 which inactivates its GAP activity. When active, TSC1/TSC2 complex serves as a GAP for Rheb, reducing levels of Rheb-GTP. Rheb-GTP activates mTOR by an uncertain mechanism (thus two arrows). ATP, phosphatidic acid (PA), and amino acids (AA) all influence mTOR activity, although the sensing mechanisms are unknown and likely indirect. Active mTOR phosphorylates 4E-BP1 and S6K1. p4E-BP1 releases from eIF4E, permitting formation of the eIF4F translation complex. pS6K1 phosphorylates S6, and together they activate the translational machinery. For simplicity only the main pathway involving TSC1, TSC2, and mTOR is shown. Arrows indicate positive actions and bars represent negative actions. TSC1 and TSC2 are used here and in the text to denote the corresponding proteins hamartin and tuberin.

clinical correlate of this model is that hamartomas that occur in TSC both in patients and animal models typically express phospho-S6 and phospho-S6K1, signposts of activation of mTOR. Two consequences of activation of this pathway are the secretion of VEGF by cells in many TSC lesions and abnormal STAT phosphorylation.

The molecular basis of epileptogenesis induced by cortical tubers is uncertain, although disorganization of the cortex in these lesions likely contributes. There is increased transcription of genes encoding glutamatergic receptors by dysplastic neurons and giant cells in tubers, with reduced expression of gamma-aminobutyric acid (GABA)-ergic receptors.[7] These expression changes may contribute to epileptogenesis in TSC. Tuber giant cells and SEN cells also express pS6 and pS6K1, like other TSC lesions, consistent with complete inactivation of TSC1 or TSC2 with activation of mTOR as in other TSC hamartomas.

The TSC1/TSC2 complex has size 330 kDa, and the GAP domain of TSC2 comprises about 10 kDa of this complex. This alone suggests that there are other functions of the complex. TSC1 has been reported to bind to ezrin and other ERM family proteins, and appears to be involved in adhesion events and rho signaling to the actin cytoskeleton.[9] TSC2 has been reported to have a role in the membrane localization of polycystin-1 in renal epithelial cells.[10] A role for the TSC1/TSC2 complex in beta-catenin signaling has also been described. Whether any of these observations are independent of or relate to the role of TSC1/TSC2 in the P13 K signaling pathway is unknown.

CONCLUSION

Tuberous sclerosis is a disorder that affects approximately 40,000 people in the United States. It is remarkable in several respects: the frequency and severity of associated epileptogenesis, the unique predisposition to autism and other behavioral syndromes, and the unusual spectrum of associated tumors including LAM. The placement of the TSC genes in the PI3 K–Akt–TSC1/TSC2–Rheb-mTOR signaling pathway explains the hallmark giant cells seen in cortical tubers and provides the possibility of several pharmacological approaches that may be helpful in TSC. This includes drugs already in use in the clinic, including rapamycin and analogues (inhibit mTOR), farnesyl transferase inhibitors (inhibit Rheb), angiogenesis inhibitors, and interferon-γ. Current ongoing and planned trials of these agents in both preclinical models and TSC patients are of considerable clinical and basic interest.

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Vibrio cholerae

Durg V. Singh
Institute of Life Sciences, Bhubaneswar, India

INTRODUCTION

Vibrio cholerae is a natural inhabitant of aquatic environments.[1] *V. cholerae*, which belongs to the O1 and O139 serogroups, is the only serogroup reported to be responsible for epidemic and pandemic forms of cholera,[2,3] whereas the *V. cholerae* non-O1 and non-O139 strains are associated with cases of sporadic cholera and extraintestinal infections.[4] Genetic and phenotypic analyses of *V. cholerae* revealed that, in addition to the genes encoding cholera toxin (CT), they require the colonization toxin-coregulated pilus (TCP) and a central regulatory protein, ToxR, which coregulates the expression of CT and TCP.[3] Polymerase chain reaction (PCR), multiplex PCR, and hexaplex PCR have been used to detect *V. cholerae* belonging to O1, O139 , non-O1, and non-O139 serogroups and virulence-associated genes, including CT (*ct*), zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), TCP (*tcp*), and outer membrane protein (*ompU*), and a central regulatory protein, ToxR.[5] Molecular techniques have been used to detect genetic changes in *V. cholerae* associated with displacement of existing serogroups by newly emerging serogroups or by other serogroups, which may provide new insights into the epidemiology of cholera. This work will focus on the current status of knowledge of epidemiology, antibiotic resistance, and virulence-associated factors of *V. cholerae*, which cause diarrhea in humans.

EPIDEMIOLOGY OF CHOLERA

V. cholerae is the causative agent of cholera, an acute dehydrating diarrhea that occurs in epidemic and pandemic forms.[2,3] Cholera has been recognized as one of the ''emerging and reemerging infections, threatening many developing countries.'' Several recent events that mark the epidemiological importance of the disease include the reemergence of cholera in Latin America in 1991, the explosive outbreak of cholera in Rwandan refugees in Goma and Zaire,[3] and the emergence of *V. cholerae* in the Indian subcontinent during 1992.[6,7] All pandemics, except for the seventh pandemic, which originated in Indonesia, arose from the Indian subcontinent, usually from the Ganges Delta region, and reached other continents.

Molecular Epidemiology

Molecular typing methods have been used to study the epidemiology of *V. cholerae*. While analyzing the nucleotide sequence of *asd* gene in *V. cholerae*, Karaolis et al.[8] demonstrated that the sixth and seventh pandemic strains and the U.S. Gulf coast *V. cholerae* O1 isolates may have derived from nontoxigenic strains, and postulated that horizontal gene transfer occurred in *V. cholerae*, resulting in the emergence of a new pathogenic strain. The use of gene probes to study restriction fragment length polymorphism (RFLP) in the *ctxAB* genes and their flanking DNA sequences, which are part of a larger genetic element (CTX element), indicated that the U.S. Gulf coast isolates of toxigenic *V. cholerae* O1 are clonal and different from other seventh pandemic isolates.[2] The diversity of CTX genotypes in *V. cholerae* O139 was reported, which may have resulted from the duplication of CTX prophage.[3] However, the toxigenic O139 strains prevalent in two endemic areas in India, including Calcutta and Alleppey, Southern India, and Bangladesh, are not clonal, and the Alleppey O139 strains are different from the Calcutta and Bangladesh strains, which contain the unique arrangement of the CTX prophage.[7]

Ribotyping and multilocus enzyme electrophoresis (MEE) analysis of the toxigenic *V. cholerae* O1 El Tor strains indicated clonal diversity, and the clones reflected broad geographical and epidemiological areas.[5,9,10] Comparative analysis of the El Tor strains of *V. cholerae* O1 and the epidemic O139 strains suggests that the O139 strains are related to El Tor strains and are derived from them by possible genetic changes in serotype-specific gene cluster.[3,9] Numerical analysis of ribotype patterns also revealed that *V. cholerae* strains belonging to the non-O1 and non-O139 serogroups are widely diverse from the O1 and O139 *V. cholerae* strains.[3,5,11] Further studies demonstrated the transient appearance and disappearance of more than six ribotypes of classical vibrios and three different ribotypes of *V. cholerae* O139. Different ribotypes often showed different CTX genotypes resulting from difference in the copy number of the CTX element and the variation in the integration site of the CTX

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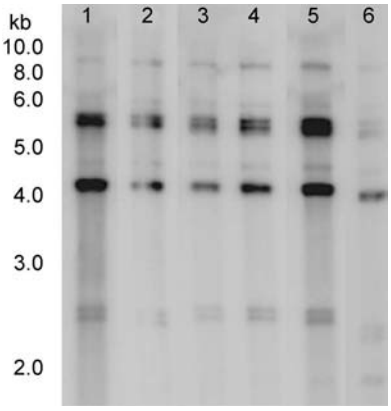

Fig. 1 Southern hybridization of genomic DNA from *V. cholerae* O139 strains digested with *Bgl*I and probed with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK 3535. Restriction pattern corresponding to ribotypes derived from strains is shown in lanes 1 and 2 (B-I), lanes 3 and 4 (B-II), and lane 5 (B-IV), and a new ribotype (B-VI) derived from *V. cholerae* O139 strain ALO95 isolated from a diarrheal patient is shown in lane 5. (From Ref. [12]. Courtesy of ASM Press, New York.)

element in the chromosome, giving rise to new clones possibly by natural selection involving unidentified environmental factors and immunity of the host.

Ribotype analysis of O139 strains isolated until 2001 showed diverse ribotype patterns[7] and, since then, one more ribotype has been detected[12] (Fig. 1). Results of pulsed-field gel electrophoresis (PFGE) analysis of clonal diversity in O139 strains showed that PFGE patterns were highly similar but nonidentical with minor differences in banding patterns of *Not*I restriction patterns. This study suggested that although O139 had a cloned origin, this serogroup is undergoing changes in a manner analogous to that seen among the El Tor strains.[13] Studies on clonality of clinical and environmental *V. cholerae* O1, O139, non-O1, and non-O139 groups by using enterobacterial repetitive intergenic consensus sequence (ERIC) PCR, box element PCR, amplified fragment length polymorphism (AFLP), and PFGE collectively indicated that *V. cholerae* O1 and O139 had a clonal origin, whereas the non-O1 and non-O139 strains belonged to different clones. The clinical isolates resembled environmental isolates in their genomic patterns, suggesting that aquatic environments are reservoirs for *V. cholerae*.[5] It was also shown that the multiple clones of *V. cholerae* O139, identical to those of O139 that caused an epidemic in 1992, were present in the aquatic environment.[14]

Antibiotic Resistance

Antibiotic susceptibility tests of O1 strains isolated from the 1979 outbreak in Bangladesh showed resistance to tetracycline, in addition to other antibiotics ampicillin, kanamycin, streptomycin, and cotrimoxazole (trimethoprim-sulfamethoxazole). The subsequent follow-up study of the epidemiological assessment of the outbreak indicated that the O1 isolates were not resistant to tetracycline, including streptomycin, chloramphenicol, amoxicillin, or nalidixic acid. All the classical *V. cholerae* strains isolated in Bangladesh during 1988 and 1989 were resistant to tetracycline, whereas strains belonging to the El Tor biotype were sensitive to the drug. Almost after a decade, tetracycline-resistant El Tor strains reemerged in Bangladesh in 1991 and in Tehran in 1998.[3,15] In October 1995, the emergence of nalidixic acid-resistant O1 *V. cholerae* El Tor strains was reported in Southern India.[16] Moreover, 80–100% of *V. cholerae* O1 isolated in Kenya, South Sudan, Peru, and Guinea-Bissau, and 65–90% of isolates from Somalia isolated between March 1994 and December 1996, were not resistant to tetracycline, whereas all the O1 isolates from Tanzania and Rwanda were resistant to this drug.[3] A number of *V. cholerae* O1 strains isolated during 1992–1997 in Calcutta showed resistance to tetracycline (in addition to ampicillin, chloramphenicol, cotrimoxazole, neomycin, and streptomycin) and emerging resistance to nalidixic acid.[17] Analyses of the antibiotic susceptibility of *V. cholerae* O1 strains isolated from Kottayam, Alleppey, and Trivandrum, Southern India, in 2000 showed that these strains are resistant to nalidixic acid, and/or neomycin, and/or streptomycin, respectively, in addition to other drugs tested, and are sensitive to tetracycline, suggesting the existence of different R-types of *V. cholerae* strains in different locations.[18] Continuous surveillance of outbreaks of cholera in Kerala, Southern India showed the emergence of nalidixic acid-resistant strains of *V. cholerae* O1 in 2002 (unpublished).

In the analysis of the O139 strains isolated during the past 9 years, Faruque et al.[7] showed that O139 strains remained largely susceptible to ciprofloxacin, tetracycline, and gentamicin, and resistant to ampicillin; susceptibility to cotrimoxazole, chloramphenicol, and streptomycin varied during this period. Recent studies have shown that O139 strains isolated from different parts of India were, by and large, resistant to ampicillin, furazolidone, streptomycin, and nalidixic acid, except for Calcutta strains that were sensitive to nalidixic acid. Recently, emergence of nalidixic acid-resistant strains of *V. cholerae* O139, which are sensitive to sulfamethoxazole, trimethoprim, and streptomycin, was observed from the outbreak of cholera in Alleppey and Trivandrum, Southern India in 2002 (unpublished). Waldor et al.[19] reported the presence of a 62-kb self-transmissible transposon-like (SXT) element encoding resistance to sulfamethoxazole, trimethoprim, and streptomycin in *V. cholerae* O139 isolated during this period. The SXT

element could be conjugally transferred from *V. cholerae* O139 to *V. cholerae* O1 and *Escherichia coli* strains, where it integrated into recipient chromosomes in a site-specific *rec-A*-independent manner. Considering the rapidly changing patterns of antibiotics observed among *V. cholerae* strains, it appears that there is substantial mobility in genetic elements encoding antibiotic resistance in *V. cholerae*.

VIRULENCE-ASSOCIATED GENES

Cholera is characterized by a severe watery diarrhea caused by toxigenic *V. cholerae*, which colonizes the small intestines and produces an enterotoxin, CT.[3] Although production of CT encoded by the *ctxAB* genes is directly responsible for the manifestation of diarrhea, cholera pathogenesis relies on the synergistic action of a number of other genes, such as colonization TCP and outer membrane protein (*ompU*), and a regulatory protein, ToxR, which coregulates the expression of CT and TCP,[3] and part of the CTX-genetic element comprising zonula occludens toxin (*zot*) and accessory cholera enterotoxin (*ace*), which are virulence factors and also play a role in the morphogenesis of the filamentous phage CTXΦ.[19]

PCR

Multiplex PCR was successfully used to detect the CT and associated virulence gene fragments and, simultaneously, to identify the O1 and O139 serogroups of *V. cholerae*.[20] From the diagnostic point of view, toxigenic–pathogenic and nontoxigenic–nonpathogenic strains of *V. cholerae* can be differentiated by the presence of *ctx* and *tcp* genes. It was difficult to know whether the CT gene-negative strain possesses other toxin genes, such as, *zot*, *ace*, part of the CTX genetic element, and *ompU*. None of these methods can be used to individually identify toxin and virulence genes. Hence, a one-step hexaplex PCR was developed for rapid detection of an array of six specific virulence genes comprising CT subunit A (*ctxA*), zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), adherence outer membrane protein (*ompU*), TCP (*tcpA*), and the regulatory protein (ToxR).[20] Results of representative strains of *V. cholerae* are shown in Fig. 2. Employing the hexaplex PCR, 97 strains of *V. cholerae* and *Vibrio mimicus* were screened for the presence of virulence genes. It was found that hexaplex PCR can rapidly detect, in a single tube, the six commonly known virulence genes in *V. cholerae*, and was specific and sensitive.

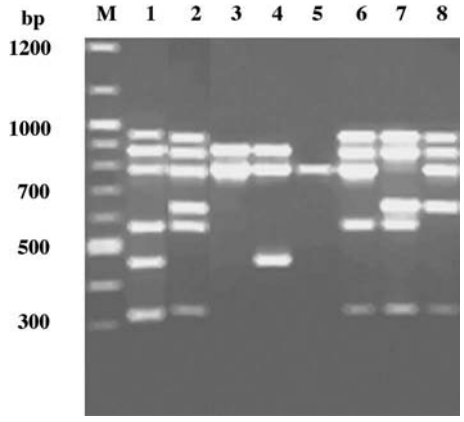

Fig. 2 Agarose gel electrophoresis of hexaplex PCR products obtained with *V. cholerae* strains. Lane M, 100-bp DNA ladder (NEB); lanes 1 and 2, toxigenic *V. cholerae* O1 El Tor strain 20 and classical strain 569B; lanes 3–5, nontoxigenic *V. cholerae* O1 El Tor strains X392, 2740-80, and 1074-78; lane 6, *tcpA*$^-$ *V. cholerae* O1 classical strain O395 RT 110-12; lane 7, *toxR*$^-$ *V. cholerae* O1 classical strain O395-12; lane 8, *ctxA*$^-$ *V. cholerae* O1 classical strain CVD 103-HgR.

DIAGNOSIS

Laboratory diagnosis of acute untreated cholera is fairly simple and straightforward as a large number of cholera vibrios are present in the stool of such cases. The stool samples or rectal swabs transported either in Cary–Blair transport medium, alkaline peptone water, or tellurite–taurocholate peptone broth in the laboratory are plated on commonly used thiosulfate–citrate–bile salts–sucrose (TCBS) medium for isolation of *V. cholerae*.[21] The sucrose-fermenting *V. cholerae* isolates are readily detected on this medium as large, yellow, smooth colonies. Suspected colonies from TCBS are inoculated into Kligler iron agar (KIA) medium. Cultures yielding an alkaline slant over acid butt with no gas or H_2S are then tested for oxidase activity and reactivity with polyvalent O1 or O139, and monospecific Ogawa or Inaba antisera using growth from the KIA slant. In case of doubtful agglutination or nonagglutination with O1 or O139 antisera, it is imperative to perform some tests such as oxidase and string test, mannitol and sucrose fermentation, lysine and ornithine decarboxylation, and arginine dihydrolation.

CLINICS AND THERAPY OF CHOLERA

Cholera is a clinical–epidemiological syndrome caused by *V. cholerae* belonging to serogroups O1 and O139. It is characterized by the passage of voluminous stools of rice water character and vomiting, which leads to

dehydration resulting from underlying deficit due to loss of large quantities of extracellular fluids. Hypovolemic shock, acidosis, and death can ensue in adults, as well as in children, if prompt and appropriate treatment is not initiated.

The key to therapy is provision of adequate rehydration until the disease has run its course in the absence of antimicrobial therapy. Rehydration can be accomplished by intravenous infusion of fluid in severe cases, or by oral rehydration with an oral rehydration solution (ORS).[2,22,23] Antimicrobial agents play a secondary but valuable role in therapy by decreasing the severity of illness and the duration of excretion of the organism. The drugs of choice are tetracycline, erythromycin, furazolidone, cotrimoxazole, norfloxacin, and ciprofloxacin. The hypoglycemia that is sometimes seen in pediatric patients should be treated with 25% or 50% glucose given intravenously.

CONCLUSION

Because *V. cholerae* is a water-borne organism, regular monitoring of this water-borne pathogen is required to protect public health particularly in developing countries where hygienic condition is poor. DNA-based molecular techniques have provided evidence to detect genetic changes in *V. cholerae* and for emergence of new clones or other serogroups. Screening of antibiotic susceptibility will possibly help to find the rapidly changing pattern of antibiotic resistance among *V. cholerae* strains and to trace the mobility of a genetic element(s), if any, encoding antibiotic resistance.

Hexaplex PCR may be used for the rapid detection of virulence genes in O1, O139, non-O1, and non-O139 serogroups from pure bacterial culture and directly from a variety of specimens, including clinical specimens, after removing inhibitors of PCR present in human feces by centrifugation and activity of remaining inhibitors by 10-fold dilution, foods by quantitative PCR, and environmental samples by extracting DNA from water samples. However, there is a need to develop a multiplex PCR for a rapid and accurate detection of *V. cholerae* belonging to O1, O139, non-O1, and non-O139 serogroups, in addition to virulence genes from a variety of samples.

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V

Vibrio vulnificus

Olivier A. E. Sparagano
University of Newcastle, Newcastle, U.K.

Brian Austin
Heriot-Watt University, Edinburgh, U.K.

INTRODUCTION

Vibrio vulnificus is a motile marine (and estuarine) gram-negative bacterium that contaminates filter-feeding shellfish, fish, and humans involved in lethal septicemia and wound infections. Outbreaks in United States, Europe, Asia, and Australia are mainly related to oyster consumption followed by clams and mussels. In addition, there have been cases of human disease in Europe (e.g., The Netherlands) associated with the handling of eels.

TAXONOMY

It was first described as *Vibrio parahaemolyticus* by the CDC in 1964 and separated in 1970.[1] The closest neighbor was thought to be *Vibrio anguillarum* type B (*Vibrio anguillarum forma anguillicida*), according to the description of Nybelin.[2] Former biotypes 1 and 2 were considered no longer appropriate,[3] and it was subsequently deemed preferable to refer to the organisms as a serovar (=serotype=serogroup) rather than biotype.[4] With serovar E being similar to biotype 2, serovar B is close to human clinical strains[5] and a new serovar A was recently described in Spain.[6]

ECOLOGY

V. vulnificus has been isolated from waters[7] with temperatures ranging from 9°C to 31°C, but proliferation was observed when the temperature exceeded 18°C[8] and will revert to a viable but nonculturable (VNBC) state if water temperatures go below 10°C and, therefore, could become undetectable by culturing.[1] Interestingly, studies on temperature resistance showed better results when *V. vulnificus* is subjected to starvation.[9] It also appears that for waters with salinity above 20 ppt, *V. vulnificus* levels decline and should not be considered as a risk for humans.[10]

PATHOLOGY

Two main pathologies have been associated with *V. vulnificus*: wound infections and primary septicemia.[1,11–15] Recently, an alarming number of human cases were reported in Japan,[16] Taiwan,[17] Singapore,[18] Chile,[19] United States,[20] New Zealand,[21] and Spain.[22] Infection occurs also in immunocompromised patients. Furthermore, snakebites were complicated when *V. vulnificus* was also present in the wound.[23]

The proliferative and degenerative symptoms could be indicated by the observation that *V. vulnificus* induces apoptosis in macrophages[24] bypassing the first defense mechanisms of the host. Proinflammatory cytokines are known to be of major importance in septicemia because of this bacterium.[25] For details of other virulence factors, see the review by Strom and Paranjpye.[1]

However, metalloproteases have been well studied in connection with virulence.[26] In addition, the expression of a capsular polysaccharide (CPS) was shown to be virulence-related.[27] Molecular studies have been carried out (to be developed further in another article in this volume) on a 68-kbp plasmid, which was found in virulent strains from fish.[28]

DETECTION

Molecular Detection

Studies on the 16S rDNA gene have already given some interesting results either by differentiating *V. vulnificus* against other representatives of the *Vibrio* or by differentiating type B from type A strains, thus allowing an understanding of mixed infections and seasonal variation between strains.[29] Indirect study with PCR-DGGE showed sensitivity based on a pure culture of *Escherichia coli* of between 10^4 and 10^5 cfu/mL.[30] A study of the 16S–23S rDNA spacers highlighted the possibility of detecting NCBV strains in oysters,[31] demonstrating the value of such molecular methods to

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identify pathogens that will not grow on artificial media whatever species they are.

DNA probing the *cth* and *tlh* genes (hemolysin-related genes) allowed authors to differentiate *V. vulnificus* and *V. parahaemolyticus*, respectively.[32] However, polymorphism is still a major problem to find strain-specific or species-specific probes not cross-reacting with other vibrios.[33]

However, other authors tried to elucidate the gene regulations of *V. vulnificus* to develop new control methods. The haem uptake receptor R (hupR) was shown to share homologies with the LysR family and could be a positive regulator of *hupA* gene to satisfy *V. vulnificus* iron requirements.[34]

Several regions such as cytolysin, *vvhA* (*V. vulnificus* hemolysin gene), pR72H (a 0.76-kb *Hin*dIII DNA fragment cloned from *V. parahaemolyticus*), and 16S–23S rRNA were simultaneously used to specifically identify this bacterium.[40] The *vvhA* gene responsible for the production of the cytolytic hemolysin of *V. vulnificus* and its activation by the cAMP receptor protein (CRP) has also been extensively studied.[35] Mutagenesis study of a virulent *V. vulnificus* strain identified four essential capsular polysaccharides (CPS) for virulence expression.[36] A seminested PCR on the *vvhA* gene showed its transcription in NCBV populations and may be very useful for assessment of water quality.[37]

Nonmolecular Detection

Pathogenic *Vibrio* spp. are usually isolated by preenrichment, for example, in alkaline peptone water (APW) with 1% NaCl. Some media have been developed specifically for *V. vulnificus* and include semiquantitative approaches[38] and selective agars.[39]

An immunofluorescence method was used successfully to detect *V. vulnificus* serovar E, showing the possibility of developing immunological tests.[40]

CONCLUSION

V. vulnificus has a bad reputation because of the tremendous debilitating impact on animals and humans. However, there is some progress toward the identification and the prevention of the diseases. For the latter, some workers have successfully protected eels against *V. vulnificus* attacks by immunization showing that capsular antigens are better for protection and should be administered by intraperitoneal injections with vaccines enriched with toxoids.[41] However, vaccination strategies gave different results according to the route of administration and the antigen preparation.[42] Nevertheless, there is optimism for controlling this bacterium, with preliminary studies already highlighting bacteriophage activities[43] and the effect of bacteriocins on *V. vulnificus*.[44] After observing that around 85% of male patients developed endotoxic shock, some studies have examined the role of estrogens for protection.[45] However, further studies of the genome could allow the identification of key factors needed to eradicate this pathogen. In addition, it would then be possible to develop new microarray techniques to identify culturable versus nonculturable or pathogenic versus nonpathogenic strains or differentiating several pathogens at the same time.[46]

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Viral Encephalitis—Japanese Encephalitis, Tick–Born Encephalitis, and St. Louis Encephalitis

Matthias Niedrig
Jens Mehlhose
Robert Koch Institut, Berlin, Germany

INTRODUCTION

Japanese encephalitis (JE), St. Louis encephalitis (SLE), and Tick–Born encephalitis (TBE) are important representatives in the group of *Flavivirus* diseases. The viruses of these diseases are transmitted by mosquitoes or ticks. Symptoms of diseases are very different; they could reach from mild over severe encephalitis to death.

The following geographical distributions are typical for each disease:

- Japanese encephalitis: Asia, Australia;
- St. Louis encephalitis: North, Central, and South America;
- Tick–Born encephalitis: Europe, Asia.

The typical diagnostic for these diseases includes virus detection by polymerase chain reaction (PCR) or isolation and detection of antiviral antibodies. Treatment is in general symptomatic. For TBE specific immunoglobulin is available.

TRANSMISSION

All three diseases are caused by flaviviruses which are RNA-viruses of the family of Toga viruses.

A majority of flaviviruses can cause encephalitis in humans, e.g., JE, TBE, SLE, Murray–Valley encephalitis, etc.

Tick–Born encephalitis virus is transmitted by infected ticks. Ticks live mainly in forests with much grass, bushes, and dry leaves. The virus enters the body by a tick bite. Direct transmission from person to person is not known. Small rodents, birds, roes, and deer are primary virus reservoirs. Transmission via unpasteurized milk or cheese is a common problem in high endemic areas.

Japanese encephalitis virus is transmitted by mosquitoes. They live in humid areas with much water, in East Asia and Southeast Asia. Direct transmission is not known. Domestic animals, rodents, pigs, equines, and birds are primary virus reservoirs.

St. Louis encephalitis virus is also transmitted by mosquitoes, but in America direct transmission has not been described.[1–12]

GEOGRAPHICAL DISTRIBUTIONS

The virus of TBE occurs in definite areas in Europe: Albania, Austria, Croatia, Czech Republic, Estonia, Finland, Germany, Hungary, Latvia, Lithuania, Poland, Romania, Russia, Slovenia, Slovakia, Sweden (Fig. 1).

In Germany TBE virus occurs in certain areas in Bavaria, Baden-Württemberg, Hess, Thuringia, and in the Rhineland-Palatinate.

There is an increase of the disease in spring, summer, and early autumn.[6,13,14]

Japanese encephalitis mainly occurs in Japan, China, Eastern Siberia, India, North Thailand, Australia; especially in areas with much water and swamps (Fig. 2). There is a periodic increase in summer.[1–4,15–22]

St. Louis encephalitis occurs in Canada (Ontario), United States (especially in western states and in the southeast), Trinidad, Jamaica, Panama, Brazil, and Argentina (Fig. 3).

An accumulation of disease is regionally limited and increases in summer and early autumn, especially in years with warm and wet weather and great population of mosquitoes.[4,5,9,10,23–30]

INCUBATION PERIOD

The incubation period of all three diseases is generally between 5 and 15 days. Single cases of 21 days (SLE) and 28 days (TBE) were described.[3,5,6]

SYMPTOMS

The symptoms are very variable. There are very mild and nearly asymptomatic but also severe and deadly cases. They are often more severe in elderly people.

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Fig. 1 Geographical distribution of tick–born encephalitis. (Courtesy of M. Bröker and D. Gniel, Chiron Vaccines, Chriron Behring GmbH.) (*View this art in color at www.dekker.com.*)

A biphasic course of disease is typical for TBE. The disease starts with symptoms, which are similar to flu: mild temperature, headache, pain in the limbs.

A typical meningo encephalitis may occur in 10% of infected people after a short interval (5–10 days) without symptoms. These are the following symptoms: high temperature; heavy headache; nausea and vomiting; meningeal irritations; occasional numbness or stupor, especially in patients older than 50 years; neurological signs (paralysis, convulsions).

Typical for TBE is persistent symptomatic over months, especially in elderly people, although there is a complete recovery in most cases. Persistent headache, weakness of concentration, and mild paralysis are very seldom after disease recovery.

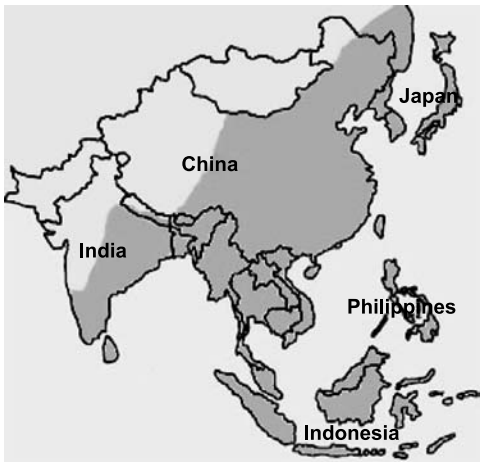

Fig. 2 Geographical distribution of Japanese encephalitis. (*View this art in color at www.dekker.com.*)

The symptoms of JE are also very variable. Most infections are asymptomatic. There are severe cases especially in small children and elderly people with the following symptoms: high temperature, headache, stiffness of the neck and other meningeal signs, vomiting,

Fig. 3 Geographical distribution of St. Louis encephalitis. (*View this art in color at www.dekker.com.*)

stupor, disorientation, paralysis, disturbance of reflexes. Temperature drops in most cases after the 10th day of disease, but the infection very often leads to persistent neurological and psychical damages (tremor, convulsions, paralysis, mental, and behavior weakness). The mortality is nearly 10%.

The following symptoms are typical for SLE: high temperature, headache, stiffness of the neck, vomiting, shunning the light, confusion, tremor, increase of unconsciousness until coma, changes in liquor (pleocytosis, increase of protein). Complications are pneumonia or gastrointestinal hemorrhages. The mortality rate is nearly 10–20%, especially in patients older than 50 years. Causes of death are failure of the kidneys or coma. Encephalitis can lead to persistent damages after enduring the disease, e.g., mild paralysis, defects of speech, memory weakness.[1,3–6,9,13,14,22,27,28,31–33]

COMPLICATIONS

Complications by TBE are very seldom and mainly found in elderly patients; typical is a cure with defects. Damage of the cardiac excitations system is reported. Tick–Born encephalitis infections could be more severe if *Borrrelia burgdorferi* causing Lyme disease infection is present in parallel with an infected tick.

Japanese encephalitis is more severe; the rate of complications is higher. Neurological and psychical damages are relatively frequent, e.g., convulsions, paralysis, mental weakness.

St. Louis encephalitis also has a higher rate of complications, typically in elderly people. There are mild and subjective consequences (tiredness, forgetfulness, weakness of concentration, defects of speech), but also dangerous complications such as convulsions, paralysis, pneumonia, hemorrhages.[1,3–6,9,11,22,27,28,30,31]

PROGNOSIS

Tick–Born encephalitis has a relatively good prognosis. Their mortality rate amounts to 1–2%, especially in elderly patients who suffer complications in the central nervous system.

The mortality rate of JE is higher; it amounts to nearly 10%, especially in patients older than 50 years.

St. Louis encephalitis has a similar mortality rate to JE (less than 5% in patients younger than 50 years; 10–20% in patients older than 50 years).[3–6,9,10,14,34,35]

DIAGNOSTICS

PCR and RT-PCR Diagnostics of Viral Encephalitis

Molecular detection of pathogenic viruses by PCR has become a very important tool in diagnostics. Although PCR provides the most sensitive and fastest method for virus genome detection, there are some very important issues that must be considered when using this method. Because of the high sensitivity, contaminations by traces of positive amplification products could cause false-positive results. These problems are partially overcome by the new real-time PCR technology which avoids contamination of the amplified viral genome sequences. The other aspect that must be considered is that the virus load could be very different in the course of the different diseases, within different organs, or different specimens (blood, liquor, sputum, etc.), which could cause problems regarding the interpretation of the results. Therefore, a negative result cannot be interpreted as an exclusion of the respective viral infection. For Flavivirus infection such as JE, SLE, and TBE the majority of patients show a very mild course of disease and very often only a low viremia. Only in severe cases with encephalitis symptomatic is it likely to get positive results by PCR in the acute phase of the disease. The introduction of PCR in the diagnosis of this kind of diseases will lead to more information on the pathogenesis and course of disease in these patients. Presently, most of the JE, SLE, and TBE infections are still diagnosed by conventional detection of IgM and IgG antibodies in serology assays such as immunofluorescence, ELISA, and/or neutralization tests.

Typical for the diagnosis of TBE are the following methods:

- Detection of virus antibody by ELISA in serum or liquor (IgM in acute phase serum; IgG);
- Virus isolation and detection of nucleic acid in blood or liquor in the first phase of the disease (PCR, cell culture);
- Western blot in special laboratories.[6,13,14]

Typical for the diagnosis of JE are the following methods:

- Virus isolation from tissues;
- Detection of viral nucleic acid from liquor by RT-PCR and confirmation of the sequenced RT-PCR product;
- Detection of virus antibody (IgM, IgG) by indirect immunofluorescence (from blood or other body fluid);
- Detection of virus antibody by ELISA (specific IgM in acute phase serum or IgG);

- Virus antibody by neutralization test;
- Immunohistochemistry and laboratory findings (mild leukocytosis).[7,36–39]

Typical for the diagnosis of SLE are the following methods:

- Virus isolation from the brain and detection of viral nucleic acid by RT-PCR and confirmation of the sequenced RT-PCR product;
- Virus antibody (IgM, IgG) by indirect immunofluorescence, by ELISA or by neutralization test;
- Immunohistochemistry findings from the brain;
- Laboratory diagnosis (mild leukocytosis).[40–42]

DIFFERENTIAL DIAGNOSIS

Tick–Born encephalitis, Japanese encephalitis, and St. Louis encephalitis have to be distinguished from other diseases of the central nervous system, especially from inflammatory diseases of the brain (other viral encephalitis, viral or bacterial meningitis, poliomyelitis, tuberculosis, Lyme disease). Because of the unspecific symptoms of these diseases, other Flavivirus infections should also be considered for differential diagnosis. In parts of the Far East where TBE is endemic the Omsk hemorrhagic fever is also endemic; in most areas where JE is also endemic dengue infections are occurring regularly as well; and since 1999, the West Nile virus is distributed in North America also in the same areas where SLE is present.

The following criteria indicate TBE:

Clinical image is compatible with TBE (biphasic disease course after a tick bite in a high-risk area; first phase with symptoms similar to flu, short interval without symptoms, neurological symptoms after 4–10 days); and

Diagnostic detection of viral nucleic acid by PCR or virus by isolation or detection of specific viral antibodies of IgM or IgG type. Differentiation of TBE-specific antibodies from other anti-Flavivirus (yellow fever, dengue, West Nile, JEV, SLE)-directed antibodies could create difficulties in serology. A fourfold rise in the antibody titre in two follow-up serum samples is a strong indicator of an infection.

The following criteria indicate JE:

Clinical image is compatible with the disease (headache, high temperature, neurological symptoms within the last 3 weeks before onset of disease, being in Eastern Asia or in India or in Southeast Asia or in Australia); and

A JE case is confirmed with one of the following laboratory diagnoses: isolation of the virus from tissues or detection of the virus nucleic acid in a sequenced RT-PCR from liquor;

Diagnostic detection of viral nucleic acid by PCR or virus by isolation from liquor and/or blood. Detection of specific viral antibodies of IgM or IgG type. Differentiation of JEV-specific antibodies from other anti-Flavivirus (yellow fever, dengue, West Nile, TBE, SLE)-directed antibodies could create difficulties in serology. A fourfold rise in the antibody titre in two follow-up serum samples is a strong indicator of an infection.

The following criteria indicate SLE:

Clinical image is compatible with the disease [headache, high temperature, neurological symptoms within the last 3 weeks before onset of disease, having been in the United States, Canada (Ontario), Trinidad, Jamaica, Panama, or Brazil]; and

A SLE case is confirmed with one of the following laboratory diagnoses: diagnostic detection of viral nucleic acid by PCR or virus by isolation from liquor and/or blood.[1,2,5,6,13,28] Detection of specific viral antibodies of IgM or IgG type. Differentiation of SLE virus-specific antibodies from other anti-Flavivirus (yellow fever, dengue, West Nile, TBE, SLE)-directed antibodies could create difficulties in serology. A fourfold rise in the antibody titre in two follow-up serum samples is a strong indicator of an infection.

TREATMENT

Treatment is symptomatic for all three diseases. For TBE, immediate vaccination and/or administration of specific hyper immunoglobulin antisera could be considered for treatment. For SLE and JE antiviral treatment is not available. The treatment is limited by symptomatic and supportive measures.

The following measures are sensible: drugs against temperature, aches, and other symptoms; intensive medical measures against complications such as stupor, unconsciousness, coma, convulsions, etc.[3–5,8,13,18,19,30,43–47]

PROPHYLAXIS

Avoidance of tick bites is an important principle of prophylaxis against TBE.

It is recommended to wear bright, long clothes on trips to forests in high-risk areas and to use repellants. Immediately after some outdoor activities in endemic areas search the body carefully for ticks, remove the ticks carefully, disinfect the wound carefully after removing the ticks, and use ticks prevention for domestic animals.

There is an indication for vaccination against TBE. The vaccination is active (consists of inactive virus) and recommended for travelers in high-risk areas and for people with outdoor activities in their profession or those on holiday (ranger, gardening, veterinary surgeon, hiking, etc.).

Complete protection against TBE is achieved after three vaccinations (one basic vaccination and two boosters). A new vaccination is recommended after 3 years. A passive immunization with globulin is possible in the first 2 days after the exposition.[7,13,14]

Prophylaxis against JE requires protection against mosquitoes (e.g. saving clothes, mosquito nets, fly screens, repellants) in high-risk areas (e.g., Eastern Asia, Southeast Asia, India, especially in summer). Vaccination is possible for people traveling to high-risk areas (e.g., trekking tours) or staying in high-risk areas for a longer time, especially in rural regions. This vaccination is active. Protection is effective 4 weeks after the vaccination for about 4 years. There is a lifelong immunity after an infection (also asymptomatic).[8,22,31,43–46,48]

Measures of protections against mosquitoes are important for prophylaxis against SLE. It is recommended to stay away from water or in wet regions especially at night in high-risk areas (United States, Canada, Trinidad, Jamaica, Brazil), wear long clothes or saving clothes, use mosquito nets, fly screens, repellants, etc. Vaccination is not available.[4,5,9,28]

CONCLUSION

Tick–Born encephalitis, Japanese encephalitis, and St. Louis encephalitis are three species of encephalitis caused by Flaviviruses (Flavivirus diseases) transmitted by arthropod vectors such as mosquitoes or ticks.

Typical for these diseases are:

- Occurrence in regions with specific geographical distributions;
- Symptomatic, very different course of diseases, more severe in elderly people;
- Treatment is typically symptomatic; specific antiviral treatment with immunoglobulin only for TBE;
- Specific diagnosis mainly based on serological assays.[1,3,5,6,13,14,31,48–50]

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Viral Hemorrhagic Fevers—Arenaviridae, Filoviridae, Bunyaviridae, and Flaviviridae

Christian Drosten
Bernhard-Nocht-Institute of Tropical Medicine, Hamburg, Germany

INTRODUCTION

With increasing international traveling activity, the importation of severe exotic viral diseases into non-endemic countries is becoming more and more likely. Terrorist attacks with highly pathogenic viruses are another scenario making diagnostic tools for these viruses more relevant than ever.

VIRAL HEMORRHAGIC FEVERS

The term ''viral hemorrhagic fever'' (VHF) describes a variety of viral diseases which are characterized by fever and bleeding in humans. This syndrome is caused by RNA viruses belonging to the families Filoviridae (Ebola virus and Marburg virus), Arenaviridae (Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus), Bunyaviridae [CCHF virus, Rift Valley fever (RVF) virus, and Hantaviruses], and Flaviviridae (yellow fever virus and dengue virus). After transmission from their reservoir host or vector to humans, these viruses cause an acute infection and there is no evidence of chronic courses.

The clinical symptoms in the early phase of a VHF are very similar irrespective of the causative virus and resemble a flu-like illness or a common enteritis. Headache, myalgia, gastrointestinal symptoms, and symptoms of the upper respiratory tract dominate the clinical picture. Hepatitis is also common. Therefore, especially in the early phase, virological testing is of utmost importance in diagnosis. The late phase of a VHF is more specific and characterized by organ manifestations and organ failure. Hemorrhage, the hallmark of a VHF, is present only in a fraction of patients depending on the virus species or even virus strain. Mild and subclinical courses seem to occur in all hemorrhagic fevers. However, if the disease is symptomatic, the case fatality ranges between 5% and 30%, but may be as high as 80% in Ebola fever.

With a few exceptions, currently, there exists no specific and effective therapy for VHF. The drug ribavirin is effective against Lassa virus and probably against CCHF virus. Vaccines have been developed against yellow fever virus,[1] Junin virus,[2] and RVF virus.[3]

Isolation of the virus in cell culture or laboratory animals, PCR, virus antigen detection, electron microscopy, and detection of specific antibodies in the serum of the patient are common methods for laboratory diagnosis of a VHF. According to the focus of this volume, this article addresses molecular diagnostic methods only. However, diagnosing a VHF always requires combined testing with more than one method.

This article reviews published VHF RT-PCR methods. Reported analytical sensitivities are compared against virus concentrations found in patients, and primers are reevaluated based on the latest GenBank entries by multiple sequence alignment (refer to table legends).

MOLECULAR DIAGNOSIS OF VIRAL HEMORRHAGIC FEVER—REVIEW OF PUBLISHED METHODS

Filoviruses

Ebola and Marburg viruses can be detected by Filoviridae-specific primers binding to the polymerase gene. These primers target sites that are highly conserved among the virus family[4] and were applied in PCRs of conventional and real-time format (Table 1, PCR 1–3). The glycoprotein gene of Ebola virus is used as a target to detect all four subtypes of Ebola virus (Zaire, Sudan, Ivory Coast, and Reston), but not Marburg virus (Table 1, PCR 4).[4] Furthermore, real-time PCRs in the glycoprotein gene for differentiating Zaire and Sudan strains, as well as for detecting Marburg virus, are available (Table 1, PCR 7 and 8).[7,8] PCR tests targeting the nucleoprotein gene detect and differentiate Ebola subtypes Zaire and Reston (Table 1, PCR 5 and 6). However, differentiation between filovirus species or subtypes is not required in the clinical situation.

Sensitivity studies have been mainly carried out using the polymerase gene-specific PCR. The clinical sensitivity of this PCR was 100% in two studies and thus higher than virus isolation or antigen capture assay.[4,5] Furthermore, even in patients with silent seroconversion, Ebola virus RNA can be detected in PBMC by a nested format of the

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Table 1 Filovirus PCR

PCR	Virus and target region	Method	Primer	Sequence	Quality score X/Y/Z[a]	Sensitivity	Reference
1	Filovirus polymerase	2-step	Filo-B Filo-A	atgtggtgggttataataatcactgacatg (g) atcggaatttttctttctcatt (ag)	11/0/0[b] 9/0/0[b] family	Clinical 100% GS: virus isolation, antigen test	[4]
2	Filovirus polymerase	2-step SB	—	—	—	Clinical 100% GS: antigen test, serology	[5]
3	Filovirus polymerase	1-step real-time	—	—	—	95% detection limit: 2647 copies/mL; 15 copies/PCR	[6]
4	Ebola GP	2-step	EBO-GP2 EBO-GP1	ttttttttagtttcccagaaggcccact (g) aatgggctgaaaattgctacaatc (ag)	16/0/0 18/0/0	Clinical 100% GS: virus isolation, antigen test	[4]
5	Ebola Reston NP	1-step	RES-NP2 RES-NP1	caagaaattagtcctcatcaatc (g) gtatttggaaggtcatggattc (ag)	2/0/0[c] 2/0/0[c]	Clinical 100% GS: antigen test	[4]
6	Ebola Zaire NP	1-step	ZAI-NP2 ZAI-NP1	gcatattgttggagttgcttctcagc (g) ggaccgccaaggtaaaaaatga (ag)	8/0/0[d] 8/0/0[d]	–	[4]
7	Ebola Zaire/Sudan GP	1-step real-time	EBOGP-1Dfwd EBOGP-1Drev EBOGP-1ZPrb EBOGP-1SPrb	tgggctgaaaaytgctacaatc (ag) ctttgtgmacatascggcac (g) FAM-ctaccagcagcgccagacgg-TX VIC-ttacccccaccgccggatg-TX	12/0/0[e] 12/0/0[e]	10–100 fg viral RNA/PCR 3–8 pfu/PCR	[7]
8	Marburg GP	1-step real-time	MBGGP3fwd MBGGP3rev MBGGP3prb	ttccccttggaggcatc (ag) ggaggatccaacagcaagg (g) Fam-cgatgggctttcaggacaggtgt-TX	6/0/0 6/0/0	2–5 pfu/PCR	[8]

[a]Homology of primers to published virus sequences (GenBank); X/Y/Z score: X, number of published sequences overlapping the primer-binding site (the more the better); Y, sequences containing ≥ 5 mismatches (the lesser the better); Z, sequences containing ≥ 2 mismatches within 5 bases from the 3′-end of the primer or a mismatch at the ultimate 3′-base of the primer (the lesser the better). Abbreviations: g/ag, genomic/antigenomic primer; p, probe; SB, Southern blot; FAM, 6-carboxyfluorescein; TX, 6-carboxytetramethylrodamine.
[b]Ebola and Marburg sequences.
[c]Only Ebola-Reston sequences.
[d]Only Ebola-Zaire sequences.
[e]Only Ebola-Zaire and -Sudan sequences.

polymerase gene PCR.[9] The viral RNA concentration in a serum of a convalescent and an acute patient was 3×10^3-fold and 3×10^5-fold, respectively, above the detection limit of the real-time format of the polymerase gene PCR.[6] Taken the data on clinical and analytical sensitivity together, the polymerase gene PCR is a reliable tool to diagnose filovirus infections.

Bunyaviruses

CCHF virus is generally detected by RT-PCR targeting the S RNA segment which is best characterized among the three genomic segments (Table 2, PCR 1–3). However, the virus could be detected by PCR in only 2/3 of retrospectively (serologically) confirmed cases.[11] RNA concentration in serum was tested in one case of CCHF virus infection and found to be 7.7×10^5 copies/mL of plasma by real-time PCR,[6,16] which is about 3×10^2 times above the detection limit of that assay. According to the current data, PCR is a useful tool in diagnosing CCHF, but must be complemented by virus isolation and serology.

For detection of Rift Valley fever virus, assays targeting the M and S segment are available (Table 2, PCR 4–7). Quantitative real-time PCR has also been developed[6,15] but has not yet been applied to clinical samples. Only one clinical evaluation study has been conducted[13] showing a sensitivity of 70.6% (Table 2, PCR 5). All samples negative by PCR but positive by virus culture had detectable IgM, suggesting that a

Table 2 Bunyavirus PCR

PCR	Virus and target region	Method	Primer	Sequence	Quality score X/Y/Z[a]	Sensitivity	Reference
1	CCHF	2-step	F2	tggacaccttcacaaactc (ag)	19/0/0	Clinical 25%	[10]
	NP	nested	R3	gacaaattccctgcacca (g)	19/0/0	GS: suspect in outbreak	
			F3	gaatgtcatgggttagctc (nested ag)	18/0/0		
			R2	gacatcacaatttcaccagg (nested g)	31/0/0		
2	CCHF	2-step	F2	tggacaccttcacaaactc (ag)	19/0/0	Clinical ~60%	[11]
	NP	SB	R3	gacaaattccctgcacca (g)	19/0/0	GS: serology	
			Probe	Nick-translated probe			
3	CCHF	1-step	CCS	atgcaggaaccattaartcttggga (ag)	70/0/0	95% detection limit:	[6]
	NP	real-time	CCAS	ctaatcatatctgacaacatttc and ctaatcatgtctgacagcatctc (1:1 g)	37/0/0	2779 copies/mL; 15 copies/PCR	
4	RVF	2-step	RVF1	gactaccagtcagctcattacc (ag)	3/0/0	0.5 pfu/PCR	[12]
	G2	nested	RVF2	tgtgaacaataggcattgg (g)	21/0/0		
			RVF3	cagatgacaggtgctagc (nested ag)	21/0/0		
			RVF4	ctaccatgtcctccaatcttgg (nested g)	21/0/0		
5	RVF	2-step	Nsca	ccttaacctctaatcaac (g)	2/0/0	Clinical 70%	[13,14]
	NSs	nested	NSng	tatcatggattactttcc (ag)	2/0/0	GS: virus isolation; 0.5 pfu/PCR	
			NS3a	atgctgggaagtgatgagcg (nested g)	2/0/0		
			NS2g	gatttgcagagtggtcgtc (nested ag)	2/0/0		
6	RVF	2-step	S432	atgatgacattagaaggga (ag)	20/0/0	100 copies/PCR	[15]
	NSs	real-time	NS3m	atgctgggaagtgatgag (g)	2/0/0		
			CRSSar	FAM-attgacctgtgcctgttgcc-TX			
7	RVF	1-step	RVS	aaaggaacaatggactctggtca (ag)	43/0/0	95% detection limit:	[6]
	G2	real-time	RVAs	cacttcttactaccatgtcctccaat (g)	21/0/0	2835 copies/mL	
			RVP	FAM-aaagctttgatatctctcagtgccccaa-TX		16 copies/PCR	

[a]Homology of primers to published virus sequences (GenBank); X/Y/Z score: X, number of published sequences overlapping the primer-binding site (the more the better); Y, sequences containing ≥ 5 mismatches (the lesser the better); Z, sequences containing ≥ 2 mismatches within 5 bases from the 3′-end of the primer or a mismatch at the ultimate 3′-base of the primer (the lesser the better). Abbreviations: g/ag, genomic/antigenomic primer; p, probe; SB, Southern blot; FAM, 6-carboxyfluorescein; TX, 6-carboxytetramethylrodamine.

combination of IgM and PCR testing seems to be at least equivalent to virus isolation.[13]

Hantavirus infections causing renal syndrome are usually diagnosed serologically because the virus is rapidly cleared. Therefore, hantavirus PCR assays are not discussed here in detail.

Arenaviruses

All current diagnostic PCRs target the S RNA segment (Table 3). For some Lassa virus PCRs, clinical evaluation data are available showing 85% and 100% sensitivity (Table 3, PCR 1 and 3). These studies also demonstrate that virus is detectable beginning at least from day 3 of fever. Quantitative real-time PCR was established (Table 3, PCR 2) and used to monitor virus RNA concentrations during the course of disease in two cases of Lassa fever.[6,23] The RNA concentrations in serum of these patients were 5×10^2–2×10^6-fold above the detection limit of the assay. Extensive sequence information for the S RNA segment of Lassa virus has recently become available and reveals considerable genetic variability of the virus.[22,24,25] In light of these new sequences, some PCR primers, which had been designed mainly on the basis of isolates from Guinea, Sierra Leone, or Liberia, require revision (Table 3, PCR 3–6).

PCR assays are published for the New World arenavirus Junin (Table 3, PCR 7 and 8). Clinical sensitivity of the combination of the two different Junin PCRs was 98%.

A PCR predictably amplifying any member of the Arenaviridae has been developed by targeting the highly conserved termini of the S RNA segment.[22] Although this PCR amplifies the whole 3.4-kb S RNA, it was able to detect Lassa virus RNA in clinical samples. The L RNA segment of arenaviruses contains highly conserved

Table 3 Arenavirus PCR

PCR	Virus and target region	Method	Primer	Sequence	Quality score X/Y/Z[a]	Sensitivity	Reference
1	Lassa GPC	2-step SB	36E2 80F2	accggggatcctaggcattt (g) atataatgatgactgttgttctttgtgca (ag)	Family 11/2/0	Clinical 100% GS: virus isolation; 1–10 copies/PCR	[17]
2	Lassa GPC	1-step real-time	—	—	—	95% detection limit: 2445 copies/mL; 14 copies/PCR	[6]
3	Lassa GPC	2-step SB	G2 G1 probe	cagaatctgacagtgtcca (ag) gtgtgcagtacaacatgagt (g) gctcccaccccaagccatcc (p)	7/0/0 7/1/4	Clinical ~85% GS: virus isolation	[18]
4	Lassa NP	2-step SB	N2 N1 N3	ctgcccctgttttgtcagacatgcc (g) ggggctcgggctgggagagatggag (ag) aatgcagagttgctcaataatcagttcgggacc (p)	7/3/5 7/4/2	10^2 $TCID_{50}$ per PCR	[19]
5	Lassa GPC	2-step SB	GPC2 GPC1 GPC3	ggatggcttggggtgggagctacat (g) ataaccgatgggagatggtctcgag (ag) ggcagtgatcttcccaggttgtatttggattatc (p)	7/0/0 7/4/3	—	—
6	Lassa NP	2-step nested	N2+ N1+ N2 N4	tgtactgcatcattcaagtcaac (g) aagtgcaggtgtctatatggg (ag) ctgcccctgttttgtcagacatgcc (nested g) caacctaagctcacagcaacttgac (nested ag)	7/1/0 7/3/0 7/3/5 7/5/4	50 $TCID_{50}$ per PCR	[20]
7	Junin S RNA	2-step	J2 1	ggcatccttcagaacat (g) cgcacagtggatcctaggc (ag)	5/0/0 Family	PCR 7 and 8 combined	[21]
8	Junin S RNA	2-step	1 J3	cgcacagtggatcctaggc (ag) caaccacttttgtacaggtt (g)	Family 43/0/1	Clinical 98% GS: serology	—
9	arenavirus	2-step	RT PCR2 PCR3 PCR4	cgcaccgdggatcctaggc (g/ag) cgcaccgaggatcctaggcatt (g/ag) cgcaccggggatcctaggcaat (g/ag) cgcaccggggatcctaggctt (g/ag)	Family Family Family Family	Positive with high-titered serum and CSF	[22]

[a]Homology of primers to published virus sequences (GenBank); X/Y/Z score: X, number of published sequences overlapping the primer-binding site (the more the better); Y, sequences containing ≥ 5 mismatches (the lesser the better); Z, sequences containing ≥ 2 mismatches within 5 bases from the 3′-end of the primer or a mismatch at the ultimate 3′-base of the primer (the lesser the better). Abbreviations: g/ag, genomic/antigenomic primer; p, probe; SB, Southern blot; FAM, 6-carboxyfluorescein; TX 3,6-carboxytetramethylrodamine.

regions which are now being used to develop a PCR assay that is able to detect various Old World arenaviruses such as Lassa virus and lymphocytic choriomeningitis virus. In conclusion, suspected Lassa virus infections will be identified by PCR with high probability after day 3 of illness, but primers may fail because of the variability of the virus.

Flaviviruses

Yellow fever is usually detected using a universal flavivirus PCR (Table 4, PCR 1–8). Most of these universal assays target the NS5 which is highly conserved among the *Flavivirus* genus. Data on clinical sensitivity of these assays for yellow fever are largely lacking. Only few yellow fever virus-specific PCR tests have been published (Table 4, PCR 9–11). A quantitative real-time PCR specific for yellow fever virus exists, and in two cases, the viral RNA concentration in serum has been determined by this assay.[6,35] It was 2×10^2-fold and 1×10^3-fold above the PCR detection limit. Therefore, yellow fever may be reliably diagnosed by PCR, but virus isolation and serology must be performed in parallel.

Dengue hemorrhagic fever (DHF) is rare compared with dengue fever (DF) and is almost exclusively seen in endemic areas. The molecular diagnosis of dengue infection has been extensively reviewed elsewhere.[36,37] Noteworthy are recently developed real-time PCR assays, which either detect all dengue virus subtypes[6] or differentiate between the four subtypes.[38–40] Because dengue virus is rapidly cleared during infection, DF and DHF are usually diagnosed by detection of specific IgM (μ-capture EIA), which may be complemented by PCR and virus isolation. In a few imported cases of dengue

Table 4 Flavivirus PCR

PCR	Virus and target region	Method	Primer	Sequence	Quality score X/Y/Z[a]	Reference
1	Flavivirus NS5/3′-NCR	2-step	1	ggtctcctctaacctctag (g)	Genus	[26]
			2	gagtggatgaccacggaagacatgc (ag)	Genus	
2	Flavivirus NS3	2-step	DV1	ggracktcaggwtctcc (g)	Genus	[27]
			DV3	aartgigcytcrtccat (ag)	Genus	
3	Flavivirus NS5	2-step	FG1	tcaaggaactccacacacatgagatgtact (g)	Genus	[28]
			FG2	gtgtcccatcctgctgtgtcatcagcataca (ag)	Genus	
4	Flavivirus NS5/3′-NCR	2-step	EMF1	tggatgacsackgargayatg (g)	Genus	[29]
			VD8	gggtctcctctaacctctag (ag)	Genus	
5	Flavivirus NS5	2-step	CFDJ9977	gcatgtcttccgtcgtcatcc (g)	Genus	[30]
			FUDJ9166	gatgacacagcaggatgggac (ag)	Genus	
6	Flavivirus NS1	2-step	DJA	tccatcccatacctgca (g)	Genus	[31]
			DJS	gacatggggtattggat (ag)	Genus	
7	Flavivirus NS5	2-step	MA	catgatgggraaragrgarrag (ag)	Genus	[32]
			cFD2	gtgtcccagccggcggtgtcatcagc (g)	Genus	
8	Flavivirus NS5	2-step	MAMD	aacatgatgggraaragrgaraa (g)	Genus	[33]
		nested	cFD2	gtgtcccagccggcggtgtcatcagc (ag)	Genus	
			FS778	aargghagymcdgchathtggt (nested g)	Genus	
9	Yellow fever NS5/3′-NCR	2-step	EMF1	tggatgacsackgargayatg (g)	Genus	[34]
		nested	VD8	gggtctcctctaacctctag (ag)	Genus	
			NS5YF	atgcaggacaagacaatggt (nested g)	17/0/0	
10	Yellow fever, West African strains Envelop	1-step real-time	269 R	tgaaaggcgcggaacg (g)	39/0/0	[Preiser, 2002, #219]
			127F	ccagttcaagccgccaaatag (ag)	25/0/4[b]	
			150T	FAM-cggtgtttggctctgcctttcagg-TX		
11	Yellow fever 5′-NCR	1-step real-time	YFS	aatcgagttgctaggcaataaacac (g)	31/0/0	[6]
			YFAS	tccctgagctttacgaccaga (ag)	32/0/0	
			YFP	FAM-atcgttcgttgagcgattagcag-TX		

[a]Homology of primers to published virus sequences (GenBank); X/Y/Z score: X, number of published sequences overlapping the primer-binding site (the more the better); Y, sequences containing ≥ 5 mismatches (the lesser the better); Z, sequences containing ≥ 2 mismatches within 5 bases from the 3′-end of the primer or a mismatch at the ultimate 3′-base of the primer (the lesser the better). Abbreviations: g/ag, genomic/antigenomic primer; p, probe; SB, Southern blot; FAM, 6-carboxyfluorescein; TX, 6-carboxytetramethylrodamine.
[b]West African strain sequences only.

fever, the diagnosis was established by PCR before the development of specific IgM.

Apart from yellow fever and dengue virus, rare flaviviruses such as Kyasanur Forest virus or Alkhurma virus can cause a hemorrhagic fever.[41,42] Use of universal flavivirus PCRs is appropriate when these infections are suspected and typical VHF agents have been ruled out.

CONCLUSION

Improved amplification and detection technologies are beginning to improve the diagnostics of even very exotic viral infections. The sensitivity of many available methods can be assumed sufficient for the clinical setting, but more sequencing of virus strains is necessary to increase the reliability of molecular tests in the detection of rare variants. For the time being, molecular diagnostics can only complement classical methods such as virus culture, antigen testing, and antibody serology. The sensitive issue of diagnosing hemorrhagic fevers must be restricted to experienced laboratories equipped with biosafety level 4 facilities that allow handling of life virus.

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von Hippel–Lindau Disease

V

Deborah J. Marsh
Diana E. Benn
Kolling Institute of Medical Research, University of Sydney, Sydney, Australia

INTRODUCTION

Von Hippel–Lindau disease (VHL; OMIM#193300) is one of the endocrine neoplasia syndromes characterized by loss of function germline *VHL* mutations that predispose specific hormone-secreting cell types to become neoplastic. The live birth incidence of this disease is in the range of 1 in 36,000 to 1 in 39,000.[1] Individuals affected with VHL develop both benign and malignant tumors, as well as cysts, in many organ systems. Given the multisystem nature of this disorder, a multidisciplinary treatment approach is required.

VHL (chromosomal location 3p26–p25) was identified in 1993 using positional cloning strategies,[2] and, subsequently, loss of the corresponding wild-type allele in affected tissue was reported, consistent with this gene having a tumor suppressor function.[3] Up to 50% of patients with germline *VHL* mutation develop only one feature of this disease; however, penetrance is believed to be approaching complete by 65 years of age (reviewed in Ref. [4]). Given its small size, consisting of only three exons encoding a 213-amino acid protein product, *VHL* is readily amenable for genetic scanning in at-risk or symptomatic individuals.

CLINICAL DESCRIPTION

Presentation

Patients with VHL are predisposed to develop tumors, predominantly retinal and central nervous system (brain and spinal cord) hemangioblastomas (HABs), clear cell renal cell carcinomas (CCRCCs), pheochromocytoma (PHEO), and, occasionally, extraadrenal paragangliomas (PGLs) (reviewed in Ref. [4]). Clear cell renal cell carcinomas are the major cause of morbidity. In addition, VHL patients may develop endolymphatic sac tumors, pancreatic islet cell tumors, papillary cystadenomas of the epididymus (males), or, occasionally, broad ligament (females) and visceral cysts of the kidneys and pancreas.

Phenotypic Expression—The Subtypes of VHL

Based on clinical expression, VHL disease has been divided into four subtypes. The majority of VHL kindreds are classified as VHL Type 1 families and manifest HABs (retinal and central nervous system), CCRCC, and other characteristic lesions but by definition, not PHEO. The presence of PHEO in association with Type 1 lesions indicates VHL Type 2 families (7–20% of all families), with a further subclassification of families as low (Type 2A) or high (Type 2B) risk for CCRCC, and Type 2C having PHEO only.[5]

Clinical Diagnostic Criteria for VHL

For patients with a family history, the occurrence of a central nervous system HAB, PHEO, or CCRCC or even multiple pancreatic cysts is indicative of VHL disease. For sporadic presentation, a diagnosis of VHL would be indicated by the presence of two or more retinal or central nervous system HABs and a characteristic visceral tumor. As epididymal or renal cysts occur frequently in the general population, they are not included as part of the diagnostic criteria. Sporadic presentation of retinal or central nervous system HAB, especially in a young patient, may also be an indication of VHL (reviewed in Ref. [1]).

Clinical Management of VHL

Clinical management of a patient with VHL requires comprehensive serial screening with regular follow-up as well as surgical intervention. Von Hippel–Lindau patients have a greater than 70% lifetime risk of CRCCs, which cause death of 15–50% of patients. Annual imaging [computed tomography (CT) and/or ultrasonography] is suggested, in addition to renal surgery. Once symptomatic, CCRCCs have already undergone metastasis and respond poorly to chemotherapy or radiotherapy. Surgical resection is also recommended for central nervous system HABs (located in the cerebellum, spine, and brain stem)

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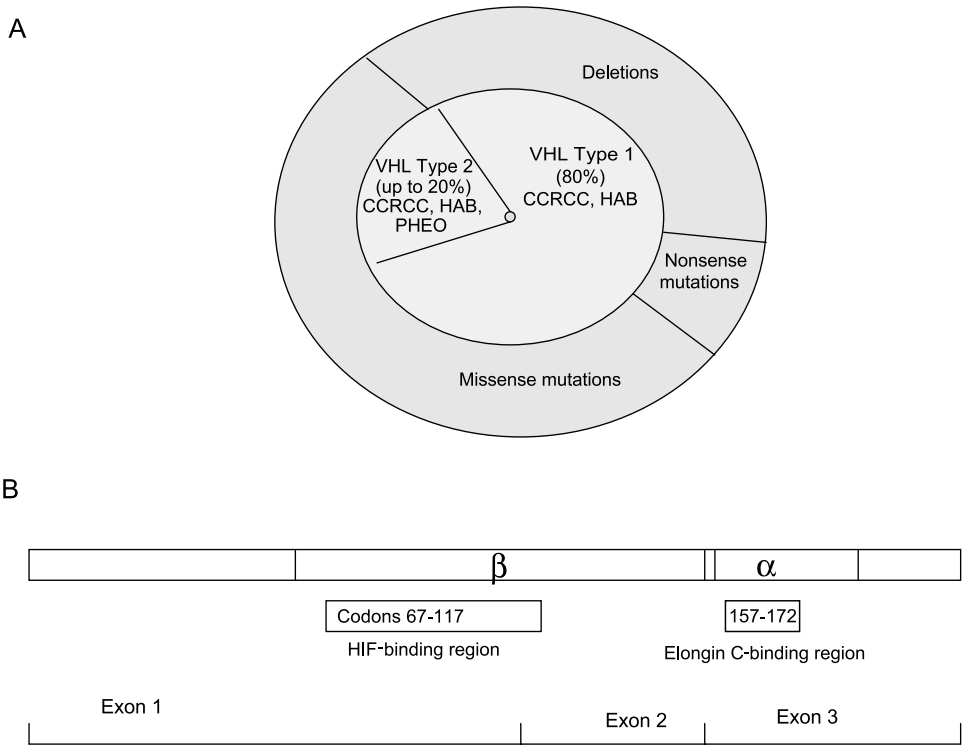

Fig. 1 A. Distribution of germline mutations in VHL Types 1 and 2. VHL Type 1 disease exhibits germline deletions (46%), nonsense mutations (10%), and missense mutations (40%), the latter frequently found in the β region of *VHL*. VHL Type 2 disease shows missense mutations (96%) and rarely deletions (4%). (Reviewed in Ref. [18].) B. Regions of VHL showing exon regions encoding the α and β domains, HIF-binding region (codons 67–117), and elongin C-binding domain (codons 157–172).

and PHEO. Unrecognized PHEO may cause hypertension at times such as surgery or pregnancy, due to sudden elevation in circulating catecholamines, leading to a life-threatening situation. Regular screening for PHEO, including urinary catecholamines, and imaging studies [ultrasonography, CT, or magnetic resonance imaging (MRI)] should be conducted. Given the early onset nature of PHEO in VHL, it has been recommended that screening begins at 5 years of age.[6]

Retinal angiomas are present in greater than half of patients with VHL. Regular opthalmological examinations from a young age are suggested as a preventative measure in VHL patients with the aim of avoiding retinal detachment and hemorrhage that may lead to blindness. Imaging techniques, such as CT or MRI, are suggested to assess for pancreatic cysts (if pancreatic islet tumors are identified, surgery is recommended) and endolymphatic sac tumors of the internal auditory canal that may lead to hearing loss.

Lifelong, regular surveillance is required to best manage the multiple and often recurrent tumors in VHL. Detailed review articles focusing on appropriate clinical management for VHL patients have been published.[1,7]

MOLECULAR DESCRIPTION

VHL—The Gene and Protein

The VHL gene is made up of 639 nucleotides, consisting of three exons that generate two mRNA species. The larger one of 4.6 kb corresponds to all three exons and generates a full-length 213-amino acid protein which is expressed in most tissues and has been implicated in a variety of functions including fibronectin matrix assembly, angiogenesis, and regulation of hypoxia-inducible genes.[8] The VHL gene product (pVHL) is a component of a ubiquitin ligase which targets the transcription factor hypoxia-inducible factor (HIF) subunits HIF-1α and HIF-2α for ubiquitination and proteolytic degradation in the presence of oxygen, by forming a ubiquitin–ligase complex with elongin B and C, as well as Cullin-2 and Rbx1 (reviewed in Ref. [9]).

There are two main domains of pVHL, the α helical domain interacting with elongin C, and the β domain where binding to HIF takes place. Generally, cells defective for pVHL (resulting from VHL inactivation) cannot degrade HIF-α subunits leading to the transcriptional activation of HIF target genes such as vascular

endothelial growth factor.[10] This is consistent with the angiogenic nature of VHL-associated tumors.

The role of pVHL in oxygen-sensing pathways and the highly vascularized nature of the tumors seen in VHL may, in the future, extend the clinical management options discussed above to include molecular drugs designed to inhibit HIF and/or its downstream targets. Early studies have suggested that these inhibitory drugs may work best in concert with other molecular drugs targeting cellular growth and survival pathways with the aim of achieving tumor regression and/or disease stabilization (reviewed in Ref. [11]).

Identified *VHL* Mutations

Over 160 different germline *VHL* mutations have been identified in over 500 kindreds worldwide and are recorded in the Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). Mutations include missense and nonsense point mutations, microdeletions, microinsertions, splice site, complex rearrangements (including inversions), whole gene, and gross deletion.[12] Intragenic germline point mutations are spread across the coding sequence and exhibit two clusters, one at the 3′ end of exon 1 and the other at the 5′ end of exon 3, but no mutations have been identified in the first 53 codons.[13] Mutation ''hot spots'' are apparent at hypermutable sequences such as CpG dinucleotides.[14] Founder mutations have also been reported,[15] as well as germline mosaicism which is a factor to take into consideration when analyzing for apparently sporadic presentations of VHL.[16] Some index cases may in fact present de novo, with no known family history.

Genotype/Phenotype Correlations in VHL

The phenotypic expression of VHL disease can vary widely between families, and specific correlations of genotype and phenotype are now recognized. Von Hippel–Lindau Type 1 families typically have deletions (46%), missense (44%), and nonsense (10%) mutations (reviewed in Ref. [17]) (Fig. 1A). These missense mutations are frequently observed in the β-domain of pVHL and are predicted to lead to loss of function of pVHL by disrupting the protein's hydrophobic core (Fig. 1B). In particular, mutations in exon 2 cause loss of the ability of pVHL to shuttle between the nucleus and the cytoplasm, leading to loss of regulation of hypoxia-inducible genes.[18]

In general, the majority of kindreds with VHL Type 2B disease and virtually all Type 2A and 2C patients have missense mutations.[5] In Types 2A and 2B, either binding to elongin C (α domain of VHL) or the HIF-α binding site (VHL β domain) is affected by mutations, thus interrupting the degradation of the target proteins such as HIF-α in the proteosome (Fig. 1B). For Type 2C families with PHEO as the only expression of VHL disease, the missense mutations are generally clustered in the VHL α domain. It has been demonstrated that mutants such as L188V with an increased likelihood of PHEO-only phenotype can downregulate HIF but cannot promote fibronectin matrix assembly. This suggests that after pVHL inactivation abnormal fibronectin assembly may contribute to PHEO pathogenesis in VHL disease.[19]

Genetic Screening

Who should undergo genetic screening for VHL?

Presymptomatic, predictive mutation analysis is recommended for all at-risk members of a family with VHL, increasing the diagnostic power of the clinician. Given the direct benefit of early, appropriate clinical screening in this condition, knowledge of young mutation carriers and immediate instigation of a clinical management program may be integral to the prevention of malignant tumor development, loss of sight and hearing. As VHL is an autosomal dominant condition, the offspring of an affected parent have a 50% chance of inheriting a *VHL* mutation. If after predictive testing the at-risk family member is shown not to carry the *VHL* mutation present in the family, no additional genetic or clinical screening is required. A recent study of VHL patients' attitudes toward presymptomatic genetic diagnosis in children as well as prenatal genetic diagnosis reports a wide range of opinions, indicating that these areas remain controversial.[20]

It should be noted that approximately 50% of PHEO-only families with no other evidence of VHL disease may have germline *VHL* mutations. Other familial syndromes where PHEO may present as the sole phenotype include multiple endocrine neoplasia Type 2 (mutations in the *RET* protooncogene) and pheochromocytoma–paraganglioma syndrome [mutations in the succinate dehydrogenase (*SDH*) genes *SDHB*, *SDHC*, and *SDHD*, encoding three of the four subunits of mitochondrial complex II] (reviewed in Ref. [21]). In addition, *VHL* germline mutations in 3–11% of patients with apparently sporadic PHEO have been reported.[22]

Standard molecular techniques used for *VHL* mutation scanning

The heterogeneity of mutation type seen in VHL means that multiple molecular techniques are required to enable a complete mutation scan. Mutations have been identified in nearly 100% of VHL families using a combination of fluorescence in situ hybridization (FISH), Southern

blotting, and sequencing of the coding and flanking regions of *VHL*.[12]

dHPLC—Suitable for detecting a subset of *VHL* mutations

More recently, denaturing high-performance liquid chromatography (dHPLC) has been used as an alternative to sequence analysis for rapid scanning for mutations in *VHL*.[23,24] This technique makes use of mismatched heteroduplex formation due to the presence of a mutant and wild-type allele. Mismatched heteroduplexes, and mutant and wild-type homoduplexes, can be distinguished after elution from a chromatographic column at a predetermined temperature based on sequence-specific thermostabilities. Given that gross germline deletion of *VHL* and its flanking intervals have been reported in VHL (predominantly those with Type 1), it is important to recognize that standard dHPLC would not be a suitable screening tool for this subset as the presence of only one allele would preclude the formation of heteroduplexes.

CONCLUSION

Von Hippel–Lindau disease is a multisystem disorder requiring a multidisciplinary approach, with regular imaging and biochemical analyses required to monitor affected patients and identify disease in its early stages. Genetic screening to identify germline *VHL* mutations, enabling targeting of affected individuals for vigilant clinical surveillance, is an important component of the clinical management of at-risk members in VHL families. As our knowledge of pVHL and the molecular pathways in which it functions increases, future treatment options in the form of molecular target drugs may provide an alternative, or an adjunct, to the surgical intervention offered today for tumors such as PHEOs and CCRCC, the latter of which is the greatest cause of mortality in VHL.

Online information specifically for the VHL patient is available at: http://www.vhl.org. Detailed reviews recommended for further reading on this subject include Maher and Eng,[13] Lonser and colleagues,[7] and Bryant and colleagues.[9]

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von Willebrand Disease

M. Said Enayat
The Birmingham Children's Hospital NHS Trust, Birmingham, U.K.

INTRODUCTION

In 1926, Eric von Willebrand described a bleeding diathesis in a series of families from Äland Islands in Finland, that although resembling hemophilia bleedings the patients have also shown platelet function abnormalities. Referred to this condition as ''pseudohemophilia,'' he clearly identified a different pattern of autosomal inheritance with varying degree of the penetrance. The pathophysiology of this disorder has been clarified during the past 40 years when it was noted the difference in bleeding tendencies between factor VIII deficiency hemophilia and von Willebrand disease (VWD) which is caused by deficiency of another clotting protein, namely, the ''von Willebrand Factor or VWF.'' In 1985, VWF cDNA was cloned, allowing major advances in the molecular genetics of VWD, and, subsequently, its structure was deduced from the cDNA and confirmed by direct amino acid sequence analysis. Von Willebrand disease is a common autosomally inherited bleeding disorder with variable clinical presentation. Von Willebrand disease is due to quantitative or qualitative abnormalities of VWF and its gene is encoded on chromosome 12.[1]

von Willebrand factor is a large, multimeric glycoprotein ($10–20 \times 10^6$ kDa) circulating in blood plasma, platelet α-granules, and subendothelial connective tissue, and secreted by the vascular endothelial cells and bone marrow megakaryocytes.[2] von Willebrand factor has two essential hemostatic functions: mediating platelet adhesion to subendothelial connective tissue and carrying FVIII in blood circulation, essential for normal FVIII survival.[3]

von Willebrand factor gene is located at the short arm of the human chromosome 12 (12p13.2), contains 52 exons, and spans about 180 kb. The structure of VWF gene, pseudogene, the protein, and its specific functional domains are summarized in Fig. 1. Exon 28 is the largest exon with 1379 bases, encoding for A1 and A2 domains. A VWF pseudogene has been localized on chromosome 22q11–13, spanning 21–29 kb and corresponds to exons 23–34 of VWF gene. The VWF cDNA encodes a primary translocation product of 2813 amino acid residues. Von Willebrand factor precursor contains 22 amino acids signal peptide, a large propeptide of 741 amino acids and a mature subunit of 2050 amino acids.[4] Amino acid residues of VWF are numbered from the signal peptide, which would be from 1 to 2813. The VWF subunit is extensively glycosylated with 12 N-linked and 10 O-linked oligosaccharides. The N-linked oligosaccharides of VWF have the unusual property of having ABO blood group determinants and this has implication in the level of VWF concentration. Following translation of a $\sim$9-kb mRNA, removal of the signal peptide, and initial glycosylation, the monomeric subunits of VWF with a molecular weight of approximately 250 kDa form dimers in the endoplasmic reticulum. In the endothelial cells, after further posttransitional modification in the Golgi apparatus, large VWF multimers are either constitutively secreted in the circulation or stored in Weible–Palade bodies[5] which release their stored VWF molecules after stimulation by a variety of stimuli, including vasopressin analog (DDAVP) treatment. Various binding functions of VWF are localized to different domains of this molecules including binding to platelet glycoprotein Ib (GP1b), ristocetin, botrocetin, heparin, sulfatides, and minor binding sites for collagen types I and III.[6]

MOLECULAR DIVERSITY OF VWD

von Willebrand disease is an extremely heterogeneous disorder, and results from phenotypic and genotypic tests form the basis of the diagnosis and classification of different types of VWD. The numerous variants of VWD generally fit in the original classification of either type I (partial) and III (total) reduction or type II with qualitative defect of VWF. Multimeric analysis of von Willebrand antigen (VWF:Ag) by SDS-gel agarose gel electrophoresis[7] results in identification of many different type II variants that have minor structural differences. The latest classification of VWD, however, reduced the total number of type 2 to four variants of 2A, 2B, 2M, and 2N on the basis of their structure/function (Table 1).[8] As type 1 VWD is now considered to be purely quantitative disorders, some previously designated variants have now been reclassified as type 2, e.g., type 2M (Table 1). The molecular basis of these six new subtypes with clear pathophysiological mechanisms has now been further defined with identification mutations in some of these

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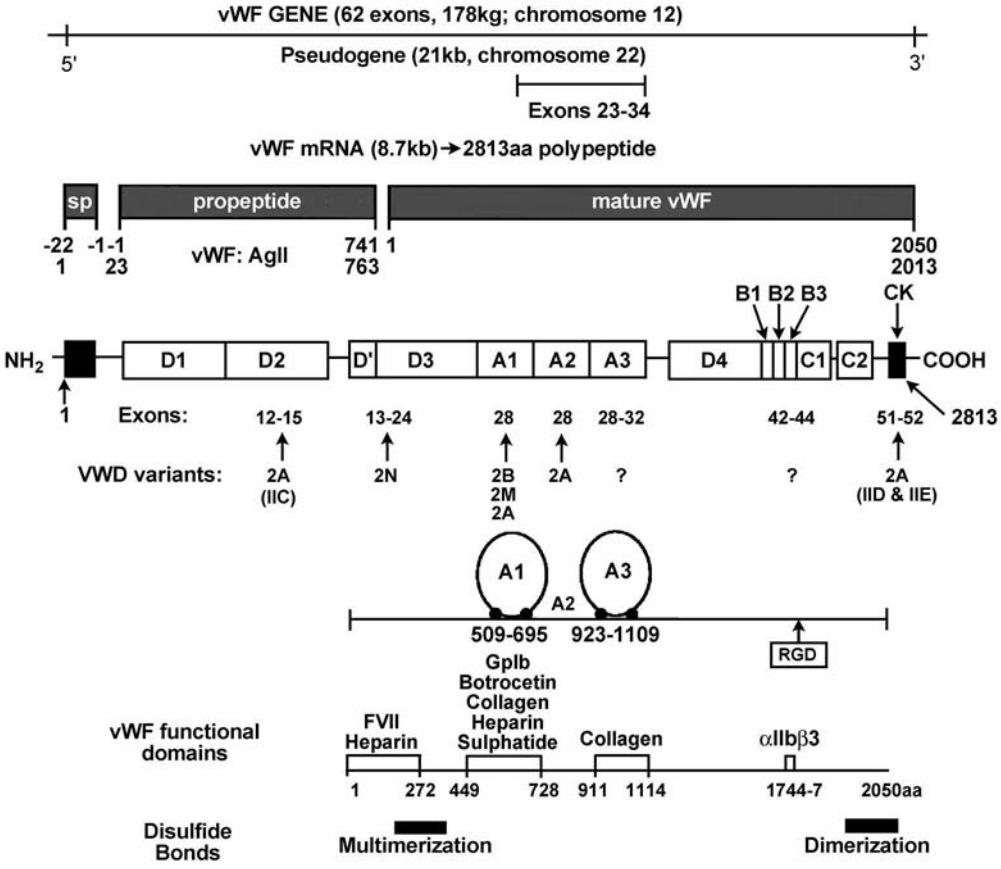

Fig. 1 Schematic presentation of the human VWF gene, pseudogene, mRNA, and protein. Numbers below the boxes representing signal peptide (sp), propeptide, and mature VWF in the upper row (in red) are that of mature VWF amino acid numbers and below them are the amino acid numbers from the ATG initiation codon (residue 1) to the carboxy-terminal lysine (residue 2813) of the pre-pro-VWF (in blue). The lettered boxes denote the series of the repeated homologous segments. The approximate localization for known VWF functional domains within the mature VWF sequence is indicated below. The general location of mutations associated with the most common type 2 variants of VWD is depicted below the domain structure of VWD together with the associated exons. (*View this art in color at www.dekker.com.*)

defects. A database of VWD lists all the point mutations, insertions, and deletions identified in this type. This site (http://www.shef.ac.uk/vwf), which has been created on behalf of the ISTH VWF Scientific and Standardization Committee, is maintained by Sheffield University and contains up-to-date information on the latest mutations and polymorphisms in *VWF* gene. In the recent 2003 XIX Congress of the International Society on Thrombosis and Hemostasis in Birmingham, United Kingdom, several presentations emphasized the need for a new classification of VWD based on molecular diagnosis and improved multimeric analysis of VWF. With so many unclassifiable patients with type 1 VWD group, it has now been suggested that such an improved multimeric method would help in future classification of these patients.

Type 1 VWD patients have reduced VWF:Ag level with normal multimer composition (Fig. 2). This is the commonest VWD type, accounting for about 70% of the clinical cases, with patients having mild to moderate bleeding. It has dominant mode of transmission with incomplete penetrance of approximately 60%. While lowering of VWF:Ag could be the result of a mutation in VWF gene, additional variations such as ABO blood group, race, and age complicate the diagnosis of this type of VWD. A broad range of VWF levels suggests several distinct molecular mechanisms could be responsible for this type of VWD, and in many affected families possession of a single mutant allele does not consistently cause symptoms, with some patients being asymptomatic and only transmitter of the disease. In some other families, exceptionally lowered VWF levels and with dominant inheritance trait and very high penetrance may be caused by dominant-negative missense mutations that impair the intracellular transport and secretion of normal VWF

Table 1 Previous and revised classification of VWD

Revised classification of VWD	Previous classification
Type 1 VWD Partial quantitative deficiency of VWF	Type I, I ''platelet normal,'' I ''platelet low'' IA, I-1, I-2, I-3
Type 3 VWD Virtually complete deficiency of VWF	Type III
Type 2 VWD Qualitative abnormalities of VWF	
Type 2A Qualitative variants with decreased platelet-dependent function that is associated with absence of high molecular weight multimers	Type IIA, B, C, D, E, F, G, H, I and I ''platelet discordant''
Type 2B Qualitative variants with increased affinity for platelet GP1b	Type IIB, I New York, I Malmö, I Sydney
Type 2M Qualitative variants with decreased platelet-dependent function not caused by the absence of high molecular weight multimers	Type B, IC, ID, Vincenza
Type 2N Qualitative variants with markedly decreased affinity for factor VIII	Normandy

subunits. Presently, a European study into molecular and clinical markers for diagnosis and management of type 1 VWD has been reporting large number of mutations associated with this type of VWD.[9]

Severe type 3 VWD has autosomal recessive inheritance with a prevalence of about one per million. Molecular analysis of this type has been more difficult, with no possibility of targeting for mutation detection. Reported

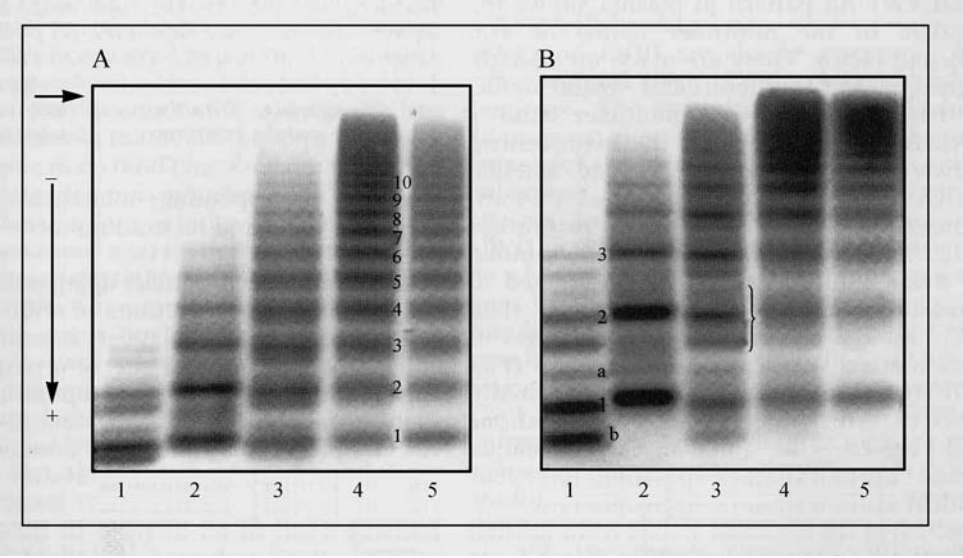

Fig. 2 VWF:Ag multimer patterns in normal and different types of VWD plasma. The plasma samples were electrophoresed in 1.2% (panel A) and 1.8% (panel B) agarose gel with sodium dodecyl sulphate, and the bands were visualized by 125I-labeled anti-VWF monoclonal antibody followed by autoradiography. Lane 1 is a type IIA phenotype; lane 2, IID phenotype; lane 3, type 2B; lane 4, normal; and lane 5, type 1 VWD plasma. The triplet structure of multimer band is demonstrated in lane 3 (panel B). The minor satellite bands ''a'' and ''b'' can be best seen in the band 1 of plasma from phenotype IIA VWD in lane 1. Arrow at the top of the gel points to the line between the stacking and separating gels. Direction of electrophoresis is from top to the bottom.

mutations for type 3 VWD are either homozygous or compound heterozygous, and although total or partial VWF deletions have been reported, this is uncommon. A large number of nonsense, frame shift, or missense mutations that predict the loss of VWF protein expression, or a markedly truncated or disrupted protein, have been identified.[10,11] Multiple substitutions in the VWF gene that mimic the pseudogene sequences have also been reported to cause type 1 and 3 VWD.[12] Haplotyping with a large battery of RFLPs including a highly polymorphic tetranucleotide repeat in intron 40 in families with type 1 and 3 VWD can have an important role in determining the inheritance of disease in these families.[13] Gene tracking of the affected allele can be used to identify the transmitters or the asymptomatic carriers of VWD, and this methodology has been found to be an essential initial step in studying such families prior to more comprehensive investigations and mutation detection.[14]

In the latest classification of the VWD, type 2 variants with qualitative abnormalities of VWF are divided into four groups of 2A, 2B, 2M, and 2N (Table 1). They are classified on the bases of phenotypic data and functional assays. These categories have distinct pathophysiological mechanisms, and their reported mutations are located in specific functional regions of the VWF gene. Each group has distinct clinical features and with specific therapeutic need. FVIII:C and VWF:Ag concentration, together with multimer distribution, ristocetin-induced platelet aggregation, and FVIII binding assay are the minimum phenotypic assays, which are needed for these classifications. Type 2A VWD patients have decreased platelet-dependent function with selective absence of high and intermediate molecular weight multimers (Fig. 2). Both recessive and dominant mode of inheritance have been reported; however, dominant inheritance accounts for the majority of cases. The reported mutations are all single-base substitutions with five in the A1 domain, and the rest in the A2 domain. However, mutations for some of the type 2A phenotypes that are now classified in this group (formally designated as type IIC, IID, and IIE VWD; Table 1) are located in other regions of VWF gene. Most of the point mutations for type 2A VWD were identified in exon 28 where VWF pseudogene is also located. Using allele-specific PCR strategies for amplification of this exon circumvents this problem.[15] Arg1597Try mutation accounts for about 1/3 of type 2A VWD patients. The mechanisms for these mutations can be divided into two categories.[16] Group 1 mutation is the result of improper folding of the VWF multimers, resulting in impairment of the assembly, storage, and retention in the endoplasmic reticulum, both in the plasma and in the platelet compartments. In group 2, the mutations do not interfere with VWF assembly or secretion, but they do render normal VWF multimers more susceptible to a specific VWF cleaving metalloproteinase or ADAMST13 present in the plasma.[17] ADAMST13 cleaves normal VWF multimer between the proteolytic site Tyr1605 and Met1606, and generates a 176-kDa fragment localized to the C-terminus, and a 140-kDa to the N-terminus of the mature VWF.[18]

In the very rare recessive IIC, IID, and IIE phenotypes, several missense, small insertions, or deletion mutations have been reported in the D2 domain. The common pathophysiological mechanism involved in all of these VWD type 2 A mutations is the absence of high molecular weight multimers and, consequently, the reduction in in vivo VWF-dependent platelet adhesion.

Type 2B VWD is characterized by increased affinity of the mutant VWF for platelet and reduction in high molecular weight multimers (Fig. 2). The remaining VWF multimers are not hemostatically effective and cause bleeding and thrombocytopenia in the patients that can be exacerbated during physical exercise, stress, and pregnancy. This defect is identified in the laboratory by enhanced ristocetin-induced platelet aggregation. Type 2B is inherited as an autosomal dominant and accounts for less than 20% of all type 2 VWD. Aside from a single amino acid insertion, the genetic defect is generally found to be a point mutation in the A1 domain of VWF gene that contains the glycoprotein Ib (GpIb) binding domain and results in a gain of function. The most frequent mutations are Arg1306Trp, Arg1308Trp, Val1316Met, and Arg1314Gln accounting for $\sim$90% of the subtype.

Type 2M variants are characterized by decreased platelet-dependent function not caused and associated with loss of high molecular weight multimers, and their phenotype can be caused by mutations that inactivate specific binding sites for the ligands in connective tissue or on the platelet surface, without affecting multimer assembly or stability. Most of these variants were previously grouped with type I VWD, including type B with absent ristocetin—but normal botrocetin-cofactor activity and normal multimers and ''Vincenza'' variant where unusually ultrahigh molecular weight multimers are present.[19] In the past few years, mutations have been identified and are now listed in the database. Some of these mutations, including Ala1374Cys and Ala1374His, have been confirmed by mutation recombinant VWF studies. A type 2M ''Vincenza'' mutation, heterozygous Arg1205His, is the latest to be reported for type 2M VWD identified in all of the affected members of seven Italian families and in one German patient.[20]

Type 2N VWD or ''Normandy VWD'' results from defective binding ability of VWF with FVIII, and these patients show disproportionately reduced levels of FVIII and normal VWF multimers.[21] As a result of description of this type of VWD, FVIII binding assay should be the differential diagnosis test for patients with mild to

moderate hemophilia A. In fact, it is possible that some previously reported apparent female hemophilia cases with unusual inheritance pattern may have been due to this type of VWD. These ambiguities can now be clarified by FVIII binding assay. Type 2N VWD mutations are all clustered in the N-terminal region (D′ and D3 domains, from exon 13 to 24) of the VWF gene, which contains the FVIII binding site (Fig. 1). Apart from one deletion, all of them are single-base substitutions. Type 2N VWD patients are generally homozygous or compound heterozygous for one or more of these mutations. The most frequently reported mutation is Arg854Gln and, along with most of the others, does not result in complete disruption of FVIII binding to VWF. Even in homozygous patients, a residual level of binding can be demonstrated and is only slightly reduced in the heterozygous state. Combination of a type 2N Cys788Arg mutation, together with another (Cys1225Gly in pre-pro VWF), can also cause a mixed phenotype associated with both mutations. This type has been classified as combined type 1 and type 2N VWD.[22] Other combinations of type 2N mutation on one allele and a frame shift mutation on the other VWF allele have also been reported.[23]

CONCLUSION

Since the initial description of VWD in 1926, we now have a better understanding of molecular biology, genetic abnormalities, and pathophysiology of this disorder. Treatment for the severely affected patients and carrier detection in these families has also been achieved. But with clarification of the molecular genetics and identifications of the mutational basis of this disease, together with insight into structure–function relationship of VWF molecule, future efforts should now be directed toward more definitive diagnostic methods, classification, and novel treatments of this common human bleeding disorder.

RESOURCES

OMIM no. 193400
Gene map locus 12p13.3
Mutation database http://www.sheffield.ac.uk/vwf/

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Wilson Disease

Juliane Halangk
Hartmut Schmidt
Heiko Witt
Charité, Berlin, Germany

HISTORICAL INTRODUCTION

Wilson disease, also referred to as hepatolenticular degeneration, was first described in 1912 by Samuel A. Kinnier Wilson as a familial progressive disorder of the central nervous system accompanied by liver cirrhosis, and by Bruno Fleischer as a ''pseudosclerosis'' with tremor, liver cirrhosis, and corneal deposits.[1,2] One year later, Rumpel demonstrated an excessive hepatic copper accumulation in Wilson disease.[3] This finding was confirmed by Lüthy, who also detected an elevated copper concentration in the basal ganglia.[4] An autosomal recessive inheritance pattern was shown by Bearn,[5] and in 1993 the disease-causing gene was identified as a metal-transporting P-type ATPase designated *ATP7B*.[6–8]

CLINICAL PRESENTATION

Wilson disease is a protean disorder with a broad clinical spectrum owing to the toxic effect of copper on the various organ systems. The disorder occurs worldwide with an estimated prevalence in Caucasians of approximately 1 per 30,000 to 1 per 100,000 live births and a carrier frequency of about 1 per 90 to 1 per 150.

Most patients with Wilson disease present between 5 and 35 years of age. However, Wilson disease should be included in the differential diagnosis in individuals with liver disease of uncertain cause even if they are younger or older. Clinical presentation of liver disease varies from acute fulminant liver failure requiring transplantation to chronic liver disease with cirrhosis and its complications, such as portal hypertension, esophageal varices, and hypersplenism, but also solely steatosis may occur.

Neurological manifestations of Wilson disease typically develop later in life, most often in the third decade, but may also occur in children. The spectrum of symptoms range widely from discrete tremor, dysarthria, and lack of motor coordination to severe pseudobulbar palsy. Because of affection of the basal ganglia clinical signs of Parkinson disease such as micrographia, hypersalivation, hypomimia, and rigid dystonia may occur. Psychiatric manifestations include behavioral changes and deterioration of performance at school or at work. Major depression, anxiety, or frank psychosis are further presentations. Many of those patients with neurological or psychiatric symptoms have liver disease, but not all of them display hepatic symptoms.

Kayser–Fleischer rings are characteristic although not mandatory findings in Wilson disease, representing deposits of copper in the Descemet's membrane of the peripheral cornea. They appear as a band of golden-brownish pigment when visible by direct inspection. However, in most cases, slit-lamp examination by a skilled examiner is required. Other ophthalmological changes such as sunflower cataracts representing copper deposition in the lens might be revealed. These clinical observations are of diagnostic value, but do not obstruct vision. Nonetheless, Kayser–Fleischer rings are not entirely specific for Wilson disease and are also observed in patients with chronic cholestatic diseases.[9] Kayser–Fleischer rings typically develop later in the course of the disease, are rare in childhood, and are mostly although not necessarily detectable in patients with neurological manifestations, but in only about 55% of patients presenting initially with liver disease.[10]

In some patients, Coombs-negative hemolytic anemia is the first clinical disease manifestation. Other rare extrahepatic manifestations of Wilson disease may include renal tubular abnormalities, arthralgias, cardiomyopathies and arrhythmia, amenorrhea, or repeated miscarriages (reviewed in Ref. [11]).

MOLECULAR GENETICS

Copper is absorbed in the small intestine and enters the liver via the portal vein. In the liver, copper is incorporated into ceruloplasmin or copper-containing enzymes and excess copper is secreted into the bile. The precise intracellular target for the toxic action of copper is unknown. Copper is potentially toxic because of its ability to promote free radical formation. Lipid peroxidation of membranes and organelles as well as modification of mitochondrial DNA and alterations of protein synthesis are described in patients with Wilson disease.

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The Wilson disease gene, ATP7B, encodes a P-type ATPase that acts as a membrane copper-transport protein. In hepatocytes, ATP7B is located in the trans-Golgi apparatus where it functions to incorporate copper into ceruloplasmin and to excrete copper into the bile.[12,13] Although the genetic defect in Wilson disease resides primarily in the liver, ATP7B is also expressed in a variety of other tissues including kidneys, brain, heart, muscle, and pancreas.

Human *ATP7B* is approximately 50 kb, has 21 exons, and is located on the long arm of chromosome 13 (13q14.3–q21.1). The gene product consists of 1465 amino acids. It forms eight transmembrane domains with most of the protein residing on the cytoplasmic side. In addition to the characteristics of all P-type ATPases such as the ATP-binding site, the phosphatase domain, and the motif containing aspartyl phosphate, ATP7B contains six N-terminal copper-binding motifs.

DIAGNOSING WILSON DISEASE: A HIGH INDEX OF SUSPICION

Diagnosis of Wilson disease is particularly challenging: no single laboratory test or clinical finding exists to establish or rule out definitive diagnosis. Early diagnosis, however, is crucial, as timely therapy may maintain normal life expectancy. If Wilson disease is suspected, the complete diagnostic battery of physical examinations and laboratory tests including liver biopsy with measurement of hepatic copper content has to be performed. Neither normal serum ceruloplasmin, urinary copper levels, nor absent Kayser–Fleischer rings exclude the disease.

Ceruloplasmin serum levels less than 20 mg/dL are suspect for Wilson disease, although normal concentrations are seen in 5–25% of patients; moreover, 10–30% of heterozygous carriers display lowered ceruloplasmin levels. Decreased ceruloplasmin is also observed in patients with chronic liver disease of other etiology[14] and can be absent in cases of aceruloplasminemia.

Reduced serum copper concentrations below 60 μg/dL may be found in Wilson disease. However, most patients show normal copper levels and up to one third of heterozygous carriers exhibit decreased serum copper.[10] In contrast, the determination of serum free copper reveals increased concentrations in Wilson disease.

Measurement of 24-hr urinary copper excretion represents a valuable diagnostic test. Individuals who excrete >100 μg (>1.6 μmol) in 24 hr are suspect for Wilson disease. Various chronic liver diseases and especially cholestatic diseases, however, might also increase urinary copper.[15] In addition, urinary copper excretion after D-penicillamine challenge should be measured in suspected Wilson disease. Urinary copper excretion is a useful marker in monitoring pharmacotherapy and compliance.

Liver biopsy with determination of hepatic copper content is the most valuable single diagnostic procedure. Liver copper >250 μg/g dry weight is highly suggestive for Wilson disease, but may be also found in cholestatic diseases and in idiopathic copper toxicosis. The major shortcoming of a single liver biopsy specimen is underestimation of hepatic copper because of inhomogeneous copper deposition in the liver. Detection of copper in histological specimens by special staining methods such as rhodamine or orcein staining depends on its intracellular localization and is therefore insensitive and rarely helpful in establishing diagnosis. The histological findings in Wilson disease may resemble the features of various other liver disorders such as autoimmune hepatitis or nonalcoholic steatohepatitis.

Neuropsychiatric examination is required in every patient with Wilson disease and magnetic resonance imaging (MRI) should be performed in patients presenting with neurological manifestations.

MANAGEMENT

In all patients with Wilson disease, whether symptomatic or not, consequently implemented treatment is obligatory for maintaining normal life expectancy. The aim of therapy is the elimination of excess body copper and prevention of copper reaccumulation. Treatment should be initiated as soon as the diagnosis is established, and the mainstay remains lifelong pharmacotherapy.[11] Cessation of therapy leads to progression of disease and can even cause acute fulminant liver failure. Thus, the patient's compliance is essential and should be monitored as well as potential side effects of medication.

Chelating agents such as D-penicillamine promote urinary excretion of excess copper. In the initial phase of therapy, penicillamine is usually administered in doses of 1000–1500 mg/day in two divided dosages. Maintenance therapy can be started when 24-hr urinary copper excretion is less than 500 μg. Maintenance dose is usually 750–1000 mg/day administered in two divided dosages. In pediatric patients, 15–25 mg D-penicillamine per kilogram body weight should be administered in two divided dosages. Food inhibits absorption of penicillamine; thus the drug should be taken 1 hr before or 2 hr after meals. D-Penicillamine antagonizes the effect of pyridoxine and daily supplementation with 25 mg is recommended. D-Penicillamine therapy has to be discontinued in 20–30% because of severe side effects including fever, rash, lymphadenopathy, neutropenia, thrombocytopenia, lupuslike symptoms, nephrotoxicity, Goodpasture syndrome, myasthenia gravis, and polymyositis. It is

crucial to realize that these side effects can occur even years after starting initial therapy. Worsening of neurological symptoms has been reported in some patients treated with D-penicillamine.

Trientine (triethylene tetramine dihydrochloride) is an alternative, rather expensive chelator with fewer side effects. Trientine is given at an average daily dose of 750–1500 mg as two to three divided dosages (in children, 15–20 mg/kg body weight). Side effects include iron deficiency anemia because of its chelating action requiring iron supplementation.

Zinc salts have few side effects, and three daily dosages of 100 mg ingested before meals are effective in maintenance therapy (in children, 3–4 mg/kg body weight). Zinc interferes with copper absorption in the gastrointestinal tract and induces intestinal metallothioneins, which act as endogenous chelators. Zinc salts can be combined with D-penicillamine or trientine. Because of interactions, chelating agents should be administered with some hours delay and not simultaneously with zinc.

Ancillary to pharmacological treatment dietary copper restriction is recommended, but its therapeutic value remains to be determined.

In patients with fulminant hepatic failure or decompensation of chronic liver disease liver transplantation is required. The molecular defect in Wilson disease is cured by a liver graft and no specific therapy in respect to Wilson disease is necessary after transplantation. The neurological course of disease after transplantation is currently under investigation.

GENETIC TESTING

The discovery of the underlying genetic defect in Wilson disease raised the hope of overcoming the obstacles in diagnosing the disease. Genetic testing in Wilson disease, however, is complicated by the fact that most patients are compound heterozygous with different mutations on each allele. More than 300 *ATP7B* mutations spread over the entire coding sequence have been described and are listed in a database that can be downloaded at http://www.uofa-medical-genetics.org. The most frequent *ATP7B* mutation found in Caucasians is a histidine by glutamine exchange at codon 1069 (H1069Q) in exon 14, which is found in 10% to 70% of Wilson's disease chromosomes.[16] It is proposed that the H1069Q mutation results in an increased degradation of mutated protein within the endoplasmic reticulum.[17] Another frequent mutation in Caucasians represent 3402delC, whereas the most frequent mutation in Asia is R778L.[18]

The large number of mutations and the high prevalence of compound heterozygotes hamper genotype–phenotype correlation analysis. In a homogeneous population from Saxonia, Germany, H1069Q was found in 87% of patients. Homozygosity for H1069Q was shown to be associated with later onset of disease, predominance of neurological symptoms, and milder hepatic disease compared to compound heterozygotes or carriers of other mutations.[16] This correlation has also been established in several other populations.[19,20] However, conflicting results have been obtained by others.[21] Genotype–phenotype correlation of other mutations is rendered difficult by their low frequency and the different ethnic backgrounds of the populations studied. The phenotypic appearance of a certain genotype is most probably influenced by a variety of genetic, epigenetic, and environmental factors that remain to be elucidated.

Genetic testing in Wilson disease is possible in affected index patients and indicated in patients in which the clinical presentation and the laboratory tests do not allow a definite diagnosis. Genetic testing is also indicated to identify asymptomatic relatives. It has to be pointed out that a negative genetic test result does not exclude Wilson disease in a suspected case.

Several methods have been described for mutation detection in Wilson disease. In areas where a specific mutation is common, such as H1069Q in Central Europe, genotyping using restriction fragment length polymorphism (RFLP) analysis or melting curve analysis with fluorescence resonance energy transfer (FRET) probes is feasible.[22]

For screening further mutations, single-strand conformation polymorphism (SSCP) analysis represents an alternative approach. However, sensitivity of SSCP is low with a detection rate of only 50–60%, and homozygous individuals may be missed by this method because of absent heteroduplex generation. Moreover, *ATP7B* contains numerous harmless polymorphisms that cannot be discriminated from disease-causing mutations by an altered SSCP mobility pattern.

Direct DNA sequencing with a sensitivity of 65–85% is available in specialized laboratories, but is time-consuming and cost-intensive.

Predictive genetic testing of family members is possible and can be generally recommended. Screening of sibs is especially mandatory, as each sib possesses a one in four chance of being affected. If the underlying mutations are unknown, haplotype analysis using microsatellite markers (*D13S314*, *D13S301*, *D13S316*) or intragenic SNPs is alternatively feasible to detect asymptomatic sibs. The risk for offspring of an affected patient is approximately 1 per 180, assuming a carrier frequency

of 1 per 90. This risk, however, is markedly higher in consanguineous families.

Although prenatal testing is principally feasible, Wilson disease is in our opinion no indication for prenatal diagnosis and induced abortion because treatment started in asymptomatic individuals after birth allows a normal life span.

CONCLUSION

Wilson disease should be considered in patients with uncertain signs of hepatic, neurological, or psychiatric disease. Ocular slit-lamp examination as well as measurement of serum ceruloplasmin, urinary copper excretion before and after D-penicillamine challenge, and liver biopsy with determination of the hepatic copper content are required. It has to be pointed out that Wilson disease is a diagnostic challenge because of the variety of clinical presentations and laboratory findings. Genetic analysis is rendered difficult by a high number of different mutations in distinct regions of the *ATP7B* gene and the high prevalence of compound heterozygotes. Genetic testing is indicated in patients in which the clinical presentation and the laboratory tests do not allow a definite diagnosis. A negative genetic test result does not rule out Wilson disease. Family screening of first-grade relatives should be done to detect asymptomatic cases. Early therapy with chelators or zinc is lifesaving and has to be continued lifelong. Special attention has to be directed to therapy compliance and occurring side effects. Liver transplantation is necessary in decompensated liver disease, but its utility in neurological disease is controversial.

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Wolff–Parkinson–White Syndrome, Familial

W

Robert Roberts
Baylor College of Medicine, Houston, Texas, U.S.A.

INTRODUCTION

Wolff–Parkinson–White (WPW) syndrome was described as a clinical entity in 1930 by the individuals whose name it bears.[1] It had been postulated the genesis of the WPW syndrome was due to an accessory pathway bypassing the AV node from the atria to the ventricle. This was subsequently confirmed by electrical cardiac mapping and this technique is now used routinely to identify and ablate the accessory pathway.[2] Wolff–Parkinson–White syndrome is the second most common cause of paroxysmal supraventricular tachycardia in the western world, and in Asian countries such as China it is the number one cause.[3] It became a model for reentry tachycardia. Thus WPW has become the intrigue of the electrophysiologist both as a mechanism for tachycardias as well as a disease that has a specific treatment.

CLINICAL DESCRIPTION

The most common presenting complaint is palpitations and/or a sense of a rapid heart rate followed by presyncope, syncope, or sudden cardiac death (SCD). Sometimes, the only feature is sudden death, which can occur without warning, usually in the setting of atrial fibrillation with a rapid ventricular response. It is also very common for WPW to be discovered as an incidental finding without symptoms on the electrocardiogram due to a routine medical checkup. The incidence of the disease increases with age. It is claimed that about 50% will be symptomatic due to associated supraventricular tachycardia. However, it should be emphasized that WPW may occur in infancy as a cause of SCD more commonly than is generally recognized. At this age electrocardiograms are frequently not performed and it would be rare at postmortem to examine the conduction system. In a recent study of SCD in the young in which pathology studies were performed and electrocardiograms were obtained, WPW exceeded that of familial hypertrophic cardiomyopathy as a cause of SCD. There is also the concern that WPW may play a role in the so-called sudden infant death although this is more commonly associated with Brugada syndrome or long QT syndrome.

It is reasonable to expect that all WPW is genetic in origin. The percentage of cases due to familial vs. sporadic remains unknown. Only one gene has yet been identified and based on our studies[4] in other families with WPW it is evident there are several other genes responsible for this syndrome. The familial form of WPW caused by the gene PRKAG2 has other clinical features, which distinguish it from the more common form of WPW. In addition to having the WPW syndrome and supraventricular tachycardias, it is often associated with hypertrophic cardiomyopathy and conduction defects. In routine WPW syndrome, atrial fibrillation is said to occur in about 20–30% of cases, whereas in the syndrome due to PRKAG2 it is more frequently occurring in up to 50% of cases.

GENETICS

It should be emphasized that all forms of WPW syndrome are likely due to genetic defects. Most cases with the WPW syndrome have an accessory pathway without conduction defects or hypertrophy. The form of WPW due to the PRKAG2 gene is inherited in an autosomal dominant pattern. The PRKAG2 gene located on chromosome 7q36 encodes the gamma 2 subunit of AMP-activated protein kinase (AMPK). AMP-activated protein kinase consists of three subunits, alpha, beta, and gamma, with each subunit encoded by a distinct gene.[5] The alpha subunit is the catalytic component, and the beta and gamma are regulatory units. The gamma subunit has the binding site for AMP which is essential for enzymatic activity. AMP-activated protein kinase is present in all tissues although there may be tissue specificity on the basis of isoforms present within the heterotrimeric enzyme. There are three isoforms of the beta and three isoforms of the gamma which are derived from alternate transcripts of the same gene. The genetic defect consists of mutations in the PRKAG2 gene which encodes for the gamma subunit. The initial mutation was a substitution of glutamine for arginine at residue 302. The PRKAG2 gene consists of 16 exons and is more than 280 kb. The AMPK subunit protein is 63 kDa.[6] Six mutations[7] have been described as listed in Table 1, all of which are missense

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Table 1 AMPK (PRKAG2) mutations responsible for WPW

Arg302Gly
Arg531Gly
Thr400Asn
Asn488Ile
His142Arg
Ins Leu

mutation, except for the leucine insertion. Expression of two of these mutations, the R302Q[8] and N488 I,[9] as a transgenic in the mouse is associated with the WPW syndrome, cardiac hypertrophy, and abnormalities in the conduction system. The penetrance based on the few families so far studied is in the range of 70% to 80%.

GENOTYPE TO PHENOTYPE CORRELATIONS

In 1930, Wolff, Parkinson, and White[1] described patients with electrocardiograms showing a short PR interval, a delta wave, and a wide QRS complex. It was postulated an accessory atrial ventricular pathway would explain the particular ECG and the recurrent tachycardias. Electrophysiological investigations, cardiac electrical mapping, surgical findings, anatomic studies, and results of ablation treatment have confirmed the presence of accessory conduction pathways (Fig. 1). The normal cardiac conduction is from the atrium to the ventricles through the AV node. In WPW syndrome, there is an accessory pathway between the atrium and the ventricle which bypasses the AV node and this prematurely depolarizes a portion of the ventricle to give you the short PR interval and the slurred upstroke of the QRS. However, the overall activation of the ventricle is slightly later due to the normal electrical impulse which passes through the AV node. A property of the AV node is to decrease the rate of transmission to the ventricle. Under certain conditions, conduction through the AV node to the ventricle can give rise to retrograde conduction from the ventricle to the atrium through the accessory pathway. Upon arrival in the atrium, the AV nodal conduction system has recovered resulting in reentry of the impulse into the AV node. Arrival of this impulse in the ventricle can now again be transmitted retrograde back through the accessory pathway generating a circus movement leading to reentry tachycardia. Wolff–Parkinson–White syndrome has long been regarded as intriguing to cardiologist and particularly to the electrophysiologist as it is the sine quo non of reentry tachycardia. The most common form of supraventricular tachycardia observed with WPW is that of antegrade conduction through the AV node with retrograde conduction through the accessory pathway. The usual result is a regular supraventricular tachycardia with a rate of about 120 to 160 beats per minute. The overall configuration is a narrow QRS. The vectors of the QRS, however, may vary depending on the anatomical location of the accessory bundle. The most common anatomical bundle is that which goes from the left atrium to the left ventricle and occurs in about 50%; a posteroseptal, right ventricular, or anteroseptal insertion of the AV pathway is found in approximately 30%, 13%, and 7% of patients, respectively. It is claimed that about 80% of the supraventricular arrhythmias associated with WPW have a regular supraventricular tachycardia with a narrow QRS. In the remaining 20% the arrhythmia is generally atrial fibrillation. The incidence of tachyarrhythmias in WPW syndrome is unknown. Reports from a variety of studies indicate 10–80%. Atrial fibrillation is very dangerous because it predisposes to failure and sudden death. This is because the rapid rate is transferred to the ventricle due to lack of the damping effect of the AV node. This rapid rate of the ventricle prevents proper relaxation during diastolic for adequate blood to return to the heart resulting in

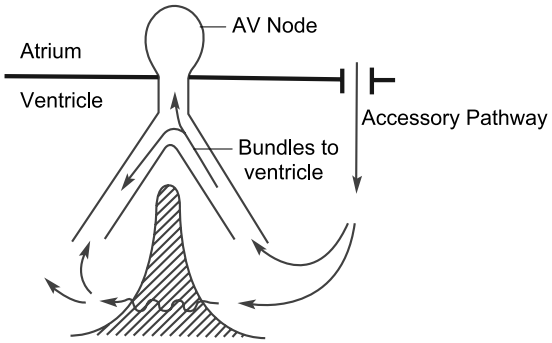

Fig. 1 Cardiac electrical conduction is normally from the SA node through the atrium and subsequently transmitted to the ventricles by the AV node and right and left bundles. In WPW, there is an accessory pathway as indicated in the figure connecting the atrium to the ventricle without going through the AV node. Thus, an impulse arising in the atrium may get conducted to a portion of the ventricle through the accessory pathway and subsequently be retrograde conducted through the normal conduction system including the AV node. Arrival of this retrograde conduction impulse is delayed in the AV node permitting adequate recovery time for the accessory pathway so that it may be conducted again through the accessory pathway giving you reentry tachycardia. Other times conduction through the accessory pathway will simply give preexcitation of only a portion of the ventricle indicated by the short PR interval and the slurred upstroke of the QRS. The remainder of the QRS is generated by conduction through the AV node and bundle branch system.

decreased cardiac output, cardiac failure, and may deteriorate into ventricular tachycardia or fibrillation and sudden death. Atrial fibrillation is particularly dangerous if there is a short antegrade refractory period as ventricular fibrillation is more likely to occur.

The genetic defect is in the PRKAG2 gene which encodes for the gamma subunit of the kinase AMPK. AMP-activated protein kinase is a sensor of the body's ATP level. AMP-activated protein kinase is activated by an increase in the ratio of AMP to ATP. AMP-activated protein kinase increases the availability of ATP by increasing glucose absorption, inhibiting glycogen synthesis, increasing fatty acid oxidation, and decreasing fatty acid synthesis. The phenotype consistently observed in addition to WPW syndrome is excess glycogen in the myocytes of the heart.[10] Hypertrophy of the heart is also observed in a percentage of the patients. Several genetic animal models expressing either the R302Q mutation[8] or the mutation N488I[9] as a transgene have been generated. All of them have the preexcitation syndrome and excessive myocardial glycogen phenotype. The model expressing the R302Q mutation has the preexcitation syndrome, increased cardiac glycogen, and inducible supraventricular reentry tachycardia as observed in the human phenotype. In this model,[8] we have shown there is loss of function of AMPK leading to loss of inhibition of glycogen synthase resulting in increased formation of glycogen in the heart. The hypertrophy is probably a compensatory feature due to less ATP available for contractility inducing formation of more sarcomeres. The mechanism whereby AMPK leads to accessory pathways or alters conduction remains to be determined. AMP-activated protein kinase with the R302Q mutation has been shown to inhibit the cardiac sodium inward current in in vitro studies.[11] This is an exciting area of research as AMPK is also one of the pivotal enzymes in obesity, insulin resistance, and the metabolic syndrome.

INCIDENCE

The true incidence of preexcitation is unknown but varies from one to three per thousand populations. As preexcitation on the ECG is often concealed it is felt the true incidence of preexcitation is grossly underestimated.

DIAGNOSIS

Patients presenting with supraventricular tachycardia or the symptoms of syncope or presyncope due to an arrhythmia should have a regular 12-lead electrocardiogram to detect preexcitation, namely, short PR interval with slurred upstroke of the QRS. If an accessory pathway is anticipated as the accessory pathway is often concealed, it may be necessary to do an intracardiac electrophysiological study. Premature atrial stimulation is performed to induce supraventricular tachycardia which would indicate the presence of an accessory pathway. Termination of the supraventricular tachycardia by single premature impulse is further evidence of an accessory pathway. The other procedure that helps to make the diagnosis is the administration of procainamide, which inhibits the accessory pathway inducing conduction through the normal AV nodal system resulting in normalization of the electrocardiogram. The presence of a family history is highly suggestive and the disease can be confirmed by myocardial biopsy showing the characteristic phenotype of excessive glycogen deposition if mutations in the AMPK gene are responsible for the disease.

TREATMENT

Incidental identification of WPW on electrocardiogram requires assessment of possible symptoms. If the patient is asymptomatic having never had palpitations or syncope, no further specific treatment is required. It is important, however, to remind the patient that they should not take digoxin or verapamil. If the individual is a pilot or makes his living by driving a vehicle, one may be concerned with the possibility of development of atrial fibrillation. It is suggested in these asymptomatic individuals that electrophysiological studies be performed to determine whether there is a short accessory pathway, which is more likely to predispose to atrial fibrillation. If that were the case, then the recommended treatment would be radiofrequency ablation of the accessory pathway. Individuals with documented preexcitation and symptoms should be considered for curative therapy, namely, ablation. If ablation is not considered, then drug therapy such as beta-blockers, amiodarone, or flecainide should be considered. In the familial form of WPW, there is also a conduction defect in the regular normal AV node system and a pacemaker is frequently required for complete heart block. Patients presenting with supraventricular tachycardia should be converted with i.v. adenosine, as seldom it is necessary to perform electrocardioversion.

GENETIC COUNSELING

The familial form of WPW is inherited as an autosomal disorder. This means 50% of the offspring will inherit the mutant gene and thus be at risk of developing the disease. As it is autosomal dominant, there is vertical transmission of the gene with each generation expected to have individuals affected with the disease. The mutant gene

will affect males and females equally, and based on experience with families so far, one can expect the penetrance to be in the 70–80% range. Onset of the disease is age dependent and increases with age, but onset can occur very early or very late. Once a proband has been identified in a family, it is important to screen all family members with at least a 12-lead electrocardiogram. Individuals in a family with segregation of the disease who are symptomatic with a normal electrocardiogram should have EP studies with provocation to exclude an accessory pathway.

GENETIC TESTING

In North America, genetic testing is not yet approved as the procedure is still research. Therefore any attempt to screen must be done on a research protocol. In some European countries, genetic screening is already a routine procedure. The gene responsible for this syndrome is the gamma 2 subunit of the AMPK protein. The official designation for the gene is PRKAG2. As there are already multiple mutations present in the PRKAG2 gene, it is preferable to sequence the complete cDNA.

CONCLUSION

Wolff–Parkinson–White syndrome is the second most common cause of supraventricular tachyarrhythmias in the western world and the most common cause in Asian countries such as China. The first gene responsible for this disorder was identified to be a mutation in the gamma 2 subunit of AMPK, which is encoded by the gene PRKAG2. This has now been confirmed in several families throughout North American and Europe. Six mutations all except one are missense mutations present in the gamma 2 subunit. The syndrome consistently exhibits preexcitation (short PV interval on the ECG), delayed upstroke of the QRS, and often is associated with impaired atrioventricular conduction as well as ventricular hypertrophy. Genetic animal models have been developed and are being explored to improve the diagnosis, prognosis, and treatment of this syndrome. Genetic testing is available through DNA sequencing but is performed at present only as a research tool.

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Wuchereria bancrofti

W

Kosum Chansiri
Pornpimon Kanjanavas
Phaisan Khawsak
Sintawee Khuchareontaworn
Srinakharinwirot University, Bangkok, Thailand

Sirichai Phantana
Ministry of Public Health, Nontaburi, Thailand

Peerapan Tan-ariya
Mahidol University, Nakorn Pathom, Thailand

INTRODUCTION

Lymphatic filariasis is a significant health problem in many developing countries. Globally, 1.1 billion people live in known endemic areas and about one-fourth of them may be infected. Lymphatic filariasis is the second leading cause of permanent and long-term disability and undermines the social and economic welfare of the affected people and communities. The World Health Organization launched a global program to eliminate lymphatic filariasis as a public health problem by the year 2020. Human lymphatic filariasis is mainly caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. Bancroftian filariasis, *W. bancrofti*, is responsible for 90% of lymphatic filariasis and widely distributed throughout the tropics and subtropics. *B. malayi* infection is endemic in Asia such as China, Korea, India, Indonesia, Malaysia, Philippines, and Sri Lanka. *B. timori* infection occurs in Indonesia (islands of Alor, Flores, and Timor).[1]

The microfilariae of *W. bancrofti* were first described by Demarquay in 1863 and the adult worms were first described in 1877 by Cobbold in Australia and by Lewis in India. In 1878, Manson completed the description of the life cycle by showing that mosquitoes acted as intermediate hosts for the parasite.[2] It has been speculated that *W. bancrofti* originated from Southeast Asia, where its closest known relative, *Wuchereria kalimantani*, parasitizes the Indonesian leaf monkey. From there, it was presumably carried by the earliest migrants to the islands of the South Pacific, perhaps as early as 2000 BC. Another migration from the same area of Southeast Asia, known to have settled in Madagascar sometime before 500 AD, may have brought filariasis to that island and subsequently to the mainland of Africa. Filariasis is known to have spread throughout Central Africa and into Arabia by the 14th or 15th century (with no evidence of its presence in Egypt in pharaonic times) and to have been imported to the New World via the slave trade in the 17th and 18th centuries. It was introduced into north-eastern Australia during the 19th century, but has since been eradicated.[3]

BODY OF TEXT

Diagnosis of Filariasis

Diagnosis of filariasis infection is frequently made on strict clinical grounds, particularly in endemic areas, but demonstration of microfilariae in the circulating blood is the only means by which one may make a certain diagnosis. The advantage and disadvantage of the detection techniques were discussed in Table 1.

Microscopic Examination

In the past, surveys for lymphatic filariasis depended on the examination of blood films, which, in most areas, had to be collected around midnight. The thick blood smear technique normally employed to detect microfilaria is intensive especially when the parasite is nocturnally periodic and a nocturnal specimen is required to increase the chance of detecting the infection. The need for a nocturnal blood sample has been obviated by the administration of 50 and 100 mg of DEC before blood examination. Furthermore, conventional microscopy is not suitable for large-scale microfilariae screening of populations in endemic areas because it is very time-consuming and labor-intensive.

Various concentration methods have been developed to increase the sensitivity for the direct demonstration of

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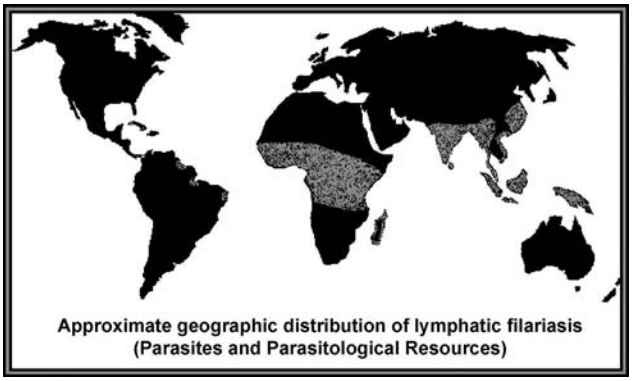

Fig. 1 Distribution of lymphatic filariasis. (From Ref. [1].) (*View this art in color at www.dekker.com.*)

microfilaria, and these include the Knott's concentration and membrane filtration techniques in which 1 mL or more heparinized blood can be processed and the microfilaria can be concentrated for easy detection.

The Knott's concentration technique, although simple and economical to perform, is rather tedious. The membrane filtration technique, which effectively concentrates microfilariae onto a membrane that can be stained with Giemsa and examined under a light microscope, is a rapid, sensitive, and easy technique to carry out. However, the membrane filter technique requires the large volume of blood collected when the carrier cases are investigated. These two techniques should nevertheless be used to detect low microfilaremic states and to monitor the effectiveness of chemotherapy where microfilaria counts may become very low.

A recent technique developed for the detection and identification of microfilaria in blood samples is the

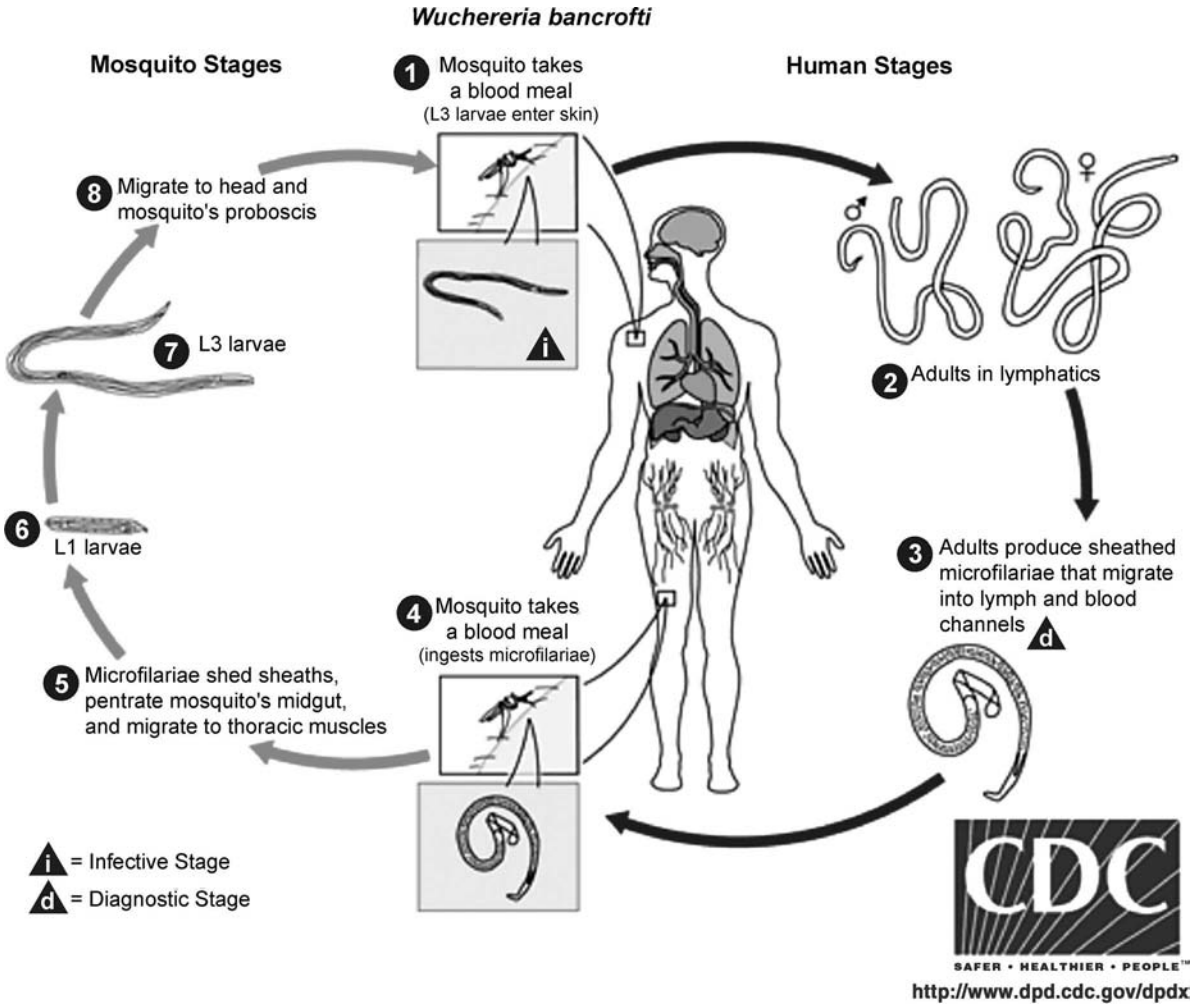

Fig. 2 Life cycle of *W. bancrofti*. (From Ref. [2].) (*View this art in color at www.dekker.com.*)

Table 1 Comparison of the methods used for detection of *W. bancrofti*

Methods	Advantage	Disadvantage
Giemsa stain of thick blood films	• Low cost • Easy	• Underestimate • Cannot detect low density of microfilariae • Blood collection at night time
Filtration test	• Easy • Sensitive	• Time-consuming • Blood collection at night time
Immunological test	• Blood can be taken at anytime • Sensitive	• Cross-reaction among closely nematode • Costly
DNA probes	• Sensitive • Specific • Can be used for detection of the parasite in mosquitoes	• Costly • Blood collection at night time
PCR-based methods	• Very sensitive • Specific • Reproducible • Can be used for detection of the parasite in mosquitoes	• Costly • Blood collection at night time

quantitative buffy coat (QBC) technique, which involves centrifugation of the blood sample collected in a micro-hematocrit tube containing heparin, EDTA, and acridine orange. Microfilariae, if present, are concentrated in the area of the buffy coat after centrifugation and can be viewed by fluorescent microscopy as acridine orange stains the DNA of the parasite.[4]

Immunological Diagnosis

Intradermal tests

Intradermal tests performed by several investigators have produced equivocal results. Early studies with purified antigenic extracts of adult *Dirofilaria immitis* (Sawada antigen) showed high positive rates in *W. bancrofti* microfilaremic and clinically positive patients.[5] However, it was found that this antigen could not discriminate between patients with and without clinical manifestations. In addition, the Sawada antigen was reported to have cross-reactivity with other filarial infection.[6] Subsequently, other investigators compared the Sawada antigen with *W. bancrofti* microfilarial and larval extracts and *B. malayi* infective larval antigen.[7] From these studies, it appears that antigen prepared from human parasites has greater diagnostic utility in intradermal tests.

Antibody detection

A number of serological assays have been developed for the immunodiagnosis of lymphatic filariasis. These include the indirect fluorescent antibody test (IFAT), indirect hemagglutination test (IHA), precipitin techniques, and the enzyme-linked immunosorbent assay (ELISA). Various stages of antigens have been employed in the IFAT. These include frozen sections of adult worm and larvae,[8] papainized microfilaria,[9] and sonicated fragment microfilaria.[10] From these studies, homologous antigens were found to be better than heterologous antigens for antibody detection. The IHA test and precipitin techniques have not been widely used for the diagnosis of filariasis in recent years as previous studies have shown their lack of sensitivity.[11] However, the ELISA is very popular in the diagnostic laboratory because its sensitivity is reported to be equal to that of radioimmunoassay (RIA).

Nevertheless, the methods that have focused on the detection of filarial antibodies in the host generally lacked sufficient sensitivity and specificity to discriminate effectively between past and present filarial infections in humans. The detection of circulating filarial antigens in the peripheral blood would thus provide a more accurate indication of active filarial infection in man than the detection of antibody.

Antigen detection

Antigen detection assays would be especially useful for diagnosing a microfilaremic prepatent and occult infection where classical parasitological tests are not useful. *W. bancrofti* represents a success to the detection of circulating antigen, in that an ELISA is commercially available for detection of this parasite (Trop-Ag

W. bancrofti ELISA kit, JCU Tropical Biotechnology Pty Ltd, Queensland, Australia). The assay is dependent on a monoclonal antibody, Og4C3, which, curiously, despite being raised against antigens of the bovine parasite *Onchocerca gibsoni*, detects circulating antigen in serum samples from individuals infected with *W. bancrofti*, but not *Onchocerca volvulus*. Negative results were obtained with sera from individuals harboring *B. malayi*. A second specific assay, which is currently undergoing commercial development, is based on the mAb AD12.1 which recognizes a 200-kDa antigen in the circulation of individuals infected with *W. bancrofti* which again is of adult worm origin. Both assays have been evaluated as diagnostic tools in a number of studies and can detect circulating antigen in virtually all (94–100%) microfilaria carriers, as antigen levels remain constant throughout the day.

However, antigen detection methods are now recognized to have advantages over other diagnostic methods for *W. bancrofti* infection. The ELISA format of assays such as the Og4C3-dependent test is not ideal for use in the field. A new rapid assay has therefore been developed (the filariasis card test produced by ICT Diagnostics using mAb AD12.1). This assay takes only 5–15 min to complete, requires no specialized equipment, and gives comparable results to ELISA. Such assays are likely to prove valuable in the global effort to eradicate lymphatic filariasis as a public health issue. The immunochromatographic test (ICT) kit has been validated and used worldwide as an effective tool in detecting infections, especially in low-level microfilariae carriers, which are not usually detectable by conventional night blood examination. The ICT filariasis card test was recently recommended as a rapid screening tool to define the prevalence and distribution of filariasis as part of the global program to eliminate lymphatic filariasis. It was found that adult filarial antigen levels persist in microfilariae-negative persons for up to 3 years after treatment.[12]

Monoclonal antibody-based enzyme immunoassays for detecting parasite antigens would therefore be more suitable for field application. Several groups of researchers have successfully used such assay in the diagnosis of lymphatic filariasis. Weil et al.[13] used two monoclonal antibodies to detect *D. immitis* in a direct sandwich ELISA and to detect *W. bancrofti* antigens in human sera.

DNA probes

During the 1980s, a substantial effort to isolate and characterize filarial species-specific DNA probes was undertaken. The main objective was the development of a diagnostic system that would allow assessment of the impact of filarial nematode control programs. Later, the adaptation of polymerase chain reaction (PCR) technology and the development of a procedure involving ELISA detection of PCR products permitted the adoption of DNA probe-based methodology from the bench to the field. It is also of interest to note that effective diagnosis, the initial objective of the DNA probe-based research, was not only achieved, but also surpassed in that a deeper knowledge of the epidemiology of filarial disease, as well as filarial phylogenetic relationships, was achieved from this work.[14] PCR development has had a major impact on filarial diagnosis. The species-specific DNA probe sequences referred to above were used to design PCR primers that allowed both specific and sensitive parasite detection. The specificity of amplified DNA was confirmed by hybridization with species-specific oligonucleotides or the full DNA probes. The use of filarial DNA probe-based research has, in addition to benefiting diagnosis, contributed greatly to our knowledge of the epidemiology and pathology of the corresponding human diseases, as well as to genomic structure and evolutionary connections between related species. *W. bancrofti* PCR detection systems were developed to identify the parasite in mosquitoes.[15]

Polymerase chain reaction assays

Polymerase chain reaction is an in vitro method for amplifying selected nucleic acid (DNA or RNA) sequences by a pair of oligonucleotide primers. The method consists of repetitive cycles of DNA denaturation, primer annealing (hybridized to their complementary sequences), and extension by DNA polymerase. The product of each PCR cycle is complementary to and capable of binding primers, and so the amount of DNA synthesized is doubled in each successive cycle.

The development of PCR-based assays for the diagnosis of many parasitic and nonparasitic infections offers the possibility of improved sensitivity and specificity. These assays were recently developed for diagnosis of infection with the filarial parasite *O. volvulus* and *B. malayi* and are more sensitive than parasitologic diagnosis. The recent identification of a *W. bancrofti* repeated DNA sequence has enabled the development of a PCR-based assay capable of detecting *W. bancrofti* genomic DNA in human blood, urine, hydrocele fluid, and mosquito vectors such as *Aedes polynesiensis* and *Culex quinquifasciatus*.[16]

Recently, a nested PCR is introduced for improvement of specificity and sensitivity of detection, involving two steps of amplification. In the first step, initial pair

of primers is used to generate a long sequence that contains the target DNA sequence. Subsequently, a small amount of this product is used in a second step of amplification, which employs primers to the final target DNA. The efficiency of the second round of amplification is enhanced because of the more rapid and more complete denaturation of the first reaction product as compared with total genome. The application of nested PCR has been used for many infectious diseases including parasite, bacteria, and virus. The seminested PCR is the use of two rounds of PCR and by the replacement of one of the two primers from the first round of PCR with a different primer in the second round. This technique was applied for various kinds of detection such as monoclonal B-cell population in plastic-embedded bone marrow biopsies,[17] bacterial diseases,[18] and viral diseases.[19]

The polymerase chain reaction assay was developed for species-specific detection of filarial parasites in the blood samples from infected individuals (*B. malayi*, *W. bancrofti*, and *Loa loa*) and even in the mosquito vectors.[20,21] The 969 nucleotides of the moderately repetitive *W. bancrofti* sequence have been described as being species-specific. This sequence has a moderate copy number (450–700) and appears to be interspersed within the parasite genome. The use of this region in PCR to detect *W. bancrofti* has been documented.[3]

CONCLUSION

Sensitivity and specificity of the detection tools for *W. bancrofti* are crucial. At present, the PCR protocol has been introduced for diagnosis of parasitic infection in human blood because it is rapid, reproducible, and has a high specificity and sensitivity. It can diminish the time consumed in detection of parasites and requires relatively few amounts of blood, and thus it is not only suitable for the screening of parasitic infection in a large blood sample numbers, but also will be beneficial for elucidation of the possible vectors of the multiple infection. However, the cost of PCR is still high and it requires sophisticated equipment that may be not suitable for manipulation in the fieldwork.

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Yersinia spp.

Luther E. Lindler
Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland, U.S.A.

INTRODUCTION

The genus *Yersinia* includes a group of organisms with a wide range in pathogenic potential. On one end of the spectrum are saprophytic organisms that do not cause animal disease, while on the other end is the organism that causes plague. Recently, we have compared the genomic sequences of the agent that causes plague on an intraspecies level. This article will present a brief description of the pathogenic *Yersinia* in terms of disease and properties including virulence factors. The currently available genomic information will also be discussed with an emphasis on the emergence of plague as a unique bacterial disease.

PATHOGENESIS

There are three species of *Yersinia* that are pathogenic for humans. *Yersinia pestis* causes plague whereas *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* cause gastroenteritis. The principal features that differentiate these organisms are listed in Table 1. A good source for differential identification of these species can be found at http://vm.cfsan.fda.gov/~ebam/bam-8.html.

Plague

The characteristic that separates *Y. pestis* infection from disease by the other pathogenic *Yersinia* spp. is the former's ability to cause serious infection of healthy animals. The disease caused by *Y. pestis*, plague, can take three forms. Bubonic plague occurs following the bite of an infected flea. The inflammation is caused by the uncontrolled growth of the organism. The organism gains access to the circulatory system from the lymphatic system such that the liver becomes infected. *Y. pestis* reaches high numbers in the liver and spleen. The organism can be recovered from the blood shortly before death. Septicemic plague occurs when the organism can be recovered from blood in the absence of a bubo. Death occurs because of multiple organ failure as a consequence of lipopolysaccharide (LPS) toxicity. Discoloration of the individual, hence the name Black Death, occurs because of hemorrhage and cyanosis as a result of disseminated intravascular coagulation precipitated by LPS intoxication. The last form of the disease, pneumonic plague, is spread by aerosol. This infection results in a fulminate pneumonia followed by spread to deeper organs. Death can occur within 24 hr.

Gastrointestinal *Yersinia*

Both *Y. pseudotuberculosis* and *Y. enterocolitica* can cause foodborne illness, although infections caused by the latter organism are more common. A thorough description of the disease and causative bacteria can be found at http://vm.cfsan.fda.gov/~mow/chap5.html. Briefly, these organisms enter the host by the oral route where they gain access to the mesenteric lymph nodes. Generally, the infection does not progress beyond this point except in immunocompromised individuals. In rare cases, the organism can infect deeper tissues and cause bacteremia. More typically, infection results in fever, diarrhea, and abdominal pain. Finally, some strains of *Y. enterocolitica* produce a heat-stable enterotoxin (Yst) as indicated in Table 1. However, the role this toxin plays in virulence is unclear because stains lacking it are virulent.

PHYSIOLOGICAL DIFFERENCES

Major physiological differences between the pathogenic *Yersinia* are listed in Table 1. One of the most striking features is the inability of *Y. pestis* to produce typical gram-negative O-side chains on LPS at any growth temperature.[1] Both of the enteropathogenic *Yersinia* are also rough when grown at mammalian body temperature but produce O-side chains on their LPS when grown at room temperature. All of these organisms are serum-resistant in the absence of O-polysaccharide.

Another notable physiological difference between *Y. pestis* and the enteropathogenic *Yersinia* is motility. *Y. enterocolitica* and *Y. pseudotuberculosis* are motile

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Table 1 Comparison of *Y. pestis* properties with *Y. pseudotuberculosis* and *Y. enterocolitica*

Characteristic	Yps[a]	Yptb[a]	Ye[a]	Function
pYV	+	+	+	YOP production and antiphagocytosis
pPst	+	−	−	Plasminogen activator production
pFra	+	−	−	Murine toxin and capsule synthesis
HPI	+	+/−[b]	+	Yersiniabactin siderophore production
pgm loci	+	+[c]	−	Pigmentation on Congo red agar
pH 6 Antigen (*psa*)	+	+	+	Putative adhesin; designated *myf* in Ye
yst	−	−	+	Heat-stable enterotoxin
YadA	−	+	+	Adhesin
LPS O antigen	−	+	+	Cell structure/envelope
Invasin (Inv)	−	+	+	M cell translocation
Ail	+/−[d]	+	+	Host cell attachment and serum resistance
IS*1541* and IS*100*	++[e]	+	+[f]	Insertion sequence elements
Low calcium response (LCR)	++[g]	+	+	Regulation of YOPs and reduced growth at 37°C in the absence of added Ca^{2+}
Motility at 26°C	−	+	+	Chemotaxis
Rhamnose fermentation	−[g]	+	−	Sugar metabolism
Melibiose fermentation	−[g]	+	−	Sugar metabolism
Urease	−[g]	+	+	Nitrogen assimilation

[a]Yps: *Y. pestis*; Yptb: *Y. pseudotuberculosis*; Ye: *Y. enterocolitica*.
[b]Nonpathogenic strains are negative.
[c]The 68-kb *pgm* locus is present in *Y. pseudotuberculosis* but is usually silent.
[d]In some strains of *Y. pestis*, *ail* is interrupted by a copy of IS*285*, but in others, this locus is intact.[2]
[e]The ++ and + refers to the higher number of these insertion sequence elements or relative intensity of the LCR seen in *Y. pestis* vs. *Y. pseudotuberculosis* and *Y. enterocolitica*, respectively.
[f]*Y. enterocolitica* does not harbor copies of IS*100*.
[g]Metabolic capability known to undergo reversion.

when grown at room temperature but nonmotile when grown at 37°C.[1] In contrast, *Y. pestis* is nonmotile at all tested growth conditions. Strain CO92 appears to have an intact set of flagella genes,[2] whereas KIM encodes a mutant *flhD* flagella regulatory element.[3] It remains to be determined if there are environmental conditions that *Y. pestis* might become motile.

Generally, the growth of *Y. pestis* is more fastidious at 37°C than is growth of the enteropathogenic species. The growth rate of *Y. pestis* is not enhanced when cultivated at 37°C compared with 26°C, whereas the rate of cell division is enhanced for the enteropathogenic *Yersinia*. This fact is partially a result of a larger number of nutritional requirements by *Y. pestis* at the higher growth temperature as well as a more pronounced low calcium response (requirement for millimolar amounts of calcium for growth at 37°C) by this species.[4]

Subclassification

Y. pestis is generally classified into biotypes according to the ability to ferment glycerol and to produce nitrate.[5] The three biotypes are Antiqua (nitrate +, glycerol +), Mediaevalis (nitrate −, glycerol +), and Orientalis (nitrate +, glycerol −). Strains of *Y. pseudotuberculosis* and *Y. enterocolitica* are generally classified according to serotypes (http://vm.cfsan.fda.gov/~ebam/bam-8.html).

THE GENOME

Early studies analyzed the relationship between *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* as well as other enteric gram-negative organisms at the whole-cell DNA hybridization level.[6] More recent experiments using multilocus sequence typing and IS*100*-based fingerprinting have clearly shown that *Y. pestis* is a recently emerged clone of *Y. pseudotuberculosis* with remarkably little sequence variability.[7] *Y. pestis* itself is extremely monomorphic at the DNA sequence level.

Two complete genomes from two biotypes of *Y. pestis* have been completed. The first was the modern biotype Orientalis strain CO92[2] and the second was the Medievalis strain KIM.[3] CO92 is a recent pneumonic plague isolate that is highly virulent, while KIM is a

Table 2 Interesting characteristics of complete sequences *Y. pestis* strains CO92 and KIM[a]

Characteristic	Strain		Comments
	CO92	KIM	
Size (bp)	4,653,728	4,600,755	Larger size of CO92 mostly because of more copies of IS elements as well as one 11 kb and other numerous smaller insertions
rRNA operons	6	7	Loss of one operon possibly due to intragenomic recombination
Number of ORFs	4,012	4,198	Numbers include identified pseudogenes
Pseudogenes	149	54	KIM (Mediaevalis) is thought to be an older biotype[5]
IS*1541*	66	58	Includes complete and partial copies
IS*100*	44	35	Used in genotyping[7]
IS*285*	21	19	
IS*1661*	9	10	Includes complete and partial copies
Chromosomal type III secretion	Intact	Mutant	*sseJ* of KIM encodes a frameshift mutation in this *Salmonella* pathogenicity island 2-like system
Phage and large islands	21	15	These are regions with significantly different G+C ratios than the 47.6% found on the majority of the chromosome
Selenocysteine-associated genes	*selB* frameshift	Intact	Suppression of opal mutations in genes with frameshifts
Secretin near *tad* loci	Absent	Split	Involved in biofilm formation; mutated in KIM, and deleted in CO92
yapB	Intact	Truncated	Encodes autotransporter apparently nonfunctional in KIM
rscA	Intact	Truncated	Locus known to be involved in systemic dissemination in *Y. enterocolitica*[18]
Single-stranded prophage	Present	Absent	Phage element near *tca* and *tcc* insecticidal toxin genes

[a]Taken from Refs. [2,3]. Complete plasmid sequences can be found in Refs. [8–13].

laboratory strain isolated from a human pneumonic case over 4 years ago. Interesting characteristics to emerge from the genomic sequencing projects of these two *Y. pestis* strains are noted in Table 2.

MOLECULAR DIAGNOSTICS

Several molecular assays have been developed based on DNA sequences specific to the various pathogenic *Yersinia*. A few of these assays are listed in Table 3.

Y. pestis Assays

Classically, the identification of *Y. pestis* has been based on Gram's stain, biochemical reactions, and reactivity of the organism with anticapsular antibody. The first and most common molecular target for the identification of *Y. pestis* is the *pla* locus (Table 3). Although not reported in the literature, false positive reactions have been seen from environmental samples (Dr. Joan Gebhardt, Naval Medical Research Center, Silver Spring, MD, personal communication). In addition, atypical strains of *Y. pestis* that lack *pla* have been found to be virulent in animal models.[14] The second locus used for specific identification of *Y. pestis* is the capsular gene *caf1*. However, *caf1*-negative strains have been reported to be virulent.[15]

Y. pseudotuberculosis and *Y. enterocolitica* Assays

The high degree of homology between *Y. pestis* and *Y. pseudotuberculosis* at the DNA level makes development of molecular assays specific for the latter organism difficult. The 16S rRNA sequence is identical for these bacterial species. A 1 base pair difference was noted in the 23S rRNA gene, and an assay has been developed based on this polymorphism.[16]

Y. enterocolitica is a more important foodborne pathogen than is *Y. pseudotuberculosis*, and therefore more assays have been developed to detect this species (Table 3). The 16S rRNA sequence is significantly different from the other pathogenic *Yersinia* so that this makes a convenient higher copy number target. One

Table 3 Partial list of published molecular diagnostic tests for *Yersinia*

Organism	Target	Method	Sensitivity	Comments	Reference
Y. pestis	*pla*	Standard PCR	10 CFU/reaction	Early molecular diagnostic developed based on *Y. pestis* specific gene	[19]
Y. pestis	*pla*	Fluorogenic PCR	~850 copies/reaction	Detection limit based on purified DNA; specific detection obtained in fleas, blood, tracheal fluid, serum, and swabs	[20]
Y. pestis and *Y. pseudotuberculosis*	*pla*, *caf1*, *inv*, and *yopM*	Standard PCR	~80 CFU/reaction	Useful assays for differentiation of closely related species of this genus	[21]
Y. pestis	*pla* and *caf1*	Standard PCR	~10–50 CFU/reaction	PCR assays developed for two species-specific genes	[22]
Pathogenic *Yersinia*	rRNA	Fluorogenic probes, PCR and in situ hybridization	~100 CFU/mL	Suitable for identification of infected tissues	[16]
Y. enterocolitica	rRNA	Fluorogenic PCR	10 CFU/mL	Sensitive method suitable for detection in blood samples	[23]
Y. enterocolitica	rRNA and *ail*	Standard PCR	~1 CFU/reaction	Duplex PCR using two specific targets	[17]
Y. enterocolitica	*yst*	Fluorogenic PCR	100 CFU/reaction	Good for identification of some pathogenic strains of the organism	[24]

group has combined this target with the *ail* gene that is specific for only pathogenic strains of *Y. enterocolitica*.[17]

CONCLUSION

The genus *Yersinia* includes completely avirulent or low virulence strains of enteropathogenic species to probably the most deadly pathogen known to humankind (*Y. pestis*). Genomic examination of *Y. pestis* has revealed interesting characteristics of this highly virulent organism and shed some light on the emergence of this pathogen. Expansion of our available information pertaining to different strains of *Y. pestis* (including atypical strains) and comparison to highly related strains of *Y. pseudotuberculosis* will undoubtedly open other doors for our understanding of the emergence of deadly pathogens as well as lead to new understanding of host specificity.

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