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Pharmacogenomic Testing in Current Clinical Practice

Implementation in the Clinical Laboratory

Humana Press

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ISBN 978-1-60761-282-7 e-ISBN 978-1-60761-283-4
DOI 10.1007/978-1-60761-283-4
Springer New York Dordrecht Heidelberg London

Printed on acid-free paper

Humana Press is part of Springer Science+Business Media (www.springer.com)

Preface

The "personalization" of therapeutics promises to deliver a more efficacious and cost-effective approach towards treating patients with chronic diseases. For many medications, the pharmaceutical industry's objective of "one drug fits all individuals" has proven to be incorrect. Pharmacogenomics is an emerging discipline that will be essential for implementing personalized medicine. While there have been dozens of contemporary review articles on the science and specific application of pharmacogenomics to particular drugs, this book, "Pharmacogenomic Testing in Current Clinical Practice: Implementation in the Clinical Laboratory" is the first compilation of the tests currently in routine clinical use. In this rapidly changing field, we recognize that a text of this type will be quickly outdated. Nevertheless, we have assembled chapters from the key authorities and investigators who have conducted the essential clinical trials necessary to justify pharmacogenomic testing today.

This book is designed as a reference to clinical laboratory directors who are contemplating or assigned the task of establishing a pharmacogenomics laboratory, and pharmacologists and clinicians who must interpret results of testing. Each author has given a pharmacologic background on the target drug, the need for pharmacogenomic testing, and how results can be translated into clinical decisions. Where appropriate, case studies are given to illustrate typical clinical scenarios. An extensive bibliography is cited so that the reader can refer to the original studies.

In planning for this book, we made a distinction between pharmacogenomic tests for genes that alter drug metabolism, transport, and excretion from "companion diagnostic tests" that are performed on tumor tissues. Pharmacogenomic tests are conducted to determine germ line mutations using DNA extracted from blood or buccal swabs. Companion diagnostics are conducted to determine somatic mutations using DNA and RNA from tissue biopsies. The US Food and Drug Administration's Center for Drug Evaluation and Research have made a similar distinction. The CDER have made *recommendations* on the use of pharmacogenomic tests in conjunction with specific therapeutics such as warfarin or clopidogrel. For many companion tests, the FDA has coapproved a drug (e.g., trastusmab) *requiring* a positive test result (Her2/neu) for its labeled

therapeutic use. We have determined that companion diagnostic tests are outside of the scope of this book.

San Francisco, CA — Alan H.B. Wu
Chicago, IL — Kiang-Teck J. Yeo
May, 2010

Acknowledgements

We like to thank all of our contributors for making the development and publication of this book possible. We also wish to thank the various companies, journals who have granted us permission to use the figures in this book.
This book is dedicated to our wives, Pamela and Tet-Kin for their understanding and selfless support of our careers and giving us the time to work on this book.

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Part I
Basic Concepts

Chapter 1
Issues in Translation of Pharmacogenomics into Clinical Practice

Kiang-Teck J. Yeo, Nikolina Babic, and Alan H.B. Wu

Keywords Pharmacokinetics • Pharmacodynamics • Pharmacogenomics • Barriers • Clinical laboratory improvement act

1.1 Clinical Laboratory Tests

Clinical laboratory tests are commonly used for supporting the diagnosis and prognosis of a disease, monitoring the efficacy progress of therapeutic management decisions, and measuring the foreign toxins. Recent advances in genomics have resulted in the generation of new assay panels to support personalized drug therapy – this offers new opportunities for clinical laboratories to make important contributions to healthcare. Pharmacogenomics (PGx) is a recognized discipline within pharmacology that involves testing relevant human genes, whose products are involved with the inter-individual variability of a drug's pharmacokinetics, pharmacodynamics, and human leukocyte antigen (HLA) system profile. The effective use of PGx testing promises to improve the therapeutic efficacy of drugs while reducing the incidence and severity of adverse drug effects, and drive the optimum drug selection for therapy.

The desire to improve the therapeutic index of drugs stems from the knowledge that the efficacy of the most widely used FDA-approved drugs averages around 50% with a range of 25% for chemotherapeutics and up to 80% for analgesics [1], and incidence of serious adverse drug reactions (ADRs) estimated to be two million per year in the US and cause 100,000 deaths [2]. In a prospective study of hospital admissions caused by ADRs at two large UK hospitals, Pirmohamed et al. found a prevalence of 6.5% with a projected annual cost of $847 million [3].

Parts of this chapter was published in Wu et al. [12] with permission from Future Medicine Ltd., http://www.futuremedicine.com/loi/pgs.

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_1,

The Institute of Medicine was instructed by the US Congress to perform a comprehensive study of drug safety and to find means to prevent or reduce medication errors [4]. The degree by which ADRs can be reduced through PGx testing is currently unknown. Errors associated with incorrect scripting or dispensing of prescriptions, or by patients not following a physician's dosing recommendations will not be corrected by PGx testing. However, adverse events due to an incorrect prescribed dose or the use of a therapeutic agent that is not optimal for a particular individual may be minimized by PGx testing. For example, the implementation of pharmacogenomic testing for abacavir [5] or carbamazepine [6] promises to greatly reduce the incidence of potentially life-threatening, delayed cutaneous hypersensitivity reactions.

There are major ongoing research efforts conducted by academia and the pharmaceutical industry in discovering new gene associations that better predict individual drug handling and the effect (therapeutic and toxic) of existing and novel therapeutics. The US Food and Drug Administration (FDA), in its commitment to advancing personalized medicine, is taking a leadership role in drafting guidance documentation for companion diagnostics. In some cases, the need for a companion laboratory test (i.e., the need for a certain test result before a drug is given) will be seen as an impediment to the implementation of that drug, as the pharmaceutical industry will naturally opt to develop safer and more efficacious alternatives that do not require companion testing. In other cases, PGx tests may well help salvage drugs that might not normally receive FDA approval or perhaps even those that have been removed from the market, if the toxicity profile can be minimized with targeted testing (Table 1.1). In the end, perhaps only a relatively small cadre of current and future drugs will require PGx testing just as today, only a few drugs require regular therapeutic drug monitoring. Therefore, important studies and decisions lie

Table 1.1 Examples of drugs undergoing pharmacogenomics rescue

Drug/company	Class	Therapeutic indications	Goal	Potential PGx biomarker	FDA status
Bucindolol/ Arca Biopharma	β Blocker	Heart failure	Efficacy	β_1-adrenergic receptor polymorphism	Rejected in June 2009, resubmission in progress
Lumiracoxib/ Novartis	COX-2 analgesics	Arthritis	Safety	MHC Class II genes	Never approved in US, pulled from foreign markets in 2007
Tremelimumab/ Pfizer	Cancer	Melanoma	Safety	Genome studies in progress	Conducting a second drug trial after finding a biomarker that identifies patients who benefit from the drug

Adapted from http://personalizedmedicinecoalition.org/membership/newsletter/PMC-Newsletter-Spring'10.pdf Accessed 5/4/2010

ahead with regard to which drugs are most appropriate for such testing, how testing is to be conducted and reimbursed, how PGx tests results are reported and interpreted, and demonstrating the medical and/or economic benefits of testing.

1.2 Terminology and Definitions

Pharmacogenetics is not a new discipline, and the importance of inheritance in the role of drug response was known since the 1950s – as demonstrated by genetic variation of pseudocholinesterase on succinylcholine effects [7] and N-acetyl transferase 2 (NAT2) polymorphisms on isoniazid [8] metabolism as reviewed by Weinshilboum [9]. However, most PGx studies in the past employed phenotypic markers of genetic variation (e.g., drug/metabolite concentrations in blood or urine, direct enzyme activities measurements), so advances in this field were somewhat limited and slow. With the completion of the Human Genome Project in 2001 [10], there was a "re-birth" and accelerated discovery in the field of pharmacogenetics and pharmacogenomics, since we now have a better and more comprehensive genetic knowledge base and new molecular technologies to study precise genotypic variations and correlate them to phenotype. Vogel [11] introduced the term "pharmacogenetics," which can be defined as the study of the role of a small number of genetic variations that are relevant to a drug's disposition or effect. Pharmacogenomics involves the study of a larger collection of genomic factors that contribute to the individual variability of drug responses. This may include genes that regulate phase I oxidative drug metabolism (especially the cytochrome P450 family of enzymes), phase II drug conjugation enzymes (e.g., glucuronosyltransferases and N-acetyltransferases), drug transporter proteins (e.g., organic anion transporters), and drug targets-enzymes or receptors [9]. In the past, the variations observed for most drugs in terms of utilization and adverse events were limited to only a few SNPs within a gene (monogenic traits) and "pharmacogenetics" may be the preferred term. However, for warfarin and clopidogrel, multiple SNPs within several genes (polygenic traits) have been determined to influence the drug's therapeutic effects, and therefore the term "pharmacogenomics" may be preferred. Despite these definitions, these terms are often used interchangeably and the acronym "PGx" is used commonly.

1.3 Current Barriers

The term "personalized medicine" was coined in the late 1990s and relates to "providing the right dose to the right person at the right time" using genetic markers of drug metabolism, transport, and receptor interactions. While this is a very attractive concept that was widely covered in the lay press, the implementation of pharmacogenomics at the bedside or in the physicians' office has been slow. The barriers to wider adoption and implementation include (a) lack of education and

understanding by prescribing physicians regarding available pharmacogenetic tests; (b) lack of consensus guidelines on interpretation and the use of pharmacogenetic results; (c) paucity of randomized controlled trials demonstrating the clinical utility of such testing; (d) lack of studies demonstrating the cost-effectiveness of using pharmacogenetic markers; (e) lack of specific reimbursement codes; (f) lack of commercial assays; (g) lack of standardization in a given PGx panel, reporting, and interpretation of results; and (h) tension between patient's awareness of media/web-based pharmacogenetic testing and physician's readiness to adopt patient-driven testing (reviewed extensively by Wu et al. [12]). However, there are additional current challenges like the Center for Medicaid and Medicare Services' (CMS) recent ruling of not supporting reimbursement of warfarin sensitivity genotyping due to a "lack of current available evidence" for improved health outcomes for Medicare beneficiaries [13], which has resulted in a temporary setback in adoption.

1.4 Potential Solutions

1.4.1 FDA Initiatives

Since physicians and patients find no value in the genotyping per se, PGx testing results must be put into the context of "actionable" decisions. The FDA continues to lead the efforts to translate recent advances in PGx into clinical practice. In this respect, the FDA has approved revised labeling requirements for selected drugs where polymorphisms have been linked to either a reduction in drug efficacy or an increased incidence of adverse events (Table 1.2). This drug re-labeling initiative now raises a medico-legal implication in cases where an ADR has occurred. For example, if a patient suffers an adverse reaction that is directly linked to the use of a drug, and the prescribing physician did not know that the individual was predisposed to that adverse event as the result of a genetic polymorphism, that physician may find it difficult to defend a medical malpractice lawsuit, given that a warning for that event was present in the drug's label [14]. These recent drug re-labeling recommendations are likely to drive the need to utilize PGx testing for optimal prescribing to a "defensive practice of medicine." Irrespective of the driver, when implemented, proper utilization of PGx promises to incrementally, and in some cases, substantially improve the safe use therapeutics.

1.4.2 Clinical Trials

Because of the ongoing debate about the clinical usefulness of warfarin genotyping – a "poster child" case for PGx – a recent multi-centered, double-blind, randomized control trial – *the Clarification of Optimal Anticoagulation through Genetics* (COAG) was launched. This study seeks to evaluate the efficacy in the use of clinical and

Table 1.2 US Food and Drug Administration relabeling initiative

Drug	Enzyme	Goal[a]	Year	Status
6-MP[b]	TPMT[c]	Safety	2003	Completed
Azathioprine	TPMT	Safety	2003	Completed
Atomoxetine	CYP[d] 2D6	Safety	2004	Completed
Irinotecan	UGT1A1[e]	Safety	2004	Completed
Warfarin	CYP 2C9, VKORC1[f]	Safety	2007	Completed
Abacavir	HLA-B*5701[g]	Safety	2007	Completed[h]
Allopurinol	HLA-B*5801	Safety	2007	Completed
Carbamazepine	HLA-B*1502	Safety	2007	Completed[i]
Phenytoin and Fosphenytoin	HLA-B*1502	Safety	2008	Completed[i]
Clopidogrel	CYP 2C19	Efficacy	2009	Completed
Tamoxifen	CYP 2D6	Efficacy	2006	Pending

[a]"Safety" goals indicate a potential for reducing drug-induced adverse events. "Efficacy" goals refer to the potential for improving the effectiveness of the drug

[b]6-Mercaptopurine

[c]Thiopurine methyltransferase

[d]Cytochrome

[e]UDP-glucuronosyltransferase

[f]Vitamin K epoxide reductase complex 1

[g]Human lymphocyte antigen

[h]Department of Health and Human Services recommendation made to genotype Caucasian patients prior to Abacavir therapy

[i]FDA recommendation made to genotype Asian patients prior to carbamzepine or phenytoin therapy

genetic information to guide warfarin therapy initiation and improve anticoagulation control for patients. The trial is sponsored by the National Heart, Lung, and Blood Institute and will recruit 1,200 patients initiated on warfarin among 12 different medical centers in the US and is estimated to take up to 4 years to complete [15]. At the University of Chicago, we are currently performing *The Clinical and Economic Implications of Genetic Testing for Warfarin Management Trial* funded by the *Agency for Healthcare Research and Quality*. The aims of this study are (a) to set up a genetic registry of a racially diverse set of patients undergoing warfarin therapy; (b) to determine the efficacy, costs and cost-effectiveness of existing pharmacogenetic algorithms for the management of warfarin therapy among hospitalized patients; and (c) to develop a clinical pharmacogenetic algorithm for the management of warfarin therapy among hospitalized African American patients [16].

With regard to another widely used antithrombotic – the antiplatelet drug, clopidogrel, two recent large clinical studies showed that individuals carrying *CYP2C19* loss-of-function alleles (e.g., *2,*3,*4, or *5) had significantly lower concentrations of the active drug metabolite, reduced platelet inhibition, resulting in threefold increase in risk of stent restenosis, and a 3.6-fold increase in rate of cardiovascular events [17, 18]. Because of these accumulating evidences, the FDA has recently issued a black-box warning to health-care professionals to consider *CYP 2C19* genotyping so as to identify patients who are poor metabolizers of clopidogrel; this allows providers to act on this information to consider alternative antiplatelet

medications or alternative dosing strategies for clopidogrel [19]. These and several ongoing clinical trials when completed may contribute significantly to the accumulating evidence that relevant PGx testing can lead to effective tailoring of target drug therapies to avoid ADRs, while maximizing efficacy.

1.4.3 Cost-Effectiveness Considerations

A key solution for uptake of PGx is to have studies demonstrating the cost-effectiveness of such testing. Detailed description of this aspect is covered in Chap. 3. A recent collaborative study by Mayo and Medco, involving 900 patients on warfarin, showed that hospitalization rates decreased by 30% when genotyping information was used in warfarin dosing versus the usual clinical dosing [20]. This should translate to cost-savings that would easily justify the costs of pharmacogenotyping, which continues to decrease with technological advance.

1.4.4 PGx Algorithms, Reports, and Interpretations

Complex genotyping results need to be readily translated into actionable decisions by providers to encourage wider use of testing. For example, warfarin sensitivity genotyping results need to be integrated and made somewhat user-friendly by using a PGx report as shown in Fig. 1.1. To further make it actionable, one needs to consolidate these results into a therapeutic output, which in this case, is the calculated dose based on the measured polymorphisms and relevant clinical factors. Thus, it would be important to have reliable and validated algorithms that can be employed for this purpose. With regard to warfarin, recently the International Warfarin Pharmacogenetics Consortium developed a warfarin dosing algorithm based on genetic and clinical information and showed that in a validation cohort of 1,009 patients, the combined algorithm identified a larger fraction of patients requiring extreme doses (≥49 mg/week and ≤21 mg/week) than the clinical algorithm alone [21]. A commonly used web-based warfarin dosing algorithm that employs clinical, co-medications, and genetic polymorphisms information (*CYP 2C9*, *CYP 4F2*, and *VKORC1*) to calculate the appropriate warfarin dose can be found at the following URL: http://warfarindosing.org/Source/InitialDose.aspx (accessed May 15, 2010).

1.4.5 Laboratory Aspects and Guidelines

All clinical laboratory tests, including PGx tests, are regulated by the Clinical Laboratory Improvement Act (CLIA) of 1988 and have to be performed in certified laboratories. CLIA certification can be obtained by a laboratory getting accreditation from an organization like College of American Pathologists, which

Patient Name	**Medical Record Number**	**Age**	**Sex**
Ordering Physician		**DOB**	
Attending Physician		**Report Notes**	
Collected	**Printed**		

Test	**Allele**	**Result**	**Interpretation**	**Estimated Warfarin Dose***
CYP450 2C9	430 C>T (*2) 1075 A>C (*3)	C/**T** A/**C**	Reduced warfarin metabolism expected	Loading dose (mg/d):_____ Therapeutic dose (mg/d):_____
				Reviewed by:
VKORC1	-1639 G>A	G/A	Normal warfarin sensitivity expected	_____ Pharm. D.

*Estimated warfarin dose is calculated and reviewed by the UCMC Clinical Pharmacist.
The dose is calculated using the algorithm published on http://www.warfarindosing.org website.

Fig. 1.1 Sample report for warfarin sensitivity genotyping

has been given deemed status by the Centers for Medicare and Medicaid Services. Documentation of personnel qualifications and training, test proficiency, satisfactory quality control, and validation of assays are critical components for accreditation. PGx assays are classified as *high-complexity* tests since all of them require extraction of DNA from blood (or oral fluids), and for many a requirement of amplification by polymerase chain reaction followed by detection on beads, silicon chips or solution-based systems. Before a PGx test can be released for clinical use, each laboratory must demonstrate genotyping accuracy by validating against a predicate assay cleared by the FDA or confirm with some definitive techniques such as bi-directional sequencing method. For less common variants, the existence of validated DNA sources containing such polymorphisms will greatly facilitate the implementation of these methods, increasing the accessibility of such technologies in the clinical laboratories. In addition, the clinical laboratory

Table 1.3 Current FDA-cleared pharmacogenomics assays

Test	Manufacturer	Testing platform	Approval date	Target drug
CYP[a] 2D6	Roche	Amplichip	1/2005	Not specified
CYP 2C19	Roche	Amplichip	1/2005	Not specified
UGT1A1[b]	Hologic	Invader	9/2005	Irinotecan
CYP2C9/VKORC1[c] (6484 allele)	Nanosphere	Verigene	9/2007	Warfarin
CYP2C9/VKORC1 (3673 allele)	Autogenomics	Infiniti	1/2008	Warfarin
CYP2C9/VKORC1 (6484 allele)	Paragon	RT-PCR	5/2008	Warfarin
CYP2C9/VKORC1 (3673 allele)	GenMark	eSensor	7/2008	Warfarin

[a]cytochrome
[b]UDP-glucuronosyltransferase
[c]Vitamin K epoxide reductase complex 1

must also be ready to provide reliable PGx results within the required turnaround time for efficacious use of the information. Inter-disciplinary collaboration between the laboratory, clinical pharmacologist/pharmacist, and the provider is essential to insure proper interpretations and integration of PGx results for timely decision-making regarding dosing or alternative drug regimen. There are now several commercial PGx multiplex assay platforms, several of which are FDA-cleared for *CYP 2D6*, *CYP 2C19*, *UGT1A1*, *CYP 2C9/VKORC1* (Table 1.3). However more extensive PGx panels for some like *CYP 2C19, CYP 2C9/VKORC1* are usually available as *research use only* or *investigational use only* products – in this case individual laboratory may have to further optimize the assay and validate them independently, much like laboratory developed tests [12, 22].

The National Academy of Clinical Biochemistry has recently published the 2010 PGx guidelines that specify the requirements for test validation, quality control, and proficiency testing [23]. Furthermore, the GeT-RM collaborative project sponsored by Center of Disease Control has recently validated a series of Coriell DNA from human cell lines that contained relevant PGx variants that can serve as a ready source of quality control materials (Table 1.4) [24]. Collectively, these multi-center collaborative efforts will go a long way in facilitating the uptake of PGx by the clinical laboratory, and by extension the clinical community, by making it easy to order, interpret, and personalize drug therapy according to the relevant genetic and clinical variables of an individual patient.

1.4.6 Reimbursements

At present, there are no specific American Medical Association Current Procedural Terminology (CPT®) reimbursement codes available for any PGx test. Currently, many laboratories bill according to the individual procedures required to produce a

Table 1.4 Genetic testing reference material coordination program[a]

Coriell cell line number	CYP2C19	CYP2C9	VKORC1 c.-1639G>A	CYP2D6
GM17289	*2/*2	*1/*1	AA	*2/*4
GM17203	(*1/*2) *2/*17	*1/*1	AA	*4/*35
GM17272	(*1/*1) *17/*17	*1/*1	AA	*4/*10
GM17246	(*1/*8) *8/*17	*1/*2	GA	*4/*35
GM17115	*1/*1	(*1/*1) *9/*9	GG	*1/*2
GM10005	(1/*1) *1/*17	(*1/*1) *1/*9	GG	*17/*29
GM07439	(*2/*2) *2/*10	(*1/*1) *1/*9	GG	*4xN/*41
GM 12244	*1/*1	*2/*3	GG	*35/*41
GM17052	*1/*3	*1/*1	AA	*1/*1
GM02016	*1/*2	*1/*1	GG	*2xN/*17
GM17296	(*1/*1) *17/*17	*1/*1	GA	*1/*9

[a]Pratt et al. Partial data from manuscript submitted to *Journal of Molecular Diagnostics* [24]

test result (DNA extraction, amplification, analysis of the DNA by probes, mutation scanning, and interpretation). Each of the specific procedure codes can be multiplied by the number of alleles interrogated, e.g., CPT 83914 (for allele-specific primer extension) × 6. However, the lack of specific reimbursement CPT codes is a real deterrent for clinical laboratory to implement such PGx tests as hospitals are concerned about the uncertainties surrounding the reimbursement of these new tests. The recent passage of the landmark healthcare reform, which contained principles of personalized medicine, will hopefully speed up the adoption of pharmacogenomics by fostering better exchanges between the various governmental agencies (CMS, FDA, NIH) to address barriers and offer solutions to translate these discoveries to clinical practice.

Various efforts are underway by nongovernment, nonprofit organization like the Personalized Medicine Coalition (PMC) to engage CMS to offer guidance on the scientific evidence needed for CMS to approve reimbursement for a given genetic test. PMC is a broad coalition of academic, industrial, patient, provider, and payer communities with a mission to advance the understanding and adoption of personalized medicine for the benefit of the patient [25].

1.4.7 Education, Training, and Pharmacogenomics Resources

A very important solution to the adoption of PGx testing is the education of all health-care professionals regarding the basic principles of pharmacogenomics and its emerging role in Personalized Medicine. There are many recent reviews on the various aspects of pharmacogenomics. Weinshilboum et al. described the evolution of pharmacogenomics as a science and summarized several of the scientific advances that are keys to this field [9]. Roden et al. assembled a glossary of terms and detailed the approaches toward discovery of new gene associations [26]. Because drug metabolism is a critical aspect of pharmacogenomics, Wilkinson

reviewed the CYP-450 system and how polymorphisms interact with the pharmacokinetics of therapeutic drugs [27]. Bolonna et al. examined the potential for pharmacogenomic testing focusing on psychiatric drugs [28], Yong et al. focused on the cancer drugs [29], and Carlquist on cardiovascular disease drugs [30]. For research, there are tools and consortia available to assist in the discovery of association of drug response with genetic variation. The Pharmacogenetics Research Network is a collaborative group of investigators who have been organized into five major clinical areas: asthma, depression, cardiovascular disease, drug addiction, and cancer [31]. For the rapid dissemination of data, there is also a publicly available knowledge base that collates information describing the relationship between drugs, diseases, and genetic variation; this is the Pharmacogenomics Knowledge Base [32]. Medical and graduate schools are beginning to incorporate pharmacogenomics in their pharmacology curricula. Even more encouraging is the recent availability of a web-based tool, called DNA Twist, to educate middle and high school students about the world of pharmacogenomics [33, 34]. These educational efforts should produce a new generation of students who are facile with the use of genomics, proteomics, and metabolomics (and other future "-omics") in future applications of personalized medicine.

1.5 Conclusion

Personalized medicine is currently attracting a lot of attention in the media, and the general public is becoming more aware of the promises of the genomic revolution. Web-based *direct-to-consumer DNA testing* services abound that promises a wealth of genetic information (costing less than $1,000) and are now widely marketed to the average consumer as tools of *empowering personal choices about your health and well-being*. Pharmacogenomics is one aspect of personalized medicine that will continue to gain ground and make inroads into clinical practice. Ultimately, a large part for the success of wider adoption of pharmacogenomics will depend on a multidisciplinary team approach. The stakeholders in this process include departments of clinical pharmacology/pharmacy, medical genetics, laboratory medicine, and researchers in the field of genetics and pharmacogenomics. Medical specialties must also be invested in this where appropriate, e.g., oncology, cardiology, and psychiatry. A major component of a successful program will be the education of physicians, who use the medications for which pharmacogenomics data will be relevant, and the incorporation of clinical pharmacogenomics in the physician training curricula both during and after medical school.

Due to rapid advances in genomics, proteomics, and metabolomics, we predict that there will be an increasing need for a clinical pharmacogenomics service – the clinical laboratory should take the lead in making these tests available when sufficient evidence is established for clinical usefulness. Most importantly, these complex results need to be made "user-friendly" to the clinical community (e.g., via improved information technology) before widespread adoption can happen.

With this revolution underway, new opportunities are now available for medical students, graduate students, medical technologists, residents, and fellows in pathology and laboratory medicine to become specialist in this area of personalized medicine.

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Chapter 2
Molecular Diagnostic Methods in Pharmacogenomics

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Keywords Real-time polymerase chain reaction • Pyrosequencing • Mass spectrometry • Microarray • Allele-specific primer extension

2.1 Introduction

Rapid advances in pharmacogenomics research have facilitated the transfer of pharmacogenomics testing into clinical laboratories. In the past several years, the US Food and Drug Administration (FDA) has begun to recognize the importance of genetic information and has required advisories on drug labels seeking to inform physicians and patients about the availability of genetic tests to guide drug dosing and prescriptions [1]. This has furthered the desire to adopt this testing into clinical laboratories. At this time, FDA has required manufacturers of 11 drugs to modify their labels to include information on pharmacogenomics testing [2]. With the advances in pharmacogenomics and information technology fields, a new concept of genomic testing has also emerged on the market – a concept of direct-to-consumer DNA testing. Web-based companies are in the market with the goal of enabling a patient to take control of his/her own genetic testing and results. One such company, 23 & Me (www.23andme.com, accessed 04/08/10), offers a variety of genetic tests, including pharmacogenomic tests for warfarin sensitivity, 5-FU toxicity, clopidogrel efficacy, and abacavir hypersenstivity. Traditional clinical laboratories are also expanding their repertoire of pharmacogenomic testing. Therefore, it is clear that there is an increased need for rapid, reliable, and cost-effective genotyping methodologies amenable to easy adoption by the clinical laboratory community. At present, the most frequently genotyped targets are the cytochrome P450 (CYP 450) super-family and phase II drug-metabolizing enzymes such as uridine diphos

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_2,

Table 2.1 Different SNP analysis approaches

Method	Mechanism	PCR requirement	Detection
Hybridization			
Microarray	Solid phase-based	Yes	Fluorescence; electrochemical signal
Verigene®	Signal amplification technology	No	End point or real-time fluorescence detection
Invader®			
Real-time PCR	Hybridization probes TaqMan® probes Molecular beacons FRET probes	Yes	Fluorescence
Sequencing			
Sequencing	Chain terminating sequencing (Sanger method)	Yes	Capillary electrophoresis fluorescence
Pyrosequencing	Sequencing by synthesis	Yes	Light
Mass spectrometry	Mass differentiation of single or multiple analytes	Yes	Mass spectrometry

Adapted from Wang et al. [3]

phate-glucyronosyltransferase 1A1 (UGT1A1), thiopurine *S*-methyltransferase (TPMT), and *N*-acetyltransferase (NAT).

A number of commercial platforms are now available for pharmacogenomics testing (Table 2.1). As shown in Table 2.1, most approaches require PCR amplification of DNA template with different single nucleotide polymorphism (SNP) detection and identification strategies. The exceptions are Verigene® (Nanosphere, Northbrook, IL) and Invader® (Hologic, Madison, WI) technologies, which are based on the principle of signal amplification. The genetic variants can be detected using hybridization, real-time PCR, sequencing, or mass spectrometry methods that involve extension of specific primers designed to detect SNPs of interest. Hybridization is generally utilized in microarray platforms, and it involves capture oligonucleotides immobilized on a solid surface that hybridize to the labeled primer extension products. In real-time PCR, as the reaction progresses, the fluorescence is generated and detected in real time. Sequencing utilizes labeled chain-terminating nucleotides that are incorporated during primer extension. Labeled fragments are then separated by electrophoresis and identified based on size. In mass spectrometry, primer extension products are detected based on differential mass-to-charge ratios between wild type and variant strands. The most frequently used approaches currently employed are microarrays and real-time PCR.

This chapter provides an overview of different platforms and technologies available, specifically focusing on their applications to pharmacogenomics testing. Virtually all methodologies described here have other applications in genetic and microbiology testing.

2.2 DNA Extraction and Quantitation

DNA quality is critical for successful genotyping regardless of the platform or methodology used. The classical method of DNA extraction involves cell lysis, followed by organic (usually chloroform/isoamyl alcohol) extraction and ethanol precipitation. Although this method is reliable, it is also time-consuming and cumbersome. Many satisfactory DNA isolation kits are available on the market today. Some examples of commercially available kits include QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA), based on solid-phase extraction technology, MagMax™ kit (Applied Biosystems, Foster City, CA) and Maxwell® 16 system (Promega Corporation, Madison, WI), which employ magnetic bead technology to extract DNA from the sample. These methods are straight-forward and can be used either for manual or automated DNA extraction. Manufacturers generally provide specific, easy to follow protocols for each kit.

The most common method for DNA quantitation is absorbance measurement at 260 nm, since DNA absorbs UV light at this wavelength. For a pure double-stranded (ds)DNA, an absorbance of 1.0 at 260 nm corresponds to a DNA concentration of 50 μg/ml. Although this method is sensitive to interferences from nucleotides, single-stranded (ss)DNA, RNA, and proteins, the amount of these potential interferents is minimal in most DNA preparation methods and does not, therefore, present a significant problem.

DNA purity is assessed by determining the ratio of DNA absorbance (260 nm) to protein absorbance (280 nm). DNA with A260/280 ratios between 1.7 and 1.9 should be sufficiently pure and free of protein interferences.

A more specific method for DNA quantification is the picogreen assay. Picogreen® is a fluorescent intercalator dye that is specific for dsDNA and will fluoresce when bound to DNA. The system is capable of detecting dsDNA at concentrations of 25 pg/ml. The picogreen assay is more labor-intensive than the UV spectrometric method; however, the enhanced sensitivity is usually not required for the majority of DNA preparations from whole blood.

2.3 Microarray-Based Genotyping Platforms

Genomic microarrays utilize solid surfaces, such as a small chip, where a large number of probes are arrayed, allowing for many SNPs to be interrogated simultaneously. Table 2.2 shows microarray-based platforms currently available on the market. Virtually, all of the assays offered on these platforms require multiplex PCR amplification prior to genotyping. One exception is Verigene, which works on a basis of signal amplification and does not require a PCR step. At present, only a handful of genotyping tests are available for in vitro diagnostic (IVD) use. Most of the IVD tests on the market today are for warfarin sensitivity testing, a result of the recent FDA decision to approve updated labeling for Coumadin, the brand name of warfarin (http://packageinserts.bms.com/pi/pi_coumadin.pdf., accessed 04/07/10), requiring the inclusion of genotyping considerations regarding patient's response to the drug.

Table 2.2 Genotyping platforms and respective pharmacogenomics tests

Platform	Manufacturer	Test	Target drug
Amplichip™	Roche (Plesanton, CA)	CYP2D6 (IVD)	Various drugs
		CYP2C19 (IVD)	Various drugs
eSensor®	GenMark (Carlsbad, CA)	CYP2C9/VKORC1 (IVD)	Warfarin
		CYP 2C19 (RUO)	Clopidogrel
INFINITI™	AutoGenomics (Carlsbad, CA)	CYP2C9/VKORC1 (IVD)	Warfarin
		CYP2D6, 2C19, 3A4, 3A5	Various drugs
		UGT1A1	Irinotecan
		MDR-1	Various
		5-FU	5-Fluorouracil
		NAT-2	Various drugs
Tag-It®	Luminex (Austin, TX)	Open system	Various
Verigene®	Nanosphere (Northbrook, IL)	CYP2C9/VKORC1 (IVD)	Warfarin
Real-time PCR	ParagonDx (Morisville, NC)	CYP2C9/VKORC1 (IVD)	Warfarin
eQ-PCR Light Cycler	TrimGen (Sparks, MD)	CYP2C9/VKORC1 (IVD)	Warfarin
Invader®	Hologic (Madison, WI)	UGT1A1 (IVD)	Irinotecan

IVD in vitro diagnostics; *RUO* research use only

The remainder of this section describes different methodologies utilized by the current microarray genotyping platforms.

2.3.1 Affymetrix® Technology

The Affymetrix technology is utilized in the manufacture of DNA chips for genotyping in the Roche CYP450 system (AmpliChip® 450 Test, shown in Fig. 2.1) that provides detection of *CYP2D6* and *CYP2C19* gene variants. This test is FDA-cleared for IVD use and is currently offered by several major reference laboratories. Affimetrix chips are oligonucleotide arrays that contain hundreds of thousands of oligonucleotide probes anchored on glass support at very high density. The basis of this technology is hybridization of the target DNA to the microarray and generation of fluorescent signal. A workstation provided by Affymetrix is necessary to collect data and perform the analysis.

2.3.2 eSensor® Technology

GenMark eSensor utilizes electrochemical detection technology (Fig. 2.2). The single-stranded capture probes are immobilized on the working electrode surface. Following PCR amplification, the target DNA is digested with exonuclease into sin-

Fig. 2.1 Roche AmpliChip®. The amplified target DNA is fluorescently labeled, fragmented, and added to the chip where a laser scans and analyzes the intensity of fluorescence. (Courtesy of Roche Diagnostics)

gle strands and combined with single-stranded signal and capture probes. Signal probes contain the polymorphism of interest and an electrochemically active ferrocene dye unique for that SNP. Once all the reagents are combined, the target amplicon hybridizes simultaneously to signal and capture probes, generating current. If there is no match between the signal probe and the DNA target, no current will be generated. Of the systems currently available, this is the only true random-access analyzer, where each sample can be loaded and unloaded independently. Upon completion of PCR and exonuclease digestion steps (~3.5 h), the analysis time on the instrument is 30 min [4]. Individual analyzers can run 1–8 samples and can be configured in such a way that a maximum of 24 samples could be analyzed at once. At present, the only commercially available IVD test is warfarin sensitivity genotyping test.

2.3.3 INFINITI™ and Luminex xTAG® Allele-Specific Primer Extension

The allele-specific primer extension (ASPE) method is utilized by the INFINITI (AutoGenomics Inc., Carlsbad, CA) and Luminex xTAG (Luminex Corporation, Austin, TX) platforms. The ASPE method involves two phases following PCR: enzymatically driven sequence specific primer extension and a capture on a solid surface, such as chip (INFINITI) or bead (Luminex), for detection. Figure 2.3 illustrates this methodology, using the INFINITI assay as an example. In general, an amplified PCR

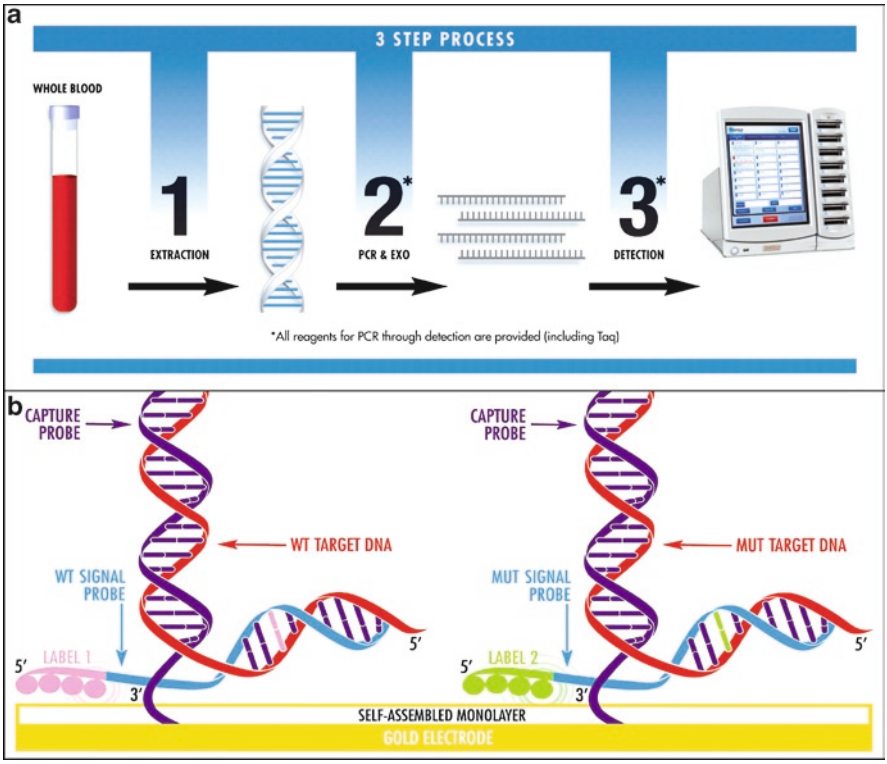

Fig. 2.2 GenMark eSensor™ Warfarin Sensitivity assay genotyping principle. (**a**) Three-step process involved in genotyping on eSensor platform: DNA is extracted from the whole blood, amplified by PCR, digested into single strands, and loaded onto a cartridge provided by manufacturer. Each cartridge contains specific capture probes on particular electrodes. Cartridges are then read by fully automated analyzer. (**b**) A close-up view of one electrode within a cartridge. The sample is heterozygous in this example, so both the wild type and mutant target molecules will hybridize to the capture probe and to the appropriate signal probe. This means that two different types of ferrocene are present on the electrodes surface. Thus, when the instrument checks for hybridization complexes, it will read current coming from both types and interpret the sample as heterozygous for this mutation. (Courtesy of GenMark)

fragment serves as a template for ASPE reaction. ASPE occurs on board the instrument where PCR amplified genomic DNA is combined with the allele-specific primers. These primers are then extended by the activity of polymerases. Extension occurs only if the 3′ end of the allele-specific primer is bound to the homologous sequence on the amplicon. A short segment at the 5′ end of the allele-specific primer remains free. This free 5′ segment is used to hybridize ASPE product to a complementary oligonucleotide sequence (called Zipcode in the INIFINITY methodology), that is immobilized on a chip or a bead. The signal is generated and detected using the fluorescent labels. In the INFINITI assay, the fluorescent label is directly incorporated into a growing primer strand as fluorescently labeled deoxycytidine triphosphate (dCTP). In the case of xMap Luminex assay, biotinylated dCTP is incorporated into

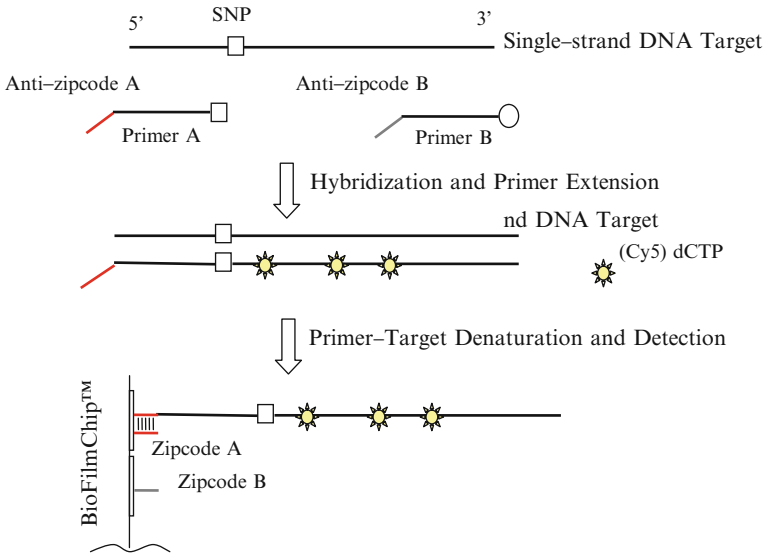

Fig. 2.3 Automated micro array method: INFINITI™ analyzer method combines allele specific primer extension (ASPE) and fluorescence detection. The primer extension occurs only when a matching primer hybridizes to the target sequence. If there is a mismatch (e.g., Primer B), no extension occurs. During the ASPE step, fluorescently labeled deoxycytidine triphosphate, (Cy5) dCTP, is incorporated into the growing strand. Unique, complementary nonhuman oligonucleotide sequences (zipcode and antizipcode) are used to capture fluorescent signal. Figure adapted from INFINITI™ Analyzer – Customer Training manual and used with permission.

a growing primer strand. The fluorescent dye is conjugated to streptavidin, which then binds to biotinylated dCTP and the hybridized beads are read by the Luminex system. Luminex xMAP is a fluidic system based on the principles of flow cytometry where the stream of suspended microspheres passes through the detection chamber single file. Once in detection chamber, each microparticle is excited by two different lasers and the fluorescence is detected.

Presently, the INFINITI platform offers the most comprehensive test menu of all the automated microarray platforms available (Table 2.2); however, the only IVD test panel currently available on this platform is warfarin sensitivity genotyping panel that includes *CYP2C9* *2 (c.681G>A), *3 (c.636G>A) and VKORC1 (c.–1639G>A). The other tests are for research use only (RUO) at this time.

It is worth noting that for IVD genotyping panels, all the primer sets and multiplex PCR parameters are provided by the manufacturer. In case of RUO, investigational use only (IUO) or analyte specific reagents (ASR) tests, the individual laboratory has to optimize the PCR conditions and occasionally design their own primer sets and independently validate these assays before they can be offered for clinical service. Luminex currently offers only IUO genotyping tests, including CYP450 2C19, 2D6 and warfarin sensitivity (2C9/VKORC1) tests. Although not all the reagents and parameters are optimized by the manufacturer, in this case the manufacturer does provide a sample protocol for ASPE and hybridization to xTAG

beads (http://www.luminexcorp.com/support/protocols/xtag_protocols.html, accessed 10/20/10). Due to the unique design of beads, one can perform up to 100 reactions within a single well. Therefore, up to 100 different SNPs could conceivably be interrogated for a single patient.

2.3.4 Verigene Technology

As mentioned previously, the Verigene platform (shown in Fig. 2.4) does not require PCR amplification to detect polymorphisms in target DNA. Here, capture oligonucleotide probes attached to a solid phase support hybridize to the target DNA. Following capture, target sequences are further hybridized to complementary oligonucleotides bound to nanometer-sized gold particles. The final step is the catalytic deposition of silver onto the gold nanoparticle resulting in the enhancement of the signal by six orders of magnitude, thus making this a highly sensitive method.

Verigene is another example of a fully automated, user friendly instrument. An additional benefit of this platform is the capability of the system to do DNA extraction on-board. The user process for Verigene System is as follows: the user places 25 μL of the DNA sample and an equal volume of the test-specific sample buffer into the test cartridge and then inserts the cartridge into the Verigene Processor unit (Fig. 2.4), or, the user inserts the test cartridge, an extraction tray, and pipette tips into the Verigene Processor SP unit and adds 1 ml of the whole blood sample into the extraction tray. The processor will then execute either DNA hybridization or extraction and hybridization, followed by the genotyping test. Currently, an IVD test for warfarin sensitivity and an RUO test for ten different potentially clinically relevant 2C19 SNPs are available on Verigene.

Microarrays are optimally suited for detection of high number of allelic variants, for example, genotyping the numerous CYP 2D variants. This technology allows for large-scale simultaneous screening and detection of thousands of SNPs that might be clinically relevant.

However, most of the microarray platforms currently available are not conducive to high-throughput testing in terms of number of patient samples that could be genotyped. For example, INIFINITI takes approximately 8 h to result a batch of 24 samples, while eSensor can provide results for 8–24 patients within 4 h, depending on the number of analytical towers used. Thus, the platforms that are designed such that the operator can potentially "walk-away" once all the samples and reagents are loaded, such as INIFINITI and eSensor, are a good fit for an average clinical laboratory that does not have staff with extensive molecular genetics background. The platforms capable of analyzing large number of samples that offer mostly RUO or ASR tests are geared more toward the labs that have some level of expertise in molecular genetic testing, since the appropriate primers and assay parameters have to be established and optimized by the user. The manufacturers do, in general, provide basic guidelines to aid the user in establishing these parameters.

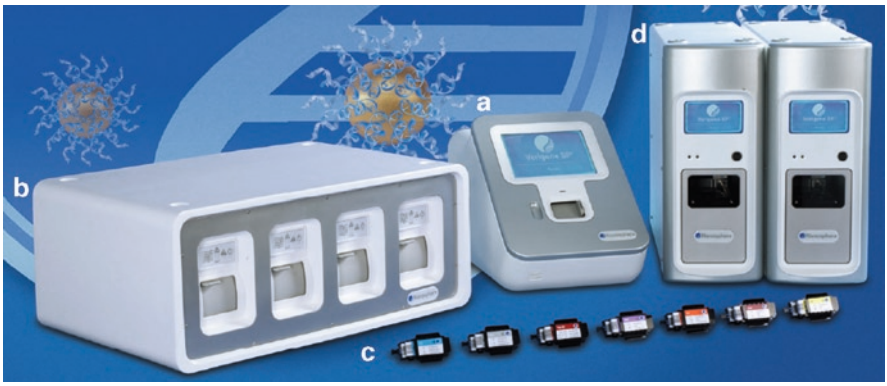

Fig. 2.4 The Verigene™ genotyping platform. (**a**) The Verigene Reader is barcode scanning and network communication-enabled central control unit and main user interface. (**b**) The Verigene Processor is comprised of four independent test modules controlled by the Verigene Reader. It facilitates the wet chemistry inside the test cartridge (**c**). Test processing in this module relies on offline nucleic acid extraction. (**d**) The Verigene Processor SP possesses all of the same test processing functionality and components as the regular processor but additionally provides capabilities to extract DNA directly from the specimen. These additional functions require the following consumables: a Verigene Extraction Tray and Verigene Tip Holder Assembly (not shown). (Courtesy of Nanosphere)

2.3.5 Real-Time PCR

In the application of real-time PCR for SNP genotyping, fluorescently labeled probes are designed to detect SNPs of interest. As the PCR progresses, the genomic DNA is amplified resulting in higher fluorescence with each consecutive cycle. Real-time PCR is performed on thermal cyclers that can detect fluorescence at different wavelengths. The most commonly used types of signal detection probes in use today for real-time PCR include hydrolysis (TaqMan) probes and hybridization probes. Both types of probes utilize fluorescence resonance energy transfer (FRET) principle, where the fluorescence is generated by the energy transfer from one probe to another. The difference is in probe design and the principle used to achieve specificity. The mechanism of each probe is described below.

2.3.5.1 Hydrolysis Probes

Hydrolysis probes are the most widely used real-time PCR probes. In hydrolysis probes, specificity lies in probe design, and the nucleotide sequence of each probe is complimentary to the target DNA region that contains a SNP of interest. Typical assay design is shown using ParagonDx real-time PCR with the TaqMan™ probe as an example (Fig. 2.5a). The probe consists of a reporter dye and a quencher. If the primer and the target amplicon match, they hybridize and Taq DNA polymerase

initiates primer extension. At the same time, the appropriate probe also hybridizes to the target. During the extension step, the exonuclease activity of polymerase cleaves the reporter dye from the probe. Once the dye and quencher are separated, the dye is free to emit its characteristic fluorescence. If there is no match between the probe and the target, the probe does not hybridize and the reporter dye fluorescence remains quenched. The change in fluorescence emitted is plotted with each PCR cycle and the amplification plot is generated (Fig. 2.5b). The genotype is determined based on the end-point fluorescence, where the fluorescence emitted by the reporter dye bound to variant allele is compared to the fluorescence emitted by the reporter dye bound to the wild-type allele. The genotype is determined based on the relative ratio of the two. Currently, a maximum of two sets of dyes can be used per reaction tube, which limits the extent of multiplexing in assays using classic hybridization probes.

An assay marketed by Fluidigm Corporation (San Francisco, CA) consists of dynamic arrays for SNP genotyping (Fig. 2.6) offering a potential for "multiplexing" through automation. This technology utilizes microfluidics-based devices called integrated fluidic circuits (IFCs) where networks of interconnected microchannels are fabricated onto miniature devices. Up to 96 patients and 96 different SNPs can be applied to a single device (96.96 dynamic array). Thus, 96 SNPs can be genotyped for each patient. This technology enables a laboratory to use the existing

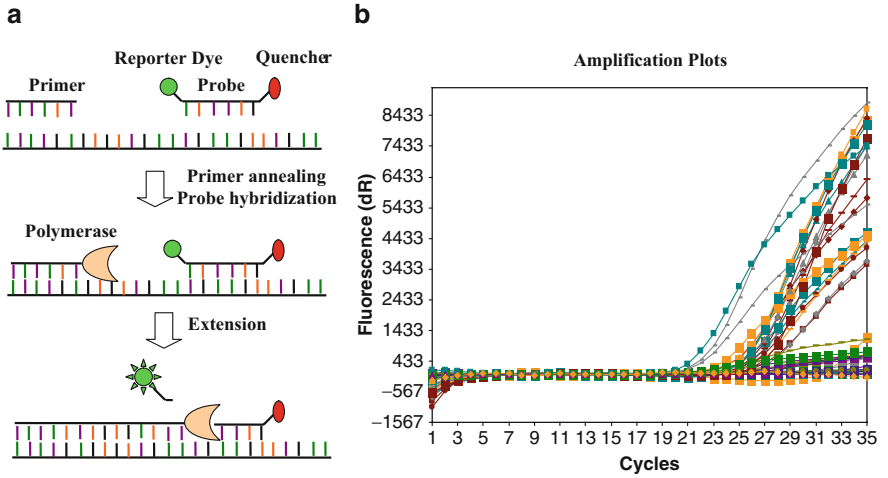

Fig. 2.5 Real-time PCR using Taqman probe method. (**a**) A schematic of PCR using Taqman probe methodology. Taqman probe consists of a reporter dye and a quencher. When the probe is intact, the reporter dye emission is quenched. The matching probe hybridizes to the target and Taq DNA polymerase initiates primer extension. During the primer extension step, the exonuclease activity of polymerase cleaves the reporter dye from the probe. The dye is then free to emit its characteristic fluorescence. If there is no match between the probe and the target, the probe does not hybridize and the reporter dye fluorescence remains quenched. (**b**) Representative amplification plots that show increasing reporter dye fluorescence with each PCR cycle. The data was generated on a Stratagene Mx3005P real-time PCR platform (Stratagene, La Jolla, CA). Figure reproduced from Personalized Medicine, Babic et al. [5] with permission of Future Medicine Ltd

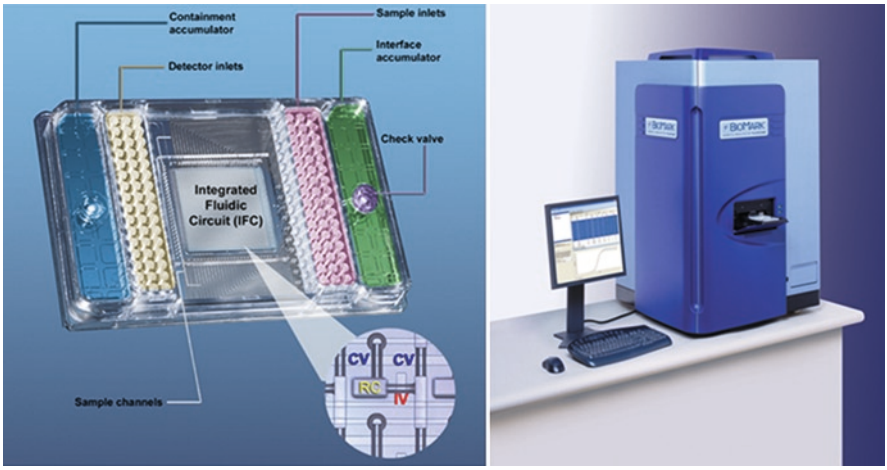

Fig. 2.6 A 48.48 dynamic array chip is shown on the *left*. The center of the chip is the integrated fluidic circuit (IFC), a network of fluid channels, valves and reaction chambers. The blow-up portion of the IFC shows one of the 2,304 individual reaction chambers and its associated containment and interface valves. Following the sample loading, the digital array is thermocycled, imaged, and analyzed on a BioMark™ system (*right*) (Courtesy of Fluidigm Corporation)

TaqMan SNP genotyping assays in a microarray configuration requiring only 192 pipetting steps within 3 h-time frame. This is in sharp contrast to a conventional 384-well microplate real-time PCR assay that would require 18,432 pipetting steps and 8 days to complete (http://fluidigm.com/applications/genotype-profiling.html, accessed 04/04/10).

2.3.5.2 Hybridization Probes

Hybridization probes are a pair of fluorescent probes of different lengths placed in close proximity (see Fig. 2.7 for illustration). In these probes, specificity is achieved by designing the shorter probe to be specific for variant allele, while the longer probe is common. In such a case, melting curves recorded by real-time PCR will be different. Primary condition for the FRET to occur is that there is a significant overlap between the emission spectrum of one fluorophore (termed donor) with the excitation spectrum of the other (termed acceptor). Thus in real-time PCR design, one oligonucleotide probe (the sensor probe) is specific for a target and contains a 5′ donor molecule, while the other (an anchor probe) is common and contains a 3′ acceptor molecule. Only when these are placed within 1–5 bp of each other, energy can be transferred from the donor to the acceptor, resulting in the emission of fluorescence.

The main advantage of real-time PCR is the speed with which samples can be analyzed, since the signal is read in real-time and there are no post-PCR processing steps. PCR can be performed in 96- or 384-well format, thus allowing a high-throughput design. Furthermore, since this is a closed-tube method of analysis, the risk of

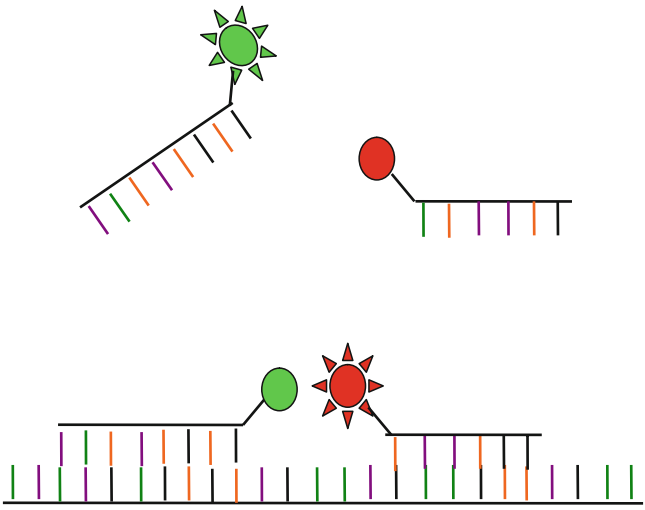

Fig. 2.7 Mechanism of action of hybridization probes. Shown are the two fluorescent dyes with overlapping spectra where the emission wavelength of one dye overlaps with the excitation wavelength of the other. When the dyes are apart "green" dye will fluoresce. When the dye are placed within 1–5 bp of each other, such as upon binding to target DNA, the emission wavelength of the "green" dye will be absorbed by the "red" dye, causing only red fluorescence to be detected

sample contamination, errors from mistakes in tube transfers, or amplicons escaping into the laboratory environment is minimal. Until recently, one of the major disadvantages of real-time PCR was the limitation in multiplexing capabilities. Since it is often necessary to test several alleles for the same patient, it would be desirable to streamline the processes such that all of the necessary genotype information could be obtained from minimal user intervention. Several manufacturers have developed sophisticated and streamlined real-time PCR instruments. One such instrument, already described at the beginning of this section, is marketed by Fluidigm (see Sect. 2.3.5.1). Another example is a BD MAX™ System (HandyLab Inc., Ann Arbor, MI), shown in Fig. 2.8. This platform combines fully automated DNA extraction with microfluidics-based real-time PCR performed in disposable microfluidics cartridges. BD MAX technology is another example of a true random-access analyzer, besides GenMark eSensor, where each cartridge can be controlled separately. Although the entire genotyping process on BD MAX instrument is very simple and elegant, unlike Fluidigm, this platform offers only limited multiplexing capabilities.

2.4 Invader® Assay

The typical workflow of the Invader assay is shown in Fig. 2.9a. The Invader® chemistry is composed of two simultaneous isothermal reactions: a first reaction that detects polymorphism of interest and a second reaction used to generate and amplify

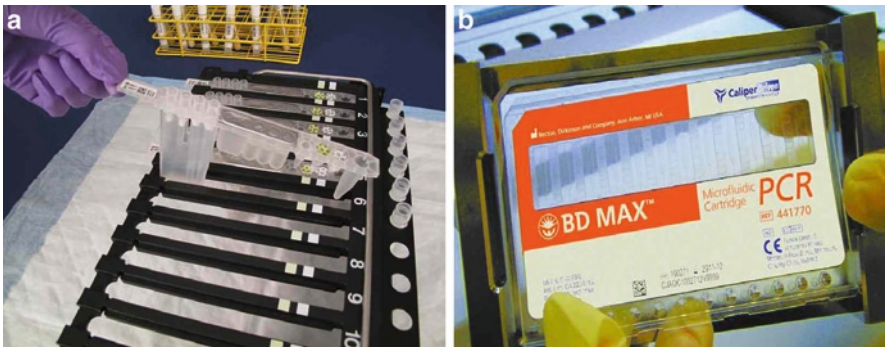

Fig. 2.8 BD MAX™ system. **a**. Unitized Reagent Strip (URS). This strip contains all the reagents and consumables required for lysis, nucleic acid extraction and PCR set-up. **b**. Disposable microfluidic cartridges, showing a total of twelve reaction chambers. Each chamber is individually controlled to enable the user to perform different PCR protocols simultaneously within a cartridge. Each chamber can be sealed off with a set of micro-valves, prior to PCR initiation, thus preventing evaporation and minimizing the risk of contamination. (Courtesy and © Becton, Dickinson and Company)

the signal. In the first reaction (Fig. 2.9b), two oligonucleotides, a primary probe, and an invading oligonucleotide are used. Both oligonucleotides contain a nucleotide complimentary to the polymorphism of interest. Therefore, both the probe and an invading oligonucleotide will bind to the variant allele, overlapping at the SNP position. The 5′ flap of the probe, including the overlapping nucleotide (i.e., SNP), is then enzymatically cleaved. Released flaps from the primary reaction serve as invading oligonucleotides for a hairpin FRET probe in a second reaction. Once the 5′ flap is bound to the FRET probe, the probe is cleaved and fluorescent signal is generated (Fig. 2.9c). Each released 5′ flap can cycle between cleaved and non-cleaved FRET probes, thereby amplifying the signal.

The advantages of Invader technology include streamlined workflow and high throughput. Since this technology does not require PCR, it only takes 30 min to genotype a 96-well plate (Fig. 2.9a). Since the only instrumentation required is a UV plate reader, the technology is not cost prohibitive. Finally, while the methodology is amenable to be automation, it could not be easily multiplexed because a maximum of two different primary probes and their complementary FRET probes can be used in a single well. Therefore, only a single SNP can be detected in each well. Since all the reagents have to be manually added to the plate, this technology is not very practical for analysis of genes that have a large number of variants, such as CYP2D6 alleles.

2.5 Sequencing

Sequencing is a definitive method of determining a DNA sequence and identifying variant(s). The DNA sequence is determined by amplification of target sequence, followed by fragmenting the genome, typically from a PCR product,

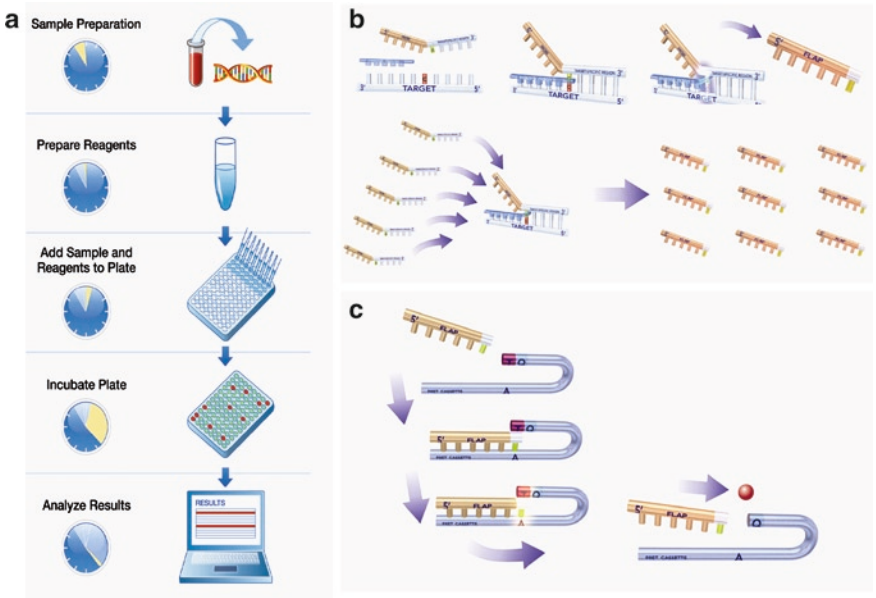

Fig. 2.9 Invader® assay. **a**. Overall workflow of the Invader assay. **b**. The scheme of the Invader primary reaction where two oligonucleotides, a primary probe and an invading oligonucleotide, bind to the target DNA simultaneously overlapping at the SNP position and subsequent cleavage of the primary probe 5′ flap. **c**. Secondary reaction involving binding of 5′ flap to the FRET probe, subsequent cleavage and signal generation. (Courtesy of Hologic)

into short fragments. In chain terminator sequencing (Sanger sequencing), primer extension is initiated by DNA polymerase. A low concentration of a chain-terminating nucleotide (most commonly a di-deoxynucleotide) is used along with the four deoxynucleotide bases. Each of the dideoxynucleotide chain terminators is labeled with a separate fluorescent dye. Limited incorporation of the chain-terminating nucleotide by the DNA polymerase results in a series of related DNA fragments that are terminated only at positions where that particular nucleotide is used. The fragments are then size-separated by capillary electrophoresis and different nucleotides within a strand are visualized as different color peaks (Fig. 2.10).

2.6 Pyrosequencing

Pyrosequencing, also known as a sequencing-by-synthesis, is a technique based on real-time detection of DNA synthesis (Fig. 2.11). The pyrosequencing platform PSQ™ 96MA (Qiagen Inc., Valencia, CA) is capable of reading 50–100 bases and genotyping virtually any SNP with high accuracy. In addition, this technology can also be used quantitatively to determine the amount of each allele in the DNA

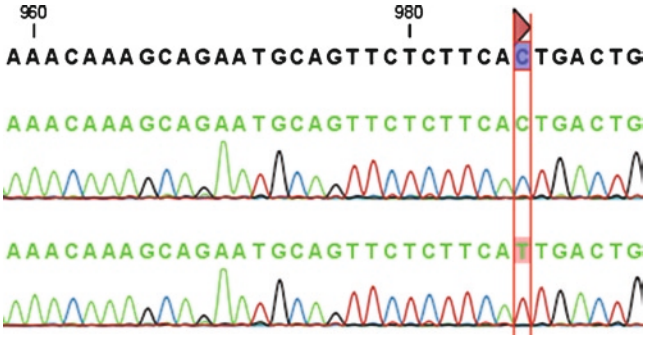

Fig. 2.10 DNA sequencing detected by fluorescence capillary electrophoresis. This figure shows 986C>T polymorphism. Modified from http://www.clcbio.com/index.php?id=785, accessed on 4/22/10

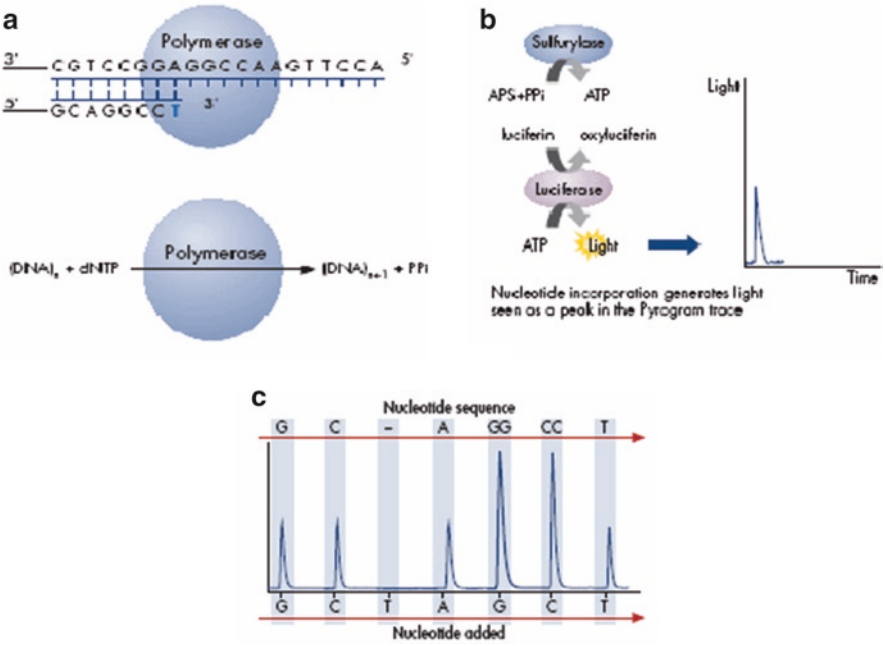

Fig. 2.11 Pyrosequencing. Target DNA ius first PCR-amplified and then reacted with deoxyribonucleotide triphosphates (dNTPs), liberating pyrophosphates (PPi) in equimolar quantities (A). In (B), each liberated PPi is reacted with a series of enzymes generating light. Each fluorescent signal is seen as a peak in a raw data output (Pyrogram), shown in (C). © QIAGEN, all rights reserved.

sample and thus estimate allelic frequency of a SNP in a population [5]. Following the PCR reaction, DNA amplicons are separated into single strands and hybridized with a sequencing primer. During the sequencing reaction, dNTPs are added one at a time, up to 50 bases until the desired region containing the polymorphism has

been sequenced. The pyrosequencing reaction also contains a mixture of enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase. In a coupled enzymatic reaction with ATP sulfurylase and luciferase, the pyrophosphate released from an incorporated dNTP generates a light signal that is proportional to the amount of dNTP incorporated. Apyrase is required for the removal of unincorporated dNTP and generation of ATP prior to the addition of next nucleotide. The light signal is detected with a CCD camera and is recognized as a Pyrogram™. Pyrosequencing has been successfully applied to CYP2C9 and CYP3A5 genotyping [6, 7]. Seatki et al. [8] used pyrosequencing to provide comprehensive genotyping of six UGT1A1 polymorphisms in the Japanese population, while Odeberg et al. studied the prevalence of different UGT1A1 polymorphisms in a Swedish cohort, encompassing 14 different ethnic groups [9]. Pyrosequencing was also used to study the role of UGT1A7 and UGT1A9 polymorphisms in prediction of response and toxicity in colorectal patients treated with irinotecan [10] and in the study of the importance of ABCB1 gene polymorphism in ovarian cancer resistance to paclitaxel [11].

2.7 Mass Spectrometry

Mass spectrometry is a widely used technology for the identification of a variety of compounds, ranging from small molecules, such as drugs, to biomolecules, such as proteins, peptides, and oligonucleotides. In this approach, the analyte is detected as a peak with a specific mass-to-charge ratio. Mass spectrometry has been successfully applied to genotyping by several groups [12–18]. The most frequently used configurations are matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) due to the fact that MALDI allows efficient ionization and fast analysis times, while TOF mass analyzers are not mass limited and do not require compound fragmentation prior to detection.

One of the implementations of the MALDI TOF MS is the MassARRAY® IPLEX® Gold – SNP Genotyping system (SEQUENOM, Sand Diego, CA), shown in Fig. 2.12. Gabriel et al. described a protocol for SNP genotyping using this platform [19]. The general workflow of MassARRAY assay is fairly simple, requiring initial PCR target amplification, followed by a specifically designed ASPE process. The resultant extension product will have allele-specific difference in masses, which is a base of detection and SNP identification by mass spectrometry. MassARRAY system has a capability of processing up to 384 samples in parallel and allows for multiplexing of up to 40 different SNPs in a single well. It takes approximately 45–60 min of instrument time to process and result for a 384 position chip. Some examples of MassARRAY technology applications include CYP2D6 genotyping and study of genetic variants in prostate cancer [14, 15]. Recently Yang et al. have reported the use of similar mass spectrometry technology called surface-enhanced laser desorption and ionization mass spectrometer (SELDI-TOF) for warfarin genotyping [12].

The advantages of using mass spectrometry for genotyping include high sensitivity and specificity, high throughput, and cost savings required for genotyping. However, the equipment itself is fairly expensive and highly specialized requiring highly trained personnel.

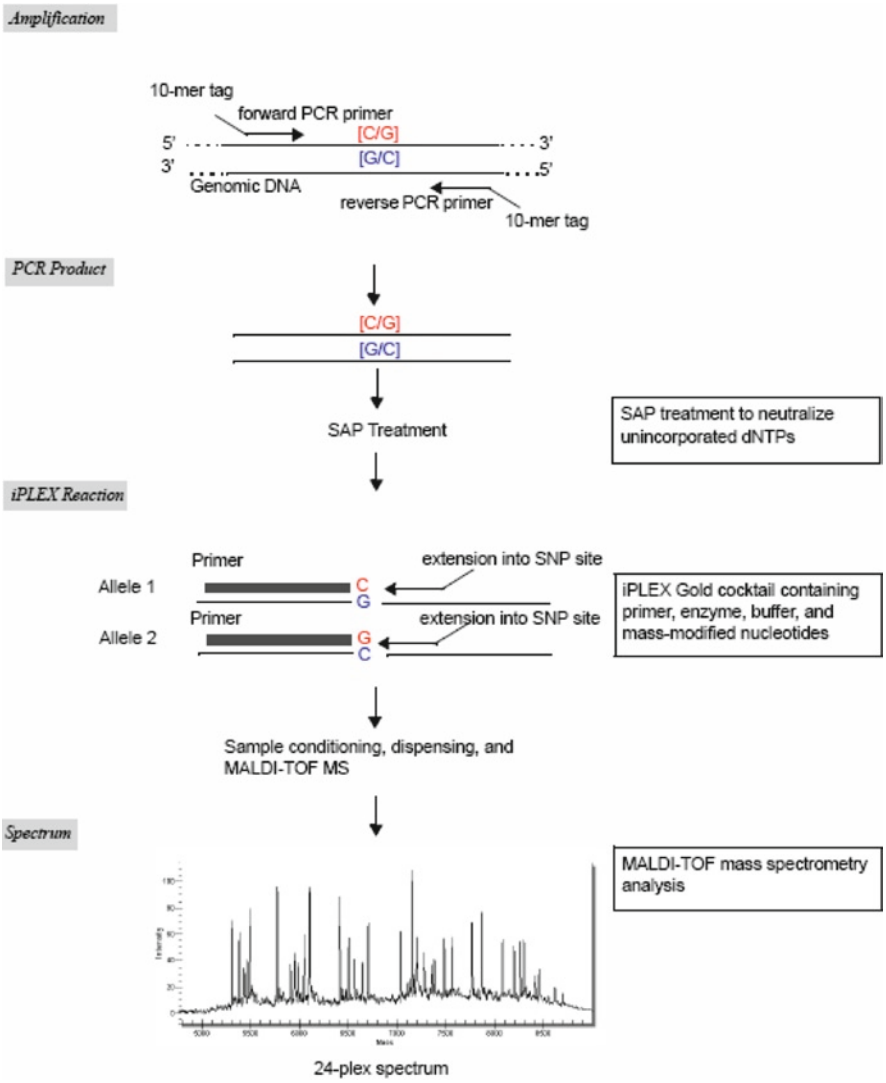

Fig. 2.12 MassARRAY iPLEX gold reaction. Following the PCR amplification of target DNA, all the unincorporated dNTPs are treated™ with shrimp alkaline phosphatase (SAP) to render them unavailable to future reaction. The iPLEX Gold cocktail is then added and the mixture is thermocycled to extend the primer by only one nucleotide (*Note*: The primers should be designed such that the 3′ end of each primer is immediately adjacent to the polymorphic site). The extended primers are then analyzed by MALDI-TOF and potential variants differentiated by differences in mass of terminating nucleotides. (Courtesy of Sequenom, Inc.)

2.8 Practical Considerations

Overall, a number of very good genotyping methodologies are available on the market today. The variety in platform designs and test menus allows the application of genotyping testing to a wide range of clinical laboratories, from small to large hospitals and specialized genetic centers. Despite a large number of platforms on the market today, there are only a few characterized DNA reference materials available for PGx testing. Therefore, running the appropriate QCs often presents additional challenge for the PGx laboratory. In the absence of reference materials, laboratories often resort to the use of unconfirmed and nonrenewable sources of DNA material, such as residual patient samples. Recently our laboratory, in collaboration with CDC, characterized a panel of 107 genomic DNA reference materials available from the Coriell Cell Repostiories for five loci (*CYP2D6*, *CY2C19*, *CYP2C9*, *VKORC1*, and *UGT1A1*) commonly included in PGx testing panels (http://wwwn.cdc.gov/dls/genetics/rmmaterials/MaterialsAvailability.aspx, accessed 4/20/2010). This will enable laboratories performing PGx testing to purchase already characterized quality control materials. Another consideration that should not be overlooked by any laboratory performing the PGx testing is the variability in allele variant definition between different assay platforms. This can lead to the discrepant results, especially when multiple SNPs are used to identify variant alleles, for example, *4 vs. *10 variant in *CYP2D6*. *CYP2D6**4 has no enzymatic function, while *CYP2D6**10 has decreased enzymatic function. The major SNP in *4 is c.1846G>A with the minor SNPs c.100C>T, c.974C>A, c.984A>G, and c.4180G>C. In *10 haplotype, the major defining SNP is c.100C>T and the minor SNP is c.4180G>C. If the assay does not define multiple SNPs of variant alleles, the potential exists, therefore, that patient could be identified as *4/*10 where the true genotype may be *1/*4. This is important because *4/*10 is assigned an intermediate metabolizer phenotype, while *1/*4 is assigned an extensive metabolizer phenotype. This discrepancy could potentially affect the clinical management of drug therapy.

Other practical aspects each laboratory should consider when selecting a genotyping platform include testing volume, specific tests, and the available personnel. If a clinical chemistry laboratory, staffed with traditionally trained medical technologists, is looking to implement warfarin genotyping test, for example, the optimal choice for such laboratory would probably be one of the fully automated microarry platforms (e.g., eSensor or INFINITI) that offers IVD products. Since these platforms allow multiplex PCR, only one PCR tube needs to be prepared for each patient. Furthermore, the manufacturers provide detailed instructions, optimized PCR parameters, and all the reagents necessary. The operator can follow the directions, load the samples onto the analyzer, and walk away. Virtually, all the automated platforms contain the software that interprets the readings and displays final genotype result for each sample. On the other hand, the laboratory that is interested in establishing their own genetic testing for various applications would benefit from the open platforms, such as Luminex or Beckman microarrays that

give the user freedom to design customized test menu. If one is interested in testing limited number of alleles, real-time PCR should be considered. This technology offers the shortest analysis time, and most of the real-time PCR instruments function as open systems allowing user-specific testing. However, one should keep in mind that open system microarrays and real-time PCR demand some previous training and experience in design of molecular diagnostic tests, which is in contrast to the fully automated platforms that could easily be operated by any medical technologists regardless of their background.

2.9 Conclusion

While it is clear that technology in pharmacogenomics field has come a long way, the data on clinical utility of pharmacogenomics testing is still lagging. According to the Secretary's Advisory Committee on Genetics, Health and Society (SACGHS [NIH]) (http://oba.od.nih.gov/oba/SACGHS/reports/SACGHS_PGx_report.pdf, accessed 04/28/10), the successful implementation of pharmacogenomic testing into a clinical laboratory requires tests that would predict a specific clinical outcome and lead to informed decision making by clinicians. There are numerous barriers currently preventing wider adoption of pharmacogenomics in clinical practice (dealt extensively by Yeo et al., Chap. 1), but solutions are beginning to appear. One prediction is certain: before these pharmacogenomics tests can become the standard of care in personalizing drug therapies, clinical trials showing utility and cost-effectiveness of such testing are necessary.

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Chapter 3
Economics of Pharmacogenomic Testing in Clinical Practice

Alan H.B. Wu

Keywords Quality-adjusted life years • Incremental cost-effectiveness ratio • Markov model

3.1 Introduction

The realization of pharmacogenomic testing or any new clinical laboratory program from the research bench into routine practice requires economic justification. It is insufficient for clinical laboratories to simply report a genotype for a particular individual. Test results must be interpreted by qualified individuals taking consideration of the clinical context of the tested patient. Ultimately, these interpretations must have the potential to lead to clinically actionable decisions. Ideally, these decisions lead to better clinical outcomes thereby justifying the cost for testing. If testing never leads to changes in patient management, pharmacogenomics will be for research purposes only. In general, there are three types of medical questions that can be answered by pharmacogenomic testing:

1. Is this the right drug for my patient?
   There are many types of drugs that can be used to treat a patient with a particular disease. The efficacy of these drugs may depend on a number of environmental, demographic, and genetic factors. For many drugs, selection of the best drug for a patient is a matter of "trial and error."

   A patient with a particular genotype might be linked to others who have previously shown to not benefit from the selected drug. While this does not guarantee nonefficacy for the patient in question, the medical decision might be to consider a different medication. For example, patients with breast cancer may be treated with an aromatase inhibitor instead of tamoxifen if they have a null genotype for

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_3,

CYP2D6. If testing identifies the patient to have a genotype where the majority of others have shown clinical efficacy, the patient and physician can get some satisfaction and assurance that this drug will be successful. While the economic benefit cannot be quantitated, these patients may be more compliant with their medications if they know that therapeutic selection was based on the individual's genetic makeup.

2. Will this drug produce a rare side effect?
   The U.S. Food and Drug Administration (FDA) and equivalent regulatory bodies in other countries are responsible for ensuring the safety of therapeutic drugs. A drug will not receive regulatory clearance or will be removed by the FDA from routine practice if a significant rate of adverse events is observed. Nevertheless, unexpected catastrophic side effect can occur with use of medications at therapeutic doses. Pharmacogenomic testing has the promise of reducing the incidence of rare adverse events for selected medications. The best example is the link between particular human lymphocyte antigen (HLA) polymorphism and prediction of Stevens Johnson syndrome (SJS) and Toxic Epidermal Necrolysis (TEN) for drugs such as abacavir, carbamazepine, phenytoin, and allopurinol. Where there are many other drugs that can produce SJS/TEN, their genetic associations have not been uncovered. As with testing for drug efficacy, the medical decision for patients determined at high risk for an adverse event would be the use of an alternative drug. The economic impact of pharmacogenomic testing would be avoidance of the medical costs needed to treat patients suffering from these side effects.
3. What is the best dosage for the drug that has been selected?
   Most medications have a narrow therapeutic window for efficacy and toxicity avoidance. Pharmacogenomic testing can be useful in predicting the dosage that results in a blood concentration that falls within that window. Predictive dosage algorithms have been established using a combination of specific patient demographic information, the history of other medications and diseases, and relevant genotypes. The best example is for warfarin where a multiethnic dosing algorithm has been created [1]. There are several areas where a dosing algorithm based in part on pharmacogenomics might have an economic impact. The biggest impact is avoidance of disease progression if the drug is underdosed, or adverse events if the drug is overdosed. Other benefits include the reducing or eliminating the costs associated with adjusting dosages (e.g., new prescription costs or reduced need for clinic visits and monitoring tests).

3.2 Economic Outcome Measures

The cost justification of pharmacogenomic tests is a major area of interest among policy makers and insurance companies and research among health economists. Like genetic diseases, pharmacogenomic testing requires testing many dozens or even hundreds of patients to identify one who will benefit from the test result [2]. The higher

the incidence of a significant polymorphism and/or the more severe the clinical consequence of an adverse drug event, the easier it is to justify pharmacogenomic testing.

Economic measures for pharmacogenomics or any medical intervention can be assessed by several means. One cost analysis approach is to estimate the actual medication expenses for providing equivalent medical care for patients with and without pharmacogenomic testing. For example, clopidogrel (Plavix®) is a prodrug that is effective in blocking platelet function and is used to prevent stenosis in patients who are treated with percutaneous coronary angioplasty. Patients who are carriers for the reduced metabolism genotype of CYP2C19 (*2 or *3) have poorer outcomes than wildtypes. Prasugrel (Efient®) is a drug that is not affected by CYP2C19 polymorphism and is an alternative to clopidogrel. Table 3.1 shows a hypothetical economic analysis comparing prasugrel drug costs, which are higher than clopidogrel, to pharmacogenomic testing for clopidogrel and use of prasugrel for carriers of the null gene.

In another economic model, the costs of providing testing for a population can be compared to the savings achieved by avoiding an adverse event using an incidence rate from published reports. The costs of an event can be estimated from reimbursements given for specific Diagnosis Related Groups (AHRQ) [3]. Table 3.2 shows a different hypothetical example for clopidogrel, whereby pharmacogenomic testing can be justified if the medical intervention of increasing the dosage from the standard 75 to 150 mg for CYP2C19 carriers can reduce the rate of adverse outcomes to that of the wildtype [4]. Clinical trials are being conducted to test this hypothesis. Both of these examples show that under the assumptions made, pharmacogenomic testing is economically justified. The advantages of pharmacogenomic testing will be further enhanced with the availability of generic formulations, as the patent for Plavix® expires in November 2011.

The more complete economic model calculates the costs associated for producing one quality-adjusted life-year (QALY) for a given medical intervention as the main criteria for cost-effectiveness. The extreme limits of QALY are 0 for death and 1.0 for an individual who is in perfect health. Individuals in varying degrees of ill

Table 3.1 Hypothetical cost-effective pharmacogenomic (PGx) models following angioplasty with stent placement: Drug expenses model for clopidogrel vs. prasugrel

Measuring parameter	No PGx (prasugrel only)	PGx (prasugrel and clopidogrel)
Number of subjects	100	100
CYP2C19 carrier rate	NA	25%
PGx testing ($150 ea)	NA	$15,000
Prasugrel annual drug costs[a]	$200,000 (100 patients)	$50,000 (25 patients)
Clopidogrel annual drug costs	NA	$125,000
Total	$200,000	$190,000
Savings		$10,000

[a]Estimated drug costs of $5.45/day for prasugrel and $4.62/day for clopidogrel, from Cohen DJ. http://www.cardiovascularbusiness.com/index.php?option=com_articles&view=article&id=18875:tct-prasugrel-costs-hospitals-less-than-clopidogrel-due-to-less-repeat-pci&division=cvb

Table 3.2 Hypothetical cost-effective pharmacogenomic (PGx) models following angioplasty with stent placement: Clinical outcomes model for standard vs. high-dose clopidogrel

Measuring parameter	No PGx (75 mg dosage)	PGx (150 mg dosage)
Number of subjects	100	100
CYP2C19 carrier rate	NA	25%
PGx testing ($150 ea)	NA	$15,000
Adverse event rate[a]	12% (12 patients)	8% (8 patients)
Annual DRG #122 for AMI[b]	$240,000	$160,000
Clopidogrel annual drug costs	$168,000	$210,000[c]
Total	$408,000	$385,000
Savings		$23,000

[a] Rates taken from Mega et al. [4]

[b] AMI DRG: $20,000 each, from Agency for Healthcare Research and Quality. http://www.ahrq.gov/data/hcup/

[c] Double drug costs for 25 patients on 150 mg dosage

health would have fractions between these limits (e.g., a bedridden individual may have a QALY of 0.5). Table 3.4 shows examples of the impact of medical interventions on QALY [7]. A calculation of the incremental cost-effectiveness ratio (ICER) is the dollar amount necessary to achieve complete health benefit for a particular intervention. It is calculated by:

$$\text{ICER} = \frac{(\text{Costs}_{\text{standard approach}} - \text{Costs}_{\text{proposed intervention}})}{(\text{QALY}_{\text{standard}} - \text{QALY}_{\text{proposed intervention}})}$$

Health economists in the United States have determined that society in general is willing to pay an ICER of up to $50,000/QALY for a proposed change in medical practices [5], although figures as high as $100,000/QALY have been cited [3]. This threshold might be higher in countries where health and family values or their willingness to pay for these services are higher than in the U.S., and lower in regions where the population is less economically developed.

There are a few studies that have calculated the additional financial resources necessary for pharmacogenomic testing relative to standard medical practices in achieving a QALY. These studies use a Markov model where the medical costs are calculated for a hypothetical patient who has a disease that can be treated with a medication whose dosage or selection is based on a pharmacogenomic test [6]. This "base case" is meant to be representative of the resources needed to treat the population as a whole. Examples of pharmacogenomic Markov decision models for a chemotherapeutic drug are shown in Fig. 3.1. Table 3.3 lists a hypothetical example of the pharmacogenomic testing for clopidogrel using the same carrier frequency rates and genotyping costs as Table 3.1and 3.2 and estimating QALY at 0.80 for a poststent patient without restenosis or complications and 0.25 for a combination of death, acute myocardial infarction, and stroke. Table 3.4 lists the ICER for other representative medical interventions that have been studied [7].

In all of these economic models, the costs for performing genotyping are presumed at the time of the analysis. In reality, such costs are not static and continue to decrease

Table 3.3 Hypothetical cost-effective pharmacogenomic (PGx) models following angioplasty with stent placement: Calculation of quality-adjusted life years (QALY) for clopidogrel pharmacogenomics

Measuring parameter	No PGx	PGx
Fraction free of events (estimated QALY = 0.80) [a]	0.88	0.92
Fraction with events (estimated QALY = 0.25) [a]	0.12	0.08
QALY [b]	0.736	0.756
Additive QALY for PGx	NA	0.020
CYP2C19 carrier rate [c]	NA	25%
Cost for PGx testing per patient	NA	$150
Cost to identify 1 carrier patient (4 patients tested)	NA	$600
Expense/QALY ($600/0.022)	NA	$27,270

[a] Estimated QALY for a patient without cardiac disease who has had a successful stent placement without (QALY = 0.80) and with (QALY = 0 25) an adverse event (death, stroke, acute myocardial infarction)

[b] QALY for no PGx: (0.88 × 0.80) + (0.12 × 0.25). PGx: (0.92 × 0.80) + (0.08 × 0.25)

[c] Rates taken from Mega et al. [4]

Table 3.4 Representative QALY

Intervention	Disease	QALY range
Mammography screening	Breast cancer	10,000–25,000
Medications	Hypertension	10,000–60,000
Dialysis	End-stage renal disease	50,000–100,000
Implantable defibrillators	Myocardial infarction and heart failure	30,000–70,000

Data from reference Neumann et al. [7]

with technology improvements and commercial competition. Even minor changes in expenses can have a major economic impact when multiplied by large numbers.

3.3 Cost-Effectiveness Studies for Specific Pharmacogenomic Testing Applications

Pharmacoeconomic studies applied to pharmacogenomic testing is a new field that will be the focus of many future studies and be of extreme interest to clinicians, laboratorians, policy makers, and payers of medical practices. In the following sections, specific published studies related to specific pharmacogenomic tests are reviewed.

3.3.1 Pharmacogenomic Testing for Thiopurine Methyltransferase

Economic assessment of thiopurine methyltransferase (TPMT) testing for the prevention of hematopoietic toxicity for patients treated with azathioprine has been

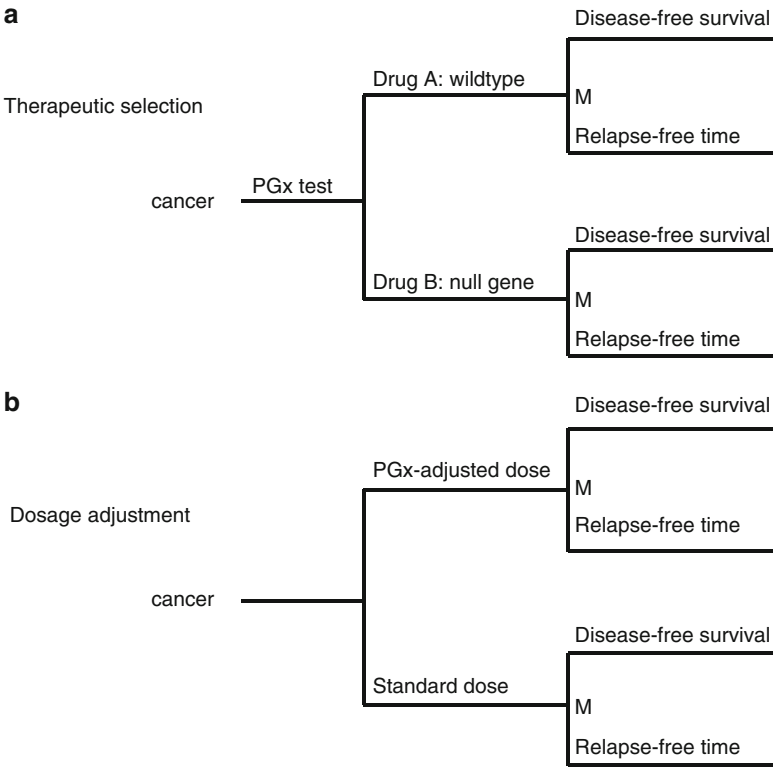

Fig. 3.1 Markov decision model for a hypothetical pharmacogenomic test to chemotherapeutic efficacy. (**a**) Pharmacogenomics to determine therapeutic selection. (**b**) Pharmacogenomics to determine dosage. *M* Markov modeling

studied by several investigators. According to Gurwitz, based on the TPMT deficiency rate, the average genotyping cost to identify one deficient individual was about $10,000 [8]. This figure must be weighed against the rate of adverse events and mortality. Priest et al. showed a cost savings of $7 and $78/patient for using a genotype vs. phenotype testing, respectively, of azathioprine for management of inflammatory bowel disease [9]. Two pharmacoeconomic studies used a hypothetical decision analysis model, comparing direct medical costs for conventional weight-based azathioprine dosing for patients with rheumatological conditions vs. a dose derived from genotyping for TPMT [10, 11]. In both the Canadian and Korean studies, the costs and drug drop-out rates were lower for the genotype-dosed model. These outcomes were largely achieved by the avoidance of dose-related toxicities. Two European studies have reported similar ICERs of $1,300 and $3,000 and for use of TPMT testing in patients with inflammatory bowel disease and acute lymphoblastic leukemia, respectively [12, 13] (Table 3.5). These reports suggest that even though the incidence of TPMT variances is low, physicians planning on using azathioprine should consider testing prior to drug administration and to use alternative medications for patients at high risk for hematopoietic complications.

Table 3.5 Summary of pharmacogenomic cost-effective studies

Drug	Genotype	Lab test costs	ICER[a]	References
Azathioprine	TPMT[b]	$50	$1,300	Winter et al. [12]
Azathioprine	TPMT	$220	$3,000	van den Akker-van Marle et al. [13]
Warfarin	2C9 and VKORC1[c]	$400	$170,000	Eckman et al. [16]
Warfarin	2C9 and VKORC1	$200	$357,000	You et al. [18]
Abacavir	HLA*B-5701[d]	$63	$10–30,000	Hughes et al. [21]
Abacavir	HLA*B-5701	$68	$36,700	Schackman et al. [22]
Clozapine	6-panel	$500	$47,000	Perlis et al. [25]
Citalopram	HT_{2A}[e]	$500	$93,000	Perlis et al. [26]
Bupropion	Dopamine receptor	$300	$3,000	Heitjan et al. [30]
Tobramycin	Mit 12s ribosome[f]	$338	$79,300	Veenstra et al. [31]

[a] *ICER* incremental cost-effectiveness ratio
[b] *TPMT* thiopurine methyltransferase.
[c] *VKORC1* vitamin K epoxide reductase complex 1
[d] *HLA* human lymphocyte antigen
[e] *HT* serotonin receptor
[f] Mit mitochondria

3.3.2 Pharmacogenomic Testing for Warfarin Dosing

Several hypothetical models have been established to determine if a warfarin dose based on the pharmacogenomic testing for CYP2C9 and vitamin K epoxide reductase complex 1 is cost-effective. The Brookings Joint Center for Regulatory Studies conducted an analysis based on a genotype cost of $350 and a 15 and 50% reduction in bleeding events and stroke, respectively [14]. Based on these assumptions, they concluded that warfarin pharmacogenomics will save nearly $2 billion dollars in the US per year as a nation. Unfortunately, the few outcome studies that have been published have not suggested that dosages determined by pharmacogenomic testing will result in these outcome improvements.

Two pharmacoeconomic studies used atrial fibrillation as the test case. Leey et al. concluded that a reduction in the incidence of warfarin complication by 0.1% would be associated with a cost benefit for pharmacogenomic testing [15]. This was based on a genotyping cost of $250 and that the use of pharmacogenomic testing would not lead to potentially harmful modification to an anticoagulation regimen. In contrast, using a genotyping cost of $400 and an assay turnaround time of 3 days, Eckman et al. determined an overall ICER of $170,000 for warfarin pharmacogenomic testing and concluded that testing under these conditions was not cost-effective (Table 3.5) [16]. In a subanalysis, these investigators concluded that an ICER of <$50,000 could be achieved if testing was restricted to patients at high risk for hemorrhage or if results were available within 24 h for under $200. The faster reporting and enactment of pharmacogenomic test results could reduce the incidence of bleeding during the immediate dosing period. The Eckman et al. models were based on three existing randomized trials of standard vs. pharmacogenomic genotyping data (total 429 patients with 11 adverse events). You et al. used the results from a single randomized trial [17] and computed a higher ICER of

$357,000 [18]. They conclude that cost-effectiveness can only be achieved if the cost per test was under $47. While the economic conclusions of these latter two trials are difficult to challenge, they were based on very small data set and the conclusions that pharmacogenomic testing is not indicated are premature [19]. Repeated cost-effectiveness estimates will be necessary with the publication of additional randomized trials, such as the one sponsored by the National Heart, Lung and Blood Institute [20].

3.3.3 Pharmacogenomic Testing for Abacavir

There have only been a few reports examining the cost-effectiveness of pharmacogenomic testing for *HLA-B*5701* to avoid delayed hypersensitivity reactions (HSR) in patients taking abacavir. Hughes et al. compared the medical costs of treating patients with HSR induced by abacavir and abacavir substitutes without pharmacogenomic testing vs. testing all subjects and the costs for abacavir substitutes for those who were positive for *HLA-B*5701* and the costs for treating HSR patients who were negative for HLA-B*5701 and abacavir substitutes [21]. Using an ICER of dollars per HSR avoided and a test cost of $63, these investigators found values ranging from $10,000 to $30,000 depending on variability in alternative medications and medical treatments for HSR (Table 3.5). Similar results were reported by Schackman et al. [22], who used a genetic test cost of $68 and reported an ICE of $36,700. In both of these models, pharmacogenomic testing is only cost-viable for the Caucasian population as other ethnicities have a low or absence incidence of the *HLA-B*5701* genotype. Moreover, the need for pharmacogenomic testing will decline with the availability of other antiretroviral drugs for treating patients with human immunodeficiency virus that are less expensive, do not produce HSR, or are more efficacious than abacavir.

3.3.4 Pharmacogenomic Testing for Antipsychotic Drugs

The major economic motivation for pharmacogenomic testing of antipsychotic drugs involves avoidance of side effects, particularly for drugs that are metabolized by CYP2D6, and therapeutic selection to maximize drug efficacy. There have been no cost-effective models studied for CYP2D6 pharmacogenomic testing. One pilot study showed that psychiatric patients seen at one facility who had a poor 2D6 metabolizer genotype had higher numbers of adverse drug events, higher costs associated with treatment, and longer duration of stay than in those who were extensive or intermediate metabolizers [23]. Although the sample sizes were small, these investigators concluded that individuals who are poor metabolizers will benefit most from genetic testing in terms of therapeutic decisions. Rodriquez-Antona et al. did a rough estimate of the additional costs needed to treat patients who are

poor metabolizers of drugs used for psychiatry [24]. At $250 for each test, they estimated a genotyping cost of $3,500 to test 14 patients in order to identify one who is a poor metabolizer. If the length of stay was on average 7 days per patient per year longer for the poor vs. extensive metabolizers, as suggested by previous studies, there would be a cost savings of $4,900, exceeding that of the genotyping costs. Such a model has not been prospectively tested.

There were two studies that performed economic modeling analysis for a specific antischizophrenic medication. Perlis et al. examined the impact of pharmacogenomic testing of neurotransmitter-receptor related genes for use of clozapine among schizophrenic patients [25]. The model compared the use of conventional first- and second-line antipsychotic drugs with no pharmacogenomic testing (and use of clozapine as the third-line) vs. pharmacogenomic testing and use of clozapine as the first-line drug for positive patients only. These investigators calculated a cost of $47,000 per QALY for pharmacogenomic testing (Table 3.5). In a more recent study, Perlis et al. constructed another model for comparing pharmacogenomic testing for response to serotonin-selective reuptake inhibitors (SSRIs) [26]. Positive responders are given citalopram vs. bupropion for negative responders. The ICER vs. conventional strategy without testing was $93,500 (Table 3.5). In both of these studies a relatively high genotyping cost of $500 was included in the model, as none of these tests are commercially available. For clozapine response, a panel of single nucleotide polymorphisms was proposed [27] in the genes for serotonin receptor (5-HT_{2A} and 5-HT_{2C}), serotonin transporter promoter (5-HTT), and histamine H_2. For citalopram, the model was based on genotyping for 5-HT_{2A}.

3.3.5 Miscellaneous Other Pharmacoeconomic Studies

There are a number of economic studies that have examined other less widely studied pharmacogenomic tests. Furuta et al. established a dosing regimen based on CYP2C19 genotyping for the use of proton pump inhibitor regimens for the eradication of *Helicobacter pylori* [28]. They found a higher rate of microbiological eradication in the tailored vs. standard regimen group (96% vs. 70%, respectively) with no significant increase in total costs ($669 vs. $657, respectively). The cost of genotyping for 2C19 was set at $83. In patients with nephropathies, Costa-Scharplatz et al. examined the cost-effectiveness of pharmacogenomic testing for an insertion/deletion variance in the angiotensin converting enzyme (ACE) gene for selection of ACE inhibitors vs. angiotensin II receptor blockers [29]. Using a very modest genotyping cost of $30, they concluded that the addition of testing resulted in a reduction of costs with the avoidance of the expenses for chronic hemodialysis. An assessment of QALY was not made in this study.

For smoking cessation, Heitjan et al. performed an economic model for the pharmacogenetic testing of an insertion/deletion variance in a dopamine receptor to select bupropion or transdermal nicotine vs. no testing for all patients for bupropion, transdermal nicotine, varenicline, or no drug at all [30]. The ICER was very

favorable at $3,000 for a genotype cost of $307. Veenstra et al. evaluated the use of a genetic test for a mutation in mitochondrial 12S ribosomal rRNA to predict hearing loss among cystic fibrosis patients treated with aminoglycosides [31]. With an ICER of $79,300, they concluded that pharmacogenomic testing was not cost-effective and could lead to worse patient outcomes with the avoidance of antibiotic treatment in falsely positive pharmacogenomic test results.

3.4 Summary

Novel therapeutic, intervention, and medical practice decisions are becoming increasingly linked to adherence to clinical practice guidelines, documentation of medical evidence, and justification by economics. Reimbursement for new services will be denied without data to support the medical and/or economic advantages. Pharmacogenomic testing is especially being scrutinized because of the higher costs associated with genetic testing and the promise and notoriety that this can enable personalized medicine. Producing accurate effective economic analyses for pharmacogenomic tests is dependent on the quality of the assumptions made. Unfortunately, there are very few randomized studies available to fuel the analyses that have been conducted. Therefore, some of the conclusions made are premature. Nevertheless, such studies have led to policy changes regarding test utilization. The announcement that the US Centers for Medicare and Medicaid will not reimburse laboratories for warfarin pharmacogenomic testing unless such testing is part of a clinical trial may be an example of a "rush to judgment [32]." These important decisions may inhibit the impetus and funding to conduct the randomized studies needed to document efficacy of pharmacogenomic testing, and such attitudes may become a self-fulfilling prophecies. A second problem for implementing pharmacogenomics is that the majority of those tested have the expected genotype and therefore no therapeutic alterations are needed. The expenses for the identification of one affected patient where a medical decision is needed must be justified by the testing of all others. These economic realities have been the burden of tests for genetic diseases as well.

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Chapter 4
From Personalized Medicine to Personalized Justice: The Promises of Translational Pharmacogenomics in the Justice System

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Keywords Personalized justice • Daubert standard

4.1 Introduction

Enabled by Pharmacogenomics (PGx), molecular imaging, and other molecular biomarkers, personalized medicine (PM) promises to optimize therapy while minimizing side effects. It may also dramatically impact the justice system in ways we are only beginning to understand.

Personalized medicine has already entered the curricula of well-regarded medical schools such as that of Johns Hopkins [1], but law schools offer no analog. Although clinical acceptance of PM has proved slow even with FDA support [2, 3], PM's legal ramifications are evident. Recently, for example, the FDA relabeled some drugs with companion PGx [2] such as warfarin with cytochrome P450 (CYP) 2C9 and vitamin K epoxide reductase complex 1 to reduce bleeding [3–5]. If PGx retrospectively reveals that the warfarin patient was at high risk and testing was not initially

*This editorial reflects the consensus of the coauthors and not the official position of opinion of their respective employers.

This chapter was published in its entirety in: Wong SHY, et al.: Wong SH, Happy C, Blilnka D, Gock S, Jentzen JM, Donald JM, Coleman H, Jortani SA, Lucire Y, Morris-Kukoski C, Neuman M, Sander TL, Wagner MA, Wynn JR, Wu AHB, Yeo J. From personalized medicine to personalized justice – assembling the framework with pharmacogenomics and assessing its applicability to the justice system. Pharmacogen 2010;11:731–737.

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_4,

performed, litigation may follow. Indeed, some lawyers advertise on the internet for cases involving warfarin-related errors [6]. Consequently, PGx may become part of defensive medicine.

Personalized Justice (PJ) complements PM and the overlapping practice of translational medicine [7–10], which hold that individual differences are caused primarily by genetic and environmental factors. The acronym "*TSPB"* captures its elements in relation to adverse drug reaction (ADR): *T*oxicity, *S*ensitivity, impaired *P*erformance (e.g., driving under the influence of drugs), and *B*ehavioral changes. Future legal applications may include molecular imaging and analyses – genomic, proteomic, metabolomics, and epigenetics/imprintomics. By comparison, molecular DNA fingerprinting for identity testing is well accepted [11]. Conceptually, Fig. 4.1 proposes a social balance relationship for PM and PJ [10]. In assessing PJ, consider two index scenarios:

4.1.1 Drug Toxicity

A 9-year-old boy, diagnosed with ADHD, obsessive-compulsive disorder, and Tourette's syndrome, was treated with methylphenidate, clonidine, and fluoxetine [12]. Over a 10-month period, he developed GI toxicity, incoordination and disorientation, and seizures. He died from a cardiac arrest. Postmortem toxicology showed high fluoxetine and norfluoxetine concentrations, and PGx revealed a poor CYP 2D6 metabolizer, resulting in fluoxetine accumulation and

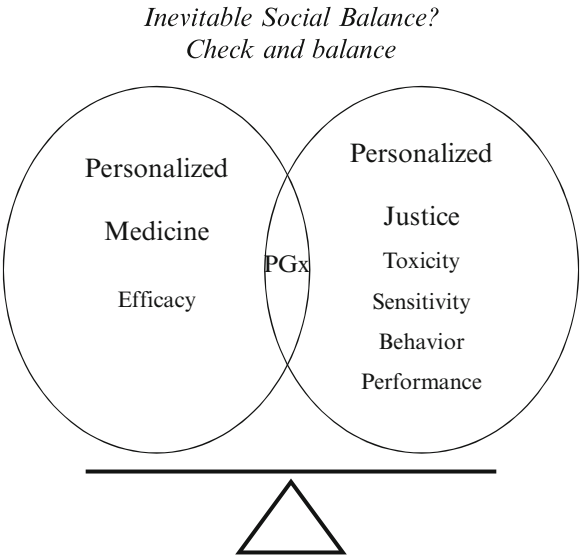

Fig. 4.1 Complementary relationship of PM and PJ. (Reproduced and modified with permission from ref. [10]. In press)

toxicity. Subsequently, the boy's parent was absolved from involvement in fluoxetine intoxication. Another example is genotyping uridine 5′-diphosphate (UDP)-glucuronyltransferase 1A1 for patients medicated with irinotecan to avoid hematopoietic toxicity [13].

4.1.2 Drug Sensitivity

In addition to warfarin, one should genotype HLA-B*5701 [14] and HLA-B*1502 [15] for patients medicated with abacavir [14] and some antiepileptics [15], respectively, to avoid Stevens Johnson Syndrome (SSJ). Lawyers use internet advertising to reach persons so affected [16].

4.1.3 Evidence Base for Personalized Justice

In establishing PJ, a firm foundation should be based on sound legal principles as well as reliable and valid evidence-based studies, not on "junk" science and unsubstantiated case reports. This lesson resonates in the deficiencies that beset various forensic sciences recently reported by the National Academy of Science [17, 18]. The American Academy of Forensic Sciences supports the National Academy of Science's 13 recommendations and the following principles: The need for strong scientific foundations; laboratory accreditation; certification of technicians; the standardization of terminology; ethical protocols; governmental oversight; and the education of legal professionals, including judges, in forensic scientific methods and principles [19–21]. It is imperative that PJ heeds these recommendations, including the study of the relationship of PGx biomarkers to TSPB and the education of interested parties including forensic pathologists and toxicologists, those engaged in molecular diagnostics, and of course, the legal community. Based on the aforementioned assessment, this editorial ushers in the practice of PJ by: differentiating between science and myth, proposing a legal framework, updating the reader on rapidly developing technological advances, and illustrating scenarios and published cases.

4.2 Legal Framework

While personalized medicine is rapidly taking root among the medical sciences, one may reasonably expect a slower, more begrudging acceptance by the legal profession. Law is innately conservative and reluctant to accommodate dramatic change. "Cutting-edge" developments of all sorts often take decades to gain a foothold [22]. It will be important, then, to educate judges, lawyers, and legal academics about the explanatory power of PJ and PM. The law's incredibly rich experience with DNA developments may, however, facilitate this task [23].

One set of barriers consists of evidence rules, particularly those involving expert opinion testimony. The vaunted "*Daubert* standard" pioneered by the federal courts and adopted by many states demands that judges serve as gatekeepers who will ensure that only "reliable" science is admitted [24]. Although courts have been distressingly inconsistent in how they scrutinize most sciences [25], DNA evidence has become the gold standard for forensic sciences. And the DNA channel may provide a helpful port of entry for PJ.

The prime questions, though, will relate to the role PJ will play in the legal system. DNA evidence thus far is narrowly confined to trace evidence: Was this biological evidence left by the defendant or someone else? A thornier problem occurs when we attempt to apply biological evidence to moral culpability, which pertains to an accused's personal blameworthiness. The Supreme Court recognized in *Penry v. Lynaugh* [26] that punishment for a criminal offense should be directly related to the defendant's personal culpability. The concept of personal culpability acknowledges that human choices are shaped by many factors: genetic, neurological, intellectual, educational, social, and environmental. It follows, then, that an individual's blameworthiness for criminal conduct may vary depending on the factors that shaped his moral development or compromised his choices.

Thus, from a PJ perspective, the question becomes something like this: Should courts consider identifiable biological conditions that predispose a person to criminal behavior in weighing moral culpability? Legal precedent suggests that it should. Consider *Roper v. Simmons* [27] where the Supreme Court held that persons under the age of 18 years could not be subjected to the death penalty because their brains were not yet fully developed. MRIs and neuro-imaging showed that neuronal changes in the brain continued into the early twenties. Because the brain affects behavior, the justices ruled that punishing a person for behavior caused by an underdeveloped brain (of which the defendant had no choice) violated the prohibition against cruel and unusual punishment. Similar logic was applied in *Atkins v. Virginia* [28], which prohibited subjecting the mentally retarded to the death penalty. *Roper* and *Atkins* illustrate the principle that criminal defendants with brain-based deficits are not as morally culpable as those without. As such, they deserve a lesser penalty. This is a legal springboard for PJ.

4.3 Forensic Pathology Perspectives

For several medical examiner/coroner offices in US and Europe, PGx has served as an adjunct for drug death certification – an emerging practice of molecular autopsy [9, 10]. Previous studies showed a higher prevalence of *CYP 2D6* genetic variations, corresponding to intermediate and slow metabolizers with decreased or without enzymatic activity, in the decedents intoxicated with methadone, oxycodone, and antidepressants [9, 10, 29, 30]. Thus, PGx might aid to interpret the effect of impaired drug metabolism due to genetic variations. If potentially lethal medications are identified at the scene with correspondingly toxic drug concentrations of the

decedent and subsequent PGx testing confirms an "extensive" (normal) metabolizer, death is certified as suicide. If the deceased's genotype is a variant – resulting in decreased drug metabolism, death is certified as accident. Recent indications of PJ for forensic pathology include a PGx section in forensic toxicology texts for medical examiners by Molina [31] and Karch [32]. Future molecular diagnostics biomarkers of interest might include epigenetics/imprintomics and gene expression in understanding suicide [33, 34], metabolomics, and proteomics [10].

4.4 Molecular Diagnostics

The detection of individual genetic variants is at the heart of PM and PJ. Single nucleotide polymorphisms (SNPs), the most common type of genetic variation, might affect drug metabolism [35]. Several SNP genotyping technologies facilitate rapid PGx testing in clinical laboratories. The three main steps – DNA extraction, amplification, and detection – may be performed by automated platforms. Biotech companies offering PGx testing platforms, some with FDA-approval, include: Luminex xTag, Roche Amplichip, Affymetric DMET chip, Autogenomics INFINITI, Osmetech eSensors, ParagonDx, and ABI SNaPshot and Taqman assays. Thus, the laboratory can rapidly develop, validate, and perform PGx testing in-house within months, further enhanced by readily available quality control products and survey programs. The limitations include: existing evidence to demonstrate significant and medically relevant correlations for many disease-causing genes and variants, limited detection of genetic variants within the context of each testing platform, clinical interpretation of genotype results including environmental factors, and transplanted organs interfering with testing.

4.4.1 Drug Hypersensitivity In Vitro Diagnostics

In vitro lymphocyte toxicity assay (LTA) compares peripheral blood lymphocytes of patients with history of ADR to control individuals who take the same drug in the same dose and do not present any ADR [36]. LTA is based upon the dysfunction of mitochondria in people hypersensitive to certain drugs such as sulfonamides, non-steroidal anti-inflammatory, protease inhibitors, and antiepileptics. This test can also detect possible drug–drug interactions. Dysfunction of mitochondria has severe cellular consequences and is linked to lack of detoxification of drugs in human. Several surveillance strategies have evolved that limit mitochondrial damage and ensure cellular integrity. Intraorganellar proteases conduct protein quality control and exert regulatory functions, allowing mitochondria to protect against apoptosis. LTA can be used in PJ when several drugs are incriminated in an ADR in order to enable distinguishing between the drug that produced the reaction and the other drugs, which have been taken in the same period of time, but did not contribute to the ADR.

4.5 Illustrative Cases and Scenarios

4.5.1 Alcohol

Alcoholism, with up to 30–40% inheritability, is a complex and controversial disease having both environmental and genetic components. Genetic variations influence pharmacokinetics/metabolism and pharmacodynamics. Alcohol dehydrogenase and acetaldehyde dehydrogenase are two main polymorphic enzymes involved in alcohol metabolism, with minor contribution by CYP 2E1. Pharmacodynamic systems influenced by PGx are: gamma-aminobutyric acid A/B receptors, glutamate (N-methyl-D-aspartic acid and α-amino-3-hydroxyl-5-methyl-isoxazole-propionate), serotonin, voltage-activated calcium channels, dopamine/norepinepherine/acetylcholine, and opioid systems. For example, naltrexone, used for detoxification, binds to opioid receptor μ1 and the variants of the candidate gene of this receptor may affect addiction treatment [37, 38].

4.5.2 Antidepressants and Antipsychotics

Personalized justice might address the effect of antidepressant and/or antipsychotics on behavioral changes. A recent review examined the relationship of violent behavior to the antidepressants: paroxetine, sertraline, and fluoxetine. Different verdicts in a series of medicolegal cases reflected the different judicial processes without considering drug-induced violence [39]. Incidentally, Lucire (Lucire, Y. (2009) Personal communication) studied patients medicated with antidepressants and antipsychotics metabolized by polymorphic CYPs and assessed the development of akathisic, suicidal and/or homicidal ideations, and relationship to CYP genes variations. The validity of these preliminary observations is pending publication in peer-reviewed journals and validation by other investigators.

4.5.3 Warfarin

Oral warfarin anticoagulation is widely used to prevent thromboembolic events. Dosing selection is due to a narrow therapeutic range with a large interindividual variation (20-fold) affected by genetic and nongenetic factors [40, 41]. About 10–17% of patients experience bleeding [40]. Genotyping of *CYP2C9, CYP 4F2, VKORC1*, and relevant clinical factors account up to ~56% of dosing variability [42, 43] In 2007, the FDA relabeled warfarin to suggest genotyping, followed by the January 22, 2010 relabeling [5]: “The patient’s CYP2C9 and VKORC1 genotype information, when available, can assist in selection of the

starting dose." Previously, in May 2009, the Centers for Medicare & Medicaid Services recommended against reimbursement [44]. Potential legal culpability was addressed in the introduction.

4.5.4 Pain Management

In addressing pain management with safety, Woodcock of the FDA discussed the balance of providing patient with efficacious analgesics and the associated risks [45]. For example, in ultrarapid metabolizers, greater CYP2D6 activity can lead to poisoning after opioid administration. A 2007 case report detailed a breast-feeding infant who suffered respiratory depression and died as a result of toxic amounts of morphine being present in the milk [46]. The mother, later identified with multiple copies of *CYP2D6* genes corresponding to an ultrarapid metabolizer, "over-converted" codeine to a "high" amount of morphine. This was excreted into breast milk, resulting in baby's high morphine concentrations identified in postmortem analysis. Consequently, guidelines were developed for breast-feeding mother medicated with codeine.

4.6 Conclusions

In recognizing the complementary, check and balance relationship of PM and PJ, translational PGx may serve the promising role of an *ADJUNCT* biomarker for interpreting drug-related toxicity and sensitivity. Currently, robust, scientific, and clinical studies are lacking to substantiate the relationship of PGx and behavioral/performance changes [39]. These desired PJ studies are challenging to perform due to ethical and legal consideration and lack of funding. Consequently, interpretations may be extrapolated from case reports and clinical behavioral and performance studies, e.g., studies related to "driving under the influence of drug." Other advances include automated platforms and potential use of oral fluid for toxicology and PGx. Oral fluid, currently being evaluated for forensic drug testing [47–50] and therapeutic drug monitoring, is easily collected for PM and PJ pending on outcome studies to demonstrate efficacy comparable to blood samples. In considering PGx for PJ, pros and cons are listed in Table 4.1.

In ushering in PJ practice with PGx, a working group should consist of colleagues from interrelated disciplines in order to probe and keep abreast of recent developments, to grade evidence of case reports and outcome studies, and to develop inclusion and exclusion criteria. With sound scientific and legal principles and correct interpretation, a firm and lasting foundation supports the emerging concept of PJ becoming a reality to enhance patient safety and to maintain social justice.

Table 4.1 Pros and Cons of using PGx as an adjunct biomarker in personalized justice

Pros
Stability of DNA in postmortem settings
Personalized approach for assessing drug response
Assist in interpretation of drug concentrations in postmortem toxicology and drug death certification
Assess patient compliance
Turn-around-time suitable for medicolegal/forensic applications
PGx cost is "low" in comparison to the legal settlement
Might differentiate chronic vs. acute toxicity
Cons
Data available in clinical cases, but limited in postmortem cases
Legal interpretation challenging due to complexity
Drug inhibitors and inducers of enzymes, and environmental factors complicating interpretation
Does not account for posttranslational modifications
Multiple enzyme systems involved in the metabolism

Acknowledgements We acknowledge permission Future Medicine Ltd., http://www.futuremedicine.com/loi/pgs.

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Part II
Chemotherapeutics

Chapter 5
Irinotecan

R. Stephanie Huang, Federico Innocenti, and Mark J. Ratain

Keywords Irinotecan • UGT1A1 • Pharmacogenetics

5.1 Pharmacology

Irinotecan (Fig. 5.1 also called CPT-11 or 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) is a semisynthetic analog of the natural alkaloid camptothecin. It was first approved in Japan in 1994 for small-cell lung cancer and hematologic malignancies, followed by approvals in France for the treatment of advanced colorectal cancer in 1995 [1]. Irinotecan was introduced in the US in 1996 and received a full U.S. Food and Drug Administration (FDA) approval for the treatment of colorectal cancer in 1998. Currently in the US, irinotecan is primarily utilized for patients with metastatic colorectal carcinoma whose disease has recurred or progressed following initial fluorouracil-based therapy. However, the drug has demonstrated efficacy in treating a wide variety of neoplasms and was approved for the use of treating lung and breast cancer in Japan [2].

The development of irinotecan started in the late 1980s in Japan. In an anticancer drug activity screen conducted in a HST-1 human squamous carcinoma cell line, SN-38, a main active metabolite of irinotecan, was found to augment anticancer activity in combination with cisplatin, mitomycin C, 5-fluorouracil (5-FU), and etoposide [3]. The *in vitro* assay also showed greater activity in colon and hepatocellular carcinoma cell lines when comparing SN-38 to cisplatin, mitomycin C, doxorubicin and 5-FU [4]. Furthermore, irinotecan demonstrated therapeutic

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_5,

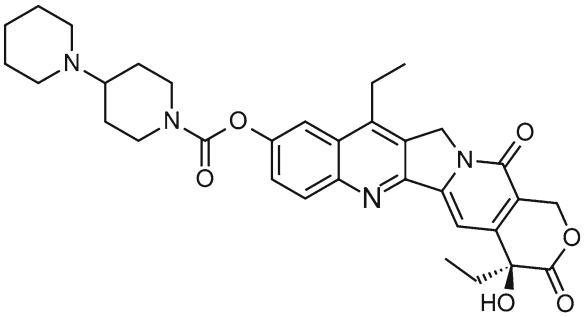

Fig. 5.1 Structure of CPT-11

efficacy when tested in a panel of human tumor xenografts derived from adult and pediatric central nervous system malignancies [5], from human testicular embryonal carcinomas [6], and from human ovarian cancer and soft-tissue sarcoma lines grown in nude mice [7].

In a series of phase II single-agent irinotecan trials conducted in Japan in the early 1990s, promising antitumor activity was observed in non-small cell lung [8], small cell lung cancer [9], uterine, cervical and ovarian cancer [10], gastric cancer [11], metastatic colorectal cancer [12], pancreatic cancer [13], breast cancer [14] as well as refractory leukemia and lymphoma [15, 16]. These results were subsequently confirmed outside of Japan [17, 18].

The primary use of irinotecan (outside of Japan) is in treating advanced colorectal cancer. The response rate to irinotecan as a single agent has ranged from 17 to 27% [19]. Efficacy was demonstrated both in chemotherapy-naïve patients and those who progressed after 5-FU-based chemotherapy [20–23]. As a single agent, irinotecan can be given at 125 mg/m^2 weekly (with intermittent breaks), or as a single 350 mg/m^2 dose every 3 weeks [24]. Combinations of irinotecan with 5-FU and leucovorin (FOLFIRI) resulted in significant improvements in objective tumor response rates, time to tumor progression, and survival when compared with 5-FU/LV alone [25–28]. Guichard et al. showed that the schedule of irinotecan and 5-FU combinations administration is a critical parameter for chemotherapeutic efficacy both *in vitro* and *in vivo* [29]. In addition to FOLFIRI, other irinotecan containing therapies, including FOLFOXIRI (irinotecan, oxaliplatin, 5-FU, and leucovorin) and the cetuximab ± irinotecan regimen, are also recommended by the National Comprehensive Cancer Network (NCCN) practice guidelines in treating advanced colon and rectal cancer [24, 30].

5.2 Pharmacokinetics

After intravenous infusion, irinotecan plasma concentrations decline in a multi-exponential manner, with a mean terminal elimination half-life of 6–12 h. The mean terminal elimination half-life of the active metabolite SN-38 is 10–20 h [31–33].

Large interindividual variability in the pharmacokinetics of irinotecan was observed. Over the recommended dose range of 50–350 mg/m^2, the plasma area under the curve concentration (AUC) of irinotecan increases linearly with dose [31–34]. Maximum concentrations of the active metabolite SN-38 are generally seen within 2-h following the end of a 90-min infusion of irinotecan [33]. SN-38 rebound concentrations were observed in many courses at about 0.5–1 h following the end of the i.v. infusion, which is suggestive of enterohepatic recycling [35]. Irinotecan exhibits moderate plasma protein binding (30–68% bound) while SN-38 is highly bound to human plasma proteins (approximately 95% bound). The plasma protein to which irinotecan and SN-38 predominantly bind is albumin.

Irinotecan is metabolized to 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin (APC) [36, 37] or 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin (NPC) [38] and potential other intermediate metabolites [39] via a cytochrome P450 mediated process [40]. CYP2A6, CYP2C9, and CYP3A4/5 show roles in irinotecan metabolism *in vitro* [41]; while CYP3A4 is the most relevant CYP isoform in irinotecan metabolism *in vivo*. Neither APC nor NPC contributes directly to irinotecan *in vivo* activity. NPC is further converted to SN-38 (7-ethyl-10-hydroxy-camptothecin) by carboxylesterase [42, 43]. SN-38 is subsequently conjugated predominantly by the enzyme UDP-glucuronosyl transferase 1A1 (UGT1A1) to form a glucuronide metabolite (SN-38G) [44] (http://www.pharmgkb.org/search/pathway/irinotecan/liver.jsp). Rivory et al. showed that the transformation of SN-38 to the glucuronide is the rate-limiting step in the elimination of SN-38 [45].

The disposition of irinotecan has not been fully elucidated in humans. The urinary excretion of irinotecan is 11–20% of the initial dose; SN-38, <1%; and SN-38G, 3%. The cumulative biliary and urinary excretion of irinotecan and its metabolites (SN-38 and SN-38G) over a period of 48 h following administration of irinotecan in two patients ranged from approximately 25% (100 mg/m^2) to 50% (300 mg/m^2) of the initial dose. The biliary excretion of the carboxylate forms of irinotecan and SN-38 and SN-38G is mediated by multiple transporters, including ABCB1 [46], ABCC2 [46], and ABCG2 [47]. The contribution of each transporter differs greatly [48, 49]. This was further supported by increased sensitivity to irinotecan and SN-38 after antisense cMOAT cDNA transfection in the HepG2 cell line [50]. Total clearance of irinotecan is about 14.6 ± 6.4 L/h/m^2, and it does not vary with increased dosage [51]. It has been reported that body mass index, age, and sex may be independent predictors of pharmacokinetic parameters of irinotecan [52].

5.3 Pharmacodynamics

Irinotecan and its active metabolite SN-38 bind to the topoisomerase I-DNA complex and prevent religation of these single-strand breaks. Research suggests that the cytotoxicity of irinotecan is due to double-strand DNA damage produced during DNA synthesis when replication enzymes interact with the ternary

complex formed by topoisomerase I (TopoI), DNA, and either irinotecan or SN-38 [53]. Mammalian cells cannot efficiently repair these double-strand breaks.

SN-38 is approximately 1,000 times as potent as irinotecan as an inhibitor of topoisomerase I purified from human and rodent tumor cell lines. *In vitro* cytotoxicity assays show that the potency of SN-38 relative to irinotecan varies from 2- to 2,000-fold. SN-38G had 1/50 to 1/100 the activity of SN-38 in cytotoxicity assays using two cell lines *in vitro*.

5.4 Toxicity

Expected irinotecan toxic effects include gastrointestinal and hematological complications, such as nausea, vomiting, diarrhea, and infection. The more severe toxicities, namely severe early and late onset diarrhea and neutropenia, occurred in approximately 30% of patients depending on dose and schedule [21]. Fuchs et al. reported 36 and 19% of grade 3/4 diarrhea occurred in patients treated with irinotecan weekly or once every 3 weeks, respectively. Furthermore, grade 3/4 neutropenia was found to occur at a similar rate in both the weekly and once every 3 weeks regimen groups (29 and 34%, respectively) [54]. In treatment combination, the rates of grade 3 and 4 diarrhea have been reported to be approximately 10 and 15% for FOLFIRI and the combination of bevacizumab and FOLFIRI, respectively [55]. Febrile neutropenia, a severe form of neutropenia that requires immediate medical attention, is observed in 3–11% of colorectal cancer patients who underwent FOLFIRI treatment [26, 56].

Diarrhea associated with irinotecan administration is probably the result of the enterocolitis caused by high levels of SN-38 retained for a long period in the intestine [57]. Gupta et al. observed high correlation between glucuronidation of SN-38 and the severity of diarrhea and suggest that modulation of glucuronidation may affect irinotecan therapeutic outcomes [58]. Furthermore, Takasuna et al. demonstrated that the inhibition of the beta-glucuronidase activity in the intestinal microflora may ameliorate the diarrhea caused by irinotecan in rats [59].

Given the potential adverse effects, careful monitoring of the white blood cell (WBC) count with differential, hemoglobin, and platelet count is recommended before each dose of irinotecan. Concomitant medications such as antiemetics, atropine, and loperamide [60] can be given to patients for prophylaxis and/or management of symptoms from treatment. The ASCO guideline for the management of treatment-induced diarrhea can be found in Eng's review [61]. Furthermore, empirical antimicrobial therapy can be given both in case of diarrhea and febrile neutropenia [62]. According to the most recent US guidelines, treatment with colony-stimulating factors is appropriate for patients with a greater than 20% risk of febrile neutropenia [63].

5.5 Pharmacogenetics

Tumor-specific somatic mutations and abnormal gene expression as well as germline genetic variations have been reported to be associated with irinotecan therapeutic efficacy and toxicity [64–68]. However, to date, the role of somatic mutations has not been confirmed to be significant in irinotecan therapeutic outcomes.

Germline DNA variations may affect both pharmacokinetics and pharmacodynamics of irinotecan. The most well-known example is between *UGT1A1* genetic variation and irinotecan-induced toxicity. The *UGT1A* gene locus has been mapped to chromosome 2q37. The entire *UGT1A* locus spans approximately 200 kb. To date, at least ten functional UGT1A proteins are known to be produced from this single gene locus composed of alternative first exons shared with four common exons [69]. The genetic organization of the *UGT1A* gene locus enables a tissue-specific gene expression of hepatic and extrahepatic UGTs and most likely ensures that a broad array of differing substrates can undergo glucuronidation in humans [70]. Genetic variations within the *UGT1A* gene locus are common with over 100 single-nucleotide polymorphisms (SNPs) within the promoter regions and the *UGT1A* coding sequence, many of which are found to be in linkage disequilibrium with each other [70]. A detailed *UGT1A1* allele nomenclature can be found at the following website http://www.pharmacogenomics.pha.ulaval.ca/webdav/site/pharmacogenomics/shared/Nomenclature/UGT1A/UGT1A1.htm (accessed on January 2010).

A case report showed that individuals with Gilbert's syndrome, a benign form of familial hyperbilirubinemia, have an enhanced risk for irinotecan toxicity [71]. This suggests a potential genetic basis for irinotecan-related toxicity in the *UGT*1.1* gene. Iyer et al. demonstrated that UGT1A1 is the isoform responsible for the glucuronidation (inactivation) of the active metabolite of irinotecan, SN-38. These findings indicate a genetic predisposition to the metabolism of irinotecan, suggesting that patients with low UGT1A1 activity, such as those with Gilbert's syndrome, may be at an increased risk for irinotecan toxicity [44]. A small study conducted in Japan supports this hypothesis that *UGT1A1* homozygote 7/7 TATA box genotype is associated with high SN-38/SN-38G metabolic ratio, which leads to impaired SN-38 glucuronidation [72]. Bosma et al. showed that the insertion of an additional repeat (TA_7) is associated with a decrease in *UGT1A1* expression and consequently decreased glucuronidation of its targets [73]. Further investigation showed that this common insertion/deletion in the *UGT1A1* promoter TATA box reduces the transcriptional efficiency of the gene, with an inverse correlation between the number of TA repeats (5, 6, 7, 8 alleles) and transcriptional efficiency [74]. Among these TATA box variations, the 6 allele is classified as *1, while the 7 allele is classified as *28. Homozygosity for the *28 allele is the most common variant associated with Gilbert's syndrome in Caucasians [73]. The 5 allele (*36) and the 8 allele (*37) repeats are rare in Caucasians, but they appear to be more common in African-Americans. Several *UGT1A1* variants are almost exclusively found in Asians. For example, *UGT1A1*6* allele is a nonsynonymous coding variant 211G>A missense in *UGT1A1* exon 1, resulting in glycine 71 alteration to arginine

(G71R) [75]. *UGT1A1*27* (P229Q) has a frequency of ~3% in Asians [76], and *UGT1A1*7* (Tyr486Asp) is very rare [77].

It has been shown that *UGT1A1*28 and *6* polymorphisms correlate with reduced glucuronidation activity toward SN-38 and bilirubin [77, 78]. Individuals who are homozygous for the *UGT1A1*28* allele commonly suffer dose-limiting neutropenia through decreased degradation and clearance of SN-38 [79]. This genotype–phenotype association has been confirmed by multiple studies with various significance and effect size [80]. For example, a prospective study of adult cancer patients who received irinotecan monotherapy demonstrated that patients who carried two (TA_7) alleles had a 50% incidence of grade 4 neutropenia, while those who were heterozygous for (TA_7) or carried no (TA_7) alleles had a 12.5 and 0% incidence, respectively [81]. A recent meta-analysis further established that the incidence of toxicity in *UGT1A1*28* patients was positively correlated with the dose used, as genotype–phenotype association was only significant at medium or high irinotecan treatment doses (>250 mg/m^2, every 3 weeks) [82]. This is further supported by pediatric studies of low-dose irinotecan, which show little association between the **28* allele and toxicity [83, 84]. A recent prospective trial conducted in Italy found that the maximum tolerated dose (MTD) of irinotecan in FOLFIRI is 310 mg/m^2 in patients with the *1/*28 genotype and 370 mg/m^2 in those with the *1/*1 genotype [85]. The relationship between higher irinotecan dose and better treatment efficiency remains to be evaluated; however, data seem to point toward the utility of *UGT1A1**28 genotype as a potential therapeutic guideline in optimizing irinotecan treatment efficacy and minimizing toxicity.

Despite the significant correlation observed between *UGT1A1* genetic variation and irinotecan induced toxicity, the *UGT1A1*28* genetic test has median positive predictive value of 0.5 and median negative predictive value of 0.85 [80]. Despite a high rate of false positives, this test can be useful to reach informed decisions about how to select among alternative effective therapies, like the ones available for metastatic colorectal cancer. Further research efforts have involved the studies of other candidate genes and more recently combination of genetic and nongenetic factors in order to improve the predictive value of the test. For example, genetic variations in other glucuronosyltransferases (e.g., *UGT1A7*, *UGT1A9*) [86–88] and transporters (e.g., *ABCB1*, *ABCC2*, *ABCG2*) [89–92] have also been suggested to contribute to variability in irinotecan pharmacokinetics and toxicity. A principal component analysis to estimate irinotecan pharmacokinetic variation confirmed the role of polymorphisms in *UGT1A1*, *1A7*, and *1A9* in the irinotecan SN-38 pathway, which involves the conversion from irinotecan to SN-38 as well as the enterohepatic recirculation of SN-38 [93]. More recently, the functional significance of *SLCO1B1* variations has been demonstrated. SLCO1B1 plays a significant role in SN-38 transportation [94]. A haplotype variation in *SLCO1B1* (*15) exhibited decreased transport activities for SN-38 *in vitro* [94]. This genetic effect was seen in Asian cancer patients who carry *SLCO1B1*15*, with decreased irinotecan clearance and subsequently increased exposure to irinotecan [95]. Increased irinotecan related toxicities were also observed in lung cancer patients who carry *SLCO1B1* variations [96]. A case report demonstrated a 66-year-old Japanese male with

pharyngeal carcinoma who carried *UGT1A1*6/*28* and *SLCO1B1*15/15* genotypes had extensive SN-38 accumulation and severe irinotecan induced toxicity after one-cycle of treatment [97]. A multivariate analysis showed *SLCO1B1 521TC* or *CC* and *UGT1A1*6/*6* genotypes were independently predictive for grade 4 neutropenia in non-small cell lung cancer patients treated with irinotecan [98]. This was further supported by a combined genetic predictors and patient characteristics model in predicting neutropenia and pharmacokinetics of irinotecan, in which genetic variations in drug transporters (e.g., *ABCC1* and *SLCO1B1*) appear to have a significant impact [99]. Although the additive effect of drug transporter genetic polymorphisms and *UGT1A1* on irinotecan pharmacokinetics and neutropenia has been reported [100], the additional effects of these transporter polymorphisms remain small [101]. Furthermore, the genetic polymorphisms in irinotecan target genes have been extensively evaluated. Recently, a *UBC9 10920CG* genotype was reported to be associated with increased irinotecan sensitivity in non-small cell lung cancer patients although these results have not yet been replicated [102]. Variations in other pharmacodynamic genes (*TOP1*, *PARP1*, *TDP1*, and *XRCC1*) were not associated with irinotecan-induced neutropenia, despite initial positive findings [103, 104]. Larger studies, either candidate gene or genome-wide association, are required for both hypothesis generation and replication.

5.6 Pharmacoethnicity

The frequency of the *UGT1A1 *1/*1*, **1/*28*, and **28/*28* genotypes varies greatly among different ethnic groups [80]. In Caucasians, genotype frequencies are 56, 28–36 and 9–17%, respectively. In Africans, they are 13–30, 38–50, and 17–33%, respectively [105]. In Asians, they are 65–84, 15–31, and 1–4%, respectively; while the *UGT1A1*6* allele is almost exclusively found in Asians (~20%) [75]. Han et al. suggested that *UGT1A1*28* testing alone may not be sufficient to predict irinotecan-induced toxicity in patients of Asian origin. Instead, a combined *28 and *6 test is more appropriate [87, 106]. In addition, it is well established that not only allele frequencies but also the composition of haplotype blocks is influenced by the population structure [107]. In Japanese patients, genetic linkage has been reported between *UGT1A1*6* and *UGT1A7* and *1A9* polymorphisms [108]. This linkage disequilibrium seems common in Asians in general. High variability of alleles and haplotypes of *UGT1A1*, *1A6*, and *1A7* was observed in the São Miguel Island population with a strong interaction between functional polymorphisms related to the alteration of the UGT enzyme activity [109]. In fact, *UGT1A* haplotype-based approach has been shown to be an efficacious strategy to predict FOLFIRI treatment outcomes [110].

Racial disparities have been observed in tumor response rate and severe adverse events in Caucasian and African-American colorectal cancer patients after adjusting for age, sex, performance status, and dose-intensity [111]. In a NCI-sponsored trial (N9741), significant racial differences in the distribution of polymorphisms in key

candidate genes were also observed between races, suggesting that this disparity may be in part due to varied genetic frequency in different ethnic groups. Interestingly, grade ≥3 toxicity was higher in whites (48%) than in blacks (34%), largely due to the higher rate of grade 3 diarrhea in the white patients, despite the fact that the *UGT1A1*28* genotype is more common in blacks than whites. The authors concluded that a single genotypic difference is unlikely to account for the observed racial variation in adverse effects and response rate; rather, if these differences are genetically determined, they are likely mediated by a complex interplay of genotypes. In contrast to their findings, Gupta et al. reported the lack of race and sex effects on the plasma availability of irinotecan, SN-38, and SN-38G as well as in the incidence and severity of toxicity when treating cancer patients with single-agent irinotecan [51]. Given the variation in genetic composition of UGTs in different ethnic groups, it is plausible that similar phenotypic outcomes may be produced by different genetic profiles. Nonetheless, it would be ideal to incorporate a wide range of ethnic groups to address unequally distributed alleles.

5.7 Clinical Utility of *UGT1A1* Genotyping

Based on pharmacogenetic evidence, the FDA approved the addition of a warning to the irinotecan label (June 2005) (http://www.fda.gov/medwatch/SAFETY/2005/Jun_PI/Camptosar_PI.pdf) [112]. The label warns that homozygosity for *UGT1A1*28* is a risk factor for severe neutropenia and that patients with this genotype should be treated with a reduced starting dose of irinotecan. A commercial genetic test (the Invader UGT1A1 Molecular Assay [Third Wave Technologies]) was also approved by the FDA in 2005 for the detection of *UGT1A1*28*, a first for any chemotherapy agent [113–115]. However, other *UGT1A1* variants (e.g., *6) that also result in reduced enzyme activity in Asians have not been included in the label warning or in the *UGT1A1* Invader assay. Baseline serum bilirubin level has been evaluated in predicting toxicity or efficacy among patients receiving irinotecan for metastatic colorectal cancer. Although modest elevations of bilirubin are associated with increased grade 3–4 neutropenia in patients treated with weekly irinotecan, baseline serum bilirubin does not reliably predict overall irinotecan-related toxicity or efficacy [116]. Later additional methods, including DNA sequencing and fragment analysis, have been compared to the Invader assay. All three methods were valuable for genotyping the *UGT1A1* (TA)n repeat, with the sequencing and size-based assays having the fewest drawbacks [117]. O'Dwyer et al. have proposed a practical approach in the utility of *UGT1A1*28* allele testing in the clinic [118]. Currently, the National Comprehensive Cancer Network (NCCN) practice guideline in oncology for colon and rectal cancers states, "Irinotecan should be used with caution and with decreased doses in patients with Gilbert's disease or elevated serum bilirubin. There is a commercially available test for *UGT1A1*. Guidelines for use in clinical practice have not been established" [24].

The discovery and validation of *UGT1A1* genetic variants and the establishment of useful genotyping methods in predicting irinotecan-related toxicity were a paradigmatic success in pharmacogenetic research and translational work. However, *UGT1A1* genotyping is not routinely performed in predicting irinotecan toxicity in current clinical practice. For example, Gardiner surveyed Australian and New Zealand laboratories that offered genetic testing and found very few performing pharmacogenomic testing [119]. Corkindale et al. looked at reasons why pharmacogenetic tests are not used and listed a number of factors [120]. Among them were lack of a clinical authority to use for interpretation, lack of peer recognition of the tests, no understanding of the cost implications of the test, and difficulty in getting practical information about the tests. In a more recent review, issues for the clinical laboratories were also pointed out, including the availability of FDA-cleared tests, the absence of reimbursement codes, the need for genotyping accuracy, and the need to find clinical expertise to interpret laboratory results [121].

Low allelic penetrance, heterogeneity in patient populations and treatment regimens, unaccounted gene–environment interactions, and differences in outcome measures across studies hamper the precise assessment of the clinical performance of the UGT1A1 genotyping test [80]. Furthermore, a complex trait like drug response is likely a result of many factors, with the combination of genetic and environmental contributions. There are many appropriate explanations for the low usage of the *UGT1A1* genotyping test [115]. For example, although genotyping has been consistently associated with hematological toxicity induced by higher doses of irinotecan, the risk is reduced at the lower doses used in combination regimens [82]. The difficulty in interpreting different genetic markers in different ethnic groups has limited the utility of *UGT1A1* genetic testing in irinotecan toxicity prediction. Furthermore, the irinotecan package insert advises that a reduction in the starting dose by at least one level should be considered for patients known to be homozygous for the *UGT1A1*28* allele. However, the precise optimal dose reduction in this patient population is not known. In fact, the prognostic impact of *UGT1A1*28* has not been established [122]. To date, no studies have been performed to demonstrate preserved efficacy in irinotecan dose reduction. Another concern is the lack of established reimbursement and the lack of education of clinicians about the potential value (and limitations) of testing [112]. In addition, to answer the question of how *UGT1A1* testing could add to the safety or efficacy of irinotecan as compared to the current protocol of adjusting drug dose on the basis of standard clinical tests such as WBC counts [123], pharmacoeconomic evaluation is needed.

5.8 Conclusion

Understanding the reasons for treatment failure and developing an ability to predict those patients who would benefit the most (and least) remain important aims in medicine [124]. Germline variations in the *UGT1A1* gene locus have an impact on

irinotecan therapy induced toxicity, and this information has been acknowledged by the FDA on the irinotecan label. However, the results of a genetic test need to be integrated in the context of the clinical picture of each individual patient. The development of pharmacogenetic models of drug toxicity risk must include independent variables related to the characteristics of the patients, the disease, concomitant medications, and other variables. The data accumulated so far and the information added to the revised label suggest that the *UGT1A1*28* testing will not be a mandatory test in the clinic but, rather, its use will be at the discretion of the treating physician, at least in the short term [80]. The future lies in the discovery of additional genetic and nongenetic markers through genome-wide association studies and combination of germline and cancer pharmacogenetics to establish more precise irinotecan toxicity and efficacy prediction models.

Acknowledgments The authors would like to thank Dr. Ryan Munoz for critical review of this chapter.

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Chapter 6
Pharmacogenomics of Tamoxifen

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Keywords Estrogen receptors • Adjuvant therapy • Breast cancer • Endoxifen

6.1 Introduction

Tamoxifen is used in the treatment of estrogen receptor (ER) positive breast cancers and in breast cancer prophylaxis for high-risk women [1, 2]. The benefits of tamoxifen are apparent, as the drug successfully reduces rates of recurrence and mortality in patients with ER-positive breast cancer [3]. However, these benefits are not without risk; adverse effects range from hot flashes to endometrial cancer and life-threatening thromboembolism [2]. The use of genetic information to predict response to tamoxifen therapy holds the potential to improve therapeutic outcome while minimizing toxicity. This chapter will discuss aspects of tamoxifen pharmacology and pharmacogenetics, with case studies to explore the clinical utility of genotype testing in tamoxifen therapy.

6.2 Tamoxifen Pharmacology

Tamoxifen is the best-known selective estrogen receptor modulator (SERM), a family of drugs, which also includes toremifene and raloxifene. SERMs affect ER activation to alter gene transcription and other sequelae of ER function [4]. Tamoxifen is FDA-approved for the treatment of breast cancer in both men and women, from carcinoma in situ to metastatic disease [5]. It is also approved for breast cancer

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_6,

prophylaxis in high-risk women. Off-label uses include treatment of infertility from anovulation or oligospermia, prevention of osteoporosis, and therapy for gynecomastia and precocious puberty.

Tamoxifen is a competitive inhibitor of estrogen binding to the ER [6], but its pharmacological activity varies between tissues and is thought to depend on the profile of transcriptional coactivators expressed [7]. In the breast, tamoxifen acts as an ER antagonist, disrupting estrogen binding and turning off ER-mediated proliferation and survival signals. In contrast, both bone and the endometrium respond to tamoxifen as an ER agonist, resulting in increased bone density and endometrial proliferation [8]. Tamoxifen also has estrogen-like effects on serum lipid profiles although no long-term cardiovascular benefit has been reported [9].

Tamoxifen is given orally and is well absorbed, with a concentration peak occurring 3–7 h after dose administration [4]. Common side effects include hot flashes, nausea, and breakthrough vaginal bleeding in postmenopausal women; these rarely necessitate cessation of tamoxifen therapy. More serious adverse responses have been reported, however, such as increased incidence of thromboembolic events and endometrial cancer. Such adverse responses may contribute to the finding that therapy with tamoxifen beyond 5 years fails to provide further benefit to breast cancer patients and may worsen outcome with additional use [7]. For these reasons, long-term tamoxifen use is generally limited to patients in whom the benefits outweigh the risks, such as those diagnosed with ER-positive breast cancer, or women at high risk of developing breast cancer.

The drug is extensively metabolized, with some metabolites possessing equal or greater antiestrogenic activity compared to the parent drug. The hepatic cytochrome P450 (CYP) family is largely responsible for tamoxifen biotransformation; the major enzymes involved include CYP2D6 and CYP3A4/5, with lesser contributions from CYP2C9 and CYP2C19 (Fig. 6.1) [10–14]. Removal of a methyl group by CYP3A4/5 creates the major metabolite *N*-desmethyltamoxifen (NDT), whereas hydroxylation by CYP2D6 results in formation of 4-hydroxytamoxifen (4-OH-TAM). Sequential metabolism by both pathways creates the highly active metabolite 4-hydroxy-*N*-desmethyltamoxifen, commonly known as endoxifen. Conversion to NDT accounts for roughly 90% of a tamoxifen dose and accumulates to higher concentrations than the parent drug, while 4-OH-TAM and endoxifen are present in much lower quantities, less than 10% of total tamoxifen metabolism [14, 15]. NDT displays approximately the same activity as the parent drug; in contrast, both 4-OH-TAM and endoxifen have much greater affinity for the ER and are 30- to 100-fold more potent inhibitors [16–18]. In most individuals, endoxifen concentrations are six- to tenfold greater than 4-OH-TAM levels, thus it is now considered the primary active metabolite of tamoxifen [13, 19, 20].

The existence of several active metabolites and potential for metabolic variability suggest that tamoxifen would be an excellent candidate drug for therapeutic drug monitoring (TDM). However, due in part to the fact that few laboratories are currently capable of measuring tamoxifen, NDT, 4-OH-TAM, and endoxifen, TDM applications remain limited and therapeutic ranges are not well established [21, 22]. Given the multi-year administration protocols found to be optimal for tamoxifen

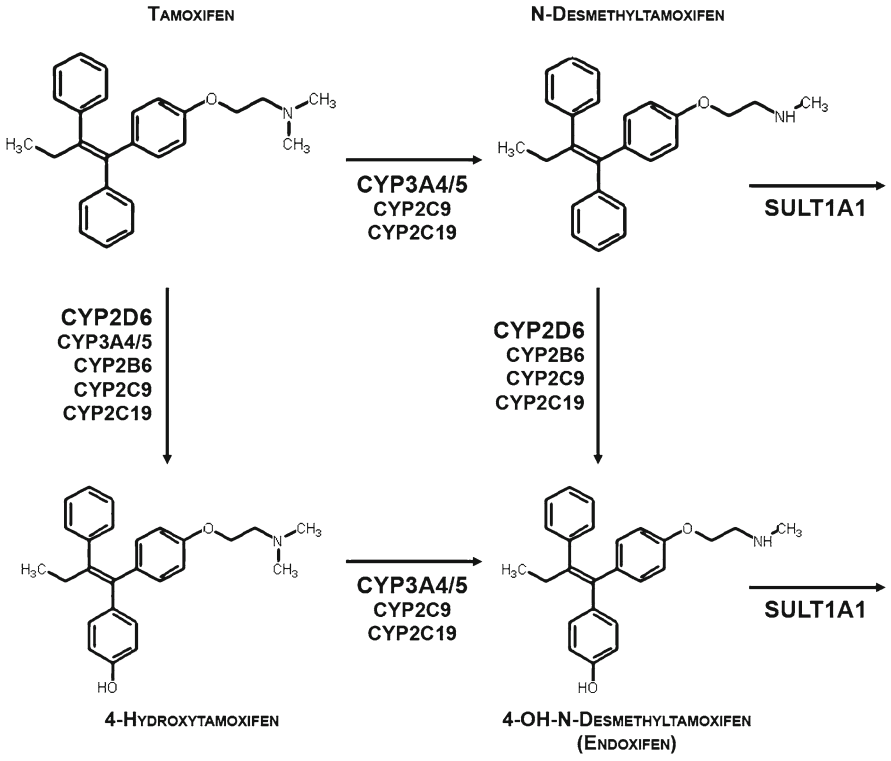

Fig. 6.1 Metabolism of tamoxifen to endoxifen

therapy, it is expected that expansion of TDM to routine practice would permit more accurate assessment of long-term compliance and might improve patient response.

For patients with poor response or contraindications to tamoxifen therapy, alternative hormonal therapy does exist. Raloxifene, for example, is another SERM that has been shown to be effective, or better than tamoxifen for use in breast cancer prophylaxis and prevention of osteoporosis [7]. For breast cancer therapy, the use of aromatase inhibitors is recommended for patients who cannot tolerate tamoxifen, or who have already received tamoxifen for 5 years. Aromatase inhibitors include anastrozole, exemestane, and letrozole, and function by disrupting endogenous synthesis of estrogen [4]. These agents are generally associated with lower incidence of adverse responses, with therapeutic efficacy that is comparable to or, in some settings, better than that of tamoxifen.

Tamoxifen is likely to remain a mainstay of hormonal therapy for breast cancer, but there remains much opportunity for optimization of its use. The metabolic pathways responsible for conversion of the drug to its active metabolites are highly variable, and it is becoming apparent that differences in an individual's ability to form endoxifen contribute significantly to therapeutic response and patient outcome.

Tamoxifen pharmacogenetics is therefore an area of intense study, with the goals of using germline polymorphisms in genes encoding metabolic enzymes to optimize dosing and to select individuals most likely to respond to tamoxifen therapy.

6.3 Tamoxifen Pharmacogenetics

6.3.1 CYP2D6 Polymorphisms

The enzymatic activity of CYP2D6 varies greatly between individuals, due in part to the high frequency of polymorphisms in the *CYP2D6* gene. Over 100 *CYP2D6* variant alleles have been described (www.cypalleles.ki.se, accessed October 20, 2009). The resulting enzymatic activity allows individuals to be categorized into poor (PM), intermediate (IM), extensive (EM), and ultrarapid (UM) metabolizers. There are significant ethnic differences in the frequency of *CYP2D6* variants; one of the most important PM alleles, *CYP2D6*4*, is present in 12–21% of individuals of Northern European descent, but is found in only 1–2% of Asians and Black Africans [23]. Variants can be quite common, as seen with *CYP2D6*10*, which confers an IM phenotype and is present in 57% of Han Chinese [24, 25]. The major CYP2D6 alleles include fully functional *CYP2D6*1*; null alleles with essentially no residual activity (*3-*8, *11-*16, *18-*20, *38, *40, *42, *44); reduced-function alleles (*9, *10, *17, *29, *36, *37, *41); and amplified alleles comprised of multiple copies of the gene (*1XN, *2XN, *35XN, and *41XN).

The influence of *CYP2D6* genotype in tamoxifen metabolism has been shown in a number of studies. During tamoxifen treatment, women with two or more fully functional copies of *CYP2D6* have higher plasma endoxifen concentrations than patients with at least one null allele (**3-*6*), or those taking known CYP2D6 inhibitors [26], which suggests that CYP2D6 function is essential for optimal conversion of tamoxifen to its highly active metabolites. Analysis of *CYP2D6* genotype and endoxifen plasma concentration in 158 patients from multiple ethnicities demonstrated that patients with IM genotypes, e.g., **10* (reduced activity) or **4* (null) heterozygotes, had endoxifen concentrations similar to PM. Similarly, a Chinese study demonstrated that patients homozygous for *CYP2D6*10* had lower serum concentrations of 4-hydroxytamoxifen [27]. All these findings support the utility of genotyping *CYP2D6* to predict formation of highly active tamoxifen metabolites in patients considering or undergoing therapy with tamoxifen.

The influence of *CYP2D6* variant alleles has also been documented in terms of treatment outcome. A retrospective study of 223 postmenopausal women examined **4* (the most common null allele associated with PM status) and **6* (a low-frequency PM variant) [28]. Women homozygous for *CYP2D6*4* had poorer outcomes than women with **4/*1* or **1/*1* genotypes, showing shorter time to relapse and worse disease-free survival. In addition, despite 20% incidence of moderate to severe hot flashes in women with zero or one **4* allele, no *CYP2D6*4/*4* patients experienced

this side effect. Borges et al. confirmed these results and expanded the analysis to include 33 different CYP2D6 variants [29]. Patients with reduced-activity *CYP2D6* alleles (**4*, **5*, **10*, **41*) had significantly poorer outcome than carriers of functional alleles, as documented by higher recurrence rates, shorter times to relapse, and worse event-free survival. Studies looking at the association of *CYP2D6*10* with clinical outcomes in Asian patients showed that patients with this IM variant have a shorter recurrence-free survival period [27, 30].

Recently, the findings of a multicenter study that included 1,325 women treated with tamoxifen for early stage breast cancer were published [31]. The study included 609 women with EM, 637 women with IM, and 79 women with PM *CYP2D6* genotypes. The recurrence rates were 15% for EM, 21% for EM/IM heterozygotes, and 29.0% for PM. This is the largest published study so far that provides sufficiently powered evidence for an association between *CYP2D6* genetics and clinical outcome of tamoxifen. This data indicates that individuals with *CYP2D6* variants conferring PM status have a substantially higher risk of tamoxifen treatment failure, and in these patients alternative forms of adjuvant endocrine therapy should be considered.

6.3.2 CYP2D6 Inhibitors

Hot flashes, a common side effect of tamoxifen treatment, are often treated with antidepressants such as the selective serotonin reuptake inhibitors (SSRI) [2]. Coadministration of the SSRIs paroxetine or fluoxetine, both of which potently inhibit CYP2D6 activity, affects metabolism in tamoxifen-treated patients: CYP2D6 EM individuals show a significant reduction of endoxifen levels when these SSRIs are added to tamoxifen therapy [26, 29]. In contrast, other SSRIs that are only weak inhibitors of CYP2D6, such as venlafaxine, did not significantly affect endoxifen levels, and thus may be preferable for the treatment of hot flashes in breast cancer patients [26, 32]. However, coadministration of any CYP2D6 inhibitors, be they strong (e.g., fluoxetine, paroxetine, bupropion, quinidine) or weak (e.g., sertraline, duloxetine, cimetidine, terbinafine, amiodarone), has the potential to lower endoxifen plasma concentrations [26, 29, 33], and may render tamoxifen less effective [34, 35]. In fact, strong CYP2D6 inhibitors have been shown to reduce endoxifen concentrations in CYP2D6 EM to drug levels comparable to those seen in CYP2D6 PM [26]. This is referred to as a phenocopy, i.e., a phenotype induced by environmental factors (in this case, enzymatic activity reduced by comedications), which mimics a different genotype.

It has been shown that the phenocopying due to the coprescription of CYP2D6 inhibitors was an independent predictor of breast cancer outcome in postmenopausal women taking tamoxifen [36] and of reduction in endoxifen plasma concentrations [37]. A recent study assessed the combined effect of genetic variation and drug-induced inhibition of CYP2D6 on breast cancer outcomes [35]. In this analysis, patients were segregated according to whether potent or weak/moderate,

CYP2D6 inhibitors were coprescribed with tamoxifen. Based on the genotype and medication history, patients were classified as having an extensive (normal) or decreased CYP2D6 metabolism. Patients with decreased metabolism had significantly shorter time to recurrence and worse relapse-free survival than patients with extensive metabolism. CYP2D6 genotype and concomitant potent CYP2D6 inhibitors are highly associated with endoxifen plasma concentration and may have an impact on the response to tamoxifen therapy [29]. All together, these studies indicate that coadministration of potent CYP2D6 inhibitors should be avoided in patients taking tamoxifen as they might jeopardize the success of the treatment.

Although SSRIs are frequently coadministered, many other drugs have been reported to inhibit the CYP2D6 enzyme system (http://medicine.iupui.edu/clinpharm/ddis/table.asp). The vast majority of theses drugs are prescription drugs, but at least two CYP2D6 inhibitors are also know for their potential as drugs of abuse, namely methadone and cocaine. Indeed, cocaine appears to have a lower inhibition constant [38] than do paroxetine and fluoxetine [39, 40], suggesting it is a more potent inhibitor of CYP2D6. It has also been suggested that 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) has a similar inhibition constant to fluoxetine [41].

The use of drugs of abuse, prescribed and nonprescribed medications, herbal supplements, and some foods can inhibit or induce enzymatic activity manyfold, to the extent that a PM phenocopy may be induced, regardless of genotype. The complexity of tamoxifen metabolism precludes the use of a probe drug for phenotyping studies, thus measurement of the parent drug and its highly active metabolites may prove a critical means of addressing both the interindividual variability and the efficacy of tamoxifen therapy in breast cancer patients.

6.3.3 Role of Other Polymorphisms in Tamoxifen Treatment Outcome

Variants in genes encoding other metabolic enzymes have also been studied for associations to tamoxifen metabolism and outcome, including *CYP3A*, *CYP2C9*, *CYP2C19*, and *SULT1A1*. Conversion of tamoxifen to NDT is primarily mediated by CYP3A4/5 [14], but the significance of polymorphisms in the genes encoding these isoforms remains unclear. The null allele variant *CYP3A5*3* was not associated with any statistically significant differences in plasma concentrations of tamoxifen or its metabolites [26, 42]. Similarly, no differences in time to relapse, disease-free survival or overall survival have been observed in CYP3A5*3 individuals, suggesting minimal influence on patient outcome [28]. However, Wegman et al. reported that breast cancer patients with a CYP3A5*3/*3 genotype show improved recurrence-free survival, an unexpected result given that this null genotype should reduce formation of NDT, the precursor of endoxifen [43].

CYP2C9 and CYP2C19 are also capable of catalyzing formation of NDT, though to a lesser extent [12, 14, 15]. An investigation of *CYP2C19* variants **2*, **3* (both null alleles), and **17* (which confers UM status) examined the association of this gene with tamoxifen treatment outcome. Compared to the reference *CYP2C19*1* allele, patients with the UM **17* allele had lower risk of relapse and prolonged time to relapse, whereas the two null variants **2* and **3* were not associated with differences in treatment outcome [44]. Similarly, the presence of low-activity *CYP2C9* variants (**2* and **3*) affected neither patient outcome nor tamoxifen metabolite concentrations [26, 44].

The Phase II enzyme SULT1A1 causes formation of sulfated tamoxifen metabolites [45]. The role of this enzyme in enhancing drug clearance would suggest that reduced SULT1A1 activity would slow clearance of active tamoxifen metabolites, and thus improve treatment efficacy [46]. Despite this, no significant differences in plasma concentrations of tamoxifen and its metabolites were found in patients with the low-activity *SULT1A1*2* allele [26]; indeed, some reports indicate that tamoxifen is less effective in patients with the **2* variant, with increased risk of cancer recurrence and death [46–48]. Despite this, a study of 677 tamoxifen-treated postmenopausal women found no association between *SULT1A1* genotype and either improved or worsened treatment outcome [43, 48].

6.4 CYP2D6 Genotyping and Tamoxifen: Clinical Practice and Case Studies

Current evidence strongly suggests that knowledge of the *CYP2D6* genotype may be beneficial when selecting a breast cancer treatment. An FDA advisory panel has suggested that the tamoxifen package insert should alert physicians of the following concerns: first, that *CYP2D6* PM patients are at increased risk for recurrence of their breast cancers if treated with tamoxifen, and second, that coadministration of certain SSRIs known to inhibit CYP2D6 can affect the metabolism of tamoxifen [49].

Knowledge of patient genotype can guide selection of alternate therapies: for example, in postmenopausal PM individuals diagnosed with breast cancer, aromatase inhibitors are a reasonable alternative to tamoxifen treatment. Randomized clinical trials in postmenopausal breast cancer patients have demonstrated superior efficacy and better overall safety for aromatase inhibitors as compared with tamoxifen [50]. For breast cancer prevention, raloxifene appears to be a viable alternative for *CYP2D6* PM; raloxifene and tamoxifen have been shown to be equally effective in reducing breast cancer incidence in high-risk postmenopausal women [51, 52].

The following case studies highlight representative examples of the clinical utility of genotyping *CYP2D6* in patients receiving or considering tamoxifen therapy.

6.4.1 Case 1

6.4.1.1 Presentation

A 69-year-old female presented after palpating a mass within her right breast, which was biopsied and diagnosed as infiltrating ductal carcinoma. She underwent right wide local excision. Findings confirmed an infiltrating ductal carcinoma, Nottingham grade III, forming a 1.8-cm mass in greatest dimension. Angiolymphatic invasion was negative, and all margins were negative. A single right axillary sentinel lymph node was negative for metastatic disease. The tumor cells were strongly positive for ER (greater than 75%) and PR (51–75%), and were HER-2 negative.

6.4.1.2 Therapy

Following surgery, the patient received adjuvant radiation but declined adjuvant chemotherapy. Five years' treatment with anastrozole was recommended; however, due to financial constraints, the patient requested tamoxifen. *CYP2D6* testing determined the patient's genotype to be *CYP2D6 *1/*1*, and she was placed on tamoxifen, 20 mg daily. After 3 months, she returned with significant vasomotor symptoms (hot flashes) leading to insomnia and decreased quality of life. She requested to go off tamoxifen. She was counseled against discontinuation and instead was prescribed Venlafaxine XR, 75 mg once daily, which improved her hot flashes substantially.

6.4.1.3 Discussion

Prospective genotyping of this patient confirmed her EM status, indicating that she is likely to be able to convert tamoxifen to its highly active metabolites. Activation of tamoxifen is suggested by the patient's presentation with hot flashes: this symptom is less common in patients with PM alleles [35] and may be associated with clinical outcome [53], although it is not recommended for use as a predictor of tamoxifen response [54]. Venlafaxine has been shown to be effective in relief of tamoxifen-induced hot flashes [55].

6.4.2 Case 2

6.4.2.1 Presentation

A 48-year-old, premenopausal female underwent a left skin-sparing mastectomy for a 1.3-cm Nottingham grade II/III invasive ductal carcinoma. Angiolymphatic

invasion was negative, and all margins were negative. One sentinel lymph node was found to be involved with metastatic carcinoma although an additional 27 axillary nodes were dissected and shown to be negative. Estrogen and progesterone receptors were strongly positive (greater than 75% nuclear staining), and HER-2 was negative.

6.4.2.2 Therapy

After surgery, the patient received doxorubicin and cyclophosphamide followed by paclitaxel using the dose-dense schedule. Following the completion of chemotherapy, the patient was rendered amenorrheic. A recommendation was given for adjuvant tamoxifen, 20 mg daily, for 5 years. The patient experienced minimal to no hot flashes while on tamoxifen. After 1.5 years on tamoxifen, she requested a *CYP2D6* genotype test. The results demonstrated *CYP2D6 *4/*4*, consistent with a CYP2D6 poor metabolizer. She was then switched to anastrozole, and shortly thereafter developed moderate vasomotor symptoms and arthralgias not requiring pharmacotherapy. She completed a total of 5 years of adjuvant anastrozole.

6.4.2.3 Discussion

This patient's status as a PM was unknown at the time of tamoxifen initiation. In the absence of *CYP2D6* genotype testing, it is likely that she would have completed the 5-year regimen as planned, thus increasing her likelihood of recurrence. The availability of genotypic information permitted selection of a more appropriate adjuvant endocrine therapy.

6.4.3 Case 3

6.4.3.1 Presentation

A 57-year-old female presented with new asymmetry in the right breast on a routine mammogram. A needle biopsy demonstrated invasive lobular carcinoma, for which she underwent wide local excision and sentinel lymph node biopsy. The sentinel node biopsy was positive, and she underwent a complete right axillary lymph node dissection. Pathology demonstrated a 4.0 cm invasive lobular carcinoma in greatest dimension, Nottingham grade I, with 8 of 13 lymph nodes positive for metastatic carcinoma. Angiolymphatic invasion was negative, and all margins were negative. The tumor cells were ER and PR positive (51–75% nuclear staining) and HER-2 negative.

6.4.3.2 Therapy

The patient received postoperative doxorubicin and cyclophosphamide followed by paclitaxel using the dose-dense schedule, followed by adjuvant chest wall radiation. A recommendation was given for adjuvant anastrozole; however, the patient refused because of concerns regarding osteoporosis. A bone mineral density demonstrated osteopenia, with a total hip *T*-score = −2.1. The patient's *CYP2D6* genotype was obtained and determined to be *CYP2D6*1/*2A*. She began tamoxifen therapy, but after 2.5 years could not tolerate the drug and was switched to anastrozole. After 6 months of anastrozole, the patient had developed disabling arthralgias, carpal tunnel syndrome, and hair thinning; she thus discontinued anastrozole and went back on tamoxifen. After 5 total years of adjuvant hormonal therapy, a recommendation was given to switch to letrozole.

6.4.3.3 Discussion

A heterozygous **1/*2A* genotype suggests that this patient's metabolic phenotype would be in the range of extensive to ultra-rapid. Such individuals would have successful conversion of tamoxifen to active metabolites, but may in fact be predisposed to adverse responses due to the increased efficiency of drug activation. However, the use of tamoxifen is not contraindicated in such patients, so long as the side effects are tolerable or can be managed with SSRIs or other therapeutics.

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Chapter 7
Thiopurines

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Keywords Thiopurine methyltransferase • Leukemia • Inflammatory bowel disease • Azathioprine • Mercaptopurine • Thioguanine

7.1 Introduction

Thiopurines are antimetabolite prodrugs used clinically as antineoplastics and as immunosuppressants. The first clinically effective thiopurines, thioguanine and mercaptopurine, were developed in the 1950s by Wellcome Research Laboratory scientists Gertrude Elion and George Hitchings [1]. After the approval of mercaptopurine in 1953 by FDA for the treatment of leukemia, it was discovered that when it was combined with other anticancer agents, childhood leukemia could be cured. The development of these compounds contributed to Elion and Hitchings being awarded the Nobel Prize in medicine in 1988. Today, there are three thiopurine drugs (mercaptopurine, thioguanine, and azathioprine) (Fig. 7.1) that are widely used in the treatment of leukemia, rheumatic diseases, inflammatory bowel disease, and solid organ transplant.

7.2 Pharmacology of Thiopurine Drugs

7.2.1 Clinical Indications and Target Patient Populations

For more than 50 years, mercaptopurine has been used as part antileukemic maintenance therapy in the treatment of childhood acute lymphoblastic leukemia (ALL) and has contributed to the high cure rates achieved (>80%) [2]. Thioguanine

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_7,

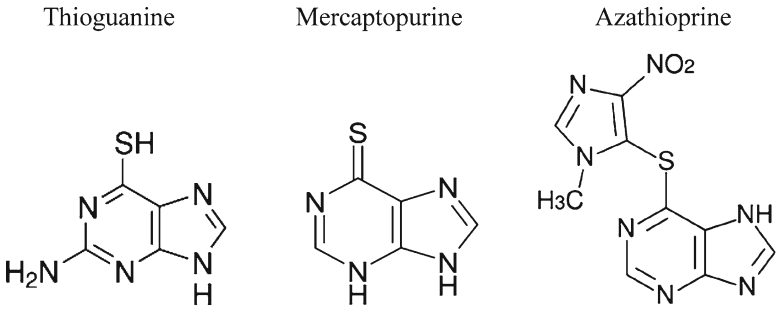

Fig. 7.1 Chemical structures for the clinically used thiopurine drugs

is indicated in the treatment of myelogenous leukemia, while azathioprine is indicated as adjunct therapy in solid organ transplant and in rheumatoid arthritis (Table 7.1). Although the labeled indications for these agents are limited, there are many unlabeled investigational uses that have proven to be beneficial.

Both azathioprine and mercaptopurine have shown great promise in the treatment of inflammatory bowel disease (i.e., Crohn's disease, ulcerative colitis) by lowering steroid requirements and prolonging remission [3]. In Crohn's disease, azathioprine has been shown to prevent relapse as well as decrease the dependence on and resistance to steroids [4, 5]. These agents have also proven to be beneficial in ulcerative colitis therapy through effectively inducing remission in as many as 70% of patients [6]. In autoimmune hepatitis, when azathioprine is combined with corticosteroids, the 20-year life expectancy increases to 80% and the incidence of hepatic fibrosis can decrease by as much as 79% as compared to corticosteroids alone [7]. Azathioprine has also been used to treat inflammatory eye conditions to include uveitis and dysthyroid orbitopathy [8].

7.2.2 Pharmacodynamics/Pharmacokinetics

Thiopurines are purine analogs (Fig. 7.1) requiring intracellular activation to exert their cytotoxic effects. Mercaptopurine (a hypoxantine analog), azathioprine (a prodrug of mercaptopurine), and thioguanine (a guanine analog) (Fig. 7.1) are all subject to activation by hypoxanthine phosphoribosyl transferase (HPRT) and other enzymes to form the cytotoxic thioguanine nucleotide (TGN) metabolites or inactivation by thiopurine methyltransferase (TPMT) (Fig. 7.2).

TGN metabolite incorporation into DNA constitutes the primary mechanism of thiopurine cytotoxicity. Thioguanine is more directly converted to TGNs bypassing many of the enzymatic steps that are required for mercaptopurine activation (Fig. 7.2). As the predominant inactivation pathway, TPMT catalyzes the S-methylation of thiopurine drugs. Unlike thioguanine, mercaptopurine (and azathioprine) can also undergo inactivation by xanthine oxidase.

Table 7.1 Labeled indications for clinically used thiopurines

Drug (brand name)	Clinical indications	Dosage[a]	Place in therapy	Toxicities	Monitoring considerations
Mercaptopurine (Purinethol®)[b]	Acute lymphoblastic leukemia	Induction: 70–100 mg/m^2/day (children); 100–200 mg/m^2/day (adults)	Remission induction; maintenance therapy	Bone marrow suppression, hepatotoxicity, renal toxicity, gastrointestinal toxicity, hypersensitivity reactions	CBC with differential and platelet count, serum transaminases, alkaline phosphatase, bilirubin, urinanalysis, hemoglobin, and hematocrit
	Acute myelogenous leukemia	Maintenance: 50–75 mg/m^2/day (children); 80–100 mg/m^2/day (adults)			
Thioguanine (Tabloid®)[c]	Acute myelogenous leukemias; chronic myelogenous leukemias; granulocytic leukemias	Children <3 years: 3.3 mg/kg/day Children >3 years and Adults: 2–3 mg/kg/day	Remission induction; consolidation; maintenance therapy		
Azathioprine (Imuran®)[d]	Kidney transplant; solid organ transplant (non-renal)	Initial: 3–5 mg/kg/day Maintenance: 1–3 mg/kg/day	Adjunct therapy to prevent transplant rejection		
	Rheumatoid arthritis	Initial: 1 mg/kg/day × 6–8 weeks, increased by 0.5 mg/kg every 4 weeks until response or up to 2.5 mg/kg/day Maintenance: reduce by 0.5 mg/kg every 4 weeks until lowest effective dose is reached	Disease modifying antirheumatic agent		

[a]Actual dosages may vary 2–3 fold from label doses. Many uses of thiopurines are "off-label"

[b]Labeling for Purinethol as of August 2003

[c]Labeling for Tabloid as of June 2009

[d]Labeling for Imuran as of May 2008

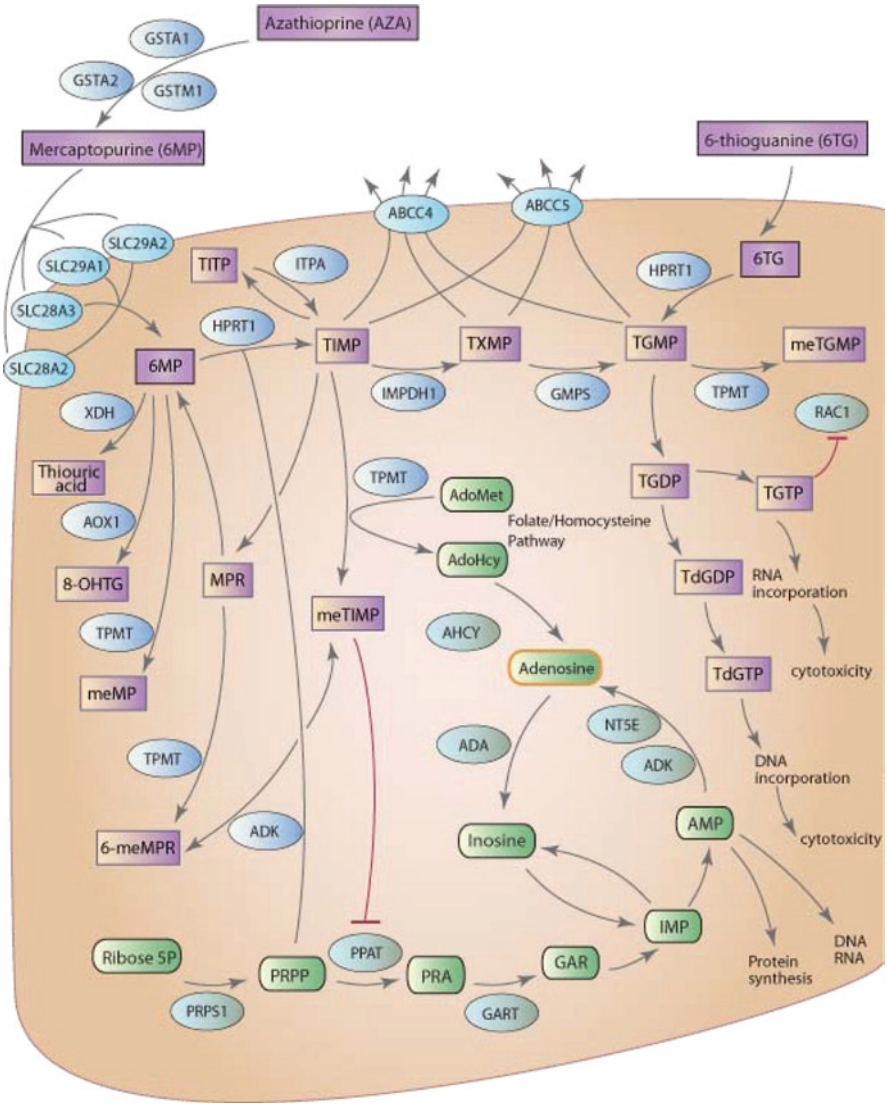

Fig. 7.2 Thiopurine pathway, reproduced with permission www.pharmgkb.org. Azathioprine is a prodrug that is metabolized to mercaptopurine; mercaptopurine is susceptible to direct inactivation via methylation by the polymorphic enzyme TPMT, leaving more substrate available for anabolism to the active metabolites, TGNs (thioguanine nucleotides). The secondary mercaptopurine metabolite, thioinosine monophospate, can also be methylated by TPMT, leading to the formation of methylmercaptopurine nucleotides (MeTIMP), which have some antitumor and immunosuppressant properties. Thioguanine is also directly methylated by TPMT to methylthioguanine and is more directly metabolized to TGN, with no secondary methylated active metabolite analogous to MeTIMP

TPMT can form another toxic metabolite, methylthioinosine monophosphate (meTIMP) or methylmercaptopurine nucleotides (MeMPN), which may exert its cytotoxic effects through inhibition of de novo purine synthesis [9, 10]. It is unclear to what extent meTIMP metabolites contribute to the overall cytotoxicity of mercaptopurine; active methyl metabolites do not exist for thioguanine (Fig. 7.2). TPMT is subject to a few well-studied deactivating genetic polymorphisms that are responsible for significant interpatient variability in response to thiopurine therapy [11]. When TPMT enzymatic activity is low, excessively high TGN levels can result and lead to life threatening toxicities (i.e., myelosuppression, second cancers).

7.2.3 Dosing, Toxicity, and Monitoring Considerations

Thiopurines have a narrow therapeutic index requiring careful dosage and monitoring considerations regardless of the disease state being treated. Although general dosing recommendations are available for approved indications (Table 7.1), thiopurines are typically dosed based on the track record of their use in treatment protocols, especially in the treatment of neoplasias.

The most serious acute toxicities associated with thiopurine therapy are myelosuppression and hepatotoxicity (Table 7.1). When myelosuppression is severe, life-threatening complications can occur. When conventional therapy is administered, patients who inherit dysfunctional TPMT can have excessively high concentrations of the active TGNs in blood cells. Hence, routine monitoring of blood cell counts is important to both ensure that adequate immunosuppression is achieved, as well as to monitor for excessively low blood counts that could warrant dose reductions or temporary withholding of therapy. Potentially life-threatening complications include infection, anemia, and bleeding complications.

Thiopurine associated hepatotoxicity can present in many forms such as intrahepatic cholestasis, focal centralobular necrosis characterized by hyperbilirubinemia, increased alkaline phosphatase and liver aminotransferases (aspartate and alanine), jaundice, ascites, and encephalopathy [12, 13]. Hepatotoxicity is most often seen after 2 months of therapy but can also occur very early in therapy (within 1 week) or may be delayed for several years post therapy. Chronic thioguanine administration has been linked to veno-occlusive disease of the liver, which appears to be dose-related [14]. The meTIMP metabolite contributes to toxicity caused by mercaptopurine and azathioprine [15]. Routine monitoring of liver aminotransferases, uric acid, and bilirubin is recommended for early detection of liver toxicity.

A serious delayed complication associated with thiopurine therapy is the risk of developing a secondary cancer [16–19]. This poses a real challenge for clinicians because it is impractical to manage or monitor a disease that has not yet developed. Second cancers that have been associated with thiopurine therapy are brain, skin, and myelogenous leukemia [16, 20, 21]. Because secondary cancers may be associated with high levels of thiopurine active metabolites, thiopurine testing may be indicated to minimize these high exposures (see below).

It is well established that polymorphisms in *TPMT* account for a significant degree of interpatient variability in response to thiopurine therapy. At St. Jude Children's Research Hospital, all newly diagnosed ALL patients are assessed for TPMT status prior to initiating therapy. The goal of this approach is to achieve comparable active metabolite levels and toxicity profiles for all patients, regardless of TPMT genotype or phenotype. However, determining a patient's TPMT status prior to initiating thiopurine therapy has not been universally employed. In a study that assessed azathioprine efficacy and toxicity in patients with rheumatoid arthritis, it was concluded that *TPMT* genotyping prior to initiating azathioprine therapy could potentially allow for higher doses of azathioprine and ultimately greater treatment efficacy [22]. In Crohn's disease, thiopurines are discontinued in as many as 1/3 of patients due to poor or no response and up to 1/5 of patients due to adverse drug effects [23]. In this patient population, poor response to thiopurines has been associated with low levels of the active TGN metabolites while good response was associated with higher levels [23]. Additionally, screening for TPMT status has been suggested to be beneficial in the prevention of thiopurine-associated toxicity in patients treated for inflammatory eye conditions [8].

In addition to understanding the importance of knowing a patient's TPMT status to prevent toxicity, there are a couple of drug interactions that can result in significant toxicities if coadministered with thiopurines at standard doses. First, allopurinol is a potent inhibitor of xanthine oxidase and when coadministered with mercaptopurine or azathioprine, excessively high levels of the active TGNs can result [24]. In this situation, a dose reduction of the thiopurine drug would be warranted. Thioguanine is not a substrate for this pathway so is not affected by allopurinol coadministration. Second, aminosalicylates (i.e., mesalazine, olsalazine, and sulfasalazine) are known inhibitors of TPMT, and hence patients who receive these drugs concurrently with thiopurines should be monitored closely as they are at an increased risk of severe myelosuppression.

7.2.4 Markers of Treatment Efficacy and Toxicity

Determining the best dose of thiopurines to administer is extremely important when treating diseases that are life-threatening or that could result in significant morbidity. Measuring thiopurine metabolites, TPMT activity, and *TPMT* genotypes can be helpful to minimize the risk of treatment failure or life-threatening toxicities when doses are too low or too high, respectively (Table 7.2).

When administering drugs with narrow therapeutic indices, it is important that informative therapeutic monitoring parameters are measured and utilized to help guide therapy. In the ALL patient population, the degree of myelosuppression can be used to tailor mercaptopurine therapy. Indeed, a modest level of myelosuppression is expected, and in fact desired, as it gives an indication that the target cell population is receiving adequate drug exposure.

Table 7.2 Thiopurine pharmacologic testing

Test	Relationship to dosing	Relevant thiopurines	Affected by prior erythrocyte transfusions	Utility for assessing compliance
Erythrocyte thioguanine nucleotide (TGN) metabolites	Best after steady-state dosing[a]	Mercaptopurine, thioguanine, azathioprine	Minimally[b]	Yes
Erythrocyte methyl mercaptopurine nucleotide metabolites (MeTIMP)	Best after steady-state dosing[a]	Mercaptopurine, azathioprine	Minimally	Yes
MeTIMP/TGN ratio	Best after steady-state dosing[a]	Mercaptopurine, azathioprine	Minimally	Yes
Erythrocyte thiopurine methyltransferase (TPMT) activity	Not affected	Mercaptopurine, thioguanine, azathioprine	Yes, especially if low activity recipient receives blood from high activity donor[c]	No
TPMT genotype[d]	Not affected	Mercaptopurine, thioguanine, azathioprine	No	No

[a]Most informative if patient has been receiving constant regular dosing for at least 2–4 weeks and obtained at 6–24 h from last dose

[b]Given a starting hemoglobin of 8 g/dl and a TGN of 3,000 pmol/8 × 10^8 RBCs, if a patient is transfused with enough blood to raise the hemoglobin to 10 g/dl, the erythrocyte TGN is projected to be diluted to 2,400 pmol/8 × 10^8 RBCs

[c]Given a starting hemoglobin of 8 g/dl and a TPMT of 1 U/ml PRBCs, if a patient is transfused with enough blood to raise the hemoglobin to 10 g/dl, the erythrocyte TPMT is projected to be artifactually elevated to 5.2 U/ml PRBCs, if transfused with blood cells from a donor whose TPMT status is 22 U/ml PRBCs (consistent with homozygous wild-type–90% chance)

[d]TPMT genotype might not match host phenotype if patient has undergone allogeneic stem cell transplant, or if patient has a rare inactivating polymorphism not interrogated by the genotyping test

Hematologic parameters are also used to monitor toxicity. Low platelets (<50 × 10^9/L), WBC (<1,000/mm^3), and ANC (<300/mm^3) could potentially lead to bleeding complications and infection and often warrant withholding therapy to allow levels to return to acceptable values. It is important to point out that when therapy is withheld for any reason, the potential for treatment failure (i.e., disease progression or recurrence) can be increased. Thus, it is likely beneficial to avoid a period of profound myelosuppression that may compromise therapy.

Thiopurine metabolite levels (i.e., MeTIMP and TGNs) can be used as parameters to assess and guide therapy (Table 7.2). As discussed later in this chapter, medication compliance can be assessed in patients who are wild-type for TPMT receiving mercaptopurine by the MeTIMP/TGN ratio. And finally, as mentioned previously, elevated liver transaminases, total bilirubin, and uric acid are markers of liver toxicity and should be monitored routinely while patients are receiving thiopurines.

7.3 Pharmacogenetics and Pharmacogenomics of Thiopurines

It is well known that interindividual differences in drug response exists, and that genetic variation plays an integral role in the observed drug phenotypes. *TPMT* is one of the best examples of the importance of pharmacogenetics in providing optimal drug therapy. The genetic variations in *TPMT* result in a trimodal population distribution in TPMT activity [25]. TPMT protein activity is directly correlated to the level of TPMT protein expressed, and inversely correlated to intracellular levels of TGNs (Fig. 7.3) [26]. Approximately 90% of the population inherit homozygous wild-type *TPMT* alleles (*TPMT*1/*1*) and have high levels of protein activity, ~10% are heterozygous and have intermediate activity, and <1% are homozygous variant and have low to no detectable activity (Fig. 7.3a, b). Individuals with low TPMT protein expression are at risk for having high intracellular concentrations of active TGN metabolites, and hence are at an increased risk of life threatening myelosuppression and secondary cancers (Fig. 7.3c). Those who are TPMT deficient may require as much as a 15-fold dose reduction in order to have comparable intracellular TGNs and toxicity as patients with wild-type TPMT [27]. Low TPMT protein activity caused by variant alleles is a result of increased protein degradation as compared to wild-type *TPMT* (Fig. 7.3d).

7.3.1 Relevant TPMT SNPs and Haplotypes

TPMT is located on chromosome 6 (locus 6p22.3), is 34 kilobases in length [28], and consists of 10 exons (Fig. 7.4). There are 28 known variant alleles [29], many of which have been associated with decreased protein levels in *in vitro* studies [29].

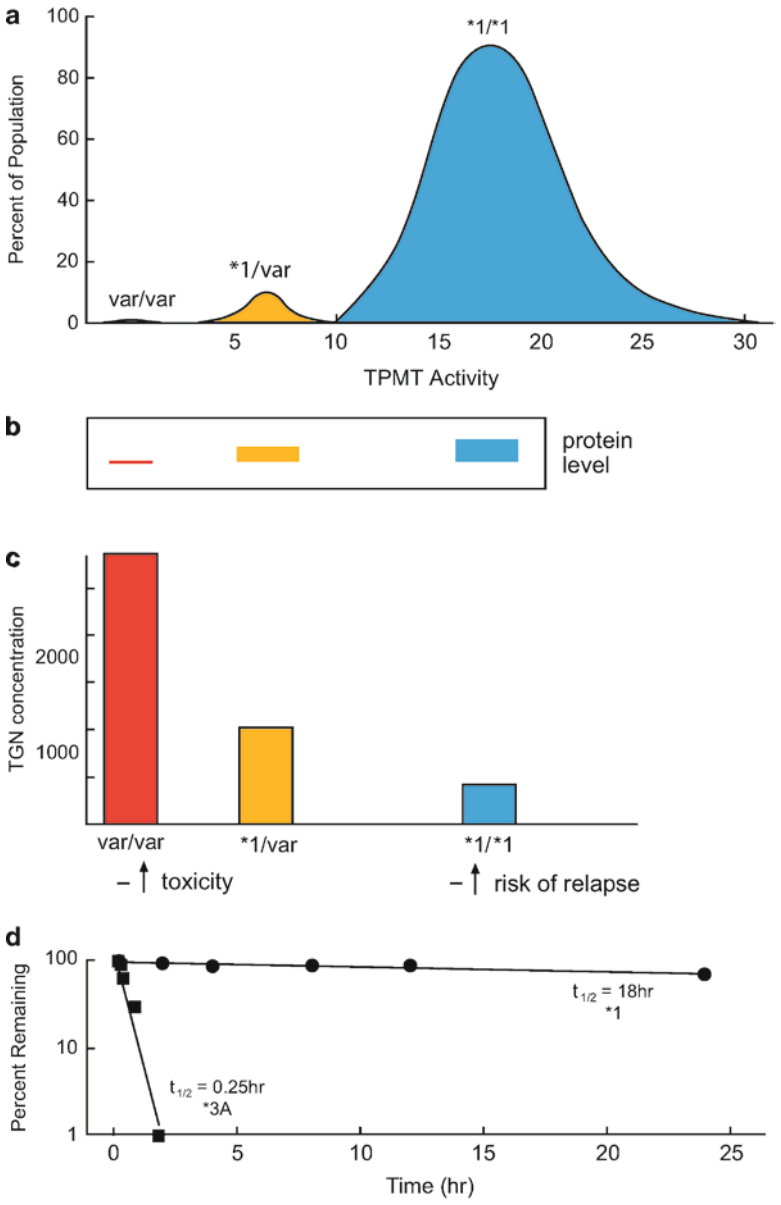

Fig. 7.3 Thiopurine methyltransferase and the clinical consequence of the genetic polymorphism, reproduced with permission from Clinical Pharmacology and Therapeutics [26]. (**a**) The genetic polymorphism in *TPMT* results in a trimodal population frequency distribution in TPMT activity with deficient activity caused by inheritance of two variant (var) alleles, intermediate activity caused by heterozygosity, and high activity associated with homozygous wild-type (*1/*1) genotypes. (**b**) TPMT activity is directly proportional to the amount of TPMT protein; (**c**) and is inversely related to intracellular concentrations of active TGN metabolites. Low TPMT is associated with toxicity and high TPMT may result in increased risk of relapse. (**d**) The biochemical basis for low protein conferred by the most common variant polymorphism (*3A) is illustrated by the longer half-life for in vitro expressed *TPMT*1* when compared to the variant protein

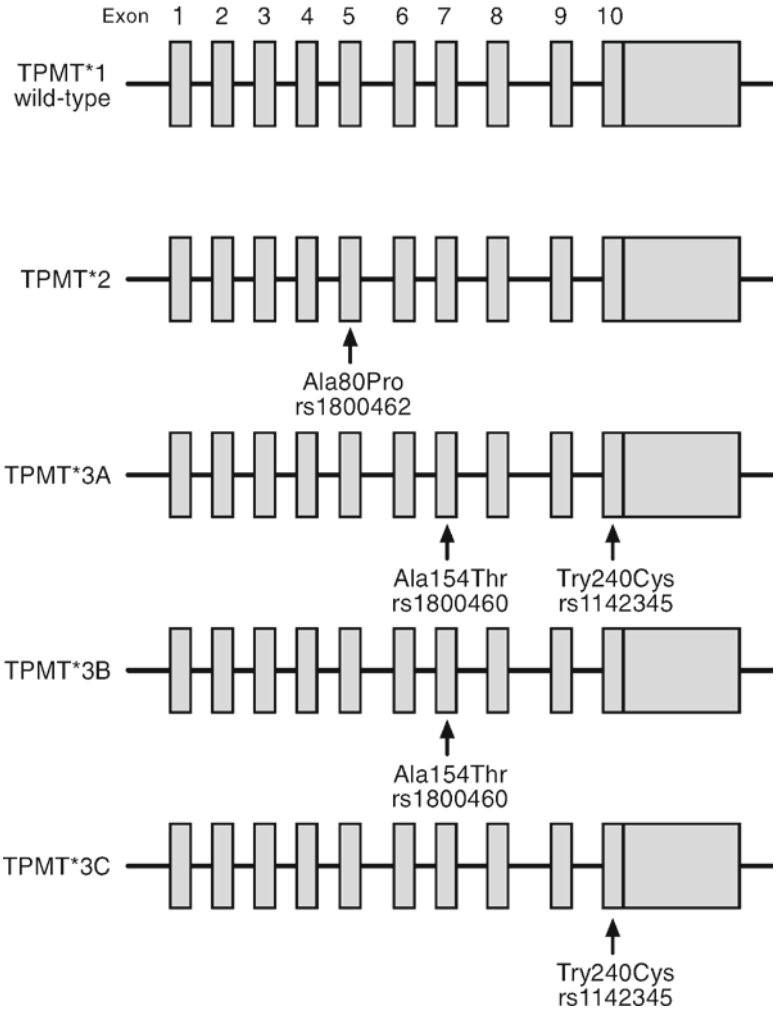

Fig. 7.4 *TPMT* genetic variants representing ~90% of the intermediate and low enzymatic activity. Exonic locations are shown with the amino acid substitutions and SNP identifiers (from dbSNP) listed

The level of TPMT enzymatic activity is currently the best predictor of how patients will respond to thiopurine therapy. Although there are 28 known *TPMT* variants, the level of TPMT protein activity is most often influenced by a few well-studied deactivating genetic polymorphisms: *TPMT*2*, *TPMT*3A*, *TPMT*3B*, and *TPMT*3C* (Fig. 7.4). Importantly, the frequency at which these variants occur will vary based on race or ethnic background.

To date, the *TPMT*3A* variant, consisting of two nonsynonymous coding single nucleotide polymorphisms (SNPs) located on exons 7 (ala154Thr) and 10

(Tyr240Cys), is the major clinically relevant haplotype that is predictive of TPMT activity and is the most common *TPMT* variant in whites [30]. The *TPMT*3C* allele consists of a single nonsynonymous coding SNP located on exon 10 (Tyr240Cys) and occurs most commonly in individuals of Eastern Asian and African decent (Fig. 7.4). Although the *TPMT*2* (exon 5) and *TPMT*3B* (exon 7) alleles have been well studied; they are much less common than *TPMT*3A* or *TPMT*3C* variants. Overall, these variants account for ~90% of the *TPMT* inactivating alleles. Although *TPMT* genotype is predictive of *TPMT* activity and thiopurine tolerability in homozygous variant individuals (these patients have no measurable protein activity), there is a great degree of variability in TPMT activity based on genotype in wild-type and heterozygous individuals.

As discussed later in the chapter, there are three clinical tests used to assess TPMT phenotype. The importance of using TPMT phenotypes to guide thiopurine therapy is reflected in the fact that patients who receive standard therapy and have low intracellular TGN metabolite levels have an increased risk of treatment failure, and patients who have excessively high TPMT metabolites (i.e., TGNs) have an increased risk of second cancers and life threatening myelosuppression [10, 25, 31–34].

7.3.2 Future Gene Associations and GWAS Studies

Although TPMT is well known to be important in thiopurine therapy, identifying other genetic predictors of thiopurine toxicity and response to further optimize therapy is the next step. Candidate gene or genome wide association studies (GWAS) are the key to determining novel genes that can help further delineate other predictors of thiopurine-associated toxicities as well as better predictors of TPMT activity in individuals who have a wild-type TPMT genotype. Even TPMT activity itself appears to be influenced by genetic variation outside of the TPMT gene [26].

Recent studies have implicated *inosine triphosphate pyrophophatase* (*ITPA*) as being predictive of the risk of thiopurine toxicity. ITPA is the enzyme responsible for catalyzing the conversion of inosine triphosphate to inosine monophosphate (an intermediate in purine metabolism) (Fig. 7.2). *ITPA* deactivating genetic polymorphisms result in decreased ITPA protein activity, which can lead to high levels of a potentially cytotoxic metabolite, ITP, when thiopurines are given [35, 36]. Some studies have found a correlation between ITPA and adverse events after thiopurines in the treatment of inflammatory bowel disease [35] whereas others have failed to show significant correlations in this patient population [37]. Interestingly, Stocco et al. found that when the thiopurine dose is adjusted based on *TPMT* genotype in ALL patients, individuals having the deactivating *ITPA* variant, rs41320251, were at an increased risk of severe febrile neutropenia [36]. However, additional studies in independent populations are needed to elucidate what role *ITPA* will play in predicting thiopurine-associated toxicities and optimizing therapy.

7.4 Clinical Utility of Pharmacogenetic Testing for Personalized Medicine

There are only three clinically used thiopurines (mercaptopurine, thioguanine, and azathioprine), and the metabolism of all three are affected by the *TPMT* polymorphism (Fig. 7.1) [25, 31, 32, 38, 39]. The clinical utility of genetic testing for these agents is perhaps more widely adopted than for any other group of medications or single genetic test. The product labeling for all three thiopurines includes language on the impact of pharmacogenetics on adverse effects and availability of testing (Table 7.3).

This group of drugs, paired with the genetic polymorphism in *TPMT*, represent a "perfect storm" of findings that lend themselves to incorporation into clinical medicine, such that the pharmacogenetics of thiopurines is often held up as one of the most compelling examples in clinical pharmacology [33, 40]. First, the agents have a narrow therapeutic index: in any one patient, the difference between a dose that is effective and tolerated and a dose that is unacceptably toxic can be very small. Second, the toxicities can be life-threatening (acute myelosuppression and secondary cancers) [27, 39, 41–43]. Third, some of the toxicities (e.g., leukemogenesis) have a delay of years [16–19, 44], and therefore cannot be monitored in real time and used to adjust doses. Fourth, the diseases that are being treated with thiopurines can also be life-threatening (e.g., leukemia) or extremely serious (e.g., Crohn's disease) and thus rapid introduction of the most effective dose of these agents is crucial to cure the patient [34, 45–48]. Fifth, these medications are often used in combination with other agents that cause overlapping toxicities (e.g., other immunosuppressants or anticancer drugs that can cause infection) and thus clinical monitoring parameters (such as blood counts) may not differentiate which agent is the major culprit in causing acute toxicity. Sixth, a small number of polymorphisms account for the vast majority of the inactivating alleles in the TPMT gene [11, 49–52], making genetic testing feasible with a small number of interrogated polymorphisms. Seventh, a single blood sample can be used to measure pharmacologic phenotypes of interest (erythrocyte thiopurine metabolite levels and TPMT enzyme activity) that complement the genetic testing result. Finally, the TPMT polymorphism has no known consequences in the absence of thiopurine exposure, thus, testing for it does not carry any societal concerns outside the context of medication use, as is true for some other polymorphisms that may also affect "constitutive" disease risk implications.

There are three types of tests available for thiopurine monitoring (Table 7.2): erythrocyte TGN metabolites, erythrocyte TPMT activity, and *TPMT* genotype. For azathioprine and mercaptopurine, erythrocyte MeTIMP can also be monitored.

Measurement of thiopurine metabolites allows for an assessment of patient compliance or adherence with the oral thiopurine therapy [53–55]. Patients who have been taking thiopurines will have detectable thiopurine metabolite levels in erythrocytes, even when plasma levels are not detectable (Fig. 7.5) [56]. Those with wild-type *TPMT* will have MeTIMP/TGN ratios that are high, whereas if such patients have been noncompliant, their ratios will be low and the absolute level of TGN will be very low (e.g., <50 or 100 pmol/8 × 10^8 RBCs). There are data to indicate that at least some patients who are wild-type for *TPMT* when they receive

Table 7.3 Labeling related to pharmacogenetics of thiopurines as related to the TPMT polymorphism

Drug	Label sections	Dosing recommendations	Testing
Mercaptopurine[a]	Clinical pharmacology, warnings/ precautions, laboratory tests, dosage and administration	Substantial dose reductions for homozygous-TPMT deficient patients to avoid life-threatening bone marrow suppression. Optimal starting dose for heterozygotes not established	TPMT genotyping or phenotyping can identify patients who have homozygous deficient or intermediate TPMT activity
Thioguanine[b]	Laboratory tests, dosage and administration, warnings	Substantial dosage reductions may be required to avoid the life-threatening bone marrow suppression in those with inherited deficiency of the enzyme TPMT	Some laboratories offer testing for TPMT deficiency
Azathioprine[c]	Clinical pharmacology, warnings/ precautions, laboratory tests, adverse reactions	Patients with low or absent TPMT activity are at an increased risk of developing severe, life-threatening myelotoxicity if receiving conventional doses Dosage reduction is recommended in patients with reduced TPMT activity	It is recommended that consideration be given to either genotype or phenotype patients for TPMT

[a] Labeling for Purinethol as of August 2003

[b] Labeling for Tabloid as of June 2009

[c] Labeling for Imuran as of May 2008

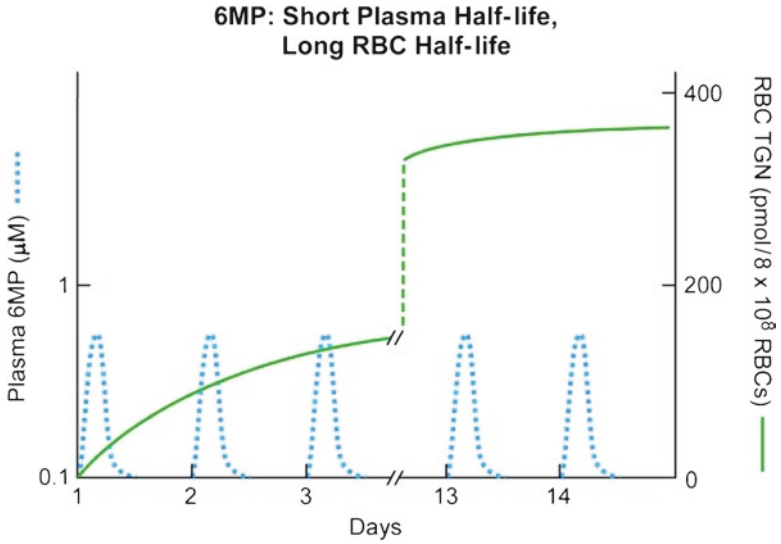

Fig. 7.5 Plasma concentrations of mercaptopurine (*left y* axis, *dashed line*) after daily doses of mercaptopurine demonstrate a rapid plasma half-life and no accumulation. Erythrocyte (RBC) concentrations of thioguanine nucleotide active metabolites (RBC TGN) indicate the slow accumulation over a period of ~2 weeks of dosing to an eventual steady state concentration

intrapatient dosage escalations of mercaptopurine, shunt the medication toward higher MeTIMP levels with little change in TGN concentrations [57, 58].

Patients receiving thioguanine have no MeTIMP levels to measure, as this metabolite is formed from an intermediate of mercaptopurine that is not formed after administration of thioguanine. Patients receiving thioguanine tolerate much higher levels of TGN than do patients receiving mercaptopurine. Thus, any putative target range of TGNs must account for the thiopurine being used. Moreover, as most of the indications for thiopurines (e.g., inflammatory bowel disease, ALL) are treated with a number of agents in addition to thiopurines, putative therapeutic ranges are likely to be influenced by the doses of the concurrent medications.

TPMT activity is widely expressed, and thus activity in any one tissue (e.g., erythrocytes) reflects the activity in tissues more important for drug disposition (e.g., liver) and in drug action (e.g., bone marrow) [59–62]. However, TPMT activity appears to be higher in certain fractions of erythrocytes than others (and thus can be affected by the proportion of immature erythrocytes in the sample) [63], by age [64], and by "time on therapy [34, 65]," although the factors that contribute to such intraindividual variability are poorly defined. Because patients with low TPMT activity are at higher risk of receiving blood transfusions than those with wild-type activity, and the majority of red cell donors will have wild-type TPMT status, erythrocyte TPMT activity can be artifactually elevated by recent transfusions (Table 7.2) [66]. Nonetheless, TPMT activity is a useful adjunct, along with thiopurine metabolite levels and *TPMT* genotype, to impute the TPMT status for any given patient.

TPMT genotypes are imputed based on the most likely haplotypes, given the interrogated SNPs [50, 67–69]. Complementary phenotype data are extremely helpful

for several reasons. There is always a chance for one sample or another to be mislabeled somewhere in the testing process, and this is particularly concerning when dealing with genotypes, which are based on a single sample. A wild-type TPMT activity is simply not consistent with a homozygous or even heterozygous low-activity genotype; one sample or the other would be suspect and solved with repeat sampling. In addition, most commercial genotyping assays test for only the three most common inactivating SNPs; although rare, it is possible for a patient to have a rare inactivating SNP that could result in a spurious wild-type genotype accompanied by low TPMT activity or low MeTIMP/TGN ratio. Another rare possibility would be that two inactivating SNP variants (e.g., Ala 154 Thr and Tyr 240 Cys, rs1800460 and rs1142345), each of which is present in the heterozygous state, which normally are assumed to be in linkage disequilibrium and thus allelic with each other could actually be present on opposite alleles; this would lead to a genotype call of heterozygote (*1/*3A) but in fact represent a compound homozygote deficient genotype call (*3B/*3C). Whereas, when a *1/*3A genotype would be consistent with heterozygous TPMT activity (and a low but detectable MeTIMP/TGN ratio), and *3B/*3C genotype would be consistent with undetectable or very low TPMT activity and undetectable MeTIMP levels – phenotypes that would be readily distinguished with one of the two phenotyping tests.

Multiple types of "variant" and "wild-type" alleles exist for every gene, and the frequency of variant TPMT alleles differs substantially by racial or ancestral background [70–73]. Because the cost of determining DNA sequence at every nucleotide is still prohibitively high, genotyping tests depend on technologies that survey a patient's DNA at particular target genetic sequences. One of the reasons that TPMT genotyping has been more widely adopted than other pharmacogenetic tests is that a relatively small number of variant alleles account for the vast majority of TPMT inactivating variants (Figs. 7.4 and 7.6) [11, 67, 74], and accurately categorize patients according to three major TPMT phenotypes: homozygous variant or deficient, heterozygote, or homozygous wild-type. However, there is

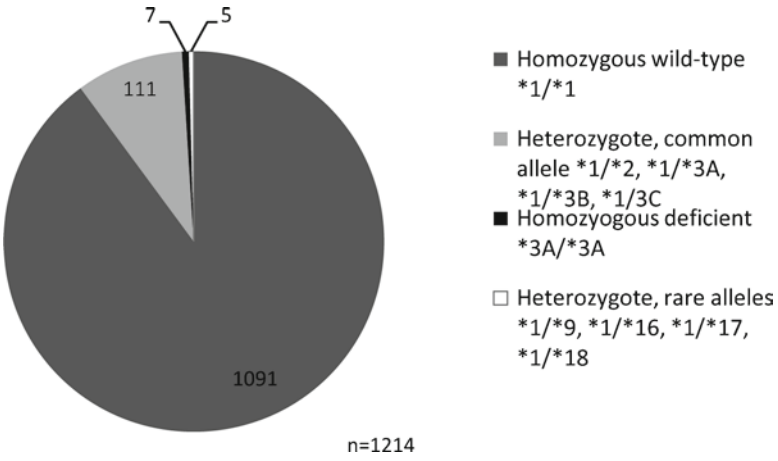

Fig. 7.6 Distribution of TPMT genotypes from a large group of individuals of European ancestry. Figure illustrates data from Shaeffeler et al. [51]

always the possibility for false negatives: a genotyping test cannot reveal any information about areas of the gene not interrogated by the test (e.g., one can only know that the patient is "wild-type" at the loci tested), and rare patients will have rare or novel inactivating variants that will be missed by standardized genotyping tests. The number of false negatives depends on the proportion of inactivating variants accounted for by the tested variants (which must be disclosed by the test).

Another complication is that most genotyping tests cannot experimentally determine haplotype, although such information is available using rather labor-intensive long-range PCR tests [68, 75]. Thus, standard genotyping tests rely upon comparing the results at individual loci with the population probabilities that polymorphisms are allelic with each other. If a patient is "heterozygote" at more than one polymorphic site in a gene, and there is a high probability that the polymorphisms are allelic (e.g., as is true for the Ala 154 Thr and Tyr 240 Cys variants, rs1800460 and rs1142345), the genotype for the patient is a likely heterozygote. If the genotype at two separate sites is "heterozygote," but there is a high probability that the polymorphisms are not allelic to each other (as would be true for the Ala 80 Pro coupled with the Tyr 240 Cys variants, rs1800462 and rs1142345), then the patient likely has a homozygous variant genotype.

7.4.1 Dosage Adjustments

Although the importance of TPMT status to thiopurine tolerance is extremely well documented, some clinicians do not test patients prior to starting thiopurine therapy. Our group has long advocated testing for TPMT status prior to initiating thiopurine therapy so that dosages can be adjusted accordingly. One reason to test everyone is that even a short course of thiopurines given to the rare (1 in 300) homozygous deficient individuals can result in serious myelosuppression [76, 77], which could be avoided by starting with dramatically decreased doses (>10-fold lower) or choosing an alternative therapy. Although it is true that only ~35% of heterozygous patients receiving normal thiopurine doses experience myelosuppression so severe that doses must be decreased, there are disadvantages of beginning thiopurine therapy and adjusting doses downward only in those who experience toxicity. In treating cancer, some regimens have so many myelosuppressive agents given relatively early in therapy that profound myelosuppression early on may delay subsequent therapy – making thiopurine dose "titration" less feasible. Moreover, because some serious long-term adverse effects (second tumors) have been associated with defective TPMT activity [16–19], even in patients who do not experience acute toxicity [78], some advocate capping doses of thiopurine in heterozygotes at some dose lower than recommended for homozygous wild-type patients (Fig. 7.5), although there are no data to indicate that such a strategy decreases the risk of second cancers. However, there are data to indicate that when TPMT status is used, in conjunction with clinical myelosuppression, to adjust mercaptopurine doses, relapse rates in childhood ALL are not adversely impacted [45].

Whereas TPMT status has been convincingly linked to tolerance of thiopurines, specific target levels of erythrocyte thiopurine metabolites have not been well established. Although a precise threshold TGN level that is consistent with noncompliance has not been established, our practice is to counsel patients regarding the dangers of noncompliance to TGN levels <100 pmol. Moreover, although MeTIMP and high TPMT activity have been clearly implicated as associated with higher risk of hepatic toxicity [12, 79, 80], the tests have a poor predictive power, as most patients with high MeTIMP do not develop hepatotoxicity. At least in ALL, the presence of elevated serum aminotransferases has been associated with improved cure rates [13], and thus it is not clear whether mild hepatic dysfunction should be considered an indication to modify therapy. Some have advocated an approach of combining allopurinol or thioguanine with mercaptopurine in order to maximize formation of TGN and minimize formation of MeMPN [24], which may obviate some of the hepatotoxicity that may be associated with MeMPN. It should also be acknowledged that thioguanine (as opposed to mercaptopurine) has been associated with a higher risk of veno-occlusive disease of the liver [81–83] and thus the use of thioguanine may be associated with its own set of liver toxicity.

Therefore, the algorithm we use for adjusting mercaptopurine doses in patients with ALL at St. Jude (Fig. 7.7) uses *TPMT* status to institute lower-than-normal starting doses of thiopurines in the rare homozygote-deficient patients and the heterozygous patients – but titrates final dosages based on tolerance to thiopurines, without targeting specific therapeutic ranges for TGN or MeTIMP metabolites. Although only about 35% of heterozygotes require a dosage reduction based on acute myelosuppression, the median daily dose for those who do experience myelosuppression is ~40% lower (44 mg/m^2) than that tolerated in wild-type patients (75 mg/m^2) [79, 84]. After instituting lower starting dosages in those with at least one variant *TPMT* allele, we then titrate up the thiopurine dose to achieve the desired level of myelosuppression, generally leaving the nonthiopurine therapy unadjusted. In heterozygotes, we generally cap the mercaptopurine dose somewhat lower (60 mg/m^2) than the normal mercaptopurine dose in wild-type patients because of our concerns for secondary tumorigenesis. In those with wild-type *TPMT*, we adjust the doses of mercaptopurine plus any other myelosuppressive agents based on an algorithm that places no special emphasis on thiopurines as the culprits (Fig. 7.7). Other polymorphisms, such as *ITPA* for thiopurines [36, 85] or *SLCO1B1* for methotrexate [86], may have an important role in acute toxicity of therapy, and as these are validated, may be incorporated into dosage regimens.

7.5 Case Report

JB is a 6-year-old child with high-risk ALL. A phase of intensification chemotherapy included cytarabine 50 mg/m^2/day × 5 days; mercaptopurine 50 mg/m^2/day PO × 10 days; and cyclophosphamide 500 mg/m^2 IV on days 1 and 5. JB was neutropenic for 6 weeks whereas the median period neutropenia is only 10 days.

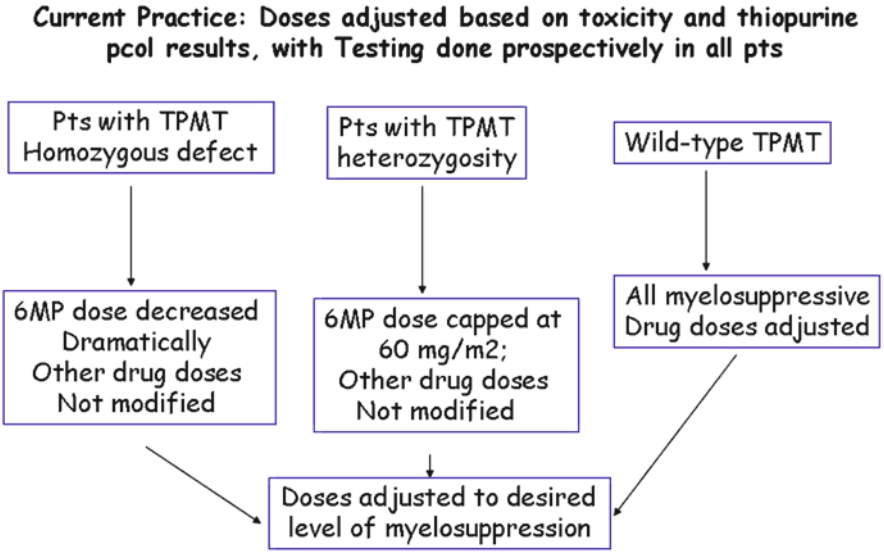

Fig. 7.7 Overview of algorithm for dosing of acute leukemia continuation therapy including mercaptopurine among children at St. Jude. For the 1 in 300 patients with homozygous TPMT deficiency, mercaptopurine doses are reduced to less than 10% of the standard dose (e.g., 10 mg/m^2/day 3 days/week instead of 75 mg/m^2/day daily). For the ~10% of patients with heterozygote TPMT status, we attempt to cap the mercaptopurine dose at 60 mg/m^2/day; if myelosuppression is observed, the preference is to reduce the mercaptopurine dose rather than that of other myelosuppressive agents (such as methotrexate). For the ~90% of patients who are wild-type for TPMT, there is no reason (based on TPMT status) to preferentially decrease the dose of mercaptopurine vs. any other myelosuppressive agent. Doses of chemotherapy in ALL are generally titrated to achieve a target level of myelosuppression; in patients with a defect in TPMT activity, there is a pharmacologic basis for focusing on decreasing the dose of thiopurine and attempting to give the other myelosuppressive agents at least full dose

A maintenance phase of chemotherapy started with methotrexate 20 mg/m^2 orally every week and mercaptopurine orally at 75 mg/m^2/day. On day 14 of maintenance, the child was admitted for fever and neutropenia, with a platelet count of 20,000/μl and hemoglobin of 6 g/dl. Both mercaptopurine and methotrexate were stopped. He has required multiple RBC transfusions over the last 8 weeks. On day 17 of maintenance, blood was drawn for thiopurine testing and showed a RBC TPMT activity of 6 U/ml PRBCs (heterozygote range = 5–14 U/ml), RBC TGN = 800 pmol/8 × 10^8 RBCs, MeMPN was undetectable, and the genotype was *1/*3A.

7.5.1 How Do We Interpret JB's Findings?

Only 10 days of thiopurines were given initially, along with other myelosuppressive therapy, so it is possible that the 6 weeks of myelosuppression was caused at least partly by agents other than thiopurines. However, the repeat of severe pancytopenia

(platelets, red cells, and neutrophils all suppressed) after only 2 weeks of maintenance raises suspicions that thiopurines are the culprits.

The RBC TPMT activity of 6 U/ml PRBCs would be consistent with a TPMT heterozygote; however, because the patient has received multiple RBC transfusions within the 90 days leading up to the test, the activity may be artifactually altered (probably increased) by allogeneic transfusions, so perhaps the TPMT activity is lower than 10 U/ml.

The fact that the RBC TGN is 800 pmol/8 × 10^8 RBCs (whereas after just 14 days of therapy, wild-type patients would generally have levels 100–400 pmol/8 × 10^8 RBCs) [53, 87, 88] would be consistent with low TPMT activity. Because most "normal" ranges are based on samples drawn <24 h from the last dose of a regimen of at least 2 weeks of therapy (i.e., "steady state") [89], and this patient actually received no mercaptopurine in the 72 h prior to this sampling, this TGN may actually be lower than reflective of the patient's true steady-state. The fact that the MeMPN levels are undetectable, in the presence of relatively high TGN, is highly indicative of absent (homozygous deficient) TPMT activity in this patient.

The genotype was *1/*3A, consistent with a heterozygote. Based on the frequency of the *3A allele, this is the most likely genotype. However, because of the very severe toxicity, the absent methyl metabolites, and the high TGN despite modest dosing, in this case, the genotype interpretation of *3B/*3C (the low activity genotypes on different alleles) is more likely. On further investigation, it turns out that JB is of African ancestry, a group for whom *3B and *3C alleles are more common than in those of European ancestry.

Thus, our ultimate interpretation is that this patient is a rare *TPMT* homozygous deficient patient. When counts recover, mercaptopurine is restarted at 10 mg/m^2/day on 3 days per week, along with methotrexate at 40 mg/m^2/week. After 8 weeks of tolerating this regimen, no transfusions are required. A repeat TPMT activity measure comes back below the limit of detection of 1 U/ml, and TGN are at 760 pmol/8 × 10^8 RBCs, and MeMPNs remain undetectable. Later in therapy, the mercaptopurine dose is titrated (based only on the desired level of neutrophil count) up to 10 mg/m^2/day 7 days a week, and no transfusions are required for the remainder of therapy.

Acknowledgments Supported by NCI grant CA 21765 and the NIH/NIGMS Pharmacogenetics Research Network and Database (U01 GM61393, and by the American Lebanese Syrian Associated Charities (ALSAC).

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Part III
Specific Pharmacogenomic Targets: Cardiovascular Drugs

Chapter 8
The Pharmacogenetics of Vitamin K Antagonist Anticoagulation Drugs

Charles Eby

Abstract Since the first report on warfarin pharmacogenetics in 1999, genetic variants have emerged as an important predictor of warfarin maintenance doses before therapy is initiated, raising expectations of greatly improved clinical outcomes. However, much of the information on warfarin sensitivity conveyed by genetic variants is captured by early international normalized ratio values traditionally used to guide dose titration. Thus, inclusion of early international normalized ratios in prediction models reduces the contribution of genetics. Moreover, in large population cohorts, genetics explained only 20–30% of variance in warfarin doses. Finally, even pharmacogenetic prediction models did not predict doses reliably in the majority of at-risk patients with warfarin requirements at the low or high end of the dose range. Currently, the clinical utility and cost–effectiveness of pharmacogenetic – based dosing are being assessed in large prospective trials in various settings. In the interim, enthusiasm for warfarin pharmacogenetics should not supersede strict adherence to traditional measures used to optimize coumarin anticoagulation.

Keywords Warfarin • Vitamin K epoxide reductase complex • Cytochrome P450 2C9

8.1 Introduction

Until very recently, three derivatives of dicoumarol, warfarin, acenocoumarol, and phenprocoumon, were the only available drugs for oral anticoagulation therapy. While they differ in half-life and potency, all three coumarins have similar pharmacogenetic, pharmacokinetic, and pharmacodynamic properties, and warfarin, the drug of choice in most countries, will be the focus of this chapter. Warfarin is effective for prevention of arterial and venous thromboses and emboli. Oral

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_8,

anticoagulation therapy with warfarin reduces the relative risk of stroke in patients with atrial fibrillation by 68% [1]. Extended warfarin therapy prevents recurrent thromboses in patients with DVTs [2] and reduces DVT complications after hip replacement surgery [3]. It is estimated that two million people start warfarin annually in the United States [4]. However, warfarin has a narrow therapeutic window and unpredictable anticoagulant dose response, which is reflected in one of the highest rates of reported adverse drug events [4].

8.2 Pharmacology of Warfarin

Warfarin's anticoagulant effect is due to inhibition of hepatic posttranslational modifications to coagulation factors X, IX, VII, and II (prothrombin) (Fig. 8.1). Six to thirteen glutamic acid residues near the N-terminus of these coagulation factors undergo carboxylation of the gamma carbon atom, producing calcium ion binding sites, increasing the affinity of the coagulation factors for negatively charged phospholipid surfaces, and accelerating the rate of thrombin generation and formation of fibrin clot. The gamma-glutamyl carboxylase (GGCX) enzyme requires a cofactor, reduced vitamin K, which undergoes oxidation to vitamin K epoxide during gamma carboxylation, and subsequent reduction by vitamin K epoxide reductase (VKOR) to regenerate reduced vitamin K. Warfarin competitively inhibits VKOR activity to deplete the availability of reduced vitamin K, diminish GGCX activity,

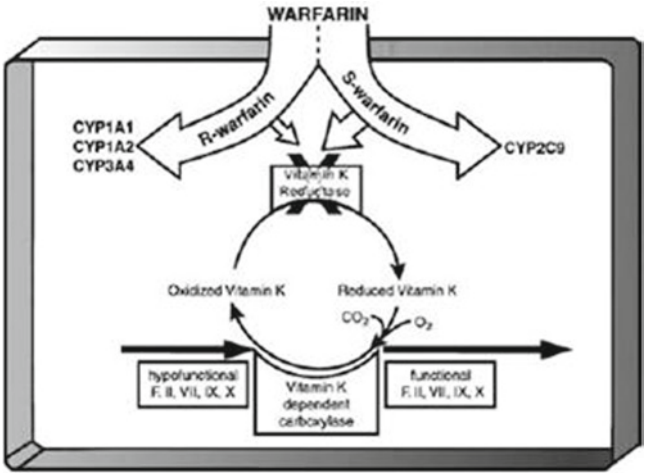

Fig. 8.1 Warfarin pharmacodynamics: Warfarin indirectly produces an anticoagulated state by preventing posttranslational modifications to selected coagulation factors. The S-enantiomer strongly blocks vitamin K oxide reductase (VKOR) conversion of vitamin K epoxide to reduced vitamin K. Reduced vitamin K is a cofactor for γ-carboxylation of glutamic acid residues on factors X, IX, VII, and II (prothrombin). *The Pharmacogenomics Journal* 2004; *4*, 224–225, reproduced with permission

and decrease the activity of circulating coagulation factors X, IX, VII, and II, thus delaying the rate of blood clot formation [5].

Knowledge of warfarin's unpredictable anticoagulant effect and bleeding risk necessitated therapeutic monitoring with the prothrombin time (PT) clotting test when warfarin was licensed in the United States in 1954 under the brand name Coumadin®. The PT initiates clotting of plasma by adding thromboplastin, a phospholipid/protein extract from brain tissue. The rate of clot formation is dependent upon the levels of coagulation factors V, X, VII, and II: the later three are vitamin K dependent, and their activities are reduced by warfarin. A PT ratio (patient PT/mean normal PT) of 1.5–2.0 produced acceptable efficacy and safety [6]. Over several decades, clinical management and therapeutic monitoring of warfarin therapy improved. Recognition of variable sensitivity of commercial thromboplastins to vitamin K-dependent coagulation factor depletion led to standardization by calibrating PT reagents to a World Health Organization reference thromboplastin to obtain an International Sensitivity Index (ISI) factor, and subsequent conversion of PT ratios to International Normalized Ratios (INR) (PT patient/PT mean normal)^{ISI} [6]. For most oral anticoagulation indications, an INR target range of 2–3 provides the best balance of safety and efficacy [6]. However, to maintain a therapeutic INR, patients require warfarin doses that vary approximately 20-fold. The graph in Fig 8.2 shows the distribution of daily warfarin doses to obtain stable therapeutic INRs (2–3) in 5,701 patients from all over the world. The average therapeutic warfarin dose per week (INR 2–3) also varies among racial and ethnic groups: people of African descent 40 mg, Caucasians 31.5 mg, and East Asians 21 mg [7]. However, determining patients'

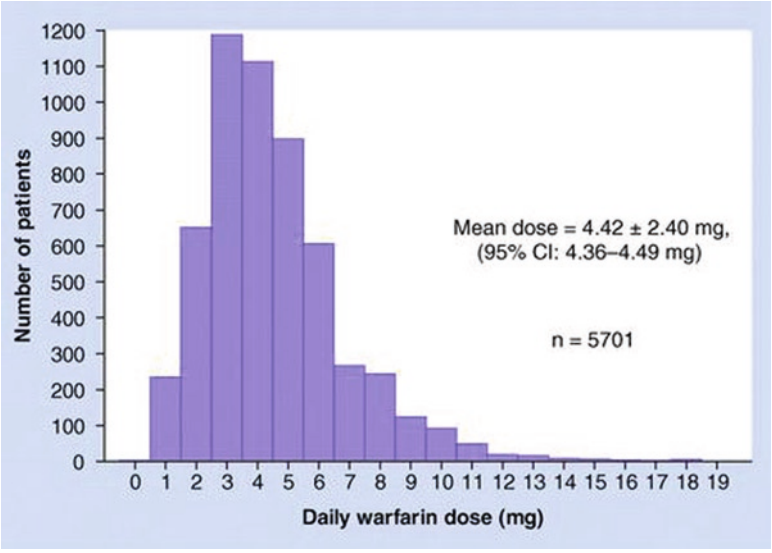

Fig. 8.2 Distribution of therapeutic warfarin doses (target INR range of 2–3) from patients whose data were pooled by the International Warfarin Pharmacogenetic Consortium investigators to derive dosing algorithms [94]. Ref. [96], reproduced with permission

therapeutic warfarin dose by "trial and error" adjustments produces unpredictable fluctuations in INRs, delays in obtaining a stable dose, and bleeding complications, particularly during the first weeks and months of therapy [8].

During the last decade, discoveries of genetic variation impact on warfarin pharmacokinetics and pharmacodynamics have been translated into improved predictions of patients' therapeutic warfarin dose. The results of preliminary clinical applications of pharmacogenetic (PGx)-based warfarin dosing have produced a range of responses from laboratory, practitioner, regulatory, and reimbursement stakeholders, ranging from skepticism to conviction that genetic-guided personalized medicine has found an ideal candidate in warfarin pharmacogenetics.

However, new oral anticoagulant drugs are poised to challenge warfarin [9]. Designed to inhibit the active form of coagulation factors X (direct factor Xa inhibitors), or factor II (direct thrombin inhibitors), clinical trials with rivaroxaban and dabigatran etexilate, respectively, have demonstrated similar efficacy and safety compared to warfarin, while not requiring therapeutic drug monitoring. If approved by regulatory agencies for prevention of thromboembolic events in patients, several factors will likely effect how quickly and to what degree oral direct coagulation factor inhibitors replace warfarin: their comparative costs and reimbursement rates, convenience, and postmarketing efficacy and safety of fixed dosing without laboratory monitoring of anticoagulant effect. Continued utilization of warfarin would be supported if ongoing clinical trials investigating PGx-based dosing demonstrate improved safety.

8.3 Pharmacogenetics of Warfarin

8.3.1 CYP2C9

Warfarin consists of a racemic mixture of S and R enantomers. Both forms are efficiently absorbed from the small intestines, circulate bound to albumin, and are taken up by hepatocytes [5]. However, S-warfarin accounts for approximately 80% of the VKOR inhibitory effect. Therefore, a reduced rate of S-warfarin metabolism has greater consequences than a similar change in R-warfarin pharmacokinetics. S-warfarin undergoes hydroxylation by cytochrome P450 (CYP) 2C9 and eventual excretion in the bile [10]. R-warfarin metabolism is more complex, involving CYP1A1, CYP1A2, and CYP3A4 to produce an inactive alcohol which is excreted in the urine [10]. The other coumarins, acenocoumarol and phenprocoumon, are also racemic mixtures, with more potent S enantomers which undergo CYP2C9-mediated metabolism. Despite some differences in half lives and metabolism, the anticoagulant effect of all three coumarins is potentiated by single nucleotide polymorphisms which reduce CYP2C9 activity [11]. There are two notable CY2C9 single nucleotide polymorphic (SNP) variant alleles: *2 (420C>T, exon 3, substituting cysteine for arginine at amino acid 144) and *3 (1075A>C, exon 7, substituting leucine for isoleucine at amino acid 359). Both in vitro and in vivo experiments confirm

Table 8.1 Variations in CYP2C9 *2/*3 and VKORC1 haplotype frequencies among different populations. Adapted from Marsh et al. [13] with permission

	European–American	African–American	Asian
CYP2C9*2	0.14	0.02	0.0
CYP2C9*3	0.06	0.01	0.04
VKORC1 haplotype A group	0.42	0.21	0.85
VKORC1 haplotype B group	0.57	0.58	0.14
VKORC1 haplotype other	0.01	0.21	0.01
Low-dose group	0.55	0.22	0.86
Haplotype A and 2C9 variant	0.18	0.01	0.06

that CYP2C9*3 expression markedly reduces S-warfarin clearance, while CYP2C9*2 has a more modest impact [12]. The minor allelic frequencies of *2 and *3 SNPs are ~14 and 6%, respectively, in Caucasians [13] and much lower in African Americans and Asians (Table 8.1). Retrospective cohort [14] and case control studies [15] associated lower therapeutic warfarin doses, delayed attainment of therapeutic doses, more supratherapeutic INRs, and higher rates of major and minor bleeding complications in patients with one or more *2 or *3 alleles.

8.3.2 Vitamin K Epoxide Reductase Complex 1

In 2004, two groups independently discovered the gene for VKOR enzyme activity [16, 17]. Located on chromosome 16, the VKOR Complex 1 (VKORC1) 11 kb gene contains three exons and has no homology to other known genes. Rieder and colleagues sequenced the VKORC1 gene, introns, and 5 and 3′ flanking regions from 186 Caucasians with known therapeutic warfarin dose requirements and identified ten common SNPs (frequency >5%) [18]. Based on linkage disequilibriums between SNPs, Rieder inferred 9 VKORC1 haplotypes. Group A haplotypes (H1, H2) were associated with lower therapeutic warfarin doses, and Group B haplotypes (H7, H8,H9) were associated with higher warfarin doses, independent of CYP2C9 *2/*3 status. Four informative SNPs were used to infer VKORC1 haplotypes in an independent cohort of Caucasians with known therapeutic warfarin doses, confirming a significant difference in maintenance warfarin dose: lowest for haplotype AA, intermediate for haplotype AB, and highest for haplotype BB. In addition, VKORC1 mRNA levels in liver tissue correlated with VKORC1 haplotype [18]. Subsequent investigations have shown that genotyping patients for one of two SNPs in high linkage disequilibrium (−1639G>A rs9923231 and 1173C/T rs9934438) account for nearly identical percentages of warfarin dose variability and can substitute for haplotype A [7]. In vitro expression experiments support SNP −1639 in the 5′ promoter region as the likely functional SNP due to decreased gene transcription [19], providing a mechanism for increased sensitivity to warfarin in patients who inherit one or two −1639G>A alleles. Investigators have confirmed the associations between VKORC1 haplotypes or tagSNPs and CYP2C9*2/*3

SNPs and therapeutic warfarin dose in various populations and clinical settings [20–23]. For example, patients who are extremely slow metabolizers (CYP2C9 *3/*3) and very sensitive to warfarin (VKORC1 −1639AA) require therapeutic warfarin doses in the range of 0.5–2.0 mg/day while patients whose genotype is CYP2C9*1*1, VKORC1 −1639GG require doses of 5–7 mg/day [24]. Collectively, there is ample biochemical, molecular, and clinical evidence supporting a genetic contribution to warfarin pharmacokinetic and pharmacodynamic interindividual variation [25, 26]. Citing its mandate to promote personalized medicine and patient safety [4], in August, 2007, the United States Food and Drug Administration (FDA) revised the package insert for Coumadin® to provide CYP2C9 and VKORC1 pharmacogenetic information and to alert prescribers that patients with variations in these genes may require lower doses compared to patients without them.

8.4 Dosing Algorithms for Warfarin

8.4.1 Initial Algorithms

By combining clinical and demographic data with CYP 2C9 and VKORC1 SNP genotypes obtained from patients with known therapeutic warfarin doses, investigators have determined the percent of warfarin dose variability attributable to different variables and derived algorithms to predict the therapeutic dose for a warfarin-naïve patient initiating anticoagulation treatment [20, 22, 27–34]. Despite heterogeneity regarding sample size, therapeutic warfarin dose criteria, clinical, demographic, and medication information ascertainment, statistical analysis, and subjects' race and ethnicity, combining clinical and pharmacogenetic data, i.e., PGx-based dosing algorithms, accounts for 50–60% of warfarin dosing variability. VKORC1 haplotype or −1639G>A genotype consistently has the most effect, accounting for 25–34% [27, 28, 35] of dosing variability, followed by age, body size, CYP2C9*3, and CYP2C9*2. Other minor, but statistically significant in some cohorts, variables include amiodarone which inhibits CYP2C9 activity [27], smoking [27, 31], indication for anticoagulation [27, 31], INR target [27, 31], statin therapy [27], gender [28], race [27], and enzyme-inducing drugs [28, 31]. However, most algorithms were derived from small, homogeneous, predominantly Caucasian populations. When applied to multiethnic [36] or African American patients [27, 37], these models accounted for a lower percent of warfarin dose variability, although Wu and colleagues reported comparable performances for five Caucasian-derived PGx algorithms when applied to a multiethnic population in San Francisco [38].

8.4.2 IWPC and Advanced Algorithms

To address these limitations, a group of investigators formed the International Warfarin Pharmacogenetics Consortium (IWPC) to derive and validate a PGx

dosing algorithm based on clinical and pharmacogenetic data from 4,043 and 1,009 subjects, respectively, from many countries and continents and whose racial makeup was 55% White, 30% Asian, 9% Black, and 6% mixed or unknown [39]. The IWPC PGx dosing algorithm included age, height, weight, VKORC1 −1639 genotype, CYP2C9*2/*3 genotypes, Asian and African race, enzyme-inducing drugs, and amiodarone and accounted for 47 and 43% of the dosing variability in the derivation and validation cohorts, respectively.

To facilitate use of PGx genotyping and dosing algorithms, Gage and colleagues created a nonprofit website: www.WarfarinDosing.org. A clinician can obtain an estimated therapeutic warfarin dose for a patient based on clinical and demographic information or a more accurate PGx-based dose estimate if CYP2C9*2/*3 and VKORC1 −1,639 genotypes are available. The PGx algorithm was derived by Gage's group at Washington University in St. Louis [27]. Alternatively, one can select the IWPC PGx algorithm to calculate a dose; however, the two algorithms provide nearly identical initial dosing estimates.

Including a patient's INR response to the first few doses of warfarin improves the accuracy of a PGx dosing algorithm. Based on data collected from patients prescribed warfarin for VTE prophylaxis after hip and knee arthroplasties, Millican et al. derived a dose refinement algorithm which explained 79% of the variability in therapeutic warfarin dose [40] and included the following independent variables: INR after three doses, first and second warfarin doses, postoperative blood loss, smoking, liver disease, CYP 2C9 *2/*3, and VKORC1 −1639 SNPs. Coagulation factor levels fall with peri-operative blood loss causing a temporary INR prolongation which is accounted for in the algorithm. Slow metabolism SNPs (CYP 2C9 *2/*3) continued to have a strong impact on the revised dose estimate, while warfarin sensitivity genotype (VKORC1 −1639G>A) decreased in importance since the INR response after three doses incorporated most of patients' warfarin sensitivity phenotype. Improved warfarin dosing accuracy by applying a dose refinement PGx algorithm after 4 days of anticoagulation therapy has been prospectively validated in orthopedic patients [41]. Investigators extended this approach further in a large, multicenter retrospective cohort of patients starting warfarin for various indications by developing a PGx dose refinement algorithm accounting for 42–58% of therapeutic warfarin variability after four or five doses, compared to 26–43% for a clinical dose refinement algorithm [42]. Two important conclusions can be drawn from these studies. First, using a clinical dosing algorithm for the first 3–4 warfarin doses followed by a PGx dose refinement algorithm improves dosing accuracy and allows more time to perform genotyping. Secondly, patients who have a genetic sensitivity to warfarin display it quickly in their INR response while the effect of slow metabolizing genetic variants is delayed. In a retrospective analysis of the participants in the PREVENT trial [43], Ferder et al. determined the contribution of CYP2C9*2/*3 and VKORC1 −1639G>A genotypes to explaining therapeutic dose variation 0, 7, 14, and 21 days after starting warfarin [44]. PREVENT was a prospective randomized trial comparing low intensity warfarin therapy (INR 1.5–2.0) to placebo in patients with idiopathic venous thromboembolic events who had completed ≥3 months of warfarin (INR 2–3) and who had stopped anticoagulation therapy for ≥1 month. At enrollment, all patients took an initial dose of 3 mg, which was adjusted, using a study nomogram, based on weekly

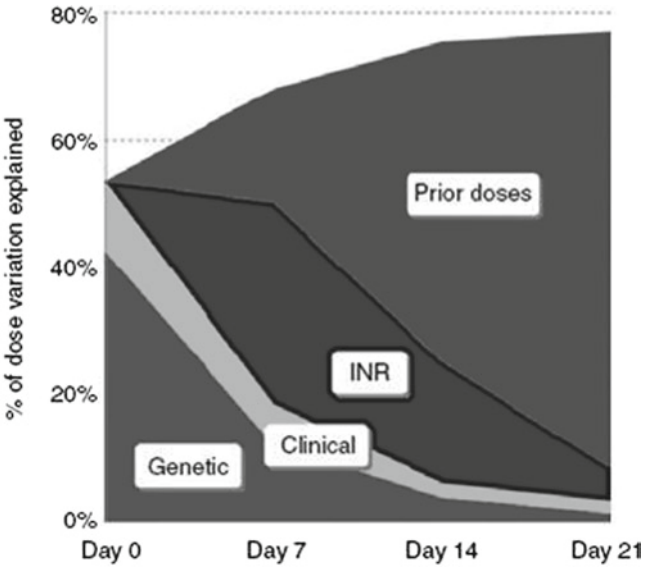

Fig. 8.3 Percentage of warfarin dose variability explained by PGx genotype (CYP2C9 *2/*3 and VKORC1–1639G>A), clinical variables (age, body surface area, target INR, gender, race, smoking, amiodarone, statin), most recent INR, and prior week's average warfarin dose at weekly time points. Ferder et al. [44], reproduced with permission

INRs until a therapeutic dose was achieved. Figure 8.3 shows the individual and total contributions to explaining warfarin therapeutic dose variability for clinical data, PGx genotype, INR, and prior warfarin dose history. Initially, PGx genotype accounted for 43% of the variability, declining to 12, 4, and 1.4% after 1, 2, and 3 weeks, respectively, as warfarin dosing history captured more of the PGX genetics phenotype. Similar findings were reported by Li et al. in a cohort who underwent more frequent early INR monitoring with a therapeutic target of 2–3 [45]. We can conclude that PGx testing can improve dosing accuracy, although with diminishing impact, for approximately a week after starting warfarin, while warfarin dosing history and INR response steadily eclipse genetic information. And consequently, once a patient's therapeutic warfarin dose has been determined by "trial and error" empiric dosing, there is no apparent utility in performing PGx testing.

8.4.3 Limitations of Dosing Algorithms

Despite the advances in clinical research to date, approximately 50% of warfarin dosing variability prior to starting therapy cannot be accounted for in Caucasians and Asian, and even more variability is unexplained in African Americans [46–48]. Potential sources of missing information include additional genetic variation, epigenetic factors, and patient behaviors including diet and compliance. Considerable

efforts have been directed at discovering polymorphisms in genes whose products are involved in the vitamin K cycle and its inhibition, but the results have not been dramatic. Comparisons of SNPs or other genetic variations in gamma-glutamyl carboxylase (GGCX) [49, 50], calumenin, a GGCX regulatory gene [49–51], vitamin K-dependent clotting proteins (factors X, VII, II, and protein C [31, 49, 53, 54]), and CYP2C18 and CYP2C19 [49] to therapeutic warfarin doses have produced inconsistent, positive associations with very weak clinical impact, in small retrospective studies. Other investigated candidate genes include apolipoprotein E, ABCB1 (ATP-binding cassette transporter B1), EPHX1 (epoxide hydrolase 1 microsomal gene), and ORM1+2 (orosomucoid 1 and 2 genes) [49, 55]. Inconsistent findings are likely due to differences in patient selection, SNP minor variant allele frequencies, statistical methods, and therapeutic warfarin dose criteria. However, when Wadelius and colleagues compared 183 SNPs in 29 candidate genes to therapeutic warfarin doses in 1,496 Swedes, only VKORC1 −1639GA and 11173C>T, and CYP2C9*2/*3 SNPs were significantly associated with warfarin dose [28]. Some SNPs do have a clinically important effect on warfarin dosing, but only in ethnic and racial groups with high frequencies for the variant. For example, a VKORC1 SNP, 5417G>T, replacing aspartic acid with tyrosine (D36Y), is associated with moderate warfarin resistance, but this SNP is only prevalent in Ashkenazi (4%) and Ethiopian (14%) Jews [56, 57]. Inclusion of this mutation on a PGx panel would improve warfarin dosing accuracy in communities including these ethnic groups. Additional rare VKORC1 nonsynonymous nucleotide substitutions altering amino acids in the cytoplasmic loop of VKOR have been discovered in patients requiring very large maintenance warfarin doses [17, 58].

8.5 Genome-Wide Association Studies

Investigators have performed several genome-wide association studies (GWAS) to discover novel SNP candidates associated with therapeutic warfarin dose. Using a drug metabolizing enzyme and transporter array chip, Caldwell and colleagues identified a SNP in CYP4F2 (rs2108622; 1297G>A;V433M) in 951 Caucasians with an allelic frequency of 30% which was associated with higher therapeutic warfarin doses and accounted for 2% of warfarin dosing variation [59]. Subsequent studies reported V433M minor allelic frequencies ranging from 30 to 45%, and the contribution to explaining warfarin dosing variation ranged from no effect [60] or 1–7% in predominantly Caucasian samples [61–64]. In vitro kinetic experiments using recombinant supersomes expressing wild type or V433M CYP4F2 in liver microsomes confirmed CYP4F2 V433M allele has a reduced capacity to metabolize vitamin K which would produce higher hepatic vitamin K concentrations and require higher warfarin doses to achieve an anticoagulant response [65]. A GWAS using a chip with approximately 550×10^3 SNPs did not identify any SNPs other than CYP2C9*2/*3 and known VKORC1 polymorphisms in a sample of 181 Caucasians [66]. Two larger GWAS projects did identify a few additional candidate

SNPs. Takeuchi and colleagues screened 1053 Swedes with a 326 × 10^3 SNP chip and identified an association between CYP4F2 and warfarin dose, in addition to CYP2C9 and VKORC1 SNP clusters [67]. Finally, Teichert and colleagues in Rotterdam performed a GWAS with a 550 × 10^3 SNP chip on DNA from 1,451 patients who took acenocoumarol. They confirmed an association for CYP4F2 V433M and a SNP in CYP2C18 (rs1998591) with warfarin dose plus previously noted SNP clusters in CYP2C9 and VKORC1 [68]. Based on these GWAS results, it is unlikely that there are other undiscovered genetic variants in Caucasians with the clinical impact of CYP2C9 *2/*3 and VKORC1 haplotype A. However, adding SNPs which marginally improve dosing accuracy to PGx dosing algorithms is feasible and has been done for CYP4F2 V433M at www.WarfarinDosing.org.

Improving the accuracy of warfarin PGx algorithms for patients of African descent is a priority. While nongenetic factors may be involved, genetic differences clearly are important. There are at least 12 common VKORC1 haplotypes in populations of African descent compared to four in Caucasians. VKORC1 haplotype A, inferred from SNPs −1639G>A or 1173T>C, accounts for only 4.2% of warfarin dosing variability in African Americans compared to 22.5% in Caucasians, based on analysis of the IWPC data [7]. However, this is not due to other VKORC1 haplotypes or SNPs unique to African Americans, but to the low allelic frequency of haplotype A in African Americans compared to Caucasians, since the impact of a VKORC1 −1639A SNP on lower warfarin dose requirement is the same for any individual, regardless of race [7]. The impact of CYP2C9 *2/*3 SNPs on warfarin dose in African American populations is negligible compared to Caucasians [69], but this is also most likely due to the very low frequencies of these alleles in African Americans [48]. On the other hand, there are several SNPs in CYP2C9 with higher allele frequencies in African Americans compared to Caucasians which are associated with lower warfarin doses. CYP2C9*5 (1080C>G) substitutes glutamatic acid for asparagine at amino acid 360 (G360R), which is next to the Ile359Leu substitution coded by CYP2C9*3, and markedly reduces S-warfarin metabolism [70]. CYP2C9*6 is a null mutation due to deletion of adenine at nucleotide 818 and is associated with lower warfarin doses [71]. CYP2C9*11 introduces an arginine to tryptophan substitution at amino acid position 335 (R335W) and is associated with a 33% reduction in warfarin dose and in vitro evidence of decreased enzyme stability [72]. Recently, investigators reported an association between CYP2C9*8 (817A>G), coding for substitution of histidine for arginine at amino acid position 150 (R150H), and reduced warfarin dose [71, 73]. These four SNPs have frequencies of <1% in Caucasians, while minor allele frequencies are higher in African Americans: 0.7–1.5% for *5, 0.7–1.3% for *6, 4.7–6.5% for *8, and 1.3–1.8% for *11 [46, 71, 73]. In a cohort of 226 African Americans, 52 (23%) had a CYP2C9 variant (*2, *3, *5, *6, *8, or *11), which accounted for 6% of warfarin dosing variation, compared to 7% for VKORC1 − 1639G>A SNP [71]. Two commercial genotype platforms, Autogenomics Infiniti and GenMark Dx (formerly Osmetech) eSensor, currently offer extended warfarin PGx assays which include CYP2C9 *5, *6, and *11. A GWAS with warfarin dose using DNA from people of African descent is likely to identify additional genetic variants leading to more accurate PGx dosing algorithms and more SNPs to add to genetic testing panels.

8.6 Warfarin Genotype Testing Technologies

While PGx-based algorithms continue to evolve with the addition of more SNPs and refinements based on early INR response, investigators have confirmed the analytical validity of genotyping methods for the three core SNPs: CYP2C9 *2/*3 and VKORC1 −1639G>A or 1173C>T. In their assessment of analytical and clinical validity of warfarin PGx, McClain and colleagues' summary of published and unpublished genotyping methods showed analytical sensitivity and specificity results of 100% for CYP2C9 *1, *2, and *3 genotype combinations when compared to sequencing or PCR-RFLP reference methods [74]. Molecular diagnostic manufacturers have responded to the interest in warfarin PGx, and to date, there are five FDA 510K approved medical devices for CYP2C9 *2/*3 and VKOCR1 – 1639G>A or 1173C>T: Nanosphere (Verigene®), Autogenomics (INFINITI®), GenMark Dx (eSENSOR®), Paragon Dx reagents with Cepheid Smart Cycler®, and TrimGen reagents with Roche Light Cycler®. Several groups have independently confirmed commercial platforms' analytical validity [75–77]. Using archived DNA from 112 previously genotyped patients, King and colleagues evaluated INFINITI automated allele specific primer extension (ASPE) microarray instrument, Invader® cleavase-based fluorescence assay, and Luminex ASPE and Tag-it® bead hybridization platform. All methods were 100% accurate for CYP2C9 *1,*2, and *3 genotypes. INFINITI was 100% accurate, and Invader and Luminex 97% accurate for VKOCR1 SNP −1639G>A genotypes [75]. Babic et al. compared INFINITI, eSENSOR, and ParagonDx reagents/Stratagene® real time PCR instrument platforms using 100 DNA samples. Once again, CYP2C9 *2 and *3 genotype concordance was 100%. VKORC1 SNP −1639G>A genotype accuracy was 100 and 97% for eSENSOR and INFINITI instruments, respectively, and 100% for VKORC1 SNP 1173C>T using Paragonx/Stratagene and INFINITI platforms [76]. In addition to the FDA-approved warfarin PGx SNPs, Autogenomics and GenMark offer partially overlapping extended genotype panels for cytochrome P450 and VKORC1 SNPs. Babic's comparison of the two instruments' CYP2C9 *5,*6, and *11 SNP genotypes was 100% concordant [76]. Only GenMark's eSENSOR extended panel includes CYP4F2 1297G>A SNP (V433M), and compared to direct sequencing, eSENSOR CYP4F2 1297G>A genotyping was 100% accurate.

While analytical accuracy is method independent, other factors such as technical complexity, instrument and reagent costs, turnaround time (TAT), reliability, and versatility for other molecular diagnostic testing will influence a laboratory director's decision when selecting a platform for wafarin PGx testing. While attention is often focused on analytical TAT [75, 77], it is important to view this from a wider perspective. First, molecular diagnostic laboratories are not staffed or organized to perform STAT genotyping. At best, clinicians should expect warfarin PGx results the next working day if testing is done locally, and within 2–3 days if performed at a reference laboratory. All currently validated methods and platform TATs are ≤8 h from DNA isolation to genotype results and would be adaptable to most laboratories' work flow. Complying with external proficiency requirements from accreditation

organizations can be challenging for molecular diagnostic laboratories, yet this quality assurance indicator is an important component of analytical validity. In 2007, the College of American Pathologists addressed this need and introduced a pharmacogenomics survey which includes CYP2C9*2/*3 and VKORC1 −1639G>A or 1173C>T SNPs.

8.7 Clinical Trials on Pharmacogenetic Testing Efficacy

8.7.1 Published Trials

While pharmacogenetic-based algorithms do improve warfarin dosing accuracy, despite the previously mentioned shortcomings, this is insufficient evidence of clinical utility to warrant routine use [78]. The next step in warfarin PGx research has been prospective trials to determine if PGx-based warfarin dosing improves clinically meaningful patient outcomes. Clearly, the most important outcomes are bleeding and thromboembolic events. However, based on the low frequencies of these events in previous trials of warfarin in patients with atrial fibrillation and venous thromboembolic events [79], investigators have typically used INR response as a surrogate endpoint for clinical utility. There is evidence to support associations between bleeding complications and elevated INRs [79, 80], and thrombosis and lower INRs [81].

To date, there are three completed, prospective randomized trials comparing INR results between a PGx dosing group and a study-specific nomogram dosing control group (Table 8.2). Major hemorrhages were uncommon among patients enrolled in these studies. In the Caraco and colleagues' trial, time to first therapeutic INR, time to stable therapeutic dose, and percent of time INR was within therapeutic range (% INR TTR) significantly favored the PGx dosing arm even though VKORC1 genotyping was not performed [82]. Subjects in the PGx arm with CYP2C9 *1/*1 genotype (63%) were started on 1.25 × the control warfarin dose, which may account for the superior results in this group. Two other studies did not demonstrate a difference in primary INR endpoints. Hillman performed CYP2C9 *2/*3 genotyping and started control subjects on 5 mg of warfarin [83], while the Couma-Gen trial PGx algorithm included CYP2C9 and VKORC1 genotypes and patients in both arms received loading warfarin doses on days 1 and 2 [32]. However, when outcomes in the Couma-Gen trial were analyzed based on subjects' genotype status, there was a significant reduction in out-of-target-range INRs in the PGx-dosed subjects who were wild type or had more than one variant SNP. In addition, the difference between stable therapeutic warfarin dose and initial dose was significantly smaller for the PGx arm compared to the control arm for subjects with wild type or >1 variant SNP, which constituted 59% of the study population [32]. While these observations require independent confirmation, they suggest PGx-based algorithms improve initial warfarin dosing accuracy in Caucasian patients compared to a uniform, empiric starting dose by increasing the dose for wild type

Table 8.2 Summary of three small prospective, randomized trials of pharmacogenetic-based initial warfarin dosing

			Initial Warfarin dosing		INR time in therapeutic range (%)	
Author (Reference)	Subjects	Genotyped SNPs	PGx algorithm arm	Control arm	Genotype arm	Control arm
Hillman et al. [83]	38	CYP2C9*2/*3	d.1: algorithm dose d.≥2: INR-based nomogram both arms	5 mg	41.7	41.5
Caraco et al. [95]	191	CYP2C9*2/*3	d.1: *1/*1: 1.25 × control dose. Proportionately lower doses for CYP2C9 variants based on % reduction in S-warfarin metabolism d.2–8: INR-based nomogram both arms	5 mg	80.4	63.4‡
Anderson et al. [32]	206	CYP2C9*2/*3 VKORC1 1173CT[a]	d.l–2: 2 × algorithm dose d.3–4: algorithm dose d. ≥5: INR-based nomogram both arms	10 mg 5 mg	49.8	51.9

‡$P < 0.001$

[a]In high linkage disequilibrium with VKORC1 promoter SNP – 1639AG

patients and decreasing the dose for patients with multiple variant alleles, while PGx-based algorithms do not improve dosing accuracy or % INR TTR for patients with single variant SNPs. Therefore, all patients would undergo the cost of genotyping in order to identify a modest majority of patients who may potentially benefit from the information.

8.7.2 *Regulatory and Reimbursement Issues for Warfarin Pharmacogenomics*

Based on the limited prospective PGx-based warfarin dosing data, it is not possible to accurately estimate the economic cost-benefit of routine warfarin PGx genotyping [84], and clinical specialty societies [85] and laboratory science organizations [86] advocate waiting for more definitive evidence. Reflecting this perspective, in August, 2009, the Centers for Medicare and Medicaid Services (CMS) announced it would not reimburse for warfarin pharmacogenetic testing of Medicare patients except when done in a CMS approved randomized clinical trial setting. However, in January, 2010, the FDA revised Coumadin labeling again, adding a table of expected ranges for therapeutic warfarin dose based on CYP2C9 *2/*3 and VKORC1 −1639G>A SNP status [87] (Table 8.3). While the label change does not explicitly require genetic testing, it does provide initial dosing guidelines for patients with all combinations of these three SNPs, which may encourage more clinicians to order warfarin PGx testing.

8.7.3 *Ongoing Clinical Trials*

Meanwhile, three large multicenter trials are underway, comparing clinical dosing algorithms to PGx dosing algorithms in patients starting a coumarin anticoagulant: Clarification of Optimal Anticoagulation through Genetics (COAG) trial (ClinicalTrials.gov: NCT00839157); European-Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial [88], and the Genetics Informatics Trial (GIFT) (ClinicalTrials.gov:NCT01006733)(Table 8.4). All three trials are prospective, randomized, fully or partially blinded designs to minimize potential

Table 8.3 Range of expected therapeutic warfarin doses based on CYP2C9 and VKORC1 genotypes

	CYP2C9					
VKORC1	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3
GG (mg)	5–7	5–7	3–4	3–4	3–4	0.5–2
AG (mg)	5–7	3–4	3–4	3–4	0.5–2	0.5–2
AA (mg)	3–4	3–4	0.5–2	0.5–2	0.5–2	0.5–2

Bristol-Myers Squib Coumadin® prescribing information, revised January 2010

Table 8.4 Comparison of three large prospective randomized trials to evaluate efficacy of PGx algorithms for initial warfarin dosing

	COAG	EU-PACT	GIFT
Study population	Newly dx'd VTE or AF	Newly dx'd VTE or AF	Hip or knee arthroplasty DVT prophylaxis
Recruitment target	1,238	2,955	1,600
Oral anticoagulant	Warfarin	Warfarin, acenocoumarol, or phenprocoumon	Warfarin
Target INR	2–3	2–3 or 2–3.5	1.8 or 2.5
Design	Double blinded	Patient blinded	Double blinded
Treatment arms	Clinical algorithm vs. PGx algorithm	Clinical algorithm vs. PGx algorithms	Clinical algorithm vs. PGx algorithm
Loading dose	Day 1	Days 1–3	Days 1–2
Revision algorithm	Yes	Yes	Yes
Primary outcome	INR % TIR	INR % TIR	VTE, major bleed, death, vascular death
Follow-up	4 weeks	3 months	4–6 weeks

biases and to determine whether adding CYP2C9 *2/*3 and VKORC1 −1639G>A genotype information to a clinical-based algorithm will affect clinically meaningful outcomes. Despite recruitment goals of 1,238 and 2,955 for COAG and EU-PACT, respectively, the primary endpoint will be % INR TTR rather than major hemorrhagic or thrombotic complications because the incidence of these events is low, and in order to have statistical power to detect a difference for those outcomes, recruitment targets would be many times higher and prohibitively expensive. Subjects participating in GIFT are at high risk for DVTs after hip or knee arthroplasties, and in this study, the efficacy of PGx-based warfarin dosing will in part be based on the rates of both symptomatic and asymptomatic DVTs detected by Dopplar ultrasound after 4–6 weeks of warfarin therapy. Despite numerous study design differences, these trials use similar algorithms and collect uniform clinical, genetic, and outcome data to permit analysis of pooled data in the future. However, it will be several years from now before we can more accurately judge the efficacy and cost-effectiveness of warfarin PGx based on the outcomes of COAG, EU-PACT, GIFT, and possibly other prospective randomized trials.

In the interim, other factors will influence utilization of warfarin PGx tests including new genetic discoveries leading to improved dosing accuracy, test costs, laboratory charges, third party reimbursement patterns, recent FDA label changes, and results from less well-controlled studies performed in general practice environments. For example, a Medco Research Institute and Mayo Clinical Laboratories collaboration offered PGx testing to Medco insured outpatients beginning warfarin and sent CYP2C9 *2/*3 and VKORC1 −1639 SNP results with interpretive and management guidelines to participating patients' physician [89]. Compared to 2,688 historical controls from the same insured pool who started warfarin a year earlier and were not offered PGx testing, 896 patients who underwent warfarin PGx

testing had a 28% reduction in hospitalization rate (25.5 v. 18.5%) and 27% reduction in hospitalization rate for bleeding or clotting complications (8.1 v. 6.0%) during the 6-month follow-up period after starting warfarin. While the "real world" management environment is a strength, there is potential for uncontrolled variables to confound these results. Due to the logistical barriers of informed consent and sample collection, the median time from patients starting warfarin to sending PGx genotypes to their physicians was 32 days which would likely diminish the impact of genetic information after 4 weeks of trial and error warfarin dosing. In addition, the investigators did not compare INR results between the PGx and control populations or correlate INR control with hospitalization rates. Nevertheless, the preliminary findings are noteworthy and may convince more clinicians to order PGx genetic testing instead of waiting several years for more definitive results from prospective, randomized controlled trials.

8.8 Pharmacologic Alternatives to Warfarin

For 56 years, there were no alternatives to warfarin for oral anticoagulation therapy, until recently. Now, two oral direct coagulation factor inhibitors are poised to challenge warfarin, and other compounds are in the pharmaceutical pipeline [9]. Rivaroxaban is a direct factor Xa inhibitor, and dabigatran etexilate is a direct thrombin inhibitor. The large, prospective, randomized RE-LY trial compared warfarin therapy (INR 2–3) and two doses of dabigatran in atrial fibrillation patients and demonstrated similar rates of stroke and systemic emboli and lower major bleeding rates for dabigatran 110 mg twice daily, and lower stroke and systemic emboli rates with similar major bleeding rates for dabigatran 150 mg twice daily [90]. The RE-COVER trial compared warfarin (INR 2–3) to dabigatran, 150 mg twice daily in patients with acute VTEs, and demonstrated similar low rates of recurrent thromboses and major bleeding [91]. In November 2010, the FDA approved dabigatran 150 mg BID to prevent thromboembolic events in patients with atrial fibrillation. In the RECORD series of randomized prospective trials, rivaroxaban, 10 mg per day, was more effective than low molecular weight heparin for DVT prophylaxis after hip or knee arthroplasty with similar bleeding complication rates [92]. Dabigatran and rivaroxaban are administered as fixed doses in adults, do not require therapeutic monitoring of anticoagulant effect to ensure efficacy and safety, and lack major pharmacogenetic or drug interactions [9]. The safety and efficacy of direct inhibitors have not been investigated in patients with prosthetic heart valves or children, or patients with moderate to severe renal insufficiency since both drugs are partially eliminated by the kidneys. It is anticipated that direct coagulation factor inhibitors will be much more expensive than warfarin, but the convenience of dispensing with periodic INR monitoring will make them attractive to many patients and physicians. How warfarin and PGx testing will be integrated into the approaching competitive oral anticoagulation drug era is unclear.

8.9 Case Report

The following description of our experience in managing initiation of warfarin therapy demonstrates the challenges and successes one can achieve with PGx dosing algorithms [93]. An internist referred a man to our anticoagulation service on a Friday afternoon. The patient consented to participate in a PGx-based dosing algorithm trial, and provided the following information: age 74, race white, weight 180 lbs, height 6″4″, and medications amiodarone 400 mg and pravastatin. His baseline INR was 1.1, and the target therapeutic INR was 2–3. Since CYP2C9 *2/*3 and VKORC1 −1639G>A genotyping would not be performed until the following Monday, a pharmacist entered the clinical and demographic data into an online dosing calculator at WarfarinDosing.org and obtained a predicted dose of 3.0 mg/day, reflecting the negative impact of his advanced age and two medications on warfarin requirements. Monday afternoon, the patient returned to clinic and after taking 3 mg of warfarin for 3 days his INR3 was 1.7. Using a "trial and error" approach to warfarin initiation, most clinicians would continue the current dose, or increase it, and repeat an INR in a few days. However, his genotype was CYP2C9 *3/*3 and VKORC1 −1639AA, indicating he was an extremely poor metabolizer of S-warfarin and very sensitive to warfarin inhibition of VKOR. Adding the patient's genotype, three doses of 3 mg and INR3 of 1.7 to WarfarinDosing.org generated a revised estimated therapeutic warfarin dose of 1.7 mg/day. With empiric dose adjustments during the subsequent 4 weeks, all INRs were therapeutic, there were no bleeding or embolic complications, and the patient's average warfarin dose was 0.7 mg/day. This case illustrates several important features of PGx algorithm warfarin dosing. First, it is not necessary to have warfarin PGx genotype results when determining an initial warfarin dose since the impact of slow metabolizing genotypes on INR is delayed and can be incorporated into a revised PGx algorithm dose estimate after three or 4 days of treatment. Secondly, PGx algorithms do not always produce accurate estimates of a patient's eventual therapeutic dose (1.7 vs. 0.7 mg/day respectively in this case), but are a definite improvement over starting all patients on 5 or 10 mg/day followed by INR-based trial and error dose adjustments. And finally, PGx dosing algorithms are an adjunct to, not a substitute for, anticoagulation management expertise in order to safely and effectively care for patients like the man in this case study.

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Chapter 9
Clopidogrel and Salicylates

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Keywords Antiplatelet therapy • Cardiovascular disease • Clopidogrel

Antiplatelet therapy is at the cornerstone of coronary disease management and is also indicated for long-term management of peripheral vascular and cerebrovascular diseases. Currently, the most widely used antiplatelet medications are aspirin and clopidogrel with a combination of these two medications as dual therapy for patients undergoing percutaneous coronary intervention with stent implantation.

9.1 Clopidogrel

9.1.1 Pharmacology and Clinical Efficacy

9.1.1.1 Mechanism of Action

Clopidogrel is an oral thienopyridine. It is a prodrug, 85% of which is metabolized by esterases to form an inactive carboxylic acid derivative; the remaining 15% is activated by hepatic metabolism by the cytochrome 450 system [1]. The active thiol metabolite irreversibly binds the $P2Y_{12}$ component of the adenosine diphosphate (ADP) receptor, leading to inhibition of ADP-dependent platelet activation and aggregation. Peak plasma concentrations of the active metabolite can be reached after several hours [1–3], with increased peak metabolite concentration and platelet inhibition when a single loading dose of 600 vs. 300 mg is administered [4]. Inhibition of platelet aggregation and activation lasts for the lifetime of the platelet, and recovery of platelet function requires approximately 5 days off therapy [5].

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_9,

9.1.1.2 Clinical Efficacy

The addition of clopidogrel to aspirin as *dual* antiplatelet therapy has been shown to prevent death and ischemic complications in patients with acute coronary syndrome (ACS) [6–8] as well as in patients undergoing percutaneous coronary interventions (PCI) [6, 7, 9]. In the management of stable coronary artery disease, the addition of clopidogrel to aspirin in patient for primary prevention of ACS is not indicated [10], but subgroup analysis suggests that patients with a prior myocardial infarction may benefit from dual clopidogrel plus aspirin therapy for secondary prevention of ACS [11].

In regard to stroke prevention, dual antiplatelet therapy combining aspirin and clopidogrel is an effective (though inferior) alternative to warfarin for primary stroke prevention in patients with atrial fibrillation who are indicated but not candidates for warfarin therapy [12, 13]. In the secondary prevention of stroke, clopidogrel monotherapy has been shown to be as effective as aspirin plus clopidogrel [14] and as effective as Aggrenox (dipyridamole plus aspirin) [15], and that clopidogrel monotherapy carries less bleeding risk than combination therapies [14, 15].

In the management of mixed atherothrombosis (defined as recent ischemic stroke, recent myocardial infarction, or symptomatic peripheral arterial disease), clopidogrel monotherapy (at dose of 75 mg daily) was modestly more effective than aspirin (325 mg daily) in reducing the combined risk of ischemic stroke, myocardial infarction, or vascular death without significant difference in bleeding events [16].

9.1.1.3 Dosing and Duration of Therapy

The PCI-CURE and PCI-CLARITY studies demonstrated a significant reduction in ischemic events in patients who were pretreated with clopidogrel before PCI versus those who were not [6, 7] The optimal loading and maintenance dose of clopidogrel is a subject of active research. The CURRENT-OASIS 7 trial [17] reported that a loading dose of 600 mg with post-PCI maintenance dose of 150 mg for the first week followed by 75 mg daily thereafter reduced stent thrombosis and CV events (primarily nonfatal MI) compared to a loading dose of 300 mg followed by maintenance dose of 75 mg daily. The high-dose clopidogrel arm was associated with a modest increase in CURRENT-defined major bleeds, but with no increase in TIMI major bleeds, intracranial hemorrhage, fatal bleeds, or CABG-related bleeds.

The duration of dual antiplatelet therapy is another area of active research. Currently, the ACC/AHA guideline recommends dual antiplatelet therapy with aspirin and clopidogrel for at least 1 month for those with bare metal stent implantation and for 12 months for those with drug-eluting stent (DES) implantation [18]. The DES-LATE trial examined the benefit and risks of extending dual antiplatelet therapy beyond 12 months, but was underpowered to draw definitive conclusions [19]. The ongoing DAPT trial [20] will provide further insights into the benefits and safety of long-term dual antiplatelet therapy after DES implantation.

9.1.2 *Variable Response, Drug–Drug Interactions, and Pharmacogenetics of Clopidogrel*

Despite established clinical efficacy, variability in clopidogrel response has been observed: inhibition of platelet aggregation with clopidogrel therapy approximates a bell-shaped distribution and individuals with the least degree of platelet inhibition have higher rates of ischemic events including stent thrombosis [21]. These observations have led to investigations of clopidogrel's absorption, metabolism, and action pathway.

Investigators have examined drugs such as statins and proton-pump inhibitors (PPIs) that are competitive substrates for or inhibitors of key CYP450 enzymes in clopidogrel metabolism (Fig. 9.1) [22]. Statins are predominantly metabolized by CYP3A4. Concurrent statin use was found to attenuate platelet inhibition by clopidogrel in a dose-dependent manner but not to increase major cardiovascular events [23–25]. PPIs inhibit the CYP2C19 enzyme crucial for clopidogrel metabolism [26]. Observational data initially raised concerns about the PPI omeprazole decreasing clopidogrel's clinical efficacy [27]. A subsequent analysis of large clinical trial database noted concomitant PPI and clopidogrel to be associated with a trend toward a modest attenuation of platelet aggregation inhibition, but *not* to be associated with adverse clinical outcomes [28]. Data from the prospective, randomized-controlled COGENT trial reported no difference in the risk of cardiovascular events or ischemic complications and a benefit in reduction of gastrointestinal bleeding in patients concomitantly taking PPI and clopidogrel [29].

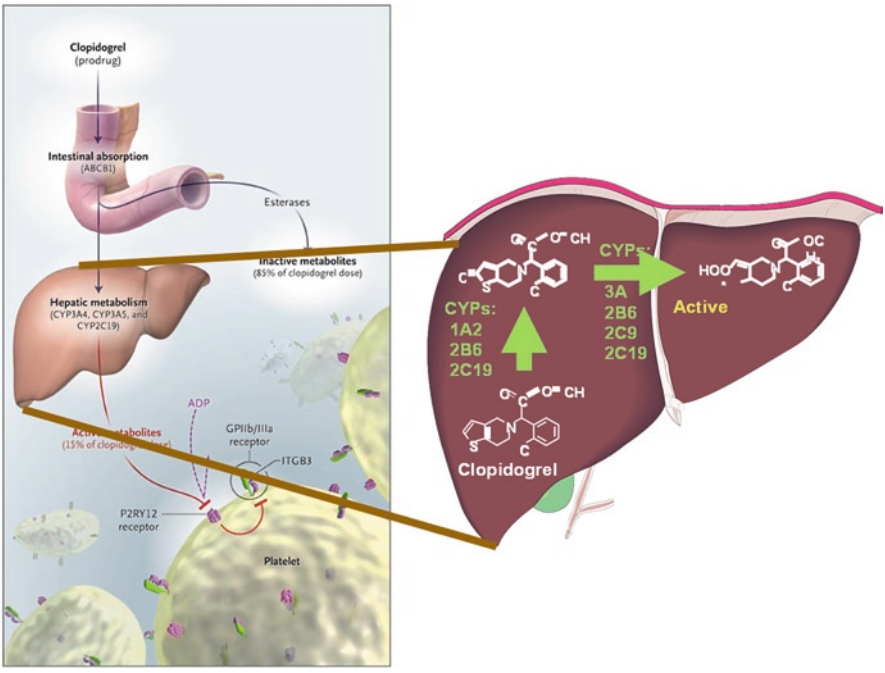

Fig. 9.1 Clopidogrel absorption, metabolism, and action pathway, adapted from Simon et al. NEJM [22] and used with permission

Investigators have also examined genes encoding the CYP450 enzymes, which are highly polymorphic with certain alleles conferring greatly reduced enzymatic function [30]. CYP2C19 is involved in both steps of the CYP450 conversion of clopidogrel to its active metabolite. Subgroup analyses of clinical trial and registry databases [31, 32] and a genome-wide association study (GWAS) [33] have identified CYP2C19 polymorphism to be independently associated with diminished inhibition of ADP-induced platelet aggregation upon clopidogrel administration. Of the reduced-function allelic variants of the CYP2C19 enzyme (including *2, *3, *4, *5), the *2 variant is the most common [31] and is carried by approximately 30% of Whites [31], 40% Blacks, and 55% of East Asians [34]. The *2 variant rs4244285 involves a single base-pair mutation of G->A at position 681 [35] which creates an aberrant splice site, leading to the synthesis of truncated nonfunctional CYP2C19 protein [36].

Compared to noncarriers, carriers of at least one copy of CYP2C19*2 allele have approximately 30% lower levels of active clopidogrel metabolite and approximately 25% relatively less platelet inhibition [31]. Moreover, among patients with ACS and planned PCI, carriers of at least one copy of CYP2C19*2 variant had a 50% increase in the risk of cardiovascular death, MI, or stroke and a threefold increased risk of stent thrombosis [31]. These observations have been seen in a total of nine clinical studies presented or published to date [31–33, 37, 38]. Based on the totality of the data to date, both heterozygotes and homozygotes appear to be at increased risk.

On the other hand, the CYP2C19*17 allelic variant, involving a single base-pair mutation of C->T at position 808, has been linked to increased transcriptional activity of the CYP2C19 enzyme, leading to extensive clopidogrel metabolism with enhanced production of active metabolites and subsequently exaggerated platelet response to clopidogrel therapy. CYP2C19*17 variant carrier status has been associated with greater inhibition of ADP-induced platelet aggregation, increased risk of bleeding, but without significant influence on stent thrombosis [39].

Polymorphism of other genes involved in the clopidogrel absorption, metabolism, and action pathway (Fig. 9.1) has also been explored. Specifically, polymorphism of the ABCB1 gene (encoding for P-glycoprotein involved in intestinal absorption of clopidogrel) [31], the CYP3A4 gene (encoding for eponymous protein involved in clopidogrel metabolism) [40], and the P2RY12 gene (encoding for the receptor for active metabolite of clopidogrel) [41] has generated interests. However, only CYP2C19 polymorphism has been consistently replicated in studies and was the only significant polymorphism noted in a GWAS investigating gene variants that influence clopidogrel response [33].

9.1.3 Therapeutic Implications of Pharmacogenomics of Clopidogrel and Novel Antiplatelet Agents

The replicable associations between CYP2C19 polymorphism and decreased platelet inhibition as well as worsened cardiovascular outcomes have led the U.S. Food and Drug Administration (FDA) and the manufacturer to revise the clopidogrel prescribing

information in May 2009 to include mentions of the CYP2C19 genetic polymorphism. Limited evidence is available to guide potential therapeutic modifications for individuals with CYP2C19*2 allele. Potential therapeutic modifications considered include escalation of clopidogrel dosage or switching to an alternate agent.

While earlier studies comparing clopidogrel loading dose of 300, 600, and 900 mg in patients undergoing PCI found no improved benefit in administration of loading dose higher than 600 mg [4], data from a study incorporating CYP2C19 polymorphism genotyping suggest that higher clopidogrel loading and maintenance dose (of 1,200 mg as loading and 150 mg daily as maintenance) may improve platelet inhibition in carriers of reduced-function CYP2C19 alleles [42].

Switching to an alternate antiplatelet agent may also be a logical modification for carriers of CYP2C19*2 alleles indicated for clopidogrel therapy for ACS and PCI. Prasugrel is a third-generation thienopyridine that also confers antiplatelet activity by binding to the platelet P2Y12 receptor [43]. Like clopidogrel, it is an irreversible inhibitor. Unlike clopidogrel, it achieves a much higher degree of platelet inhibition. The clinical efficacy of prasugrel for ACS and PCI was established in the TRITON-TIMI 38 trial which found prasugrel to have superior efficacy compared with clopidogrel in reducing all-cause mortality or vascular complications including stent thrombosis, albeit with an increased risk of bleeding [44]. Prasugrel was approved for use in PCI for ACS by the FDA in July 2009. Retrospective subgroup analysis of the TRITON-TIMI 38 trial found common functional CYP polymorphisms (including CYP2C19 polymorphism) not to affect active drug metabolism levels, platelet aggregation inhibition, or clinical cardiovascular event rates in individuals treated with prasugrel [45]. The differential roles of esterases and CYP metabolism in the activation of clopidogrel and prasugrel likely mediate this differential impact of CYP polymorphism. Whereas esterases shunt the majority of clopidogrel to a dead-end inactive pathway with the remaining prodrugs requiring a 2-step CYP-dependent oxidation to produce active clopidogrel metabolites, esterases are part of the activation pathway of prasugrel and prasugrel is oxidized to its active metabolite in a single CYP-dependent step [45].

Another potential antiplatelet alternative, not yet approved by the FDA, is ticagrelor, an oral reversible direct antagonist of platelet ADP PGY12. The PLATO trial reported ticagrelor to be superior to clopidogrel (loading dosing 300–600 mg with 75 mg daily maintenance dose) in reduction of vascular death, myocardial infarction, or stroke, but with an increase in the rate of non-CABG-related bleeding in ACS [46]. Ticagrelor is an active compound and not a prodrug. However, it is metabolized to inactive compounds by CYP3A4/5 and the impact of variants in those genes on safety and efficacy remains undefined.

9.1.4 Pharmacogenetic Testing

Should individuals with planned clopidogrel therapy undergo pharmacogenetic testing to guide therapeutic management, given the significantly increased morbidity and mortality in CYP2C19*2 carriers? The most recent Plavix boxed warning

includes "Tests are available to identify a patient's CYP2C19 genotype and can be used as an aid in determining therapeutic strategy. Consider alternative treatment or treatment strategies in patients identified as CYP2C19 poor metabolizers [47]."

Currently, pharmacogenetic testing in patients being treated with clopidogrel is not part of the standard of care. Although genetic testing for CYP2C19 can be done in a clinical pathology laboratory, the turnaround time for such testing would likely exceed the length of stay for most cardiology patients, thereby precluding integration of such information during the loading phase of clopidogrel therapy. To that end, several companies are now working on a point-of-care genotyping platform using whole blood. If and when genetic data did become available, it remains unclear how clinicians would use such information. Small studies have suggested that CYP2C19 reduced-function allele carriers have a greater response to increased loading and maintenance doses of clopidogrel than do wildtype individuals [42, 48]. However, the specific doses of clopidogrel needed to achieve bioequivalence to the platelet inhibition seen with a 300-mg load and 75 mg daily in wildtype individuals remains to be defined. Moreover, some data suggest that it may require prasugrel rather than higher doses of clopidogrel to reliably achieve adequate platelet inhibition in CYP2C19*2 carriers [49].

The relative clinical value of genetic testing and platelet function testing remains to be defined; some studies suggest that they offer complementary predictive values [50]. Lastly, how such testing would be reimbursed by insurance companies is unknown. In summary, prospective clinical trials are needed to further evaluate if testing indeed improves outcome, if pharmacogenetic testing is superior to platelet function testing, and if testing of all-comers versus only the high-risk population is most feasible. These questions will need to be answered before pharmacogenetic testing of CYP2C19 polymorphism can be recommended as part of the standard of care for clopidogrel therapy.

9.2 Salicylates

9.2.1 Pharmacology and Clinical Efficacy

9.2.1.1 Mechanism of Action

Aspirin irreversibly inhibits cyclooxygenase-1 and 2 (COX-1 and 2) enzymes. COX enzymes catalyze the conversion of arachidonic acid to PGG_2 and PGH_2, resulting in downstream synthesis of prostanoids and TXA2. Therefore, administration of aspirin decreases downstream production of PGD2, PGE2, PGF2, PGI2, and TXA2. Key to aspirin's clinical utility as an antiplatelet agent is the observation that inhibition of TXA2 predominates, whereas inhibition of PGI2 appears to be clinically irrelevant [51]. Together, this balance contributes to aspirin's net antithrombotic effects primarily by inhibition of TXA2-mediated platelet aggregation which leads to not only its clinical utility in prevention of thrombosis, but also its adverse effects of bleeding.

9.2.1.2 Metabolism, Pharmacodynamics, and Pharmacokinetics

Absorption of enterally administered aspirin is rapid, with bioavailability of about 50%, reaching peak serum level in 1–2 h and lasting 4–6 h, and with dose-dependent half-life of elimination of salicylates [51, 52]. Enterally ingested aspirin is activated by hydrolysis of gastric esterase. In ACS, patients are often instructed to chew the aspirin, allowing for sublingual absorption with subsequent activation via esterase hydrolysis in the bloodstream and earlier peaking of serum salicylate levels. Metabolism of salicylate occurs primarily by hepatic glucoronidation; the metabolite of aspirin is ultimately renally excreted [53].

9.2.1.3 Clinical Efficacy and Dosing

Aspirin's broad array of clinical use can be divided into four general categories: vascular-antiplatelet, anti-inflammatory, analgesic, and anti-pyretic. Cardiovascular indications of aspirin as an antiplatelet agent are well-established in the treatment [54–60] and secondary prevention [61] of ACS. Aspirin therapy is paramount in the setting of coronary revascularization with clinical efficacy in the peri- and postprocedural management of PCI with [6, 7, 9, 44, 62] or without [63, 64] stent implantation and in patients who have undergone coronary artery bypass surgery [65–67]. Although the use of aspirin for primary prevention of myocardial infarction is common, clinical efficacy has not been consistently demonstrated. Data from primary prevention trials demonstrate efficacy in reduction in risk of myocardial infarction in patients with chronic stable angina [68, 69]. Without selecting for patients with chronic stable angina, aspirin primary prevention trials yielded gender-specific results; while combined analyses of the Physicians' Health Study and the British Physicians' Study noted reduction in myocardial infarction events in men [70, 71], the Women's Health Study found aspirin to lower the risk of stroke without affecting the risk of myocardial infarction in women [72]. While controversial, low-dose aspirin was found to be inefficacious in primary prevention of atherosclerotic events in patients with type-2 diabetes [73].

In primary prevention of cardioembolic stroke in patients with atrial fibrillation, high-dose aspirin alone is reasonable for lone atrial fibrillation [74] and low-dose aspirin in combination with clopidogrel has been found efficacious for patients with atrial fibrillation with moderate stroke risk who are not candidates for warfarin therapy [12]. Aspirin is also indicated in the management of acute ischemic stroke [75, 76], transient ischemic attack [76–79], peripheral artery disease [77, 80], and in peri- and postprocedural management of percutaneous stents to peripheral vessels [81]. Additionally, peripheral artery disease patients on aspirin therapy were found to have reduction in stroke event rates [80].

Therapeutic indications capitalizing on aspirin's anti-inflammatory effects include very high-dose therapy for pericarditis and acute rheumatic fever. Although aspirin also has analgesic and antipyretic effects, its uses as the principal agent in these areas are more limited. Due to potential association with Reye's syndrome, aspirin should not be used in children for viral infections [82].

9.2.1.4 Variable Response, Pharmacogenetics, and Pharmacogenomics of Aspirin

Variability in aspirin response has been noted [83–87] with potential associations with worsened cardiovascular outcomes [85]. Notable contributors include noncompliance [88], drug–drug interaction especially with nonsteroidal anti-inflammatory drugs [89], female gender [90], and diabetes mellitus [40, 91]. Genetic polymorphisms may play a role in aspirin response.

The PIA polymorphism involves a Leu (PI^{A1}) to Pro (PI^{A2}) substitution at position 33 of the GP IIIa subunit of the platelet GP IIb/IIIa receptor, which has been most commonly studied [92]. However, studies have yielded conflicting results as to whether there is an association between PIA2 allele and impaired aspirin response [93] or not [94].

More recently, a large candidate gene study found SNP rs2768759 (located 10 kb upstream of the platelet endothelial aggregation receptor-1 (PEAR1) gene) to be associated with decreased inhibition of platelet aggregation with aspirin therapy. Given that rs2768759 is quite far upstream from the PEAR1gene, the observed association with rs2768759 may reflect the effect of a SNP in the PEAR1 gene in linkage disequilibrium with rs2768759 or that rs2768759 may confer the observed effects by being part of the immediately upstream NTRK1 (neurotrophic tyrosine kinase receptor type 1) instead [95].

A subsequent genomic array study noted SNPs in PEAR1 to be associated with increased platelet response to collagen-related peptide and that PEAR1 protein expression increased after platelet degranulation. Neither rs2768759 (which was felt to be part of the NTRK1 gene by this genomic array study) nor SNPs in linkage disequilibrium with rs2768759 were included in the genotyping of the genomic array study; subsequent genotype of rs2768759 by TaqMan revealed no association with platelet response [96]. Thus, while genetic influences of aspirin response are likely present, large well-replicated studies are needed to establish potential genetic determinants of aspirin variability.

9.2.1.5 Therapeutic Implications and Pharmacogenetic Testing

Until further large-scale studies are able to demonstrate reproducible links between specific genetic determinants and biochemical evidence of aspirin variability and effects on clinical endpoints, no clear therapeutic implications or strategies for pharmacogenetic testing can be made based on current data.

9.3 Case Study

A 45-year-old man with diabetes mellitus, hypertension, and dyslipidemia presented with substernal chest pressure radiating to left jaw 3 months after implantation of a DES to the circumflex artery for non-ST-segment elevation myocardial infarction.

Patient endorses compliance with dual antiplatelet therapy of 75 mg clopidogrel daily and aspirin 81 mg daily (after switching from 325 mg daily 2 months ago). The patient also takes glyburide, metoprolol, atorvastatin, and omeprazole, the last of which was prescribed years ago upon diagnosis of gastritis. Posterior EKG found patient to have ST segment elevation in posterior leads. Prompt cardiac catheterization revealed stent thrombosis of the previously placed DES, which was well deployed. Thrombus aspiration was performed and recanalization was achieved by placement of a new DES.

In addition to providing the current standard of care for ST-segment elevation myocardial infarction [97], should this patient undergo clopidogrel pharmacogenetic testing, and how should we proceed with this patient's medication regimen?

Given that the patient presented with stent thrombosis despite compliance with clopidogrel therapy and despite a well-deployed stent, empirically increasing the maintenance dose of clopidogrel to 150 mg daily would be reasonable, as would changing to prasugrel. Such recommendations would hold regardless of the patient's genotype, given the patient's clinical presentation. More problematic is whether all patients should undergo genetic (or platelet function) testing prospectively at the time of initiation of ADP receptor blockade therapy. For now, such a recommendation cannot be made. Data from additional studies, some of which are ongoing, will be needed to define the benefits of such an approach.

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Chapter 10
Genotype-Guided Statin Therapy

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Keywords HMGCoA reductase • Hydroxymethylglutaryl coenzyme A reductase • Atorvastatin • Simvastatin • Rosuvastatin • Pravastatin • Low-density lipoprotein cholesterol • Myalgia • Myopathy • Myositis • Creatine kinase • Neuromuscular side effects • Statin-induced neuromyopathy • Physiogenomics • *SLCO1B1* • *KIF6* • *CLMN* • *APOE* • *HMGCR* • Major adverse cardiovascular event

10.1 Introduction

One of the promises of the Human Genome Project is individualization of patient care based on highly heterogeneous innate metabolic factors determined by DNA typing of gene polymorphisms. Translation of such gene polymorphisms into clinical decision support for personalized healthcare is the basis for DNA guided medicine. Pharmacogenetics serves as the foundation for the most clinically advanced application of DNA-guided medicine. Statin responsiveness is an area of high research interest given the success of the drug class in the treatment of hypercholesterolemia and in primary and secondary prevention of cardiovascular disease. Ongoing genetic inquiry into response variability indicates probable multigenic determinants for statin efficacy and safety. It is the vision that knowledge of the patient's genetic status for a number of common variants will soon guide hyperlipidemic intervention.

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_10,

10.2 Genotype-Guided Drug Therapy

It is a worthy goal to predict variability in drug responsiveness. Such prediction has the potential to reduce medication errors in general, trial and error on the part of clinicians, risks of therapy, and the resources needed to treat. Statins belong to a drug class that selectively and competitively inhibit the intracellular enzyme hydroxymethylglutaryl Coenzyme A reductase (HMGCoA reductase) that is expressed to different degrees in various tissues. In addition to the desirable inhibition of cholesterol synthesis, the inhibition of HMGCoA activity reduces synthesis of geranyl and farnesyl products as shown in Figure 10.1, leading to decreased isoprenylation of proteins and possible impairment of many varied cellular functions. Statin entry into cells can be gated, and metabolic pathways for the drugs of this class are varied and drug dependent.

The goal of identifying the genes modulating statin response is challenging. In contrast to warfarin, for which variants in two genes alone determine 30–40% of the variability in effective drug dose, or clopidogrel, whose activation depends on the *CYP2C19* gene, many more genes have roles in statin pharmacogenetics discovery.

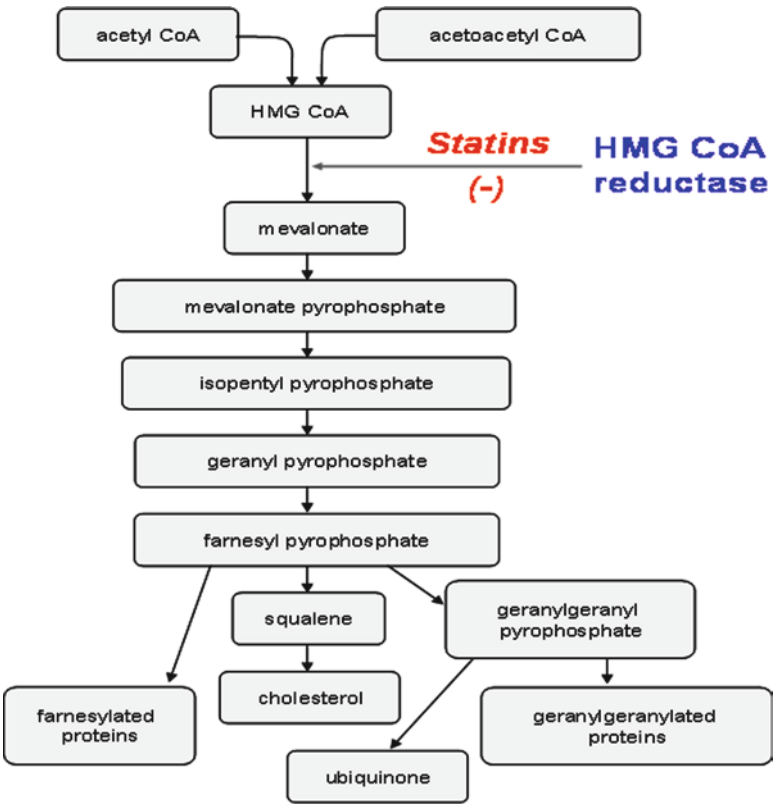

Fig. 10.1 Inhibition of cholesterol and isoprenoid synthesis by statins

Each will have a small contribution to the variability of the statin response. As we will show, the story is evolving.

10.3 Clinical Status of Statins

Statins are the most prescribed drugs in the United States [1] and the world [2]. In the United States, prescriptions for atorvastatin (Lipitor®), simvastatin (Zocor®), and rosuvastatin (Crestor®) comprise 85% of the market share [1]. The HMG CoA reductase inhibitors reduce cardiac events in coronary heart disease patients and in previously healthy subjects [3]. This success has fostered increasingly aggressive usage and dosing. Their main clinically relevant safety risk is statin-induced neuromyopathy (SINM) evidenced as a constellation of *n*euro*m*uscular *s*ide *e*ffects (NMSEs). NMSEs are disabling to 3–20% of patients on statins, require alteration of therapy, and reduce compliance [4–7]. NMSEs include myalgias (pain, weakness, aches, cramps) and myositis (typically monitored by elevation of serum creatine kinase [CK] activity) [4]. NMSEs vary in extent among drugs and from patient to patient. Were there a system to predict the safety and efficacy of the preeminent statin drugs according to the genome of each patient, a clinician could optimize the selection from among atorvastatin, simvastatin, and rosuvastatin. Alternatively, a patient's genomic profile may prove incompatible with statins, and the clinician could decide to avoid the drug class.

10.4 Statin Effectiveness

10.4.1 Low-Density Lipoprotein Cholesterol Lowering

Statins are the most effective medications for managing elevated concentrations of low-density lipoprotein cholesterol (LDLc). As LDLc lowering coincides with decreases in major cardiovascular events [3], it is a metric useful for evaluation of statin efficacy in the short-term. Administered at maximum dosages, the most common statins – atorvastatin, simvastatin, and rosuvastatin – lower LDLc by 46–57% in patients with primary hypercholesterolemia [8–10]. Pravastatin is less widely prescribed and less potent, with a mean peak response of 34% [11]. The magnitude of the LDLc response differs according to phenotypic, demographic, and as yet unexplained characteristics [12].

Approximately 50% of the variability in plasma LDLc is estimated to be attributable to inheritance [13]. Because baseline LDLc predicts the magnitude of LDLc lowering with statins [14], there may be overlap in the genes that regulate LDLc metabolism in the drug-free state and statin-mediated LDLc lowering. In the absence of statin therapy, variants in genes such as (in approximate order of their predictive strength) *APOE* cluster [15], *APOB* [16], *LDLR* [16, 17], *SORT1/CELSR2/*

PSRC1 [16], *B4GALT4* [16], *B3GALT4* [16], *NCAN/CILP2* [16], *NPC1L1* [18], and *PSCK9* [16, 19] affect LDLc. *APOE* cluster, *LDLR*, *APOB*, *SORT1/CELSR2/PSRC*, *B4GALT4*, *B3GALT4*, *PSCK9*, and *NCAN/CILP2* have been confirmed by genome wide association studies (GWAS) in more than one population [16].

Pharmacogenetic studies of LDLc lowering associated with statin therapy have focused on ~40 genes, mainly those in cholesterol synthetic, lipoprotein lipid transport, and pharmacokinetic pathways. Single nucleotide polymorphisms (SNPs) in genes of cholesterol metabolism such as *HMGCR* [20–23] and lipoprotein transport such as *APOE* [14, 24], *CETP* [25, 26], *LIPC* [27], *APOA5* [28], *LDLR rs1433099* [22], *ABCA1* [24], and *LEPR* (223A>G, rs1137101) [29] can influence the statins' ability to lower LDLc levels. Individual variants in these genes explain significant differences in statin response ranging up to about 10 mg/dL. Otherwise, only a small number of common and multiple rare gene variants that contribute to the phenotype are known [17, 30–33]. The more heavily researched genes with the greatest effects are highlighted below.

APOE harbors three common haplotypes, by convention labeled ε2, ε3, and ε4, which are the result of nonsynonymous SNPs rs429538 and rs7412 in exon 4. Genotype frequencies are shown in Table 10.1.

The presence of ε2 is significantly associated with a 4–7% greater decrease (roughly 6–10 mg/dL) in LDLc response in 1984 patients who received atorvastatin 80 mg/day and were genotyped for 291,998 SNPs [14], and in 509 patients receiving atorvastatin, simvastatin, or pravastatin and genotyped for 489 SNPs [24]. However, studies demonstrating a null or opposite effect also have been reported [35, 36]. In general, the ε4 allele is associated with a greater need for statin treatment but poorer response, while the ε2 allele has been associated with greater response [37].

HMGCR polymorphisms have been shown to be responsible for up to an 8 mg/dL difference in LDL cholesterol-lowering through simvastatin [21]. The influence of *HMGCR* haplotype 7 (Hap7), which is defined by three intronic SNPs, rs17244841, rs3846662, and rs17238540, exerts an effect that is more prominent in combination with a second haplotype (Hap2) that also includes rs3846662 [21]. The rs3846662 SNP G>A allele is also responsible for a splice variant in which exon 13 is omitted and the resultant isoform has reduced statin sensitivity [38, 39]. The rs3846662 A allele is less common in Africans (3–15%) [40] and African Americans (16–17%) compared to persons of European (50–60%) and Asian (43–50%) origin [39, 40].

Table 10.1 *APOE* genotypes

APOE genotype	rs429538 (c.388 T>C, p.C130R)	rs7412 (c.526 C>T, p.R176C)	Population frequency[a]
ε2/ε2	T/T	T/T	0.007
ε2/ε3	T/T	C/T	0.116
ε2/ε4	T/C	C/T	0.022
ε3/ε3	T/T	C/C	0.623
ε3/ε4	T/C	C/C	0.213
ε4/ε4	C/C	C/C	0.019

[a]From a meta analysis of 86,000 patients [34]

Gene-specific contributions to statin response may be dependent on the interactions of several haplotypes. For example, the combined presence of the *LDLR* L5 haplotype (presence of major allele at rs14548, rs1433099, rs7254521, rs5742911,and rs2738467) and the *HMGCR* Hap2 further attenuates the apolipoprotein B response to simvastatin in African Americans [41].

There are other examples of divergent effects on LDLc response ascribed to variants within a single gene. Of the multiple haplotypes in the *LPL* gene encoding lipoprotein lipase, some were associated with increased statin-mediated lipid changes and some with decreased changes [42]. Our work has shown that the *ACACB* gene harbors SNPs with dual effects on LDLc [43]. The rs2241220 SNP in exon 33 is a marker for a greater decrease in LDLc in patients taking statins and rs34274 in intron 1 is a risk marker whose minor allele is associated with increased LDLc [43]. As more systematic analyses that may include deep sequencing are completed, candidate gene associations that include more complete genetic coverage will emerge.

Recent findings have extended the repertoire of gene variants associated with statin efficacy to new mechanisms of drug action. A genome wide association (GWA) study combining nearly 4,000 patients from three industry sponsored studies (CAP, PRINCE, TNT) is the first GWA study to discover the candidate gene *CLMN* (through the rs8014194 SNP in intron 1) that is associated with the change in LDLc due to statin treatment [33]. *CLMN* is expressed in the liver and adipose tissue [44], and encodes the protein calmin. Calmin's function is not known [45]. However, it contains a calponin-like binding domain that suggests actin-binding activity [45]. Another novel finding from a GWA study is *KIF6* [46], which encodes a cytoskeletal protein involved in intracellular transport of protein complexes, membrane organelles, and mRNA [47] (see Sect. 10.5, *Pharmacogenetic dosing*). Ultimately, confirmation of genetic contributions to the cholesterol-lowering efficacy of statins will require functional understanding of the genotype-induced alterations in lipoprotein metabolism.

Pharmacokinetic genes. The hepatic transporter genes *SLCO1B1*, *ABCG2*, and *ABCB1* harbor genotypes known to affect the systemic exposure to various statins [48]. The concentrations of rosuvastatin, atorvastatin, and simvastatin are elevated 1.8 to 3-fold in the presence of *SLCO1B1* c.521CC (rs4149056) and 1.5 to 2.4-fold in the case of *ABCG2* c421AA (rs2231142) [48, 49]. Interest has focused on these variants in relation to differential LDLc responses to statin therapy. In Chinese patients treated with rosuvastatin, the *ABCG2 c*.421A genotype affected response in a dose-wise manner with a 6.9% greater reduction in LDLc in AA vs. CC [50]. Simvastatin acid, but not lactone, and atorvastatin acid and lactone are substrates of the intestinal P-glycoprotein efflux transporter, which pumps the drugs back into the intestinal lumen during drug absorption and is the product of the *ABCB1* gene. Though *ABCB1* haplotypes affect the pharmacokinetics of the active acid forms of simvastatin and atorvastatin in vivo [51], differential effects on LDLc lowering are statistically significant but small (~4%) [52, 53] and by themselves not enough to warrant pharmacogenetic prescribing.

The *SLCO1B1* gene, which encodes organic anion transporter protein 1B1 (OATP1B1), harbors four haplotypes *1A, *1B, *5, and *15 that are relevant to drug

Table 10.2 Pharmacokinetic genes harboring variants that affect LDLc response to statins

Statins (in order of lipophilicity)	Drug transporters			Drug metabolizers			
	SLCO1B1	*ABCB1*	*ABCG2*	*CYP3A4/5*	*CYP2C9*	*CYP2C19*	*CYP2D6*
Simvastatin	✞ [14, 55, 59]	• [52]		✞ [52, 60]			• [36]
Atorvastatin	○ [14]	• [53]		• [60, 61]			
Rosuvastatin	○ [62]		• [50]	• [62]	○ [62]	○ [62]	
Pravastatin	• [56–58]			• [63]			
Fluvastatin	✞ [59, 64]			• [60]			• [36]

• Significant effect according to current data; ○ no significant effect according to current data; ✞ results are conflicting according to current data; no symbol indicates data unavailable

transport [54]. The *1B haplotype carries the rs2306238 492A>G variant and *5 corresponds to rs4149056 625T>C on reference sequence NM_006446.4, and is commonly referred to as T521C, encoding OATP1B1:V174A [54]. The *15 variant carries both SNP minor alleles [54]. The **5* variant product demonstrates deficient transport activity and it raises risk of elevated CK activity [55] (discussed below, Sect. 10.6). However, its relationship to either statin-mediated LDL cholesterol lowering or CVD risk reduction are conflicting; current evidence based on atorvastatin [14], pravastatin [56–58], and multiple statins [59] remains weak as shown in Table 10.2.

Pravastatin and fluvastatin are less lipophilic and expected to be affected by transporting peptide variants. Heterozygous carriers of *SLCO1B1**15 allele treated with pravastatin for 8 weeks had poor LDLc reduction (-14.1%) relative to noncarriers (28.9%) [56] in a study of 33 patients. In 45 Chinese patients treated with pravastatin for 30 days, those who were heterozygous for the *SLCO1B1* 521T→C (Val174Ala) functional genetic polymorphism, experienced total cholesterol lowering of 14% compared to 22% in those who were homozygous wild type (521TT). Results are consistent with decreased hepatic uptake due to the polymorphism. The common *14 allele of *SLCO1B1*, which is distinguished by the presence of the c.463C>A polymorphism, was associated with enhanced lipid-lowering efficacy with hydrophilic fluvastatin in a study of 400 patients receiving the drug [64].

Simvastatin and atorvastatin are lipophilic and expected to be susceptible to hepatic metabolism [65]. Variants in *CYP3A4* and *CYP3A5* genes affect atorvastatin and simvastatin effectiveness [60]. The *CYP3A5*3* variant is a poor metabolizer of simvastatin, atorvastatin, and lovastatin compared to the wild type *CYP3A5*1* resulting in a greater statin concentration [66]. LDLc was 19% lower in *CYP3A5*3* carriers receiving those forms of therapy but no such effect is seen for pravastatin, which is hydrophilic, or fluvastatin. In patients treated with atorvastatin 10 mg/day, the *CYP3A4* A-290G variant (*1B, rs2740574) was significantly associated with higher levels of posttreatment LDL cholesterol, whereas the *3 variant M445T (rs4986910) was associated with lower levels of LDL cholesterol before and after treatment [61]. Homozygous carriers of the *CYP7A1* -204C allele or heterozygotes for both *CYP7A1* -204C and *APOE* ε4 alleles showed significantly poorer LDLc reduction compared to that seen in other combinatorial genotype groups after 1 year of pravastatin treatment (-24.3 vs. -33.1%) [56].

10.5 Mortality and Prevention of MACE

Long-term statin efficacy has been evaluated by determining mortality rate and/or major adverse cardiovascular event rate (MACE). MACE may include fatal coronary heart disease and nonfatal myocardial infarction and combined CHD. Mechanistically, in addition to decreasing the circulating lipid substrate (LDLc) for arterial plaque progression, SNPs may influence the ability of statins to (1) stabilize plaques, (2) modulate coagulation, (3) reduce inflammation, and (4) correct endothelial function [67]. So far, more than 30 genes have been examined, most with ambiguous results [68]. The contribution of individual polymorphisms is poorly understood.

CETP gene: In the REGRESS cohort, 812 patients with coronary artery disease (CAD) were followed for 10 years, the last eight with pravastatin therapy [69]. The efficacy (atherosclerotic disease death) of statin therapy was dependent on *CETP* genotype and associated plasma CETP levels. Those carrying the TaqIB-B2 allele, which is tagged with SNP rs17231506, had higher hazard ratios for MACE (1.53–1.59) and all caused mortality (1.30). The B2 allele effect was confirmed in another study [70]. Pravastatin therapy slowed the progression of coronary atherosclerosis in B1B1 carriers but not in B2B2 carriers, which comprised 16% of patients taking pravastatin. This common DNA variant appears to predict whether men with CAD will benefit from treatment with pravastatin to delay the progression of CAD. Another *CETP* variant, the I405V (rs5882) polymorphism, can modify the effect of simvastatin on TG reduction and HDL-C elevation with carriers of the I allele responding better to treatment [25]. In a study of 82 SNPs in genes that were selected for previously having polymorphisms associated to statins (*ABCB1*, *CETP*, *LDLR*, *LIPC*, nitric oxide synthase [*NOS*], and *HMGCR*), two SNP-statin interactions on MI were observed (one *ABCB1*, one *LIPC*) and five interactions on stroke (one *CETP*, four *LIPC*). The strongest SNP-statin interaction was for synonymous *CETP* SNP rs5883 on stroke ($P = 0.008$) [71]. In a randomized study of 1,400 renal transplant patients receiving fluvastatin or placebo, tests of reported associations between *CETP* and CVD yielded varying results [72]. All of these findings need to be confirmed in larger studies.

LDLR gene: The rs1433099 and rs2738466 SNPs were shown to significantly affect the primary outcome of CHD death or nonfatal MI or fatal or nonfatal stroke in response to high dose pravastatin [22]. The T allele for the C44857T *rs1433099* was associated with lower risk for CHD. In haplotype analysis, those carrying the C44857T[T]–A44964G[A] haplotype had a lower risk for primary endpoints (HR 0.69, CI 0.52–0.90) and cardiovascular events (HR 0.74, CI 0.57–0.95) than the C44857T[C]–A44964G[G] haplotype carriers. This was not affected by pravastatin [22].

SNPs in the coagulation factors V (Arg506Gln G>A, rs6025) and VII (Arg353Gln G>A, rs6046) influenced pravastatin's ability to prevent fatal CAD and nonfatal myocardial infarction in >9,000 patients randomized to pravastatin or placebo in the GenHAT study [73]. The combined interaction (pravastatin-SNP) hazard ratios were 1.33 and 1.92, respectively. Polymorphisms in genes in the homocysteine pathway

(*MTHFR* 677 C>T and CBSins) appear to modify the efficacy of pravastatin in reducing risk of cardiovascular events [74].

The stabilization of plaques by statins can be affected by variation in the matrix metalloproteinases (MMP) secreted by inflammatory cells. These enzymes degrade the extracellular matrix, undermining structural integrity and predisposing fibrous caps to rupture [75]. The Ala227Pro polymorphism (rs428785, NM_006988: +1134G>C) in the *ADAMTS1* metalloproteinase gene is associated with significantly increased risk of CAD or myocardial infarction [76]. Pravastatin decreased the risk of fatal CAD/myocardial infarction to a threefold greater extent in patients homozygous for 227Pro compared to those who were heterozygous [76].

Pharmacogenetic dosing: Selection of the optimal statin *dose* may also be genetically determined, at least in part. The *KIF6* gene encodes kinesin-like protein 6, a member of the molecular motor superfamily. Two prospective trials (CARE and WOSCOPS) including more than 28,000 subjects [77, 78] have shown the Trp719Arg variant (*rs20455*) in *KIF6* to be associated with coronary events [77, 78]. *Intensive* compared to *moderate* statin therapy imparted greater protection from coronary events in carriers of *KIF6* 719Arg compared to noncarriers (10.0 vs. 0.8% reduction, respectively, for absolute rates) [46]. The mechanism is unknown but appears distinct from lipid or C-reactive protein lowering [46]. Functional studies of the *KIF6* kinesin are warranted, given the consistent association of Trp719Arg with risk of coronary events and statin benefit [46].

In summary, evidence that statin-mediated reductions in CVD risk exceed levels expected from LDLc lowering have resulted in examination of pleiotropic or nonlipid lowering effects of statin treatment [67]. Large studies will be necessary because the predictive effects of new biomarkers, the number of which will be large, appear to be small.

10.6 Statin Safety

Statins are well tolerated by the majority of patients at low starting dosages. However, they can produce SINM and their usage is ultimately limited by toxicity. NMSEs occur in >10% of patients [6], affecting compliance to statin therapy. These may include myalgia (muscle aches, cramps), weakness, fatigue, heaviness, myositis, neuropathy, and other forms of intolerance to statin therapy, as shown in Fig. 10.2.

There are serum and clinical surrogate markers for SINM that we have collectively termed as NMSEs. Serologically, increased activity of serum CK provides the predominant means for assessing the degree of myopathic severity. The elevation of CK activity to tenfold ULN (upper limit of normal) has been suggested as indicating severe SINM [79]. The pharmacokinetic gene *SLCO1B1* *5 variant rs4363657 SNP is strongly associated to the high degree of CK elevation [55], likely through linkage disequilibrium with the nonsynonymous SNP rs4149056 (Val174Ala) or the rs4149080 SNP in the 13th intron [80], and there is independent confirmation of the SLCO1B1*5 relationship to elevated CK [80]. Pharmacokinetic

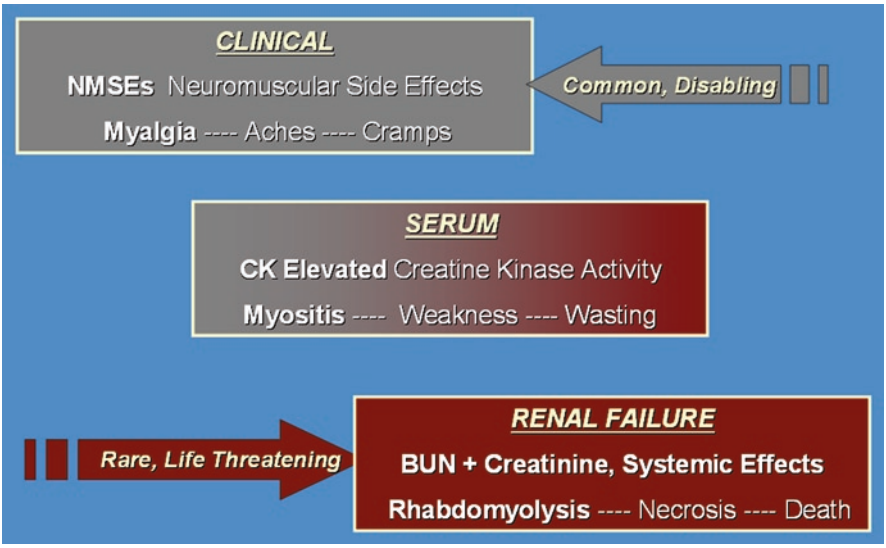

Fig. 10.2 The nature and severity of statin-induced neuromyopathy (SINM) symptoms and neuromuscular side effects

Table 10.3 Pharmacokinetic genes harboring variants that affect myalgia or CK response to statins

Statins (in order of lipophilicity)	Drug transporters				Drug metabolizers			
	SLCO1B1	*ABCB1*	*ABCG2*	*ABCG8*	*CYP3A4/5*	*CYP2C9*	*CYP2C19*	*CYP2D6*
Simvastatin	• [55, 80]	• [52]			• [52]	□ [36]		✞ [36, 81]
Atorvastatin					• [82] □ [36]	□ [36]		□ [36]
Rosuvastatin								
Pravastatin	• [83]							
Fluvastatin								

• Significant CK elevating effect according to current data; ○ no significant CK elevating effect according to current data; ■ significant myalgia effect according to current data; □ no significant myalgia effect according to current data; ✞ results are conflicting with respect to CK elevation and myalgia according to current data; no symbol indicates data unavailable

gene-focused hypotheses are founded on the increased plasma concentrations resulting from decreased hepatic entry (variation in drug transporters) and metabolism (variation in cytochrome p450 and possibly glucuronidation pathways), as mentioned (Sect. 10.4, *Pharmacokinetic genes*). Table 10.3 summarizes the findings.

CK and myalgia: Myalgias occur in patients often with no or little CK elevation and CK is not necessarily elevated in the presence of histopathological evidence of statin associated muscle damage [84, 85]. Biopsy studies of the vastus lateralis in a

series of 44 statin myalgia patients revealed damage specific to the t-tubules and the appearance of vacuoles within the muscle fibers in 60% of the patients (≥2% of muscle fibers damaged). CK activity was within normal limits in 66% of the patients with damage. Breakdown of the t-tubular system and subsarcolemmal rupture has also been observed in asymptomatic patients taking statins and with normal CK [85]. Only in the clinically rare condition of rhabdomyolysis is the relationship between myopathy, extremely elevated CK, and clinical severity, incontrovertible [86]. There is a need to identify novel surrogate markers that can better predict high risk of myalgia in patients taking statins.

Pharmacodynamic genes: The pharmacodynamic-based mechanisms that have been advanced have incorporated diverse and complex pathways and with a focus mainly on myofibers. Thompson and colleagues [4, 87] have provided thorough reviews of mechanisms of statin myopathy. Statin interactions with HMG-CoA reductase homolog proteins may interfere with energy transduction processes [88, 89]. Some mechanisms find their basis in the possibility that statin inhibition of nonsteroidal molecules such as ubiquinone and isoprenoids triggers disruption in normal mitochondrial function, cell signaling, cell proliferation, and cell repair [4, 88, 90–99]. Specific proposed myalgia etiologies include decreased sarcolemmal [4] or sarcoplasmic reticular cholesterol [85], reduced production of ubiquinone or coenzyme Q10 [100], decreased production of prenylated proteins [4], changes in fat metabolism [101], increased uptake of cholesterol [102] or phytosterols [103], failure to replace damaged muscle protein via the ubiquitin pathway [104], disruption of calcium metabolism in the skeletal muscle [84, 105], and inhibition of selenoprotein synthesis [106]. Phenotypic expression is quite variable. As a result, genetic markers are generally unknown though progress to identify candidate markers has been made [107].

Statin exposure that produces myopathy sometimes unmasks a latent muscle pathology with known genetic basis [107]. Severe statin myopathy is more frequent in the presence of variants in muscle metabolic disease genes *COQ2* (CoQ10 synthetic pathway), *CPT2* (carnitine transferase Type II deficiency), *PYGM* (McArdle disease) [108], and *AMPD1* (myoadenylate deaminase [MADA] deficiency) [109, 110]. As examples, the prevalence of heterozygosity for any of 20 mutations in *PYGM* is 1/170 (0.6%) in the general population, but 12.3% in patients with statin myopathy [109]. Similarly, the carrier frequency for CPT II deficiency is estimated at 0.4% in the general population [109] and 3.8% in patients with statin myopathy [109]. MADA deficiency is an autosomal recessive disorder traceable to the *AMPD1* gene that impairs conversion of 5-adenosine monophosphate (5′AMP) to inosine monophosphate (IMP). MADA deficiency caused by homozygosity for a mutant allele in exon 3 of *AMPD1* occurred in 6.5% of a series of patients with severe statin myopathy compared to 2% estimated for the general population [111]. The increased carrier frequencies among patients with severe statin myopathy suggest that these normally rare disorders are potentially common risk factors for drug-induced myopathy when present in the carrier state [107]. Candidate genes for deficiency of the muscle CoQ10 pathway [112] include *PDSS1*, *PDSS2*, *COQ2*, *ETFDH*, and *APTX1*. Two genes overexpressed in damaged muscle of severe statin

myopathy regardless of CK status were *SERCA3* (sarco endoplasmic reticulum transporting Ca^{+2} ATPase type 3) and *RYR3* (ryanodine receptor type 3) [86], both of which participate in calcium homeostasis. Calcium homeostasis is also impaired in malignant hyperthermia, an inherited condition that may be more common in patients with severe statin myopathy, based on the contracture test results [105].

Muscle-based symptoms of statin myalgia may reflect origins outside of the myofiber. Statins alter vascular function through decreased isoprenylation of G-proteins and disturbance of the NOS system [113]. Cell culture studies have shown that statins induced apoptosis in various cell types, including vascular smooth muscle [114, 115]. Atogin-1 (encoded by *FBXO32*) is an ubiquitin ligase that increases early in skeletal muscle atrophy and is also induced by statins [98]. The transfer of geranylgeranyl isoprene units but not farnesyl units prevents atrogin-1 induction in cell culture [116]. There are also reports of neuropathy, peripheral neuropathy, and polyneuropathy and in addition, two reports of aggravation of existing polyneuropathy associated with the use of HMG-CoA-reductase inhibitors [117], suggesting a neural basis. Other new hypotheses support a link between myopathy during statin therapy to genes affecting vascular smooth muscle [7] and serotonergic transduction [118] and raise the possibility that statin side effects arise through different pathways.

Without a unifying mechanistic understanding of the NMSE, the most relevant clinical scenario is the percentage of patients who develop myalgias and muscle weakness disabling enough to trigger a referral to a physician for neuromuscular assessment and alternative drug therapy. Therefore, it has been our goal to model clinical and genetic parameters for the analysis of statin response and the demarcation of symptomatic and metabolic NMSEs including phenotype distribution, drug regimen, sample size, predictive power, and allele frequency.

10.7 Physiogenomic Research: Simultaneous Prediction of Efficacy and Side Effects

To date, there exist only two published genome wide studies of statin response [33, 55]. Yet, based on emerging successes in predicting the balance of efficacy vs. safety [119], genomic studies are expected to eventually guide drug selection. Particularly at higher doses required for advanced disease, statins can induce myopathy and their usage is ultimately limited by toxicity [120].

An upper limit of ~3% of variance in LDLc attributable to any single SNP association has been hypothesized [41], based on findings in increasingly larger study populations. In view of small contributions of many genes to the LDLc lowering, and of the paucity of information on safety markers, our efforts are directed to simultaneously derive SNP biomarkers of statin safety and efficacy. Physiogenomics (PG) is a medical application of sensitivity analysis and systems engineering [121]. Sensitivity analysis is the study of the relationship between input and output from a system as determined by system components. PG utilizes the genes as the

components of the system. The gene variability, measured by SNPs, is correlated to physiological responses, the output, of a diversely responsive human population [122]. This approach determines how the SNP frequency varies among individuals similarly responding to the input over the entire range of the response distribution. Previously, PG has been utilized to identify genes relevant to dietary weight reduction [123, 124], exercise response [125], and drug-induced side effects [126].

We operationally defined statin efficacy as LDL cholesterol lowering [119] and statin safety as the presence or absence of myalgia [118, 119] and the level of serum CK's activity [7, 119]. In clinical populations of up to 466 patients receiving either simvastatin, atorvastatin, or rosuvastatin, we employed genotyping using the PG Array, which consists of 384 SNPs among 222 genes representing metabolic, inflammatory, and cell regulatory genes [122].

In outpatients receiving statin therapy, SNPs rs2276307 and rs1935349 in the serotonin receptor genes *HTR3B* and *HTR7*, respectively, significantly associate with statin-induced myalgia [118], and SNPs rs12695902 and rs1799983, in the *AGTR1* (angiotensin receptor type I) and *NOS* genes, respectively, significantly associate with myositis [7]. The physiogenomic approach has identified SNP markers distributed across several gene pathways that suggest neural [118] and vascular [7] components to augment the primarily myocyte-based hypotheses of statin induced myopathy. With respect to LDLc lowering, we found a dual effect within the *ACACB* gene [43] as discussed earlier.

The data indicate that SNP markers for myalgia, myositis (CK elevation), and LDLc lowering are largely phenotype specific. Our working hypothesis is that statin response phenotypes are modulated by separate gene pathways. Our findings and others will permit construction of a prototype of a physiogenomic-based safety/efficacy model that consolidates the myalgia, myositis, and LDL cholesterol reduction components. Pending further validation, we predict the existence of genotypes to help clinicians prescribe statins so as to minimize side effects and maximize efficacy.

10.8 Conclusion

Genetic variants modulate the statins' capacity to lower LDLc and induce myalgia and myositis, all of which are factors that strongly influence drug selection in the clinic. The strongest predictors of LDLc response are variants within the *APOE* and *HMGCR* genes. Taken individually, these explain up to 7% of the variance in change in LDLc but this figure is expected to decrease to 3% of the change in LDLc with statin therapy as large diverse populations are studied. Other genes examined have lesser effect sizes or have not been sufficiently studied to warrant mention. Still, it is safe to predict that numerous markers taken together will have additive effects in explaining the variance in LDLc response. With respect to CK elevation, the *SLCO1B1* gene harbors the single most important marker during simvastatin therapy. Progress to identify other markers for myositis and myalgia is hindered by the absence of a unifying pathophysiological hypothesis for toxicity as well as

the hurdles inherent to assembling large patient databases with well characterized phenotypic data.

In the long-term, statins decrease MACE and all cause mortality in part through pleiotropic mechanisms. Pharmacogenomic studies have provided new insights revealing that nonsynonymous SNPs in *KIF6* (rs20455, Trp719Arg), *ADAMTS1* (rs428785, Ala227Pro), and coagulation factors V (rs6025, Arg506Gln G>A) and VII (rs6046, Arg353Gln G>A) affect patients' risk of coronary events through pravastatin [46, 73, 76, 77]. Whether other statins have the same effects is not known. With relatively firm clinical endpoints serving as phenotypes, examination of existing large clinical databases using pharmacogenomic approaches may be useful.

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Chapter 11
The Statin Response Gene: KIF6

H. Robert Superko, Tom White, James Forrester, and Spencer King III

It is not good to settle into a set of opinions. At first putting forth great effort to be sure that you have grasped the basics, then practicing so that they may come to fruition is something that will never stop for your whole lifetime. Do not rely on following the degree of understanding that you have discovered, but simply think,

… ***This is Not Enough***

Tsunetomo [1]

Keywords Statins • Cholesterol • Lipid lowering • Kinesin proteins

11.1 Statin Therapy, Clinical Events and the Need for a New Approach

For over two decades, substantial National efforts have been directed toward reducing blood cholesterol levels of the American population with the intent of reducing the burden of cardiovascular events. This initiative was initially prompted by the successful results of the Lipid Research Clinic-Coronary Primary Prevention Trial (LRC-CPPT) that first proved reducing LDL-C resulted in a statistically significant reduction in cardiovascular events [2]. Since then an abundance of monotherapy cholesterol-lowering drug trials have consistently reported an approximate 25% relative risk reduction (RRR) for cardiovascular events [3].

While a 25% RRR in large population studies is laudable, a danger lies in the assumption that blood cholesterol reduction has similar beneficial effects in all people. Indeed, the cholesterol-lowering clinical trials left large groups of

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_11,

patients still experiencing clinical events despite successful LDL-C reduction [4]. This has now been termed, "residual risk." A clue that approximately 50% of statin treated patients may not receive benefit from LDL-C reduction was reported in 2003 utilizing single photon emission computed tomography (SPECT) myocardial perfusion imaging (MPI) [5]. In this investigation, patients achieved a 32% statin induced LDL-C reduction and the mean stress perfusion defect improved in 48% but was unchanged in 43% and worsened in 9% and change in lipid levels did not correlate with the change in perfusion defect. This chapter will review the findings regarding the impact of a polymorphism in the kinesin-6 gene that helps to identify a large group of patients that have greater than previously thought clinical event reduction benefit from statin treatment, and, a second group of patients that obtain significantly less benefit despite identical LDL-C reduction.

11.1.1 Blood Cholesterol Lowering History

In the twenty-first century, atherosclerosis is well established as the leading cause of death in most industrialized nations [6]. Large population based studies have identified several risk factors as targets of intervention that may reduce CV disease [7, 8]. This approach to identification of "high risk" populations was used in order to focus efforts on a population subset that could derive the most benefit from a treatment targeted to disorders such as hypertension or elevated blood cholesterol [3, 9, 10].

In the field of cholesterol and heart disease research, early efforts with thyroid, estrogen, and clofibrate provided some evidence that cholesterol lowering was of benefit but were marred by adverse drug interactions [11, 12]. The LRC-CPPT that used a combination of moderately low-fat diet and cholestyramine in men with hypercholesterolemia, first established that reducing elevated LDL-C resulted in fewer coronary heart disease (CHD) events (significant by a one tailed t-test) [2]. Following the success of the LRC-CPPT, HMGCoA reductase inhibitor medications (statins) became available that achieved greater LDL-C reduction with fewer side effects [13]. These remarkable compounds made achieving reduced LDL-C values relatively easy. Their success forms the basis of the paradox that easily achieved LDL-C reduction may, in part, be responsible for the failure to substantially stem the tide of CHD.

Based on multiple clinical trials, the ATP-I recommended a LDL-C goal of <130 mg/dl to assist CHD risk reduction [14]. Since the publication of ATP-I in 1988, multiple other clinical trials have reported that even greater LDL-C reduction in both primary and secondary prevention populations can achieve even greater reductions in CHD relative risk [15]. The success of these trials prompted ATP-II and ATP-III to adjust LDL-C goals downward to the current recommended levels [3].

One danger of a medical therapy, deemed to be statistically significant in large clinical trials, is the tendency to assume that almost all patients will benefit in a similar manner from that single therapy, and thus ignore other potentially beneficial treatments. Recently, the results of a series of statin-induced cholesterol-lowering trials have been used to suggest that a new LDL-C goal of <70 mg/dl should be

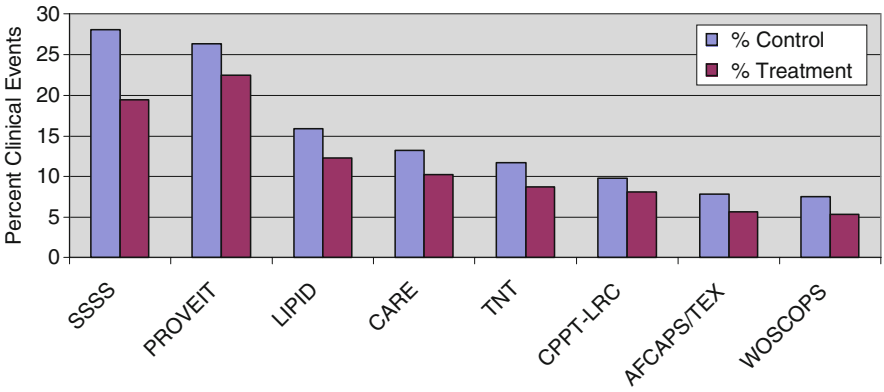

Fig. 11.1 Percent clinical events reported in monotherapy statin clinical trials in the control group compared to the treatment group. While an approximate 25% relative risk difference was obtained, significant numbers of patients treated with statin therapy continued to have cardiovascular events (Adapted from [4])

nationally embraced [16]. However, a large number of patients taking cholesterol-lowering medications, and achieving lower LDL-C values, continued to have clinical events (Fig. 11.1) [4]. This continues to be true even with the substantially greater absolute LDL-C reduction achieved in recent trials. At this point in the history of cholesterol reduction, it is important to pause and discuss the possibility that subgroups of patients can be identified that respond to a greater or lesser extent in regard to clinical event reduction attributed to statin therapy.

11.1.2 Clinical Relevance of RRR, ARR, and NNT

Many individuals in clinical trials have cardiovascular events despite statin treatment that was successful in reducing LDL-C levels [4]. The effectiveness of a treatment can be described by three concepts, relative risk reduction (RRR), absolute risk reduction (ARR), and number needed to treat (NNT) (Table 11.1). RRR assesses the reduction in risk in one group relative to another group. For example, the risk reduction noted in the treatment group compared to the placebo group. Therefore, if the total study size is 2,000 placebo and 2,000 treatment subjects, and 100 placebo patients have an event (5%) relative to 75 events in the treatment group (3.8%), the RRR is 25% (25/100).

ARR assesses the absolute reduction difference in risk in one group compared to the absolute reduction in another group. In the example above, the ARR would be 1.2% (5.0–3.8%). It is not uncommon for the public to misinterpret this 25% RRR as meaning that 25% of the entire population was saved from an event due to the treatment. In fact, if there were 1,000 subjects in the treatment group and 1,000 subjects in the placebo group, and 100 events were experienced in the placebo group

Table 11.1 Relative risk reduction (RRR), absolute risk reduction (ARR), and number needed to treat (NNT) in statin-CHD trials in populations separated by *KIF6* carrier status

Study	RRR (%)	ARR (%)	NNT
CARE Original	–24	–3.0	34
CARE Genetic Substudy			
KIF6 Carriers	–37	–4.9	20
KIF6 Noncarriers	–20	–1.4	72
WOSCOPS Original	–31	–3.5	46
WOSCOPS Genetic Substudy			
KIF6 Carriers	–50	–5.5	18
KIF6 Noncarriers	–9	–0.1	>100
PROSPER Original	–15	–2.1	47.6
PROSPER Genetic Substudy			
KIF6 Carriers	–33.6	–6.3	16
KIF6 Noncarriers	–6	–1.2	83
PROVE IT-TIMI 22 Original	–16	–3.9	26
PROVE IT Genetic Substudy			
KIF6 Carriers	–41	–10	10
KIF6 Noncarriers	–6	–0.8	125

and 75 events in the treatment group, the difference between 100 and 75 is the 25% RRR in events ($n = 25$), not 25% of 1,000 subjects ($n = 250$).

Numbers needed to treat (NNT) is a method that can assess the efficiency of different therapies. NNT is the number of subjects that are needed to be treated in order to prevent one event in a defined time period and is the inverse of the ARR. In general, the higher the NNT, the less efficient and the lower the NNT, the greater the efficiency of the treatment. For example, in the example above, it was necessary to treat 2,000 subjects in order to prevent 25 events. Thus, the NNT is (100/1.2) or 83. A lower NNT maximizes the benefits of the treatment, while lowering the overall exposure of the population who derive little or no benefit from therapy. This has implications for both population-based costs of therapy, risk: benefit, and sample sizes in future trials.

11.1.3 Atherosclerosis Pathophysiology

Not just obstruction. The impact of atherosclerosis on vascular disease can be quantitatively expressed either anatomically as the magnitude of vascular obstruction as assessed by arteriography or, as the absolute number of clinical events. In the past, it was thought that the amount of arterial obstruction correlated with the potential for clinical events. This relationship between anatomic obstruction and clinical events is tenuous, however, because in most individuals experiencing a coronary event the culprit lesion caused less than a 50% stenosis [17]. Clinical events are caused by plaque rupture with subsequent thrombosis, and rupture is largely independent of the magnitude of stenosis [18, 19]. This important concept helps to explain why large

numbers of patients appearing in the emergency department with an acute coronary event have no prior symptoms [20]. While severely stenotic lesions can rupture and thrombose, approximately twice as many events are attributed to moderately stenotic (<80%) lesions. In recognition of this, the process is now referred to as "atherothrombosis" and the plaque itself is referred to as a "vulnerable" plaque that is prone to rupture or erosion that can result in a thrombotic event [21].

Classic well established CHD risk factors such as elevated blood cholesterol, hypertension, and smoking are associated with the development of atheroma. The factors that precipitate plaque rupture are less well defined [22]. Since the etiology of plaque rupture may be related to factors other than the "classic" risk factors, it should not be surprising that many patients can significantly improve a risk factor such as elevated LDL-C and yet suffer a cardiovascular event. It is important to distinguish the slow gradual process of atherosclerosis development and progression, from the factors that may contribute to an unstable plaque prone to rupture and cause a cardiovascular event. Genetic polymorphisms may play a role in determining risk for an event, and identifying response to a specific therapy.

11.2 Kinesin Proteins

To sustain their specific functions and morphology, cells have an intracellular transport mechanism controlled by microtubule-dependent motor proteins [23]. Microtubules are intracellular structures and play specialized roles in a variety of cellular events including molecular transport which is dependent on proteins such as kinesins [24]. People walk by alternating left and right movements of the "legs." Recent work shows that kinesins share the hallmarks of bipedal walking [25]. They "walk" intracellular cargo along the microtubules with each alternating foot taking an alternating 16 nm "step" [26]. Recently, all KIF genes in the mammalian and human genomes have been systematically identified [27]. The kinesin superfamily, is composed of at least 14 families, based on phylogenetic analysis and each may transport different cargoes. The most common cargo for N-kinesins are membranous structures [28]. For example, Kinesin II transports a large protein complex that contains the tumor suppressor adenomatous polyposis coli protein (APC). The APC protein is commonly mutated in colon cancer, giving rise to versions of APC that do not function properly.

KIF6 encodes a kinesin, a class of motor proteins involved in the intracellular transport of cargo that includes membrane organelles, protein complexes, and mRNAs [27, 29, 30]. The kinesin proteins consist of a conserved motor domain that propels the kinesin along microtubules in an ATP-dependent manner and a nonconserved tail domain that binds to either directly its cargo or to other cargo-binding proteins. The tail domain of kinesin is responsible for cargo recognition and binding (Fig. 11.2) [30]. Thus, this amino acid change might affect the cargo binding or transporting activity of the kinesin protein encoded by *KIF6*. Interestingly, *KIF6* is expressed in coronary arteries and several other kinesins have been implicated in the

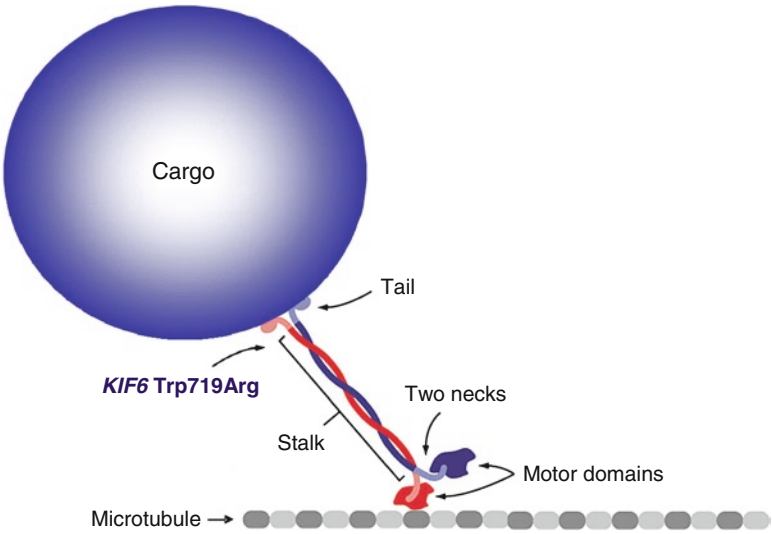

Fig. 11.2 The kinesin 6 protein walks along microtubules dragging a cargo. The KIF6 Trp719Arg polymorphism is located at the binding site of kinesin 6 protein

response of cardiac stroke volume to regular exercise and in the pathogenesis of chronic diseases, such as neurodegenerative diseases, type 2 diabetes, and Alzheimer's disease [31–33]. Functional biology studies of *KIF6* in coronary artery disease, and a potential role in vulnerable plaque, are underway using antibodies that can detect the *KIF6* protein in histopathological specimens. The polymorphism Trp719Arg in the kinesin-like protein 6 has been associated with the presence of late outgrowth endothelial progenitor cells in acute myocardial infarction [34]. Like the chromosome 9p21 single nucleotide polymorphisms (SNPs) which are also not yet characterized in terms of functional biology, an understanding of the biological process(es) involved promise to provide new insight into the pathology of CHD [35].

11.3 Discovery of KIF6 and Relationship to CHD risk

The initial discovery of the KIF6 719Arg allele and the relationship to CHD was first noted in a case-control study of myocardial infarction patients at the University of California, San Francisco [36]. The allele frequencies of 11,053 SNPs were determined in pooled DNA and the frequency of KIF6 719Arg was higher (p <0.05) in cases compared to controls. This discovery study was then followed-up by assessing in placebo groups of the two prospective randomized clinical trials CARE and WOSCOPS the association with CHD of 10 SNPs in 9 candidate genes, reported in the literature to be associated with CHD and twenty-five SNPs that Celera had found to be associated with MI in the case control study. Of the 35 SNPs

tested, only KIF6 719Arg was significantly associated with increased risk for CHD (p <0.05 after Bonferroni correction).

11.4 *KIF6* 719Arg Polymorphism is Associated with Coronary Heart Disease Event Risk

The association of the KIF6 719Arg polymorphism with cardiovascular risk has been established in seven prospective studies, in over 50,000 subjects, including the Atherosclerosis Risk in Communities (ARIC) Study, the Women's Health Study (WHS), the Cardiovascular Health Study (CHS), and the placebo arms of the West of Scotland Coronary Prevention Study (WOSCOPS), the Cholesterol and Recurring Events (CARE) study and the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) study. In the placebo arm of these clinical trials, the effect of a polymorphism impacted by statin therapy could be fully appreciated since randomization to placebo assured that statin use would not impact the analysis. In the community free-living populations of ARIC, WHS, and CHD, statin use in the community has the potential to blunt the ability of the KIF6 polymorphism to identify risk due to the existing treatment of a portion of the population with a statin medication.

11.4.1 The KIF6 Polymorphism is Associated with Coronary Heart Disease in the ARIC Study

The ARIC study is a prospective investigation of atherosclerosis and its clinical sequelae involving 15,792 individuals aged 45–64 years at recruitment [37, 38]. CHD cases (n = 1,452) were defined as participants with either myocardial infarction (MI), CHD death, or coronary revascularization procedures. In this study, carriers of two copies of the *KIF6* 719Arg risk variant were at increased risk for incident CHD with a hazard ratio of 1.22 in a model adjusted for age and sex [39].

11.4.2 The KIF6 Polymorphism is Associated with Coronary Heart Disease in the Women's Health Study

The WHS is a large prospective, placebo controlled, 2 × 2 factorial designed trial of aspirin and vitamin E in the prevention of cardiovascular disease [40]. The study was conducted among 26,274 initially healthy women aged 45 years or older who were followed for approximately 13 years for cardiovascular events including

cardiovascular death, myocardial infarction (MI), ischemic stroke, and revascularization procedures. In Caucasians, carriers of the *KIF6* 719Arg risk variant were at greater risk of CHD with a hazard ratio of 1.24 and associated with MI with a hazard ratio of 1.34. The risk for ischemic stroke did not differ between carriers of the *KIF6* 719Arg risk variant and noncarriers. In addition to long follow-up time, extensive baseline information was collected for all participants, including traditional risk factors and emerging risk factors for cardiovascular disease [41]. No substantial or significant difference was detected between *KIF6* 719Arg *carriers* and noncarriers in regard to baseline values and traditional CHD risk factors, suggesting that the risk conferred by the *KIF6* risk variant is not only independent of traditional risk factors, but also that of the other risk factors measured, such as ApoA, ApoB, CRP, ICAM, and Fibrinogen.

11.4.3 The KIF6 Polymorphism is Associated with Coronary Heart Disease in the Cardiovascular Health Study

The *KIF6* 719Arg risk variant was also associated with disease in the CHS, a prospective study of risk factors for cardiovascular disease for men and women aged 65 years and older [42, 43]. CHD was defined as nonfatal MI or definite fatal MI, angina pectoris, angioplasty or coronary artery bypass surgery or death due to atherosclerotic CHD. In the genetic substudy of CHS, the CHD endpoints were fatal and nonfatal MI. In Caucasian patients for whom genetic analysis was possible, 539 (12%) of 4,522 CHS participants had an MI during the 13 years of study follow-up for a rate of 11.9 incident events per 1,000 person-years. The *KIF6* 719Arg risk variant was associated with events with a hazard ratio of 1.29 in a model adjusted for age, sex, race, and traditional risk factors such as LDL-C and hs-CRP [44].

11.4.4 The KIF6 Polymorphism is Associated with Coronary Heart Disease in the Placebo Arms of the CARE and WOSCOPS Studies

The prospective statin trials, CARE [45] and WOSCOPS [46], assessed the effect of pravastatin in the prevention of myocardial infarction (MI) and CHD events. In the placebo arm of both trials, Iakoubova et al. [47] assessed the association of *KIF6* with risk for coronary events. The genetic study of CARE comprised 2,913 Caucasian patients and assessed risk for fatal or nonfatal MI. The genetic study of WOSCOPS, which was derived from a previously published (prospective) nested case-control study [48], included the 481 on-trial CHD events as cases and the 1,086 controls for whom DNA was available for analysis. As previously reported for the WOSCOPS nested study, the controls were matched to cases for age and

current smoking status and the CHD endpoint studied was death from CHD, nonfatal MI, or revascularization procedures.

In the placebo arm of CARE, carriers of the *KIF6* 719Arg variant had a hazard ratio of 1.50 for recurrent myocardial infarction in a model adjusted for (and independent of) age, sex, smoking, history of hypertension, history of diabetes, body mass index, LDL-C, and HDL-C. This hazard ratio for the *KIF6* 719Arg risk variant was not only found to be independent of these traditional risk factors, but also to predict risk of a similar magnitude compared to the traditional risk factors. In the placebo arm of WOSCOPS, carriers of the *KIF6* 719Arg variant had a similar odds ratio for CHD of 1.55 in a model adjusted for history of hypertension, history of diabetes, body mass index, LDL-C, and HDL-C. The WOSCOPS and CARE studies provide information in primary prevention patients with elevated LDL-C (mean LDL-C = 192 mg/dl, WOSCOPS) and secondary prevention patients with moderate LDL-C levels (mean LDL-C = 139 mg/dl, CARE). Thus, an odds ratio of approximately 1.5 has been established and reproduced in the placebo arms of two classic statin studies.

11.4.5 The KIF6 Polymorphism is Associated with Coronary Heart Disease in the PROSPER Study

In the placebo arm of PROSPER, carriers of a single *KIF6* 719Arg risk variant, with a prior history of vascular disease, were at increased risk for coronary events with a hazard ratio of 1.36 in a model adjusted for sex, age, smoking, hypertension, diabetes, LDL-C, HDL-C, and country of recruitment [49]. Carriers of the *KIF6* 719Arg variant, without prior vascular disease, were not associated with increased risk of coronary events, a finding that is consistent with results from the original trial.

11.4.6 KIF6 Polymorphism Predicts Risk for Coronary Heart Disease as Shown in Six Prospective Studies

The preponderance of published data strongly favors the conclusion that the *KIF6* variant is associated with CHD risk as seen in the six prospective studies incorporating >50,000 subjects (Fig. 11.3). Three of the studies, CARE, WOSCOPS, and PROSPER, were in the placebo arms of statin clinical trials, while the other three studies included some subjects who were treated by their physician with a statin or other CHD therapies. In the placebo arms of the statin trials, the *KIF6* 719Arg variant was associated with an approximate 50% increase in risk. Some subjects in the free living population studies (CHS, ARIC and WHS), were treated with statin medications by their physicians which may help to explain the lower hazards ratios observed in the three population studies since, as described

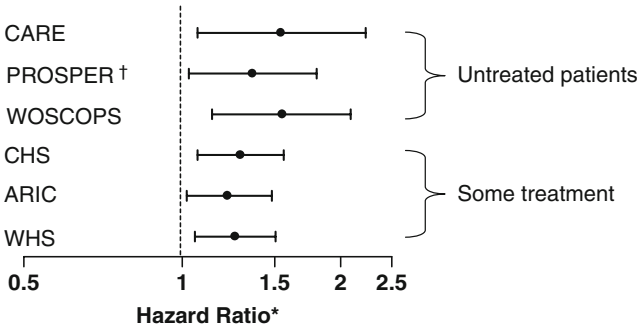

Fig. 11.3 *KIF6* variant predicts risk for coronary heart disease (CHD) in *six prospective studies with ~50,000 participants. KIF6* variant is associated with a significant increase in the hazard ratio for MI, recurrent MI, and CHD in six large clinical investigations following adjustment for traditional risk factors. †*PROSPER patients with prior vascular disease*

in the next section, the *KIF6* 719Arg variant predicts both risk for CHD and response to statin therapy.

11.4.7 KIF6 Risk Compared to Other Risk Markers

The clinical utility of *KIF6* testing as a marker of CHD risk can be compared to the utility of traditional risk factors in the placebo group of clinical trials. In the placebo arm of CARE, following statistical adjustment for all the risk variables, the *KIF6* variant was an independent and significant predictor of CHD risk and more informative than age, HDL-C <40 mg/dl, hypertension, or LDL-C >130 mg/dl (Fig. 11.4). In the placebo arm of WOSCOPS, following statistical adjustment for all the risk variables, the *KIF6* variant was an independent and significant predictor of CHD risk and more informative than HDL-C <40 mg/dl, LDL-C >189 mg/dl, and hypertension (Fig. 11.5). Both the CARE and WOSCOPS populations may be considered at high risk for CHD events. CARE subjects had a prior history of CHD and WOSCOPS subjects had elevated LDL-C.

11.5 Lack of *KIF6* polymorphism Associated with CHD Risk in Some Studies

Some investigations have not found an association of the KIF6 polymorphism with CHD risk and may be related to clinical trial design issues. In the Wellcome Trust Case Control Consortium (WTCCC) study, KIF6 was not found to be associated with increased CHD risk [50]. In the case control designed Ottawa Heart Study, KIF6 was not associated with CHD as defined by an arteriographic endpoint [35, 51].

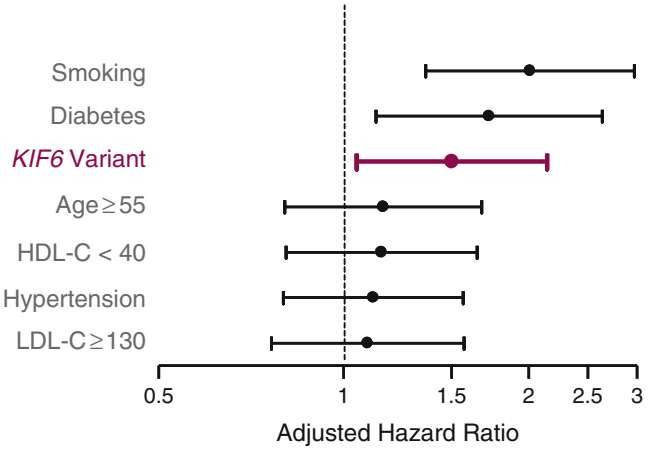

Fig. 11.4 Risk in CARE placebo arm *KIF6 variant and traditional risk factors*. *KIF6* variant and hazard ratio in relationship to classic cardiovascular risk factors. The hazard ratio is statistically adjusted for the traditional risk factors and is independent of those factors

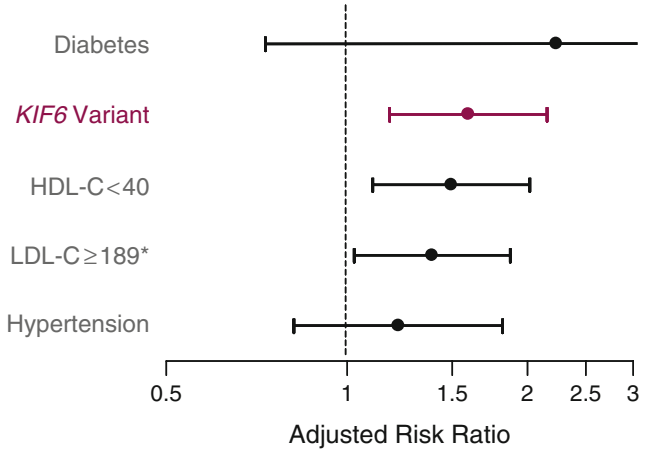

Fig. 11.5 Risk of CHD in West of Scotland Coronary Prevention Study (WOSCOPS) placebo arm *KIF6 variant and traditional risk factors*. In the placebo arm of the West of Scotland trial, the *KIF6* variant was similar in magnitude to traditional risk factors and more informative than HDL-C <40 mg/dl, LDL-C >189 mg/dl, and hypertension. In this prospective nested case and control study, patients were matched for age and smoking, and all were men. *Median level in placebo arm

The WTCCC study tested 377,857 SNPs for association with MI [50]. To adjust for multiple testing of so many SNPs in a discovery study, a large significant value of $p < 1.5 \times 10^{-8}$ was used. However, a significant association between the 719Arg allele and CHD was observed in a sex-differentiated test. Among females, the

719Arg allele was not associated with increased risk of CHD (OR = 0.91, 95% CI; 0.72–1.14). Among males, the 719Arg allele was associated with increased risk of CHD (OR = 1.18, 95% CI; 1.02–1.36). If 20% of the population from which the WTCCC cases were drawn was receiving statin therapy prior to their CHD event, the estimated risk of CHD among untreated cases for 719Arg carriers compared with noncarriers, would have been modestly higher (OR = 1.23, 95% CI; 1.02–1.42) [52]. When KIF6 was tested as a prespecified hypothesis based on its previous association with CHD risk in six prior studies, it was found to be associated with risk in men [53].

The Ottawa Heart Study is a cross-sectional, case control design study investigating the relationship of CAD as defined by arteriography in patients (onset <55 years males, <65 years females) seen in the coronary catheterization laboratory and lipid clinic (cases n = 1,540), with asymptomatic control subjects (controls n = 1,455) recruited from an elderly population without a cardiovascular disease history (>65 years males, >70 years female) [51]. In this investigation, the KIF6 719Arg variant was not associated with risk of CAD by this definition. These negative results could also be explained by the fact that 89% of the cases were on statin therapy, which would have eliminated an association with risk in *KIF6* carriers. The result of the Ottawa Heart Study supports the concept that the KIF6 variant is associated with clinical events such as MI rather than arteriographically defined CAD.

11.6 Pharmacogenomics

In addition to predicting risk, a genetic test can assist in clinical decisions, particularly in regard to drug type or dosage. Examples include the dose of coumadin and polymorphisms in the CYP450 2C9 and VKORC1 genes, and CYP450 2C19 and the use of clopidogrel. The CYP2C9*3 allele and VKORC1 381 CC and TC genotypes have been reported to explain 60.2% of the variability in daily coumadin dose requirements [54]. Simple equations have been developed to assist the physician in selecting the most effective individualized dose based on genotype differences [55]. Using such knowledge, it has been demonstrated that approximately 60% of adverse outcomes attributed to coumadin could be avoided by utilizing this genetic knowledge to determine the most appropriate dose for a given patient [56]. In 2007 the Food and Drug Administration placed a boxed warning on coumadin that indicates use of these genotypes is of clinical value (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2007/ucm108967.htm). The CYP 2C19 polymorphism is found in approximately 1/3 of the population and variants may lead to a slow or rapid rate of clopidogrel metabolism. Carriers of the reduced function allele have reduced platelet aggregation in response to clopidogrel and were reported to have a relative 53% increase in endpoints in the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis

in MI (Triton-TIMI 38) [57]. The Hazard ratio for stent thrombosis increased threefold in carriers of the reduced function allele. Similarly, the *KIF6* 719Arg polymorphism can assist physicians in lipid therapy selection that has the best chance of improving clinical outcomes for individual patients.

In addition to independently predicting CHD risk, the *KIF6* 719Arg polymorphism also predicts the response to statin therapy in regard to clinical event reduction independent of LDL-C, HDL-C, triglyceride, and nonHDL-C values. This has been demonstrated in several well-known randomized clinical trials including CARE, WOSCOPS, PROSPER, and PROVE IT-TIMI 22. In these trials, the presence of the *KIF6* 719Arg polymorphism identified a substantial and statistically significant reduction of cardiac events in the statin-treated group that was not related to changes in any of the lipid parameters including LDL. Implying a mechanism of action unrelated to classic lipoprotein metabolism.

11.6.1 KIF6 719Arg Polymorphism Predicts Response to Statin Therapy for Coronary Events in the CARE and WOSCOPS Trials

Multiple primary and secondary prevention trials have shown that statin therapy is beneficial in preventing coronary death and serious coronary events with ARRs of 3–4% and RRRs of 25–30% compared to placebo over a 5-year period. Statin therapy also reduces CHD events in some individuals who received less benefit in terms of LDL-C lowering. Identification of those individuals who may benefit from statin therapy despite less than optimal LDL-C lowering may aid with treatment compliance and reduce morbidity and mortality. Further, statins have an acceptable safety profile, but they also have dose-related side effects. Thus, it may be advantageous for physicians to use an individual's genetic information to help determine the risk/benefit of aggressive vs. standard statin treatment.

Since the *KIF6* 719Arg risk variant was associated with both risk for MI in CARE and CHD in WOSCOPS, Iakoubova et al. [47] asked whether carriers of the *KIF6* risk variant benefited from pravastatin treatment. In CARE, pravastatin treatment reduced the relative risk of MI by 37% in carriers of the *KIF6* 719Arg risk variant, and in WOSCOPS, pravastatin treatment reduced the relative risk of CHD by 50% among *KIF6* 719Arg *Carrier*s. In CARE, when genotype was not considered, the ARR was 3.5% (Fig. 11.6). When *KIF6* genotype was considered in the genetic substudy, the ARR by pravastatin was 4.9% for *KIF6* 719Arg carriers of the 719Arg risk variant and 1.4% for noncarriers. The NNT in CARE to prevent one coronary event in the original study was 34 for all patients, yet the NNT was only 20 for *KIF6* 719Arg carriers compared with 72 for noncarriers. In WOSCOPS, when genotype was not considered, the ARR was 3.5%. When *KIF6* genotype was considered, the ARR by pravastatin was 5.5% for carriers of the

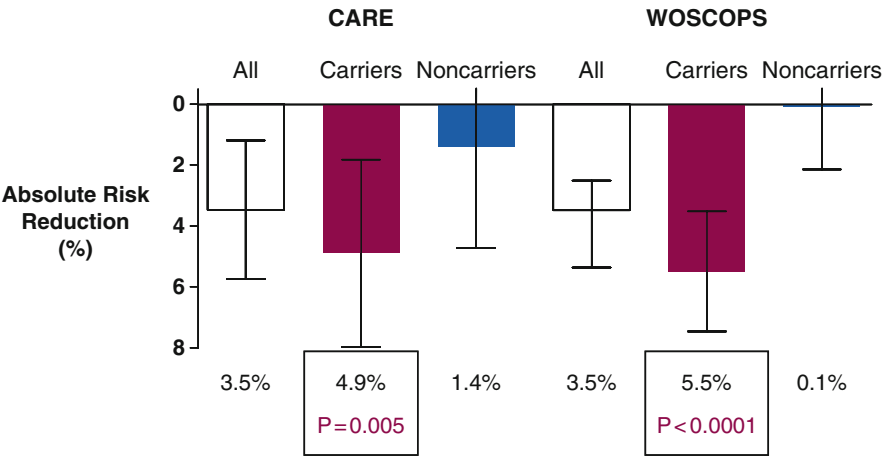

Fig. 11.6 Differential CHD Reduction by Statin Rx *KIF6 719Arg Carriers received the most benefit*. Differential reduction in CHD events, in response to statin treatment, based on *KIF6* 719Arg *Carrier* status. In WOSCOPS, risk reduction was significantly greater in carriers than in noncarriers ($p = 0.01$)

KIF6 risk variant ($p = 0.0001$), and 0.1% for noncarriers. The NNT in WOSCOPS was 46 for all patients, yet only 18 for *KIF6* 719Arg *Carrier*s compared to >100 for noncarriers.

11.6.2 KIF6 Predicts Response to Statin Therapy in Elderly Patients with Prior Vascular Disease in the PROSPER Trial

The elderly are a special population with regards to risk for coronary events and may require prevention and treatment strategies that differ from those appropriate for middle-aged patients. For example, there is evidence that plasma cholesterol levels do not predict risk of coronary events as well in the elderly as they do in middle-aged populations [58–61]. Nevertheless, observational and randomized clinical studies of statins in elderly populations have shown significant reductions of mortality and nonfatal coronary events, particularly in those with prior vascular disease [38, 58–62]. Since risk for coronary events or response to therapy may differ between the elderly and the general population, we asked whether the *KIF6* 719Arg risk variant could also predict both risk for coronary events and clinical event response to statin therapy in the PROSPER trial, as had previously been demonstrated in the CARE and WOSCOPS studies.

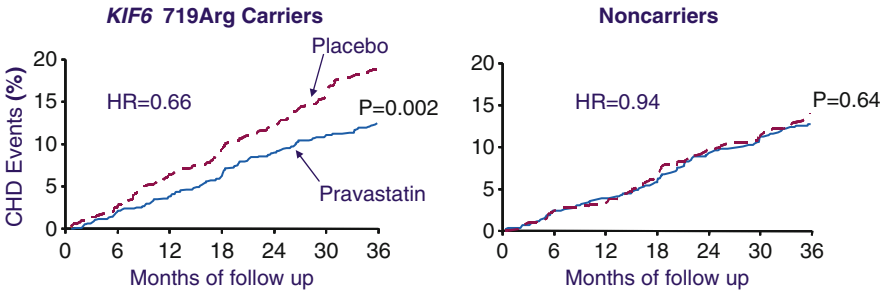

Fig. 11.7 Differential CHD reduction by statin Rx *KIF6* 719Arg *carriers received the most benefit in PROspective Study of Pravastatin in the Elderly at Risk (PROSPER).* Differential effect of statin treatment on fatal and nonfatal CHD events in PROSPER subjects based on *KIF6* 719Arg *carrier* status. Among PROSPER patients with prior vascular disease, carriers of *KIF6* Arg risk allele received significant (p = 0.002) reduction (34%) in coronary events in response to pravastatin treatment vs. placebo. Among patients without prior vascular disease there was no significant event reduction

The PROSPER prospective trial investigated the effects of pravastatin therapy on reduction of fatal and nonfatal cardiovascular events in elderly patients (70–82 years old) with and without prior cardiac and peripheral vascular disease [62]. The study cohort consisted of 5,804 patients (48% men) randomized to treatment with either 40 mg pravastatin per day or placebo and followed for an average of 3.2 years. In this study, a significant reduction of coronary events was observed only among the 42% of patients with prior vascular disease treated with pravastatin, but not among those patients without a history of prior vascular disease.

In the genetic substudy of PROSPER, carriers of the *KIF6* 719Arg risk variant with prior vascular disease received substantial and significant reduction of coronary events from statin therapy [63]. Among PROSPER patients with prior vascular disease on pravastatin therapy, the ARR was 6.3% in 719Arg carriers vs. 1.2% in noncarriers. The NNT with pravastatin in *KIF6* 719Arg *Carrier*s, was 16 compared to 83 in noncarriers. The RRR for coronary events for *KIF6* 719Arg carriers was 33.6% (HR 0.66, 95% CI 0.52–0.86). However, noncarriers received no significant risk reduction from pravastatin therapy (HR 0.94, 95% CI 0.69–1.28) (Fig. 11.7). Among those without prior vascular disease, neither the *KIF6* 719Arg carriers nor noncarriers benefited from statin therapy in terms of clinical event reduction, a finding consistent with the original PROSPER trial where significant event reduction with statin therapy was observed only in subjects with prior vascular disease [62]. Interestingly, in patients with a history of prior vascular disease, *KIF6* 719Arg carriers and noncarriers received nearly identical reductions in LDL-C on pravastatin therapy suggesting that the risk reduction observed in *KIF6* 719Arg carriers may be due to a mechanism that is independent of LDL-C lowering.

11.6.3 *KIF6 Predicts Response to Statin Therapy After Acute Coronary Syndromes in the PROVE IT-TIMI 22 Trial*

The Pravastatin and Atorvastatin Evaluation and Infection Therapy – Thrombosis in Myocardial Infarction 22 (PROVE IT-TIMI 22) study compared high-dose atorvastatin with standard-dose pravastatin treatment [64]. A genetic substudy of this cohort explored whether high-dose atorvastatin therapy, compared to pravastatin therapy, would significantly reduce coronary events in carriers of the *KIF6* 719Arg risk variant and whether the clinical benefit would be greater in carriers than in noncarriers [65].

The original PROVE IT-TIMI 22 study comprised 4,162 patients, who had been hospitalized for an acute coronary syndrome within 10 days preceding enrollment and randomized to high-dose atorvastatin (80 mg/day) compared to standard-dose pravastatin (40 mg/day) and to gatifloxacin vs. placebo using a double blind, two-by-two factorial design. Genetic analysis was possible in 1,777 Caucasian patients and the endpoint for the genetic study was the same as the original PROVE IT-TIMI 22 study: death from any cause or major cardiovascular events, which included myocardial infarction, documented unstable angina requiring hospitalization, revascularization with either percutaneous coronary intervention or coronary-artery bypass grafting, and stroke.

In the genetic study, high-dose atorvastatin therapy significantly reduced coronary events in carriers of the *KIF6* 719Arg risk variant and this clinical benefit was 6.8-fold greater in *KIF6* 719Arg *carrier*s than in noncarriers [65]. When genotype was not considered in this genetic study, the RRR for coronary events was 27%, favoring high-dose atorvastatin. When genotype was considered, in *KIF6* 719Arg *carrier*s, the RRR for death of any cause or major cardiovascular events was 41%

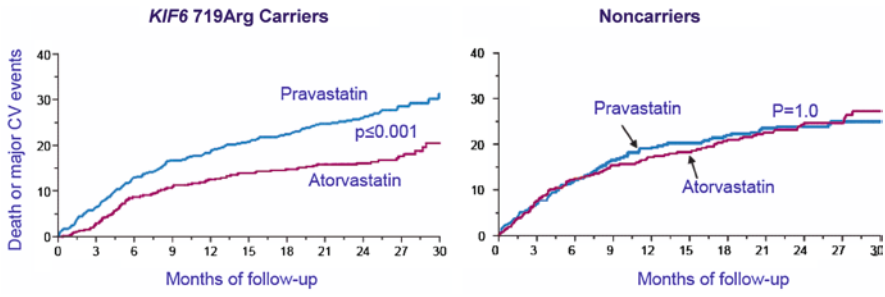

Fig. 11.8 Differential CHD reduction by statin Rx *KIF6 719Arg carriers received the most benefit in PROVE-IT.* Differential reduction in CHD events in PROVE IT-TIMI 22 based on *KIF6* 719Arg *carrier* status. *KIF6* 719Arg *carrier*s received significantly greater benefit from 80 mg atorvastatin, compared with 40 mg pravastatin, than did noncarriers. The number needed to treat (NNT) with atorvastatin (vs. pravastatin) for 2 years to prevent one event was 10 for *KIF6* 719Arg *carrier*s and 125 for noncarriers

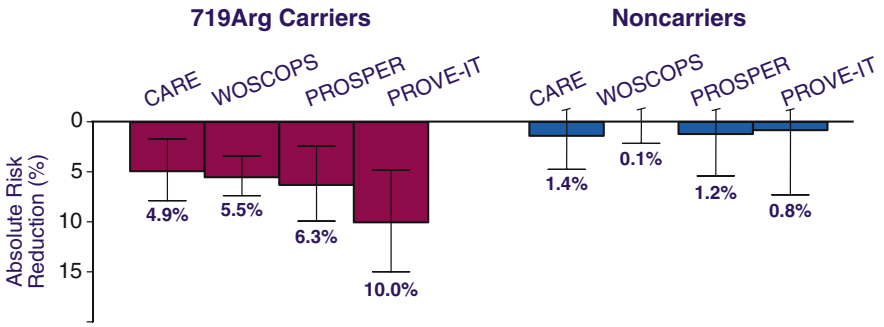

Fig. 11.9 Differential response to statin therapy based on *KIF6* 719Arg polymorphism. Differential reduction in CHD events in four clinical statin trials based on *KIF6* 719Arg *carrier* status. *KIF6* 719Arg *carrier*s received substantial and significant reduction of absolute risk. *KIF6* 719Arg *carrier*s received benefit from both a hydrophilic and lipophilic statin. No significant absolute risk reduction (ARR) was observed in *KIF6* noncarriers in these three placebo controlled, and one statin dose comparison trials

favoring atorvastatin in a model adjusted for traditional risk factors, triglyceride and CRP levels, and treatment with gatifloxacin. No significant RRR was observed in noncarriers. The ARR was 10% favoring atorvastatin therapy for carriers of the *KIF6* 719Arg variant compared to 0.8% for noncarriers. Thus, the number of patients needed to treat with high-dose atorvastatin rather than standard-dose pravastatin to prevent one acute coronary syndrome event was 10 for carriers of the *KIF6* 719Arg variant compared to 125 for noncarriers. Further, among *KIF6* 719Arg *carrier*s, this significant superiority of high-dose atorvastatin therapy was evident as early as 30 days after the start of treatment, yet in noncarriers there was no significant superior benefit of high-dose treatment at any point during the study (Fig. 11.8). This early superiority of intensive therapy in *KIF6* 719Arg *carrier*s may be due to an early plaque-stabilizing effect, a pleiotropic effect that appears not to be linked to LDL-lowering or to anti-inflammatory mechanisms related to the reduction in CRP as *KIF6* 719Arg *carrier*s and noncarriers did not differ in median LDL-C, triglyceride, or CRP levels at baseline or during the trial [65].

11.6.4 KIF6 Predicts Clinical Event Response to Statin Therapy as Shown in Four Randomized Clinical Trials

Taken together, the genetic studies of CARE, WOSCOPS, PROSPER, and PROVE IT-TIMI 22 suggest that the *KIF6* 719Arg variant influences both risk of coronary events (Fig. 11.4) and response to statin therapy (Fig. 11.9) in middle-aged men and women, and in the elderly with a prior history of vascular disease.

11.7 Clinical Examples

The clinical utility of the *KIF6* 719Arg polymorphism can be illustrated in the following two case studies.

11.7.1 Case Study #1. Elderly Asymptomatic Individual in Whom the Physician Has Discovered a Moderately Elevated LDL-C

11.7.1.1 Report

Patient CK is a 72-year-old, healthy Caucasian male with a prior history of myocardial infarction at age 64 treated with a bare metal stent (BMS) and no further events. His LDL-C was 116 at the time of the MI and he was not placed on statins.

11.7.1.2 Current Evaluation

Trig	126 mg/dl
Total cholesterol	180 mg/dl
LDL-C	109 mg/dl
HDL-C	46 mg/dl
Creatinine	1.7 mg/dl

11.7.1.3 Clinical Decision Problem

Should a statin medication be started in a 72-year-old asymptomatic male with a prior cardiovascular history and slightly elevated creatinine?

(a) If *KIF6* 719Arg = Negative → use of statins would not significantly reduce the risk of a future event and the slightly elevated creatinine suggests some degree of renal impairment and thus increased side effect risk from statin treatment.
(b) If *KIF6* 719Arg = Positive → use of statins may significantly reduce the risk of a future event and moves the statin risk/benefit decision towards the "benefit" side.

11.7.1.4 Basis for Decision

Iakoubova, O. A., Robertson, M., Tong, C. H., Roswland, C. M., Catanese, J. J., Blauw, G. J., et al. (2010). *KIF6* Trp719Arg polymorphism and the effect of statin

therapy in elderly patients: Results from the PROSPER study. *European Journal of Cardiovascular Prevention and Rehabilitation, 17*(4), 455–461.

11.7.2 Case Study #2. 58-year-old Asymptomatic Executive Treated with a Statin but With Poor compliance. No Prior History of CVD

11.7.2.1 Report

Patient PB is a 58-year-old busy male executive with moderate LDL-C elevation in the past (LDL-C = 140 mg/dl) for which his physician had prescribed simvastatin 40 mg/day. The initial LDL-C response was encouraging with LDL-C lowered to 110 mg/dl; but over the next 6 months, the physician noted that the LDL-C increased to 142 mg/dl. Upon inquiry, the physician determined the increase in LDL-C was due to poor compliance to the statin medication, in part, due to the patient's sense that he could feel no improvement and besides, he was too busy to remember.

11.7.2.2 Current Evaluation

Trig	144 mg/dl
Total cholesterol	213 mg/dl
LDL-C	142 mg/dl
HDL-C	42 mg/dl
Creatinine	1.0 mg/dl

11.7.2.3 Clinical Decision Problem

How to enhance compliance.

(a) If *KIF6* 719Arg = Positive → patient informed that he was born with a genetic status that increased his heart attack risk but more importantly, allowed him to benefit MORE from statin treatment, in regard to fewer heart attacks, than the average person and thus he should improve his compliance since he is one of the "lucky" ones.

(b) If *KIF6* 719Arg = Negative → patients informed that they were born with a genetic status that adherence to heart disease prevention methods is not only important but also indicates that combination therapy may benefit them more than statin alone and thus they warrant further evaluation and perhaps combination therapy.

11.7.2.4 Basis for Decision

Iakoubova, O. A., Tong, C. H., Rowland, C. M., et al. (2008). Association of the Trp719Arg polymorphism in kinesin-like protein 6 with myocardial infarction and coronary heart disease in 2 prospective trials: The CARE and WOSCOPS trials. *Journal of the American College of Cardiology, 51*, 435–443.

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Brown, G. B, Zhao, X. Q., Chait, A., Fisher, L. D., Cheung, M. C., Morse, J. S., Dowdy, A. A., Marino, E. K., Bolson, E. L., Alaupovic, P., Frohlich, J., & Albers, J. J. (2001). Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease. *The New England Journal of Medicine, 345*, 1583–1592.

11.8 Conclusions

The *KIF6* 719Arg polymorphism has clinical relevance for two reasons, one for risk determination, and one for statin clinical event benefit. The *KIF6* 719Arg polymorphism identifies patients at increased risk for CV events in over 50,000 research subjects, and also divides patients into two groups in regard to the benefit they may receive from statin therapy, specifically in regard to fewer clinical events. This benefit appears to be independent of LDL-C change and hs-CRP change and has been documented in multiple clinical trials.

These effects are seen in both men and women and are ethnically diverse. The pharmacogenetic clinical event response findings have been shown in groups with well controlled, moderate, and elevated LDL-C levels such as the PROVE IT-TIMI 22 study, the CARE, and the WOSCOPS study. Further, similar findings have been documented in older individuals (>70 years) who have a prior history of vascular disease but not in an older group without a prior history of vascular disease.

Individuals who are not carriers of the 719Arg allele may benefit from statin therapy in regard to fewer clinical events but the likelihood of benefit is surprisingly low, despite substantial LDL-C lowering. In this group, it seems reasonable to investigate non-LDL causes of CHD and consider treatment with combination lipid therapy such as nicotinic acid + statin based on the results of the FATS and HATS investigations that achieved 80–90% reductions in clinical events [66, 67].

Compliance to medication is a major problem faced by most physicians. *KIF6* carriers have an even stronger reason to maintain compliance with their physician prescribed statin medication.

By testing only once in a patient's lifetime, the physician gains knowledge of the presence or absence of the *KIF6* 719Arg polymorphism which allows the physician to: (1) utilize genetic SNP analysis to further refine cardiovascular risk evaluation; and (2) allow the physician to utilize pharmacogenomic information to personalize statin-induced lipid-lowering therapy with the goal of reducing future cardiovascular events.

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Part IV
Drugs that Cause Delayed Hypersensitivity

Chapter 12
Abacavir

Elizabeth J. Phillips and Simon A. Mallal

Keywords Abacavir • Abacavir pharmacokinetics • Abacavir hypersensitivity • Pharmacovigilance • Rechallenge • HLA-B*5701 • False positive clinical diagnosis • Abacavir patch testing • Abacavir observational studies • PREDICT-1 study • SHAPE study • Negative predictive value of HLA-B*5701 • Immunopathogenesis of abacavir hypersensitivity • HLA-B*5701 translation to clinic • HLA-B*5701 quality assurance • HLA-B*5701 laboratory testing • HLA-B*5701 screening test

12.1 Pharmacology and Clinical Use of Abacavir

Abacavir is a guanosine analog that competitively inhibits the reverse transcriptase of the human immunodefiency virus (HIV) and is used in combination antiretroviral therapy for the treatment of HIV. It was approved by the US Food and Drug Administration in 1998 and has been in clinical use since that time. Abacavir is currently most commonly used as part of a fixed-dose combination, 600 mg in combination with lamivudine 300 mg which is given once-daily as part of combination antiretroviral therapy. It is also available as a liquid formulation and has been approved for use in children. Abacavir is well absorbed with an absolute bioavailability of 83% and can be taken without regard to food [1]. Although the parent drug has a short plasma half-life

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_12,

of only 2 h, abacavir is metabolized to carbovir triphosphate which has an intracellular half-life of 20 h or longer [1, 2]. It is this long intracellular half-life of carbovir triphosphate that makes abacavir pharmacokinetically amenable to once-daily dosing and this has been supported by clinical data [3, 4]. Unlike other nucleoside analog reverse transcriptase inhibitors, abacavir is metabolized predominantly by the liver by two major pathways: alcohol dehydrogenase and uridine diphosphate glucruonyltransferase. Less than 2% is excreted unchanged in the urine [1]. In addition, unlike other antiretroviral drugs that are metabolized by the liver such as nonnucleoside reverse transcriptase inhibitors and protease inhibitors, abacavir is not significantly metabolized by cytochrome (CYP) P450 enzymes. It also does not inhibit or induce CYP enzymes which make drug interactions unlikely. Therapeutic drug monitoring has been infrequently employed for abacavir as it is the intracellular concentrations of carbovir triphosphate which would be most likely to be associated with drug effect and these are difficult and costly to measure on a routine basis. There is also very little information on validated intracellular target concentrations and their association with clinical efficacy.

12.2 Abacavir Side Effects

The major treatment limiting side effect of abacavir, which is independent of the dose of the drug, is a drug hypersensitivity syndrome. Abacavir hypersensitivity was first described in the premarketing phase of drug development. In predominantly Caucasian populations, it has been clinically diagnosed in approximately 8% of those initiating the drug [5]. On first exposure to the drug symptoms of abacavir hypersensitivity usually appears in the second week of treatment, a median of 9 days after its initiation. Initial symptoms such as fever, malaise, diarrhea, nausea, and vomiting are common but can be very nonspecific and hence challenging to separate from side effects of other antiretroviral drugs, infections, or immune restoration/inflammatory disease. Rash tends to be a late component of the abacavir hypersensitivity syndrome occurring in approximately 70% of patients prior to drug discontinuation. The rash associated with abacavir hypersensitivity is a mild to moderate maculopapular exanthema and not a blistering eruption with skin separation characteristic of Stevens–Johnson syndrome or toxic epidermal necrolysis. Symptoms of abacavir hypersensitivity should remit within 72 h of stopping the drug and this dechallenge used as one of the criteria that strengthens the likelihood of the clinical diagnosis of abacavir hypersensitivity [5]. Rechallenge with abacavir following a reaction clinically compatible with abacavir hypersensitivity can result in rapid and severe shock and mortality and is therefore contraindicated [6]. The success of abacavir as an antiretroviral drug was predicated in the early postmarketing years by a robust clinical management pharmacovigilence program that educated physicians and other healthcare workers treating patients with HIV in the early recognition and management of abacavir hypersensitivity. This included permanent discontinuation of abacavir in patients with signs and symptoms compatible with abacavir. By necessity from a drug safety standpoint, the clinical pharmacovigilence program led to many more patients being diagnosed with abacavir hypersensitivity and discontinuing abacavir than truly had the disease. Patients themselves were also engaged in this process of early recognition of abacavir hypersensitivity and

were given a warning card and a 24 h contact line should they develop symptoms or signs in the first 6 weeks of abacavir treatment.

12.3 Pharmacogenetics of Abacavir Hypersensitivity

Early clues for a genetic basis of abacavir hypersensitivity included lower frequency of the reactions described in those of African and African American descent, and a case report describing the syndrome in a father and daughter [7, 8]. In March 2002, two independent research groups reported an association between the histocompatibility class I allele, HLA-B*5701 and abacavir hypersensitivity [9, 10]. Both studies employed a candidate gene approach and had an overrepresentation of Caucasian men. The Western Australian population-based cohort study provided complete case ascertainment of 200 patients exposed to abacavir [9] whereas, the US GSK study was a retrospective case-control design [10]. In the Western Australian study the HLA-B*5701 allele was present in 78% of patients clinically diagnosed with abacavir hypersensitivity [9]. Despite the strong association between HLA-B*5701 and abacavir hypersensitivity, 22% of patients in the Australian study and 45% of patients in the US study with a clinical diagnosis of abacavir hypersensitivity lacked HLA-B*5701 [9, 10]. This less than 100% negative predictive value of HLA-B*5701 for clinically diagnosed abacavir hypersensitivity raised safety concerns about the potential clinical utility of HLA-B*5701 as a screening test to prevent abacavir hypersensitivity. These concerns were further heightened with a subsequent US case-control study that showed only 8% sensitivity of HLA-B*5701 for clinically diagnosed abacavir hypersensitivity in African American populations [11]. It later became clear that these early studies showing lower sensitivity of HLA-B*5701 for clinically diagnosed abacavir hypersensitivity were hampered by false positive clinical diagnosis. This false positive clinical diagnosis was particularly apparent in Blacks and other non-White populations with a lower carriage rate of HLA-B*5701 (Fig. 12.1). The problem with false positive clinical diagnosis has been further highlighted in randomized double-blinded controlled treatment trials where consistently 2–7% of patients found to have not even received abacavir after unbinding of the study had been given a clinical diagnosis of abacavir hypersensitivity [12–15]. The problem of the high sensitivity but low specificity of clinical diagnosis for abacavir hypersensitivity was largely overcome by abacavir patch testing, a technique whereby increasing concentrations of abacavir were applied in a petrolatum vehicle on the skin surface with standard commercial patch tape. In 2002, it was noted that a high proportion of patients with clinical syndromes compatible with abacavir hypersensitivity had clearly positive skin patch tests 24 h after application and that the histopathological picture of a skin biopsy from a skin patch test was identical to that of the rash of acute abacavir hypersensitivity reaction [16]. Three of four HLA-B*5701 negative clinical abacavir hypersensitivity patients were accessible for follow-up in the original Western Australian study returned and had negative abacavir skin patch tests and subsequently went on to tolerate abacavir [17]. Subsequent patch test studies

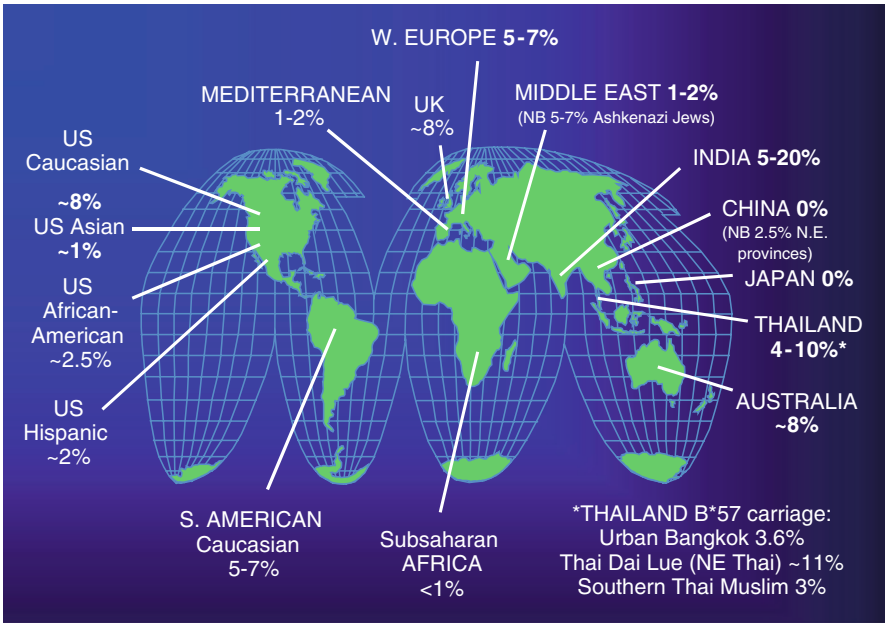

Fig. 12.1 Carriage rate of HLA-B*5701 in different populations (Adapted from Phillips, E. (2006). *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *43*, 103–105 by the University of Chicago Press)

showed 100% of patients with positive abacavir patch tests carried HLA-B*5701 [18–21]. This suggested that abacavir patch testing identifies those with true immunologically mediated abacavir hypersensitivity and could be used as a research tool to circumvent the false positive clinical diagnosis.

Between 2006 to the present, several observational studies have been published that have supported the utility of HLA-B*5701 testing in decreasing both true immunologically mediated and the false-positive clinically diagnosed abacavir hypersensitivity. The Western Australian cohort introduced routine HLA-B*5701 screening in early 2002 and subsequently published the prospective experience following the introduction of HLA-B*5701 screening between 2002 and 2005 [17, 22]. This study showed a significant reduction in abacavir hypersensitivity from 8% before introduction of screening to 2% following postscreening. All three cases of abacavir hypersensitivity occurred in HLA-B*5701 positive patients who had been prescribed abacavir despite the positive screening test. No cases of abacavir hypersensitivity were described among 138 HLA-B*5701-negative patients. This study also showed a reduction in all cause discontinuation of abacavir potential highlighting the positive downstream effects that HLA-B*5701 screening has on the confidence of the patient and provider. Several other observational studies have also shown significant reductions in patients diagnosed with clinical abacavir hypersensitivity reaction following introduction of HLA-B*5701 screening [23–28]. In the Atazanavir/Ritonavir Induction/Simplification with Epzicom Study (ARIES), the

first randomized open-label clinical trial to utilize prospective HLA-B*5701 screening only 4/517 (0.8%) of patients received a clinical diagnosis of abacavir hypersensitivity and all four had negative skin patch tests adding further support that screening essentially eliminates true immunologically mediated abacavir hypersensitivity syndrome [28].

The highest level of evidence to support the clinical utility of HLA-B*5701 for the prevention of abacavir hypersensitivity syndrome has come from the Prospective Randomized Evaluation of DNA Screening in a Clinical Trial (PREDICT-1) study [20]. The PREDICT-1 study set the stage as being the first randomized pharmacogenetic study to examine the clinical effectiveness of a pharmacogenetic marker to reduce the toxicity of a specific drug. In this study, a total of 1,956 predominantly Caucasian (84%) patients, were enrolled across 265 sites in Europe and Australia, and were randomized either to real-time HLA-B*5701 screening and exclusion from abacavir based on the HLA status or a control arm that received retrospective screening following exposure to abacavir [20]. The study was additionally unique in utilizing coprimary endpoints which included clinical diagnosis of abacavir hypersensitivity by the patient's physician and clinically diagnosed abacavir hypersensitivity which had been confirmed by positive patch testing. Abacavir patch testing was conducted in all patients 6–10 weeks following a clinical diagnosis of abacavir hypersensitivity as well as in 100 patients who were tolerant of abacavir to test the specificity of patch testing. The results of the study were striking in that patch test positive abacavir hypersensitivity was completely eliminated in the group who underwent HLA-B*5701 screening. This indicates a 100% negative predictive value of HLA-B*5701 for true immunologically mediated (patch test positive) abacavir hypersensitivity. A significant reduction in clinically diagnosed abacavir hypersensitivity from 7.8 to 3.4% also occurred. It is likely that most of these 3.4% who were clinically diagnosed with abacavir hypersensitivity but patch test negative represent false-positive clinical diagnosis. This was supported by a multivariate model which suggested that there were significantly more patients starting nonnucleoside reverse transcriptase inhibitors and those with gastro-intestinal symptoms using protease inhibitors in the HLA-B*5701 negative group that received a clinical diagnosis of abacavir hypersensitivity. These finding suggest that symptom overlap and confusion contributed to false-positive clinical diagnosis in these patients. Indeed, a 3.6% rate of false-positive clinical diagnosis had been anticipated in the study design and was used for the power calculation for the clinical outcome endpoint. The high anticipated false positive clinical diagnosis rate also motivated the use of the coprimary endpoint of patch test confirming abacavir hypersensitivity [20].

The generalizability of the PREDICT-1 study to all populations was limited by its predominant Caucasian population. To look at the applicability of HLA-B*5701 testing to more diverse populations, the Study of Hypersensitivity to Abacavir and Pharmacogenetic Evaluation (SHAPE) study was conducted to examine the sensitivity and specificity of HLA-B*5701 for clinically diagnosed and patch test positive abacavir hypersensitivity in White and Black American populations [21]. This study was a retrospective case-control study that applied patch testing to Black and

White cases with a clinical history consistent with abacavir hypersensitivity reaction and HLA-B*5701 testing to both cases and controls tolerating abacavir. In this study, 100% of both White and Black patch test positive cases carried HLA-B*5701 supporting that HLA-B*5701 has 100% sensitivity and 100% negative predictive value for immunologically mediated abacavir hypersensitivity that is generalizable across race.

12.4 Mechanism of Drug-Induced Delayed Hypersensitivity Reaction

In parallel with the strong body of clinical evidence outlined above, insights into the immunopathogenesis of abacavir hypersensitivity provides additional evidence to support that abacavir hypersensitivity is restricted to the class I MHC allele HLA-B*5701 and is associated with an abacavir specific CD8+-mediated immune response [29–31]. This process is exquisitely restricted by HLA-B*5701 as demonstrated by elegant research showing that a single amino acid change within the peptide-binding domain is enough to result in clinical and ex vivo tolerance. Any change in the peptide biding groove appears to obliterate the ability of the HLA allele to accommodate and bind the haptenated version of abacavir or its reactive metabolite [29]. This explains why patients who carry other members of the B17 serogroup such as HLA-B*58 and HLA-B*5703, that differ from HLA-B*5701 by only a few amino acids, are tolerant to abacavir. Other experiments from healthy, abacavir naive HLA-B*5701 blood donors have shown that CD8+ T-cells proliferate in response to abacavir over 11–14 days and can be restimulated by abacavir-exposed antigen-presenting cells transfected with HLA-B*5701 that are devoid of other HLA antigens [29].

12.5 Predictive Value of Genotype Testing for Patients on Abacavir

Data from the PREDICT-1 study showed the positive predictive value of HLA-B*5701 for abacavir hypersensitivity to be 55%, meaning that approximately 45% of patients carrying HLA-B*5701 would not develop hypersensitivity on exposure to abacavir. A follow up genetic study of the PREDICT-1, SHAPE and multinational Australian-Canadian-Swiss Study attempted to look at factors driving abacavir tolerance by looking at polymorphisms in several genes haplospecific to HLA-B*5701 as well as KIR, CD14, and drug metabolizing genes such as alcohol dehydrogenase comparing 95 abacavir patch test-positive patients and 43 HLA-B*5701 patients tolerating abacavir. This study did not identify any definitive abrogating factors in HLA-B*5701 patients tolerating abacavir but did suggest that it is highly likely that these lie outside of the MHC [32].

12.6 Application of HLA-B*5701 Screening to the Clinic

The process starting from discovery of the association between HLA-B*5701 to the translation of the test into widespread clinical practice that has been endorsed by international treatment guidelines illustrates the number of hurdles and steps involved in getting a pharmacogenetic test to the clinic (Fig. 12.1). Although HLA-B*5701 is most prevalent in White European populations (Fig. 12.2), current evidence suggests that the implications of HLA-B*5701 positivity are similar across race and the 100% negative predictive value of HLA-B*5701 for true immunologically mediated abacavir hypersensitivity is broadly applicable to both white and non-White race [20, 21]. This is extremely important from a practical standpoint as in the globalized world where migration and population admixture is frequent and it is not be possible to determine genetic racial background by patient appearance or self-defined ethnicity [33]. Furthermore, screening is cost effective even in races that have a very low prevalence of HLA-B*5701 because it

Fig. 12.2 A roadmap for the transplantation of pharmacogenetic research into clinical practice: the abacavir example (Adapted from Phillips, E, Mallal, S. (2009). *Personalized Medicine*, *6*, 393–408, Future Medicine Ltd)

Table 12.1 The number needed to screen for HLA-B*5701 to prevent one case of abacavir hypersensitivity (ABC HSR) is dependent on the prevalence of the abacavir hypersensitivity as well as the positive predictive value of HLA-B*5701 for ABC since there is such a high rate of false positive clinical diagnosis for ABC HSR(2–7%) which is also significantly reduced with HLA-B*5701 screening. When this is taken into account, the number needed to treat to prevent once case decreases from 32 to approximately 13. Explanatory tables are shown below. For each table a theoretical population of 100,000 has been assumed and the number of individuals with the ADR is calculated based on its reported prevalence and the number of tolerant patients is calculated by subtracting this figure from 100,000. The sensitivity and specificity of the HLA test for the ADR have been taken from the literature and the numbers in each of the four cells (a–d) calculated. The positive and negative predictive values (PPV and NPV) and numbers needed to test (NNT) were then calculated from the numbers in each cell (a–d).

	HLA +ve	HLA −ve	Total	Prevalence = (a+b)/1000
ADR +ve	a	b	a + b	Prev = a + b/a + b + c +d
				Sensitivity = a/a + b × 100%
Drug tolerant	c	d	c + d	Specificity = d/c + d × 100%
Total	a + c	b + d	a + b + c + d = 100,000	
	PPV = a/a + c × 100%	NPV = d/b + d × 100%	Number need to prevent one case = 100,000/a	

Abacavir true immunologically mediated + clinical false-positive

	B*5701 +ve	B*5701 −ve	Total	Prevalence = 8%
ABC HSR (including clinical false-positive)	8,000	0	8,000	Sensitivity = 100%
ABC tolerant	2,425	89,575	92,000	Specificity = 97.4%
Total	10,425	89,575	1,00,000	NNT to prevent one case = 12.5
	PPV = 76.7%	NPV = 100%		

Abacavir true immunologically mediated

	B*5701 +ve	B*5701 −ve	Total	Prevalence = 3.1%
ABC HSR	3,150	0	3,150	Sensitivity = 100%
ABC tolerant	2,550	94,300	96,850	Specificity = 97.4%
Total	5,700	94,300	1,00,000	NNT to prevent one case = 32
	PPV = 55%%	NPV = 100%		

prevents both true immunologically mediated abacavir hypersensitivity and false-positive clinical diagnosis, and in view of the consistent 2–7% rate of false-positive clinical diagnosis [34]. Data from all studies to-date suggest that the number needed to test to prevent one case of abacavir hypersensitivity would be 13 if taking into account both true and false-positive clinical diagnosis and 32 if considering only prevention of true immunologically mediated abacavir hypersensitivity (Table 12.1). Perhaps the most important role of HLA-B*5701 screening is improving drug safety in that the major morbidity and mortality of abacavir hypersensitivity occurs on repeat rather than initial exposure to abacavir, therefore, primary HLA-B*5701 screening prevents the population at highest risk from ever being exposed or sensitised to abacavir.

Most crucial in the successful translation of HLA-B*5701 screening into routine clinical use has been the effective development of cost-effective and quality assured laboratory testing [35]. The development of a internal quality assurance program now driven by the Asian Pacific Histocompatibility and Immunogenetics Association (APHIA) has been instrumental in the success and safety of ongoing HLA-B*5701 testing globally. The development of molecular and flow cytometric techniques was particularly important since the sequence based typing used in HLA testing is expensive, can have a long turnaround time, and has typically been performed in the specialized transplant laboratories. PCR-based techniques such as sequence specific amplification and real-time PCR melting curve analysis are sensitive and specific assays that have been developed and have now been applied in many routine diagnostic laboratories [36, 37]. Flow cytometric-based techniques have also been described and look promising as cost-effective tests that can be added on to the CD4+ and CD8+ counts that are done at baseline and follow-up in all patients on antiretroviral therapy [38]. Other PCR-based techniques have included a Taqman assay detecting the single nucleotide polymorphism HCP5 rs2390529 within an endogenous retrovirus [39]. Previous studies suggested that this marker was in complete linkage disequilibrium with HLA-B*5701 and was therefore a haplospecific marker. However, cases of patch test positive abacavir hypersensitivity have occurred in patients positive for HLA-B*5701 but negative for HCP5 rs2395029 [32] which suggests that this marker cannot safely be used as a screening test for the prevention of abacavir hypersensitivity. This underscores that any surrogate that does not have 100% sensitivity for the presence of HLA-B*5701 cannot safely be implemented as a screening test.

12.7 Case Report

A 43-year-old Caucasian HIV-positive man presents asymptomatic with a CD4+ count of 250/μl and an HIV viral load of 150,000 copies per ml. Screening test comes back negative for HLA-B*5701 and he is commenced on treatment with abacavir-lamivudine (600/300 mg) once daily and nevirapine

initially 200 mg once daily with a plan to increase to 400 mg once daily after two weeks. Two weeks into treatment, he develops a maculopapular eruption starting on his trunk and becoming generalized, but he is afebrile and otherwise well. Laboratory tests reveal normal full blood count and liver function tests; rash continues to be bothersome despite antihistamines and topical steroids; and atazanavir/ritonavir is substituted for nevirapine. Within a few days the rash has resolved.

12.8 Summary

- The patient being HLA-B*5701 negative in this case gives additional reassurance that this is not abacavir hypersensitivity syndrome, particularly when drugs such as abacavir and nevirapine that have overlapping toxicities are used together.
- Rash does not occur as the initial symptom with abacavir hypersensitivity syndrome and isolated rash without fever and other symptoms does not meet criteria for the clinical diagnosis of abacavir hypersensitivity. Nevirapine is a common cause of isolated rash. Isolated rash can occur with abacavir, however this is not known to be predicted by HLA-B*5701.
- HLA-B*5701 is most useful as a screening test for patients who are naive to abacavir. Although there is yet to be another HLA allele associated with abacavir hypersensitivity, it is still possible that a rare HLA allele may yet be discovered and HLA-B*5701 negativity should not be used as the basis for rechallenge in a patient who has experienced a clinical syndrome compatible with abacavir hypersensitivity.

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Chapter 13
Allopurinol

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Keywords Gout • Uric acid metabolism • Hypersensitivity reaction • Severe cutaneous adverse reactions

13.1 Pharmacology of Allopurinol

Allopurinol (ZYLOPRIM), chemically described as 1,5-dihydro-4*H*-pyrazolo (3,4-*d*) pyrimidin-4-one, is a xanthine oxidase inhibitor used to treat patients with gout, kidney stones, and hyperuricemia associated with cancer therapy.

Uric acid is a by-product of the breakdown of certain nucleotides/nucleosides (purines) in the body. Hyperuricemia occurs when the body produces more uric acid than can be eliminated, or when uric acid can not be adequately excreted from the body. Allopurinol prevents the production of uric acid by blocking the activity of the enzyme that converts purines to uric acid. Allopurinol is a structural analog of the natural purine base, hypoxanthine (Fig. 13.1). The drug is therefore a competitive inhibitor of xanthine oxidase, the enzyme responsible for the conversion of hypoxanthine to xanthine and xanthine to uric acid. Allopurinol is metabolized to the xanthine analog, oxypurinol (alloxanthine), that also inhibits xanthine oxidase.

Increases of serum uric acid concentration, referred to as hyperuricemia, can lead to the deposition of monosodium urate crystals in various tissues. This may result in attacks of gout, urate nephropathy, and/or tophi.

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_13,

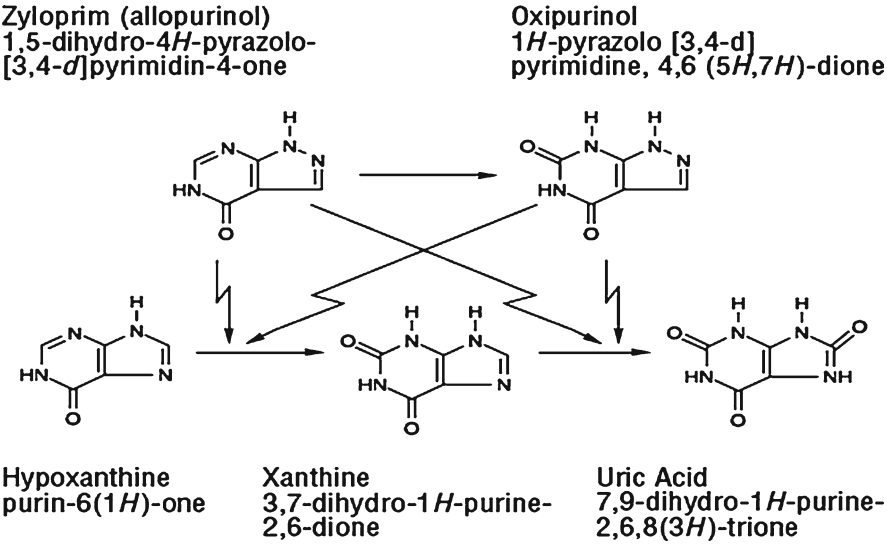

Fig. 13.1 The mechanism by which allopurinol reduces production of uric acid

13.1.1 Clinical Indications

Regardless of the cause of hyperuricemia, the first-line of pharmacologic therapy to reduce serum urate concentrations in most gout patients is inhibition of xanthine oxidase with allopurinol. When effective and well-tolerated, allopurinol is a cost-effective option [1, 2]. Allopurinol is the most frequently used antihyperuricemic agent, probably because of a convenient once-daily dosage regimen and the fact that the drug can be used to treat both urate overproduction and underexcretion [1]. Other indications for allopurinol treatment are Lesch–Nyhan syndrome, recurrent uric acid kidney stones refractory to other treatments, certain enzyme/blood disorders, and hyperuricemia associated with cancer chemotherapy [3, 4]. However, because allopurinol is not innocuous, the drug is not recommended for treatment of asymptomatic hyperuricemia.

13.1.2 Target Patient Populations

The Third National Health and Nutrition Examination Survey (NHANES III) estimated that 5.1 million patients in the USA were afflicted with gout between 1988 and 1994 [5] At present, at least three million in the US are thought to suffer from active gout, and an additional 3–6 million individuals have a history of gout [6, 7]. Data from a US managed care claims database revealed that the prevalence of gout has increased, from 2.9 per 1,000 in 1990 to 5.2 per 1,000 in 1999 [8]. Gout is also becoming more

prevalent in other countries, including New Zealand and Taiwan [9, 10]. The incidence of gout has been found to correlate strongly with serum urate concentration, increasing markedly when this exceeds 420 μmol/L (7.0 mg/dL) [11].

13.1.3 Alternative Medications

Pharmacologic urate-lowering drugs include uricosurics (probenecid, sulfinpyrazone, and benzbromarone), which increase the excretion of uric acid in the urine; xanthine oxidase inhibitors (allopurinol and the recently approved febuxostat), which inhibit uric acid production; and the experimental uricase (Rasburicase™), which degrades urate [1, 12]. Pharmacotherapy thus consists of a choice between a medication that reduces urate production and a drug that increases urate excretion. Uricosuric drugs such as probenecid and sulfinpyrazone increases renal urate clearance and are considered antihyperuricemic agents for patients with primary gout who present substantially decreased renal urate excretion. Such drugs are contraindicated in patients with high urinary concentrations of uric acid and are not recommended for patients with chronic renal insufficiency. Allopurinol remains the most frequently used antihyperuricemic agent because of a convenient once-daily dosage regimen and an ability to treat both urate overproduction and underexcretion [1]. Febuxostat, a recently developed xanthine oxidase inhibitor, is considered a reasonable alternative for patients intolerant to allopurinol. Febuxostat is now approved in Europe and the USA, but is not yet available in other countries.

13.1.4 Dosing, Pharmacokinetics, and Pharmacodynamics

Allopurinol has been approved by the US Food and Drug Administration (FDA) at doses up to 800 mg/day. The expert consensus EULAR spelled out guidelines have reinforced FDA dosing guidelines for allopurinol in patients with preserved renal function [2, 13]. These guidelines recommend that allopurinol be initiated at a dose of 100 mg/day, and increased by 100 mg/day every 1–4 weeks until the target serum urate level (<6 mg/dL) is achieved or the maximum appropriate allopurinol dose is reached. FDA dosing guidelines have also advocated daily doses of 200–300 mg allopurinol as adequate for most patients with mild gout, and an average daily dose of 400–600 mg allopurinol is expected to control hyperuricemia in patients with moderately severe tophaceous gout [13]. However, the vast majority of allopurinol prescriptions are for doses ≤300 mg/day, which often fails to adequately treat the hyperuricemia of gout. This situation has arisen from consideration of longstanding guidelines for allopurinol use calibrated to renal function and serum concentrations of oxypurinol, to avoid allopurinol hypersensitivity syndrome (HSS) [13]. These guidelines are not evidence-based and fail to adequately treat hyperuricemia and also fail to prevent allopurinol HSS [7, 13].

A longer-term goal of allopurinol therapy is to prevent further attacks, eliminate tophi, and prevent joint destruction, by consistently reducing the concentration of urate [1, 2, 14]. As urate is insoluble in physiologic solutions at concentrations exceeding 6.7–7.0 mg/dL, current guidelines for inhibiting ongoing urate crystal deposition, reducing total body urate stores, and resolving macroscopic tophi recommend continuing (lifelong) reduction of serum urate concentrations to <6 mg/dL (approximately 360 mmol/L), ideally to 5–6 mg/dL [15]. Uric acid levels usually begin to fall within 2–3 days of commencement of allopurinol treatment, but return to original levels within 7–10 days if treatment is stopped. Thus, several months of therapy may be required before gout can be controlled.

13.2 Toxicities

Although allopurinol is well-tolerated by most patients, approximately 2% develop a mild skin reaction (rash and itching) [3] and 0.4% suffer from allopurinol HSS, a severe cutaneous adverse reaction that can be life-threatening [16]. This syndrome may present with fever, eosinophilia, and rashes, ranging from maculopapular through Stevens–Johnson syndrome (SJS) to toxic epidermal necrolysis (TEN) and multiorgan involvement, with significant mortality and morbidity [17]. Potential risk factors for allopurinol HSS include Han Chinese ethnicity, older age, and underlying renal impairment [18]. The risk of allopurinol HSS may be reduced by administering smaller doses of drug from 50 to 300 mg/day, with the exact dose being directly proportional to creatinine clearance [19]. However, such regimen is not well adhered in clinical practice, and their effectiveness in reducing allopurinol hypersensitivity reactions has been challenged [20].

13.3 Pharmacogenetics and Pharmacogenomics of Allopurinol

Allopurinol is a frequent cause of severe cutaneous adverse reactions (SCAR), including drug HSS, SJS, and TEN. Although rare, the mortality rate of patients with allopurinol-induced SCAR can be as high as 26% [17, 21]. SJS is characterized by high fever; malaise; and a rapidly developing blistering exanthema of macules and target-like lesions, accompanied by mucosal involvement. TEN has similar presentations with even more extensive skin detachment and a higher mortality rate (30–40%) [22]. HSS has systemic manifestations with multiorgan involvement, in addition to exanthema [17].

A recent European multinational case–control study found that allopurinol has become the drug most commonly associated with SJS/TEN in Europe and Israel, accounting for 17.4% of 379 patients with SJS or TEN [23]. Allopurinol also plays a major role in the induction of SJS or TEN in Asian populations [18, 24, 25]. Data from 230 consecutive SJS/TEN patients assessed over a 5-year period (1997–2002)

in the Chang Gung Memorial Hospital Health System of Taiwan showed that allopurinol was the second leading cause of SJS/TEN, after carbamazepine [26]. However, because of recent awareness of carbamazepine-induced SJS, allopurinol has now become the leading drug responsible for severe adverse reactions in patients receiving compensation from the Taiwan Drug Relief Foundation (TDRF) (http://www.tdrf.org.tw).

Drug hypersensitivity is typically dose-independent and unpredictable, resulting from exaggerated immune-mediated reactions [27]. Susceptibility to such idiosyncratic reactions is thought to be genetically determined, and familial predisposition to allopurinol hypersensitivity has been reported [28]. To identify genetic factors predisposing an individual to allopurinol-induced SCAR, we assessed polymorphisms in genes related to drug metabolism and the immune response in 51 well-characterized patients with allopurinol-induced SCAR, including 30 with HSS, 13 with SJS, 5 with SJS/TEN and 3 with TEN, and compared allele frequencies in such patients with those of 228 controls, including 135 allopurinol-tolerant subjects, and 93 healthy individuals [29]. All participants were unrelated Han Chinese residing in Taiwan. We found that the HLA-B*5801 allele was present in all 51 (100%) patients with allopurinol-induced SJS/TEN/HSS, but only in 15% (20/135) of allopurinol-tolerant controls (corrected $P = 4.7 \times 10^{-24}$, OR = 580.3, 95% CI = 34.4–9,780.9), and 20% (19/93) of healthy controls (corrected $P = 8.1 \times 10217^{-18}$, OR = 393.5, 95% CI = 23.2–6,665.26) [29]. The occurrence of HLA-A*3303, HLA-B*5801, HLA-Cw*0302, and HLA-DRB1*0301 alleles of the HLA-B*5801 ancestral haplotype was also significantly associated with allopurinol hypersensitivity, as were several haplotype-specific polymorphisms near the HLA-B gene, including SNPs located in BAT3, MSH5, and MICB. Recombinant mapping data further concluded that the HLA-B*5801 allele itself was the major susceptibility gene for allopurinol-induced SCAR in the Han Chinese population [29].

As the distribution of the HLA-B*5801 allele varies among different populations (e.g., 2–4% in Africans, 1–6% in Caucasians, 3–15% in Asian Indians, and 8.8–10.9% in Chinese (http://www.ebi.ac.uk/imgt/hla/stats.html, Accessed 10 May 2007), an association between this allele and allopurinol-induced SCAR may also be present in other ethnic groups. Indeed, such an association has been observed in patients from Europe, Japan, and Thailand [30–33].

Data from a European study showed that 55% (15/27) of patients of European ancestry with allopurinol-induced SJS/TEN carried the HLA-B*5801 allele [30], compared with 1.5% (28/1,822) of controls, resulting in an odds ratio of 80 (95% CI = 34–187; corrected $P < 10^{-6}$) (Table 13.1). Among 31 patients with allopurinol-induced SJS/TEN enrolled in the European study, four were of non-European ancestry (two from Asia, one from South America, and one from Africa) and all four had the HLA-B*5801 allele [30]. In addition, all three Japanese patients with allopurinol-associated SCAR carried the HLA-B*5801 allele [31], and a moderate but statistically significant association was observed between HLA-B*5801 and allopurinol-associated SJS/TEN in Japanese patients ([32]; Table 13.1). Recently, HLA-B*5801 was found to be present in all of 27 patients with allopurinol-induced SJS/TEN from Thailand [33]. Moreover, the risk of allopurinol-induced SJS/TEN

Table 13.1 HLA-B*5801 in allopurinol-induced severe cutaneous adverse reactions (SCAR)

Study number	1		2 (European study)				3		4	
Study population	Han Chinese [a]		Caucasian [b]		Non-European ancestry (two Asians)		Japanese [c]		Thai [d]	
Case	51/51	(100%)	15/27	(55%)	4/4	(100%)	7/13	(54%)	27/27	(100%)
Control	20/135	(15%)	28/1822	(1.5%)			6/493	(1.2%)	7/54	(13%)
Odds ratio (95% CI)	580.3 (34.4–9780.9)		80 (34–187)				94.7 (24.4–367.3)		348.3 (19.2–6,336.9)	
P value	4.7×10^{-24} [e]		$<10^{-6}$ [e]				1.71×10^{-9}		1.61×10^{-13}	
Reference	Hung et al. [29]		Lonjou et al. [30]				Kaniwa et al. [32]; Dainichi et al. [31]		Wichittra et al. [33]	

[a] Case: Allopurinol-SCAR; control: Tolerant control

[b] ase: Allopurinol-SJS/TEN; control: A mixed European population

[c] Case: Allopurinol-SJS/TEN; control: Japanese population

[d] Case: Allopurinol-SJS/TEN; control: Tolerant control

[e] Adjusted using Bonferroni's correction for multiple comparisons to account for observed alleles

was found to be significantly higher in patients with than without the HLA-B*5801 allele (OR 348.3; 95% CI = 19.2–6336.9; $P = 1.61 \times 10^{-13}$) (Table 13.1).

Ethnically associated differences in the prevalence of HLA-B*5801 among patients with allopurinol-associated SCAR (55% in European populations and 100% in Thailand and Southeast Asia, including Taiwan) may be linked to the very different allelic frequencies in these populations. Indeed, the dbMHC data base shows that the allele frequency of HLA-B*5801 is significantly higher in Thais (8.6%) and Han Chinese (7.3%) than in Europeans (0.8%) or Japanese (0.61%). In Taiwan, the allele frequency is 10% with carrier prevalence of 20% [29].

Whereas, all allopurinol-SCAR patients from Southeast Asia carried at least one HLA-B*5801 allele, it was also found that additional patients tolerant to allopurinol carried the allele. This suggests that the HLA-B*5801 allele is necessary but not sufficient for development of allopurinol-SCAR. Other genes may also be involved in the mechanism of pathogenesis, such as costimulatory molecules involved in the interaction between antigen-presenting cells and T cell interaction [29].

13.4 Clinical Utility of PGx Testing in Personalized Medicine

One important goal of pharmacogenomics is to prevent severe ADRs by using a simple test to screen out patients at risk. Before any test can be used in clinical practice, however, several important issues must be considered [34, 35]. These include the incidence and severity of the adverse event; the sensitivity and specificity of the predictive marker; and whether equally effective, alternative medications are available for individuals who test positive.

Although the incidence of SCAR is relatively low compared with common diseases, SCAR is serious and even life-threatening. Many surviving patients have long-term complications, some of which result in permanent damage, such as blindness and renal failure. The medical cost associated with ADRs is also very high. Allopurinol is the most widely-prescribed urate-lowering agent in the world, with about 2.8 million prescriptions per year written for the treatment of gout in the US [6]. Recent extensive research has attempted to elucidate the roles of uric acid and oxidative stress in the development of various diseases [36]. These studies have indicated that the spectrum of conditions that can be treated with allopurinol may expand to include metabolic syndrome and related disorders, chronic kidney disease, and the adverse effects of cancer treatment [37]. As potential indications increase, the number of prescriptions for allopurinol may also increase.

Data from a retrospective case-control study in Taiwan, using an allopurinol-tolerant group as the control, found that the HLA-B*5801 allele would have 100% sensitivity and 85% specificity in detecting the risk of allopurinol-associated SCAR (Table 13.2). Assume the prevalence rate of 0.4% (four allopurinol-associated SCAR in 1,000 allopurinol users), the presence of B*5801 allele would have a 2.6% positive predictive value (PPV) for detecting allopurinol-induced SCAR, whereas absence thereof would have 100% negative predictive value (NPV)

Table 13.2 HLA-B*5801 as a test for Allopurinol-induced SCAR

	Han Chinese		European		Thai	
	Affected	Tolerant	Affected	Tolerant	Affected	Tolerant
Patients with the allele	51 (100%)	1,905 (15%)	15 (55.6%)	101 (1.5%)	29 (100%)	936 (12.96%)
Patients without the allele	0 (0%)	10,794 (85%)	12 (44.4%)	6,622 (98.5%)	0 (0%)	6,285 (87.04%)
Sensitivity (%)	100		55.6		100	
Specificity (%)	85		98.5		87	
Positive predictive value (%)	2.6		12.9		3.01	
Negative predictive value (%)	100		99.8		100	
Assumed prevalence rate (%)	0.4		0.4		0.4	
ADRs	SCAR		SJS or TEN		SJS or TEN	
Reference	Hung et al. [29]		Lonjou [30]		Wichittra et al. [33]	

(Table 13.2). Similar sensitivity, specificity, PPV, and NPV results can be found in European and Thai populations (Table 13.2).

Results from Han Chinese patients (Table 13.2) indicate that 15% of those who test positive for the HLA-B*5801 allele may never develop SCAR and may therefore be unnecessarily denied the drug. However, the life-threatening consequences of SJS/TEN, and the availability of alternative drugs, may justify the withholding of allopurinol from such patients. Hyperuricemia in HLA-B*5801-positive gout patients can be treated with uricosurics (e.g., probenecid, sulfinpyrazone, or benzbromazone), unless creatinine clearance is <50 mL/min or urate overproduction has been documented. Such patients may be treated with febuxostat, a newly approved xanthine oxidase inhibitor.

In addition to the HLA-B*5801, other factors such as renal function or virus infection may be risk factors for the development of allopurinol-induced SCAR [17, 29, 38]. Current guidelines suggest that the allopurinol dose be calibrated to renal function and serum concentration of oxypurinol to avoid allopurinol HSS. However, lower doses (e.g., ≤300 mg/day) may fail to adequately treat hyperuricemia. Moreover, these guidelines have not succeeded in prevention of allopurinol-associated SCAR. Using the presence of the HLA-B*5801 allele as a pharmacogenetic test, in conjunction with tests of renal function, the vast majority of patients at low risk for development of allopurinol-induced SCAR (test-negative patients) can be treated with higher doses of allopurinol sufficient to achieve normouricemia. Prospective studies will be very helpful to determine the effectiveness of HLA-B*5801 testing in prevention of allopurinol-associated SCAR. Such a study is ongoing in Taiwan.

13.5 Conclusion

Allopurinol is a frequent cause of SCAR, and the HLA B*5801 allele is a marker of susceptibility. The clinical severity of SCAR, the availability of alternative medications, and the high sensitivity and specificity of the HLA B*5801 marker provide a plausible basis for the development of tests to identify individuals at risk of this potentially life-threatening condition caused by allopurinol.

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Chapter 14
Carbamazepine and Its Structurally-Related Antiepileptics

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Keywords Epilepsy • Stevens Johnson syndrome • Seizures

14.1 Pharmacology of Carbamazepine

14.1.1 Clinical Indications and Patient Populations for Carbamazepine

Carbamazepine (CBZ) (Equetro®, Carbatrol®, Epitol®, Tegretol®) is one of a class of antiepileptic drugs (AEDs) that aim to prevent or reduce the severity of abnormal nerve activity in the brain, thereby blocking seizures [1, 2]. CBZ was initially approved for the treatment of epilepsy in the US in 1974. Currently, the indications for CBZ use include seizures, trigeminal neuralgia, and bipolar disorder [3]. CBZ is now first-line therapy for the treatment of partial seizures and tonic-clonic seizures. The mechanism of action of CBZ antiepileptic effects is thought to be sodium channel blocking, limiting the repetitive firing of action potentials, slowing the rate of recovery of voltage-activated Na^+ channels from inactivation, decreasing the activity of nerve cells, and preventing them from firing abnormally in the brain [1, 3]. In addition, CBZ may also prevent abnormal brain signals from spreading to other parts of the brain. Complex partial seizures most frequently arise from the repetitive firing of action potentials in the temporal lobe of the brain and cause impairment of consciousness with or without accompanying automatisms. Patients who have complex partial seizures can be treated with CBZ with a greater response and more significant improvement than patients with other types of seizures [1, 4]. Although CBZ is approved to treat various types of seizures (e.g., partial seizures,

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A.H.B. Wu and K.-T J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine,DOI 10.1007/978-1-60761-283-4_14,

generalized tonic-clonic seizures, or mixed seizure patterns), it is usually not effective at treating absence seizures (petit mal seizures) [5].

In the past decade, in addition to its antiepileptic effects, CBZ has increasingly become the medication of choice for the treatment of bipolar disorder, particularly acute mania [6]. CBZ has been approved to treat manic or mixed episodes (which include characteristics of both mania and depression) associated with bipolar disorder, also known as manic depression [6]. CBZ also has good therapeutic effects in trigeminal neuralgia (also known as tic douloureux), a nerve condition that causes episodes of facial pain (typically cheek or jaw pain) [7]. It is thought that CBZ works to treat trigeminal neuralgia by blocking the nerve signals that cause pain and other unpleasant sensations. Moreover, CBZ also has antidiuretic effects that may be associated with reduced levels of antidiuretic hormone.

14.1.2 Alternative AED Medications for Carbamazepine

Alternative medication options for CBZ in the treatment of epilepsy include a number of different AEDs. In addition to CBZ, the major drugs used in partial seizures and generalized tonic-clonic seizures comprise other first-line AEDs such as oxcarbazepine (OXC), phenytoin (PHT), pentobarbital (PHB), valproate (also called valproic acid), and lamotrigine [LTG], among others; and also second-line AEDs such as gabapentin, topiramate, vigabatrin, and levetiracetam, and the like [8, 9]. As the mechanism of action of CBZ is mainly due to its prevention of repetitive firing of action potentials by binding to the alpha-subunit of voltage-gated sodium channels in neurons [10], the alternative AEDs (e.g., OXC, PHT, PHB, valproate, LTG, topiramate, and vigabatrin) with similar mechanisms of action are good choices for the treatment of partial seizures, generalized tonic-clonic seizures, or mixed seizure patterns. In addition to the mechanism of action, the selection of alternative medications for CBZ may be determined by the effectiveness, price, side effects, cross-reactivity of skin reactions, and the practical experience of physicians. Since side effects are commonly associated with the administration of AEDs, monotherapy, and initiation with a single agent at a time are suggested. Furthermore, many AEDs are hepatic enzyme inducers; for example, CBZ is a CYP450 enzyme inducer. CBZ and its alternative AEDs can potentially interact with many other medications [11]. Therefore, prescribing CBZ or one of its alternative AEDs in combination with other medicines must be done with caution to observe for potential drug-drug interactions.

Among the alternative medications, OXC (Trileptal®) is a 10-keto analog of CBZ and acts as a prodrug [1]. The mechanism of action of OXC is similar to that of CBZ, however, it is a less potent enzyme inducer than CBZ, and OXC is metabolized by different enzymes [12]. As a prodrug, orally ingested OXC is rapidly converted to its main active metabolite, a 10-monohydroxy derivative, which is inactivated by glucuronide conjugation and eliminated by renal excretion. OXC has been approved for monotherapy or adjunctive therapy for partial seizures in adults and partial seizures in children aged 4–16.

PHT (diphenylhydantoin, Dilantin®) is a hydantoin and exerts antiseizure activity without causing general depression of the CNS [1]. PHT is effective against all types of partial and tonic-clonic seizures, but not absence seizure. The mechanism of antiepileptic action of PHT is similar to CBZ, which is mediated by slowing the rate of recovery of voltage-activated Na+ channels from inactivation, therefore limiting the repetitive firing of action potentials [1]. The pharmacokinetic characteristics of PHT are influenced by its binding to serum proteins, by the nonlinearity of its elimination kinetics, and by its metabolism by CYPs. The side reactions/toxicity of PHT includes cerebellar-vestibular effects, gastrointestinal symptoms, gingival hyperplasia, osteomalacia, megaloblastic anemia, hirsutism, and hypersensitivity reactions, among others [1].

LTG (Lamictal®) is a phenyltriazine derivative, and its antiepileptic action has been proposed to involve the inhibition of voltage-sensitive sodium channels, a mechanism similar to those of PHT and CBZ, and also involve the modulation of presynaptic transmitter release of excitatory amino acids [1]. LTG is effective against a broad spectrum of seizures, including partial and secondarily generalized tonic-clonic seizures in adults, and Lennox-Gastaut syndrome in both children and adults. In addition, LTG was also approved for maintenance treatment of bipolar I disorder. LTG is completely absorbed from the gastrointestinal tract and is metabolized primarily by glucuronidation. Common side effects of LTG include headaches, dizziness, and insomnia. In extremely rare cases, LTG has been known to cause drug reaction with eosinophilia and systemic symptoms (DRESS), Stevens–Johnson syndrome (SJS), or toxic epidermal necrolysis (TEN) [1].

14.1.3 Dosing, Pharmacokinetics and Pharmacodynamics of Carbamazepine

CBZ (Equetro®, Carbatrol®, Epitol®, Tegretol®) comes in several different formulae, including tablets, chewable tables, extended-release tablets, extended-release capsules and suspension (liquid). All are administered orally. Several different manufacturers make these drugs, and not all forms are approved for each indication. For example, Equetro® is approved to treat bipolar disorder only, while Carbatrol®, Epitol®, Tegretol®, and generic CBZ are approved to treat epilepsy and trigeminal neuralgia, but not bipolar disorder. The dosage of CBZ for treating epilepsy depends upon a patient's age and weight. For adults and children over 12 years old, the recommended starting dose is 200 mg twice daily. For children aged 6–12 years, the recommended starting dose is 100 mg twice daily. For children under 6 years, the starting dose is calculated by weight: 10–20 mg/kg total per day, divided into two or three doses per day (10–20 mg/kg/day b.i.d. or t.i.d.). When combining CBZ with existing anticonvulsant therapy, it should be added gradually while the other AEDs are maintained or gradually decreased [13].

The relationship between the dose of CBZ and concentrations of the drug in plasma is not a simple one. Because of its limited aqueous solubility and high lipid solubility, the absorption of CBZ is slow but complete following oral administration [1].

Approximately 75% of CBZ binds to plasma proteins. After oral administration of CBZ tablets, its peak plasma level can be achieved after 4–5 h [13]. CBZ is lipophilic and distributes rapidly into all tissues, including the liver, kidney, and brain. The therapeutic concentrations of CBZ for adults are reported to be between 6 and 12 μg/ml, although considerable variation occurs. The concentrations of CBZ in CSF appear to correspond to the levels of free drug in plasma. Side effects referable to the CNS are frequent at concentrations above 9 μg/ml. The elimination half-life of CBZ averages 35 h, ranging from 18 to 65 h. Since CBZ induces hepatic metabolic enzymes, autoinduction of CBZ decreases its half-life to 10–20 h with chronic administration [1, 14, 15].

CBZ is metabolized in the liver where it exhibits autoinduction. As an inducer of hepatic enzymes, CBZ can enhance the expression of the hepatic cytochrome (CYP) P450 enzymes, including CYP2C, CYP3A, and UGT, thus enhancing the metabolism of drugs degraded by these enzymes and altering clearance of these drugs [14, 15]. Hepatic P450 CYP3A4 is primarily responsible for biotransformation of CBZ. It converts CBZ to CBZ-10, 11-epoxide (10, 11-epoxyCBZ), which also has antiepileptic activity since CBZ-10, 11-epoxide is able to limit sustained repetitive firing at therapeutic concentration [16]. In the liver, CBZ-10, 11-epoxide is detoxified by microsomal epoxide hydratase to inactive compounds excreted in the urine primarily as glucuronides.

14.1.4 Side Effects and Toxicities of Carbamazepine

CBZ can cause two types of ADRs. Type A is dose-dependent and results from acute overdose or during chronic therapy. CBZ may produce common type A ADRs such as drowsiness, vertigo, fatigue, unsteadiness, dizziness, nausea, and vomiting; these ADRs require monitoring and dose adjustment [17, 18]. Less common ADRs include dry mouth, ataxia, diplopia, and blurred vision [17, 18]. In comparison, CBZ may produce idiosyncratic type B ADRs, which are dose-independent, not related to its pharmacologic reactions, often serious and life-threatening, and historically unpredictable. Type B ADRs experienced with CBZ include hypersensitivity reactions, hepatitis, and blood dyscrasias (aplastic anemia, agranulocytosis) [19, 20]. Among them, cutaneous adverse drug reactions (cADRs) are the most common hypersensitivity reactions. cADRs can present in a variety of ways from mild maculopapular eruption (MPE) with increasing severity to hypersensitivity syndrome (HSS), SJS, and TEN [21].

MPE is characterized by cutaneous itchy, erythematous macules and papules, which usually spontaneously resolve within 1–2 weeks after withdrawing the causative drugs. The rate of CBZ-induced MPE is ~3.7% [22]. HSS, also called “drug reaction with eosinophilia and systemic symptoms” (DRESS), is characterized by systemic manifestations with fever, hematologic abnormalities, and multiorgan involvement (e.g., hepatitis and nephritis) in addition to skin rash [21]. MPE and HSS are nonblistering cADRs. In comparison, SJS and TEN are two of the most

serious blistering cADRs with a 10–50% mortality rate [21]. SJS and TEN are characterized by a rapidly developing painful or burning blistering exanthema of purpuric macules and target-like lesions accompanied by mucosal involvement and skin detachment to a varying extent. SJS is defined as skin detachment of less than 10% and TEN as skin detachment greater than 30% [23]. Mucous membranes commonly affected by SJS and TEN include the eyes, oropharynx, and anogenital areas. Survivors are at risk of permanent complications such as blindness due to corneal damage [23].

14.2 Pharmacogenetics for Carbamazepine Hypersensitivity

*14.2.1 HLA-B*1502 and CBZ-SJS/TEN in Han Chinese*

As mentioned above, CBZ can cause a variety of hypersensitivity reactions, including mild MPE to severe HSS and SJS/TEN. We previously studied the genetic polymorphisms of genes involved in immune-regulation and CYP-P450 single nucleotide polymorphisms (SNPs) in 44 Han Chinese patients in Taiwan with CBZ-SJS/TEN and compared their allele frequencies with those of 101 tolerant controls [24]. We found that all 44 patients (100%) who developed CBZ-SJS/TEN carried the HLA-B*1502 allele, while only 3% of the CBZ-tolerant group (odds ratio: 2,504, corrected $P = 3.13 \times 10^{-27}$), and 8.6% of the 93 healthy subjects carried the allele [24]. Our extension study, which included 60 patients who experienced CBZ-SJS/TEN and 144 tolerant controls, revealed that 59 of 60 CBZ-SJS/TEN patients tested positive for HLA-B*1502 allele, while only 6 (4.16%) of the 144 tolerant controls carried the allele (odds ratio: 1,357, corrected $P = 1.6 \times 10^{-41}$) (Table 14.1) [25].

To determine whether the HLA-B gene itself or genes in the vicinity of the B locus contributes to the main susceptibility of CBZ-SJS/TEN, we performed fine-mapping in the MHC region using 220 SNPs, 20 short tandem repeat polymorphisms (STRPs), and HLA alleles [25]. We found that polymorphisms located between HLA-DRA and HLA-C showed strong association. In particular, the TT or GT genotypes of rs3130690, 36 kb telemetric to the HLA-B locus, were present in 98% CBZ-SJS/TEN patients, but only in 5% of tolerant controls. MICA*019, 47 kb centromeric to the HLA-B locus, was present in 95% of CBZ-SJS/TEN patients, but only in 15% of tolerant controls. The recombinant map of Cw*0801-HLABC-CA*119-rs3130690T-B*1502-MICA*019 defined the susceptible region within 86 kb (i.e., between the T allele of rs3130690 and MICA*019) flanking the B*1502 gene in the 4 Mb MHC region [25]. Within this 86 kb region, HLA-B is the only known gene. In addition, we have performed a whole genome scan using Affymetrix GeneChip Human Mapping 100K set in a case-controlled association study to identify additional markers/susceptibility genes other than HLA-B*1502 that might predispose individuals for CBZ-SJS/TEN. Using 56 cases/54 controls for CBZ-SJS/TEN, we confirmed the previous observation that the most significant association was observed in the SNPs of the HLA-B regions on chromosome 6,

Table 14.1 Genetic associations of carbamazepine hypersensitivity

Phenotype	HLA association	Population	References
SJS/TEN	HLA-B*1502: 98.3% (59/60) [$P = 1.6 \times 10^{-41}$, OR 1357]	Han Chinese in Taiwan	Hung et al. [25]
SJS/TEN	HLA-B*1502: 100% (4/4) [$P = 1.48 \times 10^{-4}$, OR = 71.9]	Han Chinese in Hong Kong	Man et al. [27]
SJS/TEN	HLA-B*1502: 100% (6/6) [$P = 0.0005$, OR = 25.5]	Thai population	Locharernkul et al. [28]
SJS/TEN	No association with HLA-B*1502 in Whites (0/8); HLA-B*1502: 100% in Asian ancestry (4/4)	Germany and France; Vietnam, China, Cambodia and Reunion Island	Lonjou et al. [32]
SJS/TEN	No association with HLA-B*1502 (0/7); weak association with HLA-B*5901 (1/5)	Japanese	Kaniwa et al. [30] and Ikeda et al. [31]
HSS	SNPs in motlin gene in HLA region [$P = 0.0046$, OR 7.11]; no association with HLA-B*1502	Han Chinese in Taiwan	Hung et al. [25]
MPE	HLA-A*3101 [$P = 0.0022$, OR 17.5]; no association with HLA-B*1502	Han Chinese in Taiwan	Hung et al. [25]
MPE	No association with HLA-B*1502	Han Chinese in Hong Kong	Man et al. [27]
MPE	No association with HLA-B*1502	Thai population	Locharernkul et al. [28]
HSS/MPE[a]	Significant association with TNF-HLA-DR3-DQ2 haplotype [$P = 0.02$, OR 3.2]; no association with HLA-B*1502	Caucasians in UK	Alfirevic et al. [34]

SJS/TEN Stevens–Johnson syndrome/toxic epidermal necrolysis; *HSS* hypersensitivity syndrome; *MPE* maculopapular eruption

[a]CBZ-hypersensitivity: only two with blistering skin rashes

and we did not find additional SNPs that were significantly associated with CBZ-SJS/TEN [26]. The above data suggested that HLA-B*1502 itself is the key genetic susceptibility to CBZ-SJS/TEN.

*14.2.2 HLA-B*1502 and CBZ-SJS/TEN in Asians and Caucasians*

The same association between HLA-B*1502 and CBZ-SJS/TEN also have been reported in independent studies from other Asian countries (Table 14.1). Man et al. reported that 4 out of 4 (100%) Chinese patients with CBZ-SJS/TEN tested positive for HLA-B*1502 compared to 14.5% in tolerant controls from Hong Kong (odds ratio: 71.9, $P = 1.48 \times 10^{-4}$) [27]. Another independent study in a Thai population showed that 6 out of 6 (100%) CBZ-SJS/TEN patients all tested positive

for HLA-B*1502, while only 8 of 50 (16%) of tolerant controls were positive for the allele (odds ratio: 25.5, $P = 0.0005$) [28]. Mehta et al. reported from India that 6 out of 8 CBZ-SJS patients had HLA-B*1502 while none of the ten controls were found to be positive (odds ratio: 71.40, $P = 0.0014$) [29]. However, a recent case series from Japan showed no significant association between HLA-B*1502 and CBZ-SJS/TEN in Japanese patients [30, 31]. None of the 12 Japanese patients with CBZ-SJS/TEN carried the HLA-B*1502 allele [30, 31]. Instead, Ikeda et al. suggested that HLA-B*5901 is one of the candidate markers for CBZ-induced SJS in the Japanese population [31]. It should be noted that the frequency of the HLA-B*1502 in the Japanese population is extremely low, almost nonexistent.

The genetic association between CBZ-SJS/TEN and HLA-B*1502 in a given population seems to correlate with the allele frequency of HLA-B*1502. For example, the allele frequency of HLA-B*1502 is ~0% in Caucasians, and a case series including 12 patients with CBZ-SJS/TEN from Europe, including Germany and France, showed no significant association between HLA-B*1502 and CBZ-SJS/TEN in Caucasian patients [32, 33]. It is interesting to note that, among the 12 patients tested, 8 were Caucasian and none of these patients carried the allele; the remaining 4 patients were from Vietnam, China, Cambodia, and Reunion Island (also people of Chinese descent), and all 4 of these patients tested positive for HLA-B*1502. This finding indicates that the genetic factor (HLA-B*1502 allele) is the main risk factor for CBZ-induced SJS/TEN (Table 14.1).

14.2.3 HLA, TNF-Alpha Alleles and CBZ-MPE/HSS

Genetic susceptibility of CBZ hypersensitivity seems phenotype-specific; HLA-B*1502 association is specific for CBZ-SJS/TEN, but not for MPE or HSS. We found that MPE was associated with HLA-A*3101 (odds ratio: 17.5, corrected $P = 0.0022$) and HSS with SNPs in the motlin gene (odds ratio 7.11, corrected $P = 0.0046$) [25]. Case-series studies in Asian countries where strong HLA-B*1502 association was found in CBZ-SJS/TEN also showed that CBZ-MPE was not associated with the allele [27, 28]. In a large case-series study of 56 Caucasian patients from the UK with CBZ–cADRs, primarily HSS, a significant association was found between HLA-B*0801 and HLA-DR3, DQ2 and TNF-308 alleles in patients with CBZ-hypersensitivity [34]. These data suggest that genetic susceptibility to CBZ-hypersensitivity is not only ethnic-specific, but also phenotype-specific (Table 14.1).

*14.2.4 HLA-B*1502 and Other AEDs-SJS/TEN*

It is interesting to note, since OXC, the prodrug of CBZ, shares structural similarity with CBZ, that three OXC-SJS cases have been reported to date, and two were

HLA-B*1502 positive [35, 37]. The OXC-SJS case who did not test positive for HLA-B*1502 had no separation of epidermis and dermis as seen in typical cases of SJS [36]. In addition, recent studies reported an increased frequency of HLA-B*1502 in patients with PHT- and LTG-induced SJS/TEN in Hong Kong and Thailand as compared to controls. Although the number of reported cases was small, many tested positive for HLA-B*1502, including 1 of 2 LTG-TEN and 1 of 1 PHT-SJS patients with Han Chinese background, and 4 of 4 PHT-SJS Thai patients (odds radio: 18.5, $P = 0.005$) [27, 28]. Furthermore, we carried out a case-control association study in Han Chinese residing in Taiwan and enrolled 26 PHT, 6 LTG-induced SJS/TEN patients, and 113 PHT-tolerant, 67 LTG-tolerant subjects, respectively, who were on the drug for more than 3 months without experiencing adverse reactions [37]. We found that HLA-B*1502 was present in 8 (30.8%) of 26 PHT-SJS/TEN subjects, while only in 9 (8%) of 113 PHT-tolerant subjects (OR=5.1 (95% CI 1.8–15.1), $P = 0.0041$). In comparison, HLA-B*1502 was present in 2 (33%) of 6 LTG-SJS patients and 6 (9%) of 67 LTG-tolerant subjects (OR=5.1 (95% CI, 0.8–33.8), $P = 0.1266$) [37]. In contrast, LTG-induced SJS/TEN or HSS in Caucasians was reported to associate with HLA-B*3801 or HLA-B*5801 weakly [33, 38].

14.3 Clinical Utility of HLA-B*1502 Testing for Personalized Medicine of CBZ

14.3.1 Pharmacogenetic Test and FDA Recommendation

The value of a pharmacogenetic (PGx) test is related to its cost-effectiveness and several other factors, including: (1) the incidence and severity of the adverse events, (2) the sensitivity and specificity of the predictive markers, and (3) whether equally effective, alternative medications are available for individuals who test positive. Although the incidence of CBZ-SJS/TEN is low, and data on the pharmaco-economic analysis of cost-benefit for CBZ-SJS/TEN are not yet available because SJS/TEN carries high mortality and morbidity and many surviving patients have long-term complications (e.g., ocular damage, renal failure), it is valuable to perform genotyping of HLA-B*1502 before prescribing CBZ for high-risk Southeast Asians in whom HLA-B*1502 allele frequency is high in order to avoid these life-threatening conditions.

Due to strong evidence of the association between HLA-B*1502 allele and CBZ-SJS/TEN in some Asian countries as described above, the US Food and Drug Administration (FDA) and similar regulatory agencies in Canada and Taiwan have relabeled CBZ with genetic information, which notes that “patients with ancestries from areas in which HLA-B*1502 is present should be screened for HLA-B*1502 allele before starting treatment with CBZ” [39, 40].

14.3.2 Sensitivity, Specificity, and Predictive Values of the PGx Test

The sensitivity, specificity, and predictive values of a pharmacogenetic test for a specific genetic marker correlate with the allele frequency and disease prevalence in the testing population. For example, considering CBZ-SJS/TEN in Han Chinese of Taiwan, if using a CBZ-tolerant group as the control in a test for SJS/TEN, the HLA-B*1502 allele would have 100% sensitivity and 97% specificity. Assuming a 0.25% prevalence rate, the presence of HLA-B*1502 has a 7.7% positive predictive value for detecting CBZ-SJS/TEN, whereas its absence has a 100% negative predictive value [41]. The low positive predicted value is because there are HLA-B*1502 carriers who are tolerant to CBZ. Although the positive predictive value of the test is low (~7.7%), in view of the severity of the diseases and availability of alternative medicines, CBZ should not be prescribed in HLA-B*1502 carriers unless the expected benefit clearly outweighs the increased risk of serious skin reactions [42].

14.3.3 Alternative Medicines Following the PGx Test

Several alternative AEDs are available that are as equally effective as CBZ, therefore it is feasible to withhold CBZ from HLA-B*1502 carriers in favor of using other AEDs. However, the choice of alternative AEDs should be made with caution as some of the aromatic AEDs with similar chemical structures may have cross-reactivity hypersensitivity reactions [43]. In the clinical setting, CBZ, OXC, PHT, LTG, and PB have a 20–30% chance of cross-reactivity of skin rashes [44–46]. About 25–33% of CBZ hypersensitive patients showed cross-sensitivity of skin rashes to OXC, and 25–70% of OXC-hypersensitivity patients showed cross-reactivity to CBZ [44–46]. Most of the cases showing cross-reactivity hypersensitivity are primarily reported as mild skin rashes such as MPE. It is suggested that if patients have ever suffered CBZ hypersensitivity, they are unfavorable for the administration of aromatic AEDs sharing a similar chemical structure, particularly prohibiting OXC. As mentioned before, several case studies reported that SJS/TEN caused by OXC, PHT, or LTG have been reported to associate with HLA-B*1502, therefore, it is suggested to avoid OXC, PHT, or LTG as alternatives to CBZ therapy in patients who test positive for the HLA-B*1502 allele.

14.4 Case Report

Recently, we have carried out a prospective study in Taiwan aimed at determining the value of screening HLA-B*1502 before prescribing CBZ to prevent CBZ-SJS/TEN. In this study, we identified individuals at risk of CBZ-SJS/TEN by using

HLA-B*1502 genotyping and CBZ was not prescribed in those test positive for the HLA-B*1502. We have enrolled more than 3,000 patients and the preliminary results suggest that application of HLA-B*1502 genotyping as a screening tool before patients taking CBZ can effectively reduce the incidence of these life-threatening adverse drug reactions in our population [47].

14.5 Conclusion

Screening for the HLA-B*1502 allele before starting treatment with CBZ in Asian countries as well as for patients in non-Asian countries who are of Asian descent is justified in view of the high frequency and seriousness of the consequences of SJS/TEN, the high sensitivity and specificity of the marker, and the availability of alternative AEDs equally effective as CBZ. Similar chemicals, such as OXC, PHT, and LTG should also be avoided in individuals who test positive for HLA-B*1502.

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Part V
Miscellaneous Drugs

Chapter 15
Pharmacogenetics of Flucloxacillin and Amoxicillin-Clavulanate Associated Hepatic Dysfunction/Injury

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Keywords Drug-induced liver injury • Anti-staphylococcal agent • Beta-lactamase inhibitor

15.1 Introduction

Drug-induced liver injury (DILI) may be life-threatening and is one of the most common reasons that prevent investigational drugs from reaching the market [1] or newly approved drugs being withdrawn post marketing [2, 3]. DILI is a term that describes various different types of hepatic damage, which includes cholestasis (accumulation of bile in the biliary canaliculi and/or cessation of bile flow) and hepatocellular damage [4]. In cholestatic hepatitis, the biochemical markers alkaline phosphatase (ALP or Alk Phos) and gamma-glutamyltransferase (GGT) would be markedly elevated. However, hepatocellular damage causes marked elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Jaundice (hyperbilirubinemia) is usually present in both types of hepatic damage, however, specific elevation of conjugated bilirubin indicates more of a cholestatic problem. Despite extensive reporting of DILI in literature, the mechanisms causing the hepatic injury remains complex and are often incompletely understood [5].

Hepatotoxicity can be predictable and dose-related, or unpredictable and idiosyncratic. Dose-related hepatotoxicity is usually detected in the preclinical stages of drug development and these investigational drugs often do not achieve regulatory approval to be marketed. Idiosyncratic hepatotoxicity usually is not observed until larger numbers of individuals are exposed to the drug, during the postmarketing phase [6]. In this chapter, two antibiotics that can cause cholestatic hepatotoxicity are discussed in detail.

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_15,

15.2 Flucloxacillin (Floxapen®)

15.2.1 General Pharmacology

Flucloxacillin was first marketed in the 1970s in Europe. It is the anti-staphylococcal agent of choice in many countries, with the exception of the USA and Canada [7]. Flucloxacillin is a semisynthetic penicillin (Fig. 15.1, I) with a narrow-spectrum of bactericidal activity. It has considerable activity against Gram-positive organisms like penicillin sensitive and penicillinase producing *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*. It is not active against Gram-negative bacilli, or *Streptococcus faecalis* and recently methicillin-resistant *Staphylococcus aureus* have become resistant to it. Flucloxacillin is available in parenteral and oral formulations and is well-absorbed from the gastrointestinal tract. Presence of food in the gastrointestinal tract delays its absorption, resulting in lower peak serum concentrations. Flucloxacillin is highly bound to serum proteins (95% bound), and 10% of it is metabolized to flucloxacilloic acid (Fig. 15.1, II) [8]. The elimination half-life of flucloxacillin is 30–60 min, and about 50% of a dose is excreted by the kidney within 6 h of administration. Indications for flucloxacillin include pneumonia, osteomyelitis, skin, soft tissue and wound infections, infected burns, and cellulitis. Flucloxacillin can commonly cause gastrointestinal disturbances (nausea, vomiting, diarrhea) and less frequently causes hematological side effects such as neutropenia and thrombocytopenia. It is contraindicated in patients with history of hypersensitivity to β-lactam antibiotics and patients with previous history of flucloxacillin-associated jaundice/hepatic dysfunction.

15.2.2 Hepatotoxicity

Hepatotoxicity due to flucloxacillin was first described in a case report in 1982 [9]. Shortly after, a number of additional cases were reported [10–12]. To date, 348 and 1,477 reports regarding suspected flucloxacillin-associated hepatotoxicity have

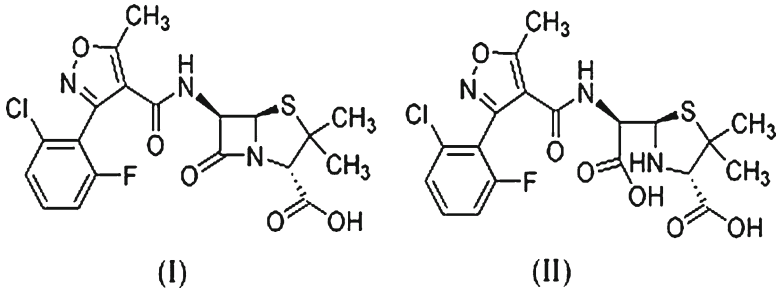

Fig. 15.1 Chemical structures of (I) flucloxacillin, and (II) flucloxacilloic acid

been registered in the Swedish Adverse Drug Reaction Database (SWEDIS) and WHO Adverse Drug Reaction Database (VigiBase), respectively [13]. Classical flucloxacillin-induced cholestasis usually present as painless jaundice with elevated bilirubin (mainly conjugated "direct bilirubin") and ALP [14]. A delay of several days to weeks usually occurred between the start of treatment and onset of clinical symptoms [11], which included nausea, abdominal pain, pruritus, and fever. The course of the hepatotoxicity was typically protracted, averaging about 11 weeks, ranging from weeks to months [7]. The risk of cholestasis due to flucloxacillin was reported by Russman et al. to be 8.5 per 100,000 (1 in 12,000) first-time users according to a cohort study using the data from the UK General Practice Research Database [14]. Devereaux et al. reported an incidence rate of 1:15,000 [7]. Susceptibility to flucloxacillin-induced cholestasis was associated with the patients' age and duration of flucloxacillin treatment. Patients over 55 years of age who received flucloxacillin treatment for over 14 days were at increased risk of developing cholestasis [14, 15]. The incidence of flucloxacillin-induced cholestasis was also found to be higher in females compared to males [14, 15].

Hepatotoxicity due to flucloxacillin was not discovered preclinically because in vitro toxicity studies by Lakehal et al. showed that drug concentrations up to 1 mM were not toxic to human hepatocytes or biliary epithelial cells [16]. However, a minor metabolite of flucloxacillin, 5'-hydroxymethylflucloxacillin, formed by CYP3A4 and initially discovered by Thijssen [17, 18], was found to be toxic to biliary epithelial cells in vitro [16], but the concentration of this minor metabolite in vivo was only 1% of the concentration of the parent drug. Several hypotheses had been proposed to explain the mechanism of DILI due to flucloxacillin, but no consistent result had been observed to prove these hypotheses. Carey et al. proposed that treatment with flucloxacillin could result in the formation of hepatic protein adducts [19]. A serendipitous discovery that patients who were rechallenged with flucloxacillin developed symptoms of eosinophilia, inflammatory infiltrates, and fever suggested the involvement of the immune system in the pathophysiology of this syndrome [9]. Maria et al. were unable to implicate an immunological basis for flucloxacillin-associated DILI using lymphocyte proliferation assays [20].

15.2.3 Pharmacogenetics

The potential genetic basis for DILI susceptibility was investigated and several candidate genes such as those for drug transporters (e.g., ABCB4, ABCB11), familial intrahepatic cholestasis type 1 gene (FIC1) [21], drug metabolizing enzymes e.g., CYP3A4 and CYP3A5 [4], and human leukocyte antigen (HLA) [22] were studied. Amongst these studies, the association between HLA and susceptibility to flucloxacillin related DILI was found to be the strongest.

The UK DILIGEN study was intended to identify genetic determinants of DILI [23]. Genome-wide and candidate gene association studies (GWAS) were performed on 51 cases with flucloxacillin-associated hepatic dysfunction and 282 controls

matched for sex and ancestry. Thirty-six out of the 51 cases were female and the average age of onset of flucloxacillin related DILI was 63 years old, consistent with the earlier studies of risk prediction where patients older than 55 years were more susceptible to hepatic injury. Most of the cases (86%) were diagnosed as having flucloxacillin-associated cholestasis. The Illumina Human1M BeadChip which contained 1,072,820 markers was used to genotype cases and controls. 206,421 markers were discarded due to failure to meet standard quality control criteria. The GWAS revealed one highly significant signal in the MHC region on chromosome 6 (Fig. 15.2) [23]. This single nucleotide polymorphism (SNP) associated with flucloxacillin-associated DILI was rs2395029, with a *P*-value of 8.7×10^{-33} (trend test) and estimated odds ratio (OR) of 45 (95% CI = 19.4–105). Among the cases, 43 of them (84%) carried the risk allele (T), which has an allele frequency of approximately 5% in the European population. In comparison, only 11% of the controls subjects carried this allele. rs2395029 is a missense SNP in the HCP5 gene which was reported by Colombo et al. to be in complete linkage disequilibrium with *HLA-B*5701* [24]. For the cases in the GWAS, *HLA-B*5701* and rs2395029 genotypes correlated perfectly. This meant that *HLA-B*5701* showed a highly significant association with flucloxacillin-associated DILI. Patients possessing this allele and treated with flucloxacillin were associated with an 80-fold increased risk of developing DILI. Data from the same study also revealed that the *HLA-DRB1*0701-DQB1*0303* haplotype were significantly more common among flucloxacillin-related hepatotoxicity compared to controls.

*HLA-B*5701* is relatively common in Northern Europe but rarer in Africa and Asia. This allele was also associated with the development of skin-hypersensitivity reactions to abacavir (see Chap. 12). The pathophysiology of abacavir-related skin reactions has been well detailed. An abacavir metabolite interacts with cytotoxic $CD8^+$ T cells, with recognition dependent on the presence of the *HLA-B*5701* antigen [25]. However, the mechanism associated with flucloxacillin-induced DILI has not been so fully elucidated. There is no obvious structural similarity between

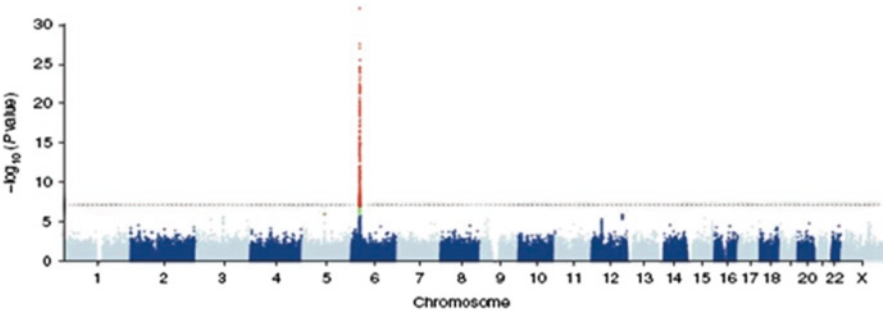

Fig. 15.2 Flucloxacillin DILI genome-wide association study data [23]. Each dot represents a SNP. The *x* axis represents the position of the SNP on chromosomes. The *y* axis represents the –log10 of Cochran-Armitage trend *P* value of the SNP in the case-control association study. The very strong signal in chromosome 6 lies in the regions coding for the MHC genes. Reproduced with permission from publisher.

flucloxacillin and abacavir to explain why both adverse drug reactions are associated with *HLA-B*5701*. Further studies are required to improve our understanding of these observations.

15.3 Amoxicillin-Clavulanate (e.g. Augmentin®)

15.3.1 General Pharmacology

Amoxicillin-clavulanate was first marketed in UK in 1981, and was FDA approved in the US in 1984, and granted marketing approval in Australia in 1986 [26]. It is widely used as an oral and parenteral antibacterial consisting of the combination of a semisynthetic antibiotic amoxicillin, and the β-lactamase inhibitor, clavulanic acid (Fig. 15.3) as its potassium salt. Amoxicillin is a 4-hydroxy analog of ampicillin, derived from the basic penicillin nucleus, 6-aminopenicillanic acid. It has a broad-spectrum of bactericidal activity against many gram-positive and gram-negative microorganisms. However, amoxicillin was found to be susceptible to degradation by β-lactamases. Clavulanic acid is produced by the fermentation of *Streptomyces clavuligerus*. It is a β-lactam that is structurally related to the penicillins and possesses the ability to inactivate a wide variety of β-lactamases by irreversibly binding to the enzymes active site. Clavulanic acid is particularly active against the clinically important plasmid-mediated β-lactamases responsible for transferred drug resistance to penicillins and cephalosporins. The amoxicillin-clavulanic acid combination protects amoxicillin from degradation by β-lactamase enzymes and extends the antibiotic spectrum of amoxicillin to include many normally resistant bacteria.

Amoxicillin-clavulanate is well absorbed from the gastrointestinal tract and can be taken without regard to meals, but absorption of clavulanic acid is increased when taken with food. Amoxicillin is 18% and clavulanic acid is 25% bound to serum proteins. Amoxicillin diffuses readily into most body tissues and fluids with the exception of the brain and spinal fluid. The half-life of amoxicillin is 1.3 h and that of clavulanic acid is 1 h. Approximately, 50–70% of the amoxicillin and 25–40% of the clavulanic acid are excreted unchanged in urine during the first 6 h post dosing. Concurrent administration of probenecid delays amoxicillin excretion but does not

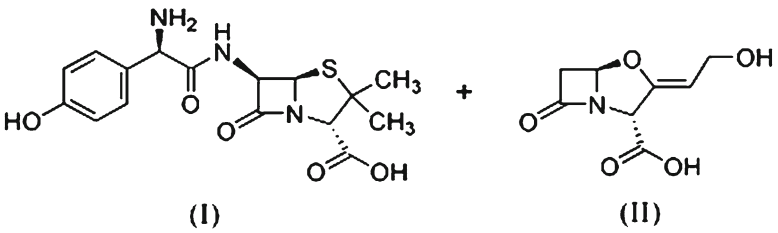

Fig. 15.3 Chemical structures of (I) amoxicillin, and (II) clavulanic acid

delay renal excretion of clavulanic acid. Indications for amoxicillin-clavulanate include lower respiratory tract infections, otitis media, sinusitis, skin infections, and urinary tract infections. Amoxicillin-clavulanate can cause side effects of mild gastrointestinal disturbances and rarely causes hemolytic anemia and thrombocytopenia. It is contraindicated in patients with a history of allergic reactions to any penicillin and in patients with a previous history of cholestatic jaundice/hepatic dysfunction associated with the antibiotic.

15.3.2 Hepatotoxicity

The first case of DILI associated with amoxicillin-clavulanate was reported in 1988 [27], and several additional case reports soon followed [26, 28, 29]. More recently, an Italian database of spontaneous reporting of suspected adverse drug reactions defined that the amoxicillin-clavulanate combination caused a higher incidence of DILI (4%) compared with amoxicillin alone (1%) [30]. The risk of DILI associated with amoxicillin-clavulanate was reported to be 1 in 10,000 patients treated with the drug [31, 32]. Similarly to flucloxacillin, the risk of hepatic injury increased with the patients' age and duration of treatment with amoxicillin-clavulanate [33]. Thomson et al. reported that patients over 55 years old had an odds ratio of developing DILI due to amoxicillin-clavulanate of 16.1 (95% CI = 2.9–88.9) compared with patients less than 30 years old [34]. The combination of advancing age and repeated intake of amoxicillin-clavulanate was reported to increase the risk of DILI to 1 in 1,000 patients [31]. Men had an increased risk compared to women in developing amoxicillin-clavulanate related DILI [34, 35].

15.3.3 Pharmacogenetics

The cellular mechanism(s) underlying DILI associated with amoxicillin-clavulanate is poorly understood. Several of the hypotheses concerning the pathophysiology of this adverse drug reaction involves drug metabolism. Firstly, patients who metabolize the drug differently compared to others or lack adequate protective mechanisms to neutralize reactive metabolites may develop DILI. Secondly, patients with immune systems that more readily recognize the neoantigens that were formed when the drug combinations' active metabolites interact with hepatocyte proteins may also develop DILI [5]. Preliminary studies have shown that amoxicillin-clavulanate associated hepatotoxicity, which is mainly cholestatic, is linked to *HLA-DRB1*1501* [36, 37], and the proposed immunological mechanism may be mediated by the HLA class II system. The HLA Class II antigens present peptides to the T-cell receptor of $CD4^+$ helper T lymphocytes, and this leads to the subsequent development of an immune response and caused stimulation of B cells and cytotoxic T cells [36]. Hautekeete et al. found that there was linkage disequilibrium

between *HLA-DRB1*1501* and *DRB5*0101*, as well as *DQB1*0602* [36]. O'Donohue et al. independently found that *HLA-DQA1*0102* was also in linkage disequilibrium with the other three alleles [37]. These researchers also reported no difference between patients who are homozygous, heterozygous, or negative for *HLA-DRB1*1501* and the severity/duration of jaundice, hepatic histology, or biochemical pattern of hepatic injury. However, the *HLA-DRB1*1501* haplotype conferred susceptibility to primary sclerosing cholangitis [37].

15.4 Conclusions

In summary, studies showed that there was an association between flucloxacillin-associated cholestatic hepatitis and *HLA-B*5701*, and a similar association was found between amoxicillin-clavulanate-associated cholestatic hepatitis and *HLA-DRB1*1501*. However, prospective screening for the presence of these SNPs will not be very useful due to the low prevalence of drug-associated cholestatic hepatitis. The genotyping test may be helpful in establishing diagnosis in jaundice patients with the suspected diagnosis of drug-induced cholestasis. However, this use of the genotyping needs to be prospectively studied to confirm such utilization.

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Chapter 16
Immunosuppressants Pharmacogenomics

Ping Wang

Keywords Immunosuppression • Transplantation

This chapter will focus on three immunosuppressants used for posttransplantation graft maintenance. These include the calcineurin inhibitors (CNIs), cyclosporine and tacrolimus, and the cell proliferation inhibitor sirolimus. These drugs share similar mechanism of action, metabolism pathway, and pharmacogenetics/pharmacogenomics. Other immunosuppressants will not be discussed here.

16.1 Clinical Indication and Mechanism of Action

Cyclosporine, tacrolimus (also named FK506), and sirolimus (also named rapamycin) are all used posttransplantation to suppress T-cell-mediated immune responses. They are frequently used in combination with steroids and antiproliferative agents such as azathioprine or mycophenolate mofetil. Tacrolimus has been shown to be 10–100 times more potent than cyclosporine [1, 2]. Both tacrolimus and sirolimus are used at much lower doses than cyclosporine. Two recent multisite randomized trials suggest that regimens containing low-dose cyclosporine or low-dose tacrolimus are safe and effective, and in the case of tacrolimus, even more advantageous for renal function, allograft survival, and acute rejection rates in renal transplant recipients than standard-dose regimens [3, 4]. The target trough levels of 50–100 ng/mL cyclosporine and 3–7 ng/mL tacrolimus in these studies are much lower than the traditionally suggested target trough ranges. See Wallemacq et al. [5] for proposed target tacrolimus trough concentration guidelines for kidney, heart, and liver transplantation by the 2007 European Consensus Conference on Tacrolimus Optimization.

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A.H.B.Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4_16,

Although chemically distinct, cyclosporine and tacrolimus both function at the same step in the immune activation cascade by blocking the serine/threonine phosphatase activity of calcineurin [6]. The binding of foreign antigens to receptors on the T cell surface triggers the activation of *ras* and the increase in the intracellular calcium concentration. Through the facilitation of calcium/calmodulin, the phosphatase activity of calcineurin is activated. Calcineurin then dephosphorylate NF-ATc, the cytosol subunit of the transcription factor NF-AT. Dephosphorylation triggers nuclear translocation of NF-ATc, which binds to NF-ATn and leads to transcription activation of cytokines and T-cell activation. Cyclosporine and tacrolimus form complexes with their respective immunophilins, cyclophilin and FKBP12, which further engage calcium/calmodulin/calcineurin to form a pentamer and inhibit the phosphatase activity of calcineurin, thereby blocking T-cell activation and cytokine production.

Sirolimus is structurally related to tacrolimus. It also binds to FKBP12, but inhibits mTOR (mammalian target of rapamycin), a key regulator of cell growth and proliferation. The cell cycle of various cell types are arrested in G1 phase as a result of mTOR inhibition [7]. The mechanism of action of the three drugs is depicted in Fig. 16.1.

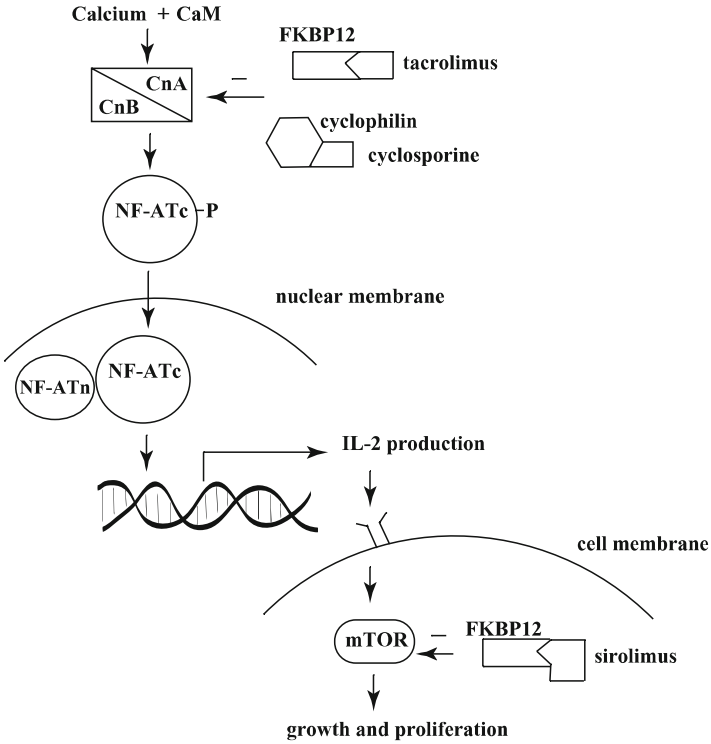

Fig. 16.1 Schematic drawing illustrating the mechanism of action for cyclosporine, tacrolimus, and sirolimus. CnA and CnB are the catalytic and regulatory subunits of calcineurin, respectively

16.2 Dosing Variability and Toxicities

Great interindividual and interethnic variability has been observed in the dose requirement of the CNIs and sirolimus [8, 9]. Because of the variability, it is a challenge in both clinical practice and clinical trials to achieve target concentration [10]. Oral bioavailability of CNIs in African Americans is 20–50% lower than in non-African Americans. Studies have shown that absorption of orally-administered immunosuppressants, rather than hepatic metabolism and clearance, is the main source of variability [9, 11]. The CNIs are substrates of the cytochrome P450 3A (CYP3A) enzymes, which are expressed in enterocytes and convert CNIs to their metabolites. Absorption is also regulated by the drug efflux pump P-glycoprotein, which is encoded by the *MDR1/ABCB1* gene and expressed on the apical membrane of enterocytes. After absorption, CNIs and metabolites are transported via the portal vein to the liver, where CYP3As are also expressed and hydroxylation and demethylation metabolism takes place. Some metabolites are excreted in the bile. The rest of the CNIs and metabolites enter systemic circulation. All three drugs are highly lipophilic and distribute primarily into red blood cells (90–95%), with minor fractions appearing in plasma, lymphocytes, and granulocytes. The pharmacokinetics of the CNIs is shown schematically in Fig. 16.2. The pathway for sirolimus is not as clearly elucidated, but is thought to be similar to that of CNIs.

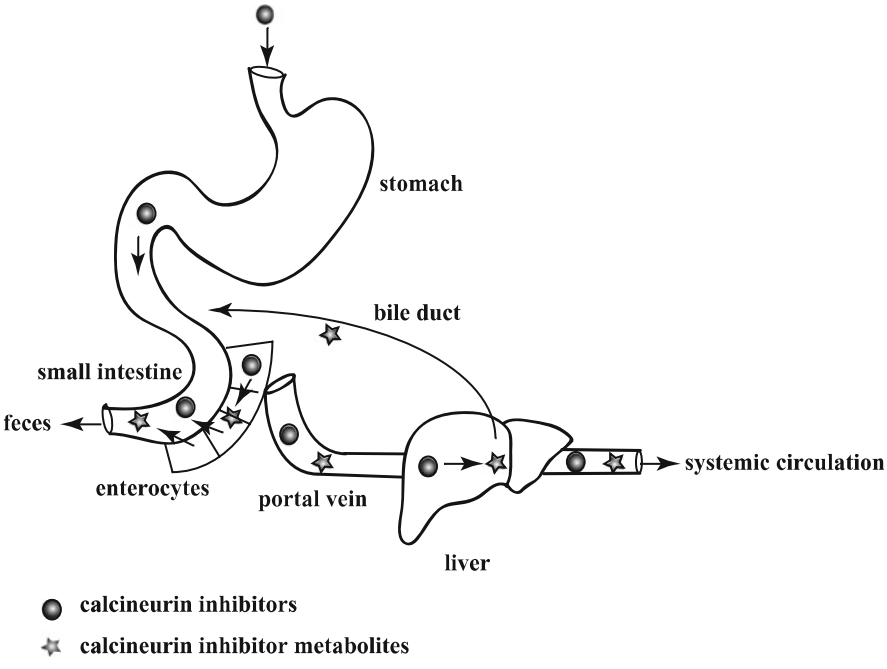

Fig. 16.2 Schematic drawing illustrating the pharmacokinetics of CNIs

The use of these immunosuppressants has resulted in dramatic improvements in the short-term graft survival rate. However, long-term outcomes did not improve to the same extent [12], especially in African Americans, who continue to have poor graft survival rates, shorter graft half-lives, delayed graft function, and increased risk for acute and chronic rejections. Part of this is due to the chronic graft dysfunction and immunosuppressants-associated side effects, attributable to the narrow therapeutic indices of these drugs. The toxicities of cyclosporine and tacrolimus are well-described, including nephrotoxicity, neurotoxicity, diabetes mellitus, hypertension, hyperlipidemia, and gastrointestinal disturbances. Both cyclosporine and tacrolimus cause renal damage, contributing to chronic graft dysfunction. Sirolimus has a different toxicity profile. When used together with cyclosporine or tacrolimus, sirolimus increases CNI exposure and synergizes with these drugs pharmacodynamically, resulting in reduction in the dose requirement of CNIs. This benefits renal function in the long term. However, sirolimus delays the clearance of circulating low-, intermediate-, and very low-density lipoproteins [13], which leads to hyperlipidemia being the most concerning side effect. A high rate of death due to cardiovascular diseases with well-functioning grafts is associated with sirolimus use [14, 15]. Due to serious drug-related toxicities, especially CNI-related nephrotoxicity, there has been great interest in minimizing or withdrawing CNI sometime after transplantation, while maintaining adequate immunosupression and low rejection rates. Two recent trials, the CAESAR Study and the ELITE-Symphony Study successfully demonstrated the feasibility and benefits of cyclosporine and tacrolimus reduction [3, 4]. However, it is also clear from the studies that complete withdrawal of CNI is associated with significantly higher incidence of biopsy-proven acute rejections [3]. For a review of CNI minimization, withdrawal, and avoidance trials, refer to Srinivas and Meier-Kriesche [16].

16.3 Therapeutic Drug Monitoring and Pharmacodynamic Monitoring

In current clinical practice, dosing of cyclosporine, tacrolimus, and sirolimus is guided by therapeutic drug monitoring (TDM). Although alternative strategies such as C2 monitoring have been advocated for cyclosporine [17] and improved clinical outcomes have been shown using such strategies [18], trough blood concentration (C0) is still the parameter most commonly measured. The goal is to maintain the whole blood trough concentration within a predefined therapeutic range, which is dependent on the transplanted organ, the time after transplantation, and the analytical method used to measure the drug. In reality, the target range also varies among different transplant centers.

The sample of choice for TDM is EDTA-anticoagulated whole blood, and the drugs need to be extracted before measurement. Commercial immunoassays are available for the monitoring of cyclosporine, tacrolimus, as well as sirolimus and

are widely used in clinical laboratories. These assays include the microparticle enzyme immunoassay (MEIA, Abbott Diagnostics, Abbott Park, IL), the fluorescence polarization immunoassay (FPIA, Abbott Diagnostics), the enzyme-multiplied immunoassay technique (EMIT, Dade Behring, Glasgow, DE and Abbott Diagnostics), the antibody-conjugated magnetic immunoassay (ACMIA, Dade Behring-Siemens, Deerfield, IL), the cloned enzyme donor immunoassay (CEDIA, Microgenics, Fremont, CA), and the chemiluminescence microparticle immunoassay (CMIA, Abbott Diagnostics,). An enzyme-linked immunosorbent assay (ELISA, the PRO-Trac assay, Diasorin, Stillwater, MN) did not gain wide use. In recent years, the use of laboratory-developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods that can simultaneously quantify several immunosuppressants in one run gradually increased. The antibodies used in the MEIA assays cross-react with some metabolites, some of which are biologically active. Therefore, immunoassays show positive biases compared with methods using LC-MS. The precision of the most widely used (MEIA) is poor at tacrolimus concentrations below 9 ng/mL [19]. In view of recent clinical trial results demonstrating benefits of decreased tacrolimus dose, the 2007 European Consensus Conference on Tacrolimus Optimization recommended that analytical methods measuring tacrolimus should have a limit of quantitation (LOQ) lower than 1 ng/mL [5]. For cyclosporine, the desired LOQ should be below 20 ng/mL [20]. Lab-developed assays using LC-MS/MS measure the parent immunosuppressants with high specificity and can achieve LOQ below the recommended levels. The CMIA assay has also been reported to conform to the recommendations [21–23]. For a comparison of the performance of current analytical methods for tacrolimus, refer to Wallemacq et al. [5].

Although monitoring of whole blood trough concentration and adjusting dose of CNIs and sirolimus according to the trough concentration has been standard clinical practice for many years, drug level can only be measured after dose is administered. Moreover, the correlation between drug dose and blood concentration is poor. It may take several months to achieve a stable dose using the "trial-and-error" dose adjustment approach. Methods that can help improve dosing of these drugs are being sought. These include research assays that measure the concentration of CNIs in lymphocytes and transplanted tissues. Studies have shown their correlation with acute rejections. However, it is unlikely that these assays will make their way into clinical practice for technical and turnaround time reasons. Pharmacodynamic monitoring has also been attempted by measuring pentamer formation, calcineurin phosphatase activity, IL-2 production, and T-cell cytometry and function. These assays are useful but are cumbersome to perform in clinical settings. A method gauging the global immune cell function has been approved by the Food and Drug Administration (FDA) for clinical use. This assay uses the increase in ATP concentration in CD4+ T cells as a marker of lymphocyte activation [24, 25]. However, the results do not correlate well with tacrolimus concentrations [26], and no data have been published relating the results to clinical outcomes.

16.4 Pharmacogenetics and Pharmacogenomics

The pharmacogenetic studies of cyclosporine, tacrolimus, and sirolimus have generally focused on CYP3A4, CYP3A5/CYP3AP1, and P-glycoprotein. Given that these proteins are expressed in small intestine, liver, kidney as well as other tissues, the genotypes of both the donor and the recipient need to be considered when relating pharmacogenetic results to transplant outcomes. The findings are summarized in Table 16.1.

The human *CYP3A* gene subfamily is located on chromosome 7 and consists of *CYP3A43*, *CYP3A4*, *CYP3A7*, and *CYP3A5*. CYP3A43 is only expressed at very low levels in some tissues and CYP3A7 is primarily a fetal enzyme.

The *CYP3A4*1B* allele is encoded by a single nucleotide polymorphism (SNP) (A392G) located in the 5′ promoter region of the gene. This polymorphism has been linked to more aggressive forms and advanced stages of prostate cancer, especially in African Americans [27, 28]. However, its functional significance is still a matter of debate. The **1B* allele is rare in the Asian population [29]. The effect of the **1B* allele on tacrolimus pharmacokinetics was shown in one study in kidney transplant recipients [30]. The association between *CYP3A4*1B* and cyclosporine reported in healthy subjects in one study [31] was not repeated in other studies involving healthy volunteers or renal transplant recipients [32, 33].

CYP3A5 contributes to at least half of the total hepatic CYP3A enzyme activity and is expressed abundantly in the small intestine [34]. It is the major enzyme responsible for the metabolism of CNIs and sirolimus.

The association between CYP3A5/CYP3AP1 and tacrolimus is more definite as it has been reported in numerous studies. *CYP3AP1* is a pseudogene that has a polymorphism G-44A that is in linkage disequilibrium with the **1/*3* polymorphism (A6986G) of *CYP3A5*. The **3* allele of *CYP3A5* causes cryptic splicing of the mRNA, which generates a prematurely terminated protein product at amino acid 109 [34]. Most studies to date have confirmed that the **3* allele results in decreased tacrolimus clearance, decreased dose requirement, and shorter time needed to achieve target trough concentration. Associations between the **3* allele and tacrolimus-related toxicities have also been reported (Table 16.1) [30, 35–51]. The *CYP3A5*6* allele results in deletion of exon 7 and loss of functional CYP3A5. The *CYP3A5 *7* allele has a T insertion between codons 345/346 and also results in a prematurely terminated protein product. Neither **6* nor **7* has been associated with tacrolimus dose requirement. Of note, the prevalence of *CYP3A5*3*, **6*, or **7* varies a lot with ethnicity. In Caucasians, 80% of individuals are **3* homozygotes, and 1 in 500 carry the **6* allele. In contrast, only 30% of African Americans are **3* homozygotes, and 3 in 20 carry the **6* allele [34, 52]. *CYP3A5*7* occurs with the frequency of 10–22% among African Americans, but is not found in Caucasians and Asians [53, 54].

Although cyclosporine is also mainly metabolized by CYP3A5, the association between CYP3A5 and cyclosporine pharmacokinetics or dose requirement is less consistent throughout the literature (Table 16.1). One hypothesis is that there is a

Table 16.1 Effects of *CYP3A4*, *CYP3A5*, and *MDR1* polymorphism on cyclosporine, tacrolimus, and sirolimus

	CYP3A4	CYP3A5	MDR1
Cyclosporine	Area under the curve (AUC)/dose ratio was affected by **1B* [31]; oral clearance was lower in **1B* carriers [76]; no association [32, 33]	No association [30, 76–79]; dose-adjusted trough concentrations were higher in **3* carriers[39]	No association [31, 33, 39, 76, 80, 81]; 3435T carriers had higher oral clearance [82], higher AUC [83]; 3435C carriers had higher dose [84]; C-G-C haplotype was associated with lower AUCs [29]; carriers of 1236C had lower dose-adjusted peak concentration and lower AUC [78]; 3435TT is a risk of nephrotoxicity [73]
Tacrolimus	**1B* carriers required more tacrolimus to reach target trough concentration [30]	**3* is associated with decreased tacrolimus clearance, decreased dose requirement, and shorter time to achieve target concentration [30, 35–51] and lower incidence of biopsy-proven nephrotoxicity in renal recipients [48], but higher incidence of nephrotoxicity in liver recipients [49]	No association between C3435T, G2677A/T, and dose [30, 38, 39, 42–44, 47, 85, 86]; 2677A/T [87, 88]or 3435C [35, 87, 88] required higher dose; 2677 SNP associated with tacrolimus-associated neurotoxicity [74]; less than three copies of T-129C, C3435T, and G2677A/T polymorphisms were associated with lower tacrolimus levels [70]
Sirolimus	**1B* carriers required higher dose [89]	No association [51]; **1* carriers had higher dose [89, 90] and higher clearance [91]	No association [51, 89]

drug absorption barrier that is saturated by the much higher dose of cyclosporine compared to tacrolimus or sirolimus [55].

The effect of *MDR1* genotypes or haplotypes on immunosuppressant pharmacokinetics is also controversial. Three genetic variants, including C1236T in exon 12, G2677A/T in exon 21, and C3435T in exon 26, are studied most extensively in the literature. These SNPs are linked with each other [56] and one study suggested that *MDR1* haplotype rather than genotypes of individual SNPs was more important with regard to pharmacogenetics [29]. However, there is controversy regarding whether these polymorphisms change either function or expression level of MDR1 [50, 56–58]. Both positive and negative findings have been reported for association between *MDR1* variants and CNIs (Table 16.1). One confounding factor for the *MDR1* association studies is the prevalence of diarrhea in the transplant population. Mycophenolate mofetil, which is often given to transplant recipients as an antiproliferative, as well as tacrolimus, can cause frequent diarrhea. Chronic diarrhea may in turn damage the intestinal epithelium and disrupt mucosal gene expression [59]. It has been reported that diarrhea can increase trough tacrolimus levels due to decreased intestinal P-glycoprotein activity [60, 61]. This confounds the association between *MDR1* genotype/haplotype and immunosuppressant dose.

The genes involved in the pharmacodynamics of immunosuppressants have not been studied extensively. One study used a single-strand conformational polymorphism assay with limited sensitivity to screen for polymorphic variations around the tacrolimus-FKBP12 binding sites in a relatively small population of Caucasians and did not detect any polymorphisms [62]. More recent studies have reported associations between genetic polymorphisms in the calcineurin gene and schizophrenia [63, 64].

A recent candidate gene association study examined the correlation between tacrolimus dose and polymorphisms in genes involved in pharmacokinetics and pharmacodynamics of tacrolimus [65]. Seven hundred and sixty-eight polymorphism in fifteen candidate genes were screened, and five *CYP3A4* and *CYP3A5* genetic variants in linkage disequilibrium emerged as significantly associated with stable tacrolimus dose. *CYP3A5 *3* was identified as the one with the most significant correlation, further pinning down the effect of this allele in tacrolimus pharmacogenomics.

Genetic variants in non-CYP450 genes have also been reported to impact CNI pharmacokinetics. For example, the C-25385T SNP of the pregnane X receptor gene has been reported to significantly influence tacrolimus clearance [66]. The pregnane X receptor is a nuclear receptor that is involved in the up-regulation of drug-metabolizing enzymes such as CYP450s and drug transporters in response to hormones and medications.

There are fewer studies focusing on the pharmacogenetics of sirolimus. Some indicated that *CYP3A4*1B* or *CYP3A5*1* carriers require higher sirolimus dose, while others found no correlation (Table 16.1). The inconsistency is at least partly due to the interaction between sirolimus and CNIs, which are sometimes coadministered. There was no association between sirolimus and *MDR1* genotype in these studies.

Large-scale SNP arrays and gene expression arrays have also been used to predict CNI-associated toxicities or to diagnose acute rejections and infections. One study identified eight SNPs that could be used to predict CNI-associated arrhythmia, ischemic heart disease, and heart failure in renal transplant recipients [67]. Using molecular signatures to diagnose rejections and infections (for example, [68, 69]) has the potential to avoid invasive biopsies in the future and helps to elucidate the biological mechanism underlying these processes.

16.5 Clinical Utility of Pharmacogenetic Testing

Although CNIs are double-edged swords in transplantation, it is clear from recent minimization/withdrawal studies that complete avoidance of CNIs is impossible at this stage. It is therefore important to improve the dosing strategy to maximize efficacy and minimize toxicities. Clinical pharmacogenetic testing may help to achieve this goal.

Most studies, except for one, did not find difference in incidence of acute rejections between carriers and noncarriers of the *CYP3A5*3* allele [30, 40, 70–72]. Some attribute this to TDM, suggesting that blood concentration monitoring is able to correct any genetic difference by reaching target trough concentration quickly. However, significant delays have been observed in reaching target concentration in *CYP3A5* expressers in the first 2 weeks after kidney transplantation [40]. In the same study, rejection episodes occurred earlier in *CYP3A5* expressers. The absence of any difference in acute rejection rates is likely due to the already low incidence of acute rejections in the first years after transplantation and the relatively small sample size in most studies. On the other hand, many reports indicate that donor or recipient genotypes of *CYP3A*s or *MDR1* may predict the risk of drug-related side effects, such as nephrotoxicity, neurotoxicity, hyperlipidemia, and hypertension [48, 49, 73, 74]. Therefore, although pharmacogenetics may not be able to further decrease the already-low incidence of acute rejection, it may help to identify patients with increased susceptibility for drug-related toxicities. For these patients, different immunosuppressive regimens or different target concentration may prove beneficial.

Another potential utility of pharmacogenetic testing is the quick prediction of the optimal initial and/or stable dose for an individual patient. One study developed an algorithm to predict the stable dose of tacrolimus based on *CYP3A5* genotype, demographics, and interacting comedications [65]. The idea is to shorten the time needed to achieve stable dose by having a prediction of what that dose should be from the very beginning of therapy. At least two prospective randomized trials are being carried out in Europe to investigate the benefits of dosing patients based on *CYP3A5* genotype. One study randomizes *CYP3A5* expressers to either a standard initial tacrolimus dose or a twofold higher initial dose [55]. The other study randomizes patients to either receive standard dose or to receive 75% of standard dose for *CYP3A5*1* carriers and 150% of standard dose for noncarriers [75].

Results of the latter study showed that a significantly higher proportion of patients reached target trough concentration 3 days after initiation of tacrolimus in the pharmacogenetics-guided group. In the same study, *CYP3A*1/*1* homozygotes were more likely to be underdosed, while *CYP3A5*3/*3* carriers were more likely to be overdosed in the nonpharmacogenetic group [75]. It is yet to be seen if clinical outcomes of these prospective studies are impacted by pharmacogenetics.

It is unlikely that pharmacogenetics of immunosuppressants will replace conventional TDM. The major advantage of pharmacogenetics is the knowledge of patient genotype before the advent of immunosuppression. Used together with TDM and possibly pharmacodynamic monitoring, pharmacogenetics may help to improve long-term transplant outcomes.

16.6 Clinical Pharmacogenomics Testing

No diagnostic assay has been approved by FDA for clinical genotyping of *CYP3A4, CYP3A5*, or *MDR1*. Genetic variants, including *CYP3A4 *1B*, *CYP3A5*3* and *MDR1* C1236T, C3435T, and G2677A/T, can be genotyped using methods such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or pyrosequencing. EDTA-anticoagulated whole blood is the preferred specimen, but epithelial cells from buccal swabs can also be used. Partial automation of testing is possible by using commercial kits (analyte specific reagents, ASRs) that can be run on automated analyzers (for example, the INFINITI analyzer, AutoGenomics, Carlsbad, CA). These assays need to be validated by clinical laboratories according to Clinical Laboratory Improvement Amendments (CLIA) requirements before they can be implemented clinically.

Since the major advantage of pharmacogenetics is the knowledge of genotype before starting therapy, genotyping results should be available to the clinicians before transplantation takes place. The turnaround time for the laboratory-developed genotyping methods is usually 24 h to several days. However, patients usually get onto the transplantation waiting list long before surgery, which leaves enough time for pharmacogenetic testing. An ideal time for such testing would be when the patient is worked up for pretransplantation HLA typing.

16.7 Cases

16.7.1 Case 1. A 6-Month Long "Trial-and-Error" Tacrolimus Dose Titration

A 34-year-old African American male received a living-donor kidney transplant with zero mismatch on 8/8/2006 due to end-stage renal disease secondary to dia-betes

mellitus and hypertension. Daclizumab was given as induction therapy. Based on a standard dosing protocol of 0.025 mg/kg tacrolimus every 12 h and a body weight of 105 kg, he was started on 2.5 mg tacrolimus every 12 h, coadministered with mycophenolate mofetil and prednisone starting from postoperative day 2. He was discharged on 8/12/2006, when his trough tacrolimus level was 12 ng/mL. The trough tacrolimus levels were then measured every time he came to a kidney clinic for checkup. Tacrolimus dose was adjusted according to the trough levels with a target concentration of 10–15 ng/mL in the first 6 months and 8–10 ng/mL in the next 6 months. The trough level and tacrolimus dose are plotted in Fig. 16.3. Due to the persistently low trough tacrolimus level, tacrolimus dose was elevated for several times. After the dose was doubled to 5 mg twice daily on 2/22/2007, the patient's trough level finally stayed relatively stable within the target range. The patient was maintained on the dose for the following months and had consistently good graft function. Genotyping for *CYP3A5* revealed that the patient was homozygous for the **1* allele.

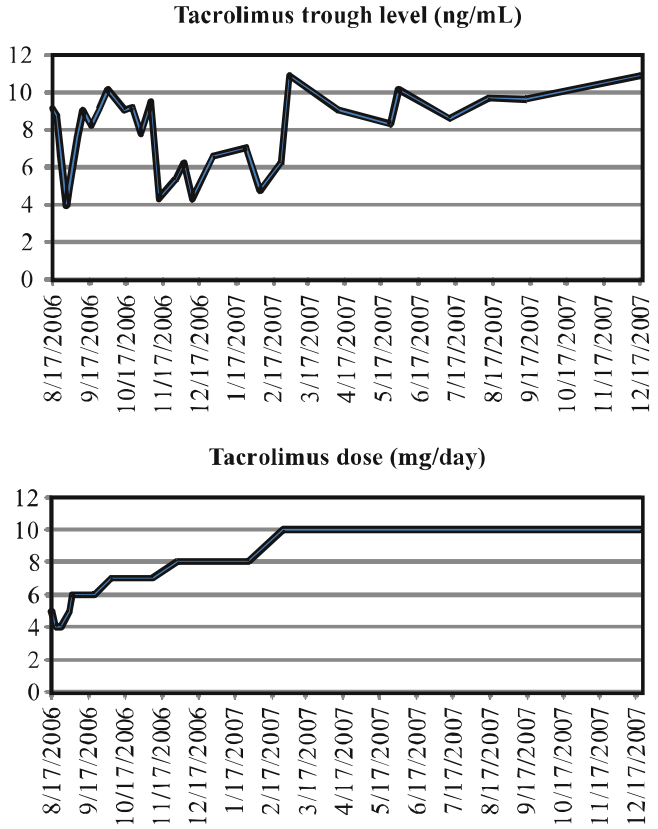

Fig. 16.3 Graphs showing tacrolimus trough concentration and dose over time for the patient in case 1

16.7.2 Case 2. Tremors and Chronic Allograft Dysfunction Due to Tacrolimus Overdose

A 20-year-old Caucasian male weighing 167 kg received a kidney transplant due to hypertension nephropathy. He was initiated on tacrolimus, mycophenolate mofetil, and prednisone. Based on his body weight and age, tacrolimus was initiated at 5 mg twice per day. He was discharged with a trough tacrolimus level of 13 ng/mL. At his checkup 2 weeks later, the tacrolimus trough level was above 20 ng/mL. Tacrolimus dose was reduced to 3.5 mg every 12 h. The patient reported to have tremors during the follow-up visits, and his serum creatinine concentration as well as urine protein/creatinine ratio gradually went up. Tacrolimus dose was further reduced to 2 mg every 12 h. The trough tacrolimus levels were mostly in the 10–15 ng/mL range, but occasionally went above 15 ng/mL. His serum creatinine levels fluctuated and he continued to have proteinuria and hypertension. Approximately 1 year after transplantation, kidney biopsy revealed tubulointerstitial fibrosis and scarring, tubular atrophy, and vascular sclerosis, without evidence of acute rejection. The histological changes were consistent with chronic allograft nephropathy. Genotyping revealed that the *CYP3A5* genotype of the patient was **3/*3*. Tacrolimus dose was further reduced to 1 mg twice per day. The patient's allograft function gradually improved after the dose reduction.

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A.H.B. Wu and K.-T.J. Yeo (eds.), *Pharmacogenomic Testing in Current Clinical Practice*, Molecular and Translational Medicine, DOI 10.1007/978-1-60761-283-4,