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Manfred Dietel
Volume Editor

Targeted Therapies in Cancer

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Targeted Therapies in Cancer

With 57 Figures and 25 Tables

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Preface

Over the past 50 years, the efficacy of cancer chemotherapy has improved considerably. Nonetheless, particularly in the metastatic situation, clinical outcome parameters, such as overall survival, have changed only modestly. This fact continues to represent a tremendous obligation for the scientific community and the pharmaceutical industry to develop new approaches in the fight against cancer.

The enormous progress in the knowledge of functional tumor cell biology, in particular of the molecular pathways and regulatory processes within tumor cells, has given researchers the chance to identify more key molecules as targets for specific drugs. These could exploit already-proven therapeutic strategies, such as humanized antibodies or small-molecule inhibitors directed against growth regulatory kinases, or completely novel approaches.

The current volume aims at providing an up-to-date report on the field of targeted cancer therapy. Contributions encompass basic and translational research as well as clinical experience and they outline functional mechanisms, diagnostic algorithms, predictive approaches, patient-related studies, and up-coming challenges in this stimulating but also controversial field of anticancer research.

Manfred Dietel
Peter Schlag

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Part III Prospects for Development

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Part I Prediction of Tumor Behavior

1 Beyond Typing and Grading: Target Analysis in Individualized Therapy as a New Challenge for Tumour Pathology

Hans H. Kreipe, Reinhard von Wasielewski

Recent Results in Cancer Research, Vol. 176

Abstract

In order to bring about its beneficial effects in oncology, targeted therapy depends on accurate target analysis. Whether cells of a tumour will be sensitive to a specific treatment is predicted by the detection of appropriate targets in cancer tissue by immunohistochemistry or molecular methods. In most instances this is performed by histopathologists. Reliability and reproducibility of tissue-based target analysis in histopathology require novel measures of quality assurance by internal and external controls. As a model for external quality assurance in targeted therapy an annual inter-laboratory trial has been set up in Germany applying tissue arrays with up to 60 mammary cancer samples which are tested by participants for expression of HER2/neu and steroid hormone receptors.

For decades, clinical cancer research was focussed on the study of empirical combinations of non-specific cytotoxic drugs. In recent years oncology has been witnessing a revolution sparked by targeted therapies, notably the chimeric monoclonal antibodies against surface molecules such as CD20 or epidermal growth factor receptor. Meanwhile almost all patients suffering from B cell lymphomas are treated with this mode of therapy (Cheson 2006). How does this revolution of therapy interfere with the classical function of histopathology to classify and to grade malignant neoplasm? Will morphological categories be replaced by a list or profile of markers which constitute potential targets for therapy? This will certainly not be the case, although the biological significance of lymphoma classification has to be reconsidered against the background of treatment response, which will potentially be more relevant than the spontaneous course of disease.

Whereas the task of typing and grading will still form the indispensable basis of cancer therapy, additional challenges with regard to reliability and reproducibility of target identification are awaiting modern pathology. Cancer ceases to be invincible—as has happened to antique heroes before—once its concealed vulnerable spot is known to the opponent. Already in the ancient myth, it required a person to uncover the secret and tell Paris to aim at Achilles‘ heel instead of his armpit (which would have been appropriate to wound Ajax). Similarly, there is good reason to believe that in the case of cancer the pathologist will be the one to reveal the secret and to guide the strike to the appropriate spot.

Targeted therapy requires the correct detection and identification of the potential molecule which might be suitable to interrupt the sustained proliferation of tumours (Savage and Antman 2002). For tissue-based analysis, immunohistochemistry provides a widely used tool to investigate cell-specific expression and to discriminate tumour cells from bystander cells. Specific mutations of potential target genes which lead to malignant transformation are best detected by polymerase chain reaction (PCR) or fluorescence in situ hybridization (FISH). Like immunohistochemistry, both methods can be applied to formalin-fixed and paraffin-embedded tissues.

In principle there are three different settings in which target molecules are detected in cancer cells:

1. Cancer cells retain some physiological properties of the normal counterpart and express

tissue-specific differentiation markers which may be used as targets (e.g. CD20, CD52, EGF-R, steroid hormone receptors).

2. Cancer cells overexpress certain molecules which could serve as targets, whereby the enhanced amount of protein provides the decisive alteration rather than the type of genetic modification (Her2/neu, c-kit, VEGF).
3. Genetic alterations induce the formation of novel non-physiological proteins which can be specifically targeted (c-kit, bcr-abl, FIP1L1-PDGFRα, EGF-R).

Whereas in the first two categories immunohistochemistry represents the method of choice, PCR and FISH dominate in the third. Potential target molecules and their detection are listed in Table 1.1.

There is little doubt that this list will grow and that pathologists will be confronted with the expanding task to specifically guide therapy by the detection of target molecules.

Are pathologists prepared to take over the task to guide targeted therapy, and are their methods reliable enough to prove the presence or absence of an appropriate target on a cancer cell? This is still an open question and a major cause for uncertainty with regard to modern therapies. In particular, quantitative parameters might be insufficiently reproducible. Principally, there are two ways to cope with this problem: centralization of diagnostics or standardization of diagnostics in a multicentric setting. In Germany pathologists have decided to opt for the second alternative; consequently, nation-wide trials for tissue-based markers in breast cancer have been set up (Rudiger et al. 2002, 2003).

Evaluation of potential targets for therapy is not a completely new challenge for pathologists, because immunohistochemical detection of oestrogen and progesterone expression in breast cancer has been used instead of the more inaccurate biochemical extract-based method of detection for more than two decades already. Therefore, the immunohistochemical detection of steroid hormone receptors has become the model system for instigating a new kind of interlaboratory trial. In these trials, tissue arrays are used for testing the reproducibility of oestrogen- and progesterone receptor assessment (Fig. 1.1;

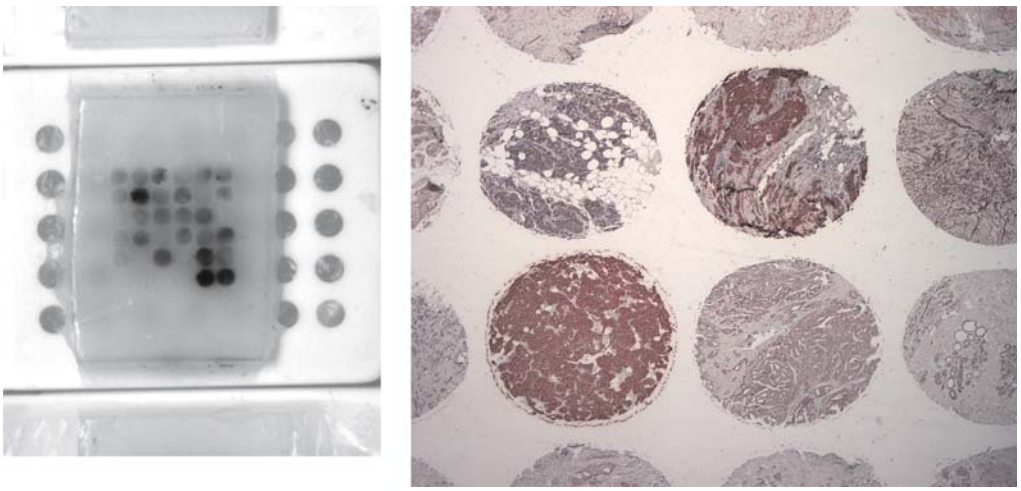

Fig. 1.1 Paraffin block of a tissue array (*left*) which is used in the immunohistochemical quality assurance trial. Thirty different tumour samples with defined target expression are assembled in one slide which has been stained for cytokeratin (*right*). Up to 200 slides can be produced from one tissue array assuring that all participants in the trial obtain almost identical material and that results among different laboratories become comparable. In the quality network of the German Society for Pathology and the Berufsverband Deutscher Pathologen („QuIP", www.ringversuch.de; http://www99.mh-hannover.de/institute/pathologie/dgp) quality assurance trials based on tissue arrays have been set up for different target molecules (ER, PR, Her2, c-kit)

Table 1.1 Potential targets for specific therapy

	Cancer type	Molecule; mode of activation	Detection	Specific drug
Steroid hormone receptors	Breast	Nuclear receptor; unknown	Immunohistochemistry	Tamoxifen, inhibitors of aromatase
Her2/neu	Breast, lung, thymus	Tyrosine kinase of membrane receptor type; gene amplification	Immunohistochemistry, FISH	Humanized monoclonal antibody against Her2 (Trastuzumab)
Epidermal growth factor receptor (EGF-R)	Colon, lung, glioma	Tyrosine kinase of membrane receptor type; gene amplification, point mutation	Immunohistochemistry	Humanized monoclonal antibody, gefitinib, erlotinib
c-kit (stem cell factor receptor)	Gastrointestinal stroma tumours, mastocytosis	Tyrosine kinase; point mutation	Immunohistochemistry, gene sequencing	Imatinib (not all types of mutation)
Bcr-abl	CML, ALL	Tyrosine kinase; fusion gene by chromosomal translocation	FISH, PCR	Imatinib
Platelet-derived growth factor receptor PDGFRα, -β	Gastrointestinal stroma tumours, chronic eosinophilic leukaemia	Tyrosine kinase; fusion gene by chromosomal translocation, point mutation	PCR, Immunohistochemistry	Imatinib
Vascular endothelial growth factor (VEGF)	Various types (e.g. colon)	Soluble cytokine; paracrine production	None	Humanized monoclonal antibody against VEGF (bevacizumab)
Vascular endothelial growth factor receptor VEGFR1–3	Various types (e.g. AML, kidney, glioma)	Tyrosine kinase of membrane receptor type; paracrine activation, amplification	Immunohistochemistry	Su11248, sunitinib
CD20	Normal and neoplastic B lymphocytes	Membrane glycoprotein; unknown function	Immunohistochemistry	Humanized monoclonal antibody (rituximab)
CD52	Normal and neoplastic T lymphocytes	GPI-anchored antigen	Immunohistochemistry	Humanized monoclonal antibody (alemtuzumab, Campath)

Mengel et al. 2003). The trials are conducted annually with up to 180 participating laboratories in Germany (http://www.ringversuch.de; http://www99.mh-hannover.de/institute/pathologie/dgp). With the help of tissue arrays it becomes possible for the first time to distribute several tumours among a high number of participating pathologists, whereby almost identical tumour areas will be studied by all participants. The first and the final slide sectioned from a tissue-array block have a distance of less than a millimetre. Furthermore, potential hazards by tumour het-

erogeneity are neutralized by the high number of samples that are encompassed by a tissue array. Only suitable material pre-tested for reproducibility enters the trials. The tissue samples are selected by a panel of three independent and experienced pathologists. Tissue microarray slides with 20–30 tissue spots either negative or expressing ER at low, medium or high levels are distributed among the participants. Whereas the majority of laboratories (>80%) usually succeed in demonstrating ER positivity in the medium- and high-expressing tissue spots, less than 50% of participants obtain the correct results in tissue samples with low expression (von Wasielewski et al. 2002). Poor interlaboratory agreement usually is based on insufficient retrieval efficacy or suboptimal immunohistochemistry. Interobserver variability, which has been tested in the trials by reviewing all immunostains, is in most instances not responsible for aberrant evaluations (Mengel et al. 2002).

Participants fill out an accompanying questionnaire in order to gather information about antigen retrieval and detection methods. To enable improvements in those institutions which scored below average, the correlation between the methods applied and performance in the trial is communicated to all participants .

The trials to assess interlaboratory reliability of steroid hormone receptor evaluation are conducted each year. Recently, a comparable trial was unleashed for Her2/neu, which yielded satisfactory results with regard to immunohistochemistry as well as FISH. Only with the help of interlaboratory trials will it be possible to guarantee the reliable and standardized detection of target molecules in a non-centralized system of histopathological services.

Interlaboratory trials may be necessary, but they are not sufficient to assure reproducibility of immunohistochemistry and FISH. Additional controls have to be included and performed such as on-slide controls. The latter can be achieved with cell lines embedded in paraffin and sliced like ordinary tissue sections. Cell lines are preferable to tissue samples because a defined content of target can be attributed to individual cell lines. On-slide controls enable the correct evaluation of immunostains, even when slides are retrieved from the archive. Furthermore, clinicians and pathologists have to collaborate in order to ensure that adequate and rapid fixation of cancer tissue samples exploited for target analysis will take place according to standardized procedures.

In conclusion, pathology is facing a new challenge and will integrate more closely with therapy planning in oncology than ever before. In order to guide tumour therapy beyond typing and grading, new methods and standards of quality assurance have to be established in histopathology.

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2 Oncogenic Signaling Pathways and Deregulated Target Genes

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Abstract

A limited number of somatic mutations are known to trigger malignancy via chronic activation of cellular signaling pathways. High-throughput analysis of gene expression in cancer cells has revealed a plethora of deregulated genes by far exceeding the number of known genetic alterations. Targeted tumor therapy takes advantage of deregulated signaling in cancer. However, cancer cells may evade successful therapy, e.g., targeting oncogenic kinases, due to mutation of the target protein or to resistance mechanisms acting downstream of or parallel to the therapeutic block. To improve therapy and molecular diagnostics, we need detailed information on the wiring of pathway components and targets that ultimately execute the malignant properties of advanced tumors. Here we review work on Ras-mediated signal transduction and Ras pathway-responsive targets. We introduce the concept of signal-regulated transcriptional modules comprising groups of target genes responding to individual branches of the pathway network. Furthermore, we discuss functional approaches based on RNA interference for elucidating critical nodes in oncogenic signaling and the targets essential for malignancy.

2.1 Diversity of Molecular Lesions Triggering Cancer Formation and of Correlative Information Obtained by Tumor Expression Profiling

Modeling the multistep conversion from normal to malignant cells has determined much of our current understanding on the molecular mechanisms underlying the formation of cancer. The cancer-inducing molecular events have been classified by dysfunction in as many as six different regulatory systems in which normal functions must be perturbed for malignancy to occur (Hanahan and Weinberg 2000; Khalil and Hill 2005). Such alterations are also present in multiple human tumors (Kinzler and Vogelstein 1996). Very recently, sequencing efforts aimed at assessing the entire coding region of tumor genomes revealed that the number of mutations is even higher (Sjoblom et al. 2006), suggesting that additional cellular pathways are disrupted and/or chronically activated. However, the functional consequences of the gene mutations, particularly their impact on cellular networks, are not understood. The increased complexity of cancer-associated alterations is also reflected in the results of high throughput analysis of gene expression. Numerous studies have revealed vast alterations of the transcriptomes in cancer cells affecting hundreds of genes (DeRisi et al. 1996; Perou et al. 1999; Zhang et al. 1997). Similarly complex sets of data are also beginning to emerge from proteome studies (Kolch et al. 2005). Distinct patterns of gene expression, called „molecular signatures," have been defined for classifying tumor types, tumor diagnosis, and even therapy response predictions (Golub et al. 1999; van't Veer et al. 2002). As is the case with very recently detected mutations, the relationship between the critical oncogenic lesions and the genetic program is poorly understood. It is generally accepted that the products of oncogenes and tumor suppressor genes together with the signaling pathways controlled by them impinge on the genetic program in cancer cells. Several reasons are

responsible for the current knowledge gap. First, gene expression profiling provides essentially correlative information, and causal relationships cannot be inferred from them. Second, the vast number and heterogeneity of transcriptional alterations precludes a detailed analysis of the roles of the corresponding gene products. Third, in view of the complexity of tumor phenotypes, the perturbed genetic program is very likely to encompass elements that act in concert rather than as individual factors, thus further complicating any kind of gene-based functional assessment. Fourth, a thorough analysis of gene functions requires multiple assays for modeling cancer cell-specific properties, e.g., proliferation and survival assays, motility, adhesion, and invasion tests, as well as in vivo assays based on tumor cell xenotransplantation.

Ras oncogenes were the earliest factors identified in human cancer that mediate cellular transformation. The products of Ras oncogenes serve as molecular switches in intracellular signal transduction and connect extracellular stimuli such as growth factors with the transcriptional machinery. The upstream and downstream components of the membrane/cytoplasmic system of signal transduction systems have been recognized as important players in models of cancer reconstruction and equally in human cancer. The membrane-bound receptor tyrosine kinases (RTKs), members of the Ras family of small GTases, and Raf signaling kinases are frequently activated in various types of cancers and exhibit oncogenic activity in appropriate cell culture-based and animal-based models (Hanahan and Weinberg 2000; Hann and Balmain 2001; Van Dyke and Jacks 2002). In view of their wide prevalence in human cancer, the receptors and their downstream effectors serve as therapeutic targets, and rational therapies aiming at blocking them have been developed (Sebolt-Leopold and Herrera 2004; Tibes et al. 2005; Fig. 2.1). Gene expression profiling studies using genetically engineered cell lines and transgenic mice have identified complex sets of transcriptional targets responding to activation of RTKs (Fambrough et al. 1999), Ras (Zuber et al. 2000), and Raf (Schulze et al. 2001) either early or in a delayed fashion. The responder genes are prime candidates for systematically assessing the individual contribution to the transformed state in the cellular models in which they were recovered. Moreover, functional studies are thought to be decisive as to whether these genes are essential for malignancy to occur or rather are deregulated as a consequence of the malignant process. Both groups of genes potentially represent important biological markers for tumor diagnosis and therapy.

2.2 Toward Determining the Rate-Limiting Steps of Cancer Cell Growth for Improving Cancer Diagnosis and Therapy

Determining the rate-limiting pathways triggering malignant growth in individual tumors is an important prerequisite for successful targeted therapy. For example, membrane-bound, overexpressed RTKs provide a rational target (Fig. 2.2). If oncogenic activity of the RTK can be blocked by a therapeutic antibody or small drug, malignancy is likely to be counteracted effectively. Furthermore, if targeting of RTK provides a cytotoxic effect, the tumor cell population is likely to be eliminated (Gschwind et al. 2004). However, tumor cells will evade this therapeutic effect in cases in which the RTK expression fails to be rate-limiting to either the tumor or a subpopulation of the primary tumor. In such cases, mutations downstream of the RTK may allow unconstrained cancer cell proliferation. Alternatively, tumor cells may become addicted to yet another signaling pathway due to its aberrant activation or crosstalk (Fig. 2.2). Mutations in the signaling network occurring downstream of the targeted signaling molecules may preclude successful therapy. For example, targeting of the RTK R_1, schematically shown in Fig. 2.2, will be without effect if effectors E_1, E_2, or others trigger malignancy. As a consequence, molecular diagnostic procedures need to be established that will detect such genetic alterations, preferably also in heterogeneous cancer cell populations. While alternate activating mutations in a single signal transduction pathway can overcome therapeutic blocks, the complexity of downstream events further complicates the diagnostic and therapeutic problem.

Several levels of the signal transduction network downstream of the membrane-bound and cytosolic elements can be distinguished. The first

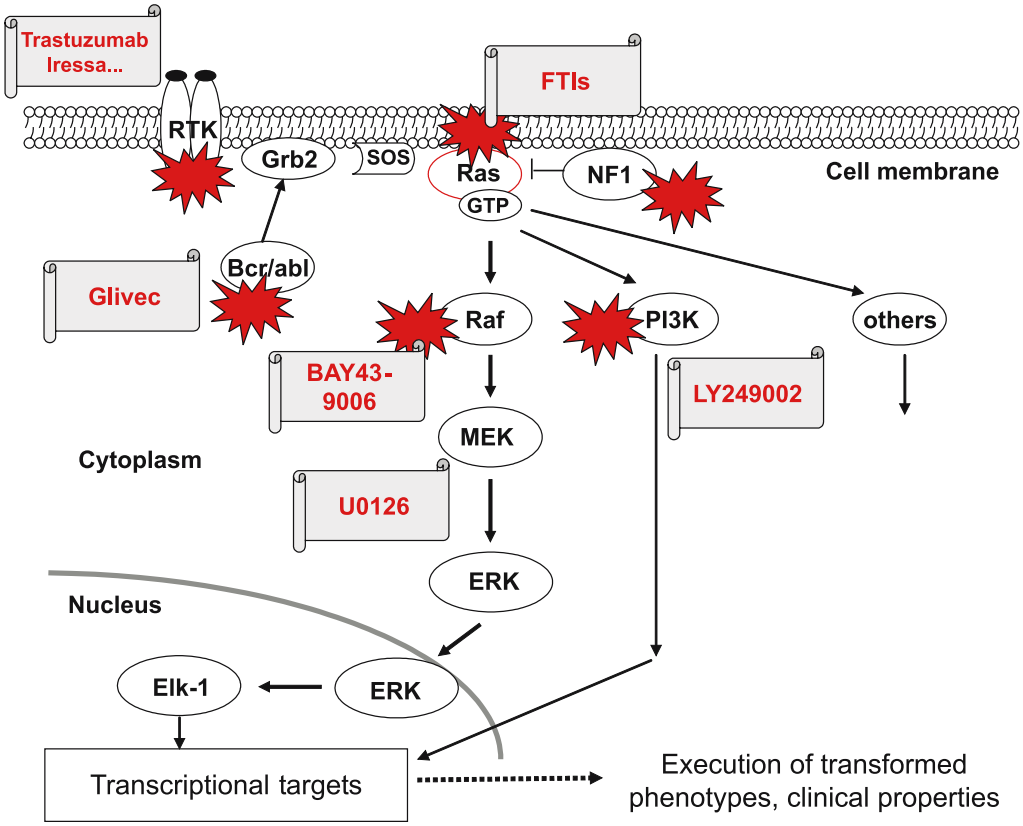

Fig. 2.1 RTK/RAS-mediated signaling and lesion-specific inhibitors. Schematic and highly simplified representation of the receptor tyrosine kinase/Ras signaling network connecting extracellular stimuli (growth factor stimulation) with gene expression via cytoplasmic signaling kinases. Components of the pathway: *RTK*, receptor tyrosine kinase (e.g., epidermal growth factor receptor, EGFR); *Grb2*, growth factor receptor-bound protein 2; *Sos*, Son-of-sevenless Drosophila homolog, guanine nucleotide exchange factor; *Ras-GTP*, Ras oncoprotein (active form) bound to GTP; *Raf*, v-Raf-1 murine leukemia viral oncogene homolog 1, mitogen-activated protein kinase kinase kinase; *Mek*, mitogen-activated protein kinase kinase; *Erk*, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK); *Elk-1*, ets-2 family transcriptional regulator; *PI3K*, phosphatidylinositol-3-kinase. Other effectors: RAS-GTP is known to bind to more than 20 different effector proteins depending on cell type (not shown). *Explosions* highlight components of the pathway that have frequently sustained genetic alterations and mutations in cancer. Lesion-specific inhibitors (therapeutic antibodies or small drugs) used in clinical and experimental cancer comprise: Trastuzumab, targeting the receptor tyrosine kinase HER2; Iressa, targeting EGFR; farnesyl transferase inhibitors (FTI), targeting HRAS and other farnesylated proteins; Glivec (STI 571), targeting the bcr-abl fusion kinase; BAY43-9006, Raf inhibitor; U0126, Mek inhibitor; LY294002, PI3K inhibitor

level comprises transcription factors that are rapidly and transiently activated (phosphorylated) by upstream effectors and generate the immediate-early response to exogenous stimulation, e.g., by growth factors (Almendral et al. 1988). Activation of these factors does not require new protein synthesis. The second level consists of factors that are activated after a delay, require new protein synthesis, and require immediate-early transcription factor activity (Lanahan et al. 1992). Some of them act as transcription factors as well, thus further increasing the complexity of the overall transcriptional response. Tumor cell populations consist, at least in part, of cycling

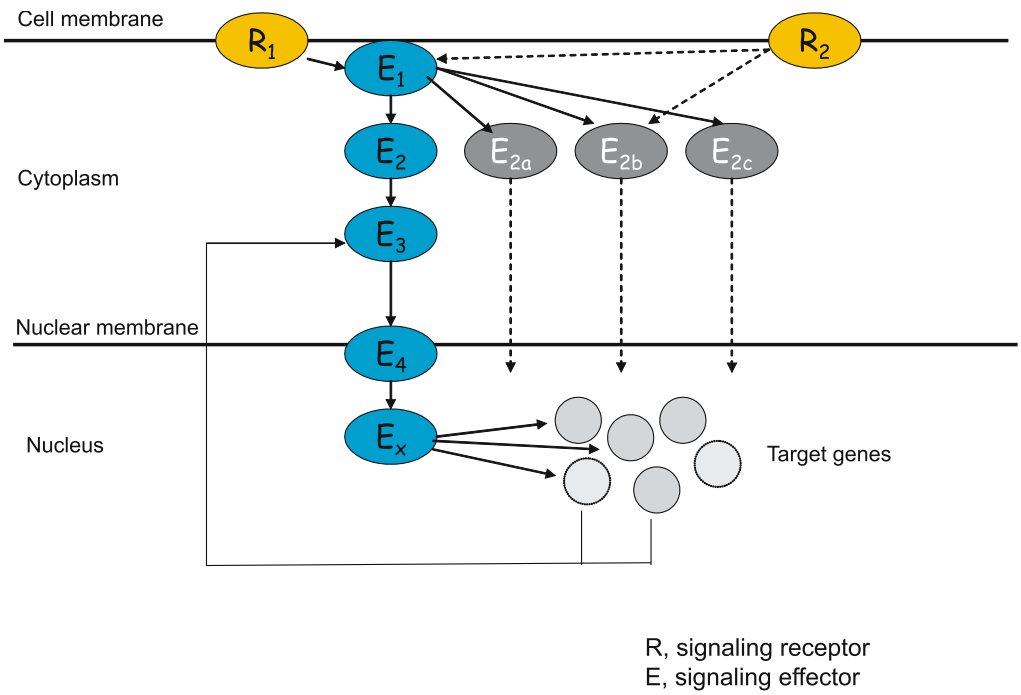

Fig. 2.2 General model of cellular signaling from the periphery to the nucleus and implications for molecular diagnosis and targeted therapy. The receptor R1, located in the cell membrane and stimulated by ligand, signals to the effector E1, located at the inner face of the membrane. Alternatively, E1 may be activated by R2. E1 interacts with its predominant substrate E2 and with other effectors (E2a, E2b, E2c, etc.). E2 interacts with activated E3; downstream effectors are subsequently activated (E4, Ex). Ex translocates into the nucleus and directly or indirectly stimulates the expression of target genes (*dark gray circles*) or represses target gene transcription (*light gray circles*). The products of target genes may be involved in feedback mechanisms that impinge on cytoplasmic signaling (*arrows*). The alternative effectors, E2a, E2b, and E2c, may stimulate transcription via other signaling cascades (not shown). See text for more details

cells. It can be speculated that the entire tumor cell population is not characterized by frequent transitions from the quiescent state to the proliferating state. This transition reflects an early step in tumorigenesis and most often an experimental condition that has proved to be instrumental for the identification of proliferation-associated genes and their regulators. Therefore, differentially expressed factors, upregulated and downregulated in tumors, of which the steady-state messenger RNA (mRNA) levels are consistently altered relative to normal cells, provide a third, decisive level of signaling response. The fourth and last level encompasses factors that are regulated in response to upper level factor activity and ultimately execute the properties of tumor cells. Notably, transcription factors or signaling molecules deregulated permanently, are capable of exerting feedback control over the primary signaling events and thus have to be taken into account as regulators or modifiers of oncogenic signal transduction (represented as target genes, Fig. 2.2). Detailed mechanistic insights into the function and relationships of all these factors in tumorigenesis are needed. These insights are only evident through a systems biological perspective.

To proceed toward the goal of developing such a perspective and to ultimately understand tumor formation and clinical tumor behavior at an individualized level, well-defined model systems triggered by well-established oncogenes

are indispensable. These are instrumental for cataloguing factors involved in tumorigenesis, to define the critical nodes of their regulation, and to assess their phenotypic contribution and precise function. Here we discuss the results of transcriptomic profiling of a model system for ovarian cancer and the strategies for elucidating the function of signaling target genes by RNA interference (RNAi). This model serves as a paradigm for better understanding the genome-wide impact of a common oncogenic pathway in the tumorigenic conversion of epithelial cells.

2.3 Effects of Oncogenic KRAS Signaling on the Genetic Program of Surface Ovarian Epithelial Cells

The precursor cells predominantly affected by genetic damage to any of the RAS isoforms, HRAS, KRAS, and NRAS, give rise to carcinomas. Therefore, we analyzed RAS-induced transcriptional alterations in transformed epithelial cells relative to their phenotypically normal counterparts. We chose to analyze the transcriptional program mediated by expression of mutated KRAS in a rat cell culture model of ovarian surface epithelium (ROSE). Immortalized rodent cells, unlike human cells (Hahn et al. 1999), can be transformed by RAS oncogenes in one step. To identify differentially expressed genes associated with KRAS-induced transformation, we chose subtractive suppression hybridization (SSH), a highly efficient and sensitive PCR-based technique independent of pre-selected gene sets represented on microarrays (Diatchenko et al. 1996; Zuber et al. 2000). The immortalized ROSE 199 cell line, originating from the continuous passage of primary rat ovarian surface epithelial cells, expresses epithelial and mesenchymal characteristics, does not transform spontaneously in vitro, and exhibits normal $p21^{Waf1}$ and very low p53 protein levels (Adams and Auersperg 1981, 1985). When cultured densely, ROSE 199 cells form multilayers resembling histologically serous papillary cystadenomas of borderline malignancy. Ras-transformed ROSE cells represent a late stage in ovarian transformation (Auersperg et al. 1999). We generated a stable KRAS-transformed derivative of ROSE 199 cells, designated ROSE A2/5. These cells exhibit epithelial-mesenchymal transition (EMT), anchorage-independent proliferation, and elevated levels of phosphorylated $p42/44^{ERK}$ (Tchernitsa et al. 2004). Stable KRAS-transfectants were particularly useful for establishing a catalog of deregulated target genes, indicative of both primary and secondary alterations related to the transformed phenotype. Although conditional oncogene expression is thought to exclusively detect the immediate effects of signaling (Schulze et al. 2001), the transcriptional response may be obscured by rapidly induced chromosomal abnormalities as well as transient genomic instability (Denko et al. 1994; Saavedra et al. 2000). Despite these potential caveats, we were able to show that the key features of mRNA expression profiles in conditionally HRAS-transformed fibroblasts significantly mimic those in stable transfectants (Zuber et al. 2000), although systematic time-resolved analysis partially revealed more complex patterns of target gene regulation (Tchernitsa et al. 2006a). To avoid transcriptional alterations reflecting differences of density-inhibited versus proliferative cells and the immediate effects of growth factor stimulation (Fambrough et al. 1999; Iyer et al. 1999; Zavadil et al. 2001), we strictly maintained ROSE 199 and A2/5 cells in exponential growth phase prior to contrasting their mRNA expression profiles.

We recovered fragments of differentially expressed genes from two subtracted complementary DNA (cDNA) libraries. In the forward subtraction, we mixed tester cDNA prepared from ROSE 199 mRNA, digested with the restriction enzyme RsaI, and ligated to adapter sequences with excess driver cDNA synthesized from KRAS-transformed A2/5 mRNA. Sequences recovered by PCR using adapter primers represent genes expressed in normal ROSE cells and downregulated in the KRAS-expressing derivative. In the reverse subtraction, we permutated tester and driver cDNAs and obtained sequences upregulated or expressed de novo in A2/5 cells. After cloning of subtracted PCR products, we determined the nucleotide sequence of 1,070 cDNAs and identified 568 individual gene sequences. Differential expression of related mRNA was confirmed by reverse northern analysis (von Stein et al. 1997) and by conventional Northern

blot analysis (Tchernitsa et al. 2004). Overall, we identified 192 expressed genes, 140 expressed sequence tags without known function at the time of analysis, and 35 sequences without a match in public sequence databases. About 20% of the transcriptional changes had been independently identified in a cell culture model of normal and HRAS-transformed fibroblasts (Zuber et al. 2000).

Stable KRAS-expression in the ROSE model of ovarian carcinoma stimulated transcription of genes capable of controlling cell signaling, gene activity, antioncogenic fail-safe programs, proteolysis, angiogenesis, invasion, and metastasis. For example, the matrix metalloproteases MMP1, MMP3, and MMP10, the small GTPase RhoC, and the glycoprotein CD44 featured prominently. The most prominent and perhaps unexpected feature of KRAS-transformed ROSE cells was the complete downregulation of genes capable of adversely affecting growth factor-induced signal transduction, transformation, and tumor progression (Table 2.1). The reduction of mRNA expression below the detection limit of highly sensitive Northern hybridizations indicated total functional loss of encoded targets. For ten of the downregulated KRAS-responsive genes with a putative transformation-suppressive role, functional impairment had been previously shown in ovarian cancer. Moreover, the remaining genes of this group were implicated as negative growth regulators in other types of cancer. However, their role in the neoplastic transformation and progression of the ovarian epithelium is yet unknown. Overall, the transcriptional profile of KRAS-transformed epithelial cells supports the notion that a cellular oncogene, in the absence of functional antioncogenic fail-safe mechanisms, can affect the regulation of multiple target genes to ultimately execute transformation and tumor progression (Bernards and Weinberg 2002). Complete downregulation very likely equals the phenotypic effects caused by disruptive target gene mutations (Sager 1997).

Of note, while we present evidence for a large-scale transcriptional downregulation of RAS-responsive genes, target gene inactivation may occur also at the level of protein stability (Sears et

Table 2.1 Transformation-suppressive genes downregulated in KRAS-transformed ROSE cells. (The original gene list is published in Tchernitsa et al. 2004. The functional characterization of transformation-suppressing genes listed here was mostly performed by forced expression in transformed fibroblasts. Growth-suppressive activity has not been demonstrated in ovarian epithelial cells, with exceptions)

Target gene	Gene product	Expression impaired in	Function	Reference(s)
HTS1/ST5	HeLa tumor suppressor	HeLa cells	Suppresses MAPK/ERK2 activation in response to EGF	Majidi et al. 2000
TGFβIIR	TGF-β receptor	Progressed carcinomas	Mediates growth inhibition by TGF-β	Evangelou et al. 2000; Paterson et al. 2001
TSC-36	Follistatin-related protein precursor	Oncogene-transformed cells	Causes growth arrest and inhibits invasion	Johnston et al. 2000; Sumitomo et al. 2000
Gas-1	Growth arrest-specific protein	Proliferating cells	Controls serum-dependent transition from G_0- to S-phase	Delsal et al. 1992, 1994
Cdh1	E-cadherin	Metastatic ovarian carcinoma	Suppresses invasion	Auersperg et al. 1999; Boyer et al. 2000
Cdh3	P-cadherin		Like E-cadherin	Lewis et al. 1994

Table 2.1 *(continued)*

Target gene	Gene product	Expression impaired in	Function	Reference(s)
Timp2	Tissue inhibitor of metalloproteases 2	Mucinous ovarian borderline tumors; Oncogene-transformed cells	Reduces tumor growth and invasion, inhibits MMPs	Furuya et al. 2000; Giunciuglio et al. 1995; Imren et al. 1996
Col1A1	Collagen α1 type 1	RAS-transformed cells	Cytoskeletal tumor suppressors	Slack et al. 1992, 1995; Travers et al. 1996
Col3A1	Collagen α1 type III			
Col4A1	Collagen α1 type IV			
Pcol3A1	Pro α1 coll. type III			
Parva	α-Parvin, related to α-actinin		Cytoskeletal tumor suppressor	Glück et al. 1993; Olski et al. 2001
Tpm2	Tropomyosin α2	Ovarian carcinoma, many transformed cell lines	Establish normal growth control in transformed cells	Alaiya et al. 1997; Gimona et al. 1996
Tpm3	Tropomyosin α3			
Tpm5a	Tropomyosin α5a			
Tpm5b	Tropomyosin α5b			
Lox	Lysyl oxidase	Many tumors, oncogene-transformed cells	Transformation suppressor (see text)	Giampuzzi et al. 2001
Gja1	Connexin 43	Adenocarcinomas, transformed cell lines	Controls gap-junctional communication in ovarian epithelial cells	Hanna et al. 1999; Umhauer et al. 2000
Thbs1	Thrombospondin 1	Downregulated in Ras-transformed cells	Inhibits angiogenesis	Alvarez et al. 2001; Zabrenetzky et al. 1994
Lot-1	„lost in ovarian tumors", zinc finger protein	Ovarian tumors	Candidate tumor suppressor	Abdollahi et al. 1997; Abdollahi et al. 1999
WT1	Wilms tumor suppressor	Papillary serous carcinomas of the peritoneum, advanced ovarian carcinoma	Suppresses Ras-induced transformation	Luo et al. 1995; Schorge et al. 2000
Sparc	Osteonectin, extracellular protein	Ovarian carcinoma	Suppressive activity in ovarian epithelial cells	Mok et al. 1996
IP6K2	Inositol hexakis-phosphate kinase 2	Ovarian carcinoma	Interferon-regulated, growth-suppressive, and anti-apoptotic	Morrison et al. 2001
NO3/DAN		Src-transformed fibroblasts	Candidate tumor suppressor	Ozaki and Sakiyama 1994
Arpp-19	Highly homologous to C4-2	Brain tumors	Candidate tumor suppressor	Sehgal et al. 1997

EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor

al. 2000), posttranslational modification (Bonni et al. 1999; Rivedal and Opsahl 2001), and protein degradation (Saha et al. 2001). The cellular targets affected by RAS-mediated transcriptional and posttranscriptional deregulation may be identical. For example, intercellular gap-junctional communication in ovarian epithelial cells can be perturbed by downregulation of connexin 43 transcription and by mitogen-activated protein kinase kinase (Mek)-dependent phosphorylation of the protein (Rivedal and Opsahl 2001). Oncogenic Ras is capable of subverting transforming growth factor (TGF)-β signaling, which functions to constrain cellular growth, in two different ways: by downregulating TGF-β receptor expression (Tchernitsa et al. 2004) and by degrading the tumor suppressor Smad4, a downstream effector protein (Saha et al. 2001).

KRAS mutations are rare in serous ovarian tumors, but frequent in ovarian tumors of the mucinous type, including cystadenomas, tumors of borderline malignancy, and carcinomas (Cuatrecasas et al. 1997; Enomoto et al. 1991; Ichikawa et al. 1994; Mok et al. 1993). The relevance of the ROSE model system for human ovarian cancer is emphasized by the finding that the RAS signaling pathway may be alternatively activated by mechanisms not involving genetic damage to KRAS, e.g., by enhanced post-receptor signaling as observed in ovarian cancer cell lines (Patton et al. 1998). Alternatively, alterations in factors capable of regulating RAS function (van Engeland et al. 2002) and activating mutations in the downstream effector B-Raf, shown in many tumors including ovarian carcinomas, can possibly result in equal deregulation of target gene expression (Davies et al. 2002). The results of KRAS-related transcriptional profiling in ROSE cells were compared with those obtained in a selected microarray-based analysis of differential gene expression in ovarian epithelial malignancies (Welsh et al. 2001). To obtain carcinoma-specific gene signatures, Welsh et al. used whole ovary-derived RNA rather than ovarian epithelium RNA. Of 29 genes, 14 (48%) underexpressed in serous papillary ovarian carcinomas were also transcribed in normal ROSE cells and transcriptionally repressed in KRAS-transformed ROSE A2/5 cells. Of 40 genes, 26 (65%) overexpressed in ovarian carcinomas relative to normal ovaries were transcriptionally stimulated in transformed ROSE cells. Although the data published by Welsh and colleagues may not be representative for all types of ovarian cancers, it is tempting to speculate that many of the commonly recovered targets represent a RAS-responsive gene signature in tumors. The decisive role of RAS signaling in epithelial transformation is further underlined by cancer reconstruction experiments based on the sequential activation of telomerase (hTERT) and the introduction of SV40 large tumor antigen—causing inactivation of TP53 and pRB tumor suppressors—and mutated HRAS into human embryonic kidney cells (HEK) and mammary epithelial cells (HMEC) (Hahn et al. 1999).

2.4 Detecting Regulatory Principles in Complex Gene Signatures as a Prerequisite for the Functional Analysis of Critical Target Genes

In view of the large number of deregulated targets, we sought to narrow down the number of targets related to executing transformed phenotypes. Usually, co-expression is interpreted as an indication of similar function (Eisen et al. 1998), but the diversity of gene products precluded a classification into functionally related groups that would aid in prioritization of targets for subsequent functional analysis. However, a clue to target gene functionality was provided by defining subsets of RAS-responsive genes related to individual branches of the RAS signaling pathway and to distinct phenotypic characteristics. Subsets of target genes were sensitive toward the inhibition of effector kinases, suggesting target organization in signal-regulated transcriptional modules (SITMs) (Tchernitsa et al. 2004). When we blocked the Raf/Mek/extracellular signal-regulated kinase (Erk) pathway in KRAS-transformed ROSE cells with the Mek inhibitor PD 98059, 58 targets, identified to be deregulated in A2/5 cells, exhibited mRNA levels indistinguishable from those in normal ROSE 199 cells, while 40 genes reached pretransformation mRNA levels only partially. This means that pretransformation RNA levels were obtained despite the continuous expression of the KRAS oncogene. Most importantly, the reversion of transcriptional alterations was coupled with the reversal of EMT and loss of anchorage

independent proliferation. This result indicated that the Mek/Erk-sensitive gene set formed a regulatory module (designated Erk module 1 for upregulated genes and Erk-module 2 for downregulated genes) and assigned a direct functional role of these modules in the control of neoplastic phenotypes. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY 249002 did not significantly affect the transformed characteristics of A2/5 cells, suggesting that the transcriptional changes dependent on the PI3K pathway are irrelevant or neutral for the analyzed phenotypes. The PI3K-module comprises 27 upregulated targets, as indicated by the restoration of transcription levels similar to the pretransformation state in response to inhibition of the PI3K pathway by LY 249002. Target gene repression by the PI3K pathway did not occur. Possibly, the signal-dependent regulators of the response were insensitive toward the experimental conditions used in ROSE A2/5 cells, or the lack of effects on repressed targets caused by PI3K inhibition was due to crosstalk between Mek/Erk and PI3K effector kinase signaling (Zimmermann and Moelling 1999). Mek signaling co-regulated 19 genes grouped in the PI3K module. This allowed for the further narrowing down of the number of critical targets in Erk modules 1 and 2: Execution of EMT and anchorage independence were mainly achieved by Mek/Erk signaling in conjunction with the deregulation of 79 target genes.

The target genes whose expression was unaffected by inhibition of Mek- and PI3K-signaling are involved in functions related to various aspects of growth control and tumor progression. Representative examples are the metastasis-enhancing GTPase RhoC, the negative growth regulators and tumor suppressor genes *TSC36*, *Gas1*, *E-cadherin*, *WT1*, *TGF-βII* receptor, *Cx43*, and four tropomyosin isoforms. The signaling pathways downstream of RAS involved in their deregulation are still unknown.

2.5 How Do Differentially Expressed Genes Contribute to KRAS-Mediated Transformation?

The inhibitor studies are helpful in narrowing down the number of target genes responsible for phenotypic properties of KRAS-expressing ovarian epithelial cells. However, they fall short of proving any causal relationships between individual deregulated genes and cellular phenotypes. The conventional way for assessing the functional role of KRAS pathway-responsive genes would be the forced expression of given candidates in appropriate recipient cells. Blocking the transformed phenotype in A2/5 cells or inducing certain aspects of transformation in normal ROSE cells could be used as a phenotypic readout. However, the expression levels observed in the ROSE A2/5 cell system are difficult to mimic in gene transfer experiments. Therefore, we decided to use the recently described technique of RNAi in mammalian cells (Elbashir et al. 2001) for assessing the functional role of differentially regulated KRAS pathway-targets.

RNAi describes a mechanism of posttranscriptional gene silencing in which double-stranded RNA (dsRNA), complementary to target mRNA, mediates its destruction (Fig. 2.3). Target mRNA degradation is catalyzed by the RNA-induced silencing complex (RISC), a multiprotein complex able to recognize dsRNA and cognate mRNA. This results in the reduction of the encoded protein and partial or complete loss of gene function (Paddison and Hannon 2002; Tijsterman et al. 2002). First detected in nematodes and plants, RNAi serves as a defense mechanism for the protection of cells against viral genomes. In the mean time, RNAi was recognized as a powerful genetic tool, permitting genome-wide RNAi screens of gene functions in *Caenorhabditis elegans* (Kamath et al. 2003). Mammalian genes can be effectively silenced by virtue of incorporation of short 19–21 nucleotide dsRNA into somatic cells (Caplen et al. 2001; Elbashir et al. 2001). This approach extended earlier reports that had suggested sequence-specific gene silencing after injection of 500 nucleotide dsRNA into mouse embryos and embryonal cell lines (Paddison and Hannon 2002). Unlike in invertebrates and plants, however, dsRNA more than 30 nucleotides in length provoked antiviral interferon-responses in somatic mammalian cells and a generalized repression of translation and cell death, even if recipient cells were devoid of interferon pathway effector proteins such as dsRNA-dependent protein kinase (PKR) and interferon-regulatory factor 1 (IRF1). Short dsRNAs were able to overcome nonspecific responses. The siRNAs can

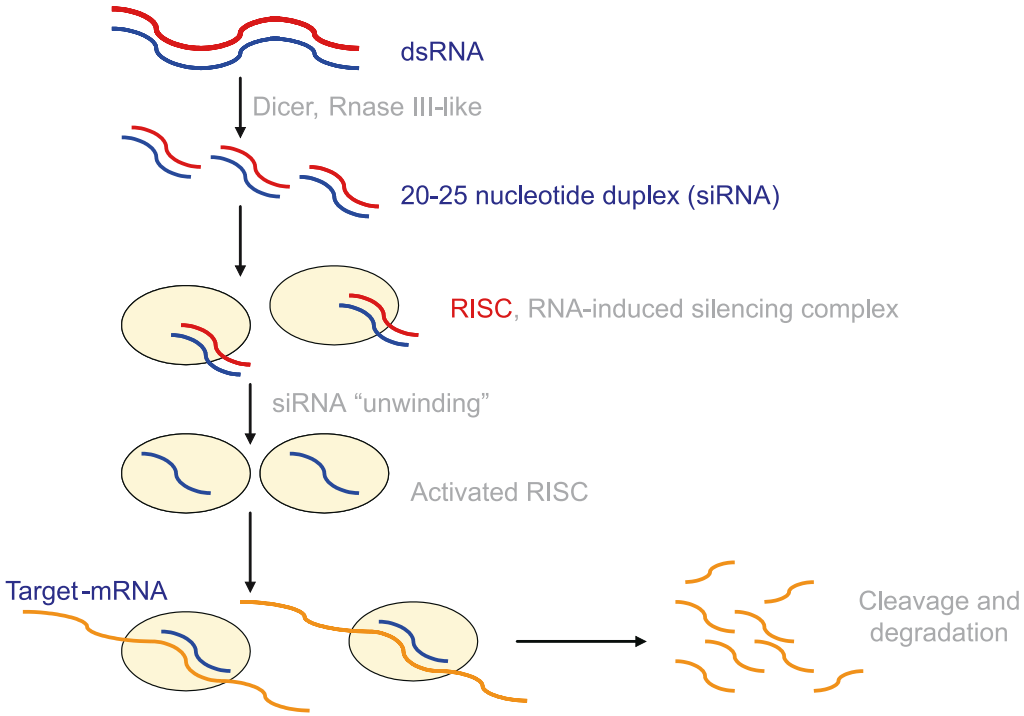

Fig. 2.3 RNA interference pathway and rules for siRNA design. We used algorithms for designing siRNAs that are available on public and commercial Web sites (http://www.ambion.com/techlib/tb/tb_506.html). As initially suggested, we did not target regions of the cognate mRNA potentially binding to regulatory proteins, i.e., sequences close to the start site and to 5′ and 3′ untranslated regions (UTR). From the mRNA we selected 23 nucleotides conforming to the consensus sequence 5′-AA[N19]UU-3′ (where N is any nucleotide). The selected sequences were subjected to basic local alignment search tool (BLAST) analysis to exclude targeting of closely related mRNA sequences (http://www.ncbi.nlm.nih.gov/BLAST). We also followed the rule of choosing an optimal GC-content of siRNA between 30% and 70%. We then constructed the siRNA by designing sense and antisense N19 sequences, each with two 3′ 2-deoxythymidine residues at the end. Systematic investigation based on targeting multiple siRNAs to the same exogenous or endogenous genes resulted in the establishment of additional rules. These obey the positioning of A/U at the 5′-end of the antisense strand, G/C at the 5′-end of the sense strand, allowing at least five A/U residues in the 5′ terminal one-third of the antisense strand, and avoidance of any GC stretch of more than nine nucleotides in length (Ui-Tei et al. 2004). Targeting the middle of the coding sequence exhibited a poorer silencing effect than targeting the 3′-UTR. Pooling of four or five duplexes per target gene was particularly efficient, and efficient duplexes showed nucleotide preferences at positions 11 and 19 of the siRNA duplex (Hsieh et al. 2004). Further characteristics for good siRNA functionality include low G/C content, a lack of inverted repeats, and sense strand base preferences at positions 3, 10, 13, and 19 (Reynolds et al. 2004)

be synthesized chemically as 19 nucleotide fragments with two 3′-overhangs on each strand or transcribed in vitro from DNA oligonucleotide templates by T7 polymerase followed by RNA strand annealing. Chemically or enzymatically synthesized siRNAs are transiently transfected into mammalian cells using lipofection, electroporation, or a combination of both methods (Schäfer and Schramme 2006).

2.6 Prioritization of Target Genes: Transcription Factors and Beyond

Signal-regulated transcriptional modules comprise target genes associated with defined branches of the Ras signal transduction network and, ideally, with defined cellular phenotypes which can be assessed in a medium-throughput manner. Despite the reduction of candidate

genes, some prioritization prior to functional assays is still required. We consider genes encoding transcriptional regulators very important, since they potentially represent nodes in the signaling and transcriptional network. The transcriptional regulators Elk1, SRF, ATF2, Myc, Fos, and others integrate postreceptor signaling via Ras proteins and gene expression (Campbell et al. 1998; Downward 1998; Malumbres and Pellicer 1998). Undoubtedly, these factors contribute significantly to the stimulation of proliferation and initiation of neoplasia. In addition, the contribution of differentially regulated transcription factors may be essential to maintain transformed phenotypes, particularly because the experimental conditions chosen for their recovery were based on normal and KRAS-transformed cells cultured in logarithmic growth phase. Thus, proliferation-associated effects on the transcriptional program were minimized. Differentially expressed transcription factors such as Fra-1 and WT1 are prime candidates for subsequent functional analysis, since they may provide novel clues to elucidating specific mechanisms of transformation-related deregulation of target gene activity.

Fra-1, a member of the Fos protein family, is upregulated more than 100-fold in HRAS-transformed fibroblasts (Zuber et al. 2000) and in KRAS-transformed ovarian epithelial cells (Tchernitsa et al. 2004) relative to their normal cellular counterparts. Fra-1 upregulation was also described in tumors (Risse et al. 1998; Zajchowski et al. 2001). Although the transforming activity of Fra-1 is weaker than that of other Fos proteins (Jochum et al. 2001), Fra-1 can induce morphological transformation, invasiveness, and motility in normal cells (Kustikova et al. 1998; Vial et al. 2003). We transiently introduced RNA duplexes targeted against endogenous Fra-1 into A2/5 cells by lipofection. We confirmed the silencing effect in cellular lysates by analyzing Fra-1 proteins 24 and 48 h after introduction of siRNA (Tchernitsa et al. 2004). The downregulation of Fra-1 protein levels reduced proliferation of A2/5 cells by 50%. In contrast, silencing KRAS [C12V] expression with a specific siRNA resulted in an almost complete growth inhibition. We did not observe any effect of Fra-1 silencing on EMT. This suggests that the target gene Fra-1 partially contributes to cell growth and survival, but does not affect the transformed properties of KRAS-expressing ROSE cells. We concluded that the upregulation of the Ras-responsive gene Fra-1 is necessary but not sufficient for the proliferative capacity of A2/5 cells. Silencing of co-regulated Ras-responsive genes is likely to complement Fra-1 knockdown to achieve full reversion of transformed phenotypes and growth inhibition. The potential role of Fra-1 in gene regulation can be inferred from the protein domain structure. The Fra-1 protein shares a DNA-binding domain with other Fos-related proteins, but lacks a transcriptional activator domain. The high abundance of Fra-1 proteins in cancer cells may reduce active activator protein (AP)-1 transcriptional complexes consisting of Fos/Jun heterodimers by shifting their composition in favor of Fra-1/c-Jun heterodimers. This can result in the attenuation of Fos-regulated gene expression (Kessler et al. 1999) and possibly cause the excessive downregulation of target genes.

The Wilms tumor suppressor gene (*WT1*), another downregulated target, encodes a Cys-His zinc finger transcription factor, which functions as a transcriptional repressor and activator and is negatively regulated by RAS signaling via the non-Erk/non-PI3K-module. The *WT1* gene is a known suppressor of Ras-mediated transformation (Luo et al. 1995). Interestingly it was reported that the *WT1* protein binds to and stimulates the E-cadherin promoter and that E-cadherin acts as a target to mediate tumor-suppressing activity (Hosono et al. 2000). Although a Ras-suppressible E-box is located in the E-cadherin promoter potentially causing E-cadherin suppression (Grooteclaes and Frisch 2000), the downregulation of *WT1* may account for the loss of E-cadherin expression in transformed ROSE cells. Since impairment of *WT1* function has been observed in ovarian carcinoma (Schorge et al. 2000), other targets critical for ovarian tumor pathogenesis may be controlled by the same mechanism.

In addition to the transcriptional regulators, other Ras-responsive genes have the capacity to control neoplastic phenotypes as well. On of the most interesting candidate genes is *lysyl oxidase* (*Lox*) (Table 2.1). This gene encodes a copper-dependent amine oxidase that catalyzes the oxidative deamination of peptidyl lysine in procollagen and proelastin. In nonenzymatic reac-

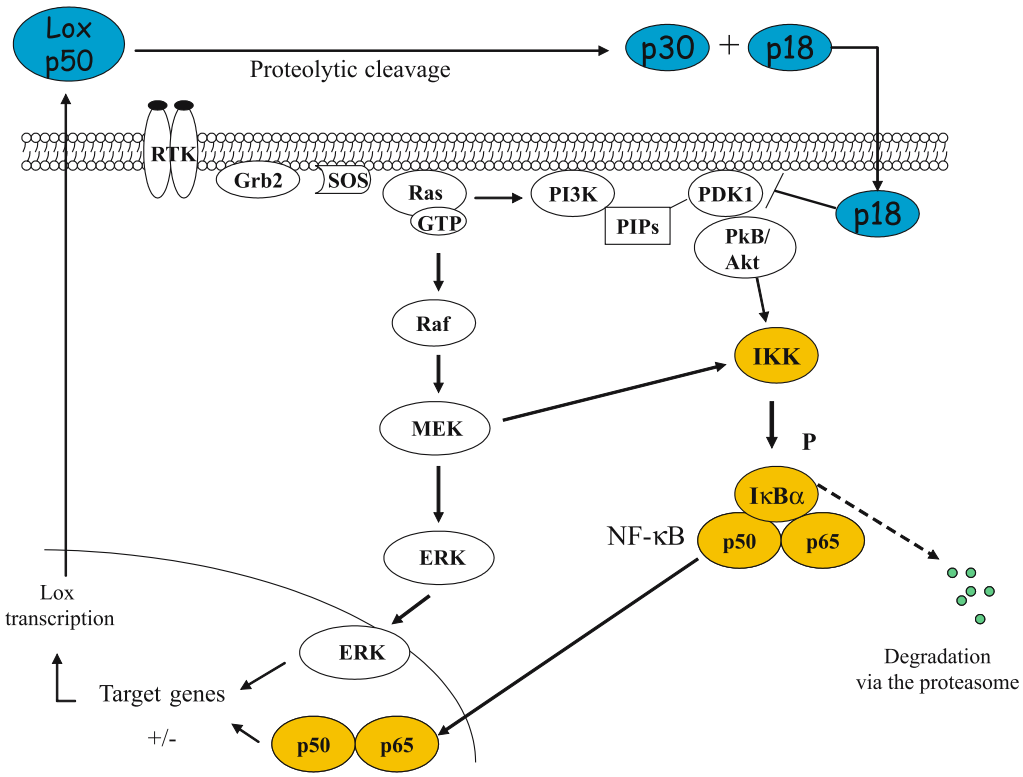

Fig. 2.4 Schematic representation of the RTK/Ras/MAPK and nuclear factor (NF)-κB pathways, showing crosstalk between RTK/Ras and NF-κB signaling via the lysyl oxidase (Lox) propeptide. Both pathways contribute to transformation, possibly through deregulation of critical transcriptional targets. Downregulation of the target of the Ras pathway, Lox, permits localization of PDK1 at the plasma membrane and the subsequent phosphorylation of inhibitor of NF-κB (IκB) via PkB/Akt and IKK. IκB is then degraded via the proteasome, and NF-κB is no longer sequestered in the cytoplasm. NF-κB translocates into the nucleus and activates transcription of its targets. In nontransformed cells, the 50-kDa Lox precursor is secreted and proteolytically cleaved into the 30-kDa enzyme and the 18-kDa propeptide. The arginine-rich propeptide is thought to penetrate the cell membrane, due to its highly basic structure, and to prevent the localization of PDK1 at the membrane and activation of the kinase (Palamakumbura et al. 2004). For further explanations, see text. *PDK1*, 3-phosphoinositide-dependent protein kinase 1; *PIP*, phosphatidylinositol-3,4,5 triphosphate, phosphatidylinositol-3,4 bisphosphate; *PkB/Akt*, protein kinase B, murine thymoma viral oncogene homolog 1; *IKKs*, IκB-kinase interacting protein; *IκB*, nuclear factor of κ light chain gene enhancer in B cells inhibitor; *NF-κB*, nuclear factor of κ light chain gene enhancer in B cells inhibitor; other abbreviations as in Fig. 2.1

tions, intra- and intermolecular condensations form crosslinks that insolubilize these matrix proteins and, hence, stabilize the extracellular matrix (Kenyon et al. 1991). Lox was recognized as one of the classical targets downregulated in Ras-transformed cells and re-expressed in phenotypic revertants derived from them (Hajnal et al. 1993). While Lox downregulation might contribute to matrix destabilization, a feature of oncogene-transformed cells, a signaling-related function of Lox was recognized more recently. The anti-Ras function of Lox was linked with the prevention of NF-κB activation. Lox expression decreased both the rate of IκBα, which is an inhibitor of NF-κB turnover, and the activity of the upstream kinases IKKα and IKKβ. Lox downregulated the PI3K and Akt kinases and partially inhibited Mek (Jeay et al. 2003). Since oncogenic Ras mediates transformation partially through the activation of NF-κB (Finco et al. 1997), the downregulation of Lox enables positive crosstalk between the Ras and NF-κB pathways to achieve

full cellular transformation (Fig. 2.4). The active component of Lox involved in signaling crosstalk is an 18-kDa propeptide derived from the 50-kDa precursor (Palamakumbura et al. 2004).

2.7 An Integrated Approach for Elucidating Ras Pathway-Dependent Gene Expression

In summary, we have described an integrated approach for elucidating the functional relationship of Ras-mediated signal transduction and transformation with the genetic program. The first step was to establish a genome-wide survey of Ras pathway-responsive target genes. In the second step, we defined subgroups of target genes (signal-regulated transcriptional modules) controlled by individual effector pathways of the Ras signaling network. Finally, we have embarked on an RNAi approach to test individual targets for their contribution to cellular transformation. Priority was given to differentially expressed transcriptional regulators. However, the example of Lox indicates that target genes other than transcriptional regulators may be of equal importance for regulating transcription and phenotypic changes. Besides studying the phenotypic consequences of ablating upregulated target genes, we have assessed the impact of gene silencing on the transcriptional program. For this purpose, we have established tailor-made oligonucleotide microarrays representing approximately 300 validated Ras-responsive genes (Tchernitsa et al. 2006a, b). These will help to elucidate the transcriptional network downstream of the cytoplasmic signaling cascade.

Besides the specific contributions of transcriptional regulators such as Fra-1 and WT-1 in tumor suppressor gene downregulation, methylation may be involved as a major epigenetic mechanism (Rountree et al. 2001). Ras signaling can indeed mediate enhanced DNA methylation activity (Macleod et al. 1995). A very recent study suggests that treatment of Ras-transformed cells with the demethylating agent 5-aza-2-deoxycytidine partially restored pretransformation mRNA levels of some target genes both in a MAPK-dependent and -independent manner (Lund et al. 2006).

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3 Predictive Pathology of Cytostatic Drug Resistance and New Anti-cancer Targets

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Recent Results in Cancer Research, Vol. 176

Abstract

Due to continuous technical developments and new insights into the high complexity of many diseases, in particular the pathogenesis of cancer, molecular pathology is a rapidly growing field gaining centre stage in the clinical management of tumours as well as in the pharmaceutical development of new anti-cancer drugs. Activated signalling components are the targets for classical therapeutic agents and newly developed inhibitors. The application of the compounds in clinical trials has revealed promising results; however, the current diagnostic procedures available for determining which patients will primarily benefit from rational tumour therapy are insufficient. To read a patients' tissue as "deeply" as possible, gaining information on the morphology and on genetic, proteomic and epigenetic alterations will be the new task of surgical pathologists experienced in molecular diagnostics, in order to provide the clinicians with information relevant for an individualized medicine. Among the different high-throughput technologies, DNA microarrays are now the first array approaches close to entering routine diagnostics. Technically advanced and well-established microarray platforms can now be evaluated by distinct bioinformatic tools capable of both identifying novel genes associated with disease development and also clusters of genes predicting the clinical outcome of an individual tumour. DNA microarrays have been efficiently used for the classification of tumour subtypes, the prediction of metastatic potential and drug response. In the current review we will focus in particular on the new possibilities of predicting the efficacy of anti-neoplastic drugs as a diagnostic tool of pathologists seeking an efficient individualized therapy.

3.1 Introduction

Identification of human tumours by applying histopathology immunohistology and nuclear acid-based techniques (in situ hybridization, comparative genomic hybridization, etc.) in relation to clinical data almost always gives precise information on dignity, tissue origin, type of tumour, stage, and grade as well as on the completeness of surgical tumour removal. Up to now, these data have provided the most relevant information on patient prognosis and are a rational basis for planning of therapy. Since the high diversity of genetic and proteomic alterations in tumour cells is not completely mirrored by tumour morphology, pathologists and clinicians often observe that two patients harbouring the "same" histological tumour type have different clinical outcomes with respect to survival and response to therapy.

In addition to conventional clinicopathological parameters, several molecular approaches have been developed to predict patients' prognosis and response to therapy. However, considering the fact that cell function is controlled by a complex network of signalling pathways and other interactions, it is unlikely that the determination of the expression of single proteins will help in the precise prediction of the clinical response of individual tumours. Hanahan and Weinberg (2000) have described six essential al-

terations in cancer cells that are shared by most human tumours: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. However, each of these alterations may be achieved by different cellular changes in gene expression and signal transduction. Thus, a more functionally orientated characterization of malignant tumours should reflect the consequences of multiple genetic changes typical for malignant cells. These are e.g. activation of oncogenes, inactivation of tumour suppressor genes, elevated growth factor production, loss of growth inhibitory cytokines, imbalance of apoptosis, adhesion and/or DNA repair regulating genes, up-regulation of drug resistance-mediating genes and many more.

The technique of genome-wide profiling using DNA microarrays can at least partially reflect the complexity of tumour signal transduction networks and by this can mirror the interaction related to multi-drug resistance. The great advantage of the array technology is the possibility to quantitate the expression of thousands of genes simultaneously. By the detection of characteristic expression patterns related to the individual biological behaviour of a certain tumour, it may become possible to predict e.g. the metastatic potential or the response to anti-proliferative drugs with a much higher accuracy than is possible today.

3.2 Technology

The generation of gene expression profiles from cultured cells and tissue samples is a well-established method. In the majority of experiments, DNA fragments specific for individual genes on activated glass surfaces are used (Brown and Botstein 1999; Pollack et al. 2002). RNA is prepared from the biological material to be analysed, reverse transcribed into complementary DNA (cDNA), labelled with fluorescent dyes and hybridized to the array. A laser scanner detects the hybridization signals, the images are normalized in various ways and the relative expression levels obtained for individual genes are further clustered into groups of genes with a similar or identical expression patterns.

The target DNA usually is a cDNA derived from tumour cell messenger RNA (mRNA) which is amplified and simultaneously labelled by RT-PCR. The RNA must be prepared from rapidly processed cell cultures or freshly frozen tissue, since the formalin-fixed, paraffin-embedded tissue stored in pathology department archives has to date failed to prove suitable for use with microarrays. Target DNA of course can also be prepared from genomic DNA fragments. For labelling, fluorescent dyes are currently used. The expression intensity of each gene is determined after hybridization of the target DNA to the immobilized array DNA.

The hybridization signal generated on each spot reflects the expression of the corresponding gene. The quantification of the signals is performed using special software which allows researchers to correct for spot integrity and technical deviations during array production and hybridization, and to interpret the signal intensity. For this purpose, cDNA of normal tissue or any other reference cDNA has to be introduced. The final results provide a genetic expression profile indicating over-expression, under-expression, no change or complete absence for each gene in the tissue samples to be compared to each other (for review see MacGregor and Squire 2002). Just recently new techniques have been developed which allow RNA preparation from formalin-fixed, paraffin-embedded tissue using special procedures of RNA purification. The RNA quality has proved sufficient e.g. for TagMan high-throughput PCR analyses.

3.3 Bioinformatics

The enormous amount of data obtained from each high-throughput array experiment, data documentation, data processing and data interpretation is a unique challenge requiring a close co-operation between pathologists and bioinformatic specialists. Therefore, methods and bioinformatic tools have been developed for the extraction of complex information, the evaluation via statistical methods and the translation of molecular information into clinically relevant data. Today, cDNA microarrays have been used for a variety of different objectives. To choose the method of statistical analysis that is appropriate

for each study, it is helpful to distinguish different types of array analysis. Simon et al. (2003) have distinguished three different types of study objectives: "*class discovery*" (find a new class), "*class comparison*" (find differences between two predefined classes) and "*class prediction*" (find a predictive gene set for a certain pre-defined class). For each type of study, different statistical strategies for evaluation should be used. According to Simon et al., using methods incompatible with the goal and the design of the study is one of the most common errors in the analysis of microarray data.

The implementation of bioinformatic procedures—which will have to undergo further standardization together with highly parallel tissue analyses—is opening the door for a broad profiling of human cancer tissue, thus improving the possibility of predicting the biological behaviour of single tumours.

3.4 Response to Therapy/ Drug Resistance

Drug resistance remains a major problem in therapy of systemic cancer diseases. Due to the high potency to adapt to therapeutical approaches, malignant tumour cells frequently develop escape mechanisms in response to radiation or anti-cancer drugs. Although many attempts have been made (review see Lage and Dietel 2002), currently there exist no reliable and practically applicable techniques to predict prior to therapy the tumour's reaction to drugs or radiation. The reasons for this stem from multiple cellular events which at the same time are often involved in the development of resistance, such as increased DNA repair, elevated levels of drug transporters, over-expressed detoxifying enzymes, decreased rates of apoptosis and several more (Fig. 3.1). To monitor the multiple altera-

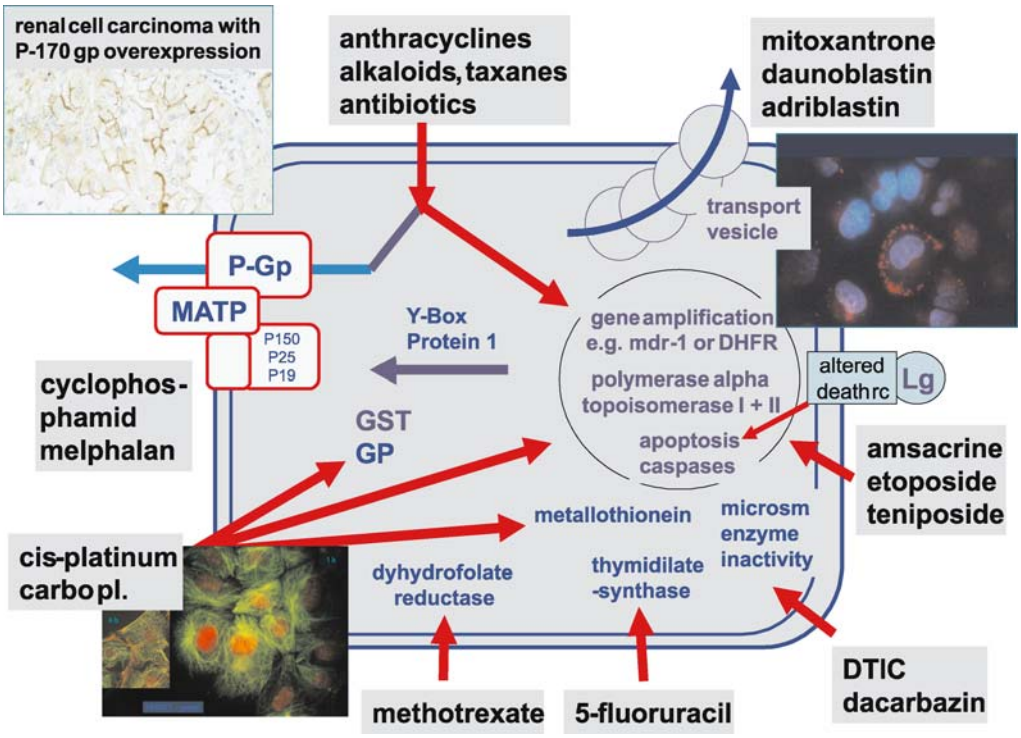

Fig. 3.1 Complex mechanisms of drug resistance in malignant tumour cells

tions, highly parallel analyses, such as the DNA microarray technique, are required. This opportunity opens new dimensions to predict therapy resistance and sensitivity.

A recent NIH-study (Scherf et al. 2000) investigated 60 tumour cell lines (NCI60) which have been treated with more than 70.000 different agents, one at a time and independently. Among other results, the study linked bioinformatics and chemoinformatics by relating cellular drug response with genetic information derived from DNA microarrays. The aim was to associate clusters of drugs with clusters of gene alterations and to define drug–gene relationships. This approach may contribute to the establishment of a defined expression database on which an individualized molecular pharmacology of tumour drug response can be established.

By comparing wild-type cell lines with derivatives resistant for thymidylate synthase (TS) inhibitors, Wang et al. (2001) were able to identify certain patterns of genetic alterations due to TS resistance. Remarkably, the associated gene expression profile was partly tissue dependent, e.g. *YES1* was over-expressed in the epithelial cell lines while it was not up-regulated in a lymphoblast cell line. In DNA array experiments, *cis*-platinum (cDDP) resistance was shown to be accompanied by altered expression of genes coding for membrane proteins and a glycoprotein hormone subunit (Higuchi et al. 2003), up to then not known to play any role in cDDP resistance.

In an excellent study by Zembutsu et al. (2002), 85 human cancer xenografts were tested with regard to altered expression profiles due to nine anti-cancer drugs often used in clinical therapy. More than 1,500 genes were identified whose expression correlated in some way with chemosensitivity. The authors identified a set of genes which could partly be associated with chemosensitivity of a particular tumour type [colon, breast, non-small cell lung cancer (NSCLC), etc.] to the different drugs applied.

To predict efficacy to adjuvant therapy of oesophageal tumours, the DNA microarray technique was applied to 20 cancer specimens with clinically known responses (Kihara et al. 2001). Identified were 52 genes which were likely to correlate with patient outcomes and possibly with chemosensitivity and chemoresistance. This approach shows some potential to determine drug response in advance.

Using DNA microarrays, bone marrow samples from 19 patients with acute lymphoblastic leukaemia were investigated with regard to resistance for an ABL tyrosine kinase inhibitor (Hofmann et al. 2002). On the basis of 95 differentially expressed genes, it appeared to be possible to distinguish responder from non-responder.

Our own lab performed experiments to define resistance-associated signatures for common cytostatic drugs. Tested for sensitivity to 5-fluorouracil, cisplatin, cyclophosphamide, doxorubicin, etoposide, methotrexate, mitomycin C, mitoxantrone, paclitaxel, topotecan and vinblastine—at drug concentrations that can be systemically achieved in patients—were 30 cancer cell lines (Györffy et al. 2005). The resistance index defined previously (Dietel et al. 1993) was used to designate the cell lines as sensitive or resistant. The subset of resistant versus sensitive cell lines for each drug was then compared. Gene expression signatures for all cell lines were obtained by interrogating Affymetrix U133A arrays. The Prediction Analysis for Microarrays approach was applied for feature selection. An individual prediction profile for the resistance against each chemotherapy agent was constructed, containing 42 to 297 genes. The overall accuracy of the predictions in a leave-one-out cross validation was 81.7%. A list of the top 67 multi-drug-resistance candidate genes which were associated with the resistance against at least four anti-cancer agents was identified. Moreover, the differential expressions of 46 selected genes were also measured by quantitative RT-PCR using a TaqMan microfluidic card system. As a single gene can be correlated with resistance against several agents, we sought associations with resistance by examining, altogether, 76 different genes and resistance phenotypes.

In a pre-clinical study, we contrasted the expression profiles of 13 different human tumour cell lines of gastric (EPG85-257), pancreatic (EPP85-181), colon (HT29) and breast (MCF7 and MDA-MB-231) origin and their counterparts resistant to the topoisomerase inhibitors daunorubicin, doxorubicin or mitoxantrone (Györffy et al. 2006). For identifying predictive genes, the Prediction Analysis for Microarrays algorithm

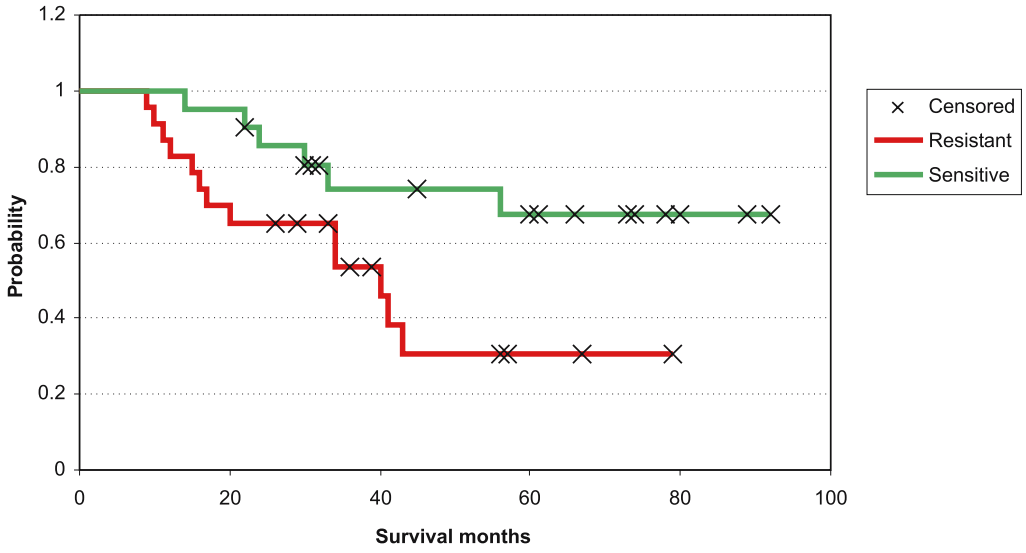

Fig. 3.2 Survival analysis of 44 breast cancer patients. The tumour samples are divided into resistant and sensitive groups based on similarity to the expression profile of cell lines resistant to doxorubicin treatment compared to their parental cells. Log-rank test (Cox-Mantel), $p = 0.0334$ (Györffy et al. 2005)

was used. The analysis revealed 79 genes best correlated with doxorubicin resistance and 70 genes with mitoxantrone resistance. In an independent classification experiment, we applied our model of resistance for predicting the sensitivity of 44 previously characterized breast cancer samples. The application of gene expression signatures derived from doxorubicin-resistant and -sensitive cell lines allowed us to effectively predict clinical survival after doxorubicin monotherapy (Fig. 3.2; for further details see Chap. 5 in this volume).

3.5 New Targets

A second major challenge has arisen that molecular pathology can fulfil. The efforts devoted to unravelling intracellular oncogenes, as well as distinct intracellular signalling pathways and their role in growth control, have uncovered a large number of potential therapeutic target structures. Herceptin and Glevec targeting the *HER2/neu* and *BCR/ABL* oncogenes, respectively, are the first designer drugs which are already used with considerable success in the clinic. In the meantime, further drugs which act more or less specifically against a broad range of receptors and signalling components have entered clinical trials (Table 3.1). This development is intimately connected with the expectation that tumour therapy will be dramatically improved in the near future. To date, the percentage of patients responding to the new inhibitors is often below 30%. This indicates that the current portfolio of diagnostic methods based on immunohistochemical or DNA analysis (e.g. fluorescence in-situ hybridization, FISH) of single target molecules is still insufficient. For the identification of those patients who will benefit from novel therapies, special methods capable of detecting the entire spectrum of rate-limiting oncogenic pathways in tumours prior to and during therapy have to be developed and adapted to routine diagnostic pathology. Clearly this will play an increasing role in the future tasks of pathological institutes.

3.6 Discussion

Gene expression profiling can be helpful for the pre-treatment assessment of anti-cancer therapy.

Table 3.1 Examples of targeted therapy approaches

Drug	Tumour type	Target	Known action	Detection method	Remarks
Trastuzumab	Metastatic breast cancer	HER-2/neu	Rc blocking	IH, FISH, ISH	[a]
Cetuximab+ irinotecan	Metastatic colorectal cancer	EGFR	Rc blocking/ immunologic response	IH, FISH,	[a]
Lapatinib GW572016	Breast cancer, e.g. trastuzumab refractory BC	ErbB1+ ErbB2 (truncated)	Dual rc blocking by tyrosine kinase inhibition	IH	
Imatinib Mesylate (Gleevec)	CML, GIST with activated c-kit receptor tyrosine kinase, other sarcomas	bcr/abl–positive (activated PK)	Tyrosine kinase inhibitor	IH	[a]
Bevacizumab (Avastin) (+5FU)	Colorectal cancer	VEGF	Rc blocking	IH	[b]
G3139 (Genta, Berkley)	Haematologic malignancies and malignant melanoma	Antiapoptotic gene *bcl*-2	Bcl-2 antisense oligonucleotide decreasing Bcl-2 mRNA	Immunophenotyping by IH	[b]
Bortezomib, epoxomicin	Multiple myeloma	Proteasome	Proteasome inhibitor		
Gefitinib (Iressa)	Non-small cell lung cancer	Mutated EGFR	Kinase inhibitor	Mutational analyses, immunoblotting	[d]
Erlotinib (Tarceva)	Non-small cell lung cancer	Mutated EGFR	Kinase inhibitor	Mutational analyses, immunoblotting	[d]
Rituximab (+CHOP), Y90-ibritumomab, I131-tositumomab	Non-Hodgkin lymphoma	CD20	Lympholytic	Immunophenotyping by IH	[c]
Gemtuzumab-ozogamicin (calicheamicin)	AML (>60 years)	CD33	Anti-CD33 guided cytotoxic antibiotic, reduction of P-glycoprotein	Immunophenotyping by IH	[c]
Alemtuzumab (Campath)	B-CLL, T-NHL, osteogenic tumours [e]	CD52	Lympholytic	Immunophenotyping by IH	[c]
Rapamycin RAD001	Breast, prostate, renal cancer	TOR	Kinase inhibitor		[d]
BMS 354825	GIST	Kit	Tyrosine kinase inhibitor	IH	[d]
Sorafenib tosylate (Nexavar BAY43-9006)	Melanoma, renal cell carcinoma	RAF kinase	Multi-kinase inhibitor		[d]

FISH, fluorescence in situ hybridization; IH, immunohistochemistry; ISH, in situ hybridization; Rc, receptor

[a] FDA-approved drugs requiring a pre-therapeutic diagnostic eligibility test

[b] FDA-approved drugs targeting specific pathway—no specific tests available

[c] FDA-approved antibody targeted therapies for haematologic malignancies guided by immunophenotyping

[d] Under development

[e] Patent No. EP:03029464.9, V. Krenn, Inst. of Pathology; submitted

Although a vast number of experiments still have to be conducted, it might become possible to predict chemoresistance and to avoid non-effective drugs and unnecessary side-effects for patients. The discrimination between responders and non-responders prior to therapy will allow an individualized strategy with a personalized combination of drugs. The translation of research on molecular tumour characterization into daily clinical practice will be the task of the upcoming years.

New technical developments will contribute to speed up the process of a more complex characterization of each individual tumour. For example, the automatic high parallel analysis of proteins and complex protein lysates has developed rapidly. Here, the opportunity of routine early detection of cancers such as breast, prostate and ovary as proteomic patterns in the serum appears at the horizon. In addition, an improved analysis of tumour samples via antibody or reverse phase protein arrays is likely to provide the pathologist in the future with information about over-expressed signalling proteins, activated oncogenic signalling pathways and other cell functions, such as drug response or the potential to metastasize. While expression microarrays and proteomic analysis rely on relatively unstable material that is incompatible with paraffin-embedded tissue samples, investigation of DNA methylation using specialized high-throughput platforms has revealed the potential of DNA methylation use in future diagnostics.

Such a "multiplex approach" described above will contribute to bolster the possibilities of improving and stimulating molecular medicine to predict tumour-associated features including tumour progression, drug response, and metastatic potential, which will have an important impact on tumour prognosis and adequate individual therapy. However, the demonstration that there exists a real benefit for the patients with, e.g. longer survival times or better response to therapy, is still open to debate. Only when well-designed clinical studies prove molecular tumour profiling in individualized therapeutic strategies as efficacious will it be integrated in routine cancer diagnosis and treatment. One major prerequisite for an advantageous path towards this eventuality is the interdisciplinary co-operation of basic scientists, clinically orientated physicians, diagnostic pathologists and clinicians, who must all speak the same "language"; they must have an understanding of their partners. The "translation" between different groups of researchers will be one of the most challenging tasks in modern experimental and diagnostic pathology.

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4 Prediction of Response to Neoadjuvant Chemotherapy in Carcinomas of the Upper Gastrointestinal Tract

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Recent Results in Cancer Research, Vol. 176

Abstract

Increasingly, multimodal treatment protocols are being employed to improve the survival of patients with locally advanced adenocarcinomas of the upper gastrointestinal tract; however, only 30%–40% of the patients respond to 5-fluorouracil (5-FU) and cisplatin-based neoadjuvant chemotherapy. The goal of our studies is the identification of reliable genetic markers—on the genomic DNA, messenger RNA (mRNA), or protein level—that could predict response of upper gastrointestinal carcinomas prior to neoadjuvant chemotherapy. In esophageal carcinomas, a higher gene expression of methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in folate metabolism, was more frequently found in responding patients. In addition high gene expression of caldesmon and of the two drug carrier proteins MRP1 and MDR1 was associated with response to therapy. By performing a genome-wide profiling on the protein level in a small group of patients, new potential markers were identified that will have to be validated in ongoing studies. In gastric carcinomas, mutations of the *p53* gene revealed no association with response or survival, but tumors with a high rate of loss of heterozygosity, as determined by microsatellite analysis, showed a better response to a cisplatin-based chemotherapy. Analysis of the expression of 5-fluorouracil (5-FU) (*TS, DPD, TP*)- and cisplatin (*ERCC1, ERCC4, GADD45A, KU80*)-related genes demonstrated an association of *DPD* expression with response and survival. The combined consideration of TP and GADD45 gene expression showed the most obvious association with therapy response in this tumor. Our studies point to promising markers with potential use for chemotherapy response prediction of adenocarcinomas of the upper gastrointestinal tract, but prospective studies for validation are necessary.

4.1 Introduction and Objective

Multimodal treatment protocols are being increasingly employed to improve the survival of patients with locally advanced adenocarcinomas of the esophagus and stomach. Neoadjuvant chemotherapeutic treatment, mainly based on cisplatin and 5-FU, has been used since 1989 in several clinical trials, and recently a statistically significant improvement in respect to resectability, progression-free survival, and overall survival in operable gastric and lower esophageal cancer has been demonstrated in a large randomized, controlled phase III trial (MAGIC trial) (Cunnigham et al. 2005). However, only 30%–40% of the patients respond to therapy, and the majority of patients undergo several months of toxic, expensive therapy without survival benefit. In particular, in the case of esophageal carcinomas, it has been shown that patients with nonresponding tumors seem to have an even worse prognosis than patients treated by surgery alone, which may be related to therapy-induced side effects, selection of chemotherapy-resistant, more aggressive tumor cells, and delay of surgery (Zacherl et al. 2003). Thus, the identification of reliable genetic markers that could predict response is highly demanding.

Several molecular markers have been investigated as potential response predictors. Thymidylate synthase as the target enzyme for 5-FU has

been widely studied for 5-FU-containing regimens in gastrointestinal cancer, but the results are inconsistent (Yeh et al. 1998; Metzger et al. 1998; Napieralski et al. 2005). Dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) are two other important regulatory enzymes involved in the degradation of 5-FU, and low levels of DPD have been shown to be associated with response in gastric carcinoma (Napieralski et al. 2005; Ishikawa et al. 2000), whereas conflicting results have been reported for TP.

The other major component used for the treatment of carcinomas of the upper gastrointestinal tract is cisplatin, which supposedly directly damages DNA. A significant association of the gene expression of the nucleotide excision enzyme ERCC1, which is involved in DNA repair, with response to neoadjuvant chemotherapy has been reported (Metzger et al. 1998).

Other markers such as glutathione S-transferase, vascular endothelial growth factor, and apoptosis-related genes such as *bcl-2*, *bax*, and *p53* have been studied, mostly by immunohistochemistry. The results have been inconclusive, so that no markers has been found to be clinically relevant at present (Yeh et al. 1998; Boku et al. 1998).

Thus, the goal of our studies is to identify effective molecular markers for response prediction for patients with esophageal and gastric carcinomas treated by a neoadjuvant chemotherapy. We are using different strategies based on the one side on targeted approaches to characterize pretherapeutic biopsies for tumor-specific molecular alterations on the genomic DNA and mRNA levels. We also analyze constitutional genetic factors, e.g., DNA polymorphisms in therapy-related genes. On the other side, we perform genome-wide profiling on the protein level to identify new marker proteins.

4.2 Results

4.2.1 Characterization of Pretherapeutic Biopsies of Esophageal Carcinomas

4.2.1.1 Analysis of mRNA Expression of Therapy-Related Genes

In this study, paraffin-embedded, formalin-fixed endoscopic esophageal tumor biopsies of 38 patients with locally advanced esophageal adenocarcinomas (Barrett's adenocarcinoma) were included. All patients underwent two cycles of cisplatin and 5-FU therapy with or without additional paclitaxel followed by abdominothoracic esophagectomy. RNA expression levels of 5-FU-metabolism-associated genes *thymidylate synthase*, *thymidine phosphorylase*, *dihydropyrimidine dehydrogenase*, *methylenetetrahydrofolate reductase*, *MAP7*, and *ELF3*, as well as of platinum and taxane-related genes *Caldesmon*, *ERCC1*, *ERCC4*, *HER2-neu*, *GADD45*, and multidrug resistance gene *MRP1* were determined using real-time RT-PCR. Expression levels were correlated with response to chemotherapy histopathologically assessed in surgically resected specimens.

The results demonstrated that the responding patients showed significantly higher pretherapeutic expression levels of *methylenetetrahydrofolate reductase* (*MTHFR*) ($p = 0.012$), *Caldesmon* ($p = 0.016$), and *MRP1* ($p = 0.007$). In addition, patients with high pretherapeutic MTHFR and MRP1 levels had a survival benefit after surgery ($p = 0.013$ and $p = 0.015$, respectively) (Langer et al. 2005). Additionally, investigation of intratumoral heterogeneity of gene expression of relevant genes (*MTHFR*, *Caldesmon*, *Her2-neu*, *ERCC4*, *MRP1*)—verified in 9 untreated Barrett's adenocarcinomas by examination of five distinct tumor areas—revealed no significant heterogeneity in gene expression, indicating that expression profiles obtained from biopsy material may yield a representative genetic expression profile of total tumor tissue (Langer et al. 2005).

In conclusion therefore, the results indicate that the determination of the mRNA levels of a few genes may be useful for the prediction of the success of neoadjuvant chemotherapy in individual cancer patients with locally advanced Barrett's adenocarcinoma.

4.2.1.2 Differential Quantitative ProteoTope Analysis of Fresh Frozen Biopsies

A comprehensive protein profiling approach, using the ProteoSys platform, has been performed until now using a small group of patients. Quantitative and qualitative protein expression analysis was performed using 2D ProteoTope techniques

after radioactive labeling of the protein extract with I-125 and I-131. The results so far point to an interesting group of proteins that may be associated with response. Validation of specific proteins by immunohistochemical analysis in a high number of cases is now part of ongoing studies.

4.2.2 Characterization of Pretherapeutic Biopsies of Gastric Carcinomas

4.2.2.1 Microsatellite Analysis and *p53* Mutation Analysis

We evaluated microsatellite instability (MSI) and loss of heterozygosity (LOH) in 53 pretherapeutic gastric carcinoma biopsies using 11 microsatellite markers. The entire coding region of the p53 gene (exons 2–11) was analyzed for mutations by DHPLC (denaturing high pressure liquid chromatography) and sequencing. The expression of p53 was evaluated by immunohistochemistry. Patients were treated with a cisplatin-based, neoadjuvant chemotherapy regimen. Therapy response was evaluated by computed tomography (CT) scan, endoscopy, and endoluminal ultrasound (Grundei et al. 2000; Ott et al. 2003).

We identified *p53* mutations in 19 of the 53 (36%) analyzed tumors. No significant association with response or survival was found for *p53* mutation or for p53 protein expression. Microsatellite instability (either MSI-H or MSI-L) did not show a correlation with response. With respect to LOH, LOH at chromosome 17p13 showed a significant association with therapy response ($p = 0.022$), but did not reach statistical significance in terms of patient survival. The global LOH rate, expressed as fractional allelic loss (FAL) was assessed and tumors were classified into tumors with a high (>0.5), a medium (>0.25–0.5), and a low (0–0.25) FAL value. A statistically significant association of FAL with therapy response was found ($p = 0.003$), with a high FAL being related to therapy response.

Thus, a high level of chromosomal instability (high FAL value) defines a subset of patients who are more likely to benefit from cisplatin-based neoadjuvant chemotherapy. The *p53* mutation status is not significantly associated with therapy response and is not a useful marker for response prediction (Grundei et al. 2000; Ott et al. 2003).

4.2.2.2 Analysis of mRNA Expression of Therapy-Related Genes

For gastric carcinomas we performed gene expression analysis, focusing on genes related to the effects of 5-FU or cisplatin. Pretherapeutic, formalin-fixed, and paraffin-embedded biopsies of 61 patients who received a 5-FU and cisplatin-based chemotherapy were included. The expression of the 5-FU-related genes *TS*, *DPD*, and *TP* and of the cisplatin-related genes *ERCC1*, *ERCC4*, *KU80*, and *GADD45A* were analyzed by quantitative real-time PCR. The expression levels of single genes and of various combinations were tested for an association with response and overall survival (Napieralski et al. 2005). High DPD levels were more frequently found in nonresponding patients and were associated with worse survival. *GADD45A* and *TP* levels demonstrated weak associations with response, but *GADD45A* expression correlated with survival. There was no association with response for TS expression, but tumors with a high TS level were associated with worse survival. The combination of *GADD45A* and *TP* revealed the strongest predictive impact. High expression values of *TP* and/or *GADD45A* were exclusively found in nonresponding patients ($p = 0.002$) and were associated with a significantly poorer survival ($p = 0.04$).

In conclusion therefore, the combined gene expression levels of *TP* and *GADD45A* represent a new parameter to predict the clinical outcome after neoadjuvant chemotherapy in gastric cancer. The association of *DPD* expression with response and survival underlines a predominant role of *DPD* to predict 5-FU sensitivity. The association of *TS* expression levels with survival, but not with response, suggests an importance of this gene for tumor progression (Napieralski et al. 2005).

4.3 Outlook

Although some of our studies point to promising markers with a potential use in chemotherapy response prediction for adenocarcinomas of the upper gastrointestinal tract, prospective studies for validation are necessary before they may be used in clinical practice. As chemotherapy response is considered to be highly complex,

depending on tumor-specific characteristics as well as on the constitutional genetic makeup of the individual patient, integrative approaches for response prediction might be necessary. In addition, the incorporation of early response evaluation by positron emission tomography (PET) for the therapeutic decision together with molecular markers might result in superior sensitivity and specificity for a successful application of an individual therapy-strategy for patients with upper gastrointestinal malignancies.

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5 Resistance-Associated Signatures in Breast Cancer

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Recent Results in Cancer Research, Vol. 176

Abstract

A major obstacle in the treatment of breast cancer is the lack of adequate methods for predicting patient response to a particular chemotherapy regime. To date, single tumour markers have provided limited success. DNA array technologies identifying thousands of genes simultaneously can help to solve this problem. We investigated cancer cell lines sensitive and resistant to the topoisomerase inhibitors doxorubicin and mitoxantrone. These drugs are used in several different breast cancer treatment protocols. We have identified the top genes best associated with resistance against each cytostatic agent. We applied our gene expression signatures to a set of pre-characterised patients receiving doxorubicin monotherapy. The patients classified as sensitive to chemotherapy exhibited longer survival than the resistant ones. In summary, in our study we have successfully transferred experimental results to a clinical problem, and managed to perform a predictive test for a selected monotherapy protocol. However, many different studies have been performed using microarrays, each producing a different gene list for the same classification problem. It is likely that future diagnostic tools will include the results of several different laboratories, focus on genes validated on different technological platforms and use large cohorts of patients.

5.1 Predicting Treatment Response

Most breast cancer patients whose cancer has not reached to the lymph nodes are cured by surgery and tamoxifen, but a significant minority will develop distant metastases and die. Systemic chemotherapies are widely used for the treatment of breast cancer, as they can reduce the risk of distant metastases by approximately one-third (Early Breast Cancer Trialists' Collaborative Group 1998). There are multiple combinations of cytotoxic drugs currently accepted as standard care. They are applied empirically despite the observation that the available regimens are not equally effective across the population of patients with a particular type of breast cancer. The major cause of failure of successful cancer treatment is primary drug resistance or the development of secondary anti-neoplastic drug resistance. Many different mechanisms of resistance to chemotherapy have been identified, which may act simultaneously, be interconnected and mutually influence each other

5.1.1 Single Markers

For optimum patient management, it is desirable to know in advance the likelihood of a tumour responding to the treatment under consideration. Predictive markers are factors that are associated with response or resistance to a particular therapy. The prototype predictive tests in oncology are oestrogen receptor (ER) and progesterone receptor (PR), which are used to select patients with breast cancer likely to respond to hormone therapy (Kuerer et al. 1999; Chollet et al. 2002). Patients containing as few as 1%–10% of cells staining for ER respond to hormone therapy (Harvey et al. 1999). Additional proteins possessing possible predictive power include HER-2, apoptotic proteins, MDR1/P-gp, metallothioneins

and more. The HER-2 protein (c-erbB-2, neu) tyrosine kinase receptor initiates intracellular signalling via the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3′ kinase and phospholipase C pathways. HER-2 was recently introduced as a predictive marker for selecting patients with advanced breast cancer for treatment with trastuzumab (Herceptin), a monoclonal antibody against HER-2 (Carter et al. 1992).

Although many different anti-cancer drugs appear to mediate tumour regression by inducing apoptosis, there is currently no consistent evidence that any of the molecules implicated in this process—such as p53, bcl-2, bax, CD95, or specific caspases—can be used as a predictive marker (Duffy 2005; Debatin and Krammer 2004). The ability of cancer cells to acquire simultaneous resistance to different drugs is commonly designated as multi-drug resistance (MDR). MDR was associated with the over-expression of ATP-dependent membrane transporters such as P-glycoprotein (P-gp, MDR1, ABCB1), the multi-drug resistance proteins MRP1 and MRP2 (ABCC1, ABCC2) or the mitoxantrone resistance protein/breast cancer resistance protein (MXR, BCRP, ABCG2). P-gp is involved particularly in drug resistance of colon, kidney, adrenocortical and hepatocellular cancers as well as of acute myelogenic leukaemia (AML) (Goldstein et al. 1989). In vivo imaging studies of breast carcinomas using the P-gp substrate 99mTc-sestamibi, developed for monitoring cardiac function, showed that transporter activity is increased in breast tumours and suggested its potential as a predictive marker for tumour response towards treatment with anthracyclines and vinca alkaloids (Alonso et al. 2002; Sciuto et al. 2002). The MRP1 gene is over-expressed in leukaemias, oesophageal carcinoma and non-small-cell lung cancers, among others (Nooter et al. 1995). However, the relationship between pre-treatment in breast cancer chemotherapy and response to therapy was not significantly correlated with ABC-transporters concentrations (Trock et al. 1997). Thus, the MDR1/P-gp expression could be merely a measure of malignancy or advanced disease, rather than indicator of chemotherapy resistance (Wang et al. 1997).

In a recent study we demonstrated that elevated metallothionein carried an ER status-independent unfavourable predictive value as far as results of tamoxifen treatment were concerned (Surowiak et al. 2005). Many clinical studies have correlated alterations in the expression of individual genes with breast cancer disease outcome, often with contradictory results. Additional examples include cyclin D1 (Steeg and Zhou 1998), urokinase-type plasminogen activator, plasminogen activator inhibitor type 1 (Janicke et al. 2001) and c-myc (Bieche and Lidereau 1995).

In summary, besides ER, to date no other tumour marker has been validated as possessing a sufficient predictive value to render it clinically useful. To achieve greater predictive power, multiple markers need to be examined and correlated with response to chemotherapy (Győrffy et al. 2005b).

5.1.2 Gene Expression Signatures

Using high-density gene expression microarrays, simultaneous assessment of thousands of genes is now possible, which allows for the identification of expression patterns in different breast cancers that might correlate with survival or response to treatment. As drug resistance or response almost certainly depends on the interplay of multiple genes, the investigation of large gene-sets will produce reliable predictive tests (Evans and Relling 2004).

In breast cancer, the first studies have addressed the important issue of molecular differences in hormone responsive and non-responsive breast tumours (Bertucci et al. 1999; Hilsenbeck et al. 1999). In these studies, cell lines were mostly used and tumour samples were rarely tested and generally in small numbers.

By investigating patients with large operable or locally advanced breast cancers on Affymetrix HGU133A microarrays, it is possible to divide mammary tumour cells into three groups based on steroid receptor activity: luminal (ER+ and androgen receptor+), basal (ER– AR–) and molecular apocrine (ER– AR+) (Farmer et al. 2005). Thus, high-density microarrays can help to identify relevant genes involved in tumour pathogenesis and to classify different tumour entities or even tumour subtypes. Using gene expression signatures it was possible to identify the third group where androgen signalling replaces oestrogen signalling as a major determinant of

the steroid-related expression profile of the cells. These findings were additionally verified in previously published data sets (Farmer et al. 2005).

Van 't Veer et al. used microarray analysis on primary breast tumours of 117 young patients, and identified gene expression signatures predictive for short interval to distant metastases in patients without tumour cells in local lymph nodes at diagnosis (van 't Veer et al. 2002). Their gene expression profiling of the primary tumour was reported to outperform all clinical parameters in predicting disease outcome. Interestingly, none of the individual genes previously described in the literature were present in their predictive gene set of 70 genes. Although it is assumed that patients most at risk of metastases would benefit most from chemotherapy, predictive tests are needed to select responsiveness or resistance to a specific treatment. Patients with features of poor prognosis would be candidates for treatments other than standard chemotherapy, avoiding loss of time and side-effects related to the unadjusted chemotherapy protocols.

To date, only a few preliminary studies have been published on the use of microarrays for predicting clinical response in breast cancer. Chang et al. investigated core biopsy samples from primary breast tumours in 24 patients before treatment and then assessed tumour response to neoadjuvant docetaxel using Affymetrix HGU95-Av2 chips (Chang et al. 2003). The taxanes, docetaxel and paclitaxel, can be more effective than anthracyclines (Hortobagyi 1997; Chan et al. 1999), but only a small subset of patients benefit from the additional treatment (Aapro 2001). They identified a 92-gene predictor, which could allow a test for docetaxel sensitivity. The authors published a new follow-up analysis of docetaxel treatment response in these patients 2 years later (Chang et al. 2005). The molecular profiles may allow for the development of microarray-based assays for docetaxel sensitivity and help in the selection of optimal breast cancer treatment.

5.2 Application of an In Vitro Classification Model to Clinical Specimens

The results of microarray analysis aiming at elucidating gene patterns associated with cytotoxic drug resistance may be obscured by the presence of various cell types in a heterogeneous cancer specimen. Therefore, in our recent study we first aimed to generate gene expression patterns of well-defined chemotherapy-resistant and sensitive cancer cell lines in order to identify discriminatory genes associated with drug resistance in four different types of solid tumours (Györffy et al. 2005a).

We have investigated two different drugs used in the therapy of breast cancer: doxorubicin and mitoxantrone. Doxorubicin is an anthracycline antibiotic, the primary mechanism of its action is likely to be the inhibition of DNA biosynthesis via binding to topoisomerase II and consequently conferring a S/G2 cell cycle arrest (Fisher et al. 1993; Miyashita and Reed 1992; Gerwitz 1999). Doxorubicin is used in breast, lung, gastric and ovarian cancer therapy, and in treating acute lymphomas. The American Cancer Society has developed six protocols for the treatment of breast cancer, four of which include doxorubicin (these are the CAF, AC, AC+ and A-CMF protocols, where A=adriamycin). Mitoxantrone is an anthracenedione-derivative, it intercalates into DNA and fragments it via topoisomerase II blockade. Mitoxantrone is less toxic than doxorubicin; it is used in the therapy of metastatic breast cancer and acute myeloid leukaemia.

In a related bioinformatics procedure, we contrasted the cell line-derived gene expression profiles with those of a set of pre-characterised mammary cancer patients (published in Sorlie et al. 2003) to use the molecular signatures for predicting the patient response to chemotherapy in conjunction with prognosis.

5.2.1 Methods

5.2.1.1 Cell Culture

The doxorubicin/daunorubicin and mitoxantrone-resistant derivatives of the human breast cancer cell line MCF7 (Soule et al. 1973) and MDA-MB-231 (Cailleau et al. 1974), the gastric carcinoma cell line EPG85-257P (Dietel et al. 1990), the pancreatic carcinoma cell line EPP85-181P and the colon carcinoma cell line HT-29 (Chen et al. 1987) were established in

our laboratory and have been described previously (Lage et al. 2000; Lage and Dietel 2002). Culture media of resistant cell lines were supplemented with daunorubicin, doxorubicin and mitoxantrone as described in Table 5.1. Daunorubicin and doxorubicin were obtained from Farmitalia Carlo Erba (Freiburg, Germany); Mitoxantrone was purchased from Lederle (Wolfratshausen, Germany).

5.2.1.2 Sample Preparation

We re-examined the drug resistance of each resistant derivative relative to the sensitive parental line by culturing the cells in the presence of drug concentrations ranging from 0.0001 μg/ml to 100 μg/ml for a period of 5 days. Proliferation was assessed using an XTT (3´-1-{(phenylamino)-carbonyl}-3, 4-tetrazolium-bis (4-methoxy-6-nitro) benzenesulphonic acid hydrate) cell proliferation kit following the manufacturer's instructions (Roche, Basal; Roehm et al. 1991).

RNA isolation was performed 24 h after cell seeding from all cell lines. The log-phase growing cells were lysed with TRIzol and RNA was isolated using Fast Track 2.0 messenger RNA (mRNA) isolation kit (Invitrogen Life Technologies, Karlsruhe) according to the manufacturer's instructions. RNA quality of each sample was assessed by visualisation of the 28S/18S ribosomal RNA ratio using the Agilent 2100 Bioanalyser.

5.2.1.3 Array Hybridisation

We interrogated complementary DNA (cDNA) arrays constructed at Stanford University containing 43,009 cDNA clones, representing approximately 30,000 unique genes. The hybridisation was performed as described previously (Alizadeh et al. 2000). The fluorescent images of hybridised microarrays were obtained us-

Table 5.1 Overview of the cell lines investigated in our analyses

Cell line	Array ID	Tumour origin	Relative fold resistance to agent	Selecting agent	Drug concentration of selecting agent	Reference for fold change
MCF7	shcg156	Breast	1		-	
MCF7/Adr	shcg157		192	Doxorubicin	0.05 μg/ml	Fairchild et al. 1987
MDA-MB-231	shcg149	Breast	1		-	
MDA-MB-231RNOV	shcg150		93	Mitoxantrone	0.02 μg/ml	Győrffy et al. 2005a
HT29	shcg143	Colon	1		-	
HT29RDB	shcg146		18	Daunorubicin	0.125 μg/ml	Sinha et al. 1999
HT29RNOV	shcg144		100	Mitoxantrone	0.2 μg/ml	Sinha et al. 1999
EPP85–181P	shbv233	Pancreatic	1		-	
EPP-181RDB	shbv235		1,800	Daunorubicin	2.5 μg/ml	Lage et al. 2002
EPP-181RNOV	shbv234		8	Mitoxantrone	0.02 μg/ml	Lage and Dietel 2002
EPG-257P	shbv236	Gastric	1		-	
EPG-257RDB	shbv225		1,857	Daunorubicin	2.5 μg/ml	Lage et al. 2000
EPG-257RNOV	shbv237		457	Mitoxantrone	0.2 μg/ml	Dietel et al. 1990

ing a GenePix 4000 microarray scanner (Axon Instruments, Sunnyvale, CA). The images of all scanned slides were inspected for artefacts, and aberrant spots and slide regions were flagged for exclusion from analyses.

5.2.1.4 Statistical Analysis

Log2 (Cy5/Cy3) was retrieved for each spot prior to further analysis. Cy5/Cy3 is the normalised ratio of the background-corrected intensities. To reduce the effect of non-specific fluorescence, we filtered spots as follows: the mean background for the red and green signals of each array was determined by calculating the average of the mean background of the corresponding colour intensities of all spots in the array. The net signal was determined by subtraction of this local background from the average intensity of each spot. Then the signals were normalised by applying a single multiplicative factor to all intensities measured for the red dye (Cy5). The normalisation factor was chosen in order to achieve the mean log(Cy5/Cy3) for the spots of 0.0, this effectively defined the signal-intensity-weighted 'average' spot on each array as having a Cy5/Cy3 ratio of 1.0. For identifying predictive genes, the Prediction Analysis for Microarrays (PAM v.1.12) package was used as described previously (Tusher et al. 2001). PAM uses soft thresholding to produce a shrunken centroid, which allows the identification and ranking of genes with high predictive potential. Hierarchical clustering was performed using the Genesis software (Sturn et al. 2002). Univariate Kaplan-Meier analysis was performed by using Winstat for Excel (R. Fitch Software, Staufen, Germany). All hybridisation data are stored in the Stanford Microarray Database (http://genome-www5.stanford.edu/) and in the NCBI Gene Expression Omnibus database (http://www.pubmed.com, GEO dataset: GSE3034). A summary of the applied statistical approach is depicted in Fig. 5.1.

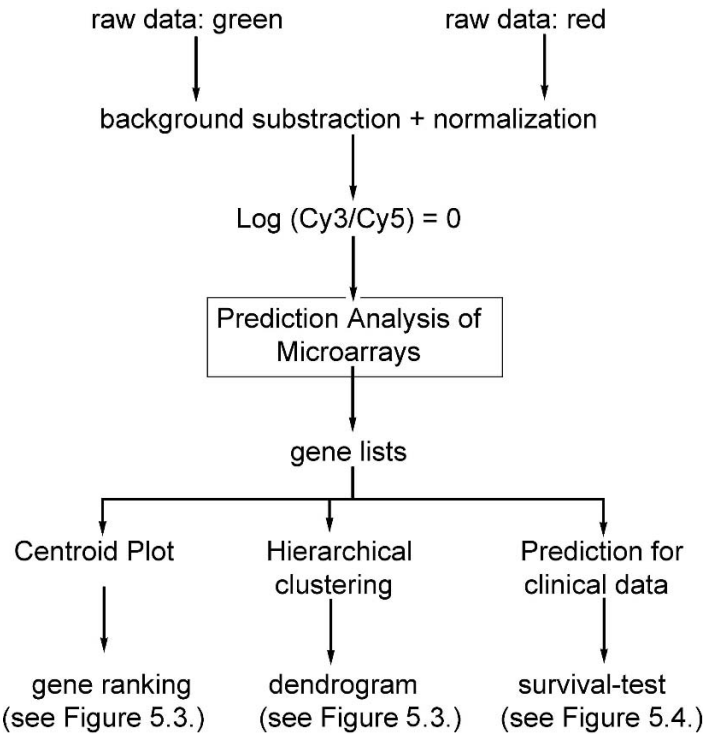

Fig. 5.1 Overview of the applied statistical analysis

5.2.3 Experimental Results

5.2.3.1 Identification of Discriminatory Genes

To identify discriminatory genes for predicting sensitivity to doxorubicin and mitoxantrone, we contrasted the gene expression profiles of closely related drug-resistant and sensitive cancer cell lines. The proliferation characteristics of the HT29 colon cancer cells at increasing drug concentrations is depicted in Fig. 5.2. We confirmed previously published data (Lage et al. 2000; Lage and Dietel 2002) indicating that the resistance pattern is a robust feature upon prolonged cultivation. Table 5.1 summarises the characteristics of the entire set of multi-drug-resistant human cancer cell lines used in our study. For gene expression profiling, RNA was prepared from non-treated parental cells and from resistant derivatives cultured in the presence of daunorubicin and mitoxantrone at a drug concentration which eliminated the majority of sensitive cells.

We divided gene expression profiles obtained for all cell lines into two sets: we compared the expression patterns of the daunorubicin/doxorubicin-resistant cell lines and the mitoxantrone

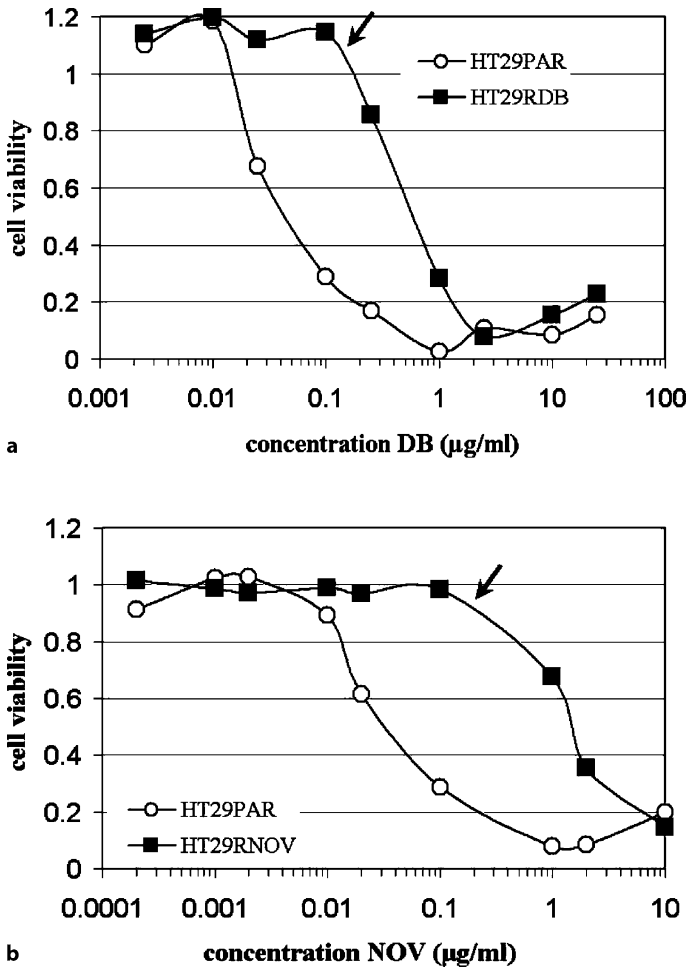

Fig. 5.2 a, b Sensitivity of the resistant HT29RDB, HT29RNOV and the sensitive HT29 colon cell lines against topoisomerase inhibitors daunoblastin (**a**) and mitoxantrone (**b**), as assessed by the XTT cell proliferation kit (Györffy et al. 2005a)

resistant cell lines independently of the parental cell lines. We selected genes that were present in at least three RNA samples within a sample set of four. Remaining in the doxorubicin resistance set were 37,771 sequences; there were 32,943 in the mitoxantrone-resistance set. To select discriminatory genes, we compared the expression profiles of the resistant and parental cell lines. The prediction analysis of microarrays was performed with the genes differentially expressed independently of tissue origin. Centroid plots for the top 30 genes associated with doxorubicin and mitoxantrone resistance are shown in Fig. 5.3A and B. To classify discriminatory genes, we have performed hierarchical clustering on the genes associated with drug resistance. The clustering dendrograms show the 30 genes best associated with doxorubicin resistance and the 30 genes best associated with mitoxantrone resistance.

On our Fig. 5.3 we have combined the centroid plots obtained by the PAM analysis with the clustering results to better visualise and explain the role of individual genes in the resistance signature. Thus, we present the direction, the magnitude and significance side-by-side in one setting. Three different cDNA clones representing the gene encoding the ABCB1 transporter consistently clusters in the doxorubicin-resistant group. This proves the robustness of our results. Additionally, a selected set of genes—including the ABCB1 mRNA—was also validated using TaqMan real-time quantitative RT-PCR (qRT-PCR).

5.2.3.2 Prediction Testing on Breast-Cancer Specimens

We used the gene expression profiles derived from doxorubicin-resistant cell lines for an independent classification of clinical specimens. As a test set for this classification we used 44 breast cancer expression profiles described previously (Sorlie et al. 2003). The data were obtained from the Stanford Microarray Database (http://genome-www5.stanford.edu/). The tumour specimens were selected because the patients had received doxorubicin monotherapy and the survival parameters were reported. Moreover, gene expression profiling of breast cancers were performed on the same technical platform. Recently, a considerable divergence across different platforms has been reported (Tan et al. 2003). Although Sorlie et al. used the same cDNA microarray facility, the proportion of common cDNA clones represented on their arrays was only 16% compared to our study, and most of the selected top sequences were not present. For this reason we performed our prediction independently of the previously selected gene list: first we constructed a matched gene list between our arrays and the arrays used by Sorlie et al. This matched gene list contained 5,923 sequences. Gene sets obtained by comparing the parental and resistant cell lines were used as a training set for the prediction of the clinical samples (test set). For the prediction we applied PAM to classify the patients rather than to build a new predictor based on this limited gene set. Therefore we set the threshold to 1, having about 1,500 genes analysed for the classification. Based on the corresponding expression profiles, we then grouped the patients' tumours as predicted to be doxorubicin-resistant or doxorubicin-sensitive. Afterwards, we performed a survival analysis (see Fig. 5.4). The patients whose tumours were classified as being resistant had a mean survival time of 32.9 ± 18.7 months ($n = 23$), while in the group of patients whose tumours were classified as being sensitive, the mean survival was 49.7 ± 26.1 months ($n = 21$, resistant vs sensitive: $p = 0.034$). Only three patient tumours (7% of total) were classified as sensitive, while the patients died earlier than 2 years after tumour resection. We failed to use the training set of genes related to mitoxantrone resistance for a similar prediction; this was due to the lack of reported clinical data.

5.3 More Studies, More Lists

5.3.1 Doxorubicin Resistance-Associated Signatures

The development of a full chemotherapy-resistant phenotype can be associated with many mechanisms including increased extrusion from the cell, metabolism, phase II conjugation and DNA damage repair. As it is one of the major anti-cancer drugs applied for breast cancer, gene

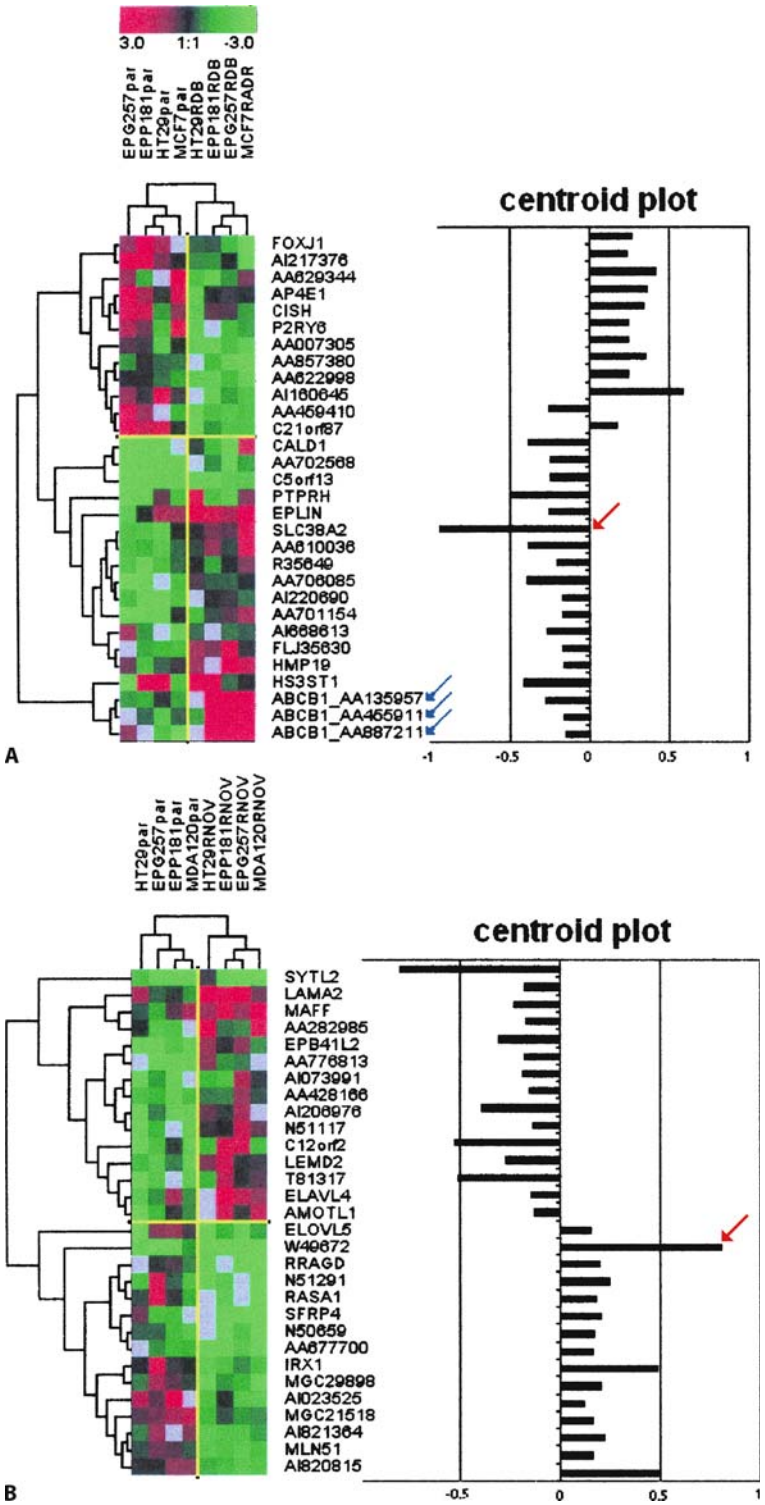

Fig. 5.3 a, b Hierarchical clustering of the top 30 genes associated with doxorubicin (**a**) and mitoxantrone (**b**) resistance. On the *right side the bars* correspond to the centroid plot values of the depicted genes. The most significant gene (with the highest deviation on the centroid plot) is identified by a *red arrow*. Three clones of the ABCB1 gene clustered consequently together in the doxorubicin-resistance associated gene list (*blue arrows*)

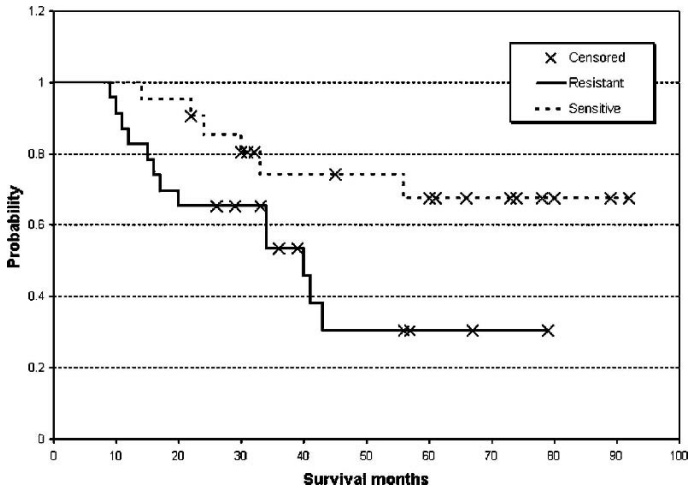

Fig. 5.4 Survival of 44 breast cancer patients receiving doxorubicin monotherapy. The classification of the patients as resistant or sensitive was made using the gene expression signatures established in cell lines (Györffy et al. 2005a). Censored: patients alive at the time of analysis

expression signatures associated with doxorubicin resistance have been extensively studied (Turton et al. 2001; Kudoh et al. 2000).

Kang et al. performed global gene expression analysis using Affymetrix HG-U133A microarrays and identified differentially expressed genes associated with acquisition of chemoresistance to the commonly used drugs 5-fluorouracil, doxorubicin and cisplatin in different human gastric cancer cell lines (Kang et al. 2004). The gene expression patterns of 10 chemo-resistant gastric cancer cell lines were compared with those of four parent cell lines using fold-change and Wilcoxon's test for data analysis. They identified 74 genes differentially expressed in doxorubicin-resistant gastric cancer cell lines.

In another study we tested 30 cancer cell lines for sensitivity to 5-fluorouracil, cisplatin, cyclophosphamide, doxorubicin, etoposide, methotrexate, mitomycin C, mitoxantrone, paclitaxel, topotecan and vinblastine at drug concentrations that can be systemically achieved in patients (Györffy et al. 2006). First, using a resistance index the cell lines were designated as sensitive or resistant, then the subset of resistant vs sensitive cell lines for each drug was compared. Gene expression signatures for all cell lines were obtained by interrogating Affymetrix U133A arrays. An individual prediction profile for the resistance against each chemotherapy agent was constructed, containing 253 genes associated with doxorubicin resistance. The overall accuracy of the predictions in a leave-one-out cross validation was 86%.

Suganuma et al. investigated gene expression signatures related to chemoresistance towards cisplatin, doxorubicin, mitomycin C and 5-fluorouracil in gastric cancer (Suganuma et al. 2003). However, the 20 specimens analysed in their study exhibited drug resistance, and none of them was sensitive to drug treatment. Therefore, a list of candidate genes specifically associated with doxorubicin resistance could not be established.

Ayers et al. have developed a multi-gene predictor of pathologic complete response to sequential weekly paclitaxel and fluorouracil+doxo rubicin+cyclophospahmid (T/FAC) neoadjuvant chemotherapy regimen for breast cancer using 42 patients' clinical results and cDNA arrays containing approximately 31,000 genes (Ayers et al. 2004).

Another recent microarray study published by Troester et al. correlated in vitro response to

chemotherapy in four cell lines to response in the breast tumours (Troester et al. 2004). The common discriminatory genes in their study do not overlap with our above-described results. The reason for this is probably due to the study design: While they have investigated treatment response after 12-, 24- and 36-h drug administration, we have focussed on the pre-existing characteristics of the resistant cell lines. Although short- and long-term drug administration may have different effects on gene expression, both sets of responsive genes could be used in predictive tests.

5.3.2 Overlapping Gene Lists

We have compared all these published sets of differentially regulated genes with each other, and we have detected a set of 52 genes described in more than one study. These results are in line with the findings of a recent study demonstrating that different gene signatures can achieve similar prediction success for the same classification problem (Ein-Dor et al. 2005). Additionally, alternative microarray platforms may yield different results (Tan et al. 2003).

Similar results were obtained in several papers describing different experimental problems. These raised concerns about the reliability of experimental results obtained using different microarray platforms. However, a recent study demonstrated that although there are relatively large differences in data obtained in labs using the same platform, the results from the best-performing labs agree rather well (Irizarry et al. 2005). Moreover, biological treatment has a far greater impact on measured expression than has the use of different platform for more than 90% of genes. In the small number of cases in which different platforms yield discrepant results, qRT-PCR generally did not confirm either set of data, suggesting that sequence-specific effects may make expression predictions difficult using any technique (Larkin et al. 2005). Another study demonstrated that microarray results are comparable across multiple laboratories, especially when a common platform and set of procedures are used. Reproducibility is the highest when analysis is based on biological themes defined by enriched gene ontology (GO) categories (Bammler et al. 2005).

In summary, we can expect to have different gene lists when using different microarray platforms for the same biological samples. Therefore, repeated observations and application of established signatures for prediction and classification of new test sets require the use of the same microarray platform and the same protocols. Thus, the detailed description of the spotting, hybridisation and scanning characteristics is desirable instead of using a short reference to another publication.

5.4 Summary and Perspectives

In our study we compared gene expression profiles of drug-sensitive and closely related drug-resistant cell lines of different origin. We identified the top genes associated with doxorubicin and with mitoxantrone resistance. We grouped the already identified genes involved in resistance against doxorubicin and mitoxantrone into functional classes comprising elements involved in signalling, cell migration, transport, apoptosis and detoxification. We retrieved additionally a high proportion of differentially expressed sequences without known function. Of the sequences, 49% that were associated with doxorubicin resistance and 44% that were related to mitoxantrone resistance were expressed sequence tags (ESTs), which might represent novel genes associated with cytostatic multi-drug resistance. This confirms previous data (Turton et al. 2001) and supports the notion that MDR is highly complex, and the combination and co-operation of several pathways is necessary to acquire the chemoresistance. Thus, the investigation of individual genes will not provide enough information for effective clinical classification.

We have designed our study to set up a gene expression signature that could be used in a predictive test for the investigated chemotherapy agents. Our study shows that DNA microarray technology can be effectively used for predicting the response to chemotherapy and indirectly patient prognosis. However, we must note that a large cohort of patients needs to be investigated before actual clinical tests can be developed based on current microarray results. Finally, we have to emphasise the role of different microarray platforms: A clinical test can only be developed on

the platform which was applied for the investigation of the training set.

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6 Resistance to Chemotherapy in Ovarian Carcinoma

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Abstract

Resistance to cytotoxic chemotherapy is the main cause of therapeutic failure and death in women suffering from ovarian carcinoma. The standard first-line chemotherapy of ovarian cancer consists of a combination of a taxane and a platinum-containing drug. Thus, the cellular and molecular mechanisms involved in resistance against these compounds are of vital importance in the context of chemotherapy of ovarian cancer. This review will discuss the current state of knowledge of drug resistance-associated factors and their impact on clinical chemotherapy response in ovarian carcinoma as well as different strategies for reversal of drug resistance.

6.1 Introduction

Although ovarian cancer has less than a 1% lifetime risk, it remains the No. 1 gynaecological killer in the Western world (Stewart and Kleihues 2003). Epithelial ovarian cancer comprises the majority of malignant ovarian neoplasms. They are classified into distinct morphologic categories based on the appearance of the epithelium into tumours of serous, mucinous, endometrioid, clear cell, transitional, squamous, mixed and undifferentiated type (Lee et al. 2003).

Only 15%–30% of women in advanced stages, i.e. Federation Internationale de Gynecologie et d'Obstetrique (FIGO) stage III or IV, will survive 5 years, whereas 95% of the women in FIGO stage I at the time of diagnosis are likely to be alive in 5 years, and most are cured following surgery (Ozols et al. 2001). Most ovarian cancer patients suffering from advanced-stage disease are treated with cytoreductive surgery followed by a combination chemotherapeutic regimen. To date, the standard first-line chemotherapy consists of a combination of a taxane, i.e. paclitaxel, and a platinum-containing drug, i.e. carboplatin. Optimal cytoreductive surgery and this combination chemotherapy results in a response rate of 75%, a clinical complete remission rate of approximately 50% and a pathologic complete response rate of 25%–30% (Ozols 2002). However, the median time to progression is only 18 months, and most of these patients still suffer relapse and die of the disease.

The high mortality of ovarian carcinoma is in part due to the rapid progression of the malignancy resulting in diagnosis in an advanced stage of disease, as well as due to the emergence of drug resistance, which limits the treatment success of recurrent disease. The development of drug resistance is a common observation following chemotherapy, although most ovarian carcinomas are sensitive to drug treatment at the beginning of the therapy. Resistance of ovarian carcinoma to taxanes and platinum drugs will be discussed in detail.

6.2 Taxanes Used for Treatment of Ovarian Carcinoma

Two taxanes are currently available for treatment of ovarian cancer, paclitaxel (Taxol) and docetaxel (Taxotere). Paclitaxel was originally isolated from the bark of the Pacific yew tree

Taxus brevifolia (Wani et al. 1971). Docetaxel comes from the needles of the yew tree. Although there are several differences in the pharmacokinetics and pharmacologic actions of the two taxanes, they share action mechanisms. They exert their anti-neoplastic effects primarily by binding to polymerized β-tubulin and stabilizing microtubules against disassembly. This causes cell cycle arrest at the G_2-M stage and ultimately leads to apoptosis in p53-dependent and p53-independent pathways (Gligorov and Lotz 2004). Both taxanes induce a number of other molecular events. For example, they induce phosphorylation and, therewith, inactivation of the anti-apoptotic protein Bcl-2 and dephosphorylation of the pro-apoptotic factor BAD (Bcl-2-associated death protein) leading to increased tumour cell apoptosis (Haldar et al. 1997). Furthermore, it was demonstrated that microtubule-associated proteins (MAPs) (Wahl et al. 1996) and the microtubule-bound ATPase LC8 (Puthalakath et al. 1999) are involved in taxane-dependent triggering of cellular pathways leading to apoptosis.

6.3 Platinum-Containing Drugs Used for Treatment of Ovarian Carcinoma

In the last few years, the platinum-containing drug cisplatin was replaced in the treatment of ovarian carcinoma by its analogue carboplatin. Carboplatin differs from cisplatin in a closed cyclobutane dicarboxylate moiety on its leaving arm instead of the chloride ligands of cisplatin. The result is an altered DNA binding kinetics, though carboplatin forms the same reaction products at equivalent doses. After the entry of the neutral square-planar platinum (II) complex cisplatin or carboplatin into the cell, binding of the ligands is destabilized. In the case of cisplatin, a water molecule displaces one of the chlorides. As a consequence, the drug becomes a monoaquated, charged electrophile that can react with nucleophilic sites of intracellular macromolecules such as DNA. The second reactive site of cisplatin can lead, with lower kinetics, to the formation of several types of bifunctional platinum adducts with DNA bases. Although only about 1% of intracellular cisplatin reacts with nuclear DNA, this is presumably the critical event in cisplatin-mediated cytotoxicity (Gonzalez et al. 2001). The two major adducts are Pt-d(GpG) and Pt-d(ApG) intra-strand cross-links, representing about 90% of DNA platination. Other lesions include Pt-(dG) monoadducts, Pt-d(GpNpG) intra-strand cross-links and $Pt(dG)_2$ inter-strand cross-links (Fichtinger-Schepman et al. 1985). Formation of these platinum–DNA adducts per se may not be sufficient to cause cell death, whereby the exact cascade of the downstream events leading to cell death is not clear. However, it is generally accepted that formation of platinum–DNA adducts and the subsequent triggering of cellular signal-transduction pathways leading to apoptosis may be the primary cytotoxic mechanism of platinum-containing drugs (Kaufmann and Vaux 2003).

6.4 Mechanisms of Drug Resistance

In practice, the phenomenon of drug resistance becomes a crucial problem when toxicity of the drugs at dosages necessary to kill cancer cells increases to a non-manageable clinical situation. Since most anti-neoplastic agents, including taxanes and platinum-containing drugs, have a low therapeutic index, even a weak decrease in the sensitivity of the ovarian cancer cells can manifest a clinically drug-resistant phenotype. The mechanisms underlying this therapy failure can be classified into two broad categories, pharmacological and cellular factors.

6.5 Pharmacological Mechanisms of Drug Resistance

Chemotherapy should be given at the maximum tolerated dose (MTD) to achieve maximum tumour cell killing. A tumour is deemed clinically resistant to the MTD if the effective drug dosage in the tumour, given as the area under the curve (AUC=drug concentration×time of drug exposure), is not efficient at achieving a cytostatic or cytotoxic effect within the neoplastic cells. This therapeutically insufficient drug dosage may be due to different physiological mechanisms that can be summarized as pharmacological mechanisms of drug resistance. These mechanisms include (1) the application of the drugs, e.g. inadequate infusion; (2) low metabolic activation in

the case of utilization of pro-drugs; (3) the pharmacokinetics in the plasma, i.e. metabolisms and excretion of the drugs; (4) the tumour microenvironment, e.g. vascularization, diffusion, hypoxia; and (5) the availability, i.e. the architecture of barriers. However, herein we will focus on the cellular mechanisms of drug resistance.

6.6 Cellular Mechanisms of Drug Resistance

In addition to these pharmacological mechanisms of drug resistance, various cellular mechanisms taking place directly within the tumour cell have been described. In the last four decades dramatic progress has been made in the understanding of cellular drug resistance-associated genes, proteins and their mechanisms of action (Gottesman 2002). Due to the inherent difficulties of investigation drug resistance directly in the patient, a large number of in vitro models have been developed and characterized from different tissue origins including ovarian carcinoma. Commonly, drug-sensitive ovarian carcinoma cells were exposed to stepwise increasing, sublethal dosages of a single anti-cancer drug, e.g. cisplatin or paclitaxel. The result was an acquired drug-resistant phenotype, often with cross-resistance to a variety of structurally and functionally unrelated drugs, a phenomenon commonly called multidrug resistance (MDR).

Transferred to the clinical situation, it was assumed that an acquired cellular drug resistance develops as a result of progressively gained somatic mutations and epigenetic alterations in

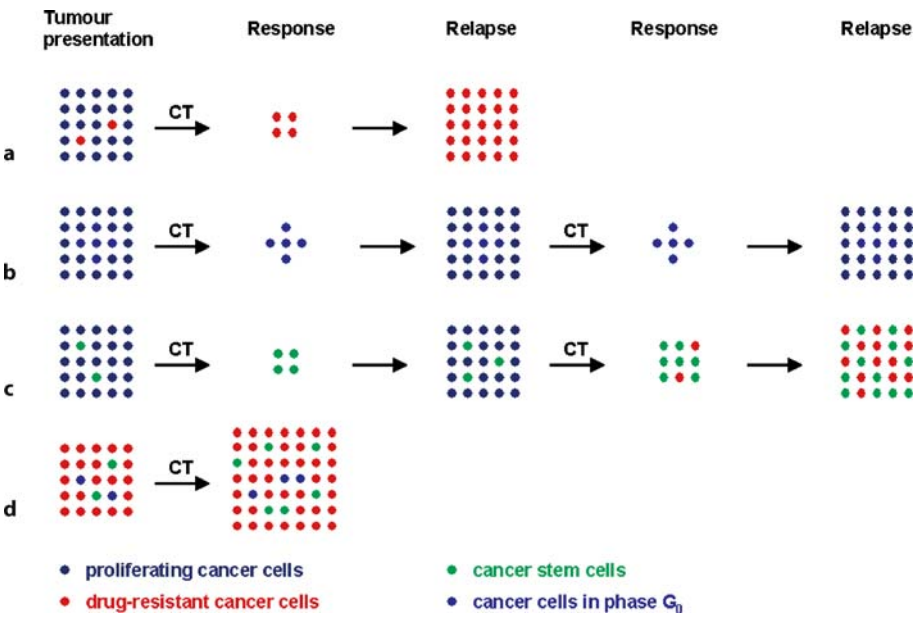

Fig. 6.1 a–d Models of cellular drug resistance in ovarian carcinoma. **a** The conventional somatic mutation model. Genetically changed cancer cells exhibiting a drug-resistant phenotype (*red*) can persist in the proliferating, drug-sensitive tumour cell population (*blue*) or can occur following drug exposure. **b** The model of quiescent cells starts from the assumption that cancer cells in phase G_0 (*lilac*) show a drug-resistant phenotype because cytotoxic anti-cancer drugs commonly attack proliferating cells (*blue*). **c** In the stem cell model, the tumour consists of cancer stem cells (*green*) exhibiting a drug-resistant phenotype due to their ABC-transporter content, their capacity for DNA repair, and the high proportion of cells in phase G_0, and cycling cancer cells (*blue*). **d** In the model of intrinsic cellular drug resistance, the tumour tissue contains different drug-resistant cell types, proliferating naturally drug-resistant cancer cells (*red*), cancer stem cells (*green*) and cells in phase G_0 (*lilac*). In reality, probably all processes are occurring simultaneously, but the relative percentages vary from tumour to tumour. Thus, an individual clinical response to chemotherapy and an individual pattern of relapse may be determined. *CT*, chemotherapy

the tumour over the time (Nowell 1976). In this conventional somatic mutation model of cellular drug resistance (Fig. 6.1A), one or more cancer cell clones acquire genetic changes that confer a drug-resistant phenotype by activation of various molecular mechanisms (see below). These genetic alterations can be coincidentally present within the tumour cell population or can be induced by the drug treatment. The genetically altered cancer cells have a selective advantage that helps them to sustain exposure to cytotoxic anti-cancer drugs.

An alternative model, the model of quiescent cells takes into account that anti-cancer drugs, including taxanes and platinum-containing compounds, primarily attack proliferating cells. However, a tumour is heterogeneous and consists of cycling cells and a significant contingent of cells in a quiescent state. Thus, these cancer cells in phase G_0 exhibit a higher level of drug resistance relative to proliferating cells (Schwartz and Shah 2005). The non-dividing tumour cells can survive chemotherapy and the tumour can relapse (Fig. 6.1B). This alternative model can explain the observation that patients who relapse after first-line chemotherapy can often be retreated with the same chemotherapeutic regiment and achieve a complete response.

A further model considers that a cancer cell population of a solid tumour can contain cells with stem-cell qualities (Al-Hajj et al. 2004). These cancer stem cells have many properties that separate them from differentiated cancer cells. They have the ability to self-renew, to differentiate, and most important in the context of drug resistance they express high levels of MDR-associated ATP-binding cassette (ABC)-transporters (Dean et al. 2005). Basing on this tumour stem cell concept, the alternative model supposes that the cancer stem cells are intrinsically drug-resistant through their ABC-transporter content, their capacity for DNA repair and the high proportion of cells in quiescent phase G_0. Thus, drug treatment does not kill the cancer stem cells and the cells support relapse of the tumour (Fig. 6.1C).

In the model of intrinsic cellular drug resistance, the tumour primarily consists of different drug-resistant cell types, intrinsic drug-resistant cancer cells that already express one or more drug resistance-mediating proteins or pathways, cancer stem cells, and cells in phase G_0. As a result, chemotherapy has no effect and the cells can proliferate (Fig. 6.1D).

In clinics, the processes of all four models may occur simultaneously and in concurrence, but the relative percentages vary from tumour to tumour. In this way, differences in the clinical response to chemotherapeutic treatment and an individual pattern of progression and relapse may be determined.

6.7 Resistance to Taxanes

As natural product-derived compounds, taxanes are good substrates of ABC-transporters, in particular MDR1/P-glycoprotein (MDR1/P-gp) or ABCB1 (Fig. 6.2A) (Gottesman 2002). MDR1/P-gp causes the so-called "classical" MDR. This phenotype is characterized by a typical cross-resistance pattern against natural-product anticancer agents, such as Vinca alkaloids, anthracyclines or taxanes, and the reversibility by the calcium channel inhibitor verapamil and cyclosporin A derivatives (Lage 2003). The membrane embedded ABC-transporters can act as energy-dependent xenobiotic efflux pumps. The drug extrusion activity of these transporters results in a decreased intracellular concentration of anti-cancer drugs. Although various studies demonstrated that MDR1/P-gp confer cellular drug resistance to ovarian carcinoma cells in vitro (Bourhis et al. 1989; Schondorf et al. 2003), the impact in clinical drug resistance of ovarian cancer remains ambiguous (Arts et al. 1999; Surowiak et al. 2006a). Other important MDR-mediating ABC-transporters such as MRP1 (ABCC1) or BCRP (ABCG2) do not seem to play a role in the cellular resistance against taxanes.

Furthermore, resistance of ovarian carcinoma cells against taxanes can be mediated by decreasing the concentration of the target molecules and by alterations in the binding affinity of the anticancer drug to the cellular target molecules. Thus, in vitro experiments using paclitaxel-resistant ovarian carcinoma cell lines identified a decrease of tubulin expression, as well as changes in the isotypes of tubulin subtypes (Kavallaris et al. 1997). Furthermore, paclitaxel resistance in ovarian carcinoma cells was conferred by mutations within the β-tubulin encoding gene that result in altered paclitaxel-mediated tubulin po-

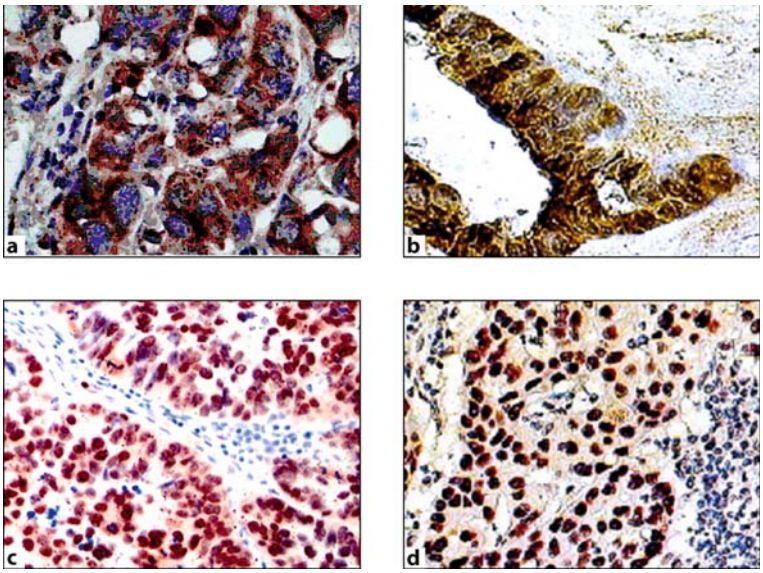

Fig. 6.2 a–d Examples of immunohistochemical staining of drug resistance-associated factors in specimens of ovarian carcinoma (kindly provided by Dr. P. Surowiak). **a** MDR1/P-gp; **b** TRAG-3; **c** MRP2; **d** MLH1

lymerization (Giannakakou et al. 1997). Since no mutations within the β-tubulin gene have been described to date in patients suffering on ovarian cancer, the clinical impact of these in vitro observations is unclear (Sale et al. 2002).

Another factor found to be over-expressed in paclitaxel-resistant ovarian carcinoma cell lines is taxol resistance-associated gene 3 (TRAG-3) (Fig. 6.2B), a protein of unknown biological function and with an unclarified role in the resistance mechanism (Duan et al. 1999). Probably, it may be part of a multi-component mechanism leading to paclitaxel resistance. Though preliminary data showed that TRAG-3 expression may be unfavourable for clinical response to paclitaxel-based treatment regiments in ovarian carcinoma patients (Materna et al. 2007), a potential clinical impact has to be confirmed in further studies.

6.8 Resistance to Platinum Drugs

The platinum-containing drugs cisplatin and carboplatin are both substrates of the ABC-transporter MRP2 (ABCC2) (Fig. 6.2C). Over-expression of this drug transporter was found in various cisplatin-resistant ovarian carcinoma cell lines (Taniguchi et al. 1996; Materna et al. 2005). Recent data obtained in cisplatin-resistant ovarian carcinoma cells indicate that not only the over-expression of MRP2 is relevant for the drug-resistant phenotype, but also the subcellular localization (Surowiak et al. 2006b). If MRP2 was localized in the nuclear membrane of ovarian carcinoma cell lines, this localization correlated with the level of cisplatin resistance. Furthermore, the expression level of MRP2 (Materna et al. 2004) as well as the nuclear localization of MRP2 (Surowiak et al. 2006b) correlated with the clinical response of ovarian carcinoma patients to treatment with platinum drug-based chemotherapeutic schemes. However, alternative studies using specimens obtained from ovarian carcinoma patients could not confirm the impact of MRP2 expression (Arts et al. 1999; Ohishi et al. 2002). Thus, the clinical importance of MRP2 expression and its sub-cellular localization in ovarian carcinoma tissue has to be elucidated in further studies.

By conjugation of platinum drugs to glutathione, these anticancer compounds can be inactivated in ovarian carcinoma cells (Godwin et al. 1992). The conjugation reaction forming the drug-glutathione complexes is catalysed by members of the family of glutathione S-trans-

ferases (GST). Since MRP2 pumps its substrates preferentially as drug conjugates, especially as drug-glutathione complexes, it is not astonishing that elevated glutathione levels were found in cisplatin-resistant MRP2-positive ovarian carcinoma cells (Materna et al. 2005). Controversial data are available concerning whether glutathione concentration (Cheng et al. 1997) or GST expression (Ferrandina et al. 1997; Surowiak et al. 2005) plays an important role in the clinical management of ovarian carcinoma. However, recent data indicate that the reasons for these discrepancies may be functional polymorphisms that alter GST activity (Beeghly et al. 2006).

The cytotoxic effects of platinum-containing drugs in ovarian carcinoma cells are mediated by the formation of platinum–DNA adducts that are triggering cellular death pathways. Thus, resistance against these anti-cancer agents can also be mediated by DNA–platinum adduct-induced activation of DNA repair pathways. These include enhanced nucleotide excision repair (NER) (Dijt et al. 1988) and decreased activity of the DNA mismatch repair (MMR) system (Lage and Dietel 1999), e.g. by diminished expression of the MMR enzymes MLH1 (Fig. 6.2D) or MSH2. The phenomenon of drug resistance by loss of MMR activity is caused by a decreased MMR-dependent stimulation of signal-transduction pathways causing apoptosis. However, the impact of these DNA repair systems, NER (Dabholkar et al. 1992) and MMR (Samimi et al. 2000), for clinical platinum resistance in ovarian cancer remains to be better clarified.

Furthermore, quite a number of additional mechanism of platinum drug resistance have been described. These mechanisms include an increased capacity for platinum–DNA adduct tolerance (Parker et al. 1991; Mamenta et al. 1994), detoxification by metallothioneins (Kelley et al. 1988), or alterations in regulatory proteins, e.g. in the oncogene encoded factor c-fos (Scanlon et al. 1991).

6.9 Common Mechanisms of Taxanes and Platinum Drug Resistance

As discussed previously, both classes of anti-neoplastic agents commonly used in chemotherapy of ovarian cancer, taxanes and platinum drugs, in the end induce cellular pathways leading to apoptosis. Consequently, inhibition of pro-apoptotic pathways and activation of anti-apoptotic pathways can lead to drug resistance in ovarian carcinoma. Various in vitro models demonstrate that a critical balance exists between cell cycle arrest allowing DNA repair and drug resistance, and triggering of apoptosis and thereby sensitivity against drug treatment. In the tumour cells, an intricate network of factors and pathways has evolved to control this balance. Response to chemotherapy may also be affected through enhanced synthesis of additional anti-apoptotic factors, or the reduced activity of pro-apoptotic proteins.

A central molecule involved in the regulation of cell cycle arrest and apoptosis is the most frequently mutated gene in human cancers, *p53*. As expected, in vitro experiments using ovarian carcinoma cell lines demonstrated that *p53* mutations conferred resistance to cisplatin as a consequence of loss of the ability of p53 to transactivate apoptosis-triggering genes (Perego et al. 1996). Active p53 can induce the expression of mitochondrial pro-apoptotic genes, e.g. *BAX*, *NOXA*, *PUMA* and *p53AIP1*, and those in the death receptor pathway including *CD95*, *TRAIL-R1* and *TRAIL-R2*. Furthermore, mutations in the p53 gene of patients receiving cisplatin therapy were found to be associated with lack of clinical response (Righetti et al. 1996). On the other hand, it was demonstrated that ovarian carcinoma cell lines with *p53* mutations were resistant against cisplatin as expected, but showed increased sensitivity against taxanes (Cassinelli et al. 2001). This in vitro observation was confirmed in a clinical setting (Lavarino et al. 2000). Accordingly, it should be taken into consideration that platinum drug-resistant ovarian cancers with dysfunctional p53 may be preferential suitable for taxane-based therapy. In further investigations the potential impact of this option have to be clarified.

An important role for the clinical outcome of ovarian carcinoma following chemotherapy has also been associated with various other apoptosis-regulating genes including *Bcl-2* (Mano et al. 1999), *Bcl-XL* (Williams et al. 2005), *BAX* (Schuyer et al. 2001), *CDK1A* (*p21*) (Scambia et al. 1997), *CDK1B* (*p27*) (Goff et al. 2001), *survivin* (Zaffaroni et al. 2002), *XIAP* (Yang et al. 2005) and components of the phosphatidylinositol 3-kinase (PI3K)/AKT

signalling pathway (Mills et al. 2001; Vivanco and Sawyers 2002). However, more studies analysing the exact role and the clinical importance of these factors for the chemotherapeutic treatment of ovarian carcinoma will provide more information in the future.

6.10 Strategies for Overcoming Clinical Drug Resistance of Ovarian Carcinoma

Since MDR1/P-gp was identified as the first important cellular factor mediating multi-drug resistance including resistance against taxanes to cancer cells, this trans-membranous ABC-transporter has become the most investigated drug resistance-associated protein. The early studies of MDR1/P-gp gave rise to the optimistic assumption that reversal of drug resistance might be a realistic option. As a consequence, different strategies for reversal of drug resistance have been extensively studied for more than two decades, originally aimed at the inhibition of MDR/P-gp. In the meantime, several reversal approaches have been made to target different drug resistance-associated factors simultaneously. Strategies to influence function or expression of drug resistance factors include low molecular weight pharmacological active compounds inhibiting the drug transport, and experimental approaches, e.g. the employment of specific antibodies, or modulation of gene expression with antisense oligonucleotides and ribozymes, or the triggering of the gene-silencing RNA interference (RNAi) pathway (Lage 2006).

For clinical applications, trials were made with small molecule first-, second-, and third-generation inhibitors of MDR1/P-gp. First-generation inhibitors, i.e. the calcium channel blocker verapamil and the immunosuppressive agent cyclosporine A, are substrates for MDR1/P-gp, and compete with anti-neoplastic agents for binding and efflux by the pump protein. These inhibitors worked very well in vitro, but they produced disappointing results in clinical settings, dampening the initial optimism of potentially overcoming drug resistance (Kellen 2003).

The second generation inhibitors, e.g. the non-immunosuppressive cyclosporine analogue valspodar (PSC833), and biricodar (VX710), were developed to specifically inhibit MDR1/P-gp. They act as competitive substrates, but showed less toxicity and greater efficacy. Although these compounds reversed drug-resistant phenotypes in clinical trials, problems arose, including toxicity and interaction with additional transporter proteins (Thomas and Coley 2003).

The newest class of drug resistance modulators, the third-generation inhibitors tariquidar (XR-9576) or zosuquidar (LY335979), show fewer side-effects than their predecessors. These compounds are not MDR1/P-gp substrates. They bind non-competitively to the transporter and thereby cause conformational changes of the molecule, and hinder ATP hydrolysis. Consequently, drug transport out of the cell is prevented, leading to increased intracellular drug concentration and enhanced cytotoxicity. Currently, tariquidar is under evaluation in several clinical trials including a National Cancer Institute (NCI) phase II study for ovarian carcinoma (ClinicalTrials.gov identifier NCT00069160).

6.11 Conclusions

Taken together, the emergence of drug resistance still remains the major cause for failure of chemotherapeutic treatment of ovarian cancer. In the last few years, basic research approaches have elucidated several molecular mechanisms contributing to cellular drug resistance. Up to now, however, there is no molecular marker or signature available that is able to predict the development of drug-resistant phenotypes in clinical practice. Furthermore, several attempts to reverse resistance to chemotherapy by pharmacological interventions have not been successful in clinics. Further studies should focus on translational projects associated with clinical studies to investigate putative molecular predictive markers or signatures.

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7 Clinical, Biological, and Molecular Aspects of Metastasis in Colorectal Cancer

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Abstract

Colorectal cancer is one of the most frequent malignant tumors with a still increasing incidence in Western countries. Currently, colorectal cancer is the second most common cancer in Europe both in terms of incidence and mortality. Approximately 90% of all cancer deaths arise from the metastatic dissemination of primary tumors. Thus, metastasis is the most lethal attribute of colorectal cancer. Today, colorectal cancer and metastasis thereof are understood as the results of early changes during tumor progression that determine the metastasis capacity. Much is known about molecules contributing to the metastasis phenotype, the pathways they control, and the genes they regulate. However, patient prognosis is mainly defined by histopathological staging, a static description of the anatomical extent of tumor spread within a surgical specimen. This review demonstrates the need for and possibilities of molecular-based staging as an essential prerequisite for improved diagnosis, prognosis, and therapy. Molecular determinants for progression and metastasis of colorectal cancer are discussed representing both potential markers for metastasis prognosis and targets for intervention strategies aiming at the ultimate goal of metastasis prevention.

7.1 Abbreviations

- APC Adenomatous polyposis coli
- CAM Cell adhesion molecule
- DCC Deleted in colorectal cancer
- ECM Extracellular matrix
- EMT Epithelial-mesenchymal transition
- FAP Familial adenomatous polyposis coli
- HGF/SF Hepatocyte growth factor/scatter factor
- MMP Metalloproteinases
- N Lymph node metastasis
- M Distant metastasis
- sLe^X Sialyl Lewis X antigen
- T Tumor infiltration depth
- VEGF Vascular endothelial growth factor

7.2 Introduction

7.2.1 Colorectal Cancer: Incidences and Mortalities

Colorectal cancer is one of the most frequent malignant tumors with a still increasing incidence in Western countries (Parkin et al. 2005). In the United States, this disease accounts for 10% of all cancers, and is currently the second and third leading cause of cancer-related death of men and women, respectively (both sexes combined, 10%) (Jemal et al. 2006). In Europe, colorectal cancer was the second most common cancer both in terms of incidence (376,400 cases, 13%) and mortality (203,700 deaths, 12%) in 2004 (Boyle and Ferlay 2005). In Germany, colorectal cancer was also listed among the 20 most frequent causes for cancer deaths in 2003, with 18% in men, second after lung cancer, and with 11% in women, following breast and lung cancer (www.dkfz-heidelberg.de). It was calculated that about 5 million people in Germany, or 6% of the popu-

lation, will suffer from colorectal cancer in the course of their lifetime. Thus, increased efforts for understanding and preventing the disease are extremely desired.

7.2.2 Colorectal Cancer Metastasis: The Major Cause of Death

Approximately 90% of all cancer deaths arise from the metastatic dissemination of primary tumors (Christofori 2006). Although about 50% of the tumors can be treated curatively by surgery alone, metastatic growth is the major factor that compromises the successful treatment and outcome of colorectal cancer. Today, patient prognosis is mainly defined by histopathological staging, a static description of the anatomical extent of tumor spread within a surgical specimen. Tumor infiltration depth (T), lymph node metastasis (N), and distant metastasis (M) are the characteristics defining the UICC/AJCC tumor stages (Table 7.1; Beahrs 1992):

I. T1–2 N0 M0
II. T3–4 N0 M0; subdivided into IIa (T3 N0 M0) and IIb (T4 N0 M0)

All early tumor stages are without metastasis in either the lymph nodes or distant organs.

III. T1–4 N1 M0; with development of lymph node metastases, subdivided into IIIa (T1–2 N1 M0), IIIb (T3–4 N1 M0)
IV. T1–4 NO-1 M1; the late tumor stage with distant metastases formation

Although lymph node metastasis represents the primary indicator for systemic disease spread, distant metastases formation constitutes the most important prognostic factor in colorectal cancer. We demonstrated the disadvantageous prognosis and mostly lethal impact of distant metastases formation in our study by analyzing survival data of 1,132 colorectal cancer patients over 60 months, classified according to the histopathological staging parameters (Fig. 7.1). Remarkably, development of distant metastasis was the main criterion that significantly determined the survival of colorectal cancer patients ($p = .0001$) despite the considerable prognostic heterogeneity of patients—not all patients without metastasis could be cured and not all patients with metastasis died from their disease. Thus, metastasis is the key event in colorectal cancer. It is the process that needs to be examined in further detail for generation of intervention or even prevention strategies, but is also the decisive parameter for outcome and success of cancer therapy.

Colorectal carcinomas possess preferences regarding the target organs of metastasis and show distinct frequencies for time point and target organs of distant metastasis formation. At the time of presentation, about 25% of patients have detect-

Table 7.1 Classification of colorectal cancer according to T-N-M categories

Tumor stage	T	N	M
I	1, into submucosa or	0	0
I	2, into muscularis propria	0	0
II	3 or 4	0	0
IIa	3, into subserosa	0	0
IIb	4, through serosa	0	0
III	1, 2, 3, or 4	1	0
IIIa	1 or 2	1	0
IIIb	3 or 4	1	0
IV	1, 2, 3, or 4	0–1	1

M, distant metastasis, spread to distant organs; **N**, lymph node metastasis, spread to nearby lymph nodes; **T**, tumor infiltration depth, extent of spread through tissue layers of colon and rectum

able liver metastases (synchronous metastases). A further 25% of patients will develop later metastases during the course of their disease (metachronous metastases), usually within a 2-year period following initial surgery of their primary tumor (Millikan et al. 1997). Besides liver metastasis (metastasis frequency 30%–70%), colorectal carcinoma spreads with 20%–40% frequency into the lung, with 5%–10% into the bone, and with lower frequencies into brain, adrenal gland, and ovary. The stable frequencies for metastases formation in specific target organs suggest a molecular background that determines metastasis tropisms. These observations point to the necessity for a more specific and molecular-based classification system for colorectal cancer, supporting diagnosis, prognosis, as well as treatment planning, which will finally improve outcome.

7.3 Metastasis: Both Process and Outcome of the Process

7.3.1 Hallmarks of Cancer Cells

Hanahan and Weinberg have summarized the most important hallmarks of cancer cells as being immortality due to limitless replicate potential, abnormal growth regulation due to insensitivity to antigrowth signals, self-sufficiency in growth signals, evading apoptosis, sustained angiogenesis, and, invasion and metastasis (Hanahan and Weinberg 2000). However, do all criteria hold true for all cancers? Although metastasis is the most lethal attribute of cancer, is metastasis an inherent feature of all cancer cells? Some malignant tumors are invasive and/or metastatic, others are highly aggressive developing metastases

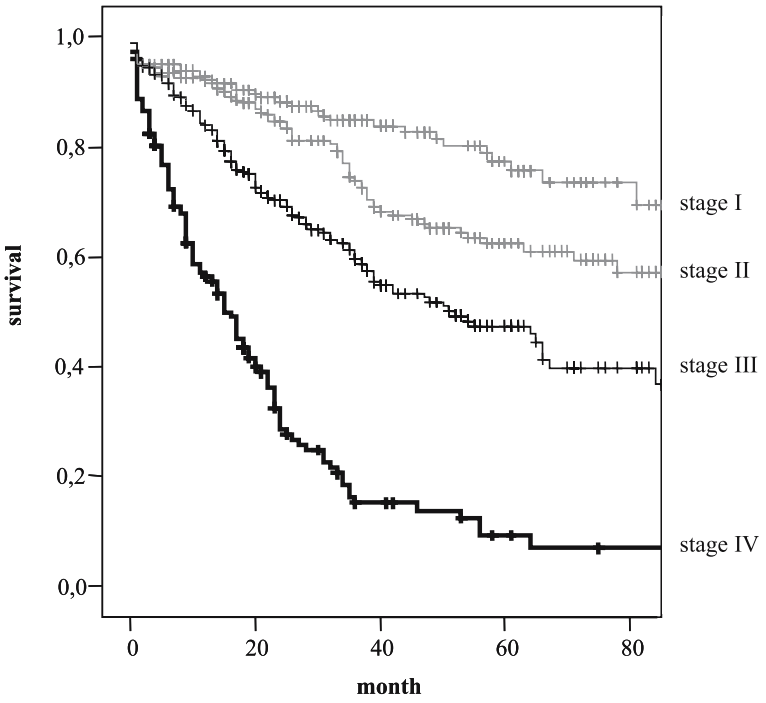

Fig. 7.1 Distant metastasis formation significantly determines survival of colorectal cancer patients. Survival of 1,132 colon cancer patients, Berlin-Brandenburg, 1996–2000, classified according to UICC/AJCC stages. *I*, T1–2 N0 M0; *II*, T3–4 N0 M0; *III*, T1–4 N1 M0; and *IV*, T1–4 NO-1 MO. Development of distant metastasis (*stage IV*) significantly determines the survival of colorectal cancer patients ($p = .0001$)

with high frequency, whereas further cancers are rarely metastatic despite being locally invasive. Furthermore, colorectal cancer represents a heterogeneous group of malignant tumors resulting from accumulating mutations over many years. As a consequence, subgroups of tumors constitute what will either metastasize almost immediately, or will never relapse or metastasize, despite being histopathologically similar. Thus, metastasis is not an inherent property of all neoplastic cells (Welch 2006). Additional myths about cancer metastasis have been recently unveiled, discussed, and clarified by the NIH Metastasis Working Group: e.g., metastasis and invasion are *not* equivalent phenotypes, tumor cells at secondary sites are *not* necessarily metastases, and extravasated cells are *not* essentially metastases. All of these metastasis-related terms have been misleadingly used in the literature; defining and clarifying them provides the basis for correct interpretation and description of biological processes and interventions.

7.3.2 Hallmarks of Metastatic Cells: The Metastatic Cascade

Metastasis is both a process and the outcome of the process. However, only small subpopulations (e.g., 0.01%) of a mostly heterogeneic tumor harbor metastatic capability (Welch 2006). Metastatic cells are characterized by specific hallmarks, enabling them to spread and colonize other tissues. Metastatic cells are able to invade; they enter and exit the vasculature. Metastatic cells are characterized by deregulated adhesion, enabling them to detach from the primary mass and then re-adhere at a target organ(s) distant from the primary tumor. Metastatic cells evade immune cell killing and possess resistance to anoikis, the programmed cell death induced by the loss of cell matrix interaction. Finally, metastatic cells are able to modulate the secondary site (e.g., reorganization of the extracellular matrix, ECM) and, most importantly, proliferate.

Cancer cells can disseminate from the primary tumor via lymphatic or hematogenous routes. They can form solid metastases in the lymph nodes, and subsequently, can disseminate from the established lymph node metastases to distant sites, where they form distant metastases. This ability might be gained during the progression in the lymph node environment. Hematogenous dissemination can occur from the primary tumor, lymph node metastases, or distant metastases. Crucial biological steps for the process of metastasis can be distinguished, constituting the so-called metastatic cascade (Fig. 7.2; for review see Chambers et al. 2002; Fidler 2003; Bogenrieder and Herlyn 2003; Van't Veer and Weigelt 2003; Pantel and Brakenhoff 2004):

- Detachment, characterized by ECM proteolysis, loss of adhesion and cell migration from a primary mass (involved genes e.g., *E-cadherin* and members of the Wnt pathway such as *β-catenin*, *APC*)
- Invasion and intravasation, based on matrix degradation and motility (proteinases, e.g., MMPs, u-PA, heparanase)
- Arrest and attachment, resulting in adhesion and survival at secondary sites
- Extravasation (selectins, integrins, chemokines)
- Proliferation at the secondary site (bFGF, TGF-β, VEGF).

7.3.3 Defining (a) Cancer Metastasis

The NIH Metastatic Working Group defines cancer metastasis as follows: "The dissemination of neoplastic cells to discontiguous nearby or distant secondary (or higher order) sites where they proliferate to form an extravascular mass." This definition is based on the following principles of metastasis: Metastasis is distinct from tumorigenicity; metastatic potential varies between people, by tumor type, and within a given tumor; metastases are clonal in origin, but heterogeneity can redevelop during proliferation; metastases are nonrandomly distributed; and metastasis is an extraordinarily inefficient process, with every step in the metastatic cascade rate-limiting (Welch 2006). Typically, 5 to 6 cell divisions are required to qualify as a colony (2^6=64 cells); thus, a metastasis must constitute at least 50 cells.

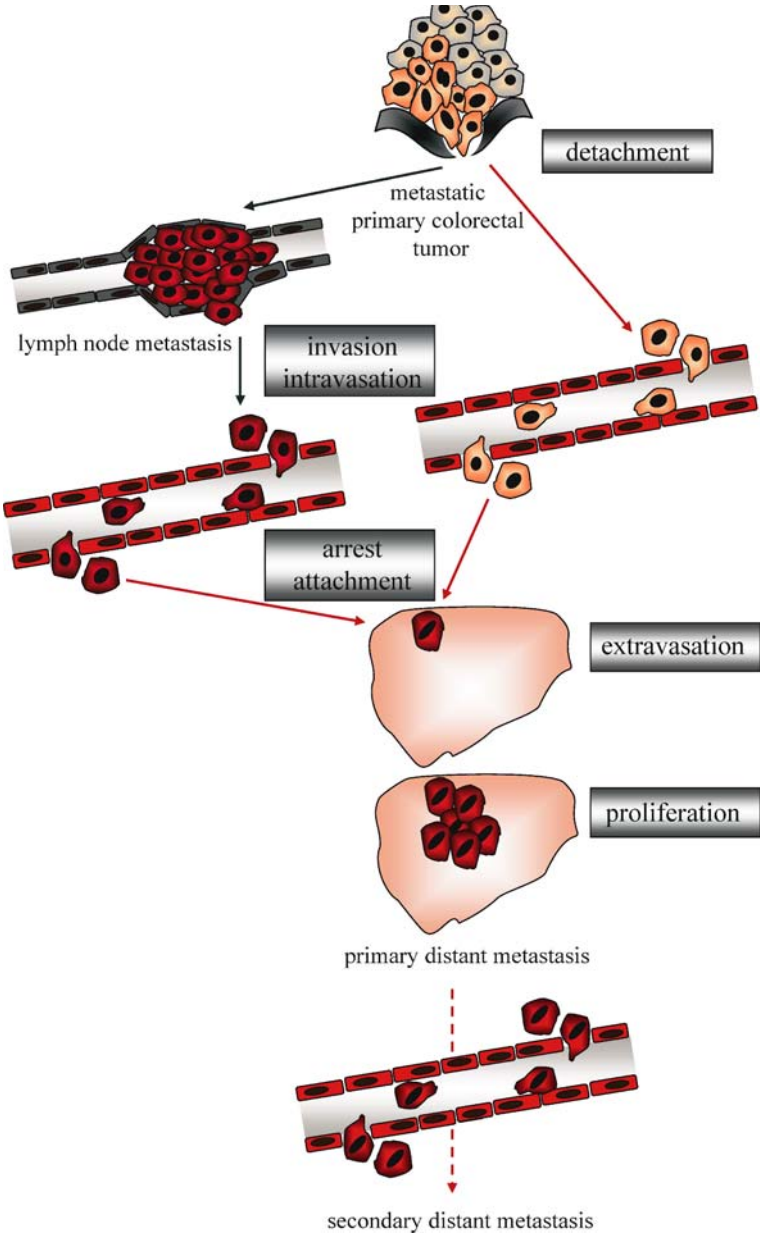

Fig. 7.2 Model for the metastatic cascade of colorectal cancer metastasis, showing lymphatic (*gray*) and hematogenous routes (*red*) of tumor cell dissemination. Hematogenous dissemination can occur from the primary tumor, lymph node metastases, or distant metastases. Crucial biological steps for the process of metastasis are indicated

7.4 Molecular Determinants for Outcome in Colorectal Cancer

7.4.1 The Adenoma-Carcinoma Sequence and Metastasis

Tumor progression and metastasis are results of sequential genetic alterations leading to accumulation of mutations in different genes and modulated downstream events. Single molecular events are meanwhile attributed to specific steps during the so-called adenoma-carcinoma sequence of tumor progression for colorectal cancer, first described as the Fearon-Vogelstein model (Fearon and Vogelstein 1990; Fig. 7.3). This adenoma-carcinoma sequence represents the stepwise progression from early lesions to carcinomas in a linear manner. Although nonlinear systems have been described as well, the Fearon-Vogelstein model pioneered the combined consideration of single, but accumulating molecular events and biological consequences during tumor progression. Today, colorectal cancer still represents the best-examined tumor entity, with genetic additions that have been made, driving the progression of the adenoma-carcinoma sequence, and with biological detours that have been unveiled (e.g., Arends 2000; Smith et al. 2006).

7.4.1.1 Oncogenes

Several genes that are assigned to defined steps of the adenoma-carcinoma sequence of colorectal cancer are affected by classic mutations. Members of the Ras gene family encode small guanosine triphosphate (GTP)-binding proteins that act as molecular switches mediating extracellular signals to nuclear transcription factors. Mutant *K-Ras* remains constitutively active. *K-Ras* mutations may occur early in the development of precancerous adenomas in the colon and rectum. Point mutations of *K-Ras*, mainly occurring at codons 12, 13, and 61, are thought to be prognostic for survival. However, the presence of only one mutation on codon 12, glycine to valine, had a statistically significant impact on survival and cancer progression, as was demonstrated by analyzing more than 3,400 patients (RASCAL II study; Andreyev et al. 2001). This study also suggests that the presence of this mutation may predispose to more aggressive biological behavior in patients with advanced colorectal cancer. Analyzing mutations in *K-Ras* and in tumor suppressors like adenomatous polyposis coli (APC) and p53 in parallel, patients with *K-Ras* mutations had significantly poorer overall survival (Conlin et al. 2005). Numerous

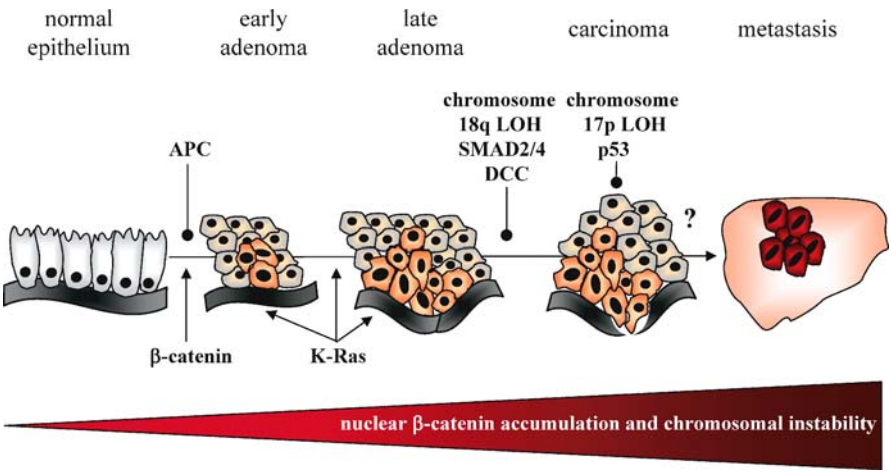

Fig. 7.3 Model for the adenoma-carcinoma sequence of colorectal cancer. According to Fearon and Vogelstein (1990) and Fodde et al. (2001), the stepwise actions of exemplified tumor suppressors (●) and oncogenes (▲) are marked

studies have been performed to demonstrate the prognostic role of mutant *K-Ras* for progression and metastasis formation of colorectal cancer (reviewed in Smakman et al. 2005; Castagnola and Giaretti 2005). However, although extensively examined, not all investigators confirmed the association between mutant *K-Ras* and unfavorable prognosis, in particular when analyzing smaller numbers of patients.

β-Catenin exerts a dual role, being involved in cell–cell adhesion and as a transcription factor in the Wnt pathway. The activation of mutations in the β-catenin gene and subsequent nuclear accumulation of β-catenin are important in the development of many cancers. About 10% of all colorectal carcinomas harbor mutations in the β-catenin gene, mostly in exon 3 affecting codons for amino acids like serine and threonine, which can be phosphorylated for proteasomal degradation (Morin et al. 1997; Ilyas et al. 1997). Nuclear β-catenin accumulation has been observed as an early event during tumor progression in up to 75% of colorectal cancers (reviewed in Doucas et al. 2005), and the prognostic value of nuclear accumulation has been described repeatedly (e.g., Ougolkov et al. 2002). Nuclear β-catenin acts as a transcriptional activator and represents an important effector in the Wnt pathway, modulating downstream molecules which then might have prognostic significance (Doucas et al. 2005; Fuchs et al. 2005). Interestingly, β-catenin target genes such as *matrix metalloproteinase-7* (*MMP-7*) (Crawford et al. 1999) and *EphB2*—a receptor tyrosine kinase whose reduced expression parallels invasion and metastasis in colorectal tumors (Guo et al. 2006)—have also been described as prognostic markers for metastasis. Recently, the melastasis promoting gene S100A4 was identified as transcriptional target of ß-catenin (Stein et al. 2006). In order to strengthen the prognostic value of the discussed oncogenes that are independently involved in the adenoma-carcinoma sequence, their combined analysis was used for identification of a subset of patients with poorer outcome (Zhang et al. 2003). It was recently demonstrated that cytoplasmic staining of p120ctn, an additional β-catenin family member, corresponds with loss or cytoplasmic localization of E-cadherin, correlates with later-stage tumors, and is prognostic for poor patient outcome (Bellovin et al. 2005).

7.4.1.2 Tumor Suppressor Genes

Many promising candidate genes that belong to the adenoma-carcinoma sequence are tumor suppressors, characterized by allelic losses on chromosome 5q (APC), 17p (p53), and 18q (*DCC*, *SMAD4*; Fodde et al. 2001). *APC* is involved in complex formation for β-catenin destruction. Mutations in *APC* prevent binding to β-catenin and its subsequent degradation. *APC* mutations are responsible for familial adenomatous polyposis coli (FAP), and APC mutations have been frequently observed in colorectal cancers at early stages (Rowan et al. 2000; Lamlum et al. 2000). APC mutations might lead to constitutive transcriptional activation by a β-catenin T cell factor complex (Korinek et al. 1997). Thus, not only APC itself, but also APC-dependent genes, such as *c-Myc* and *ornithine decarboxylase*, may be useful as genetic markers of risk and as targets for chemoprevention and therapy for colorectal cancer (Gerner et al. 2005). Several combined analyses have been performed to evaluate the prognostic significance of molecules belonging to the adenoma-carcinoma sequence. For instance, mutations of *APC* and *K-Ras* were more frequently observed in patients with locoregional metastasis, while mutant *p53* was usually detected in cases of peritoneal metastasis (Hsieh et al. 2005).

The tumor suppressor gene *p53* is located on chromosome 17p13.1. Mutations of *p53* occur in more than 50% of sporadic colorectal cancers as a late step in the adenoma-carcinoma sequence (Rodrigues et al. 1990; Fodde et al. 2001). p53 regulates central cell cycle check points, controls apoptosis through activation control of Bax, and promotes angiogenesis through regulation of vascular endothelial growth factor (VEGF) and thrombospondin (summarized in Neal et al. 2006). Higher *p53* mutation rates and *p53* overexpression in early stages have been described for metastasizing tumors vs nonmetastasized colorectal carcinomas (Sory et al. 1997). However, the role of p53 for prognosis in colorectal cancer remains, despite the numerous studies

performed, inconclusive. A large body of reports described correlations of mutation status, nuclear p53 staining, and outcome. However, another group of studies did not find significant relations of molecular and clinical parameters. A recent study evaluated several reports published in the context of *p53* abnormalities and prognosis in patients with colorectal cancer. It was stated that patients with abnormal *p53* were at increased risk of death determined by immunohistochemical staining and by mutation analysis. No effect of abnormal *p53* was described on outcome in patients treated with chemotherapy (Munro et al. 2005). Since the use of more than one tumor marker may successfully aid in the prognosis of colorectal cancer, p53 is often included when combining several potential markers for prognosis. Thus, a p53-negative/Bcl-2-positive phenotype was recently described as an independent indicator of good prognosis in colorectal cancer (Watson et al. 2005). Recently, it was shown that the prognosis of colorectal cancer is influenced by sex, p53, carcioembryonic antigen (CEA) reactivity, and Duke's stages (Nasif et al. 2006). Furthermore, a correlation of mutant p53 with CEA and epidermal growth factor receptor (EGFR) in serum of colorectal cancer patients was shown (Abdel-Aziz et al. 2006).

A further, very promising candidate as a prognostic and predictive marker for colorectal cancer is involved in late stages of the adenoma-carcinoma sequence, and is located at chromosome 18. The "deleted in colorectal cancer" (DCC) gene was identified as an important prognostic marker, in particular for the tumor stages II and III (Shibata et al. 1996).

Results from 27 studies with about 2,200 cases confirmed the relationship between colorectal cancer survival and chromosome 18q allelic imbalance/loss of DCC expression (Popat and Houlston 2005). Furthermore, DCC protein expression was found to be similar in the primary tumor and the metastasis, and more importantly, represents not only a prognostic marker but also a dominant predictor of survival in patients with unresectable, advanced colorectal cancer who are undergoing adjuvant/palliative 5-fluorouracil-based chemotherapy (Aschele et al. 2004; Gal et al. 2004).

Another candidate gene, also belonging to this chromosomal instability pathway (adenoma-carcinoma sequence) is SMAD4. This putative prognostic marker for colorectal cancer also harbors tumor suppressor properties and is also located at chromosome 18 (18q21). It belongs to the SMAD family, which mediates the transforming growth factor-β (TGF-β) signaling pathway suppressing epithelial cell growth. SMAD4 gene mutations in human cancer interfere with the homooligomer formation of the SMAD4 protein and the heterooligomer formation between SMAD4 and SMAD2 proteins, resulting in disruption of TGF-β signaling (Miyaki and Kuroki 2003). Loss of SMAD activation and/or expression together with poor prognosis have been observed in approximately 10% of colorectal cancers (Xie et al. 2003). Furthermore, SMAD4 has also been identified as a predictive marker for adjuvant chemotherapy in colorectal cancer (Watanabe et al. 2001; Boulay et al. 2002). Patients with SMAD4 retention showed a greater benefit of a 5-fluorouracil-based therapy. Further genes located on chromosome 18q (DNAM-1, SOCS6 and CADH-7) might also be of prognostic and predictive value for colorectal cancer (Storojeva et al. 2005).

These examples illustrate that much has been learned in recent years on mutations that control the initiation and progression of colorectal cancer, but less is known about molecular events that are crucial in metastasis formation. Stephen Paget, an English surgeon, wrote a century ago, "an attempt is made in this paper to consider metastasis in malignant disease, and to show that the distribution of the secondary growths is not a matter of chance" (Paget 1889). Besides the molecular events necessary for tumor progression, additional molecular modulations are needed to enable a tumor cell to become metastatic. It is meanwhile widely accepted that metastatic behavior seems to be determined at the earliest stages in tumorigenesis (Bernards and Weinberg 2002). However, even these metastatic prerequisites might require further molecular alterations, finally resulting in distant metastasis formation (Fig. 7.4).

7.4.2 Prognosis/Prediction of Outcome

Since metastasis is the main cause for cancer-related death, prognosis for metastasis and predic-

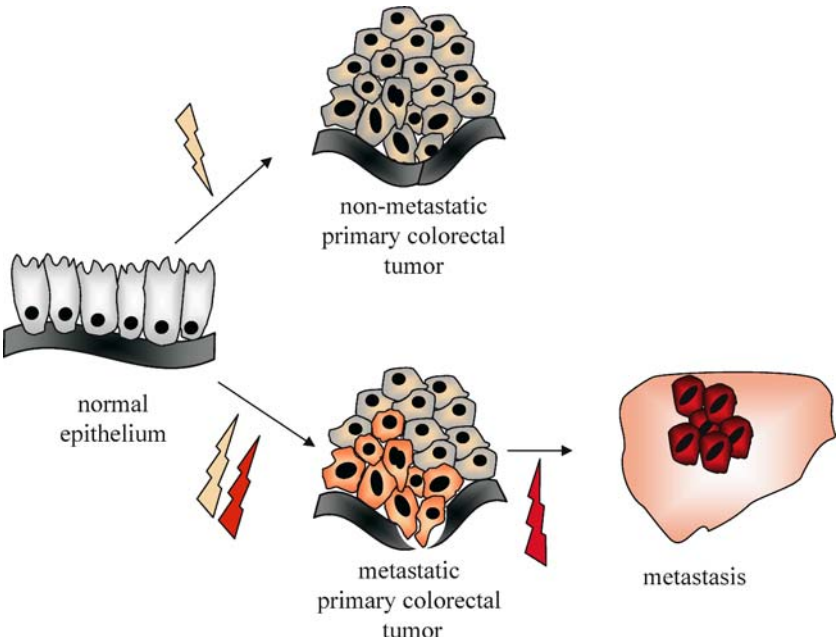

Fig. 7.4 Model for molecular events leading to tumor progression and metastasis of colorectal cancer. *Single lightning icon at top*: Molecular events leading to tumor progression. *Double lightning icons*: Molecular events determining metastasis in cells of the primary tumor. *Single lightning icon at bottom*: Molecular events leading to metastasis

tion of therapy response by expression analysis of candidate genes is of central importance. Both metastasis prognosis and response prediction at the molecular level are based on two different main strategies: the employment of single genes known to be associated or even causal for the metastatic process (Table 7.2) or the use of gene signatures.

7.4.2.1 Prognostic/Predictive Single Markers

For decades, efforts have been aimed at the identification of genes that are involved in or might be even causal for (organ-specific) metastasis. However, these features alone do not qualify a gene as metastasis prognosis marker. In accordance with McLeod and Murray, "a prognostic marker is a variable that provides information on patient outcome which is complementary to the data obtained by the pathologist from the diagnostic sections and on which therapeutic decisions can be guided" (McLeod and Murray 1999). Predictive markers, however, provide information about the chance of a patient responding to a particular treatment. Furthermore, criteria have to be defined that are the "readouts," e.g., mutation status and, most of all, expression levels, but also posttranslational events. Moreover, where and when should the marker analysis be performed? Marker detection in the early stage primary tumor, prior to any metastasis, might represent the most meaningful approach. Finally, the potential prognosis marker should be evaluated in the context of clinical parameters with respect to independence (e.g., on TNM classification). Based on that, high-risk patients could be identified prior to any metastasis, alternative/more intense screenings can be applied, and treatment protocols might be adjusted. Furthermore, targeted intervention strategies for metastasis prevention can be developed in the lab, and subsequently applied in the clinic.

7.4.2.2 Proteolysis

Proteolytic enzymes degrade the ECM, thereby facilitating the detachment of tumor cells from the primary site, and cause invasion of surround-

Table 7.2 Selected molecules with prognostic/predictive value for colorectal cancer

Function/gene family	Gene	Main readout	Related reference(s)
Oncogene	*K-Ras*	Mutation	Andreyev et al. 2001
			Conlin et al. 2001
			Smakman et al. 2005
			Castagnole and Giaretti 2005
Oncogene	*β-Catenin*	Mutation/nuclear accumulation	Morin et al. 1997
			Ilyas et al. 1997
			Ougolkov et al. 2002
			Doucas et al. 2005
			Fuchs et al. 2005
Tumor suppressor	*APC*	Mutation	Korinek et al. 1997
			Rowan et al. 2000
			Lamlum et al. 2000
			Fodde et al. 2001
			Gerner et al. 2005
			Hsieh et al. 2005
Tumor suppressor	*p53*	Mutation	Rodrigues et al. 1990
			Sory et al. 1997
			Munro et al. 2005
			Watson et al. 2005
			Nasif et al. 2006
			Abdel-Aziz et al. 2006
			Neal et al. 2006
Tumor suppressor	*DCC*	Mutation	Shibata et al. 1996
			Aschele et al. 2004
			Gal et al. 2004
			Popat and Houlston 2005
Tumor suppressor	*SMAD4*	Mutation	Watanabe et al. 2001
			Boulay et al. 2002
			Miyaki and Kuroki 2003
			Xie et al. 2003
Proteolysis/ metalloproteinases	*MMP-7*	Expression	Luo et al. 2005
			Kurokawa et al. 2005
			Ogawa et al. 2005
			Pesta et al. 2005
			Rudmik and Magliocco 2005

Table 7.2 *(continued)*

Function/gene family	Gene	Main readout	Related reference(s)
			Deryugina and Quigley 2006
Adhesion/integrins	*alphavbeta6*	Expression	Bates and Mercurio 2005
			Bates et al. 2005
			Sheppard 2005
			Enns et al. 2005
Adhesion/cadherins	*E-cadherin*	Expression	Shiozaki et al. 1996
			Mohri 1997
			Cavallaro and Christofori 2004
			Roca et al. 2006
			Shioiri et al. 2006
Adhesion/sialyl Lewis antigens	*sLeX*	Expression	Bird et al. 2006
Angiogenesis	*VEGF*	Expression	Ellis 2004
			Cascinu et al. 2000
			Ferroni et al. 2005
			Tsai et al. 2006
			Ogata et al. 2006
			Kaklamanis et al. 2006
Migration/invasion	*HGF/SF-Met*	Expression	Birchmeier et al. 1997
			Fazekas et al. 2000
			Birchmeier et al. 2003
			Takeuchi et al. 2003
			Zeng et al. 2004
			Boon et al. 2005
			Bauer et al. 2005
			Fassetta et al. 2006
Ca-binding proteins	*S100A4*	Expression	Takenaga et al. 1997
			Taylor et al. 2002
			Flatmark et al. 2003
			Gongoll et al. 2002
			Cho et al. 2005
			Helfman et al. 2005
			Hemandas et al. 2006
			Garrett et al. 2006
			Li and Bresnick 2006
			Stein et al. 2006

ing tissues and vessels. It is widely acknowledged that overexpression of MMPs is associated with a poor prognosis in colorectal cancer (Zucker and Vacirca 2004). MMP-7, a prominent member of the MMPs, also known as matrilysin, is one of the most studied proteinases associated with colorectal cancer metastasis. It was shown recently that MMP-7 is able to degrade VE-cadherin and accelerates nuclear accumulation of β-catenin (Ichikawa et al. 2006). It is overexpressed in the majority of colorectal cancers, and its expression was correlated to metastasis (Rudmik and Magliocco 2005; Deryugina and Quigley 2006). MMP-7 has been demonstrated to serve as a prognostic marker for metastatic potential and prognosis in colorectal cancer (Luo et al. 2005; Kurokawa et al. 2005; Ogawa et al. 2005; Pesta et al. 2005). Moreover, elimination of matrilysin, e.g., by ribozyme technology, is associated with low invasiveness and slow tumor growth, suggesting MMP-7 as target for therapeutic approaches (Jiang et al. 2005). Further proteinases include urokinase plasminogen activator (uPA), ADAMs (A disintegrin and metalloproteases), and heparanase (reviewed in Bogenrieder and Herlyn 2003).

7.4.2.3 Adhesion

Adhesion deregulation is an essential prerequisite for metastasis formation. Loss of intracellular adhesion allows tumor cells to escape from their primary site. Molecules such as integrins, the immunoglobulin family, and the cadherins belong to the cell adhesion molecule (CAM) family of transmembrane glycoproteins (Johnson 1999). CAMs mediate cell–cell and cell–ECM adhesion, and have been implicated in metastasis (Kawaguchi 2005; Christofori 2006; Eble and Haier 2006). Integrins are transmembrane cell-surface receptors that connect ECM ligands, such as fibronectin, laminin, and collagen, to the intracellular actin cytoskeleton. The causal involvement of integrins in early steps of metastasis, and in particular of organ-specific metastasis to the liver, has been repeatedly described (Enns et al. 2004, 2005). Based on the demonstrated integrin-mediated activation of TGF-β, it is likely that integrins play important roles in tumor growth and metastasis (Sheppard 2005). The integrin $\alpha_v\beta_6$, a receptor for fibronectin and tenascin, was identified as a novel marker of the epithelial-mesenchymal transition (EMT) and as prognostic factor for aggressive colorectal carcinoma (Bates and Mercurio 2005). Thus, the identification of $\alpha_v\beta_6$ integrin as prognostic marker validates the EMT model as a valuable tool for the identification of prognostic markers (Bates et al. 2005). Moreover, $\alpha_v\beta_6$ integrin represents an important therapeutic target for selective killing of invading and metastasizing cells in early stage disease (Bates 2005).

E-cadherin is a member of transmembrane glycoproteins involved in cell–cell and cell–ECM adhesion of tumor cells and participate in the development of an invasive and metastatic phenotype (Cavallaro and Christofori 2004). The E-cadherin/β-catenin complex plays a crucial role in cell–cell adhesion, and loss of E-cadherin results in reduced cell adhesiveness, increased invasion, and advanced stage in several tumor entities (e.g., Shiozaki et al. 1996; Mohri 1997). Recently, a correlation of increase in the number of adhesion proteins (E-cadherin, α-, β-, and γ-catenins, p120ctn), altered expression in the primary tumor, and increasingly impaired prognosis for colon carcinoma patients was described. However, no individual, independent prognostic value of each of these proteins was found. The results suggest that the entire cadherin-catenin complex should be evaluated when assessing its prognostic value (Bondi et al. 2006). Moreover, a relevance of E-cadherin and tissue inhibitors of MMP (TIMP)-2 expression was determined for prognosis of colorectal cancer patients, providing a more accurate mechanism for their classification (Roca et al. 2006). Furthermore, Slug, a member of the Snail transcription factor family, plays a crucial role in EMT regulation by suppressing several adhesion molecules including E-cadherin. Positive Slug expression was significantly associated with Dukes' stage, distant metastasis, and patient overall survival. Patients with positive Slug expression and reduced E-cadherin expression showed the worst prognosis (Shioiri et al. 2006).

Sialyl Lewis X antigen (sLe^X) and its isomer, sialyl Lewis A, are glycoproteins found on the cell surface of metastatic tumor cells. sLe^X expression is associated with poor prognosis in colorectal cancer (reviewed in Bird et al. 2006).

Interestingly, the degree of sialylation together with the expression of the antigen is suggested to be a stronger indication for aggressiveness than sLe^x expression alone.

7.4.2.4 Angiogenesis

Tumor growth and metastasis are essentially dependent on angiogenesis. Angiogenesis at the invading tumor edge, assessed by intratumoral vascular density, is suggested as an independent prognostic marker to identify subgroups of colorectal cancer patients with an unfavorable post-operative outcome (Georgiou et al. 2006). Assessment of angiogenesis is also performed by expression analysis of angioregulatory genes such as *VEGF* and thrombospondin. VEGF is the most potent regulator of angiogenesis. VEGF is associated with angiogenesis in colorectal cancer, and its pattern of expression in adenomas is maintained in the arising carcinomas, suggesting a defined place for VEGF in the adenoma-carcinoma sequence (Kaklamanis et al. 2006). Elevated levels of tumor and serum VEGF may be used as independent prognostic markers, and may discriminate between early and late stages of colorectal cancer (Cascinu et al. 2000; Ferroni et al. 2005; Tsai et al. 2006). Moreover, VEGF represents a potential predictive marker for chemotherapy regimens (Ellis 2004; Ogata et al. 2006). Different intervention strategies have been developed that target VEGF, leading to increase in overall survival and reduction in the risk of death (bevacizumab), reversal of cellular resistance (cetuximab) and activity as second-line therapy in patients who have exhausted other available treatment options (cetuximab, ABX-EGF, PTK-787, gefitinib, erlotinib) (Mancuso and Sternberg 2005).

7.4.2.5 Migration/Invasion, Survival

Hepatocyte growth factor (HGF)/scatter factor (SF) induces invasive growth, a biological program that enables tumor cells to invade and metastasize based on enhanced cell proliferation, motility, and survival. HGF/SF binds to its receptor, the tyrosine kinase c-Met (Weidner et al. 1993). Thereby, c-Met is connected to two pathways, the Ras pathway promoting migration and invasion, and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway suppressing apoptosis and promoting survival (reviewed in Birchmeier et al. 2003). Thus, deregulation of HGF/SF-Met signaling plays a very crucial role for invasive growth and metastasis of many human malignancies. Moreover, the requirement of c-Met for insulin-like growth factor I-mediated migration and invasion was published (Bauer et al. 2005). c-Met is considered as a marker of the metastatic potential in colorectal cancer due to its involvement in the generation of the motility signal. Overexpression of HGF/SF and/or c-Met was described for numerous tumor entities including colorectal carcinomas (summarized in Birchmeier et al. 2003). In primary colorectal tumors, c-Met overexpression has been repeatedly described as an important prognostic marker for early stage invasion and metastasis (e.g., Birchmeier et al. 1997; Fazekas et al. 2000; Takeuchi et al. 2003; Zeng et al. 2004). Recently, the role of HGF/SF-Met signaling pathways for survival, invasion, and transformation has been further elucidated (Boon et al. 2005; Fassetta et al. 2006). Furthermore, transcriptional targets of HGF/SF-Met signaling in colorectal cancer have been identified (Seiden-Long et al. 2006). Thus, HGF/SF and/or Met, but also molecules of HGF/SF-Met signaling cascades or genes transcriptionally driven by these cascades, represent therapeutic targets for intervention strategies.

S100A4 (also known as metastasin, mts1, FSP1, 18A2, pEL98, p9Ka, 42,A, CAPL, calvasculin) is a member of the multigene S100 family of EF-hand, calcium-binding proteins. It has long been known to be associated with cancer metastasis. Besides other effects of S100A4 contributing to metastasis, S100A4 can bind, in a Ca-dependent manner, to cytoskeletal proteins such as f-actin, nonmuscle tropomyosin, and myosin IIa, which contribute to the effects of S100A4 on cell motility (for review see Helfman et al. 2005; Garrett et al. 2006; Li and Bresnick 2006). High levels of S100A4 expression play an important role in metastasis and correlate with a negative prognosis in several types of cancer. In colorectal cancer, S100A4 expression was found to be elevated during tumor progression; with increased levels in the primary colon cancer when compared to normal mucosa, and the expression

was even further elevated in the liver metastases (Takenaga et al. 1997; Taylor et al. 2002; Flatmark et al. 2003). Furthermore, the presence of S100A4 protein was correlated with a significant decrease in survival time (Gongoll et al. 2002). Recently, correlations of S100A4 expression with progression and outcome of colorectal patients were described (Cho et al. 2005; Hemandas et al. 2006). Morover, S100A4 is of prognostic value for formation of distant melastases of colon carcinomas and for patients survival, when quantitatively determined in the not (yet) melastasized primary tumor (Stein et al 2006). Thus, high levels of S100A4 correlate with aggressive tumor growth, reduced patient survival, and poor prognosis, suggesting S100A4 as a prognostic marker for colorectal cancer.

7.4.2.6 Prognostic/Predictive Gene Signatures

Since the value of single marker genes might be limited, much effort has been made for identifying gene/protein signatures that might be useful for disease prognosis and response prediction. Thus, genome-wide expression profiling by using microarrays have been meanwhile employed for many tumor entities and treatment regimens. For colorectal cancer, many of these studies have been summarized, making array technologies—despite all current limitations and discussions—a valuable tool for understanding the cell biology of colorectal cancer (Shih et al. 2005). Studies aiming at the identification of prognostic signatures were grouped according to the tissues they compared. Thus, several reports described signatures that are able to distinguish between normal tissue and tumor, or between primary tumor and metastasis. Furthermore, prognostic signatures have been identified for defined tumor stages. With respect to the adenoma-carcinoma sequence, signatures for specific steps in tumor progression have been found. Certainly, when evaluating data from several studies, genes have been extracted known for their involvement in carcinogenesis and metastasis. Recently, further reports contributed to this rapidly evolving field, identifying signatures for survival prediction of colorectal cancer patients (Eschrich et al. 2005), describing differentially expressed proteins between metastatic and nonmetastatic human colorectal carcinoma cell lines (Ying-Tao et al. 2005), and describing prognostic factors in resected colorectal carcinomas and lung metastases (Shiono et al. 2006; Lyall et al. 2006).

Moreover, treatment options for progressive and metastatic colorectal tumors are still limited, and tumors including colorectal cancer differ in sensitivity to radiation and chemotherapy. Therefore, patients who will not benefit from chemotherapy or radiation still receive standardized, aggressive therapy, which can result in an unjustified loss in quality of life and in an inappropriate treatment. Thus, identification of signatures for response prediction of several treatment regimens have been performed (reviewed in Shih et al. 2005).

However, the overlap of single genes and signatures within the different studies analyzing comparable tissues with comparable aims is quite small, pointing to the need for a standardized methodology. Thus, future challenges of microarray technologies still remain for improved analysis, reproducibility, and validation of the identified genes/signatures (reviewed in Ransohoff 2004). Generating gene signatures will be an essential prerequisite and indispensable tool for future tailor-made, patient-individual diagnosis, prognosis, and therapy in colorectal cancer.

7.5 Conclusion and Future Perspectives

Today, metastasis of colorectal cancer is understood as the result of early changes during tumor progression which determine the metastasis capacity. Prognosis of metastasis will definitely depend on individualized, tailor-made array technology employing validated selected single genes and gene signatures. Much is known about molecules contributing to the metastasis phenotype, about pathways they control, and about genes they regulate. All of these molecules might represent potential targets for intervention strategies aiming at the reduction or even prevention of metastases formation. Thus, inhibition of metastasis seems possible by targeting the tumor cell at the primary site, employing, e.g., antiproliferative factors, tissue inhibitors of proteolytic enzymes, and inhibitors of angiogenesis.

However, reviewing all findings, whether recently published or well recognized for decades, it is clear that almost all current knowledge focuses on the capabilities of the "seed"—the metastatic cells, representing only one side of the coin. "When a plant goes to seed, its seed are carried in all directions; but they can only live and grow if they fall on congenial soil," was stated by Paget over 100 years ago (1889; Chambers et al. 2002). The importance of the "soil" side has, so far, been inadequately examined, neglected, and probably underestimated. The most limiting step in metastasis formation is, importantly, growth after extravasation at the secondary site, as demonstrated in murine models, underlining the impact and the potential of the "soil" for metastasis formation (Chambers et al 2002). Thus, the writings of Paget, in his seminal publication on the "seed-and-soil-theory of metastasis" a century ago, are still applicable: "The best work in the pathology of cancer is now done by those who, like Mr. Balance and Mr. Shattock, are studying the nature of the seed. They are like scientific botanists, and who turns over the records of cases of cancer is only a ploughman, but his observations of the properties of the soil might also be useful" (Paget 1889; Fidler 2003).

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8 Prediction of Efficacy and Side Effects of Chemotherapy in Colorectal Cancer

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Abstract

Treatment of gastrointestinal cancers has significantly advanced over the last several years with the introduction of effective chemotherapeutic and targeted drugs. Providing individual treatment with low toxicity but maximum benefit is still an unsolved problem. Inter-individual variation of drug toxicity and efficacy is mainly determined by genetic polymorphisms. The genetic approach of pharmacogenetics and pharmacogenomics is developing as a valuable tool to design tailored therapy. This review focuses on clinically significant polymorphisms in genes involved in metabolism of chemotherapy used in gastrointestinal cancer: fluoropyrimidines, irinotecan, and platinum. In addition, the first results of pharmacogenetics in targeted therapy including cetuximab and bevacizumab are discussed.

8.1 Introduction

Treatment options in colorectal cancer (CRC) have systematically advanced over the last several years with the introduction of effective chemotherapeutic and targeted drugs. But providing individual treatment with low toxicity and significant benefit is still an unsolved problem. The phenomenon of inter-patient variation of drug toxicity and efficacy as seen in our daily clinical practice is mainly determined by genetic polymorphisms. The genetic approach based on single-gene (pharmacogenetics) or multi-gene (pharmacogenomics) analyses is developing as a promising tool to design tailored therapy.

Pharmacogenetics analyzes associations between genetic polymorphisms in metabolic genes and clinical endpoints such as toxicity, response to therapy, and survival. Genetic polymorphisms are variations of the DNA sequence that may, depending on their localization within the gene, significantly alter the function of the transcribed protein. These polymorphisms may alter various processes within the cell, e.g., influx, activation, or detoxification of the substance. Several forms of genetic polymorphism have been described including single nucleotide polymorphisms (SNPs), deletion, and repeat polymorphisms. Depending on the investigated clinical endpoint by pharmacogenetic tools, it is important to choose the correct source of DNA. If associations between potential drug toxicity and genetically determined metabolic pattern are analyzed, germline DNA should be used. For analysis of drug efficacy, the DNA of the tumor appears to be the right source, since several tumor-specific aberrations that would not be captured otherwise appear in colorectal tumors.

This review focuses on pharmacogenetic knowledge of substances routinely administrated in patients with CRCs: fluoropyrimidines, irinotecan, and oxaliplatin. In addition, the first results of pharmacogenetics in targeted therapy, including cetuximab and bevacizumab, are discussed.

8.2 Pharmacogenetics and Chemotherapy

8.2.1 Fluoropyrimidines

8.2.1.1 Thymidylate Synthase

Thymidylate synthase catalyzes the intracellular conversion of deoxyuridylate to deoxythymi-

dylate—the sole de novo source of thymidylate in the cell (Kundu and Heidelberger 1974). The active metabolite of 5-fluorouracil (5-FU), namely 5-fluorodeoxyuridylate (5-FdUMP), binds to TS and inhibits it by forming a stable ternary complex (Danenberg 1977). Horie et al. (1995) first described a 28-bp sequence of the 5´-region of the TS gene to be polymorphic with either two (2R) or three (3R) repeats. Caucasians and African Americans almost exclusively possess double repeats (2R) or triple repeats (3R) for this polymorphism. Multiple repeats (McArdle and Hole 1991; Kawakami et al. 2001) have also been reported within certain Asian and African populations (Luo et al. 2002; Kawakami et al. 2001; Marsh et al. 1999), but their functional significance has not yet been determined.

The impact of the double repeats and triple repeats of this 28-bp sequence of the gene on TS function has been debated for years now. It was postulated that this 2R/3R polymorphism is implicated in modulating TS messenger RNA (mRNA) expression (Kaneda et al. 1987) and TS mRNA translational efficiency (Kawakami et al. 2001). Currently, no report definitively answers the question whether the polymorphic site in the 5´-region alters mRNA expression, translational efficacy, or both.

A higher mRNA expression was described in TS 3R carriers as compared to TS 2R carries. For example Pullarkat et al. (2001) found an almost fourfold increased TS mRNA level in metastatic colorectal tumor tissue for homozygous carriers of the 3R TS variant when compared to individuals homozygous for the 2R variant with heterozygotes demonstrating intermediate TS mRNA levels. Since Leichman et al. and other groups demonstrated a direct link between TS expression and clinical outcome to 5-FU based chemotherapy, several investigators aimed to correlate the TS polymorphism directly with efficacy and survival (Leichman et al. 1997).

One example was provided by Villafranca et al. who demonstrated that the 2R/3R TS polymorphism is associated with tumor downstaging in rectal cancers. T-downstaging ($T_{Pretreatment}$ vs $T_{Posttreatment}$) was correlated with the TS variants among 65 rectal cancer patients who received preoperative 5-FU-based chemoradiation (Villafranca et al. 2001). Patients with a homozygous 3R/3R genotype showed a lower probability of downstaging compared to the 2R/3R+2R/2R group (22% vs 60%, $p = 0.036$). This information may be crucial for determination of the optimal treatment schedule in those patients. In addition, the TS 3R/3R genotype was more often associated with local recurrence of locally advanced rectal cancers (Lu et al. 2002). Other groups could confirm these observations in retrospective evaluations. However, there are two important aspects: The prospective investigation of this association between TS polymorphism and 5-FU efficacy and toxicity was not as clear as expected. A study by Etienne et al. (2002) with 103 CRC patients who received 5-FU-based chemotherapy identified the 2R/2R TS genotype as the most favorable for survival (median survival was 19 months for 2R/2R, 10 months for 2R/3R, and 14 months for 3R/3R, $p = 0.025$). Interestingly, the group with the TS 2R/3R genotype demonstrated the shortest survival. Although Iacopetta et al. (2001) demonstrated in an analysis of 221 Dukes' C CRC patients who received 5-FU that patients possessing two 3R alleles derive no survival benefit from chemotherapy in contrast to patients who harbor at least one 2R allele (RR = 0.52, 95% CI 0.33–0.82, $p = 0.005$). It was also mentioned that a sizeable fraction of patients with a 3R/3R genotype gained some short-term benefit from 5-FU chemotherapy. Moreover, a study among 135 Japanese patients with CRC, who received 5-FU based oral adjuvant chemotherapy, failed to confirm a prognostic value of this 5´TS polymorphism (Tsuji et al. 2003).

These observations raise the question whether other modulators impact TS expression and thereby the clinical outcome to 5-FU-based chemotherapy. An analysis by Mandola et al. (2003) shed further light on our understanding of the regulation of TS function. The authors identified a new G→C SNP within the 28-bp repeat polymorphism that appears within the 3R variant of the TS gene and disrupts a binding site for the transcription factor upstream stimulating factor (USF)-1. The authors postulated that this may alter TSmRNA expression. Although the exact mechanism by which this additional SNP influences TS function is not completely understood, the importance of the TS polymorphism is currently acknowledged in clinical trials. McLeod

and co-workers designed a clinical trial in T3/T4 rectal cancers using the TS2R/3R polymorphism to stratify patients (see Fig. 8.1). Patients with the 3R/3R TS genotype and therefore an expected increased failure rate to 5-FU received and additional drug, irinotecan. This example illustrates the first steps of how pharmacogenetic knowledge may be used in the clinic.

At the moment, less information is available regarding a recently identified 6-bp deletion polymorphism in the 3´-region of the TS gene (1494-99del). This polymorphism might alter mRNA stability, secondary structure, or expression, as has been demonstrated for alterations of the 3´-region in other genes. Further analyses are needed identifying the regulators and/or mechanisms by which this deletion/insertion polymorphism alters TS expression. This may help to interpret these preliminary conflicting data (Mandola et al. 2004).

8.2.1.2 Dihydropyrimidine Dehydrogenase and Orotate Phosphoribosyl Transferase

Dihydropyrimidine dehydrogenase (DPD) represents a key enzyme of 5-FU metabolism. This rate-limiting enzyme of 5-FU catabolism inactivates more than 80% of the drug in the liver. Retrospective analyses of gene expression revealed that low DPD levels may be important for superior response to fluoropyrimidine treatment (Heggie et al. 1987). It has been suggested that low levels of DPD may increase bioavailability of the drug, thereby improving response.

Within the dihydropyrimidine dehydrogenase gene, 17 different mutations have been identified that are associated with decreased activity of the enzyme (McLeod et al. 1998). Consequently, impaired 5-FU catabolism leads to accumulation of active 5-FU metabolites. This genetically determined decrease of DPD activity can be the cause of increased hematopoietic and gastrointestinal toxicity (Diasio et al. 1988; Johnson et al. 1999; van Kuilenburg et al. 2001). An allele frequency of 0.91% for a G→A substitution in the invariant GT splice donor site flanking exon 14 (IVS14+1G>A) was reported for Caucasians, which appeared to be the most common known DPD variant. This G→A mutation causes a lack of the exon in corresponding mRNA, thereby dramatically diminishing DPD activity (van Kuilenburg et al. 1997). Patients harboring one or two of these nonfunctional DPD alleles have been shown to experience severe and even lethal toxicities of 5-FU-based regimens. Isshi et al. (2002) demonstrated that high levels of orotate phosphoribosyl transferase (OPRT) may be associated with increased sensitivity to 5-FU-based chemotherapy. OPRT catalyzes the reduction of 5-fluorouridine-5´-diphosphate (FUDP) to the actively TS inhibiting metabolite FdUMP. A recent study by Ichikawa et al. (2003) indicated that a newly identified SNP of OPRT exon 3 may be critical to predicting toxicity to 5-FU-based chemotherapy. A 213G substitution increases OPRT activity. In a small cohort of 52 CRC patients that received 5-FU chemotherapy, those who possessed one or two OPRT A alleles experienced a higher frequency of adverse events, in-

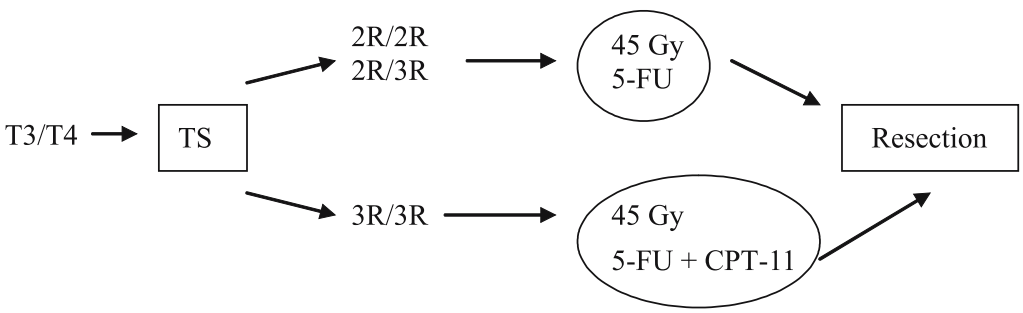

Fig. 8.1 Design of TS-stratified trial in rectal cancer

cluding diarrhea and leucopenia ($p < 0.0001$). If confirmed in a larger cohort, this polymorphism of the OPRT may also be a considerable candidate for predicting toxicity to fluoropyrimidines.

8.2.3 Irinotecan

Irinotecan is an inhibitor of topoisomerase I, an essential nuclear component for DNA replication of all eukaryotic cells. The metabolism of irinotecan is highly complex. One of the crucial enzymes in metabolism of SN-38, the active metabolite of irinotecan, is the hepatic uridine diphosphate glucosyltransferase (UGT) 1A1 (Fig. 8.2). A 2-bp insertion polymorphism $(TA)_7TAA$ (UGT1A1*28) within the TATA box in the 5´-UTR [UGT1A1 $(TA)_n$] diminishes enzyme activity and thereby results in a significant increased chance to experience grade 3 or 4 toxicities, mainly diarrhea and leucopenia (Iyer et al. 1998). Based on the analyses presented to the Food and Drug Administration in November 2004 and summarized in Table 8.1, the label for irinotecan was revised. The revised label informs the user of an increased risk of homozygotes (UGT1A1*28) for leukopenia if started with a normal dose of CPT-11. Although it is not known at the moment how to reduce the drug, this is another example how pharmacogenetic testing could help to tailor chemotherapy in the future.

Additionally, polymorphisms of UGT1A1, UGT1A9 (hepatic), and UGT1A7 (nonhepatic) have also been shown to be predictive with regard to response and toxicity following irinotecan/capecitabine treatment in CRC. However, current studies suggest that the strongest alterations in CPT-11 metabolism occur based on the UGT1A1 $(TA)_7TAA$ polymorphism.

8.2.4 Oxaliplatin

8.2.4.1 DNA Repair

The platinum drug oxaliplatin is standard treatment in the adjuvant and palliative setting of CRC patients. Oxaliplatin blocks DNA replication by the formation of different types of adducts. There are first hints that members of the DNA repair family (e.g., ERCC1, ERCC2) and glutathione S-transferases (e.g., GSTP1) determine efficacy and toxicity of this platinum drug.

The excision repair cross-complementing (*ERCC*) gene family is essential for the removal of platinum-mediated DNA adducts, and gene expression data in CRC confirm the inverse relationship between impaired DNA-repair capacity (e.g., low ERCC activity) and superior responses to platinum compounds (Shirota et al. 2001). Consistently, polymorphisms that alter nucleotide excision repair (NER) function could be

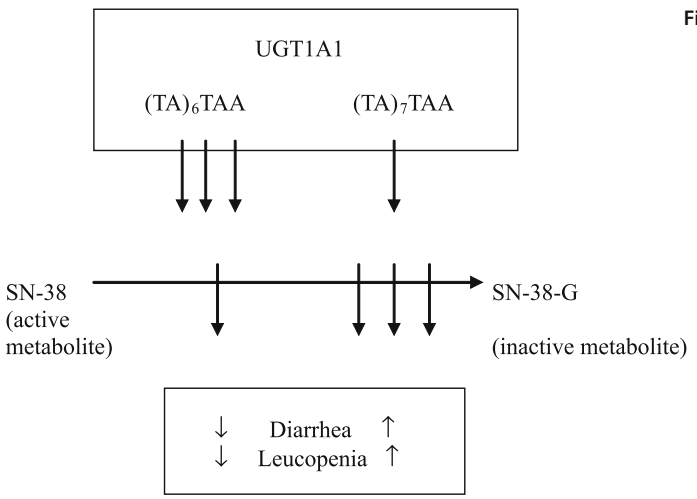

Fig. 8.2 UGT1A1 and CPT-11 toxicity

Table 8.1 UGT1A1 polymorphism and severe neutropenia risk following CPT-11

Authors	*n*/N (%)	*n*/N (%)	Odds ratio	95% CI
	7/7	6/7+6/6		
Innocenti et al. 2001	3/6 (50%)	3/53 (6%)	16.7	2.3–120.6
Rouits et al. 2004	4/7 (57%)	10/66 (15%)	7.5	1.4–38.5
Marcuello et al. 2004	4/10 (40%)	18/85 (21%)	2.5	0.6–9.7
Ando et al. 2000	4/7 (57%)	22/111 (20%)	5.4	1.1–25.9

linked to clinical outcome in patients receiving platinum-based chemotherapy. A SNP within the ERCC2 gene (codon 751) causes the amino acid exchange from lysine (Lys) to glutamine (Gln). An improved response rate of 24% was observed for patients possessing two 751Lys alleles compared to 10% in Lys/Gln or Gln/Gln carriers ($p = 0.015$) (Park et al. 2001). The survival benefit for 751Lys homozygotes was reconfirmed in a study of 106 patients. All patients received a 5-FU/oxaliplatin combination chemotherapy (Stoehlmacher et al. 2004). Conversely, McLeod and colleagues could not verify this association in a cohort of patients that received oxaliplatin (McLeod et al. 2003).

8.2.4.2 Glutathione S-Transferases

The isoenzyme GSTP1 of the family of glutathione S-transferases is highly expressed in CRC, and biochemical experiments delivered information for the direct involvement of GSTP1 in the sufficient detoxification of platinum (Goto et al. 1999; Ban et al. 1996), GSTP1 being responsible for both intrinsic and acquired resistance to platinum. The 313A→G SNP within the GSTP1 gene causes a replacement of isoleucine (ILE) by valine (Val) in the GSTP1 protein. The Val-variant has been demonstrated to be associated with decreased GSTP1 activity (Srivastava et al. 1999).

In a retrospective analysis among patients that received 5-FU/oxaliplatin chemotherapy, those who harbored two Val alleles experienced the most favorable survival (median, 25 months, 95% CI 9.4, +25 months) while the Ile/Ile genotype was associated with the least favorable survival (median, 7.9 months, 95% CI 5.4, 9.6 months). Intermediate survival outcome was noted for the Ile/Val carriers (median, 13 months, 95% CI 8.4, 24 months; $p<0.001$) (Stoehlmacher et al. 2002).

This hypothesis is further supported by data of Grothey et al. who showed an increase in oxaliplatin toxicity according to the number of GSTP1 Val alleles (Grothey et al. 2005). Patients who possessed two Val alleles stopped FOLFOX4 treatment due to neurotoxicity (which is the most limiting toxicity of oxaliplatin) in 24% of the cases as compared to only 10% (heterozygotes) and 9.2% in the Ile/Ile group ($p = 0.039$). Patient harboring one or two GSTP1 Val alleles developed neurotoxicity earlier than Ile/Ile patients. Recent data further indicate that polymorphisms of voltage-gated sodium channels may be linked to neurotoxicity in oxaliplatin-treated patients with CRC. Additional markers are urgently needed for this important drug for both efficacy and toxicity, in order to spare patients such severe side effects in the future.

8.2.5 Cetuximab and Bevacizumab

The EGFR and VEGF antibodies cetuximab and bevacizumab have successfully been introduced into treatment strategies of CRC patients. However, only a few data are available at this time concerning pharmacogenetic aspects of these drugs. Zhang et al. investigated several polymorphisms of molecules involved in the EGFR pathway including cyclin D1, cyclooxygenase 2, EGF, EGFR, VEGF, and interleukin 8 in patients treated with a single agent, cetuximab (Zhang et al. 2006). This pilot study suggests that patients with favorable genotypes of the cyclinD1 A870G polymorphism and the EGF A61G polymorphism may benefit more from cetuximab treatment than others. Although the data showed

significance, there were only 39 patients included in that trial. There are no pharmacogenetic data available on bevacizumab at this time.

8.3 Conclusion

The differential function of genes affecting drug metabolism, interactions with cellular targets, or transport can cause a dramatic shift in the toxicity profile and efficacy of drugs. Analyses with a high predictive value for drug response should include not only the individual's genetic background, but also gene–gene interactions, somatic mutations of the tumor cell, and dynamic characteristics such as variations of RNA expression. Nonetheless, the individualization of chemotherapy based on pharmacogenetic profiling is attractive.

The first practical examples, e.g., TS and UGT1A1 polymorphisms, demonstrate how pharmacogenetics could change our way of thinking about and treating disease. We are only at the beginning of the implementation of pharmacogenetics in the clinic, and the true benefits of this new tool will have to be proved during the next few years in prospective clinical trials.

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9 Proteomic Expression Profiling of Breast Cancer

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Abstract

Breast cancer is one of the most common cancers observed in women in industrialized Western countries. The development of novel diagnostic methods and the application of modern systemic therapies have significantly optimized early detection and therapy of breast cancer. However, many patients are currently overtreated. Traditionally, tumours have been categorized on the basis of histopathological criteria. However, staining pattern and intensity of cancer cells are not sufficient to reflect the molecular events driving tumour development and progression. Therefore, new genomic, transcriptomic and proteomic techniques are applied to clinical samples aiming to identify new targets for a therapy tailored for an individual patient. After an introduction to common genomic and transcriptomic profiling technologies and their relevance for clinical use, we will focus on analytical and preanalytical applications for the identification of new therapeutic targets by protein profiling, with a special emphasis on two-dimensional gel-technologies (2D-PAGE), particularly as they apply to the study of breast cancer.

9.1 Introduction

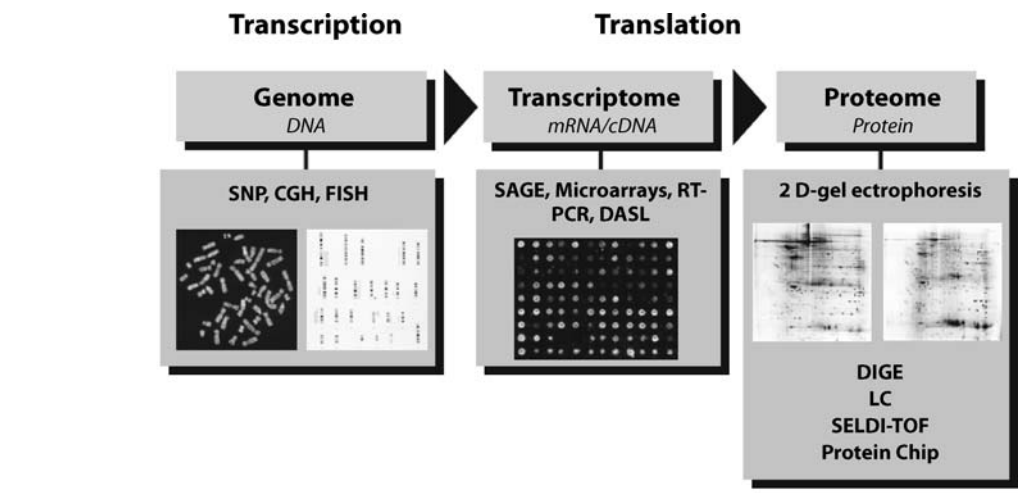
In the United States approximately 213,000 new cases of invasive breast cancer have been diagnosed in 2006, constituting approximately 31% of all new cancer cases among women; 41,000 disease-related deaths were expected (Jemal et al. 2006). In the past 10 years, improvements in diagnostic procedures for early detection and their broad application, together with the introduction of modern systemic therapies, have resulted in significant progress in early diagnosis and breast cancer therapy. In a new development, it has become feasible in clinical oncology to consider tailoring cancer therapy to an individual level of complexity by the use of suitable biomarkers. The current St. Gallen guidelines for the selection of adjuvant systemic therapy for early breast cancer patients include tumour size, grading, lymph node (LN) status, menopausal status, peritumoural vessel invasion, hormone receptor status and epidermal growth factor receptor 2 (HER2/neu) status. These markers can be classified into two major classes (Biomarkers Definitions Working Group 2001):

1. *Prognostic markers*. These provide information about the malignant potential of tumours, thereby predicting the outcome of a disease
2. *Predictive markers*. These are used to choose between different alternative treatment modalities. For instance, breast cancer patients with oestrogen receptor-positive tumours are usually treated with anti-oestrogen drugs such as tamoxifen and aromatase inhibitors, whereas oestrogen receptor-negative tumour patients are treated with chemotherapy. The overexpression of Her2/neu is predictive for the use of trastuzumab (Herceptin) at the same time as being a "drug target".

The use of these mainly histology-based prognostic parameters performs reasonably well using group-based statistical analyses. However, regarding outcome predictions for the individual

patient, these parameters should be supplemented with molecular parameters to reduce the uncomfortably high degree of uncertainty. Additionally, for prediction of therapy outcomes, the classical biomarkers provide only limited resolution of the manifested phenotype and have a limited capacity for individualizing a therapy. A more precise stratification of patients into responders versus non-responders to therapeutic agents is urgently needed by utilizing additional parameters. Therefore, new biological markers must be sought at all levels which are used to store the holistic biological information in a cell or tissue. These have been artificially classified into categories of convenience, such as the genome, the transcriptome, and the proteome and are dynamic, overlapping, and continuous in living systems (Fig. 9.1).

The introduction of microarray technology for nucleic acids opened the way to simultaneously analyse many genes—in contrast to classical histopathology—providing their specific profile of expression in a panoramic view. Therefore, a whole molecular profile is able to depict the polygenic origin of cancer, the multi-step process of tumourigenesis and the progression of cancer, which are reflected by genetic alterations that drive the transformation of normal human cells into highly malignant derivatives (Hanahan and Weinberg 2000). This concept of "molecular portraits" for each patient's tumour, first discussed by Perou et al., transcends histologic boundaries and indicates how array analysis can—compared to individual tumour markers—also provide new insights into breast cancer classification enabling a more refined stratification of the patients (Perou et al. 2000). Each of these molecular subtypes may be associated with a distinct clinical behaviour and treatment response as was shown for breast cancers of basal-like, HER2/neu over-



Modifications	Epigenetic e.g. Methylation	Genetic Point Mutation Gene Deletion Translocation Amplification	Posttranscriptional Splicing Editing	Posttranslational (>200) Splicing Phosphorylation Glycosylation etc.
Possible Amplification	Yes	Yes	Yes	No

Fig. 9.1 Different levels of biomarker research. Schematically depicted are the levels in a cell or tissue which are used to store biological information, possible modifications of genomic DNA, messenger RNA (mRNA) and proteins, and methods developed to identify them. DNA and mRNA can be amplified by polymerase chain reaction (PCR) and linear amplification methods, respectively

expressing, luminal A, and luminal B subtypes (Sorlie et al. 2001).

Besides the huge progress achieved in transcriptomics high-throughput analysis methods, recent technological progress has provided the tools to begin systematic development of comprehensive molecular pictures on the proteome level, investigating the component directly related to the phenotype and function of a cell (Hoheisel 2006).

9.2 Molecular Levels of Analytical Profiling

Since the publication of the draft sequence of the human genome (Lander et al. 2001; Venter et al. 2001), the aim of using information derived from genome analysis to tailor care to individual patients has gained prominence (Ginsburg and McCarthy 2001; Meyer and Ginsburg 2002; Snyderman and Williams 2003). Having access to the entire human genome sequence is a necessary prerequisite for molecular-based medicine. However, it is equally important to have the technology at hand to reliably visualize individual genomes, transcriptomes and proteomes providing information that, in combination with clinical data, can contribute to assessment of individual risks and guide clinical management and drug development.

9.2.1 Genomic Approaches

Genetic changes supporting oncogenesis can be point mutations, gene deletions ("loss of heterozygosity", LOH), translocations or amplifications. The exact number of genes in the entire human genome is currently estimated at about 35,000. Although humans, from a genetic point of view, are very similar to one another, there are single base exchanges in the DNA called single nucleotide polymorphisms (SNP) between individuals. It has recently been proposed that such SNPs arise by oxidation of genomic DNA, particularly involving 8-oxo-guanine, and that this is one of the largest sources of genomic diversity in human beings (Ohno et al. 2006). On average, one SNP is found every 1,500 base pairs, allowing the potential use of broad-based screens to pinpoint disease susceptibility genes to within a few 10,000ths of base pairs. Followed by sequencing of such short stretches, a specific genetic defect can be identified. Large-scale genotyping of SNPs is one new technology, assaying genotypes at thousands of loci (Hoheisel 2006). Other changes of the genome include cancer-specific variations in gene copy number, either through gene amplification or deletion. They initially highlighted the direct connection between such changes and disease, and are useful in diagnosis (Lichter et al. 1990; Feuk et al. 2006). One example in breast cancer is the amplification of the oncogene HER2/neu—methodologically determined on the genomic level by molecular methods such as polymerase chain reaction (PCR) and/or fluorescent in situ hybridization (FISH) (Benohr et al. 2005; Slamon et al. 1987; Sjogren et al. 1998). Another technology applied to analyse variations in gene copy number is comparative genomic hybridization (CGH), which has evolved from the standard hybridization of genomic DNA on metaphase spreads (Kallioniemi et al. 1992) to microarray-based CGH (array-CGH) (Pinkel et al. 1998). This technique facilitates the localization of copy-number changes very precisely by arraying probes derived from genomic sequences [e.g. BAC (bacterial artificial chromosomes) contiguous sequences (contigs)] that are tiled across a locus of interest. Such continuous coverage has been achieved for the entire human genome (Ishkanian et al. 2004; Hoheisel 2006). Beyond these genomic alterations, epigenomic modifications imposed onto the DNA, by e.g. environmental effects, can change gene expression and modify gene products in ways that initiate, accelerate or retard progression of pathologic processes without changing the coding nucleotide sequence of the genomic DNA (reviewed in: Laird 2005; Tlsty et al. 2004). The important implication is that in addition to the genetic analysis based upon nucleotide sequences, alternative approaches will be necessary to account for such environmental influences. A big practical advantage of genomic analysis is that the cellular component in question—the chromosomal DNA—is a fairly stable macromolecule enabling its convenient amplifi-

cation and analysis from formalin-fixed paraffin-embedded (FFPE) tissue archived in comprehensive tissue banks.

9.2.2 Transcriptomic Approaches

Analysing messenger RNA (mRNA) has always been an important and technology-enabled approach to examine the expression of genes. As an alternative to classical low-throughput Northern technologies, different high-throughput processes such as serial analysis of gene expression (SAGE) and microarray analysis have been developed to analyse global gene expression at the transcriptomic level (Velculescu et al. 2000; Schena et al. 1998). Both screening techniques provide the possibility to simultaneously evaluate the relative expression levels of large numbers of different mRNA transcripts in a panoramic view. Thereby, entire expression profiles themselves become a tumour marker that mirrors the polygenic nature of carcinogenesis. The increasing list of microarray experiments published each month reflects the straightforwardness of this technology, which is based on relatively inexpensive and easy-to-synthesize but sensitive and specific nucleic acid sequences. These can be arrayed into miniaturized standardized platforms and function as docking sites for complementary nucleotide sequences in the analyte (see several reviews: Butte 2002; Brentani et al. 2005; Hoheisel 2006). During the past decade, the microarray molecular profiling of breast tumours has produced a much more detailed classification scheme and has identified gene signature sets. One of the first attempts to characterize the variation in gene expression between sporadic breast tumour samples was published by Perou and co-workers (2000). In their groundbreaking study the authors showed that by differences in the expression profiles, breast cancer can be classified into so-called basal-like, HER2/neu overexpressing, luminal A, B, and C-, and normal-like tumours (Sorlie et al. 2001; Sorlie et al. 2003). These subtypes were correlated with overall survival and did not strongly reflect other clinical features such as LN status, tumour size or menopausal status, underscoring the importance of the molecular characterization of tumours. Another seminal DNA microarray analysis by van 't Veer and colleagues identified a 70-gene "profiler set" strongly predictive of a short interval to distant metastasis in LN-negative patients over 55 years of age with primary breast cancer and that can be used to classify primary breast carcinomas as having a gene-expression signature associated with either a poor or a good prognosis (van 't Veer et al. 2002; van de Vijver et al. 2002). Strikingly, this prognostic profiler set is independent of LN involvement, but is rather based upon its improved predictive power with respect to metastasis to non-lymphatic tissues. To prospectively evaluate this gene set which to date has only been retrospectively validated, a randomized clinical trial was launched in Europe ("MINDACT"). Prior to its launch the TRANSBIG Network embarked on an external, independent validation of the signature using frozen archival material of node-negative patients who are less than 60 years old. Preliminary analysis of approximately 300 samples from 6 different institutes shows that the overall performance of the 70-gene profiler set has a slightly reduced prognostic power in this external validation series compared to the original series published by van 't Veer et al. but it still outperformed the clinicopathologic risk assessment (Piccart et al. 2004; Buyse at al. 2006). Nevertheless, the "70-Gen-profiler set" from van 't Veer provides the platform for an already commercially available test. In addition to these studies further publications assigning the risk of patients with LN-negative breast cancer have been published recently (Wang et al. 2005; Pawitan et al. 2005). In one of these experiments, Wang et al. used oligonucleotide microarrays from Affymetrix to analyse a patient cohort with almost the same parameters as the cohort van 't Veer has used. They identified a 76-gene signature for untreated node-negative patients; it performed better in the multi-variate analysis compared with classical breast cancer prognostic factors. In summary, the results from van 't Veer et al. and Wang et al. indicate that gene-expression profiles are more powerful predictors of the outcome of disease in patients with breast cancer than the 2001 St. Gallen or the NIH consensus criteria and provide means for identification of patients needing adjuvant therapy (Eifel et al. 2001).

Other microarray studies have focussed on determining gene signatures of potential response of patients to specific chemotherapy and hormonal therapy regimens (Chang et al. 2003; Iwao-Koizumi et al. 2005; Ayers et al. 2004; Jansen et al. 2005). In the work by Chang et al. a discriminatory set of 92 genes was published which can be used to identify breast tumours responding to docetaxel. If the UICC (International Union Against Cancer) criteria had been applied to the same patients, some of the tumours in the gene expression-predicted resistant group would have belonged to the UICC-responding group, despite the fact that they had a completely different expression profile for the selected gene signature.

These exemplarily selected studies impressively show the power of microarrays in improving prognostic and predictive conclusions about breast cancer subtypes, but alternative test systems, which might be more suitable for routine use in the clinic, have been developed. For instance, to profile a small set of genes the application of quantitative RT-PCR (qRT-PCR) would be the most cost-effective and easy-to-handle method. This approach has been realized with the Oncotype DX test developed by Paik et al. and provided by the company Genomic Health (Paik et al. 2004). This test is based on real-time RT-PCR quantification of 16 cancer-related and 5 reference genes and is designed to identify patients benefiting most from adjuvant treatment with tamoxifen. Based on published data, 250 candidate genes were evaluated using three independent cohorts from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B.20 study to select the gene set mentioned above. After establishing a "recurrence score", the set was validated using a completely independent cohort from the NSABP B-14 trial. The assay allows for a better and/or more reproducible prognosis with oestrogen receptor (ER)-positive tumours in node-negative patients than the age of the patient, the size of the tumour or the histologic grade. Additionally, its quantitative read-out is also vastly superior to that of another commonly employed alternative method: immunohistochemistry.

An advantage of the RT-PCR approach is that it can be used with degraded RNAs derived from FFPE tumour samples, the most important and abundant source of clinical material. In contrast, microarray analysis is limited to frozen tumour samples imposing severe limitations on access to samples. Therefore, many laboratories are trying to develop methods that allow a similar degree of high-throughput gene-expression profiling using FFPE tissue as the starting material (Paik et al. 2005). This is not a trivial problem as RNAs extracted from FFPE tissues are chemically modified and fragmented and are therefore not ideal substrates for gene-expression profiling assays. Arcturus Biosciences (Mountain View, CA) has started marketing a reagent system called Paradise that has a combination of optimized RNA extraction and linear RNA amplification reagents. When used together with a specially designed oligonucleotide array, the GeneChip Human X3P array from Affymetrix (http://www.affymetrix.com/products/arrays/specific/x3p.affx), it promises to provide adequate gene-expression profiling data from FFPE-tissue. However, the "present call" rate for fresh paraffin blocks of breast cancer are reported to be below 30%, and it decreases to below 20% if the tissue block is more than 5 years old (Paik et al. 2005). Additionally, it has not been definitively clarified if gene sets obtained from cryo-preserved tissue correlate with gene sets from identical paraffin tissue (Sgroi et al. 2004; Ding et al. 2004).

Recently, a new method was developed—DASL (cDNA-mediated annealing, selection, extension and ligation) for gene-expression profiling to generate data from degraded RNAs such as those derived from FFPE tumour samples. This assay is a combination of microarray and qRT-PCR technologies into one platform that can be formatted to analyse the expression of a set of selected genes in a single clinical sample. It uses a minimal amount of total RNA (≤ 200 ng total RNA per assay) without prior linear amplification. The characteristics of DASL are (1) the use of FFPE samples as old as 24 years (unpublished data from Illumina); (2) high throughput with up to 96 clinical samples on one array plate and (3) the use of a custom gene panel with at least 512 genes per array (Bibikova et al. 2004; Fan et al. 2004).

The analysis of the transcriptome has already highlighted its potential for clinical usefulness by

generating promising results, but there are also big challenges waiting to be overcome. Especially regarding the use of microarrays, it is now evident that different platforms perform differently, resulting in only a marginal overlap of their gene-expression data, which is for instance the reason that to date no internationally accepted "risk-gene set" has been developed for "high-risk" breast tumour patients (Marshall 2004; Jenssen and Hovig 2005; Ein-Dor et al. 2005). This phenomenon is not restricted to breast cancer, but is also characteristic for gene sets of other diseases (Miklos and Maleszka 2004). Factors responsible for this phenomenon might include:

1. The application of different methodological standards
2. The application of different technical aspects including the type of array used (cDNA or oligonucleotide) (Marshall 2004), the gene sequences represented on the chips, the selection of tissue samples, the RNA extraction, what kind of probe was used for chip-hybridization (cDNA or cRNA), how the probe was labelled [cyanine (Cy) dyes, radioactivity, or biotin] and the conditions used for hybridization
3. The mode used for data processing, including the setting of instruments used for scanning the chips and the way the data were normalized and pre-processed
4. Differences in the study design and the number of samples included (Alizadeh et al. 2000)
5. Biological factors (Jarvinen et al. 2004; Ein-Dor et al. 2005)

9.2.3 Proteomic Approaches

The term "proteome" was coined in 1994 and is defined as the entire protein complement expressed by a cell line, tissue or organism. Proteomics, in analogy to genomics, is the study of the proteome, i.e. of all proteins—including their relative abundance, distribution, post-translational modifications, functions and interactions with other macromolecules—in a given cell or organism within a given environment at a specific stage in the cell cycle (Wasinger et al. 1995; Cai et al. 2004). Additionally, the study of the proteome also aims at the identification of protein isoforms. Proteomics has been classified into different sub-disciplines:

1. "Discovery-oriented" proteomics. Here, investigators are not able to impose their knowledge of biology on the experimental design. Such an experiment provides both known and unknown proteins.
2. "System-oriented" proteomics (MacBeath 2002; Choudhary and Grant 2004). In such an experiment a subset of proteins (e.g. a protein family) is directly analysed characterizing its biological functions, protein-protein or protein-DNA/RNA interactions, and protein post-translational modifications such as phosphorylation, sulphation or glycosylation.

Like the transcriptome, the rather dynamic nature of proteomes differs from individual to individual, and even from cell to cell. However, the sheer variety of potential modifications means that proteomic complexity dwarfs that of the transcriptome. Proteins undergo chemical modifications after they have been expressed, changing properties such as enzymatic activity, binding ability and activity. This myriad of modifications might give rise to 10–20 million chemically distinct polypeptides in a single tissue—a huge number compared to approximately 35,000 genes per cell of which around 6,000 are actively transcribed (Vuong et al. 2000). Despite this complicating aspect, the interest in applying proteomics to the identification of disease markers is increasing, because transcriptional activity does not necessarily reflect the activity of the proteins, which do all the work of the cell. When the same cells of tumours have been examined by both cDNA arrays and proteome methods, the correlation between mRNA transcript profiles and corresponding protein abundances has been reported to be only moderate (Anderson and Seilhamer 1997; Alaiya et al. 2000; Chen et al. 2003; Izzotti et al. 2004; Nishizuka et al. 2003). Therefore, it is obviously necessary to assess protein levels in instances where only protein expression levels correlate to disease. This is an important issue to consider, as most licensed tests that are available for disease detection are protein-based assays. In addition, proteomics provides the unique opportunity to develop serum markers to

be used for early disease detection and to follow treatment effects and disease progression.

The obvious advantages in analysing the cellular proteome, however, come with several complicating issues which are a consequence of its vast dynamic range of up to 10 orders of magnitude (Anderson and Anderson 2002), the plethora of post-translational modifications, boundless tissue, developmental and temporal specificities, disease and drug perturbations, and problems of sample degradation. Additionally, the chemistry of amino acids is much more complex to handle than that of nucleic acids: for proteins there is no amplification step that is analogous to the polymerase chain reaction (PCR). Hence, low-abundance proteins are often obscured by highly abundant proteins and separable protein species, e.g. cytoskeletal proteins, chaperones, endoplasmic reticulum proteins, proteasomal components and extra-cellular matrix proteins.

In order to get a deeper insight into the proteome of a cell, increasingly more sophisticated separation techniques have to be combined with highly sensitive mass spectrometry (MS) technologies for protein identification.

9.2.3.1 Mass Spectrometry

In recent years, MS has become almost a routine tool for identifying the proteins separated by different proteomic methods (Aebersold and Goodlett 2001; Domon and Aebersold 2006). Different types of mass spectrometers are used to support a range of research strategies in the protein sciences such as the determination of molecular weight, primary and higher order structure, post-translational modifications, quantitation, and localization. They differ in their physical principle, performance standards, mode of operation and ability to support specific analytical strategies. Matrix-assisted laser desorption/ionization (MALDI) ion sources are most commonly coupled with a time-of-flight (TOF) mass analyser. The method is very sensitive and quite tolerant to the presence of contaminants such as detergents or salts. However, these and other methods of ionization, separation and detection can be considered as modular units. Manufacturers are currently experimenting with various combinations of such modules to achieve powerful and sensitive separation and detection of peptide ions. There is an extensive variety of such combinations on the market, and it is difficult for the non-expert to maintain an overview, or for the expert to judge which system performs best. Therefore, this review aims to present an elementary level of introduction. In MALDI-TOF MS, peptides derived from proteolytic digested proteins are ionized from a plate into the spectrometer, and the mass to charge (m/z) ratios of peptides are measured based on the length of time for the peptides to move in a vacuum tube to reach a detector, and then a list of mass spectra is produced. Another method of protein ionization—electrospray ionization (ESI)—is most often coupled with ion-trap or triple quadrupole MS/MS spectrometer (Wilm et al. 1996). By applying database search algorithms, MS spectra are then matched to calculate masses in a sequence database, resulting in identification of target proteins, a method known as peptide mass fingerprinting (PMF; see Patterson and Aebersold 2003). Tandem mass spectrometry (MS/MS) can be thought of as a two-stage MS experiment whereby an ionized peptide is selected and allowed to pass into a collision cell for further fragmentation to determine sequential m/z values representing a series of ion fragments of the specific peptide (Arthur 2003).

The performance of TOF analysers has greatly improved in terms of resolution and accuracy, achieving mass accuracies in the low parts per million (ppm) range with appropriate internal calibration. However, for MS/MS measurements with TOF devices, such as the Bruker Ultraflex, calibration of the second MS step is not available, and the mass accuracy is up to 100 times poorer in MS/MS mode. While this is sufficient for confirmation of predicted fragment patterns from sequence databases to confirm peptide identity, it is frequently insufficient to permit de novo sequencing. The newer ion cyclotron resonance detectors offer the possibility of excellent mass accuracy for all measurements. These improvements offer levels of sensitivity and mass accuracy never before achieved for the detection, identification, and structural characterization of proteins. It is now possible to routinely measure molecular weights above 200 kDa as

well as obtain low parts per million mass measurement accuracy for the determination of peptides and proteins. Modern mass spectrometers can now rapidly map and fragment peptides that result from protease digestion to identify proteins and—supported by the rapid expansion of protein and gene databases—to obtain sequence information.

In addition to the MS applications combined with prior separation of proteins, imaging mass spectrometry (IMS) has been developed as a new technology enabling proteomic profiling direct on a tissue section (Chaurand et al. 2004). Briefly, molecules are desorbed from a sample that has been coated with an energy-absorbing matrix, which is a low molecular weight organic crystalline compound. The profiles recovered have been found to be extremely specific to a given tissue type and, when analysing serial sections, very reproducible. IMS offers the potential for the simultaneous analysis of many molecular species present in a single tumour regardless of the availability of specific antibodies or knowledge of the identity of the specific protein. However, its exact sensitivity is hard to estimate because exact amounts of proteins within a specific tissue are generally not well known. To date, profiling and imaging MS have been applied to multiple diseased tissues, including human non-small cell lung tumours, gliomas, and breast tumours (Yanagisawa et al. 2003; Schwartz et al. 2004; Chaurand et al. 2001) but this technique is still in its developmental phase.

One aspect of protein identification by MS that may not be apparent to more clinically oriented scientists is that the proteins are most often identified by comparing the pattern of ions measured in the mass spectrum with ion patterns predicted by comparison with sequence databases. This is true at the levels of measuring peptide masses (the process of peptide mass fingerprinting) as well as for measuring the size of ions generated when individual peptides are fragmented into smaller pieces. It is relatively infrequent that proteomics resorts to de novo interpretation of the amino acid sequence of an unknown protein without the assistance of sequence database information. This field has been reviewed recently (Domon and Aebersold 2006).

Traditionally, analysis of the proteins coded by genes was performed on single proteins at a time using techniques such as Western blots and immunoprecipitation. However, with the completion of the human genome project, proteomic technologies to identify and quantitate proteins on a global scale were developed, which are so far not as robust as those available for genomics and sometimes are still in their infancy and therefore constantly evolving. These technologies can be classified as gel-based and non-gel-based approaches and include two-dimensional (2D) gel electrophoresis-based methods including classical 2D-PAGE or 2D-difference gel electrophoresis (2D-DIGE); chromatographic separation techniques such as isotope coded affinity tag (ICAT) (Gygi et al. 1999b; Li et al. 2003) or multiple dimension protein identification technology (MudPIT) (Washburn et al. 2001; Chen et al. 2006); and recently the application of antibody and protein arrays (Table 9.1; Somiari et al. 2005).

9.2.3.2 2DE-Based Strategies

Despite several new technologies that have been introduced for high-throughput protein characterization and discovery, 2D-PAGE continues to be an affordable analytical methodology. Methods of 2D-PAGE can be divided into the conventional "one sample per gel" 2D techniques and the more recently developed DIGE (O'Farrell 1975; Patton 2002; Shaw et al. 2003; Somiari et al. 2005). They have recently been reviewed by Gorg et al. (2004), so we will limit our discussion to late technical developments and to applications to breast cancer. After visualizing by Coomassie blue, silver or fluorescent dye staining, each observed protein spot is quantified by its staining intensity. The major advantages of 2D-PAGE are the biochemical separation of intact polypeptide molecules, and their repertoire of post-translational modifications, including splicing variants. Any two species can be separated that differ in isoelectric point and molecular weight sufficiently to be separated with the resolution of the gel system used. Despite the utility of 2D-PAGE, its advantages are associated with several technology-related inherent disadvantages:

Table 9.1 Comparison of proteomic technologies and their contributions to biomarker discovery and early detection (Schrattenholz 2004; Wulfkuhle et al. 2003)

2D-PAGE[a]	2D-DIGE[b]+ radioisotopes[c]	2D-DIGE+Cy dyes[d]	LC[e]+/−s-isotopes[f]	SELDI[g]	Protein microarrays
Names of specific technologies with associated companies					
Various suppliers and companies	ProteoTope, ProteoSys	DIGE, Amersham Biosciences	ICAT[h], Applied Biosystems MudPIT[i]	SELDI Ciphergen	
Sensitivity					
Low (particularly for less-abundant proteins)	High (sub-attomole)	Low	Medium (~5 fmol)	Medium (femtomole) (diminishing yield at higher molecular weights)	Medium/high (picomole, depending on antibody)
Limited by detection methods (~1 fmol)					
Direct identification of markers					
Yes	Yes	Yes	Yes	No	Possible when coupled with MS technologies (Washburn 2003; Ouyang et al. 2003)
Pros					
Tried methodology	High resolution	Resolution	High throughput	Very high throughput	Flexible format
Good separation power	High dynamic range	No systemic error	Low molecular weight proteins	Low molecular weight proteins	Robust performance
	Differential quantification			Protein IDs not necessary for diagnostic pattern analysis	
	No systemic error				
	<1% crosstalk of labels				
Cons					
Low through put	Radioisotopes	Limited linear dynamic range of 2–3 orders of magnitude	Systemic error	Systemic error	Requires prior knowledge of analyte being measured
All IDs require validation and testing	Unlabelled protein for mass spectroscopy		False negatives	False negatives	Limited by sensitivity and specificity of antibody

Table 9.1 *(continued)*

2D-PAGE[a]	2D-DIGE[b]+radioisotopes[c]	2D-DIGE+Cy dyes[d]	LC[e]+/−s-isotopes[f]	SELDI[g]	Protein microarrays
Cons					
Time consuming			Limited dynamic range of 2–3 orders of magnitude	Very limited dynamic range	
Performs poorly for glycosylated proteins				Reproducibility issues need to be addressed	
Limited dynamic range (2–3 orders of magnitude)				Need for validation	
Use					
Discovery and identification of biomarkers	Discovery and identification of biomarkers	Discovery and identification of biomarkers	Discovery and identification of biomarkers	Diagnostic pattern analysis in body fluids and tissues	Multiparametric, systematic analysis of many analytes simultaneously
Throughput					
Low	Medium	Medium	High	Very high	High

[a] Two-dimensional polyacrylamide gel electrophoresis

[b] Two-dimensional difference gel electrophoresis

[c] Radioactive isotopes

[d] Cyanine dyes

[e] Liquid chromatography

[f] Stable isotopes

[g] Surface-enhanced laser desorption ionization

[h] Isotope-coded affinity tag

[i] Multi-dimensional protein identification technology

1. Standard protein staining in gels has moderate sensitivities (approx. 1 fmol) and clear disadvantages in terms of dynamic range of protein concentrations (Vuong et al. 2000). Neither conventional 2D gel electrophoresis methods nor any other proteome technology has the sensitivity of gene chip arrays (Gerling et al. 2003). The two to three orders of magnitude of linear dynamic range of 2D-procedures also do not match the protein reality, and thus a considerable amount of information is lost. Therefore, 2D-PAGE traditionally requires a large amount of protein starting material, and it can hardly be used to reliably detect and identify low-abundance proteins such as transcription factors. This leads to a bias in the presence of high-abundance "housekeeping" proteins in every protein database.
2. Some highly abundant proteins are not detectable by 2D-PAGE. Because of their high hydrophobicity they are not soluble in the detergent/urea buffer employed for isoelectric focussing (IEF) in the first dimension. Other proteins co-migrate with higher abundance proteins and are therefore not quantifiable by staining alone.
3. Applying 2D-PAGE is a time-consuming and labour intensive method.
4. Complex protein patterns of related samples, or even of multiple aliquots of the same sam-

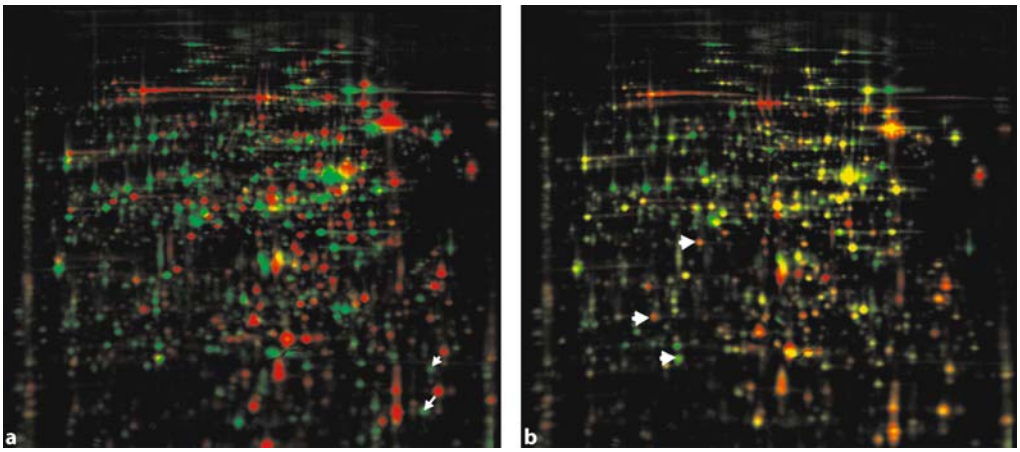


Fig. 9.2 a, b Differential analysis of 2D-PAGE. Depicted are overlays of two 2D-PAGE images in false colour. **a** Overlay of gels before warping. Protein spots of gel 1 are coloured in *green*, protein spots of gel 2 are in *red*. *Arrows* indicate orientation of warping. **b** Overlay of gels after warping. Protein spots equally represented on both gels result in a *yellow* colour. *Arrowhead* indicates a differentially displayed protein spot

ple, analysed in different gels are variable, i.e. matching of independent experiments is a severe problem. There are sophisticated software packages on the market such as Delta2D (Decodon, Greifswald, Germany) or ProteomeWeaver (Definiens) which permit gel-to-gel variations to be warped to each other. They are capable of quantifying the levels of proteins resolved on 2D-gels and have incorporated excellent spot detection algorithms and features that facilitate gel alignment and matching. Often included are commonly used bioinformatics tools such as principal component analysis, hierarchical clustering analysis or similar methods. Despite elaborate software, the inherent methodological variability necessitates some degree of manual workup for accurate spot matching and it binds a lot of computer power, making complex matching of independent experiments still quite laborious (Fig. 9.2).

5. One of the limitations of 2D-PAGE as a tool for biomarker discovery remains the sensitivity of MS characterization of protein spots, and particularly of post-translational modifications.
6. 2D-PAGE performs poorly in identifying heavily glycosylated proteins, since they tend to diffuse into clouds which are typically below the level of identification at any position in the gel.

The methodology of 2D-PAGE has also been improved by innovative modifications and sophisticated approaches: (1) the resolving power of the first dimension separation can be increased by the use of narrow pH-range immobilized pH gradient. A variation of this theme is the use of so-called "zoom gels" in which the protein contents of an individual sample are first fractionated into narrow pH ranges under low resolution, and then each fraction undergoes high-resolution separation by 2D-PAGE. Modern large "zoom gels" can reproducibly and reliably resolve thousands of different proteins from complex mixtures, which is superior to any other method (Gorg et al. 2004). Also, (2) sub-cellular fractionation of cells is able to increase the number of spots detected.

These different technological modifications together with advances in image analysis, data-mining and image storage have encouraged investigators to continue to apply 2D-PAGE for the analysis of complex samples leading to successful proteomic studies (Vercoutter-Edouart et al. 2001; Hondermarck et al. 2001; Bini et al. 1997;

Franzen et al. 1996a, b). These approaches have also been applied to biological fluids including serum (Goufman et al. 2006) and nipple aspirate fluids (NAF) (Alexander et al. 2004). In an exemplary study, Wulfkuhle et al. focussed on the identification of potential biomarkers in the early breast cancer lesion, ductal carcinoma in situ (DCIS), by analysing four cases of patient-matched, normal ductal epithelial cells and DCIS cells, specifically isolated from primary tissue by microdissection (Wulfkuhle et al. 2002, 2003). The proteomic profiles were compared by 2D-PAGE, differentially expressed spots were selected and sequenced by MS. The differential expression pattern for a subset of the identified proteins was validated by immunohistochemistry with a small, independent cohort of patient-matched normal/DCIS specimens. Very recently an analysis that combined 2D-PAGE with silver staining and MALDI-TOF and/or immunoblotting in sets of microdissected malignant breast epithelium and corresponding adjacent normal breast epithelia from 5 patients with invasive breast carcinoma was published (Hudelist et al. 2006).

9.2.3.3 2D-Difference Gel Electrophoresis

DIGE is a fairly recent advancement of 2D-PAGE technology, improving sample throughput and greatly enhancing gel reproducibility (Tonge et al. 2001; Von Eggeling et al. 2001). By using this method, which analyses several protein samples in one experiment, i.e. in one 2D-gel the systematic error of variable gel images can be avoided. Protein samples are labelled prior to electrophoresis with spectrally resolvable fluorescent cyanine dyes (Cy2, Cy3, and Cy5), Alexa dye, or with radioactive isotopes ("ProteoTope", ProteoSys, Mainz, Germany). The samples are then mixed prior to IEF and resolved on the same 2D-PAGE. The gel is scanned measuring the different labels, and multiple images corresponding to different samples are generated. Sample multiplexing in DIGE greatly refines the detection of changes at the protein level between samples (Tonge et al. 2001). Variation in spot intensities due to experimental factors, for example protein loss during sample entry into the strip, will be the same for each sample within a single DIGE gel. Therefore, the relative amounts of a protein between samples in a gel will be unchanged, thus increasing the confidence with which protein differences can be both detected and quantified. It reduces the amount of experimental variation due to a combination of multiple sample analysis in a single gel and internal standard correction (Alban et al. 2003; Lilley and Friedman 2004; Chen et al. 2005).

The fluorophores used for labelling are structurally similar and undergo nucleophilic substitution with the ε-amino group of lysine residues forming an amide. They have very similar molecular masses and are positively charged to match the charge that is replaced on the lysine residue. This matching of charge and mass ensures that all the samples essentially co-migrate to the same point during electrophoresis. In the labelling reaction, the dye/protein ratio is low. This ensures that protein molecules are only labelled with a single dye molecule. Quantitative cysteine alkylation such as DIGE labelling requires the correct stoichiometry of cysteine-reactive reagent, as well as correct reaction conditions to avoid generating artefactual spots caused by under- or over-alkylation, and these effects become critical for limited substrate levels in small volumes (Cahill et al. 2003; Sitek et al. 2005).

The fluorescence conventional strategy, with sensitivities similar to silver staining yet producing much better quantitative data, provides a linear dynamic range of almost three orders of magnitude; however, this range is reduced under conditions of signal multiplexing, so that direct multiplexing within one gel is not advisable with DIGE saturation labelling, due to fluorescence resonance energy transfer between the CyDyes (Patton 2002; Gruber et al. 2000). Accordingly, the considerable improvement in estimation precision for the ratio of abundance that can be ideally achieved by measuring intra-gel multiplexed protein spots cannot be realized with CyDyes (Poznanovic et al. 2005b). Recently the so-called saturation labelling method was introduced, which is now gaining in acceptance, and it has been applied to microdissected breast cancer samples (Wilson et al. 2005). Therein, one dye provides a reference standard to align spot patterns from multiple replicate gels (Kondo et al. 2003; Sitek et al. 2005). The disadvantage is that to reliably obtain statistically robust differential

expression data between microdissected samples sufficient multiple gels, with associated inter-gel variability, are required with micrograms of protein.

An alternative DIGE-method is radioactive labelling of the proteins using ProteoTope. Proteins from two samples are iodinated under chemically identical conditions with either 125I or 131I, mixed, co-electrophoresed by 2D-PAGE, and the signals from each isotope are differentially detected by ProteoTope imaging. To optimize the differential quantification of radioactive 2D-PAGE protein spots and to decrease the overlap in signals detected from different spots, high-resolution, 54-cm, immobilized pH gradients (IPGs)—IEF either in the continuous 54-cm IPG format or as serially connected 3×18 cm daisy chain IPG format—was developed (Poland et al. 2003; Poznanovic et al. 2005a). ProteoTope was intentionally designed for optimal protein spot quantification by radioactive detection and to achieve the most efficient analytical application of protein samples from extremely small sources, such as dissected tissue samples (Neubauer et al. 2006). It can reach a sensitivity of sub-attomole levels, with a dynamic range of over six orders, which is at least two orders of magnitude superior to fluorescent techniques. The linear dynamic range of detection of individual multiplexed, differentially abundant protein spots is typically greater than 15,000-fold, and cross-talk between the signals measured from the two samples is less than 1%. Under typical conditions, the labelling stoichiometry iodinates only approximately one tyrosine per 4,000 kDa, which generates sufficient radioactive signal, and provides a potential margin of error to establish reaction labelling conditions to avoid over-labelling of extremely limited samples. With ProteoTope, a statistically significant quantification of changes in the 15%–20% range can be distinguished with high certainty (Cahill et al. 2003), enabling quantification of even subtle protein changes in kinetic experiments. This greatly increases the accuracy of estimation of abundance ratios of identical proteins from different samples without the notorious complication of the inter-gel variability associated with conventional 2D-PAGE. Using this highly sensitive method also enables crosslabelling experiments which are performed for all labelling reactions to exclude the detection of false-positive proteins obtained by labelling or processing artefacts (Fig. 9.3).

ProteoTope was applied to a set of microdissected invasive breast cancer samples, all of which were ER+. Sub-pools were compared that were either positive or negative for the progesterone receptor (ER+/PR+ versus ER+/PR-). Employing a sample pooling strategy, several proteins differentially abundant, depending upon the presence or absence of PR, were found. In this experiment, approximately 180 ng of labelled protein was loaded per 54-cm analytical gel (Neubauer et al. 2006).

One issue using radiolabelled proteins is that the protein spots in analytical gels typically cannot be used for MS because of the vanishingly small amounts loaded. Therefore, preparative 2D-tracer-gels have to be run with high microgram amounts of unlabelled protein spiked with radioactive analytical protein, consuming highly valuable cryo-conserved tumour tissue. In the experiment mentioned above, 240 µg protein was required for the preparative tracer-gel (Neubauer et al. 2006).

9.2.3.4 Proteome Platforms Not Involving 2DE

As an alternative to gel-based separation approaches, attempts are being made to develop separation methods not involving 2D-PAGE. Liquid chromatography (LC) is one such technology that can successfully resolve a mixture of proteins and allow the isolation of individual proteins based on biochemical property. Some commonly used LC columns include size exclusion LC, ion exchange LC and reverse-phase LC. 2D LC, which combines pH gradient and reverse-phase columns, is a new proteomics technique that promises to extend the range of protein separation. Alternative "non-gel-based" approaches, such as MudPIT, have already been used effectively to catalogue many polypeptides in total protein mixtures from several organisms (Koller et al. 2002; Whitelegge 2002). However, while MudPIT is an excellent means of generating an exhaustive catalogue of proteins present in a particular protein sample, it does not yield reproducible quantitative information (Rose et al. 2004). MudPIT involves tryptic digestion of protein mixture followed by multi-dimensional

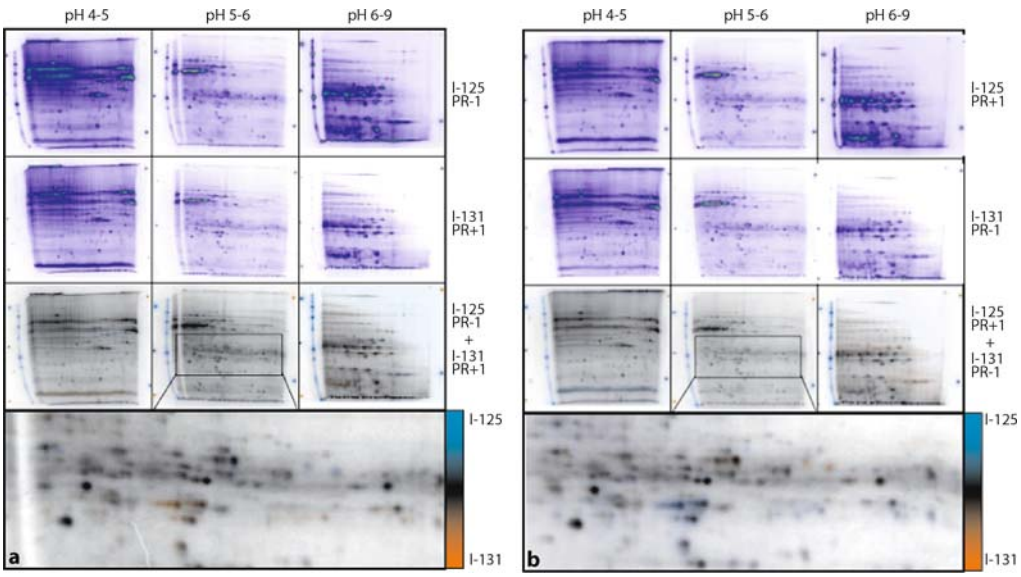


Fig. 9.3 a, b A 54-cm daisy chain IEF differential ProteoTope analysis of pooled LCM samples. The panels show actual images from an inverse replicate labelled ProteoTope experiment for one sample pair. **a** Analysis of sample PR-1 labelled with 125I, differentially compared with sample PR+1labelled with 131I. The *upper panels* show the signal detected for each isotope, depicted in false spectral colour. The signals for each isotope have been normalized against each other for total relative intensity in the *lower* dual channel images, where the signal for 125I is *blue*, the signal for 131I is *orange*, and equal amounts of both signals produces *grey* or *black* signal. Two pure sources each of 131I and 125I, as well as a 50% mixture of both isotopes, are measured on round, 2-mm pieces of filter paper placed next to each gel as imaging controls. Cross-talk between the signals from each isotope is <1%. The pH ranges of the 18-cm IPGs used for serial IEF are indicated *above* the panels, and the radioactive iodine isotope signals depicted in each panel are indicated on the *right*. Approximately 180-ng protein from each sample was loaded to each gel, and the above result was obtained by labelling approximately 3.6 mg protein from each sample. **b** The *top panels* show the inverse replicate experiment of **a**, where sample PR-1 is labelled with 131I, and sample PR+1 is labelled with 125I. (Reproduced with publisher's permission from Neubauer et al. 2006)

LC separating proteins by size-exclusion or cation exchange chromatography and in the second dimension by reverse-phase HPLC (Wall et al. 2000). Followed by MS measurement and database searching, the fractionated proteins can be directly analysed. This method shows advantages over gel-based techniques in speed, sensitivity, scope of analysis and dynamic range and it could be amenable to automation. Unfortunately, LC/MS is not capable of determining protein abundance. MudPIT has recently been associated with enzyme activity profiling in human tumour tissues, including breast tumours, and has generated functional signatures that correlate with previously described molecular subtypes (Jessani et al. 2005).

For quantitative comparisons of proteomes without the use of 2D-PAGE, ICAT technology has been developed recently (Gygi et al. 1999a). It is based on labelling a pair of samples simultaneously at the cysteine residues with differentially deuterated d0- and then d8-ICAT reagents ($^{13}C/^{12}C$ pairs are also available) (Gygi et al. 1999a). Unlike 2D-PAGE and SELDI-TOF (discussed later), which comparatively profile the naturally occurring forms of peptides and proteins, ICAT analyses the relative amounts of cysteine-containing peptides derived from e.g. tryptic digestion of protein extracts. The samples are then combined and analysed by LC-MS/MS. Each cysteinyl peptide appears as a pair of signals differing by the mass differentially encoded in the mass tag. The ratio of these signal intensities precisely indicates the ratio of abundance of the protein from which the peptide originates and the MS/MS spectrum of the peptide allows

the protein to be identified. ICAT has greatly expanded the range of proteins that can be analysed, quantified, and identified using these techniques. It has been applied to compare NAF from tumour-bearing and contralateral disease-free breasts of patients with unilateral early-stage breast cancer (EBC), identifying and quantifying differences in various specific protein expressions (Pawlik et al. 2006).

LC methods resolve hundreds to many thousands of peaks, and the limitations to the method involve not only generating reproducible analysis conditions, but also being able to process the vast reams of data that can be generated by e.g. MudPIT. The use of mass spectrometers with high-resolution Fourier transform ion cyclotron detectors reduces the stringency required of pre-mass spectrometric biochemical separations, and greatly increases the confidence with the protein identifications that are obtained (Haas et al. 2006). LC or chromatographic arrays have moderate sensitivities (~5 fmol) and a clear disadvantage in terms of dynamic range of protein concentrations—with moderate dynamic ranges of approximately three orders of magnitude (Schrattenholz 2004).

In a recently published study by Komatsu et al., 2D-LC and 2D-DIGE were compared with 2D-PAGE in combination with Coomassie brilliant blue (CBB) staining for their ability to identify proteins regulated by gibberellin (GA) in rice (Komatsu et al. 2006). Using 2D-LC and 2D-DIGE, many more proteins were detected compared with 2D-PAGE followed by CBB staining. Additionally, the two former methods detected proteins that were not reported previously. The difference between 2D-DIGE and 2D-LC was that minor GA-responsive proteins were detected only by 2D-DIGE and the low-molecular-weight proteins were detected only by the 2D-LC system. This suggests that the 2D-LC technique is the preferred method for detecting low-molecular-weight proteins. However, poor reproducibility and the large number of replicates required to establish statistical significance are problems that still must be resolved. Further, the 2D-DIGE technique is more sensitive and is able to make exact quantitative comparisons.

An often-cited technical disadvantage of "shotgun" proteomics methods such as MudPIT and ICAT, which reduce polypeptides to peptides before separation, is that modifications such as splice variants and post-translational protein modifications often escape detection. Additionally, although these methods are frequently designated as being high-throughput, they are typically expensive in terms of machine time, and experimental repetitions to gain meaningful sample sizes for the assessment of the statistical significance of differences are rarely performed for reasons of sample availability, analysis time and running costs. Nevertheless, these methods do generate considerable amounts of data in a short time.

9.2.3.5 Protein Chips

The successful application of DNA microarrays to genome and transcriptome research demonstrated the value of array-based measurements, and it was soon recognized that the ability to perform such experiments to measure proteins likewise would be very valuable. However, protein microarray technology is not as straightforward as DNA-based microarrays owing to the complex structure of proteins and e.g. the absence of a protein amplification method (Haab et al. 2001; MacBeath and Schreiber 2000). Protein microarrays are generally of two types (Speer et al. 2005): (1) antibody arrays, also known as forward phase arrays (FPA), in which the bait molecule—typically an antibody—is spotted onto suitable surfaces, and bound antigens are detected using radioactivity, fluorescence and chemiluminescence (Haab 2005). In FPAs, each spot represents only one type of bait molecule. The array is incubated with only one test sample that contains several different analytes of interest. The captured analytes are detected with a second tagged molecule or by labelling the analyte directly; and (2) non-antibody or reverse phase arrays (RPA) where sets of labelled proteins or even entire proteomes are spotted onto a slide (Liotta et al. 2003; Speer et al. 2005; Sheehan et al. 2005) (Fig. 9.4). The RPA design enables high-throughput analysis in the sense that every single spot comprises the entire protein pool of one patient/sample. Dozens of samples can be spotted on one slide in parallel and therefore can be probed with an antibody against the endpoint of interest in one experiment. This leads to excellent comparability

among samples and reduces errors. In contrast to FPAs, where different antibodies with various binding characteristics and capacities need to be combined on one platform, the RPA enables the user to choose the optimum binding conditions and dilution for the chosen antibody in order to detect the antigen of interest. This format allows multiple samples to be analysed under the same experimental conditions for any given analyte. As little as 30–60 µl of cell lysate is sufficient to print 50 or more arrays (Espina et al. 2003). Recently, a two-colour comparative fluorescence strategy has been used to compare protein levels between malignant and normal breast tissues from the same patient (Hudelist et al. 2004). Therein, a reference sample was co-incubated with a test sample to normalize for variation between spots in capture antibody concentration. The assay is competitive and generates a linear response according to the concentration of the analyte.

The application of protein microarrays provides unique advantages. It offers high-throughput capabilities because of the low sample volumes needed. Protein chips also offer high robustness, sensitivity, inter-sample comparability and the possibility of a quantitative analysis of protein expression. Depending on the individual affinities of the immobilized antibodies, antigens can typically be detected in picomole (pmol) amounts, which is currently more than 1,000

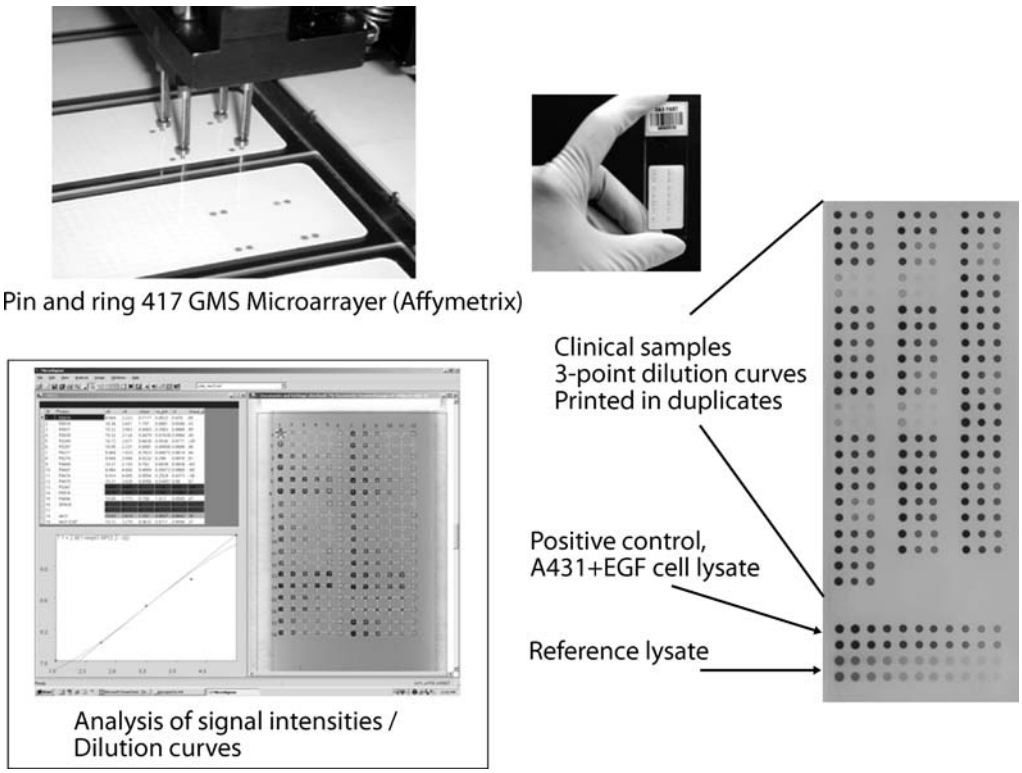


Fig. 9.4 Gene expression profiling by reverse-phase protein microarrays. The cell lysates are printed in duplicates at distinct positions on nitrocellulose coated slides using, for instance, a pin and ring 417 GMS Microarrayer (Affymetrix). Samples are arrayed in three-point dilution curves. The slides are then incubated with an antibody against a target protein of interest, such as phosphorylated endoplasmic reticulum kinase in this case. The antibody is detected by chemiluminescent, fluorescent or colourimetric assays. The intensity of the signal is proportional to the concentration of the target protein. A positive control lysate (A431 squamous carcinoma cell line) is printed on the array for monitoring immunostaining performance. Phosphorylation-specific reference peptides are printed in a 12-point dilution curve on the bottom of the array for comparative, precise quantification of patient samples between arrays. Image analysis is performed with, e.g., Microvigene (VigeneTech). *EGF*, epidermal growth factor

times less sensitive than 2D-PAGE or MS-based methods. However, with the development of suitable high-affinity antibodies and sensitive detection techniques, improvements are possible. For example, considering the specificity, affinity and cross-reactivity, only about 5% (MacBeath 2002) to 30% (Haab et al. 2001) of commercial antibodies are suitable for microarray-based analyses. Furthermore, as in any format of antibody array used, the specificity of antibody binding should be characterized, and the binding levels observed by microarray should be validated by independent methods. Appropriate assays like Western blots work well to confirm binding to a single target in a complex mixture, or to confirm changes in the level of certain targets (Orchekowski et al. 2005; Sreekumar et al. 2001). A method of validation that does not require purified antigen is immunoprecipitation and MS analysis of the captured proteins. This procedure should reveal both the specific and non-specific proteins bound by a particular target. The observation of the same biological information from different antibodies that bind to different epitopes on a protein can be a strong confirmation of the validity of the result.

Another characteristic of reverse phase protein microarrays is their flexibility, considering the fact that the design enables the user to analyse protein samples in denatured and non-denatured conditions. This flexibility ensures that protein microarrays can be used for a plethora of applications, such as drug discovery, biomarker identification, molecular profiling, developing "circuit maps" of on-going signal transduction in cell and tissue samples and assessment of response profiles for new drugs. Protein microarrays also provide a well-controlled in vitro way to study protein function, including protein–protein, protein–lipid and protein–nucleic acid interactions on a genome-wide basis (Jessani et al. 2002; Bulyk et al. 1999; Zhu et al. 2000). Already antibody arrays have been used to measure phosphorylation states and to study signalling in networks of interacting proteins (Gembitsky et al. 2004; Nielsen et al. 2003). Another novel use of antibody microarrays is to profile enzyme activity in complex proteasomes (Sieber et al. 2004). The measurement of enzyme activity rather than just abundance is important for determining the functional state of certain proteins and may be valuable for cancer research. Complex protein samples are treated with fluorescent activity-based probes and the labelled enzymes are captured and detected on antibody microarrays targeting those enzymes. Recently, expression profiling using reverse-phase protein microarrays has been applied investigating breast cancer tissue (Cowherd et al. 2004; Hudelist et al. 2004).

9.2.3.6 Surface-Enhanced Laser Desorption Ionization

The ProteinChip System (Ciphergen, Fremont, CA) is an alternative array system which has the advantage of its direct integration with MS. It is a rapid and sensitive analytical method which allows the quantification of proteins with different masses originating from complex protein mixtures such as body fluids or cell and/or tissue extracts by surface-enhanced laser desorption ionization (SELDI) at the femtomole level (He and Chiu 2003). However, SELDI cannot directly determine the identity of proteins. The SELDI protein chip platform is based on the principle that proteins from crude mixtures are selectively attracted to specific biochemical surfaces. Potential biomarkers may show a higher binding affinity to certain surfaces than serum albumin, haptoglobin and other abundant serum proteins. The current platform, based on nine different capture agents per chip, is claimed to achieve comprehensive coverage of the proteome. The SELDI-protein chip surfaces are chemically or biochemically modified to enable the capture of a certain group of proteins: they include (1) weak cation exchange, (2) strong anion exchange, (3) immobilized metal affinity chromatography, (4) reverse phase and normal phase and (5) biochemical affinities allowing proteins or antibodies to be bound directly to the chip (Arthur 2003). The retained proteins are then ionized and analysed using TOF/MS. The great advantages of this approach are its sensitivity to analyse small amounts of raw protein samples and its ability to detect proteins with molecular weights lower than 6 kDa. These characteristics make SELDI ProteinChip technology very attractive for biomarker discovery, especially because new biomarkers should be able to detect early forms of

cancer before it has metastasized throughout the body and should monitor a patient's response to therapy or the potential of recurrence in real time. Beyond that, for routine clinical use a reliable minimally invasive diagnostic test would be highly preferable. Serum has the advantage of being a readily accessible body fluid that is protein-rich and that is well-suited to proteomic analysis. Differences in proteomic patterns in serum from cancer patients can be due to: (1) serum proteins that are differentially expressed in patients with cancer, or serum proteins that are cleaved or modified in cancer patients; (2) proteins that are secreted by tumour cells; or (3) intracellular tumour proteins that are released when tumour cells die. Molecules released by tumour cells subsequently enter the blood and/or other fluids. Analysis of serum is pretty straightforward: it requires minimal sample preparation and only a very small amount of sample (1–20 μl). As an approach to serum biomarker discovery, proteomic pattern analysis has been developed to identify novel markers by comparing samples from patients with disease with those from healthy subjects. It is based on the analysis of large amounts of mass spectrometric data derived from complex protein mixture and does not per se require that the proteins involved are identified. Differentially displayed MS signals can then be identified and confirmed as potential biomarkers. In breast cancer, SELDI-TOF MS was used to investigate serum/plasma (Li et al. 2002; Vlahou et al. 2003; Becker et al. 2004; Hu et al. 2005), but also NAF (Mendrinos et al. 2005) and tumour tissues (Ricolleau et al. 2006) as a potential source for diagnostic biomarkers, and as a potential tool to predict outcome (Heike et al. 2005). Recently, SELDI has been applied to examine proteomic changes that occur in response to paclitaxel chemotherapy or 5-fluorouracil, doxorubicin and cyclophosphamide chemotherapy in plasma of 69 breast cancer patients and normal volunteers (Pusztai et al. 2004).

As with many of the initial SELDI-studies, standardization and independent validation using larger numbers of specimens is required to ensure the performance of these selected biomarkers. Attempts to validate serum proteome patterns are currently being made by collaborating research groups, where each sample is analysed by all participating laboratory. Encouraging results have been reported in reproducing separation patterns and disease classification in different laboratories (Grizzle et al. 2003–2004). Even though these results are promising, SELDI-TOF-MS screening for serum biomarker discovery limited the performance of this approach for biological fluids because proteomic technologies based on MS may not be sensitive enough to detect low-abundance molecules that are released by a few tumour cells or their microenvironment into the circulation. Profiling tissue extracts might therefore be more suitable for such a biomarker screening technology, as dilution effects are reduced (Ricolleau et al. 2006). Additionally, the clinician should be aware that doubts have been expressed concerning the reproducibility of the SELDI methodology itself (Diamandis 2004; Baggerly et al. 2004; Bons et al. 2005). Major issues to be solved are variations in serum collection and sample handling and how they will affect the analyses. Therefore, initiatives for standardizing the pre-analytical methods on different operational levels might help us to exploit the full power of the increasingly powerful high-throughput analytical technologies (Carr et al. 2004; Bradshaw et al. 2005).

9.3 Standardization of Pre-analytical Methods

The growing application of molecular diagnostic techniques in the clinic and in translational oncologic research has made it necessary to consider standardization of the pre-analytical methods used to collect, store and catalogue valuable human tissue. Therefore, guidelines for "banking" fresh tumour and normal tissues as a part of the routine activity in surgical pathological laboratories, specific for the requirements of molecular-based tests and quality control measures, have to be established. Additionally, various data about the tissue, the patient and the tissue inventory have to be managed and made available to the different co-operating disciplines and departments at different locations. For molecular analysis in the laboratory, guidelines for specimen handling, tissue enrichment strategies and quality controls must be established that are appropriate for the requirements of molecular-based tests. Finally, new experimental strategies

might have to be adapted to changes in clinical processes such as new tissue sampling methods to perform experiments.

9.3.1 "Tissue Banking"

For molecular research and the analysis of genomic DNA, mRNA, or proteins, high-quality human tissue is fundamental. Although for some of the morphology-based procedures, as well as for many applications of the PCR, FFPE tissue is adequate, most other diagnostic and research applications, which are based on intact genomic DNA, mRNA or protein, require frozen tissue samples, making the establishment of a frozen tissue bank a valuable diagnostic and research asset. In a clinical environment, usually several samples from one cancer patient—such as primary tumour, normal tissue, precursor lesions—are collected and stored at different time points with differing quality (paraffin tissue, serum, plasma) by different departments at different locations. Thus, it is necessary to consider standardization of the methods used to retrieve, freeze, store and catalogue tissue specimens by implementing standard operating procedures (SOP). Particularly, with regard to the collection and cryo-preservation of tissue, it is especially important to establish a rapid logistic chain for achieving optimum tissue preservation. This must be performed as soon as possible after ex-

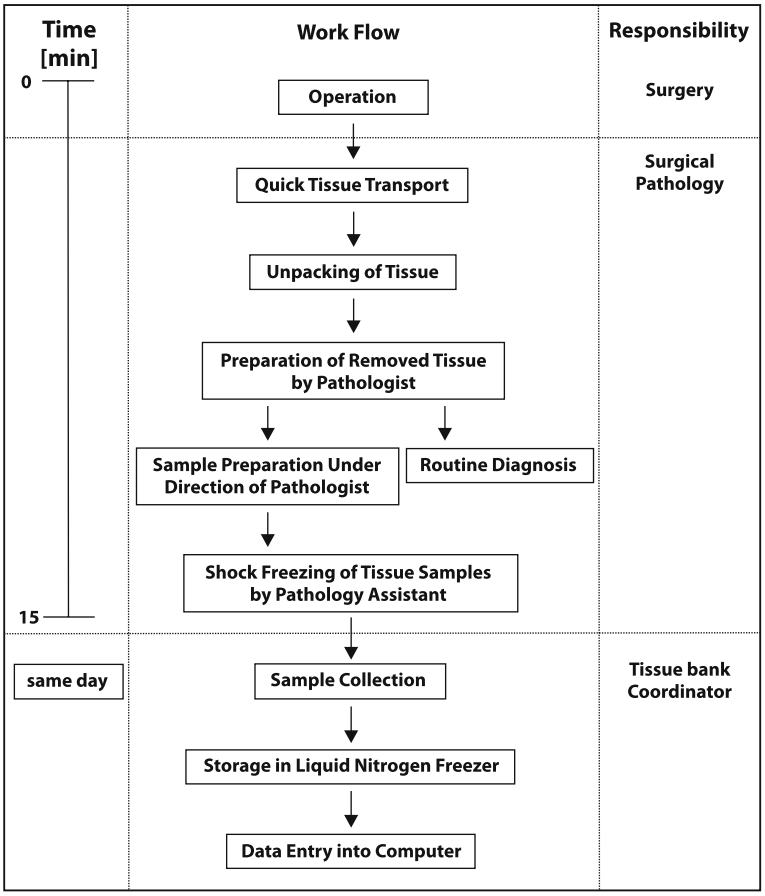


Fig. 9.5 Organizational structure of a frozen tissue bank. This diagram depicts the areas of responsibility for the surgeon, the surgical pathologist and the tissue bank co-ordinator and shows the workflow for sample collection and storage. Also depicted is the time after operation during which the tissue sample should be frozen to guarantee sufficient quality

cision of the tissue, optimally between 10 and 20 min afterwards. A responsible pathologist is required for the collection, maintenance of the tissue bank and its integration into the routine surgical pathology activities (Fig. 9.5).

Rapid freezing of the tissue samples can be achieved by several means and a variety of methods are in use in different laboratories. These include dropping freshly excised tissue into liquid nitrogen (−196 °C) or a cooled isopentane bath (−160 to −78 °C) after placing it into a 2.0-ml cryogenic vial without cryo-protection for subsequent use in procedures requiring the extraction of genomic DNA, mRNA or protein. To preserve tissue architecture and cytologic features for immunohistochemistry and in situ hybridization, freezing with a cryo-protectant such as Tissue-Tek OCT (VWR Scientific, Bridgeport, NJ) should be chosen. The mechanisms of tissue damage by freezing are complex; however, rapid cooling of the tissues retards the development and growth of ice crystals as well as abruptly halting enzymatic activity. Equally critical is the method used to store the frozen tissue for extended periods. Long-term storage of frozen tissues that may be used subsequently for molecular studies should be carried out at temperatures of −70 °C or lower. Long-term storage of the frozen tissue is recommended in the gas phase of liquid nitrogen which is under constant temperature surveillance. This is preferred because no chemical reactions take place below approximately −130 °C. Messenger RNA and proteins may undergo slow degradation in unfixed tissue stored at warmer temperatures, and growth of ice crystals is favoured, causing damage to the architectural and cytologic features of the tissue. Tissue sampled and stored under these conditions has been used successfully in molecular techniques analysing the transcriptome and proteome of tissue from breast cancer patients (Schütz et al. 2006; Neubauer et al. 2006). An alternative to cryo-preservation might be the storage of fresh tissue in high salt solutions (RNAlater, Ambion, Austin, TX) to preserve macromolecules. Ultimately the best way of monitoring the effectiveness of tissue storage conditions is to test the integrity of mRNA, because it is more labile than genomic DNA or proteins. Thus, the isolation of intact RNA is a good indication of how well the tissue has endured the frozen state (Fig. 9.6).

9.3.2 "Data Banking"

While tumour banking has been recognized for over a decade as a needed tool to advance the molecular science of oncogenesis and tumour progression (Naber et al. 1992; Naber 1996), only in the last 4–5 years has the linkage to clinical outcomes been regarded as crucial for achieving this goal (Qualman et al. 2004). Therefore, the development of databases represents an independent scientific field, which is separated from molecular and clinical research and demands high logistic and financial investment.

The introduction of databases into the clinic is increasingly important for the optimization of

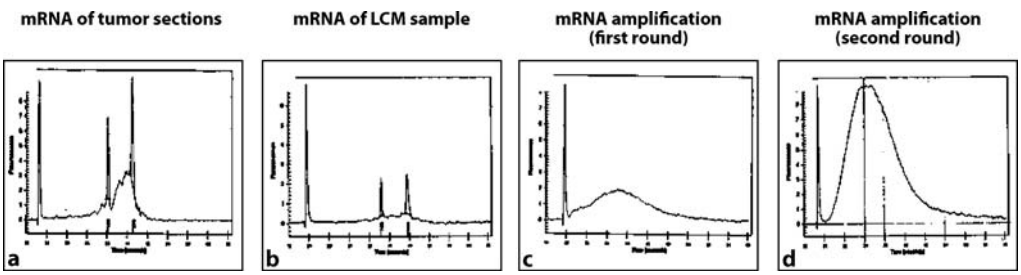


Fig. 9.6 a–d Quality control of tissue and analyte. Depicted is the control of rRNA integrity by capillary electrophoresis (Bioanalyzer, Agilent). **a** profile of rRNA from the whole tumour section; **b** profile of rRNA from laser dissected tumour cells; **c** profile amplified mRNA after first round of linear amplification; **d** profile amplified mRNA after a second round of linear amplification, before hybridization onto a microarray chip. The electropherograms in **a** and **b** show the 28S/18S rRNA peaks. The presence and a 2:1 ratio of the peaks indicate good quality. The *x-axis* indicates the time in seconds; *y-axis* indicates the measured fluorescence intensity

documentation, for quality assurance, to improve patient care, to gain reliable results and to control costs. Hence, database systems are not only important for bioinformatic data analysis but are required to develop a successful experimental design. In spite of complex database structures and functions, up to now no available database alone satisfies all medical requirements. In the field of specialized oncology databases are needed for the management of tissue banks for oncological research and care. To obtain exact results, small but defined sample collectives have to be selected out of tumour banks by combining several selection criteria. This attempt is impeded by the fact that different kinds of samples (e.g. fresh frozen, paraffin-embedded) and tissue pools, which are in general very heterogeneous because of different clinical progression and histology, might be needed. Therefore the combination of clinical data, histopathological data and data describing the course of the disease and therapy has to be enabled. In contrast to databases used in routine clinical practice for clinical documentation and quality assurance, tissue banks require tumour databases for molecular biological and scientific research. Therefore, not one single bank is needed but a collection of different banks, which are controlled by one database. The database has to provide the opportunity for flexible combined searches to gather the relevant information needed for stratification of samples. It must be pointed out that in addition to the logistic challenges collecting and storing tumour samples and associated data, there are numerous methodological, ethical and legal questions and challenges (Oosterhuis et al. 2003). In some countries co-operative or even nationwide tissue banks were established providing large numbers of annotated cancer specimens to investigators (Qualman et al. 2004; Schilsky et al. 2002; Melamed et al. 2004). Other nationwide tissue banks restrict the distribution of tissue to specifically funded projects. Recently, TumorAGENT, a new database, was developed for breast cancer research (Babel et al. 2006; Kurek et al. 2006). This dynamic relational database is unique in its structure and complexity; it has a flexible structure, architecture and user interface developed especially for the medical information and knowledge management field. It is able to collect,

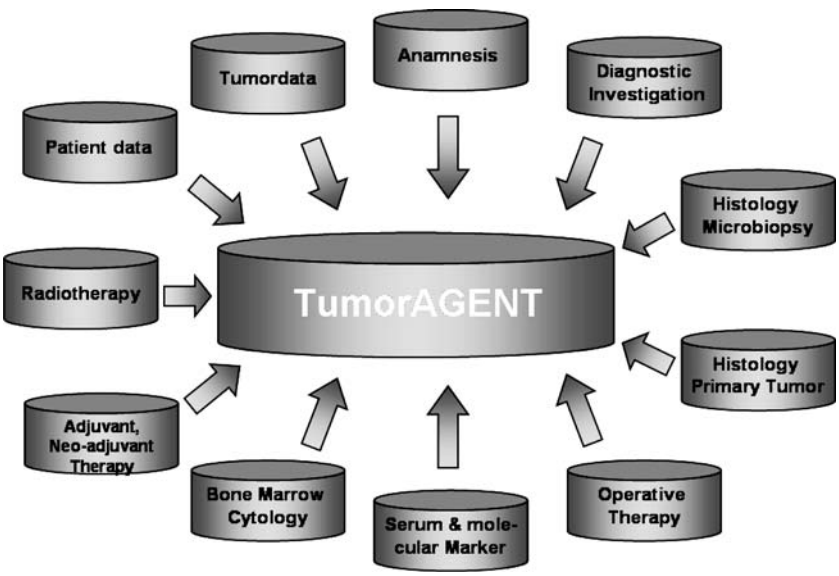


Fig. 9.7 Combined tissue and data management for oncologic research. Depicted are data modules implemented into TumorAGENT. This database consists of patient and sample management. In patient management all clinically relevant tumour data related to a patient can be collected and stored. Storage data of tissue samples are recorded in sample management. A flexibly designed search module permits data recall facilities over all data fields of patient and sample management. Even complex questions can be answered by the AND/OR relation

administrate, release and evaluate all relevant patient, tumour and sample data. The applied Web technologies offer maximum temporal and local availability. In TumorAGENT, approximately 800 attributes can be documented in about 100 forms and 30 entities, and it provides a flexible search module for molecular biological and scientific reporting (Fig. 9.7).

9.3.3 Sample Enrichment Strategies

The improvement of modern clinical screening programmes leads to the detection of tumours at an earlier stage. Consequently, profiling methods with increasing sensitivity have constantly to be improved. A challenging problem is the heterogeneity in tissue morphology accounting for the fact that the cell population of interest may constitute only a tiny fraction of the total tissue volume. For instance, breast cancer specimens are extremely heterogeneous with the tumour cells being mixed with many other cell types. Therefore, strategies for sample dissection, enrichment and amplification have to be employed to obtain specific expression profiles (Kunz and Chan 2004; Burgemeister 2005; Wulfkuhle et al. 2002). These techniques include e.g. magnetic beads, coupled with specific antibodies to label the cells of interest and to separate them by magnetic force. Alternatively, enrichment methods by the use of centrifugal forces applied to density gradients can be used (Jechlinger et al. 2003). For tissue sections, different macroscopic ("scratching") or microscopic techniques have been developed and should be applied by an experienced pathologist. One of these methods is microdissection assisted by a laser beam (Emmert-Buck et al. 1996; Wiltshire et al. 1995; Craven and Banks 2001; Bonner et al. 1997; Bichsel et al. 2000). It permits the isolation of single cells or single populations of cells from thin tissue sections (typically 5–10 µm in thickness) mounted on a glass slide (Fig. 9.8; Neubauer et al. 2006; Schütz et al. 2006).

It is reported that its application is able to reduce co-isolation of contaminating cells to as little as 0.6% (Nishidate et al. 2004). In one common type of this technology, laser capture microdissection (LCM), a narrow laser beam (7.5–30 µm in diameter) is fired at a heat-sensitive transparent polymer film on a cap that is in contact with the tissue. When the polymer is heated, it adheres to the cell(s) of interest and these cells are subsequently removed from the section when the cap is lifted (PixCell II LCM System, Arcturus, Mountain View, CA). An alternative approach is the laser microdissection and pressure catapulting (LMPC) system of PALM Microlaser Technologies (Bernried, Germany). In this system a pulsatile nitrogen laser is fitted to a modified research microscope and focussed on the sample. Specimens from different selected locations can first be laser microdissected and later catapulted by laser-induced pressure towards a collection device, such as a microtube cap (Poznanovic et al. 2005b).

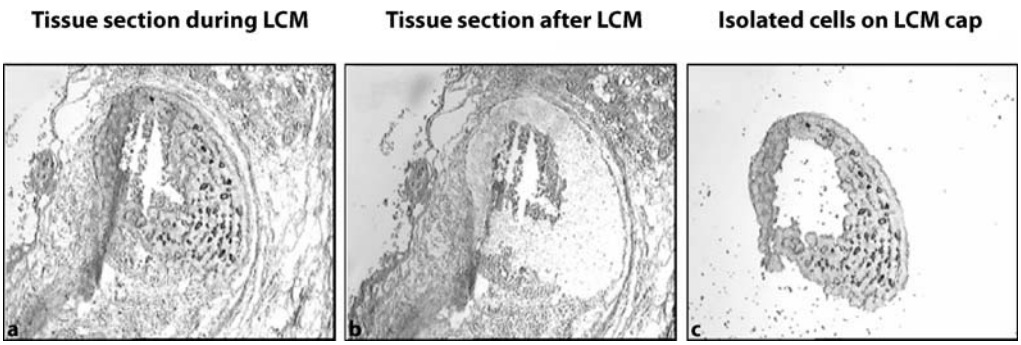


Fig. 9.8 a–c Tissue separation by Laser capture microdissection. Depicted is the isolation of epithelial cells of a breast ductal carcinoma in situ (DCIS). **a** The tissue section during LCM. Epithelial cells in the DCIS are dissected using a laser beam. The central necrotic area is not captured. **b** The tissue section after removal of the LCM cap with the epithelial being removed and the central necrotic area and the surrounding tissue being left on the slide. **c** The dissected epithelial cells on the LCM cap. The tissue section is stained with haematoxylin and eosin

The application of microdissection enables the analysis of cells from the same patient sample, e.g. different breast cancer progression stages, reducing the problem of genetic variability between individuals (Schütz et al. 2006). The other side of the coin is that the amounts of material that can be harvested by microdissection are exceptionally limited. This exacerbates the problems associated with sample yield and makes it difficult to standardize experiments, leading to reproducibility problems. Finally, experiments must be repeated to gain statistical sufficiency. The numerous replicates that are required to achieve sufficient sample sizes for acceptable statistical analysis is impossible for all but the most abundant proteins, which is exceedingly problematic with rare samples. For genomic and transcriptomic analyses, linear amplification or PCR-based amplification of the nucleic acid allows even single cells to be profiled (Schütz et al. 2006; Klein et al. 2002). But one has to acknowledge that the composition of the analyte might be biased, for instance, to the loss of the 5′-end of long transcripts. The issue of sample amount is ironically made worse by the success of contemporary clinical screening programmes, which detect tumours at an earlier stage. Therefore, high sensitivity detection methods are extremely desirable for the quantification of the small amounts of proteins often available in these samples, and the most successful studies represent the cutting edge of technology.

An important issue in using microdissection is to preserve the DNA, mRNA or the proteins. Therefore, special protocols must be established that allow quick staining of the tissue section using inhibitors for nucleases and proteinases and conserving its morphology. Eosin for example probably should be omitted or minimized from experiments investigating proteins (Craven et al. 2002). Maintaining the sections in a dehydrated state is crucial as well.

9.3.4 New Experimental Designs: Sample Pooling

Cells and their gene expression pattern are influenced by the environment, e.g. soluble factors, extracellular matrix proteins and the cell–cell communication they are deprived of when cultured. Ornstein et al. published a direct proteomic comparison between cultured clonally selected human prostate tumour cells and the patient-matched primary tumour epithelium from which they were derived (Ornstein et al. 2000). This revealed only a 25% similarity between the two protein populations even though they came from the same person. Therefore, molecular analysis of cells in their native tissue environment provides the most accurate picture of the in vivo disease state, with primary cell cultures established from fresh tissue being unable to duplicate the environment of cells in the actual tissue from which they are derived. Because of that, especially in experiments investigating clinical samples where the amount of tissue sample is particularly problematic, pooling of samples might be advantageous. Another reason for the pooling of samples is to reduce the costs of screening large numbers of samples. For proteomic analysis of microdissected clinical cancer samples, both of these situations apply, because proteins cannot be amplified. Therefore, the use of microdissection yields amounts of proteins that are difficult to reconcile with the need for greater amounts for 2D gels. In order to provide the amounts of protein obtainable from microdissection experiments on primary human samples from single patients with the amounts of protein required to achieve high quality 2D-PAGE results, a recent publication describes a sample pooling strategy (Neubauer et al. 2006). A disadvantage of pooling is that individual variations among pooled subjects are lost within a pool, so that all relevant clinical classifications are essential prior to pooling.

9.4 Conclusion

Due to technical progress of analytical methods and the knowledge of the human genome, the field of molecular medicine has become the basis for large-scale analysis of cancer cells on the genetic, transcriptomic and proteomic levels. These new technologies might transform the clinical practice of medicine, assisting with (1) the detection of cancer when it is at its earliest stage, even in the premalignant state, and (2) the individualization of treatments, which are two of the most important challenges of the post-genomic era. To accomplish these goals new biomarkers are needed to complement the existing histopatho-

logic markers being applied. This means that focussing efforts on one or a few platforms is unlikely to uncover all, or even the best, biomarkers of a disease. Therefore, different expression profiling platforms analysing genomic, transcriptomic and proteomic variations have to be used in a concerted manner. Regarding proteomic analysis, the methods available at the moment have sensitivities, a resolving power and a speed that already provide scientists with the possibility to analyse protein expression of complex systems such as breast cancer, with each approach having its strengths and weaknesses. Although these technologies are rapidly evolving they are still not as robust as those in the field of genomics, so that there are new strategies and technical improvements needed. On the other hand, even pre-analytical methods have to be improved and standardized to provide the optimal prerequisite for high-quality analysis and the comprehensive management of tissue, patient and experimental data. Further evaluations and the characterization of genomic, transcriptomic and proteomic variations may lead to the identification of biomarkers that can specifically be applied in clinical diagnoses, or might serve as drug targets.

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Part II Targeted Therapy and Clinical Applications

10 Targeted Therapy and Blood–Brain Barrier

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Abstract

Due to defined molecular lesions and signaling pathway disruptions, brain tumors represent good candidates for targeted therapy. However, in brain tumors the blood–brain barrier may prevent the transfer of progress in the molecular neurosciences to the development of effective therapeutic strategies. In this article, the most advanced forms of targeted therapy for the treatment of brain tumors and their clinical results are presented.

10.1 Introduction

Despite advances in standard therapy, the prognosis for patients with brain tumors remains poor with a median survival of 8–30 months, depending on tumor histology. Thus, these patients urgently need improvements in the therapeutic tools. Brain tumors are highly suitable for targeted therapy because they have a set of defined molecular lesions and signaling pathway disruptions that represent clear targets. In malignant brain tumors, the targeted therapy may have potential advantages as compared to standard chemotherapy:

- A high drug concentration at tumor side is not necessarily required due to the higher affinity to the tumor.
- The high specificity in targeting the tumor cells may result in a better therapeutic index.
- Targeted therapeutics lack pharmacodynamic interactions with conventional chemotherapies and anticonvulsants.
- The combination with conventional chemotherapies may result in a synergistic therapeutic response.

The present article deals with the most advanced forms of targeted therapy for the treatment of brain tumors.

10.2 The Blood–Brain Barrier

The major problem in brain tumor therapy as compared to other tumor localizations is the protective role of the blood–brain barrier (BBB). The BBB is composed of three different „lines of defense“:

- An anatomic barrier of endothelial cells of the brain capillaries with tight junctions, the basal membrane, and surrounding astrocytes (Reese and Karnovsky 1967; Fig. 10.1)
- An enzymatic barrier of degrading enzymes localized in endothelial cells
- Transport systems actively transporting drugs from the brain back into the blood, such as P-glycoprotein (P-gp) and *multidrug resistance* (MDR) protein (MRP)

The lack of fenestrations, vesicular transport, and pinocytosis in the endothelial cells of brain capillaries represent other hindrances for the exchange between the blood and the brain.

The BBB permeability for most drugs can be predicted by their lipophilicity, molecular mass, and polarity. Additionally, the cumulative surface of all brain capillaries, the blood flow rate, the concentration of the free drug in blood, and

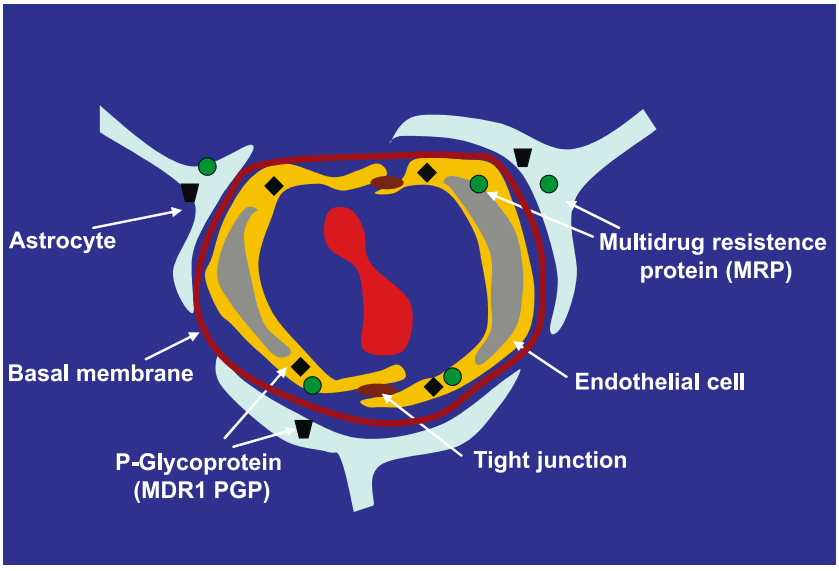

Fig. 10.1 Blood–brain barrier

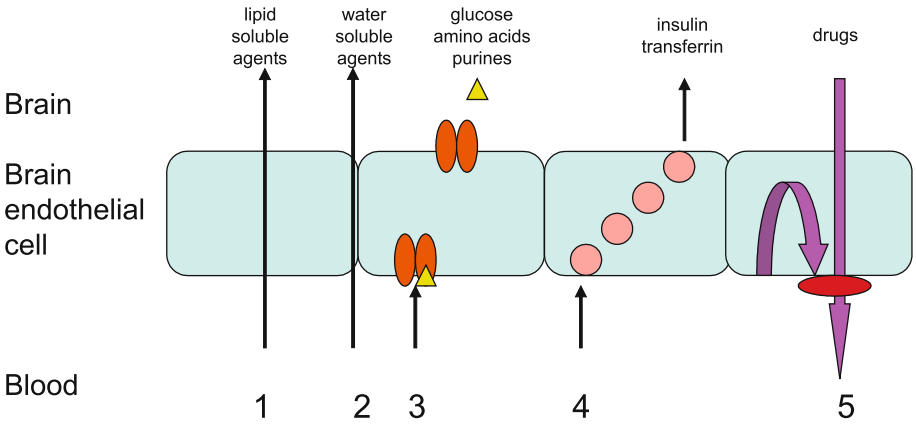

Fig. 10.2 Mechanisms of penetration through the blood–brain barrier. *1*: transcellular lipophilic pathway; *2*: paracellular aqueous pathway; *3*: transport proteins; *4*: specific receptor-mediated endocytosis; *5*: efflux pumps

the circulation time influence the penetration from the blood into the brain. Some drugs can be actively transported through the endothelial cells of BBB by membrane transporters, transcytosis, and adsorptive endocytosis (Abbott and Romero 1996; Fig. 10.2).

The role of BBB in the therapy of brain tumors is sometimes negated due to the belief that tumors do not have an intact BBB, which is illustrated by the penetration of big molecules such as contrast agents into the tumor. Indeed, the BBB of brain tumors is often locally and inhomogeneously disrupted (Schlagetter et al. 1999) with the grade of disruption being most pronounced before therapy. However, with therapy-caused tumor shrinkage the BBB can be restored. Moreover, infiltrating tumor cells surrounding the tumor bulk usually have an intact BBB. The

consideration of the inhomogeneity of the BBB of brain tumors is of major importance since the homogeneous acquisition of the whole tumor is a prerequisite of an effective treatment.

10.3 Outwitting the Blood–Brain Barrier

10.3.1 Local Therapy

The direct (interstitial) drug application into the tumor site is the simplest way to bypass the BBB. The only interstitial chemotherapy treatment approved to date for malignant glioma is the Gliadel wafer, a biodegradable polymer containing 3.85% carmustine (1,3-bis[2-chloroethyl]-1-nitroso-urea, BCNU), which is placed in the resection cavity at the time of surgery. The safety and efficacy of this approach has been demonstrated in patients undergoing resection of both recurrent and newly diagnosed malignant gliomas. In a randomized phase III study with 222 patients with recurrent malignant gliomas, a significant prolongation of overall survival with Gliadel was demonstrated (Brem et al. 1995). Also, there was a modest, however significant, survival prolongation in two double-blind, placebo-controlled, randomized trials with patients with newly diagnosed malignant glioma (Valtonen et al. 1997; Westphal et al. 2003). The therapy may be associated with local complications such as cerebrospinal fluid leakage and edema.

A continuous, long-term local drug application using stereotactically placed catheters (convection-enhanced delivery) is another possibility to bypass the BBB by large molecules such as antibodies, immunotoxins, and antisense oligonucleotides (see Sect. 4.4, below).

10.3.2 Osmotic BBB Disruption

Intraarterial mannitol application causes a temporary shrinkage of endothelial cells of brain capillaries and a passive opening of tight junctions. When cytostatics are applied shortly thereafter, 3- to 5-fold concentration increases can be reached in the tumor and 10- to 100-fold concentration increases in the surrounding brain tissue are achieved (as compared to standard intravenous application). In a study with 74 patients with primary CNS lymphoma repeatedly treated with chemotherapy after BBB disruption, a 5-year survival of 42% was reached (which is al least comparable to conventional chemotherapy), and none of the patients demonstrated evidence of cognitive loss (McAllister et al. 2000). However, BBB disruption is an invasive treatment that can only be applied by a few specialized centers.

10.3.3 Immunoliposomes and Inhibition of Drug Transporter

Conventional liposomes are not delivered to the brain in vivo because they are not transported through the BBB. However, when liposomes are conjugated to certain receptor-specific monoclonal antibodies, e.g., against transferrin receptor (antibody-directed liposomes, immunoliposomes), the complex undergoes receptor-mediated transcytosis through the BBB. Thus far, immunoliposomes have been used for delivery of antineoplastic agents to the brain in preclinical studies only (Huwyler et al. 1996).

The P-gp blocker valspodar has been used to deliver cytostatics to the brain in animals, and an improved penetration of some drugs known to be substrates of P-gp (like paclitaxel) could be demonstrated (Fellner et al. 2002).

10.4 Target Therapies in Brain Tumors

10.4.1 EGFR inhibitors

Epidermal growth factor (EGF) has been implicated in supporting oncogenesis and progression of human solid tumors and is a promising target for anticancer therapy (Table 10.1). Its receptor (EGFR) is amplified in more than 40% of gliomas, and the amplification may be associated with the activating mutation EGFRvIII (Benjamin et al. 2003). Stimulation of growth factor receptors and the Ras pathway leads to the activation of numerous signal transduction molecules instigating a cascade of downstream effectors that mediate growth and survival of tumor cells. One of the most important pathways for tumor transformation involves PI3K (phosphatidylinositol 3-kinase), Akt (phosphorylated protein kinase B), and mTOR (mammalian target of ra-

Table 10.1 Targeted therapies for malignant brain tumors

Epidermal growth factor receptor (EGFR) inhibitors	VEGFR inhibitors
Gefitinib (Iressa)	Valatanib (PTK787) (PDGFR and VEGFR inhibitor)
Cetuximab (monoclonal antibody against EGFR)	Sorafenib (VEGFR, PDGFR, and Raf kinase inhibitor
Lapatinib (EGFR and ErbB-2 inhibitor)	Sonitinib (PDGFR, c-Kit, and VEGFR inhibitor)
AEE788 (EGFR and VEGFR inhibitor)	AEE788
Erlotinib (Tarceva)	AZD2171
ZD6474 [vascular endothelial growth factor receptor (VEGFR) and EGFR inhibitor]	ZD6474 (VEGFR and EGFR inhibitor)
EKB569	
Farnesyltransferase inhibitors	mTOR inhibitors
Tipifarnib	Temsirolimus
Lonafarnib	Everolimus
	Sirolimus
	AP23573
PKC inhibitors	Other monoclonal antibodies
Tamoxifen	Rituximab (Mabthera; anti-CD20 antibody)
Enzastaurin (PKC-β2 inhibitor)	Ibritumomab tiuxetan (Zevalin; anti-CD20 antibody conjugated with Y-90)
Antisense oligonucleotides	

pamycin; Fig. 10.3). The primary event in this pathway is the activation of PI3K, which can occur from numerous agonists and receptors like platelet-derived growth factor (PDGF), EGFR, fibroblasts growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), interleukin and interferon receptors, and the Ras pathway (Newton 2004).

The results of clinical studies with EGFR inhibitors in relapsed malignant gliomas published thus far are given Table 10.2. The response rates were usually no higher than 10%, and the 6-month progression-free survival ranged between 3% and 24%. This is comparable to the results of studies with conventional chemotherapy before introduction of temozolomide (Wong et al. 1999) and with temozolomide itself (Yung et al. 1999). The toxicity of these new agents was acceptable and consisted of fatigue, skin rash, and diarrhea. Coexpression of EGFRvIII and PTEN (phosphatase and tensin homolog deleted on chromosome 10) by glioblastoma cells was associated with response to EGFR kinase inhibitors in a retrospective study (Mellinghoff et al. 2005). Studies with dual tyrosine kinase inhibitors are also under way, which include early clinical trials on lapatinib (GW-572016; GlaxoSmithKline), an EGFR and ErbB-2 inhibitor, and AEE788 (Novartis Pharmaceuticals), an EGFR and VEGFR inhibitor.

The penetration of EGFR inhibitors through the BBB is poor. The cerebrospinal fluid concentration of imatinib after intravenous application in mice was 155-fold lower than the corresponding plasma concentration (Wolff et al. 2003).

10.4.2 Angiogenesis Inhibitors

The growth and survival of malignant gliomas are dependent on an adequate blood supply. The formation of new blood vessels is coordinated

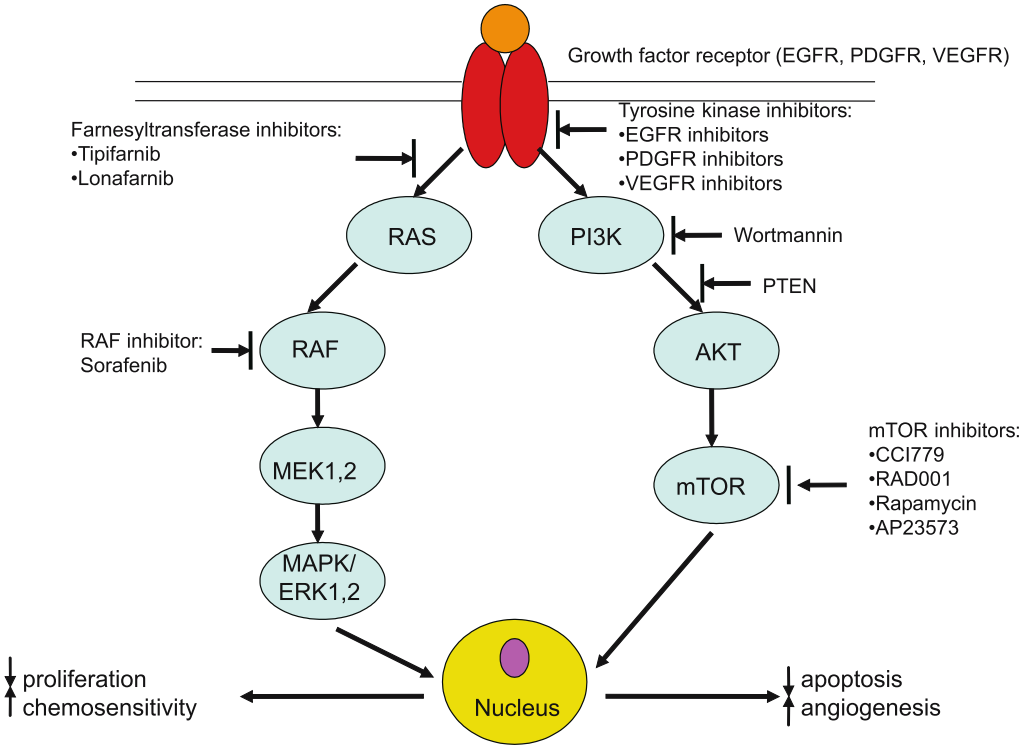

Fig. 10.3 Signaling pathways activated by the stimulation of the growth factor receptor

Table 10.2 EGFR inhibitors for therapy of malignant glioma

Agent	Responding patients/all patients	6-month PFS (%)
Gefitinib	0/53	13
(Rich et al. 2004)		
Erlotinib	2/45	13
(Raizer et al. 2004)		
Imatinib		
(Wen et al. 2004)	0/49	3
(van den Bent et al. 2005)	3/51	16
Tipifarnib	3/50	24
(Cloughesy et al. 2003)		
CCI-779	16/40	10
(Galanis et al. 2005)		

PFS, progression-free survival

Table 10.3 Inhibitors of angiogenesis for therapy of malignant glioma

Agent	Responding patients/ all patients	TTP (weeks)	OAS (weeks)
Suramin (Grossman et al. 2001)	0/12	7.8	27.3
PTK/ZK (Yung et al. 2002)	1/31	-	-
Thalidomide (Fine et al. 2000)	2/30	10	28
Enzastaurin (Fine et al. 2005)	14/70	-	-

OAS, overall survival; TTP, time to progression

by the complex interaction of many angiogenic factors, including VEGF, basic fibroblast growth factor (bFGF), and PDGF (Kesari et al. 2005). Several studies with angiogenesis inhibitors in relapsed malignant gliomas have been published thus far (Table 10.3). The response rate in these studies was in the range of 0%–20%, and the median time to progression was a maximal 10 weeks, which is not superior to the results of conventional chemotherapy. Other angiogenesis inhibitors such as the metalloproteinase inhibitor cilengitide (EMD 121974), the protein kinase inhibitor LY 317615, cyclooxygenase II inhibitors, and endostatin are currently being evaluated in clinical studies.

Remarkably, although the study with suramin was closed early as no complete or partial responders were seen at the 12-week evaluation point specified by the protocol, three patients (25% of those entered onto trial) had late clinical stabilization or radiologic response and lived over 400 days after treatment initiation (Grossman et al. 2001).

The penetration of angiogenesis inhibitors through the BBB has not been evaluated yet.

10.4.3 Inhibitors of Ras/MAPK and PI3K/Akt Pathways

Identifying and targeting common downstream mediators of growth-factor signaling, such as the Ras/mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, may yield additional potential therapeutic options. Farnesyltransferase is involved with signal transduction in the Ras pathway. Two farnesyltransferase inhibitors, tipifarnib (Zarnestra; Ortho Biotech) and lonafarnib (Sarasar; Schering-Plough), have been evaluated in clinical trials in patients with recurrent malignant gliomas. Response in 3 of 50 patients and a 6-month progression-free survival of 24% were achieved in a phase I/II study with tipifarnib (Cloughesy et al. 2003). Skin rash was the dose-limiting toxicity.

Several inhibitors of mTOR—a downstream target of PI3K signaling—are being investigated in clinical trials. Temsirolimus (CCI-779; Wyeth Research) induced response in 16 of 40 patients with recurrent malignant gliomas with a 6-month progression-free survival of 10% (Galanis et al. 2005).

10.4.4 Intratumoral Therapy

Approaches that directly target the tumor, bypassing the BBB, increase the exposure of tumor cells to the drug and reduce the probability of systemic complications. Particularly large molecules such as monoclonal antibodies used against targets in the brain (such as transferrin receptor, tenascin, urokinase-type plasminogen activator receptor, or EGFR), radiolabeled or armed with toxins, are preferentially applied by regional delivery. In a phase II study, 42 patients with recurrent primary brain tumors were treated with resection followed by application of

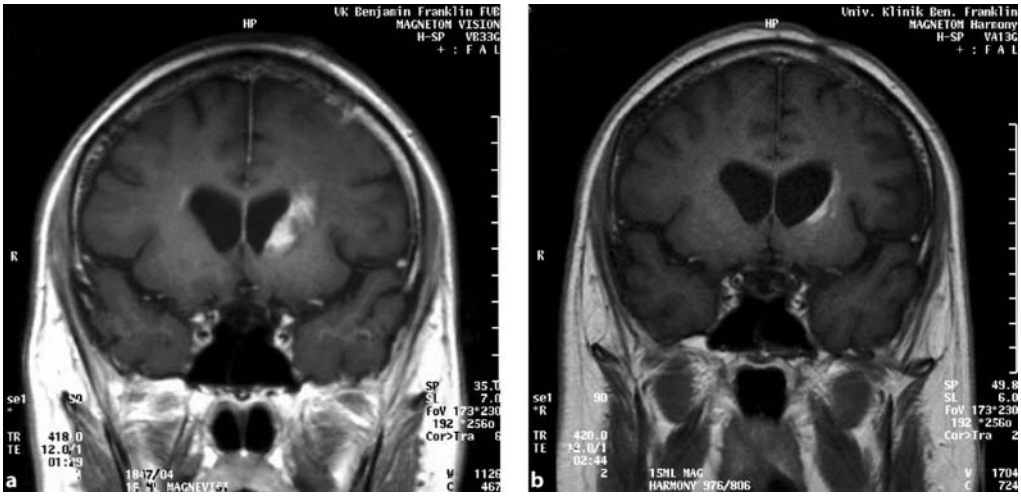

Fig. 10.4 a, b ^{111}In ibritumomab tiuxetan for therapy of primary central nervous system lymphoma (PCNSL): a 46-year-old patient with recurrent PCNSL: **a** before and **b** 6 months after ^{90}Y ibritumomab tiuxetan

an antibody against tenascin fused to iodine 131 before radio- and chemotherapy. The median survival was 64 weeks for patients with glioblastoma and 99 weeks for patients with anaplastic astrocytoma/oligodendroglioma (Reardon et al. 2006). Interleukin (IL)-13 fused with *Pseudomonas* exotoxin (IL13-PE38QQR) and transforming growth factor (TGF) fused with *Pseudomonas* exotoxin (TP-38) were safe and produced responses in patients with malignant gliomas in early phase I/II studies (Kunwar 2003; Sampson et al. 2003).

10.4.5 Other Antibodies

Rituximab (Mabthera, Roche), the monoclonal antibody against CD20, an antigen on B lymphocytes, has been used to treat relapsed primary central nervous system lymphoma (PCNSL). The results of single-patient reports with intravenous rituximab treatment were conflicting, which is not surprising given the poor penetration of the antibody through the BBB. However, a promising response rate of 53% has been reached with the combination of rituximab with temozolomide (Enting et al. 2004).

Ibritumomab tiuxetan (Zevalin, Schering), a conjugate of mice CD20 antibody with the β-emitter yttrium-90 is active in systemic lymphoma pretreated with rituximab and can be given only once. In an ongoing phase II study (E. Thiel, Charite Berlin) with patients with relapsed/refractory PCNSL, long-lasting responses have been observed (Fig.10. 4) and an accumulation of the marked antibody in the tumor has been demonstrated (Fig. 10.5). A prolonged hematotoxicity after treatment with ibritumomab tiuxetan should be taken into account.

10.4.6 Combination Regimens

Since the expression or amplification of various receptors is altered in malignant gliomas, agents targeting multiple receptors, such as sorafenib (VEGFR, PDGFR, and Raf kinase inhibitor), PTK787/ZK222584 (VEGFR and PDGFR inhibitor), SU011248 (VEGFR, PDGFR, and c-Kit inhibitor), and AEE788 (VEGFR and EGFR inhibitor), are potentially useful therapeutic combinations. Moreover, combinations of receptor inhibitors with inhibitors of downstream signaling pathways and combinations of targeted

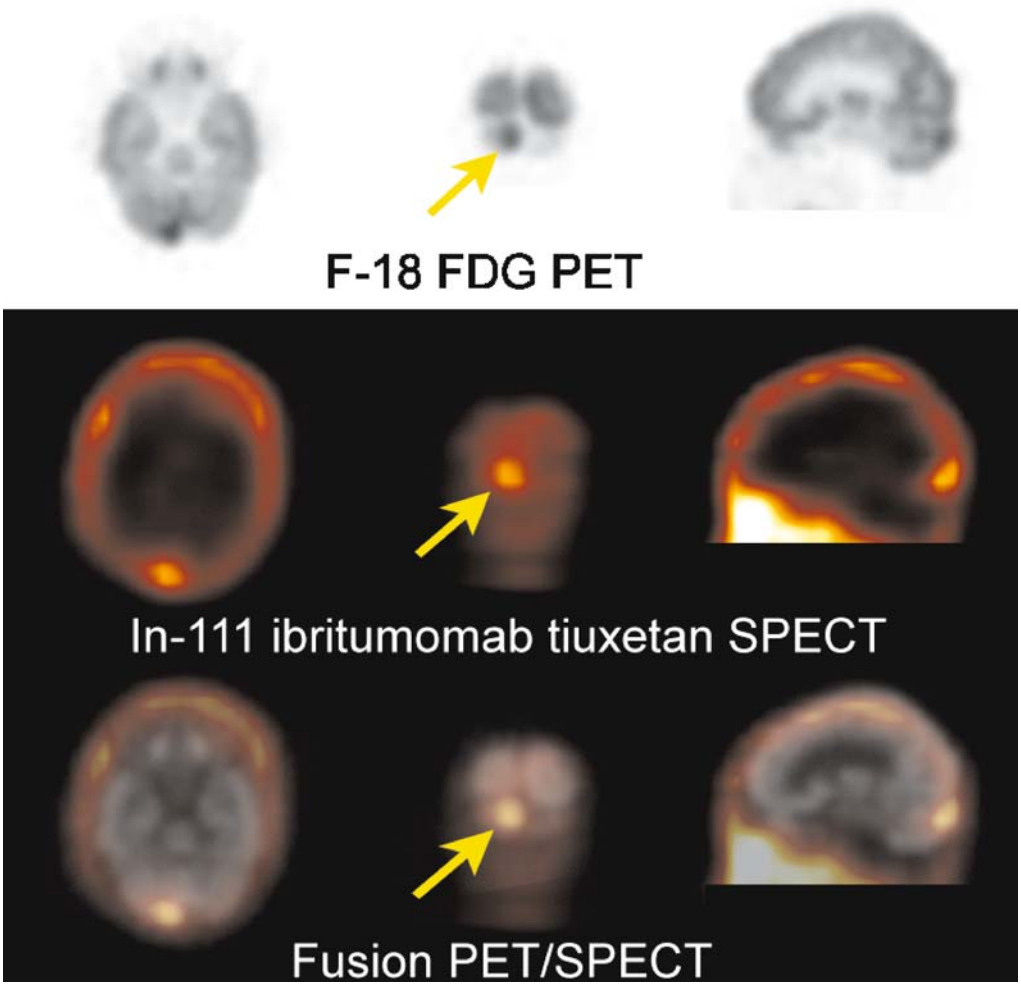

Fig. 10.5 Penetration of ^{111}In ibritumomab tiuxetan through the blood–brain (-tumor) barrier in PCNSL. PET with increased uptake of ^{18}F FDG (*upper panel*), corresponding SPECT slices (*middle panel*) with increased uptake of ^{111}In ibritumomab tiuxetan, and image fusion of both modalities (*lower panel*)

therapy with conventional therapy also hold promise for the treatment of malignant gliomas. The results of the first studies with combination regimens are promising (Table 10.4). The median progression-free survival of 14–17 weeks and the 6-month progression-free survival of 26%–39% are superior when compared to 'benchmark' studies with conventionel chemotherapy in malignant glioma (Wong et al. 1999, Yung et al. 1999). In an ongoing randomized German study, the combination of imatinib and hydroxyurea in patients with temozolomide-pretreated malignant gliomas is currently being tested (G. Dresemann, Düllen, unpublished).

10.4.7 Antisense Oligonucleotides

Due to their small molecular size, antisense oligonucleotides are potentially able to cross the BBB. However, a strong accumulation of antisense oligonucleotides in the reticuloendothelial system is usually observed after systemic application. Thus, a regional delivery into the tumor is preferred.

Table 10.4 Combination therapy versus benchmark studies in malignant glioma

Therapy	*N*	RR (%)	TTP (weeks)	6-month PFS (%)
PTK/ZK+TMZ (Reardon et al. 2004)	37	8	16.1	-
Imatinib+hydroxyurea (Dresemann 2005)	33	20	14.4	27
Imatinib+hydroxyurea (Friedman et al. 2005)	64	9 GBM	10.9 AA/AO; 14.4 GBM	26.3 GBM
Marimastat+TMZ (Groves et al. 2002)	44	13.6	17	39
Thalidomide+BCNU (Fine et al. 2003)	40	24	14.3	27.5
Bevazizumab+irinotecan (Stark-Vance 2005)	21	50	-	-
Chemotherapy without TMZ (Wong et al. 1999)	225	-	9	15
TMZ (Yung et al. 1999)	112	5.4	12.4	21

AA/AO, anaplastic astrocytoma (anaplastic oligodendroglioma); GBM, glioblastoma; PFS, progression-free survival; RR, response rate; TMZ, temozolomide; TTP, time to progression

TGF-β regulates key mechanisms of tumor development, such as immunosuppression, metastasis, angiogenesis, and proliferation. In a phase I/II study with the TGF-β2 antisense oligodeoxynucleotide AP 12009, response was observed in 2 patients and disease stabilization in 7 of 24 patients with relapsed malignant gliomas. The median survival was 146.6 (anaplastic astrocytoma), and 44 weeks (glioblastoma), respectively (Schlingensiepen et al. 2006). The value of this approach is currently being investigated in Germany in a randomized phase II study (U. Bogdahn, Regensburg, unpublished).

Summary

Major advances in molecular biology, cellular biology, and genomics have substantially improved our understanding of the genetics and proteomic changes involved in cancer, including brain tumors, and facilitated the evaluation of new treatment agents. However, crossing the BBB to reach the tumor represents a challenge for the majority of therapeutics. As a consequence, local application must frequently be used, which is invasive and has the disadvantage of locally limited efficacy without affecting the infiltrating tumor cells with an intact BBB. The first generation of studies with single-agent targeted therapy has shown only modest benefits against malignant brain tumors when „classic" effectivity criteria, such as response or overall survival, were used. The challenge for the next generation of studies with these agents is to improve our understanding of the reasons for their success or failure and to select the subgroups of patients who are most likely to respond to specific drugs. Adhering to the conventional efficacy criteria such as response or overall survival may underestimate the actual

benefit of novel therapies, so that other criteria not based on response, such as progression-free survival, positron emission tomography (PET)-analysis, or clinical benefit, may be more appropriate. Given the large number of signaling pathway abnormalities in brain tumors, a profound effect of an agent is possible even if no response is seen. Therefore, more benefit can be expected from the simultaneous use of a combination of agents targeting different pathways or from a combination with cytotoxic chemotherapy.

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11 Cetuximab: Appraisal of a Novel Drug Against Colorectal Cancer

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Abstract

Cetuximab (C225, Erbitux, Merck, Darmstadt, Germany) is a human-mouse chimeric therapeutic monoclonal antibody (mAb) that competitively binds to the extracellular domain of the human epidermal growth-factor receptor (EGFR). It has been developed out of the murine antibody M225 "from bench to bedside" in less than two decades, and is the anti-EGFR mAb furthest ahead in clinical evaluation. In Europe, cetuximab is approved for the treatment of patients with EGFR-expressing, metastatic colorectal cancer after failure of treatment with irinotecan since 2004, and for the treatment of patients with locally advanced squamous cell cancer of the head and neck concomitant to radiotherapy since 2006. We here summarize the current role of cetuximab in the treatment of colorectal cancer, give an overview on the ongoing studies, address the most important controversies, and point out the chances and challenges for the future use of cetuximab in colorectal cancer and other human malignancies.

11.1 Introduction

The human epidermal growth factor receptor (EGFR, syn. HER-1, c-ErbB-1) represents a 170-kDa transmembranal glycoprotein that is a member of the erb2-family of type 1 tyrosine kinase growth factor receptors. EGFR is constitutively expressed in many normal tissues, including the skin and hair follicles, where it is involved in the regulation of cell proliferation, differentiation, survival, and angiogenesis. Epidermal growth factor and transforming growth factor-α are the most important endogenous ligands of the EGFR, binding to the extracellular domain with low affinity. Binding of the EGFR results in homodimerization of the receptor, autophosphorylation of intracellular tyrosine residues, and activation of the RAS, ERK, phosphatidylinositol 3-kinase (PI3K)/Akt, and STAT signaling pathways (Baselga 2002; Harris 2004; Burtness 2005; Chung and Saltz 2005).

The rationale for developing a therapeutic target against EGFR was derived from the observation that the receptor is overexpressed on the cell surface of many human cancers, including colorectal and other gastrointestinal, gynecological, and urological cancers, squamous cell cancer of the head and neck, non-small cell lung cancer, and gliomas. From a recent point of view, the most important strategies to interrupt the EGFR pathway include the blockade of the extracellular receptor domain by monoclonal antibodies, or the inhibition of the intracellular tyrosine kinase (Baselga 2002; Harris 2004; Burtness 2005; Chung and Saltz 2005).

Cetuximab is a recombinant chimeric IgG1 monoclonal antibody with an molecular weight of 152 kDa that was derived from the murine antibody M225. It competitively binds to the extracellular domain of the EGFR with high affinity. Binding of cetuximab to the EGFR induces a reduction in phosphorylation and activation of receptor-associated kinases, and receptor internalization and degradation, resulting in a depletion of receptor-mediated downstream signaling. In addition, cetuximab inhibits the production of

vascular endothelial growth factor and other angiogenic factors, and induces the degradation of matrix metalloproteinases. These antineoplastic properties of cetuximab have been demonstrated to inhibit the growth of EGFR-expressing tumor cells in vitro and in vivo (Baselga 2001, 2002; Mendelsohn and Baselga 2003; Burtness 2005).

Cetuximab is administered as intravenous infusion with a starting dose of 400 mg/m² body weight, followed by a weekly maintenance of 250 mg/m². Its pharmacology follows a nonlinear, saturable kinetics; its half-life is in the range of 7 days, and neither the elimination nor the occurrence of side effects is largely influenced by concomitant chemotherapy or radiotherapy (Baselga et al. 2000; Cunningham et al. 2004; Harris 2004; Burtness 2005; Bonner et al. 2006).

In Europe, cetuximab is currently approved for the treatment of patients with EGFR-expressing metastatic colorectal cancer after failure of treatment with irinotecan, and for the treatment of patients with locally advanced squamous cell head and neck cancer concomitant to radiotherapy.

The most common clinical side effect of cetuximab is an acneiform rash that usually appears within the first few weeks of treatment, and often resolves without cessation of drug administration during the further course of treatment. The appearance of exanthema and its severity is supposed to predict the individual response to therapy in patients treated with cetuximab. Fatigue, lethargy, asthenia, or a combination of the three also commonly occur (>50%), but mostly in a mild or moderate form. The rate of severe hypersensitivity reaction has been reported in the range of 2%–5% of patients treated. It mostly occurs after the first infusion, with a rate of fatal outcomes estimated to be less than 0.001%. Pulmonary toxicity has been observed in about 0.5% of patients and includes pneumonitis and exacerbation of preexisting pulmonary fibrosis. Symptomatic hypomagnesemia or hypocalcemia have been occasionally reported. Taken together, severe side effects clearly attributable to drug administration occur in less than 5% of patients treated. Thus cetuximab can be regarded as a safe and practical treatment option (Perez-Soler 2003; Cunningham et al. 2004; Harris 2004; Burtness 2005; Perez-Soler and Saltz 2005; Bonner et al. 2006).

11.2 Early Phase II Studies in Colorectal Cancer

Results from preclinical models suggested a moderate activity of single-agent cetuximab, and a synergism with certain chemotherapeutic agents and radiation (Aboud-Pirak et al. 1988; Overholser et al. 2000; Baselga 2001; Prewett et al. 2002). The first clinical phase II studies performed in patients with metastatic colorectal cancer evaluated cetuximab either in combination with irinotecan alone or in conjunction with leucovorin/5-fluorouracil (LV/5-FU) (Saltz et al. 2001; Rosenberg et al. 2002; Laethem et al. 2003; Folprecht et al. 2006). In the largest of these trials, 121 patients with EGFR-expressing tumors were treated with concomitant cetuximab and irinotecan after they had progressed after previous treatment with LV/5-FU and irinotecan. Of those patients, 48% received disease stabilization, and 17% a partial remission (Saltz et al. 2001). In addition, three smaller phase II trials employing the combination of cetuximab and irinotecan plus LV/5-FU were performed in first-line patients. In one of those studies, 11 of 25 patients (44%) achieved a partial remission, and 16 of 25 (64%) at least a 40% reduction of tumor size (Rosenberg et al. 2002). Laethem and coworkers reported a PR in 10 of 22 (46%), and a tumor growth control rate in 19 of 22 (86%) patients. In the only trial on a first-line combination that has been published as full paper so far, a confirmed partial remission was observed in 14 of 21 (67%) patients, whereas 20 of 21 patients (95%) experienced at least disease stabilization (Folprecht et al. 2006).

The activity of single-agent cetuximab in irinotecan-refractory patients has been evaluated in a phase II-study by Saltz and coworkers. Here, 5 out of 57 patients (9%) achieved a confirmed PR, and 26 (47%) had disease-stabilization (Saltz et al. 2004).

Taken together, these early phase II studies primarily aimed at establishing a combined treatment with cetuximab and irinotecan in patients who have progressed under an irinotecan-containing schedule before. Results confirm the preclinical assumption of a modest but relevant activity of single-agent cetuximab in this setting. In addition, some smaller phase II studies on first-line treatment with cetuximab, irinotecan, and LV/5-FU revealed promising results.

11.3 The "BOND" Studies

In order to further establish cetuximab as a salvage treatment for colorectal cancer patients, the combination of cetuximab and irinotecan was compared to cetuximab alone in a large, randomized phase II trial. This "cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer" study had the identification number "EMR 62202-*007*" and thus was titled as the "BOND"-trial in the internal jargon of the manufacturer.

Patients were enrolled into the study if they had EGFR-expressing metastatic colorectal cancer, were treated with an irinotecan-containing chemotherapeutic regimen for at least 6 weeks, and had progressive disease while on treatment or 3 months thereafter. A total of 577 patients were screened before study entry, of whom 474 (82%) had tumors with positive immunohistochemical staining for EGFR. Of those, 145 were ineligible for various reasons (mostly failing other entry criteria).

Randomized into the treatment arms in a 2:1 scheme were 329 patients (218 vs 111 pts.). The median age of patients at study entry was 59. The Eastern Cooperative Oncology Group (ECOG) performance status was 0–2 in 88% of patients. Of the patients, 20% had received one and 42% had received more than two prior chemotherapeutic regimens; 63% of the patients was pretreated with irinotecan and oxaliplatin. The primary study objective was defined as the comparison of objective response rates, which was 23% in the combination, and 11% in the cetuximab monotherapy arm ($p = 0.007$). The corresponding rates of disease stabilization were 56% and 33%, respectively. The median duration of response was 5.7 months in the combination therapy group, and 4.2 months in the monotherapy group. In an exploratory analysis, median time to progression was significantly superior in the combination therapy group (4.1 vs 1.5 months, $p<0.001$). A significant difference in survival (8.6 vs 6.9 months, $p = 0.48$) was not detected, due to the study type (phase II), its small size, and the definition of response as primary objective (Cunningham et al. 2004).

The "BOND" study established the combination of irinotecan and cetuximab as an effective salvage treatment in metastatic colorectal cancer, and it served as a basis for the approval of cetuximab in the United States and Europe.

In 2005, results of another randomized phase II trial were presented in which a combination therapy of cetuximab and the vascular endothelial growth factor receptor (VEGFR) antagonist bevacizumab was compared with cetuximab, bevacizumab, and irinotecan in 75 irinotecan-refractory patients with metastatic colorectal cancer ("BOND-2" study) (Saltz et al. 2005). Of these patients, 85% had also been treated with oxaliplatin at study entry. Response/tumor growth control rates of cetuximab plus bevacizumab plus irinotecan ($n = 40$) were 35%/78%, compared with 23%/77% for cetuximab plus bevacizumab ($n = 35$). Results suggest the feasibility of the combination of the two monoclonal antibodies, and underlines the efficacy of cetuximab in heavily pretreated patients with advanced colorectal cancer. The publication of the full paper is expected to provide more detailed information. The third comparative study (labeled as the "BOND 2.5" trial) is still ongoing, and it assesses the activity of the triple combination cetuximab/bevacizumab/irinotecan in patients after failure of a bevacizumab-containing treatment.

A large single arm study has recently confirmed a relevant activity (12% PR) of single-agent cetuximab in heavily pretreated patients (Lenz et al. 2004).

11.4 Combinations of Cetuximab and Oxaliplatin

In the USA, LV/5-FU and irinotecan were the only drugs available for the treatment of colorectal cancer until oxaliplatin was approved in 2003. Here, the combination of irinotecan, leukovorin, and bolus-infusional 5-FU (IFL) was regarded as standard treatment for patients with metastatic colorectal cancer since 2000 (Saltz et al. 2000). The pivotal studies on cetuximab, as well as those on the VEGFR antagonist bevacizumab, referred to this North American standard (Cunningham et al. 2004; Hurwitz et al. 2004).

In most central European countries, the standard first-line treatment at the beginning of the century consisted of 5-FU administered as continuous infusion over 24–46 h plus LV in combination with either irinotecan ("FOLFIRI,"

"AIO-IRI") or oxaliplatin ("FOLFOX"). When applied as a first-line treatment, both regimens alone achieve response and survival rates similar to IFL plus bevacizumab (Hurwitz et al. 2004; Tournigand et al. 2004). This circumstance and the initial evaluation of cetuximab in conjunction with irinotecan delayed the initiation of studies referring to combinations with cetuximab and oxaliplatin. Therefore, only a few phase II abstracts, and no full papers, on this topic have been published so far.

In one of these phase II studies, cetuximab was added to LV/5-FU and oxaliplatin according to the FOLFOX 4-regimen in first-line treatment. Partial remissions were observed in 31 of 43 patients (72%), and the rate of tumor growth control was 95% (Díaz Rubio et al. 2005). In another trial on cetuximab combined with the weekly FUFOX protocol, a confirmed PR was reported in 65% of 49 patients treated at two different 5-FU dose-levels (Seufferlein et al. 2005).

As a conclusion, the phase II results on both oxaliplatin or irinotecan combined with continuous infusional 5-FU and cetuximab are among the best ever reported in the first-line therapy of patients with metastatic colorectal cancer (Table 11.1).

11.5 EGFR-"Negative" Colorectal Cancer

In general, the efficacy of a "targeted therapy" is based on the premise of a target structure on the tumor that is more or less consistently present in individual patients of a cohort. As monoclonal antibodies bind to defined structures on the cell surface, immunohistochemistry (IHC) has evolved as the most common approach in this context. Apart from the discussion on the technical and biological problems of this technique, it was proposed that the clinical efficacy of cetuximab in colorectal cancer patients may be correlated to the surface expression of EGFR on the tumor cells (as detected by the IHC-kit "Cytomation EGFR pharmDx" of Dako). This unproven hypothesis was drawn in analogy to other therapeutic antibodies, mainly trastuzumab (Fornier et al. 2002).

Since the approval of cetuximab as a salvage treatment for metastatic colorectal cancer, an increasing number of patients with negative EGFR-staining have been successfully treated with the drug (Chung et al. 2005). In addition, clinical studies failed to demonstrate a correlation between immunohistologically detected EGFR expression and clinical outcome (Saltz et al. 2004). From the view of most experts on this field, EGFR immunostaining using the currently available techniques does not reliably predict the efficacy of a cetuximab-based therapy, and thus should not or at least no longer serve as a basis for selection of exclusion for such treatment (Saltz 2005). Therefore, and in contrast to the approval status in colorectal cancer, cetuximab has recently been approved for a second indication (head and neck tumors, see Table 11.2) without the prefix "EGFR-expressing." Current research on the prognostic value of genetic alterations within the EGFR pathway is ongoing (Vallbohmer et al. 2005).

11.6 Cetuximab as an Adjunct to Radiotherapy

Preclinical data and the persuading clinical results obtained in patients with squamous cell head and neck cancer in principle underline that cetuximab is not only effective as a combination partner for cytostatic drugs, but also markedly enhances the antineoplastic properties of radiotherapy (Baselga 2001; Bonner et al. 2006). In the pivotal trial on head and neck cancer, the addition of cetuximab to radiotherapy—which was applied according three different optional schedules up to cumulative doses of 70, 72–77, or 72 Gy—resulted in a significant, nearly 10-month (24.4 vs 14.9) difference of duration of locoregional control (primary objective). This improvement in local control translated in a significant benefit in median survival (54 vs 29 months, $p = 0.03$), and other survival parameters. The trial was particularly criticized for its control arm (Posner and Wirth 2006), because radiotherapy alone—from a recent point of view—is an inappropriate treatment for patients with locally advanced, inoperable squamous cell head and neck cancer. However, it is impressive that the addition of cetuximab to radiotherapy results in a benefit of the same magnitude as with

Table 11.1 Phase II trials on cetuximab in colorectal cancer patients

Authors	No. of pts	Schedule	Indication	Response rate/ growth control rate
Saltz et al. 2004	57	Cetuximab	Pretreated with irinotecan	9%/47%
Cunningham et al. 2004	111	Cetuximab	Pretreated with irinotecan	11%/33%
Lenz et al. 2004	350	Cetuximab	Pretreated with irinotecan and oxaliplatin	12%/n.r.
Saltz et al. 2001	121	Cetuximab+irinotecan	Pretreated with irinotecan and 5-FU	17%/48%
Cunningham et al. 2004	218	Cetuximab+irinotecan	Pretreated with irinotecan	23%/56%
Saltz et al. 2005	40	Cetuximab+bevacizumab +irinotecan	Pretreated with irinotecan	35%/78%
Saltz et al. 2005	35	Cetuximab+bevacizumab	Pretreated with irinotecan	23%/77%
Rosenberg et al. 2002	27	Cetuximab+ILF	First-line metastatic	44%/n.r.
Laethem et al. 2003	18	Cetuximab+FOLFIRI	First-line metastatic	67%/89%
Folprecht et al. 2006	21	Cetuximab+AIO-IRI	First-line metastatic	67%/95%
Díaz Rubio et al. 2005	43	Cetuximab+FOLFOX-4	First-line metastatic	72%/95%
Seufferlein et al. 2005	49	Cetuximab+FUFOX	First-line metastatic	65%/n.r.

AIO-IRI, weekly combination of leukovorin/continuous infusional 5-fluorouracil/irinotecan; FOLFIRI, bimonthly leukovorin/continuous infusional 5-fluorouracil/irinotecan; FOLFOX, bimonthly leukovorin/continuous infusional 5-fluorouracil/oxaliplatin; FUFOX, weekly combination of leukovorin/continuous infusional 5-fluorouracil/oxaliplatin; ILF, weekly irinotecan/leucovorin/bolus infusional 5-fluorouracil (5-FU); n.r., not reported

Table 11.2 EGF-receptor antagonists (approved or evaluated in the scope of phase III-studies; by April 2006)

Drug name	Brand name	Target/class	EU approval	Indication
Trastuzumab	Herceptin	HER-2/mAb	+	HER-2 overexpressing metastatic breast cancer:
				(1) As single-agent after failure of two lines of chemotherapy and hormonal treatment
				(2) As first-line treatment combined with paclitaxel or docetaxel
Cetuximab	Erbitux	EGFR/mAb	+	(1) EGFR-expressing metastatic colorectal cancer; disease progression after prior treatment with irinotecan
			n	(2) Locally advanced squamous-cell cancer of the head and neck, concomitant to radiation
Panitumumab	-	EGFR/mAb	-	FDA "fast track"-approval for pretreated patients with metastatic colorectal cancer pending
Erlotinib	Tarceva	EGFR/TKI	+	Second- or later-line treatment in patients with non small-cell lung cancer
Gefitinib	Iressa	EGFR/TKI	-	FDA approval reversed

EGFR, epidermal growth factor receptor; HER-2, human epidermal growth factor receptor type 2; mAb, monoclonal antibody; TKI, tyrosine kinase inhibitor

the combination of cisplatin-based chemotherapy with radiotherapy. Even more impressive, this improvement is achieved without additional local toxicity.

To the opinion of the authors, cetuximab's property of acting as a potent radiosensitizer without deterioration of local side effects represents one of the most important potentials of the drug. First phase II studies on combinations of cetuximab, chemotherapy, and radiotherapy (e.g., in head and neck cancer, pancreatic cancer, and lung cancer) have recently been reported. In one of these trials, performed in patients with squamous cell cancers of the head and neck, feasibility problems occurred (Pfister et al. 2006). Thus toxicity appears to be an important topic for such "triple"-combinations, in particular in cohorts where high rates of comorbidity are typical.

Given the activity of cetuximab in colorectal cancer on the one hand, and its radiosensitizing effect on the other hand, locally advanced rectal cancer may be a promising indication for the evaluation of radiochemotherapy plus cetuximab (Machiels et al. 2006). Reports on several phase II trials on this issue are expected soon.

Summary and Future Directions

Clinical studies that constituted the basis for the approval of cetuximab in colorectal cancer patients have established a combination with irinotecan as an effective salvage treatment in patients with EGFR-positive colorectal cancers pretreated with an irinotecan-containing chemotherapy schedule. Results also demonstrate that single-agent cetuximab consistently exhibits an objective response rate in the range of 10%, even in patients pretreated with both irinotecan and oxaliplatin. For first-line combinations, few data are available yet, but phase II trials on combina-

Table 11.3 Ongoing phase III-trials evaluating cetuximab in patients with colorectal cancer (by April 2006)

NTC study number	Indication	Standard arm	Experimental arm
00079274	Stage 3 adjuvant	FOLFOX	FOLFOX plus CETUX
00265811	Stage 3 adjuvant	FOLFOX	FOLFOX plus CETUX
00265850	Met. 1st-line	FOLFIRI or FOLFOX plus BEVA	(1) FOLFIRI or FOLFOX plus CETUX
			(2) FOLFIRI or FOLFOX plus BEVA plus CETUX
00252564	Met. 1st-line	LV/5-FU plus OXALI plus BEVA	LV/5-FU plus CETUX plus BEVA
00208546	Met. 1st-line	CAPOX plus BEVA	CAPOX plus BEVA plus CETUX
00182715	Met. 1st-line	CAPOX or FOLFOX	(1) CAPOX or FOLFOX plus CETUX
			(2) Intermittent CAPOX or FOLFOX
00145314	Met. 1st-line	FLOX	FLOX plus CETUX
00061815	Met. 2nd-line	FOLFOX	FOLFOX plus CETUX
00063141	Met. 2nd-line	IRI	IRI plus CETUX
00079066	No further option[a]	BSC	CETUX[a]

BEVA, bevacizumab; BSC, best supportive care; CAPOX, capecitabine/oxaliplatin; CETUX, cetuximab; FLOX, LV/bolus-5-FU/oxaliplatin; FOLFIRI, leukovorin/continuous infusional 5-fluorouracil/irinotecan; FOLFOX, leukovorin/continuous infusional 5- fluorouracil/oxaliplatin; IRI, irinotecan; LV/5-FU, leukovorin/continuous infusional 5-fluorouracil; NTC, national cancer trials database (www.clinicaltrials.gov); OXALI, oxaliplatin

[a] Accrual completed

tions with either FOLFIRI or FOLFOX achieved the best results ever reported for patients with metastatic colorectal cancer. However, the completion of the ongoing phase III trials is necessary to finally appraise the role of cetuximab in patients with colorectal cancer (Table 11.3).

Recent experience suggests that the expression of EGFR detected in the tumor of the individual patient by IHC does not sufficiently predict the efficacy of cetuximab. Indeed, the degree of EGFR expression does not predict the clinical outcome of treatment with cetuximab in colorectal cancer patients. In addition, objective tumor responses have regularly been reported in EGFR-"negative" colorectal cancer patients. For the combined treatment with radiotherapy in patients with squamous cell cancer of the head and neck, cetuximab has recently been approved regardless of the EGFR status. Results of the respective pivotal trials suggest in a persuasive way the radiosensitizing properties of cetuximab. Mature phase II data on the combination of cetuximab with radiochemotherapy in patients with primary rectal cancer are awaited soon.

Regarding the activity of cetuximab in other gastrointestinal tumors, several phase II trials are ongoing on cancers of the esophagus, stomach, pancreas, and hepatocellular carcinoma. Phase III studies on combinations of cetuximab with chemotherapy and radiochemotherapy in patients with head and neck cancer and lung cancer have been initiated.

In conclusion, cetuximab has been introduced as the first effective therapeutic monoclonal antibody against the human EGFR in colorectal cancer. Future studies will further define its role for the different subsets of tumors, and other monoclonal antibodies targeting the EGFR are already advanced in their clinical evaluation.

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12 C-kit, GIST, and Imatinib

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Recent Results in Cancer Research, Vol. 176

Abstract

A gain-of-function mutation of c-kit is the crucial step in tumorigenesis of gastrointestinal stromal tumors (GIST). Imatinib can block the activated receptor tyrosine kinase activity of c-kit. These findings made GIST an ideal candidate for evaluation of new targeted therapeutic approaches. Clinical studies demonstrated that, with imatinib, objective responses can be reached in more than 50% of patients with advanced GIST. Furthermore, even in those patients with stable disease, long-term tumor control could be achieved. Therefore imatinib at a dose of 400 mg/day is now the standard treatment of advanced GIST in which RO-resection cannot be reached. As imatinib resistance in GIST occurs at a median of 18 to 26 months, further targeted therapies have been explored. Sunitinib, another tyrosine kinase inhibitor, seems to be useful especially in patients with exon 9 mutations of c-kit, who usually have a worse response to imatinib. This might indicate that more exactly targeted therapies in GIST might improve clinical outcomes in the future.

12.1 Introduction

When targeted therapies were introduced in the treatment of malignancies, the first successes were seen in hematological disorders such as chronic myeloid leucemia (CML) or non-Hodgkin's lymphomas. But nowadays even solid tumors can be treated successfully by a targeted therapy. One of the first examples was the introduction of imatinib in the treatment of gastrointestinal stromal tumor (GIST).

Classical oncological treatment options like chemo- or radiotherapy are of very limited use in GIST. Therefore new therapeutic approaches were needed. The finding that a gain-of-function mutation of c-kit is responsible for tumorigenesis of GIST and the fact that imatinib might block the activated receptor tyrosine kinase activity of c-kit made GIST an ideal candidate for evaluation of the new targeted therapeutic approaches. And indeed the story of imatinib and GIST prove that targeted therapies might be successful not only in hematological malignancies but also in solid tumors.

12.2 Case Report

A 61-year-old male patient was admitted to our hospital in October 2001 due to a large intraabdominal mass that was discovered during a routine check up (Fig. 12.1A and B). The patient underwent explorative laparotomy, and a tumor at the urine bladder with infiltration of the small bowl and multiple knots of the peritoneum was found. R2 resection was performed revealing a CD117-positive extragastrointestinal stromal tumor of the gastrointestinal type.

At the end of November 2001, therapy with imatinib 400 mg per day was started. Due to side effects (nightly cramps in both hands) the dose was reduced to 200 mg per day in October 2003. Under this medication the remaining postoperative tumor manifestations of maximal 4×4, 5×5, 5 cm disappeared within 1 year (Fig. 12.1C

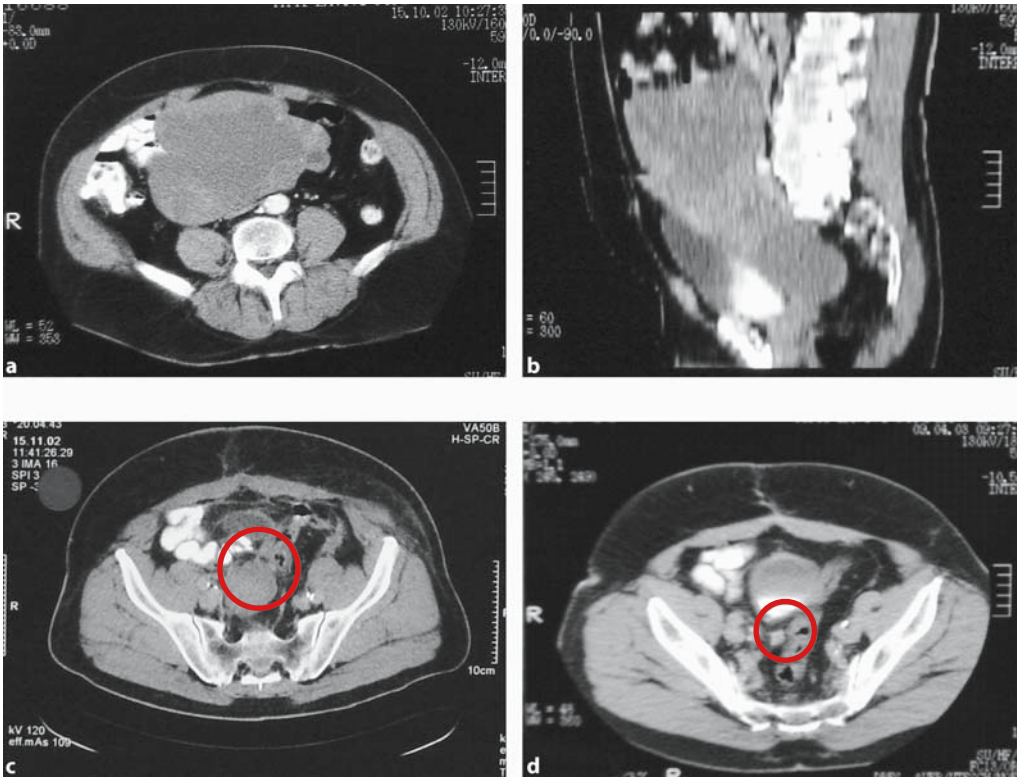

Fig. 12.1 a–d Case report of a 61-year-old patient with an extragastrointestinal tumor of the gastrointestinal type. **a** and **b**, Situation at diagnosis; **c**, postoperatively; and **d**, 5 months after start of imatinib therapy

and D) and the patient remains in complete remission 4 years later.

12.3 History and Epidemiology of GIST

GIST was first described in 1941 by Golden and Stout as "leiomyomatosal neoplasia with unusual histological features" (Golden and Stout 1941). During the following years GIST changed its name, being called leiomyoblastoma or epithelioid leiomyoma. In 1984 an additional neurogenic origin of GIST was discovered by electronic microscopy (Knapp et al. 1984) and immune histology (Saul et al. 1987), and in 1995 an overexpression of CD34 (Miettinen et al. 1995) and a gain-of-function mutation in the c-kit proto oncogene was described (Hirota et al. 1998). Today it is believed that GIST derives from the Cajal cells, which are pacemaker cells of the gastrointestinal tract.

GIST is a rare tumor that causes about 0.2% of all malignant tumors of the gastrointestinal tract. Nevertheless, it is the most common sarcoma of the gastrointestinal tract and among all sarcomas it contributes to 5% of the cases. In Germany there are roughly 250 to 300 new cases per year.

There is no sex preference, and patients are mainly in their 50s or 60s. GISTs are predominantly localized in the stomach (65%) and the small bowl (30%–35%). Manifestations at the esophagus, large bowl, rectum, or extragastrointestinal sites are rare.

12.4 Pathology and Pathophysiology of GIST

Two histological subtypes of GIST can be distinguished. There are spindle cell type GISTs contributing to about 70% of the cases, and the more

Table 12.1 Risk groups of GIST as identified by pathological features

Risk of aggressive biological behavior of GIST		
Risk	Tumor size	Mitosis
Very low	<2 cm	<5/50 HPF
Low	2–5 cm	<5/50 HPF
Intermediate	<5 cm	6–10/50 HPF
	5–10 cm	<5/50 HPF
High	>5 cm	>5/50 HPF
	>10 cm	Irrelevant
	Irrelevant	>10/50 HPF
Probably benign		
GIST of the bowl <2 cm with max. 5 mitosis/50 HPF; GIST of the stomach <5 cm with max. 5 mitosis/50 HPF		
Probably malignant		
GIST of the bowl >2 cm with more than 5 mitosis/50 HPF; GIST of the stomach >5 cm with more than 5 mitosis/50 HPF		

HPF, high-power field

rare epithelioid cell types contributing to about 30% of the cases. The main immune histological features are the positivity of CD117 and CD34. Additional neurogenic or myogenic markers can be positive.

Due to its pathological features, different risk groups can be identified (Table 12.1). Higher rates of mitosis and large tumor size are usually correlated to a more aggressive biological behavior with higher relapse rates after complete resection or higher rates of distant metastases.

The crucial pathophysiological step in the development of GIST is a gain-of-function mutation of c-kit, which can be found in 86% of GISTs. C-kit encodes for the stem cell factor receptor, the gene of which is localized on chromosome 4. C-kit encodes a transmembrane tyrosine receptor kinase with two intracellular kinase subunits. In GIST, mutations are mainly found in exon 11 (75% of cases), followed by exon 9 (20%), and exons 13 and 17 (2% each) (Heinrich et al. 2003). All mutations result in a ligand-independent activation of the intracellular kinase subunits with subsequent activation of intracellular pathways, which result eventually in antiapoptosis and mitosis. Alternatively, in about 1% of GISTs there are mutations of platelet-derived growth factor receptor A (PDGFRA), another transmembrane receptor tyrosine kinase. Only a very small minority of GISTs have no mutations either in c-kit or PDGFRA.

Imatinib inhibits the kinase subunits of both c-kit and PDGFRA by competitively binding to the ATP binding site. Interestingly, the clinical effect of imatinib therapy in GIST depends on where the mutations are, with better results in exon 11 than in exon 9 mutations (Heinrich et al. 2005).

12.5 Classical Treatment Options for GIST

As imatinib therapy does not guarantee a cure, R0 resection of the tumor was and is still the only therapeutic option in the treatment of GIST, with a confirmed curative potential. Surgery might be done by minimal invasive techniques. The role of surgery in a multimodal therapeutic approach in advanced GIST remains to be evaluated.

The role of chemo- or radiotherapy remains negligible. Different sarcoma chemotherapy protocols have been evaluated in GIST, but response rates were always less than 5%. GISTs are also typically radiation insensitive, so radiotherapy might be indicated only in a palliative setting to improve symptoms, for example pain from rare bone metastases.

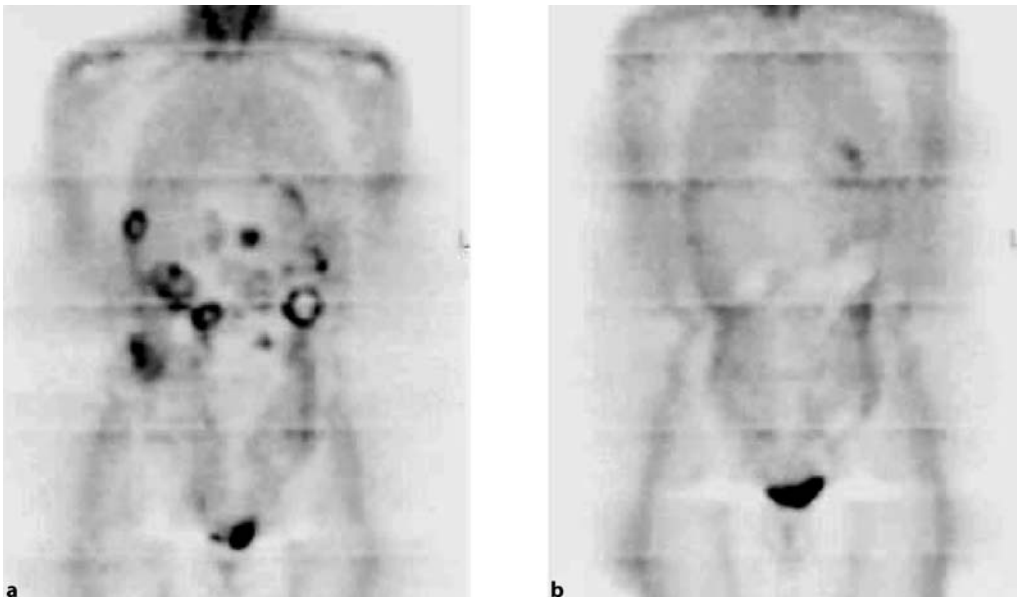

Fig. 12.2 a, b PET scan of the first patient with advanced GIST treated with imatinib. Just before starting imatinib (**a**) in March 2000 multiple intraabdominal metastases are visible, which lose their metabolic activity within 4 weeks after starting imatinib (**b**) (Joensuu et al. 2001)

12.6 Imatinib and GIST

12.6.1 The First Patient

In 2001 Joensuu et al. published a case report in the *New England Journal of Medicine* (Joensuu et al. 2001). It reports on a 51-year-old female patient from Finland who suffered from a multiple metastasized GIST. Several resections, different polychemotherapies and treatment with interferon-α and thalidomide had been unsuccessful. Within an international phase I study, imatinib at a dose of 400 mg per day was started in March 2000. Already 4 weeks later a complete metabolic remission was discovered by positron emission tomography (PET) (Fig. 12.2) and within 8 months there was a tumor reduction of approximately 75%.

12.6.2 Clinical Studies

This first patient participated in an European Organization for Research and Treatment of Cancer (EORTC) phase I study that evaluated different doses of imatinib (400–1,000 mg per day) in 35 patients with advanced GIST. Response rates were astonishingly high with 53% partial responses (PR), 17% stable disease (SD), and a progression-free survival rate (PFR) of 80% at 8 months (Van Oosterom et al. 2002). A larger phase II study with 400 to 600 mg imatinib per day confirmed these good results (54% PR, 28% SD, and PFR of 65% at 15 months) (Demetri et al. 2002). Published in 2004, a follow-up of this study compared imatinib-treated patients to a historical control group from former Southwest Oncology Group (SWOG) chemotherapy studies revealing a clearly better overall survival (Fig. 12.3) (Blanke et al. 2004). Therefore imatinib at a dose of 400 mg per day was approved for the treatment of advanced GIST by the FDA and other health authorities.

There were some randomized trials that followed. The comparison of 400 with 800 mg per day did not show higher response rates, but there was a significantly longer PFR with the higher dose (Verweij et al. 2004). Also a dose escalation to 800 mg per day proved to be of clinical benefit in some patients who progressed after treatment

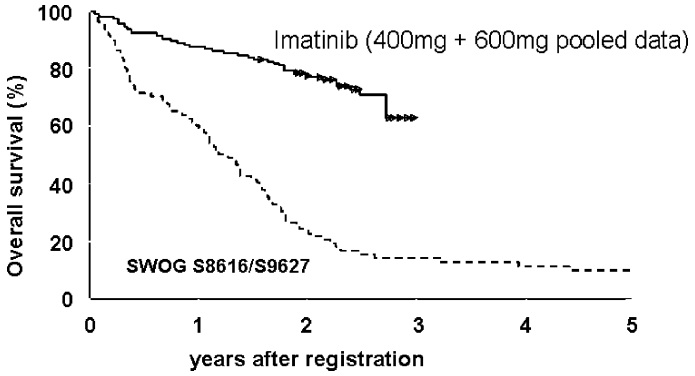

Fig. 12.3 Long-term follow-up of the first phase II study with 400 mg imatinib per day in patients with advanced GIST reveals a significant better overall survival when comparing with a historical SWOG control group treated with chemotherapy (Blanke et al. 2004)

with 400 mg per day (Zalchberg et al. 2004). Another study from France revealed that discontinuation of imatinib treatment after 1 year of PFR is associated with significantly higher rates of disease progression (Le Cesne et al. 2005). Therefore 400 mg imatinib per day is nowadays considered the standard treatment of advanced GIST. Treatment should be continued as long as there is no evidence of obvious disease progression.

The role of imatinib in multimodal treatment approaches is not yet fully clear. After preoperative imatinib treatment there are still vital tumor cells in most specimens (Rutkowski et al. 2005) and postoperative discontinuation of imatinib is associated with high rates of disease progression (Gronchi et al. 2005). A general adjuvant imatinib treatment after resection of high-risk GIST is feasible, but survival data are still lacking (DeMatteo et al. 2005).

At the moment therefore the standard of treatment of GIST should be: RO resection if possible and imatinib at 400 mg per day in advanced GIST with the aim of secondary RO resection. Adjuvant imatinib treatment and dose escalation should still be tested in clinical studies.

12.7 Imatinib Resistance

Imatinib resistance in GIST occurs at a median of 18–26 months (Verweij et al. 2004). Therefore failure of imatinib therapy becomes a new challenge. If there are single resistant clones that appear as so-called "nodule within a mass," resection and continuation of imatinib might be appropriate.

There are, however, new targeted therapies in the pipeline currently. The most mature results are available for SU 11248 (sunitinib malate) so far, which is another tyrosine kinase inhibitor (Demetri et al. 2005). Interestingly, this drug is especially effective in those patients with an exon 9 mutation (Maki et al. 2005), which is associated with worse results in imatinib therapy. Other drugs that are being evaluated are everolimus in combination with imatinib (Van Oosterom et al. 2005), AMG 706, and PKC 412 (Reichardt et al. 2005). In these cases, mature clinical results are pending, however.

Summary

Taken together, the story of imatinib and GIST is one of the first examples of a successful targeted therapy in a solid tumor. The discovery of the gain-of-function mutation of c-kit as a crucial step in pathogenesis of GIST, the availability of imatinib as a specific inhibitor of tyrosine kinases, and the lack of classical treatment options like chemo- or radiotherapy was the basis of this success. Currently, a dose of 400 mg per day with possible escalation to 800 mg per day should

be considered as the standard treatment in advanced disease. In early localized disease, RO resection should be performed. Downstaging by imatinib preoperatively might be useful.

Mature clinical results of new targeted therapies are still pending. But the possible better effect of SU 11248 in patients with the exon 9 mutation of c-kit might indicate that more exactly targeted therapies in this disease might improve clinical outcomes in the future.

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13 Antibody Therapy in Non-Hodgkin's Lymphoma: The Role of Rituximab, 90Y-Ibritumomab Tiuxetan, and Alemtuzumab

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Abstract

Targeting cancer cells with monoclonal antibodies has become an indispensable part of modern treatment against hematologic malignancies. The excitement of the first successful experimental results could be confirmed by large multicenter trials, thus paving the way for new approaches in first-line, relapse, and maintenance therapy. Three antibodies—rituximab, ^{90}Y-ibritumomab tiuxetan, and alemtuzumab—are in clinical use worldwide and are reviewed in this chapter with a focus on practical information and fundamental principles of antibody-based therapy.

13.1 Introduction

Over the past few years antibody therapy has been established as an indispensable part of modern non-Hodgkin's lymphoma therapy. And without doubt the next decade will further amplify the impact of targeted antibody therapy, either with unconjugated antibodies or with antibodies carrying additional antitumor activity like radiation or cytotoxic substances.

Virtually all antibodies focus on the cell surface with specific antigens as targets (Table 13.1). Although potential target antigens are numerous and many groups have produced mouse antibodies against a variety of epitopes, the development of a commercially available antibody with proven efficacy, availability, and tolerable side effects was cumbersome. It was up to the anti-CD20 antibody rituximab (Biogen Idec, Genentech, and Roche) to introduce the worldwide success of monoclonal antibody therapy in lymphoma therapy. Figure 13.1 gives an overview on the timeframe from the coining of the phrase "antibody" to a broad clinical use with several milestones depicted and explained.

Table 13.1 Example of target surface antigens in antibody-based therapy of non-Hodgkin's lymphoma

B cell	T cell
CD80	T cell receptor
CD20	CD4, CD8
CD23	CD3
CD22	CD25
CD52	CD52
HLA-DR	CD7

One of the essential steps in optimizing tolerability and improving lymphoma activity was the substitution of murine sequences by human fragments, a process called antibody engineering. A new terminology for chimeric and humanized antibodies was developed and is shown in Fig. 13.2.

This review focuses on clinically relevant aspects and new developments in three approved and commercially available monoclonal antibodies: rituximab (Rituxan or Mabthera), ^{90}Y-ibritumomab tiuxetan (Zevalin, Biogen Idec and Bayer-Schering Pharma), and alemtuzumab (Campath-1H/MabCampath, Bayer-Schering Pharma). It is not intended as a thorough review on the available literature and the overwhelming amount of data in antibody-based lymphoma therapy; these reviews already exist and the reader is referred to them for further study (Cheson 2006; Coiffier 2004; Maloney 2005; Robak 2005; Stern and Herrmann 2005).

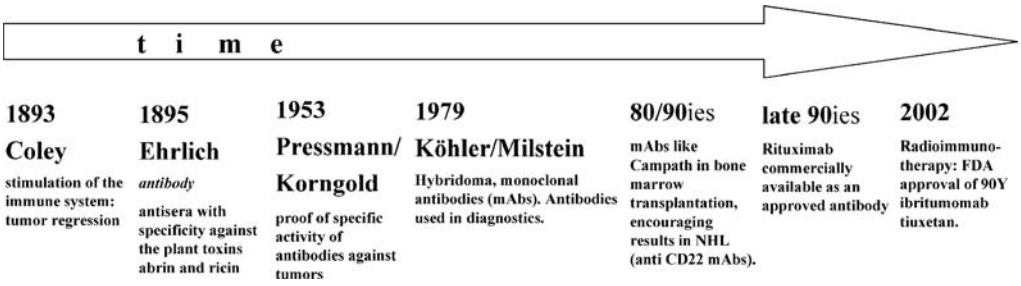

Fig. 13.1 Milestones in the development of therapeutic monoclonal antibodies

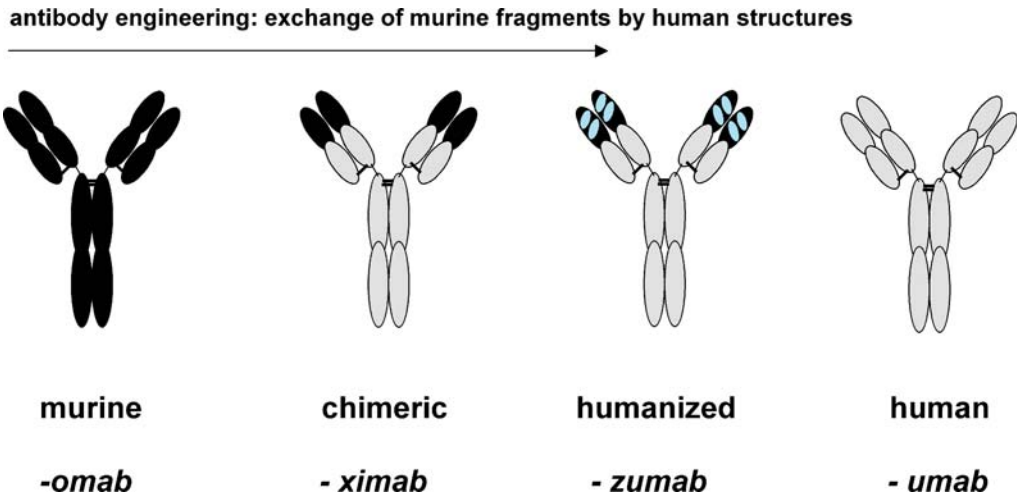

Fig. 13.2 Antibody engineering. The nomenclature of antibody structure

Apart from the former mentioned antibodies, a whole array of additional antibodies are in use or in clinical investigation: the amphipathic peptide D-(KLAKLAK)2 conjugated to anti-CD19; the primatized monoclonal antibody lumiliximab (IDEC-152) directed against CD23; the anti-CD22 antibody epratuzumab; the HLA-DR β-chain-specific antibody Hu1D10 (apolizumab) directed against the 1D10 antigen; the anti-CD80 antibody IDEC-114; the anti-CD80 antibody galiximab; the anti-CD22 recombinant immunotoxin BL22, which is successful in hairy cell leukemia; the chimeric antibody H17 that enhances rituximab activity by activation upon complement products; a humanized anti-CD22 and human ribonuclease angiogenin immunoenzyme; and the radioimmunoconjugate ^{90}Y-epratuzumab—to name only a few. The use of these antibodies will further diversify targeted lymphoma therapy, but is not discussed here.

13.2 Rituximab

The chimeric antibody rituximab (IDEC C2B8, Mabthera, Rituxan) is directed against an antigen clustered as CD20. The function of this epitope has been unknown for a substantial amount of time. We now know that CD20 is of importance in cell cycle regulation and cell differentiation. The CD20 antigen is expressed exclusively on B cells, making it an ideal target especially in the treatment of mature B cell neoplasias. The expression on late B-precursor cells is much more

unreliable, although anti-CD20 antibodies are also under investigation in the treatment of acute lymphoblastic leukemias.

After binding to the antigen, rituximab is neither split nor internalized, and there is no modulation of the antibody. The effector mechanisms are complex and not yet fully understood: Apart from a complement-activating activity (CDC—complement-dependent cell cytolysis), which in the beginning was thought to be the most important way of action, we now know about apoptotic pathways, nuclear factor-κB activation, and natural killer (NK) cell-mediated cytotoxicity (ADCC, antibody-dependent cellular cytotoxicity). The latter seems to depend at least in part on the FcγR polymorphism of the effector NK-cell.

Apart from rituximab in the setting of radioimmunotherapy and in some current chronic lymphocytic leukemia (CLL) studies, the dosing always follows the magic number of 375 mg/m². This dosage is at least in part a tribute to the availability of the antibody and logistic considerations at the time when the first studies were initiated (A. Grillo-Lopéz, oral communication, at the Expert Investigator Forum 2005); it is not exclusively based on pharmacokinetic and dose-response investigations in humans suffering from lymphoma. An increase in the rituximab dose seems to lead to increased responses at least in some instances (O'Brien S et al. 2001; Khouri et al. 2005), making a pharmacokinetic-based adjustment—especially in the setting of maintenance therapy—attractive.

13.2.1 Indolent Lymphoma

Rituximab is approved for the treatment of follicular lymphoma (FL) in most countries: as first-line therapy in combination with chemotherapy, from the second-line onwards also as monotherapy. It also is effective in other indolent lymphomas such as lymphoplasmacytic immunocytoma and mantle cell lymphoma (MCL), and an extension of the approvals is expected in several countries. Although rituximab shows activity in CLL, the first results especially with regard to the duration of remission were disappointing (Huhn et al. 2001). This was attributed in part to the dim expression of CD20 on B-CLL cells corresponding with a low antigen burden and also to the enormous number of circulating B cells. A dose escalation in B-CLL is possible, and it seems to correlate to an improved response (O'Brien et al. 2001). Consequently, several groups are currently re-testing rituximab induction therapy in CLL in combination with chemotherapy; in an attempt to achieve effective rituximab concentration on the lymphoma cells, the dose is being increased to 500 mg/m² (for example, in the CLL7 study of the German CLL Study Group).

There are now several multicentric, randomized, prospective studies from different parts of the world equivocally proving the superior activity of rituximab in first-line therapy of FL either in combination with anthracyclin-containing (Herold et al. 2004; Hiddemann et al. 2005a) or anthracyclin-free chemotherapy (Marcus et al. 2005). Of note, the M39023 study randomizing 8 cycles of MCP (mitoxantrone, chlorambucil, prednisolone) with or without 8 cycles of rituximab not only yielded to an increased event- and progression-free survival, but also showed an overall survival benefit for the rituximab arm in the intent-to-treat population (Herold et al. 2004). Clearly, the addition of rituximab has improved survival in low-grade lymphoma, which is also supported by comparison with historical cohorts undergoing an antibody-free treatment (Fisher et al. 2005).

Targeted therapy is an expensive therapy, and the discussion on pharmacoeconomics with all the upcoming new medications has only just begun. This is also true for antibody therapy in lymphoma. Several subanalyses address this issue. For the formerly mentioned M39023 trial a cost analysis has been performed retrospectively. According to an oral presentation (K. Hieke, Neos Health, at the Expert Investigator Forum 2005) with updated information on already published data (Hieke et al. 2004), treatment with rituximab increased therapy costs from €21,500 to €35,600. During the 5 years of follow-up, the subsequent costs including re-treatment were substantially smaller in the rituximab group (€17,900 compared to €30,700), neutralizing the increased initial therapeutic costs.

Antibody-based maintenance therapy in indolent lymphoma is currently under investigation in numerous trials. Although there already exists a substantial number of preliminary or already published data, we have to wait for the re-

sults of the large multicenter national and international groups to confirm the efficacy of a prolonged antibody administration, especially in the current patient population that already received rituximab in therapy induction. The worldwide PRIMA (Primary RItuximab and MAintenance) study will help finally address this question. The currently available data from several randomized clinical trials, however, strongly suggest that maintenance therapy with rituximab increases remission duration in NHL—either following induction with single-agent rituximab or chemotherapy (or both) (Hainsworth et al. 2002, 2005; Ghielmini et al. 2004; van Oers et al. 2004; Hiddemann et al. 2005b). A longer follow-up is needed to address the question of a survival advantage.

Obviously, maintenance therapy should be stratified according to the indolent lymphoma entity. In FL, for example, a prolonged administration seems to improve event-free survival (Ghielmini et al. 2004), whereas with the same rituximab schedule there was no difference in MCL (Ghielmini et al. 2005).

The dosage, the time interval, and the time frame for a maintenance therapy are undefined and differ from study group to study group. Looking at pharmacokinetics, however, a single infusion of 375 mg/m² rituximab every 3–4 months maintains a serum level of 25 µg/ml in most patients with indolent lymphoma (Gordan et al. 2005). According to the pharmacokinetic data from the pivotal trial, this is the median 3-month rituximab level associated with response (Berinstein et al. 1998).

An alternative approach to maintenance therapy is a watch-and-wait policy until disease progression, followed by re-treatment with rituximab monotherapy. One study addressed this question with an approximately fourfold advantage in progression-free survival for the maintenance therapy arm compared to the re-treatment arm. This again proves the efficacy of rituximab in preventing progression. However, the maintenance therapy did not lead to a plateau. And the actuarial 3-year survival curves for the maintenance versus re-treatment groups were not different, making an early re-treatment approach feasible and further evaluation worthwhile, especially when looking at the cost of maintenance therapy (Hainsworth et al. 2005).

13.2.2 Aggressive Lymphoma

Rituximab has activity in aggressive relapsing or refractory lymphoma even when given as a monotherapy; the remissions are in the range of 30% (Coiffier et al. 1998). However, the standard approach for younger patients relapsing after first-line therapy or with refractory disease is high-dose chemotherapy with autologous stem cell transplantation. In a chemotherapy-sensitive disease such as aggressive lymphoma, the main focus remains cure of the disease with a first-line treatment.

From a historical point of view, the disappointing results of the South-West Oncology Group data published 1993 comparing CHOP to second and third generation chemotherapy modifications like m-BACOD, MACOP-B, and ProMACE-CytaBOM, confirmed CHOP as the gold standard in aggressive lymphoma therapy for another 10 years (Fisher et al. 1993). This changed in 2002, when a French group (Group d'Etudes de Lymphomes de l'Adulte, GELA) published a significant event-free and overall survival advantage for elderly patients receiving additional rituximab to a classical CHOP therapy (median 4.2 years event-free survival with R-CHOP and 1 year with CHOP, $p = 0.00002$; Coiffier et al. 2002). The differences in event-free survival were significant for both low-risk and high-risk patients according to the IPI (International Prognostic Index) score.

An update of this study proves that this advantage is persisting over time (Feugier et al. 2005).

Meanwhile, the results of the so-called MINT trial (Mabthera International Trial) also showed a significant advantage for a young low-risk population (age-adjusted IPI 0 or 1) receiving rituximab on top of a CHOP-like regimen (2-year time-to-treatment failure 81% in the rituximab-containing arm compared to 58% for patients on chemotherapy only; 2-year survival 95% vs 85% in favor of the rituximab arm, $p = 0.0026$; Pfreundschuh et al. 2004). Interestingly, in this study the advantage of adding etoposide to CHOP was neutralized by the addition of rituximab to CHOP, which led to the coining of the phrase for rituximab as the "equalizer" in the subgroup of young good-prognosis patients.

Current results of the German high-grade lymphoma study group, which examined a 14-

day CHOP cycle (CHOP 14) leading to a survival benefit compared to the classical 21-day CHOP, show synergistic effects of both approaches: 8×rituximab given on top of 6×CHOP-14 (R-CHOP 14) led to significantly better results in elderly patients compared to R-CHOP 21 or CHOP 14 combinations (Pfreundschuh et al. 2005). These results have to be confirmed by other groups, but clearly for the *low-risk* patient with aggressive B cell lymphoma irrespective of age, R-CHOP is the new gold standard; R-CHOP 14 might further improve survival rates.

Similar to indolent lymphoma, the treatment with rituximab in aggressive lymphoma raised discussions on cost consequences and cost effectiveness. For the Netherlands, data on cost effectiveness in the year 2002 have been published (Groot et al. 2005): the incremental cost-effectiveness ratio (ICER) per quality adjusted life year was €13,983 for younger and €17,933 for older patients. According to the authors this should be considered acceptable by policymakers, especially when looking at ICER in other medical fields such as cardiology.

Although from a medical perspective especially in aggressive lymphoma with most relapses occurring during the first 2 years after diagnosis a maintenance therapy with rituximab should make sense, the first published results were disappointing (Habermann et al. 2004). In relapsed aggressive lymphomas the CORAL study (COllaborative trial in Relapse Aggressive Lymphoma, coordinated by the GELA) currently investigates in a randomized fashion the role of a maintenance therapy after high-dose chemotherapy with autologous stem cell transplantation.

13.2.3 Modification of Rituximab Activity

Several approaches are under investigation to optimize rituximab activity. One obvious approach is to increase the dose of rituximab, for example to 1,000 mg/m², which might at least in the setting of an autologous stem cell transplantation increase efficacy (Khouri et al. 2005). A combination of several B cell antibodies with different target antigens might improve the results of an antibody-based targeted therapy as well as a modification of the CD20 expression on the target cell by tumor necrosis factor (TNF)-α, interferon (IFN)-α, or interleukin (IL)-4. Other approaches including improved complement activity (e.g., with increased affinity between the Fc portion and C1q), optimized apoptosis induction (e.g., addition of glucocorticosteroids), or improved cytotoxicity (addition of IL-2, IL-12, INF-α; increased affinity between the Fc portion and the FcγR) are reviewed by Cartron et al. (2004).

13.3 Radioimmunotherapy with ^{90}Y-Ibritumomab Tiuxetan

13.3.1 Rationale of Radioimmunotherapy in Lymphoma Therapy

Lymphomas are radiosensitive proliferations. External radiotherapy in localized lymphoma—especially in FL—is an established and sometimes curative treatment. An antibody loaded with a β- or γ-emitter has the advantage of detecting occult tumor cells that cannot be identified by computed tomography (CT) or magnetic resonance imaging (MRI) scans and therefore escape external radiation—the antibody acts as a tracer carrying the radiation to the malignant cell. The so-called cross fire effect destroys neighboring cells and hence theoretically should help to reach malignant cells in bulky or poorly vascularized tumors. The hope is that radioimmunotherapy as a form of internal targeted radiation is more a potentiation than a combination of the success of immunotherapy and radiotherapy.

Two radiolabeled anti-CD20 antibodies are commercially available: ^{90}Y-ibritumomab tiuxetan (Zevalin), a β-emitter with an effective half-life time of 28 h, and ^{131}I-tositumomab (Bexxar, GlaxoSmithKline), a combined β- and γ-emitter with 59 h effective half-life time. Both antibodies show comparable, promising activity and are under investigation in numerous international studies in first- as well as second-line therapies, with or without chemotherapy including treatment with autologous and allogeneic progenitor cell support (Micallef 2004; Fietz et al. 2006). This review focuses on ^{90}Y-ibritumomab tiuxetan, which due to radioprotective issues is easier to use than the ^{131}I-counterpart and is approved in Europe.

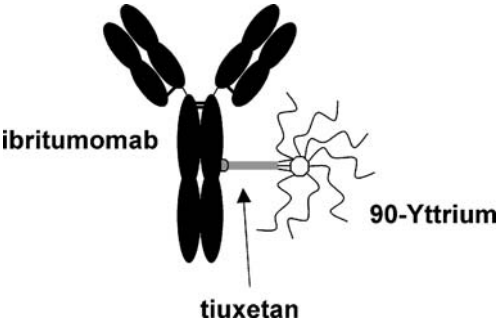

Fig. 13.3 Structure of the radioimmunoconjugate ^{90}Y-ibritumomab-tiuxetan

13.3.2 Structure of ^{90}Y Ibritumomab Tiuxetan and Clinical Handling

The ^{90}Y-labeled antibody ibritumomab tiuxetan comprises the murine monoclonal antibody ibritumomab, which is also the "mother antibody" for engineering of the chimeric rituximab. Halogens such as ^{131}I can be easily labeled to the antibody, whereas radiometals such as ^{90}Y require chelation. The linker chelator for ibritumomab is called tiuxetan. The advantage of a chelator is a more stable connection leading to a constant loss in activity over time, making a dosimetry dispensable in standard-dose ^{90}Y-ibritumomab tiuxetan in contrast to the iodine labeled tositumomab. The isotope ^{90}Y (Ytracis), a pure β-emitter, completes the structure (Fig. 13.3).

The range of the β-radiation in tissue is about 5–10 mm, leading to a 250-cell radius with radiotoxic effect. Zevalin therapy can be given as an outpatient treatment; the β-particles—once internalized—cannot travel beyond the patient's body and the radiation exposure for healthcare workers and family members is virtually zero. Two applications of reduced dose rituximab are given before the application of the radiolabeled ("hot") antibody, which is believed to positively influence biodistribution (Fig. 13.4).

Extensive data on dosing show that the CD20-positive tumor cells receive most of the radiation followed by noncritical organs such as spleen, testes, liver, colon, and heart. The bone marrow receives about 1/10 the dose of the lymphoma cells, the kidneys about 1%. The limiting toxicity for ^{90}Y-ibritumomab tiuxetan is hematotoxicity. Therefore only patients with less than 25% bone marrow involvement and thrombocytes numbering more than 150/nl receive the standard dose of 0.4 mCi/kg. Due to the unique radioimmunotherapeutic action, the spectrum of side effects is characteristic with a nadir after 6–8 weeks; here especially the prolonged thrombocytopenia requires special attention and careful introduction of this novel approach into established treatment protocols (Cheson 2005). Increasing experience with this antibody shows that repeated applications are feasible and the use of Zevalin does not interfere with any further treatment line; even stem cell mobilizations are possible (Ansell et al. 2004; Ely et al. 2005).

13.3.3 Clinical Data

Both radiolabeled antibodies seem to have improved activity compared to the native antibody rituximab as reviewed in a recent metaanalysis including 10 studies with either ^{90}Y-ibritumomab tiuxetan or ^{131}I-tositumomab (Orina et al. 2005); the rates of overall response or complete response with radioimmunotherapy were increased by 42% and 30%, respectively.

Also, ^{90}Y-ibritumomab tiuxetan seems to have a prolonged antilymphoma activity (Witzig et al. 2002). The treatment with Zevalin is very well tolerated, and even in rituximab- or chemotherapy-refractory disease, remissions have been reported (Gordon et al. 2004).

The earlier radioimmunotherapy is initiated in the course of the disease the better are the

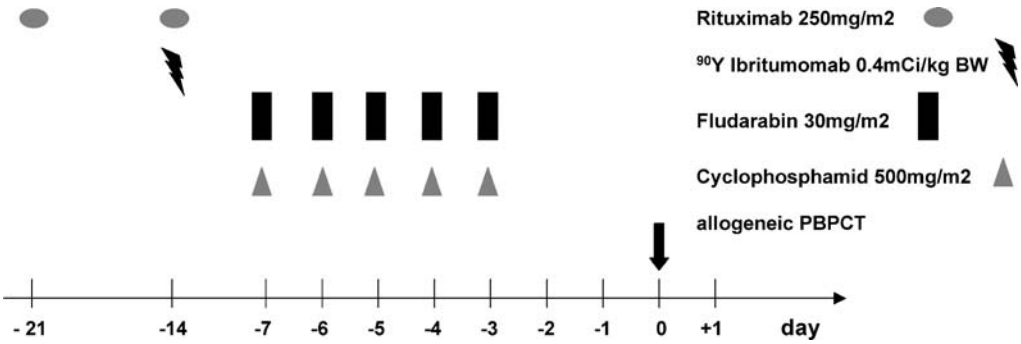

Fig. 13.4 Example of a conditioning regimen in allogeneic stem cell transplantation incorporating ^{90}Y-ibritumomab-tiuxetan. (Fietz et al 2006; reproduced with permission, http://www.tandf.co.uk/journals)

results, and the remissions last longer (Emmanouilides et al. 2003). Similar to rituximab and alemtuzumab, a bulky disease is an indicator of poor response, making a chemotherapy debulking necessary (Czuczman et al. 2002).

Currently the application of ^{90}Y-ibritumomab tiuxetan extends into allogeneic high-dose protocols (Fietz et al. 2006; Fig. 13.4), experimental indications such as primary CNS lymphoma (Kiewe et al. 2006), and consolidative therapy in follicular lymphoma (DeMonaco et al. 2005). However, ^{90}Y-ibritumomab tiuxetan is not a success story in all CD20-positive proliferations: the results in chronic lymphocytic leukemia have been disappointing (Liu et al. 2003). Moreover, as mentioned above, the β-particles of Y-90 have an estimated path length of 5–10 mm (approximately 250 cells). Since the β-emission from the bremsstrahlung evolves a certain distance apart from the nucleus with the maximal dose pointing away from isotope, it might well be that the single cell labeled with radioactive antibodies actually receives only a minimal amount of or no radioactivity. Therefore, at least from a radiophysical point of view, a radiolabeled antibody might not be of additional value compared to its nonlabeled analog in the treatment of minimal residual disease. Clearly we have to wait for the results of the ongoing studies examining radiolabeled antibodies in maintenance therapy after achievement of complete remission (CR).

More than 1,300 patients have been treated worldwide in the year following FDA approval in 2002 (Theuer et al. 2004), underscoring the urge for innovative approaches in lymphoma therapy.

13.4 Alemtuzumab

Alemtuzumab (Campath 1-H, MabCampath) is a humanized monoclonal antibody directed against the CD52 antigen. CD52 itself is a very broad marker expressed on almost every hematopoietic cell with the exception of early progenitors and the erythropoietic lineage. The Campath antibody story goes back to *Cam*bridge *Path*ology, where the antibody was developed in the early 1980s. A free recommendable description of the development of the Campath antibodies is available online (Hale and Waldmann 2000).

The first application of the IgM antibody Campath 1M was in the setting of bone marrow transplantation, most often as a means of ex vivo purging (Campath in the bag). Later the IgG2b antibody Campath 1G was used for in vivo purging. The humanized antibody 1H is the most active variant form, and the mechanisms of action—like in other monoclonal antibodies—range from ADCC to complement activation and apoptosis.

Campath-1H shows activity in many hematologic diseases—it is licensed for CLL—but response in other indolent lymphomas and rarer disease entities such as peripheral T cell lymphoma and hairy cell leukemia has been described (Rieger et al. 2004; Fietz et al. 2004).

13.4.1 Clinical Application

Compared to rituximab and ^{90}Y-ibritumomab, alemtuzumab is more tricky to apply, especially

on an outpatient basis (Rieger et al. 2004). The antibody has to be started at a reduced dose of 3 mg i.v., the second day 10 mg if the first dose was tolerated, the third day 30 mg i.v., if the second dose was tolerated. To prevent rigor, rash, hypotension, and potentially life-threatening anaphylactic reactions associated with the so-called "cytokine storm," especially for the first applications, a premedication with corticosteroids (e.g., 100 mg prednisolone i.v.) and antihistamines (e.g., clemastine) is essential. The standard dosage would be 30 mg 3×/week i.v. for 12 weeks. Due to hematologic toxicity this is rarely possible, and alternative schedules are being developed (Thieblemont et al. 2004). Also, since the duration of response after discontinuation of alemtuzumab monotherapy is often short-lived, a prolonged administration with a reduced dosing seems to be an attractive alternative (Lundin et al. 2002; Rieger et al. 2004; Thieblemont et al. 2004). Of note is that a subcutaneous injection compared to the i.v. schedule reduces the risk of the development of anaphylactic reactions, but it is almost invariably associated with local erythema at the site of injection, making a prolonged s.c. administration sometimes impossible.

The profound T and B cell depletion seen with alemtuzumab makes an infectious prophylaxis necessary: antiviral (acyclovir at least 2×400 mg/day or alternatives such as valacyclovir) and *Pneumocystis carinii* (e.g., cotrimoxazole 960 mg 4 tbls/week) medication is mandatory. Also, the CMV status has to be monitored (pp65 antigen or CMV-specific PCR). As a matter of fact, infectious complications remain the achilles' heel of alemtuzumab therapy; especially in combination with chemotherapy, the immunosuppressive effect cannot be overestimated.

13.4.2 Alemtuzumab in Chronic Lymphocytic Leukemia

Most experience has been gathered with alemtuzumab in patients suffering from chronic lymphocytic leukemia. First the antibody was applied in patients with relapsed or refractory CLL—leading to responses in about 30% of patients (reviewed in Cheson 2006).

A rapid reduction in leukocyte count is very reliably possible; also, the bone marrow infiltration responds well to therapy. A bulky disease, lymphocytic lymphoma and the type of CLL associated with predominant lymph node proliferation does not respond well to alemtuzumab monotherapy. As a matter of fact, the size of the largest lymph node is a prognostic marker for survival in alemtuzumab therapy (Moreton et al. 2005). Monotherapy with alemtuzumab can lead to sustained remissions in patients acquiring a minimal-residual disease (MRD)-negative CR; these patients clearly have a survival advantage (Moreton et al. 2005) compared to MRD-positive patients with a clinical CR.

Nowadays the combination of chemotherapy and antibody therapy (usually alemtuzumab or rituximab) leads to remissions in a range exceeding 90% of untreated CLL patients (Keating et al. 2005). A comparable rate of CR (60%) was previously only seen with allogeneic stem cell transplantation. Therefore, several international studies are investigating a purin analog-based approach with the additional application of an antibody—either rituximab or alemtuzumab. The impact on overall survival is still controversial, but the rate of complete or near CRs is impressive. Autologous stem cell transplantation especially has lost its importance, and the allogeneic approach in most countries is limited to refractory and relapsing patients as well as patients with a defined risk profile according to chromosomal aberrations or vH-mutational status.

The obvious importance of an MRD-negative status for remission duration has led to consolidative therapies with alemtuzumab in CLL. It is possible to convert MRD-positive patients into an MRD-negative state and prolong remission—but the major drawback is the rate of serious infectious complications requiring modifications of this approach (Wendtner et al. 2004). A maintenance regimen with 30 mg Campath-1H every 1–4 weeks—even an extension to a bimonthly application until disease progression is seen—seems to be effective without increasing hematologic toxicity or infectious complications (Thieblemont et al. 2004, 2005) and points toward new treatment schedules.

Summary

After years of research and careful investigation in a limited number of centers, antibody-based therapy in non-Hodgkin's lymphomas has become one of the success stories of targeted therapy today. Especially rituximab and alemtuzumab are in broad clinical use all over the world. Both act via complement activation, ADCC, and apoptosis. The combination with chemotherapy increases remission rates and remission duration. Although not a chemotherapy, these antibodies require experienced therapists, since they have a unique side effect profile including prolonged immunodeficiency. The addition of the radioisotope yttrium leads to the conversion of the "cold" anti-CD20 antibody to a radioactive β-emitter. Radioimmunotherapy with ^{90}Y-ibritumomab tiuxetan is an attractive alternative to the use of the unconjugated antibody, using fewer applications but nevertheless prolonging activity. For both targeted approaches the indications are constantly increasing, and over the next years both front-line and consolidative therapies are being evaluated.

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14 Molecular and Clinical Aspects of Proteasome Inhibition in the Treatment of Cancer

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Abstract

The proteasome is a multicatalytic threonine protease responsible for intracellular protein turnover in eukaryotic cells, including the processing and degradation of several proteins involved in cell cycle control and the regulation of apoptosis. Preclinical studies have shown that the treatment with proteasome inhibitors results in decreased proliferation, induction of apoptosis, and sensitization of tumor cells against conventional chemotherapeutic agents and irradiation. The effects were conferred to stabilization of p21, p27, Bax, p53, I-κB, and the resulting inhibition of the nuclear factor-κB (NF-κB) activation. Bortezomib is the first proteasome inhibitor that has entered clinical trials. In multiple myeloma, both the FDA (United States Food and Drug Administration) and EMEA (European Medicine Evaluation Agency) granted an approval for the use of bortezomib (Velcade, Millennium Pharmaceuticals, Cambridge, MA, USA) for the treatment of relapsed multiple myeloma. At present, clinical trials are examining the activity in a variety of solid tumors and hematological malignancies.

14.1 Introduction

During last two decades, very large, nonlysosomal, intracellular proteases have been found and further characterized in mammalian cells (Tanaka et al. 1988). Molecular examinations and structure studies have shown that they have different functional and structural characteristics than other established proteases. Due to their large molecular mass (700–800 kDa) and barrel-like structure, they were named proteasomes (Arrigo et al. 1988). The proteasome-ubiquitin system was identified as the control machinery for protein oscillation required for proper cell cycle progression, signal transduction, transcription regulation, and apoptosis. Thus it is crucially involved in neoplastic transformation of cells, inflammation, and autoimmunity. The first proteasome inhibitor that has entered clinical trials was developed by Millennium Pharmaceuticals, Cambridge, MA (formerly ProScript) and was approved by the FDA and European Medicine Evaluation Agency (EMEA) for treatment of relapsed or refractory multiple myeloma.

14.2 Structure and Function of the Proteasome

The 26S proteasomes are highly conserved, multicatalytic protease complexes that are responsible for protein degradation and maintenance of protein homeostasis. They are localized in cytoplasm and the nucleus of eukaryotic cells and are predominantly involved in the regulation of turnover of short-living proteins that regulate cell-cycle progression, apoptosis, signal transduction, antigen presentation, and inflammatory processes (Adams 2003; Kloetzel 2001). Proteins that are targeted for proteasomal degradation first must be marked by ubiquitin chains to be recognized by the proteasome as substrates. This process is carried out by a cascade of enzymes named E1, E2, and E3. E1 is the ubiquitin-activating enzyme, which binds the small molecule ubiquitin and transfers it to ubiquitin-conjugating enzyme E2. Finally, the ubiquitin-protein ligase E3 per-

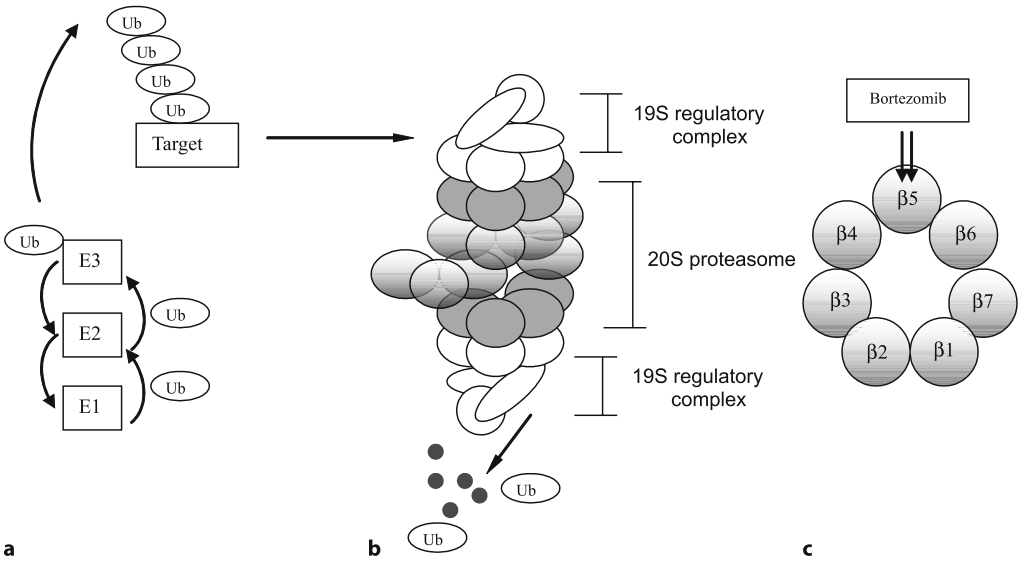

Fig. 14.1 a–c Proteasome-ubiquitin-pathway. **a** Proteins that are targeted by the proteasome for further degradation must first be labeled by a polyubiquitin chain. The ubiquitination is a highly selective process that requires the interplay of three ATP-dependent ubiquitin-conjugating enzymes E1, E2, and E3. **b** The 26S proteasome is a protein complex that consists of one 20S core (*gray*) and two regulatory 19S subunits (*white*). The lid and base components of the 19S subunits bind, cleave, and release ubiquitin and drive the unfolded protein into the proteolytic cavity. The 20S core consists of two inner β-rings (*gray shadow*) carrying the catalytic sites faced to the central cavity. Its function is to degrade the denaturated proteins. The outer α-rings (*gray*) arbitrate the interaction between the catalytic sites and the regulatory subunits. **c** Bortezomib interacts with the β5-subunit that confers chymotryptic proteolytic activity

forms the docking of ubiquitin to lysine residues of target protein (Ciechanover et al. 2000). Once the substrate has been marked with plenty of ubiquitin conjugates, it is flagged and destined for degradation by the 26S proteasome (Fig. 14.1a). Thus protein ubiquitination is a diversified and highly regulated process that defines the cellular protein turnover rate (Elliott et al. 2003). Prior to degradation, the ubiquitin-chain is removed and recycled for the further use.

Using electron microscopy, proteasomes appear as symmetrical ring-shaped particles (Tanaka et al. 1988; Walz et al. 1998). The 26S enzyme complex is composed of two major subunits, the catalytically active 20S core and one or two ATP-dependent 19S regulatory particles (Tanaka et al. 1988; Hough et al. 1987). The 19S subunits are responsible for recognition of ubiquitinated substrates and cleavage of the ubiquitin chains that precedes the translocation into the core. The catalytic core shows a four-striated cylindrical structure with a seven-fold rotational symmetry. The two outer rings are built from seven nonproteolytic α-subunits; both inner rings consist of seven β-subunits, of which each contains three active sites (Fig. 14.1b). They express post-glutamyl peptide hydrolase-like (β1-subunit), trypsin-like (β2-subunit), and chymotrypsin-like (β5-subunit) activities that are formed by amino-terminal threonine residues (Fig. 1c) (Adams et al. 1998; Groll et al. 2001; Hoffman and Rechsteiner 1994). However, the β-subunits can adapt to the functional requirements of the cell. Upon γ-interferon stimulation, β-subunits can be replaced by their immune homologs, resulting in the formation of proteasomes with different cleavage patterns (Kloetzel 2004). For appropriate hydrolysis of proteins, a conformational plasticity of the complex must be provided for the interaction between the substrates, core, and regulatory complex (Stein et al. 1996; Seeger et al. 1997).

The deregulation of the proteasome-ubiquitin system in humans can cause severe diseases, e.g.,

cancer, autoimmune, and metabolic disorders. The first evidence that proteasomes are involved in cancer was observed due to the increase of proteasome concentration in normal human mononuclear cells during blastogenic transformation induced by phytohemagglutinin (Kumatori et al. 1990). Furthermore, high levels of proteasome activities in human leukemia cells, their oscillation during the cell cycle, and their increase parallel to the induction of DNA synthesis led to the hypothesis that proteasomes may be involved in transformation and proliferation of cells (Kumatori et al. 1990). Further studies revealed that many cell cycle and cell survival regulatory proteins, such as cyclins (Sudakin et al. 1995), cyclin-dependent kinase inhibitors (p19, p21, p27 and p57) (Thullberg et al. 2000; Blagosklonny et al. 1996; Pagano et al. 1995; Urano et al. 1999), tumor suppressor proteins (p53 and Rb) (Maki et al. 1996, Boyer et al. 1996), protooncogenes (c-Myc, c-Fos, and c-Jun) (Ciechanover et al. 1991; Stancovski et al. 1995), topoisomerases (Nakajima et al. 1996), pro-apoptotic molecules (Bax, MDM 2, IAPs) (Chang et al. 1998; Yang et al. 2000), transcriptional factors (E2A, E2F, and STAT) (Kho et al. 1997; Campanero and Flemington 1997; Kim and Maniatis 1996), and inhibitor of nuclear factor-κB (NF-κB), I-κB (Palombella et al. 1994; Traenckner et al. 1994), are degraded by the proteasome (Table 14.1).

The disruption of the balance of exactly coordinated levels of pro- and antiproliferative signals leads to confounding signals in cells, which result in cell cycle arrest, activation of caspases, and induction of apoptosis. Furthermore, NF-κB was identified as a crucial factor implicated in cancer progression. In cells, NF-κB exists in an inactive form in the cytoplasm, bound to the inhibitory protein I-κB. When liberated from I-κB, NF-κB translocates into the nucleus, where it binds to DNA and controls transcription of several genes involved in growth, cell differentiation, and apoptosis. Moreover, it promotes oncogenesis and is constitutively activated in some malignant cells (Ni et al. 2001; Bargou et al. 1997), or in response to chemotherapeutic drugs, radiation, cytokines, or oxidants (Jeremias et al. 1998; Wang et al. 1996). Consistent with this, inhibition of NF-κB is a critical point in inducible chemoresistance and it seems to increase the susceptibility of malignant cells to chemotherapeutics or irradiation (Baldwin 2001; Mitsiades et al. 2003). Targeting NF-κB by using inhibitors of I-κB phosphorylation could not completely prevent cell proliferation, which indicates that effects of proteasome inhibitors are not only due to NF-κB blockade alone, but also result from targeting other signaling pathways (Hideshima et al. 2002).

14.3 Proteasome Inhibitors

The first compound with documented effects on proteasomal function was identified as the nonpeptidic *Streptomyces* metabolite lactacystin. Its intermediate clasto-lactacystin β-lactone interferes with the β5-subunit (chymotrypsin-like activity) irreversibly by selective modification of amino-terminal threonine residues and reversibly with the β1-subunit (peptidylglutamyl-like activity) and β2-subunit (trypsin-like activity) (Fenteany et al. 1995; Dick et al. 1997). In the course of further characterization of proteasome structure using X-ray crystallography, more selective and potent proteasome inhibitors were synthesized (Adams et al. 1998; Loidl et al. 1999). They included tripeptide aldehydes PSI and MG-132, which reversibly inhibit the β2- and β5-subunit by forming hemiacetal adducts with the active site of β-subunits. In addition, they inhibit thiol proteases such as calpains and cathepsin B, and display poor metabolic stability due to their configuration, resulting in low specificity and bioavailability, thus limiting their utility in vivo. The next generation of proteasome inhibitors includes boronic acid dipeptides MG-262 and bortezomib, which are capable of building stable but reversible tetrahedral intermediates with the amino-threonine residues of the catalytically active core complex of the proteasome (Adams et al. 1998). This explains their slow dissociation from the proteasome in comparison to the aldehydes, their 1,000-fold potency, and high sensitivity for chymotrypsin-like activities of the proteasome. Pharmacokinetic studies showed that after intravenous administration of PS-341, more than 90% is cleaved from the plasma compartment within 15 min. It blocks the proteasome with a K_i value of 0.6 nM (Adams et al. 1999), with a restoration of proteasome activities within 24 h (Chauhan et al. 2005). As measured

Table 14.1 Examples of cellular proteins degraded by the proteasome

	Protein	Function	Reference(s)
Cyclins	Cyclin A	Cell cycle regulation during S phase and mitosis	Sudakin et al. 1995
	Cyclin B	Cell cycle regulation during mitosis	Zhang et al. 1998
	Cyclin C	Cell cycle reentry	Cooper et al. 1999
	Cyclin D	Cell cycle regulation during G1 phase	Diehl et al. 1997
	Cyclin E	Cell cycle regulation during G1 phase and S phase	Clurman et al. 1996
Cyclin-dependent kinase inhibitors	p19	G1-S progression	Thullberg et al. 2000
	p21	Cell cycle regulation	Blagosklonny et al. 1996
	p27	Cell cycle regulation	Pagano et al. 1995
	p57	Cell cycle regulation and differentiation	Urano et al. 1999
Transcriptional factors	E2A	Cellular growth and differentiation	Kho et al. 1997
	E2F	Cell cycle regulation by gene expression control	Campanero and Flemington 1997
	STAT	Transcription of cytokine-inducible genes	Kim and Maniatis 1996
	NF-κB precursor	Maturation of NF-κB	Palombella et al. 1994
Inhibitors of transcriptional factors	I-κB	Inhibition of transcriptional factor NF-κB, which is responsible for the transcription of numerous growth factors, cell adhesion molecules, angiogenetic factors and antiapoptotic proteins	Palombella et al. 1994; Traenckner et al. 1994
Tumor suppressor proteins	p53	Cell cycle arrest, senescence, apoptosis	Maki et al. 1996
	Rb	Repressor of progression toward S phase by inactivation of the transcription factor E2F which is required for activation of S phase genes	Boyer et al. 1996
Oncogenes	c-Myc	Promotion of proliferation	Stancovski et al. 1995
	c-Fos	Promotion of proliferation, control of transcriptional factor AP-1	
	c-Jun		
Apoptosis	Topoisomerase II	Promotion of cell survival, DNA replication	Nakajima et al. 1996
	Bcl-2	Inhibition of apoptosis	Dimmeler et al. 1999
	Bax	Promotion of apoptosis	Chang et al. 1998
	MDM 2	Promotion of apoptosis	
	IAPs	Inhibition of apoptosis	Yang et al. 2000

by autoradiography in rats, PS-341 was found to be bioavailable in all organs with the exception of CNS, testes, and eyes (Adams et al. 1999). Thus, its high selectivity, stability, and low toxicity profile due to the reversibility of the proteasome inhibition allowed bortezomib to enter clinical trials.

Recently, a novel, orally bioavailable proteasome inhibitor was identified (Chauhan et al. 2005). NPI-0052 was derived from a marine gram-positive actinomycete (Macherla et al. 2005). It is a nonpeptide proteasome inhibitor that shows a structural similarity to clasto-lactacystin β-lactone (Corey and Li 1999) with some biochemical modifications. Compared to bortezomib, it displays different effects on all three proteasomal activities, with even lower EC_{50} for the inhibition of the chymotrypsin-like proteasome activity, and lower toxicity profile, suggesting a larger therapeutic index for NPI-0052 (Chauhan et al. 2005). Furthermore, other selective proteasome inhibitors are currently being tested in preclinical studies (Braun et al. 2005).

14.4 Preclinical Experience with Proteasome Inhibitors

The first evidence of the broad antitumor potential of proteasome inhibitors was given in 1999 by the National Cancer Institute screen of 60 tumor cell lines (Adams et al. 1999). Mean IC_{50} values for bortezomib were found in nanomolar range. For further evaluation of mechanisms of proteasome inhibitor mediated cytotoxicity, the authors used the prostate tumor PC-3 cell line. In an in vitro assay, the induction of apoptosis was independent of p53 status, but was accompanied by p21 stabilization, G2-M arrest, and poly-(ADP-ribose-)polymerase (PARP) cleavage. Using a PC-3/nude mouse model, treatment with bortezomib significantly suppressed tumor growth at well-tolerated doses (Adams et al. 1999).

Further investigations showed proapoptotic effects in the MCF-7 and in EMT-6 murine mammary carcinoma in vivo/in vitro assay (Teicher et al. 1999), Lewis lung carcinoma (Teicher et al. 1999), squamous cell carcinoma (Kudo et al. 2000; Sunwoo et al. 2001), pancreatic cancer cell lines (Fahy et al. 2003), ovarian cell lines HEY, A2780, SKOV3, and OVCA 429 (Frankel et al. 2000), human glioma cell lines U-87MG and T98G (Kitagawa et al. 1999), myeloid leukemia lines, and adult B and T cell leukemia cell lines (Soligo et al. 2001; Naujokat et al. 2000; Yamada et al. 2000). In multiple myeloma, it was demonstrated that bortezomib induced apoptosis directly and additionally by abrogating paracrine growth stimulation (Hideshima et al. 2001). Furthermore, the adhesion of myeloma to bone marrow stromal cells was diminished by treatment with proteasome inhibitors, which resulted in reduced mitogen-activated protein kinase (MAPK) growth signaling and protection against apoptosis induced by dexamethasone (Hideshima et al. 2001). Acting on NF-κB, subtoxic dosages of bortezomib could sensitize both drug-susceptible (Mitsiades et al. 2003) and primarily drug-resistant myeloma cell lines to doxorubicin and melphalan (Ma et al. 2003).

Interestingly, the extent of apoptosis induction in primary myeloma cells by proteasome inhibitors was independent of chromosome 13-deletion status, a subgroup with a dismal prognosis (Zavrski et al. 2003). Furthermore, treatment with proteasome inhibitors could also abrogate osteoclast differentiation and resorptional activity, highlighting its potential toward tumor microenvironment as therapeutic target (Zavrski et al. 2005). The combination therapy with gemcitabine followed by bortezomib showed the greatest induction of apoptosis in MIA-PaCa-2 pancreatic cancer cell line when compared with simultaneous incubation or preincubation with bortezomib. When the agents were given in sequence or in combination, no correlation with the modified levels of p21, p27, and Bcl-2 have been observed, which might indicate that bortezomib could block endogenous cell survival response following exposure to chemotherapy (Fahy et al. 2003).

In the human colon cancer cell lines CCD841, KM12L4, LOVO, and WiDr, pre-treatment with bortezomib for 1 h prior to addition of camptothecin suppressed camptothecin-induced NF-κB activation markedly, resulting in enhanced antitumor effects, reflected in significantly improved growth inhibition when compared with treatment with bortezomib or camptothecin alone.

When camptothecin and bortezomib at the MDT of 1.0 mg/kg were given systemically to mice bearing a LOVO cancer xenograft, a significant improvement of tumoricidal response was observed and reached greater than a 90% decrease in tumor size. The authors hypothesized that the camptothecin/topoisomerase I complex would be degraded by the proteasome under physiological conditions, since it was shown that destruction of topoisomerase I could be prevented in cells treated with proteasome inhibitors MG-132 and lactacystin (Desai et al. 1997). The inhibition of the proteasomal degradation of the complex might prolong the half-life time of the complex and result in enhanced activity of the topoisomerase inhibitors (Cusack et al. 2001).

14.5 Clinical Experience with Bortezomib in Multiple Myeloma

The first phase I trial concerning hematological malignances was performed by Orlowski and colleagues (2002). In patients with plasma cell dyscrasias, one complete remission (CR) and some partial remissions (PRs) were observed; 2 of 10 patients with non-Hodgkin's lymphoma (NHL) achieved a PR, and no response was seen in the 4 patients with a Hodgkin's lymphoma. Pharmacodynamic studies showed a time- and a dose-dependent inhibition of the 20S proteasome, with a maximum inhibition of 74%. On the basis of these results, a large multicenter, open-label, nonrandomized phase II trial (SUMMIT trial) was performed in 202 patients with relapsed or refractory multiple myeloma who had received at least three prior therapies (Richardson et al. 2003). Patients were treated with 1.3 mg/m² bortezomib intravenously twice weekly for 2 weeks, followed by 1 week rest, for up to 8 cycles. In patients with stable disease after 4 cycles or progressive disease after 2 cycles, oral dexamethasone on the day of and the day after each dose of bortezomib was added to the regimen. Of the patients, 35% achieved a complete or partial or remission or minimal response (responders); the median time to response was 1.3 months, and the median duration of response for all responders was 12 months, while for patients in complete or near-CR it was 15 months. Median survival among all patients was 16 months. The most frequent grade 3 adverse events were thrombocytopenia (28% of patients), fatigue (12%), peripheral neuropathy (12%), neutropenia (11%), anemia (8%), and vomiting (8%) (Richardson et al. 2003). In a parallel phase II trial (CREST trial), the patients with relapsed or refractory multiple myeloma were randomized to receive 1.0 mg/m² or 1.3 mg/m² bortezomib with a permission of dexamethasone in patients with progressive or stable disease after 2 or 4 cycles of single-agent therapy. The response rates (CR and PR) were 30% and 38% with 1.0 mg/m² and 1.3 mg/m² bortezomib, respectively. After addition of dexamethasone, the response rate for all patients rose to 37% and 50% in the 1.0 and 1.3 mg/m² bortezomib cohorts, which suggested some synergy between the both drugs (Jagannath et al. 2004). The first phase III trial (APEX trial) was performed in patients with multiple myeloma relapse after 1–3 prior therapies. The 669 patients were randomized in two cohorts, of which one group received 1.3 mg/m² bortezomib twice weekly for 2 weeks all 3 weeks, and the second cohort received dexamethasone 40 mg on days 1–4, 9–12, and 17–20. The results revealed a highly significant difference in response rates (38% vs 18%), benefit in time to progression, and overall survival for patients who received bortezomib. Furthermore, the rate of grade 3 or higher grade infections was lower in the bortezomib cohort (6.7% vs 11%) (Richardson et al. 2005). Bortezomib has now been approved by the FDA and EMEA for patients with relapsed or refractory multiple myeloma.

Recently, trials in upfront treatment of multiple myeloma patients were performed. A study evaluated bortezomib therapy alone or in combination with dexamethasone for previously untreated symptomatic multiple myeloma (Jagannath et al. 2005). The response rate (CR+PR) was 88%, with undetectable paraprotein (CR) in 6%, and detectable by immunofixation only in 19% (near CR). Bortezomib treatment did not affect stem cell mobilization. In another study, synergy between bortezomib and doxorubicin was utilized [PAD regimen, a combination of PS-341 (bortezomib), adriamycin and dexamethasone] (Oakervee et al. 2005). Patients with untreated multiple myeloma received four 21-day cycles of PAD, comprising a conventional bortezomib

dose and a schedule of 1.3 mg/m² on days 1, 4, 8, and 11, along with dexamethasone 40 mg on days 1–4, 8–11, and 15–18 during cycle 1 and days 1–4 during cycles 2–4. During days 1–4, patients also received 0, 4.5, or 9 mg/m² of doxorubicin at dose levels 1, 2, and 3, respectively. After PAD induction alone, 20 of 21 patients (95%) achieved at least a PR, including CR in 5 patients (24%).

14.6 Phase I and II Trials in Leukemia and Lymphoma

To investigate maximum tolerated dose (MTD) and dose-limiting toxicities (DLTs) in patients with acute leukemias refractory to or relapsing after prior therapies, 15 patients were treated with escalated doses of bortezomib ranging from 0.75 and 1.5 mg/m². The DLT included orthostatic hypotension, nausea, diarrhea, and fluid retention, all at 1.5 mg/m² bortezomib, suggesting the MDT in patients with acute leukemia at 1.25 mg/m² (Cortes et al. 2004). In a phase II trial for patients with relapsed or refractory lymphoma, 41% of the patients with mantle cell lymphoma could reach a PR or CR (Goy et al. 2005). The response rate for other types of NHL, including both high- and low-grade NHL, was 19%. Most grade 3 toxicities included thrombocytopenia (47%). For patients with indolent NHL, a further phase II trial was conducted (O'Connor et al. 2005). The overall response rate was 58%, with best responses among patients with follicular lymphoma and mantle cell lymphoma. The response duration was between 3 and 24 months (and more months), suggesting significant single-agent activity. The most common grade 3 toxicities were lymphopenia and thrombocytopenia. Only one grade 4 toxicity (hyponatremia) was observed (O'Connor et al. 2005). A further trial examined the efficacy of bortezomib in combination with pegylated liposomal doxorubicin in 42 patients with advanced hematological malignancies. Grade 3 and 4 toxicities were seen in 10% of patients and included, beyond hematological and infectious complications, fatigue and peripheral neuropathy. Responses were seen in patients with multiple myeloma, NHL, and acute myeloid leukemia. The recommended doses for further studies were fixed at 1.3 mg/m² bortezomib twice weekly for 2 weeks, and 30 mg/m² pegylated liposomal doxorubicin on day 4 of a 3-week cycle (Orlowski et al. 2005). A combination treatment within the context of a phase I trial with escalating doses of bortezomib (0.7–1.3 mg/m²) and fludarabine in patients with heavily pretreated, indolent NHL is still ongoing (Koc et al. 2005).

14.7 Solid Tumors

In humans, several phase I and II studies have been conducted. In the first phase I trial, 43 heavily pretreated patients with advanced solid tumors were treated with 0.13–1.56 mg/m² bortezomib. The DLTs were diarrhea and sensory neurotoxicity, without any dose-limiting hematological toxicity. Among all patients, there was one partial response in a patient with non-small cell lung cancer (NSCLC). Three other patients, one with renal cell carcinoma, one with nasopharyngeal carcinoma, and one with malignant melanoma, had stable disease as the best response (Aghajanian et al. 2002). In a further phase I trial, some activities were observed against prostate carcinoma (Papandreou et al. 2004). Phase II trials concerning metastatic neuroendocrine tumors (Shah et al. 2004), renal cell carcinoma (Davis et al. 2004; Kondagunta et al. 2004), and metastatic sarcomas (Maki et al. 2005) found only a little or no evidence for clinically significant activity of bortezomib in single-agent use. Therefore, combination therapies were recommended. Currently, an ongoing phase II trial is examining the efficacy of combination therapy of gemcitabine, cisplatin, and bortezomib in advanced solid tumor patients in first-line treatment. In the first 10 patients evaluated, there were 4 partial responses (2 bladder, 2 NSCLC) (Voortman et al. 2005).

14.8 Conclusions

Proteasome inhibitors represent a novel class of antitumor agents. Bortezomib, the first proteasome inhibitor introduced in clinical use, abrogates chemotherapy-induced resistance mechanisms and may act synergistically with a broad range of cytostatic agents. Results of

clinical studies revealed high activity in multiple myeloma and certain subtypes of NHL, e.g., mantle cell lymphoma and follicular lymphoma. The response rates of single-agent use in solid tumors are much lower, and appropriate combination chemotherapy should be evaluated in future. Novel proteasome inhibitors are being developed.

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15 Individualized Target Therapy of Malignant Lymphomas: An Outlook

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Recent Results in Cancer Research, Vol. 176

Abstract

Hodgkin and non-Hodgkin lymphomas represent clonal malignant expansions of B or T cells that are at various stages of maturation. As our understanding of the immunophenotype, cytogenetics and molecular biology of the lymphomas broadened, treatment of these neoplasms is evolving to include targeted therapies directed against tumour-characteristic molecules and specific signalling pathways critical to lymphomagenesis.

15.1 Introduction

Despite the great progress that has been made over the last few decades in the treatment of lymphoma, the prognosis for patients with relapsed disease, and with particular lymphoma entities such as mantle cell lymphoma, remains quite poor. The incredibly rapid pace of growth in our understanding of the molecular basis of the various lymphoma entities and sub-types is beginning to afford exciting new opportunities to both risk-stratify patients, and to identify potentially novel "drugable" targets.

The ideal target can be defined as a molecule

1. that is crucial to the malignant phenotype and is not expressed in substantial amounts in vital organs and tissues
2. that can be measured/visualized reproducibly in tumour samples
3. that correlates with clinical outcome
4. in which interruption, inhibition or interference leads to a clinical response in a substantial proportion of patients whose tumours express this target

Taking this into account, several molecules appear to be promising for the design of future individualized therapeutic strategies for malignant lymphomas, which are addressed in the following outlook.

15.2 CD30 and Classical Hodgkin Lymphoma

The recent clinical and commercial success of rituximab has created great interest in the identification for specific immunotherapeutics for malignant lymphomas. By targeting the CD20 surface receptor common to many B cell lymphomas, rituximab, a chimeric monoclonal IgG1 antibody, induces apoptosis, antibody-dependent cell cytotoxicity and complement-mediated cytotoxicity (Cartron et al. 2004; Reff et al. 1994).

Another similar molecule which is a promising target for immunotherapy is the CD30 antigen. CD30 is a 120-kDa transmembrane glycoprotein belonging to the tumour necrosis factor (TNF)-receptor superfamily (Durkop et al. 1992). It is highly expressed in the tumour cells of classical Hodgkin lymphoma (cHL) (Fig. 15.1) and anaplastic large cell lymphoma (ALCL), while it is physiologically only present in a small subset of activated B and T cells (Stein et al. 1985). Certainly, cHL has become a curable disease after the application of novel combinations of chemotherapeutic agents and extended-field radiation (DeVita and Hubbard 1993; Diehl et al. 1998). However, less than 30% of the patients who relapse after first-line treatment remain disease-free after intensified treatment (Carella 1992). This is probably due to the proliferation of therapy-re-

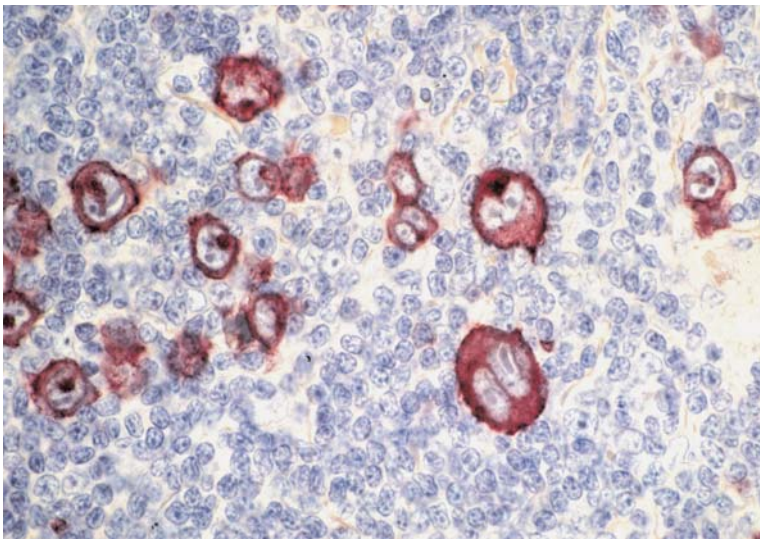

Fig.15. 1 The anti-CD30 antibody selectively highlights the neoplastic HRS cells in a case of classical Hodgkin lymphoma [immunohistochemistry employed the alkaline phosphatase-anti alkaline phosphatase (APAAP) technique and FastRed as chromogen]

sistant residual tumour cells (Schnell et al. 1996). Therefore the development of new therapeutic approaches is mandatory. In this respect, efforts to treat cHL with murine anti-CD30 monoclonal antibodies or anti-CD30 toxin conjugates have been successful in mouse models, inducing long-lasting remissions in a substantial number of animals (Schnell et al. 2003). To avoid the limitations of such therapy, a full human monoclonal antibody (5F11, also known as MDX-060) has been developed and has already demonstrated cytotoxic effects on Hodgkin and ALCL cells in vitro and in vivo (Borchmann et al. 2003).

15.3 The ZAP-70 Molecule and B Chronic Lymphocytic Leukaemia

The 70-kDa T cell receptor ζ-chain associated tyrosine kinase (ZAP-70) plays an important role in proximal T-cell receptor (TCR) signalling (Chan et al. 1992). Activation of TCR through antigen binding results in tyrosine phosphorylation of src-family tyrosine kinases such as Lck that in turn phosphorylate ZAP-70 with resulting activation of its tyrosine kinase activity (Zhang et al. 1998). The following phosphorylation of linker of activated T cells (LAT) serves to amplify the TCR signal and is critical in triggering downstream signalling events that culminate in transcriptional production of genes that regulate cell function, proliferation and death.

ZAP-70 is normally expressed in T-cells and natural killer (NK) cells and was initially thought to be exclusive in these lineages. Recently it has been shown that ZAP-70 is involved in the maturation of pro-B to pre-B cells within the bone marrow compartment (Schweighoffer et al. 2003). Interest in ZAP-70 has grown since it has been shown, through gene expression profiling, that it is expressed in a subset of cases of B-cell chronic lymphocytic leukaemia (B-CLL)/small lymphocytic lymphoma and identifies cases with unmutated immunoglobulin heavy chain genes (IgH) and a relatively poor prognosis (Crespo et al. 2003; Wiestner et al. 2003). Despite the fact that discordant expression is observed in approximately 10% of the cases, ZAP-70 is currently regarded as the best surrogate marker for mutational status of B-CLL.

ZAP-70 expression can be easily assessed by real time PCR, FACS analysis and immunohistology in bioptic specimens (Fig.15. 2), which makes it suitable for routine practice. Due to the facts that (1) ZAP-70 is part of the signalling cascade from the B cell receptor (BCR) complex to

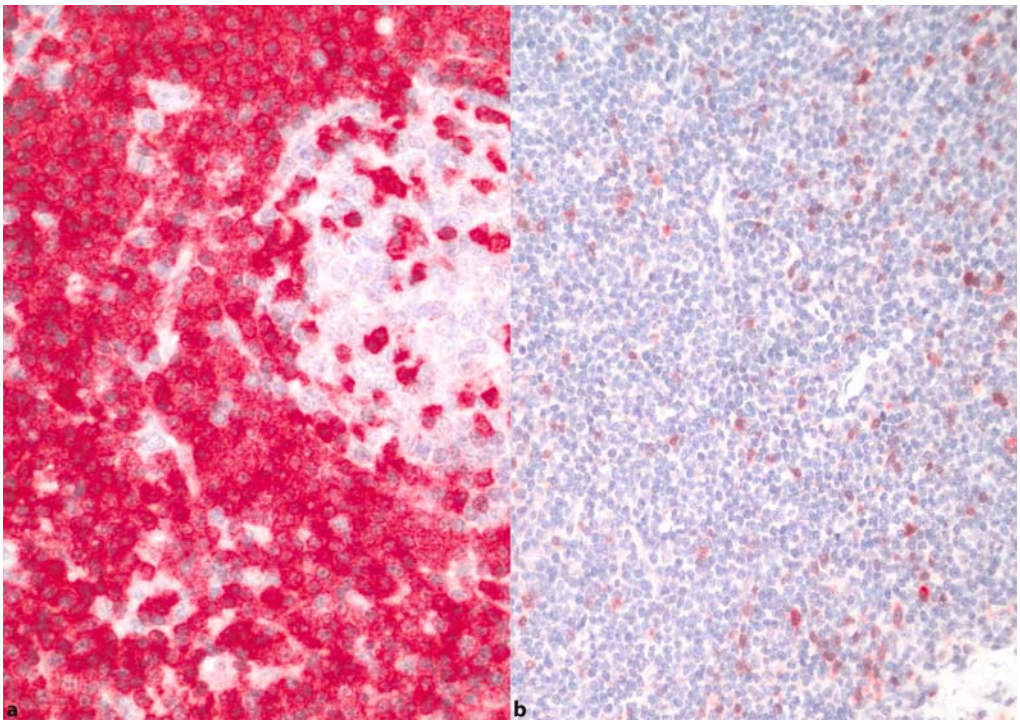

Fig. 15.2 a, b Immunohistochemical demonstration of ZAP-70 expression in two cases of B cell chronic lymphocytic leukaemia: **a** the neoplastic cells strongly express ZAP-70. The few negative cells correspond to a residual germinal centre; **b** the neoplastic cells are ZAP-70-negative. The single positive cells represent T cells normally expressing this molecule. (Immunohistochemical detection was performed with an anti-ZAP-70 monoclonal antibody using the APAAP technique)

the phosphatidylinositol 3-kinase (PI3-kinase) pathway (Chen et al. 2005) and (2) ZAP-70 is highly expressed in the neoplastic cells of unmutated B-CLL, it appears that this molecule constitutes a possible target for specific therapy in the positive B-CLL cases. ZAP-70 inhibitors such as piceatannol derivatives are already available (Soede et al. 1998).

15.4 Fusion Proteins Due to Chromosomal Translocations

15.4.1 BCL-2 and B Cell Non-Hodgkin Lymphoma

Bcl-2 is an anti-apoptotic member of a large family of genes involved in the regulation of programmed cell death (Reed 1997; Yang and Korsmeyer 1996). Pro-apoptotic (BAX and BCL-Xs) and anti-apoptotic (BCL-2 and BCL-X_L) molecules reside within the inner mitochondrial membrane and can homo- and heterodimerize upon appropriate stimulus. These interactions control the release of substances such as cytochrome C from the mitochondria into the cytosol. Cytochrome C by its turn is a central molecule in the initiation of the apoptotic process by caspase activation.

The transposition of the *bcl-2* gene to the IgH chain promoter region in the t(14;18) translocation is associated with more than 90% of follicular lymphomas at diagnosis (Fig. 15.3) and 10% of diffuse large B cell lymphomas (DLBCL), making it the most frequent event identified in non-Hodgkin lymphoma (Ong and Le Beau 1998). In

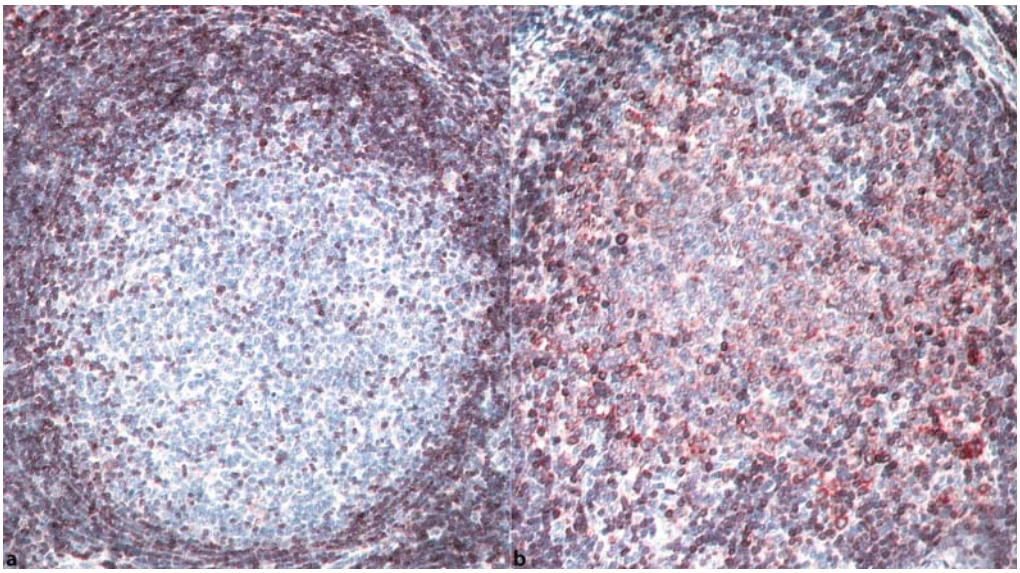

Fig. 15.3 a, b Immunohistochemical detection of BCL-2 protein. **a** The cells within a reactive germinal centre are BCL-2 negative; **b** in a follicular lymphoma with the t(14;18) translocation the germinal centre B cells strongly express BCL-2. (Immunohistochemical detection was performed using an anti-BCL-2 monoclonal antibody and the APAAP technique)

addition, 50% of DLBCL over-express the BCL-2 protein through other mechanisms and seem to be associated with a poorer prognosis (Gascoyne et al. 1997). Among the tumours of lymphoid and haematopoietic tissues, BCL-2 expression at high levels also occurs in mantle cell lymphoma, multiple myeloma, and acute myelogenous leukaemia. This over-abundance of BCL-2 can prevent or retard activation of the apoptotic machinery and allow survival under conditions that might otherwise be lethal to a cell (Reed 1997).

The obvious conclusion is that BCL-2 expression at high levels and by whatever means confers a fundamental advantage to malignant cells and that disruption of its expression, for example using antisense oligonucleotides, should have therapeutic potential.

15.4.2 Cyclin D1 and Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a distinct lymphoma entity. Clinically, it exhibits features of an indolent NHL by being an incurable disease with a median survival of only 2.5–3 years and can also show an aggressive behaviour (Argatoff et al. 1997). At the molecular level, MCL is characterized by over-expression of the G1 cyclin, cyclin D1, generally in association with the chromosomal translocation t(11;14)(q13;q32) (Rimokh et al. 1993). This translocation juxtaposes the enhancer element of the IgH region on chromosome 14 to the *bcl-1/PRAD1* proto-oncogene encoding the cyclin D1 protein on chromosome 11. As a consequence of the cyclin D1 over-expression (Fig. 15.4), there is excess progression of cells from the G1 through the S phase of the cell cycle. A similar t(11;14) translocation has been found also in a proportion of plasmacytomas. Therefore cyclin D1 appears to be a suitable target for individualized therapy. It has to be considered, however, in this setting that cyclin D1 is also present in epithelial cells, a fact that may cause some adverse effects in such therapeutic approaches.

15.5 The Nuclear Factor-κB Transcription Factors

The nuclear factor-κB proteins are a small group of closely related transcription factors which

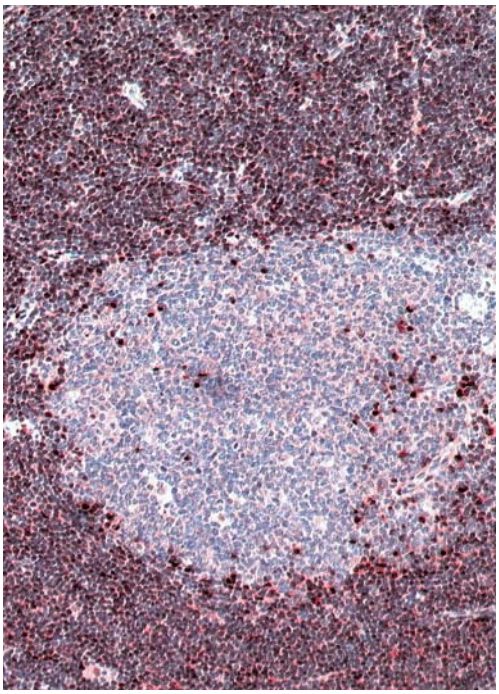

Fig. 15.4 In a mantle cell lymphoma with the t(11;14) translocation, the neoplastic cells show a strong nuclear expression of the cyclin D1 protein. (Immunohistochemical detection was performed with an anti-cyclin D1 monoclonal antibody and the APAAP technique)

consist of five members: Rel (also known as c-Rel), RelA (also known as p65 and NF-κB3), RelB, NF-κB1 (also known as p50) and NF-κB2 (also known as p52). All five proteins have a Rel homology domain, which serves as their dimerization, DNA binding and principal regulatory domain (Ghosh et al. 1998). This domain contains a nuclear localization sequence, which is rendered inactive in non-stimulated cells though binding of specific NF-κB inhibitors, known as IκB proteins. Activation of most forms of NF-κB, especially the most common form—the p50-p65 dimer—depends on phosphorylation-induced ubiquitination of the IκB proteins (Karin and Ben Neriah 2000). This sequential modification depends on two protein complexes: the IκB kinase (IKK) complex and the E3 ^{IκB}ubiquitin ligase complex. Once poly-ubiquitinated, the IκBs undergo rapid degradation through the 26S proteasome and the liberated NF-κB dimers translocate to the nucleus, where they participate in transcriptional activation of specific target genes (Ghosh and Karin 2002). Thus, the members of the NF-κB family regulate immune, inflammatory and acute phase responses and activate an anti-apoptotic gene expression programme. As signalling pathways that govern proliferation and survival are important for tumour development, NF-κB has an intrinsic oncogenic potential.

15.5.1 NF-κB and Classical Hodgkin Lymphoma

Most evidence for the role of NF-κB in human malignancies came from an analysis of cHL. Classical HL is characterized by the fact that the neoplastic cells [Hodgkin and Reed-Sternberg (HRS) cells] represent only a small fraction (2%) of the neoplastic lesions that are populated by eosinophils, neutrophils, T and B cells, plasma cells, histiocytes and others. These reactive cells are attracted by cytokines and chemokines abundantly produced by the HRS cells. While the cellular origin of the HRS cells is not homogeneous (>98% are derived from B cells and a small minority, >2%, from T cells), it has been shown that they do not resemble their normal cellular counterparts morphologically or immunophenotypically. The antigens specific or characteristic for B or T cells are more or less completely missing in the majority of cases, with the HRS cells acquir-

ing a number of antigens (CD30, CD15, CD70, TARC and IRF4) which are not usually expressed by normal B or T cells. Another intriguing characteristic of B-type HRS cells is their consistent inability to transcribe immunoglobulin despite the presence of functional immunoglobulin (Ig) gene rearrangements (Marafioti et al. 2000). Since normal B cells die of apoptosis if they lose their capacity to express Ig, the HRS cells' inability for Ig transcription points to a deregulation of the apoptotic pathway in these cells.

As stated above, cHL is the first tumour of the haematolymphoid system in which an aberrant constitutive NF-κB activation has been described (Bargou et al. 1996). More recent data indicate that the major cause of constitutive NF-κB activity is a persistently activated IKK complex (Krappmann et al. 1999). To clarify its role and to precisely identify the target genes of NF-κB in HRS cells, cHL cell lines with suppressed and unsuppressed NF-κB activity were subjected to gene expression profiling (Hinz et al. 2002). The suppression of NF-κB proved to have a significant effect as it reduced the growth and increased the rate of apoptosis in cHL cell lines. Gene expression profiling revealed that the following molecules that directly or indirectly inhibit apoptosis are expressed in dependence of NF-κB: Tumour necrosis factor receptor-associated receptor (TRAF)1, BCL-XL and cIAP2. Immunohistological analysis employing antibodies against TRAF1 revealed a selective labelling of the HRS cells (Fig. 15.5A) within the tumour lesions (Durkop et al. 1999). Thus, TRAF1 constitutes an interesting molecule for cHL target therapy.

Among the NF-κB-dependent molecules identified in cHL cell lines is CD95, which triggers apoptosis rather than protects against it. To clarify this paradox, the CD95 gene was initially analysed for any loss-of-function mutations; however, no deleterious mutations were

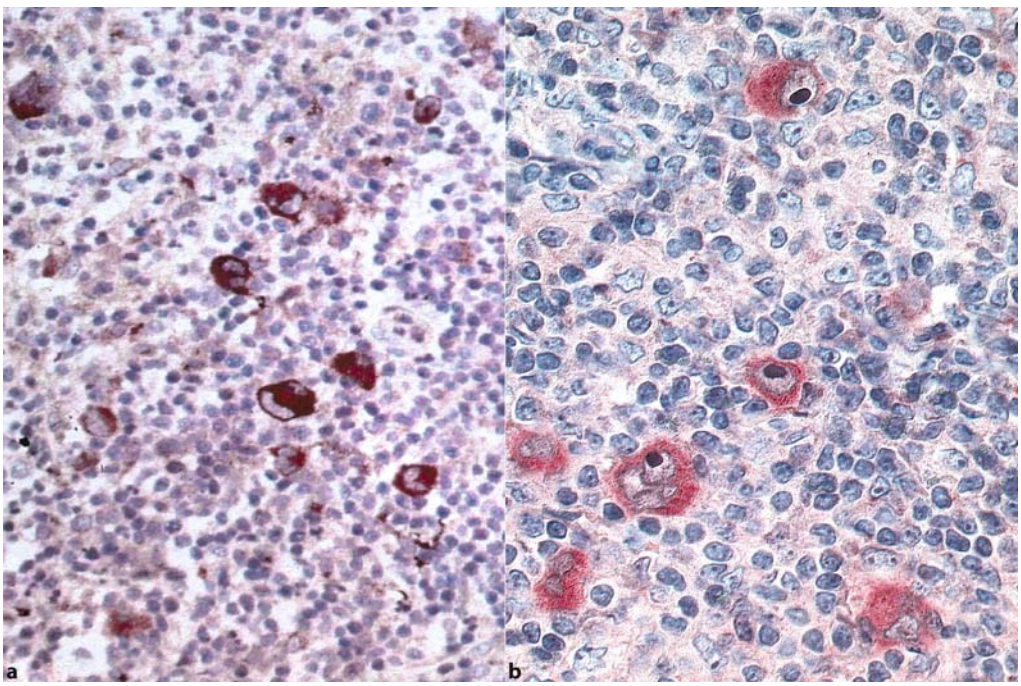

Fig. 15.5 a, b Expression of NFκB-target genes in classical Hodgkin lymphoma. **a** The neoplastic Hodgkin and Reed-Sternberg (HRS) cells selectively express TRAF1; **b** the HRS cells strongly and selectively express c-FLIP. (Immunohistochemical detection was performed with the corresponding monoclonal antibodies and the APAAP technique)

found (Muschen et al. 2000). This prompted the question as to whether CD95 is activated in HRS cells. This indeed proved to be the case, as revealed by the detection of the death-inducing signalling complex (DISC) in these cells. This complex, however, proved not only to contain the FAS-associated death domain-containing proteins (FADD) caspase 8 and caspase 10, but also the cellular FADD-like IL1B-converting enzyme inhibitory proteins (cFLIP) (Mathas et al. 2004). Expression studies showed that cFLIP is over-expressed in cultured and primary HRS cells (Fig. 15.5B). Thus, cFLIP seems to be a candidate for blockade of the death-inducing effect of CD95. Due to the facts that (1) suppression experiments could demonstrate that cFLIP exerts a very strong protective effect against apoptosis in HRS cells and (2) that cFLIP is not over-expressed by other cells in the lymphoid tissues investigated, it appears to be another interesting molecule for targeted therapy of cHL.

A further interesting NF-κB target gene is CCR7 encoding for a chemokine receptor. Studies of cultured and primary HRS identified an over-expression of CCR7 (Hopken et al. 2002). This is of special interest, as in CCR7-deficient mice the T cells do not home to lymph nodes but to the red pulp of the spleen instead. Thus, CCR7 might not only contribute to distinct dissemination of neoplastic cells into lymphoid organs, but also seems to have a more general role in tumour cell migration, as shown in the case of breast cancer metastasis.

In summary, there are several indications pointing to a fundamental importance for NF-κB in the pathogenesis of cHL as it controls a complex network of genes, which promotes the specific architecture, supports proliferation and migration, and confers resistance to apoptosis. Thus, pharmacological manipulation of the NF-κB system or of selected target genes might have a therapeutic potential for cHL (Karin et al. 2004).

15.5.2 NF-κB and Diffuse Large B Cell Lymphoma

DLBCL is the most common lymphoid malignancy in adults, accounting for nearly 40% of all non-Hodgkin's lymphomas. DLBCL is so named because the malignant lymphocytes diffusely efface the normal architecture of the lymph node or extranodal site. The cells are large transformed lymphocytes, and they have been further divided into morphologic variants: centroblastic, immunoblastic, T cell/histiocyte-rich and anaplastic.

Though DLBCL has proved to be one of he most chemotherapy-responsive human malignancies, many newly diagnosed patients will not be cured with conventional anthracycline-based chemotherapy. The variability in response to therapy suggests underlying heterogeneity in a disease that is largely treated with a homogeneous approach. Recent developments in biology are now changing the face of DLBCL. Until recently, analyses of biological heterogeneity have focussed only on individual genes with special emphasis on those associated with treatment outcome, known functions in other malignancies, and in normal lymphocyte development. The understanding of the bases of molecular and clinical heterogeneity in DLBCL could, however, be only achieved by the analysis of gene expression profiling, which delivered molecular signatures of tumours. This approach was able to stratify DLBCL patients in at least two large subgroups that had markedly different clinical outcome after multi-agent chemotherapy: the "germinal centre B cell-like (GCB)" and the "activated B cell-like (ABC)" (Alizadeh et al. 2000). The lymphomas of the subgroup designated as ABC expressed genes that are characteristically activated in blood B cells by signalling through the BCR. One of the signalling pathways prominently engaged after BCR stimulation is the NF-κB pathway, and ABC DLBCLs frequently expressed NF-κB target genes highly. In contrast, GCB DLBCLs generally had low expression of the NF-κB target genes, as did normal germinal centre B cells (Davis et al. 2001).

Thus, it can be proposed that the known ability of NF-κB to inhibit responses to chemotherapeutic agents (Baldwin 2001) may contribute to the refractory clinical behaviour of ABC DLBCLs after multi-agent chemotherapy. As it has been shown that inhibition of NF-κB can co-operate with chemotherapy to kill tumour cells, an approach combining chemotherapy with pharmacological inhibition of the NF-κB pathway might be promising in the treatment of DLBCLs with an ABC gene expression profile.

15.6 Oncogenic Tyrosine Kinases

The fusion protein oncogenic tyrosine kinases which are generated by non-reciprocal chromosomal translocations have been the focus of intensive investigation as they provide clearly defined model systems for analysis of the molecular processes leading to transformation. In many ways *BCR-ABL* has provided the paradigmatic example for such studies which have recently also included other fusion protein oncogenic tyrosine kinases, in particular the nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) that is associated with a particular lymphoma entity, the anaplastic large cell lymphoma (ALCL).

The first report of CD30 (Ki-1)-positive ALCL was published by Stein et al. (1985). This lymphoma was described as a large-cell non-Hodgkin lymphoma (NHL) with infiltration of the lymph node sinus and anaplastic morphology of the tumour cells. This particular growth pattern and the variable morphology of the ALCL tumour cells has led, historically, to the frequent misdiagnosis of this tumour as carcinoma, melanoma, malignant histiocytosis or other malignancies. ALCL has been found to arise as a primary tumour (primary systemic or primary cutaneous ALCL) or from a preceding condition such as lymphomatoid papulosis (Skinnider et al. 1999). Although relatively infrequent, accounting for only 5% of all human NHLs, ALCL comprises as many as 30%–40% of paediatric large cell lymphomas (Kadin and Morris 1998; Sandlund et al. 1994). An association between ALCL and the t(2;5)(p23;q35) chromosomal translocation was reported in the late 1980s (Kaneko et al. 1989; Le Beau et al. 1989; Mason et al. 1990; Rimokh et al. 1989). Two independent groups later identified the genes involved in this translocation as those encoding the cell cycle-regulated nucleolar protein nucleophosmin (NPM) at 5q35 and the novel kinase, named anaplastic lymphoma kinase (ALK) after the disease with which it was first associated at 2p23 (Morris et al. 1994; Shiota et al. 1994). The fusion protein NPM-ALK consists of the N-terminal region of NPM fused to the entire intracellular portion of ALK. The resultant protein is a hyperactive tyrosine kinase.

The studies of ALCL experienced rapid progress not only using RT-PCR and fluorescence in situ hybridization but also immunohistology. Since normal haematopoietic cells do not express detectable levels of the full-length ALK protein, the advent of antibody reagents specific for ALK (Fig. 15.6) has revolutionized the diagnosis of ALCL as 60%–80% of ALCLs have been found to be ALK-positive (Lamant et al. 1996; Pulford et al. 1997; Shiota et al. 1995). These ALK-posi-

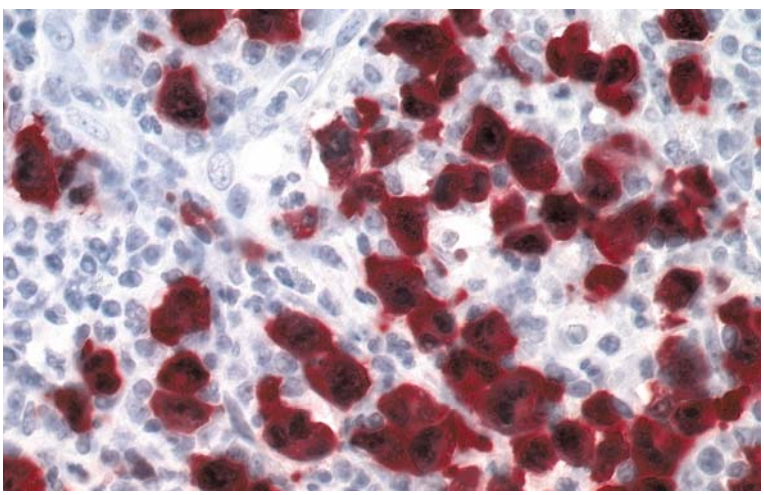

Fig. 15.6 Demonstration of the NPM-ALK fusion protein in the nuclei and cytoplasm of an anaplastic large cell lymphoma. This immunohistochemical finding corresponds to the presence of a t(2;5) translocation. (APPAP technique)

tive lymphomas exhibit a cytotoxic T cell or null (that is, lacking of both T and B cell markers) phenotype (Stein et al. 2000). The presence of ALK-positive cells in all of the described histological subtypes of ALCL suggested that these subtypes are simply morphological variants of the same disease, ultimately designated as "ALK-positive ALCL" and are included in the recent WHO classification of malignant lymphomas. These tumours tend to occur in children and young adults, with a slight male predominance.

At least 10 ALK fusion partners besides NPM have been described; with the exception of moesin-ALK and non-muscle myosin heavy chain; all of the chimeric proteins also contain the complete intracytoplasmic segment including the tyrosine kinase catalytic domain. Knowledge regarding the target substrate molecules of ALK and its fusion proteins are therefore invaluable in devising methods of treatment for the patients with tumours expressing the ALK proteins. Activation of the NPM-ALK tyrosine kinase by NPM dimerization causes autophosphorylation at multiple tyrosine residues and the consequent recruitment of a "signalosome" that couples the fusion protein to multiple downstream signalling pathways. Investigations based largely on cell line data suggest that the NPM-ALK signalosome stimulates pathways mediating the induction of growth-factor-independent proliferation, cellular transformation, protection from apoptosis and resistance to therapeutic drugs and γ-irradiation (Pulford et al. 2004).

The vast majority of studies have demonstrated ALK expression in ALCL to be a marker of favourable prognosis. Such cases have an overall 5-year survival, following CHOP-based chemotherapy, of 71%–80% compared to only 15%–46% for ALK-negative ALCL patients (Cataldo et al. 1999; Falini et al. 1999; Gascoyne et al. 1999; Shiota et al. 1995; Shiota and Mori 1996). Despite their typically favourable prognosis, the outcome of a substantial number (approximately 30%) of patients with ALK-positive ALCL is poor. The majority of them either fail to enter into remission or relapse within few months from the start of treatment. Also late and multiple relapses are not uncommon (Gascoyne et al. 1999). It has to be underlined, however, that up to now there is no one definite optimal therapeutic regimen for ALK-positive ALCL. The current approach includes the use of combination chemotherapy protocols developed for T cell lymphoblastic lymphomas/leukaemias and high grade B cell NHL. Therefore other therapeutic approaches should be considered. Certainly, tyrosine kinase inhibitors have been found to be of great value in the treatment of tumours in which oncogenic tyrosine kinases play a role. For example STI571 (GlivecTM, Novartis Pharmaceuticals, Basel, Switzerland) has been successfully used in the treatment of chronic myeloid leukaemia containing the fusion tyrosine kinase BCR-ABL. Thus far, however, no such role has been found for this compound in ALK-positive ALCL. Anecdotal evidence does suggest the likely effectiveness of another ATP-competitive small molecule inhibitor based on a report of a phase I study of the compound UCN-01. Other studies have also demonstrated the efficacy of another tyrosine kinase inhibitor, herbimycin A, in inducing caspase-dependent apoptosis in ALK-positive cell lines (Turturro et al. 2002). It is expected that the development of ALK-selective inhibitors may ultimately provide a more optimal therapeutic approach for ALK-positive ALCLs.

15.7 Final Remarks

The introduction of target therapy will have major implications for the haematopathologist. Its role will expand to include not only the precise lymphoma diagnosis but also the selection of patients who are going to receive the appropriate target therapy. The haematopathology laboratories will have to adapt to the situation and routinely employ techniques able to identify the appropriate target molecules in the tumour tissue of the individual patient. Certainly, one of the most important techniques for this purpose is immunohistochemistry. It is expected that the number of antibodies that a laboratory routinely uses will significantly increase as more lymphoma-characteristic target pathways will be identified in the future. Efforts have to be undertaken to achieve standardized immunohistochemical procedures among different laboratories, as the pathologist will not only have the task of identifying positive tumour cells, but also making quantitative estimations on the expression levels of various molecules. The routine use of fluorescent in situ

hybridization (FISH) will also become essential for the identification of specific chromosomal translocations targeted by specific antibodies or drugs. The emerging chromogenic in situ hybridization (CISH) approach also has to be evaluated to learn whether it can be used to detect translocations as efficiently as FISH. In addition, gene expression profiling and other techniques investigating the tumour genome such as micro-RNA analysis or comparative genomic hybridization will move from a pure research to a routine tool of the haematopathologist.

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16 Antiangiogenesis, Anti-VEGF(R) and Outlook

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Abstract

Tumor angiogenesis and antiangiogenesis has moved from an exotic research topic into clinical practice defining a new and promising avenue of targeted cancer therapy. Starting with a historical perspective on tumor angiogenesis, this review provides the basic concepts and classification of angiogenesis inhibition. Focusing primarily on vascular endothelial growth factor (VEGF), the biological activity and regulation of the gene expression of VEGF, its isoforms, and the receptors are summarized. Furthermore, naturally occurring modulators are listed, an overview on angiogenesis inhibitors in clinical trails is provided, and finally a brief outlook is given on this fascinating and rapidly evolving field of oncology.

16.1 History, Basic Concepts and Classification of Angiogenesis Inhibitors

Antiangiogenesis as a potential therapy option for tumors dates back to the works of Judah Folkman et al. who, in 1971, isolated for the first time a tumor factor for which they could prove an angiogenetic effect. Together with the naming "TAF" (tumor angiogenesis factor), the authors hypothesized, "It is suggested that blockade of this factor (inhibition of angiogenesis) might arrest solid tumors at a tiny diameter of a few millimeters" (Folkman et al. 1971a). TAF could be characterized biochemically only rudimentarily in those days and showed both a RNA- and a protein component. Nevertheless, Folkman pursued his vision and in the same year he published a paper about the potential possibilities of antiangiogenesis in tumor therapy (Folkman 1971b).

The particular characterization of angiogenesis factors was extremely difficult. On the one hand, suitable in vivo and in vitro procedures like isolation and cultivation of endothelial cells had to be established to analyze and quantify the activity of the factors. On the other hand, knowledge about the individual steps of angiogenesis such as degradation of the basal membrane, attraction, locomotion, and proliferation of endothelial cells was inadequate. Isolation of basic fibroblast growth factor (bFGF) only succeeded in 1979. In 1983, great progress was made in filtration, which led to the knowledge that several endothelial cell mitogens showed a binding affinity to heparin. In 1985, the amino acid sequences of bFGF and acid FGF (aFGF) were determined, two prototypes of the heparin binding endothelial growth factors, as well as those of angiogenin, a polypeptide with ribonuclease activity that does not bind to heparin. Furthermore, it was recognized that the factors of angiogenesis were in no case tumor-specific, but are found in many normal tissues endogenously. However, they are subjected to severe control mechanisms and are activated only in exceptional circumstances, e.g., the menstrual cycle or wound healing (Folkman and Klagsbrun 1987).

In 1997, Folkman et al. again received much attention by isolating endostatin, a new angiogenesis inhibitor (O'Reilly et al. 1997). Administration of endostatin in a mouse model led to a shrinking of the tumor, which grew again only

after discontinuing the administration of the inhibitor. Notably, the repeated treatment did not lead to formation of resistance to endostatin (Boehm et al. 1997).

The fact that endothelial cells and not tumor cells are the aim of the therapy entails further advantages (Fayette et al. 2005):

- No resistances in contrast to tumor cells.
- Endothelial cells are genetically stable, diploid, and homogeneous.
- Spontaneous mutations occur rarely. Long therapies are possible.
- Endothelial cells in tumors divide 50–100 times more quickly than normal endothelial cells.
- Activated endothelial cells express markers other than resting endothelial cells; or the same but on a very low expression level.
- Angiogenesis is limited in adults; this results in lower side effects.
- Endothelial cells are easily accessible through blood circulation.
- Many tumors are dependent on a few micro vessels. The destruction of those amplifies the antitumor effect.

Tumor cells as an important source of angiogenetic factors can also be the aim of the therapeutical approach. One has to differentiate between direct and indirect angiogenetic inhibitors (Kerbel and Folkman 2002). While indirect inhibitors prevent tumor cells from the synthesis of angiogenetic proteins, direct ones have an impact on the endothelial cells and try to inhibit their reaction on multiple angiogenetic factors (Table 16.1).

Endostatin belongs to a group of endogenous angiogenetic inhibitors generated through proteolytic cleavage of molecules of the extracellular matrix (ECM). Endogenous inhibitors, which do not stem from matrix molecules or are built primarily in the ECM, can be distinguished from ECM-associated proteins and peptides (Nyberg et al. 2005).

Endostatin has not had the desired clinical success so far. Nevertheless, research has made strong progress since 1971, having identified inhibitors and stimulators of angiogenesis (Table 16.2). Vascular endothelial growth factor (VEGF) and its receptors play a central role in angiogenesis.

Table 16.1 Features of direct and indirect angiogenesis-inhibitors (according to Tandle et al. 2004)

Inhibitor type	Indirect	Direct
Target	Tumor cells	Tumor endothelial cells
Action	Inhibition of expression of angiogenetic growth factors and their receptors	Inhibition of endothelial cell proliferation, migration and tube formation; apoptosis induction
Resistance	Risk of resistance	Not prone to resistance
Examples	Iressa, antibodies against endothelial growth factors, small inhibitors of receptor-TKs, interferon-α	Endostatin, angiostatin, thrombospondin-1, tumstatin, bevacizumab (Avastin)

Table 16.2 Natural angiogenesis modulators (adapted from Fayette et al. 2005, Gupta and Zhang 2005, and Nyberg et al. 2005)

Stimulators	Inhibitors
Angiogenin	Angiostatin (plasminogen fragment)[b]
Angiopoietin-1	Antiangiogenic anti-thrombin III (AaAt)[b]
AC133	Angiopoietin 2

Table 16.2 *(continued)*

Stimulators	Inhibitors
Chemokine [d]	Arrestin (collagen IV fragment) [a]
Acid fibroblast growth factor (aFGF)	Canstatin (collagen IV fragment) [a]
Basic fibroblast growth factor (bFGF)	Cartilage-derived inhibitor (CDI)
FGF	CD59 complement fragment
Del-1	Dopamine
β-Estradiol	EFC-XV [b]
Ephrins	Endorepellin (perlecan domain V) [a]
Follistatin	Endostatin (collagen XVIII fragment) [a]
Hepatocyte growth factor (HGF)	Fibronectin (Anastellin) [a]
Id1/Id3	Fibulin [a]
Epidermal growth factor (EGF)	Heparinases
Granulocyte-colony stimulating factor (G-CSF)	Human chorion gonadotrophin (HCG)
Insulin-like growth factor (IGF)	Interferons (IFN) α, β, γ [b,c]
Integrin (αvβ3, αvβ5, α5β1)	Interferon inducible protein (IP-10)
Interleukin (IL8, IL1α, IL6)	Interleukins (IL4, IL12, IL18) [b,c]
Leptin	2-Methoxyestradiol [b]
Macrophage chemoattractant protein (MCP-1)	Maspin
Matrix metalloproteases (MMPs)	Kringle 2 (prothrombin) [b]
Nitric oxide synthase (NOS)	Kringle 5 (plasminogen fragment) [b]
Placental growth factor (PLGF)	Osteopontin fragment
Platelet-derived endothelial cell growth factor (PD-ECGF)	PEX (MMP-2 hemopexin-like domain) [b]
Platelet-derived growth factor (PDGF-BB)	Pigment epithelial cell-derived factor (PEDF) [b,c]
Pleiotrophin (PTN)	Placental ribonuclease inhibitor
Proliferin	Plasminogen activator inhibitor I (PAI I)
Transforming growth factor-α (TGF-α)	Platelet factor 4 (PF-4) [b,c]
Transforming growth factor-β (TGF-β)	Prolactin (16-kDa N-terminal fragment) [b]
Tumor necrosis factor-α (TNF-α)	Retinoic acid
Vascular endothelial growth factor (VEGF)	SPARC fragment
VE cadherin	Thrombospondin-1 and -2 (TSP-1, TSP-2) [a]
	TIMPs (tissue inhibitors of MMPs) [b]
	Troponin 1 [b]
	Tumstatin (collagen IV fragment) [a]
	Vasostatin (calreticulin fragment) [b]
	Vasculostatin (BAI1 fragment)

[a–c] Endogenous inhibitors: [a] with ECM origin, [b] without ECM origin, or growth factors or cytokines according to Nyberg et al. 2005

[d] Single chemokines may partially have an opposite effect (Gupta and Zhang 2005)

16.2 VEGF and VEGF Receptors

16.2.1 Biological Activities of VEGF

Angiogenesis is a highly complex and well-coordinated process that has many modulators from natural sources (as shown in Table 16.2). The process is arranged by the sequential activation of receptors and ligands. VEGF, also know as VEGF-A, and its receptors is of very high importance since the activation of the corresponding signal pathways is a critical and limiting step in physiological angiogenesis. VEGF belongs to a gene family that comprises placental growth factor (PLGF) as well as VEGF-B, VEGF-C, and VEGF-D. VEGF-A is the main regulator of blood vessel growth, while VEGF-C and VEGF-D regulate lymphatic angiogenesis (Fig. 16.1).

VEGF is a homodimeric glycoprotein of 45 kDa that also binds to heparin (Ferrara et al. 2003). It has mitotic effects on nonendothelial cells and supports growth of arterial, venous, and lymphatic endothelia (Matsumoto and Claesson-Welsh 2001). VEGF stimulates the surfactant production in alveolar type II cells (Compernolle et al. 2002). It acts as a survival factor for endothelial cells by inhibition of apoptosis through activation of the phosphatidylinositol (PI)-3 ki-

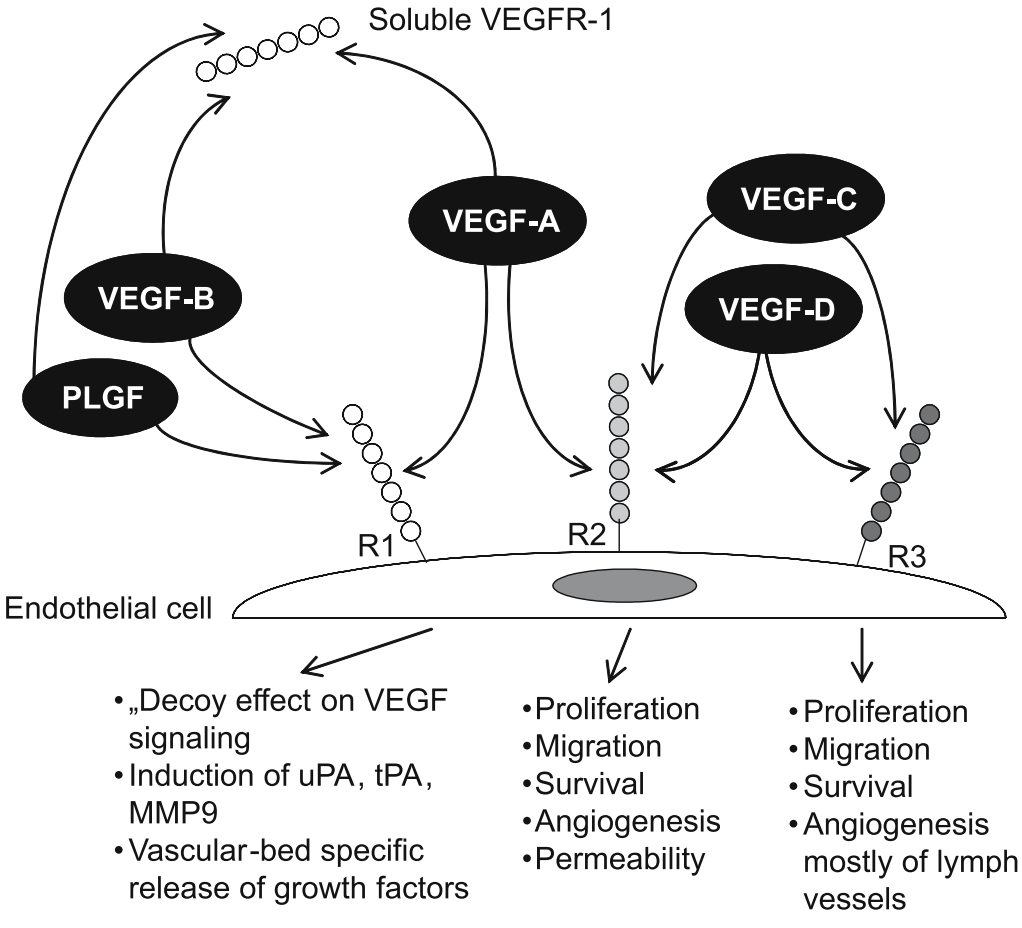

Fig. 16.1 Interaction and effect of VEGF receptors and their ligands. (Adapted from Ferrara 2004)

nase-Akt pathway and induction of Bcl-2 and A1 (Gerber et al. 1998). It is interesting to note that VEGF inhibition leads only to apoptosis in the blood vessels of neonatal mice but not in those of adult ones. A similar dependence on endothelial cells was found in newly built vessels in tumors but not in already existing ones (Benjamin et al. 1999).

VEGF also has an effect on bone marrow cells, as well as their descendants. The effect lies in the stimulation of monocyte chemotaxis, induction of ripe granulocyte and macrophage progenitor cell colonies, inhibition of the development of dendritic cells, elevation of the production of B cells, and the generation of unripe myelocytic cells. VEGF knockout mice do not survive. By selectively inducing a VEGF deficiency in hematopoietic stem cells in a mouse model, repopulation of bone marrow cells after lethal radiation was prevented (Ferrara et al. 2003).

Initially, VEGF was identified as a vascular permeability factor, hence the alternative naming VPF. Its ability to enhance the permeability of vessels is 50,000 times higher than that of histamine. This effect is shown by endothelial fenestration of the capillary bed. It is important for angiogenesis inasmuch as it leads to a deposit of fibrin in the ECM, which is the frame for the migration of endothelial cells (Ellis 2004). Moreover, it can lead to a penetration of further serum proteins, being important for blood coagulation as well as for angiogenesis (Table 16.2).

In vitro, a vasodilatory effect is shown by detachment of nitric oxide (NO) (Ku et al. 1993). Intravenous application in rats led to a transient tachykardie, hypotension, and reducing of cardiac efflux (Yang et al. 1996).

16.2.2 VEGF Isoforms

The VEGF gene consists of 8 exons that are separated by 7 introns of each other. The native protein is a heparin-binding homodimer glycoprotein of 45 kDa (Ferrara and Henzel 1989). Alternative splicing leads to formation of, in particular, four isoforms of different lengths ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$). $VEGF_{165}$ is dominant and has largely all the characteristics of the native protein. $VEGF_{189}$ and $VEGF_{206}$ are found sequestrated in the ECM, while $VEGF_{121}$ can diffuse freely and is the only isoform that does not bind to heparin. The absence of a heparin-binding domain results in the loss of mitogen stimulation of endothelial cells. Mice exclusively expressing $VEGF_{121}$ show a less pronounced lethality than those expressing the other isoforms. ECM-isoforms can be transferred to a biologically active fragment via plasmin-induced cleavage at the C-terminal end of the protein.

16.2.3 Regulation of VEGF Gene Expression

Low oxygen levels induce VEGF messenger RNA (mRNA) expression, hypoxia inducing factor (HIF-1) being the main mediator (Semenza 2002). Interestingly, it could be shown that the von Hippel-Lindau (VHL) tumor suppressor gene has a negative regulatory function in the transmission of the hypoxia inducible factor (HIF)-1-dependent hypoxia reaction concerning VEGF and other hypoxia-induced genes (Mole et al. 2001).

Many of the growth factors shown in Table 16.2 (e.g., EGF, TGF-α, TGF-β, keratinocyte growth factor, insulin-like growth factor 1, FGF, and PDGF) stimulate angiogenesis by an upregulation of VEGF. Presumably, local paracrine and autocrine spilling of these factors in the local vicinity of hypoxia regulates the expression of VEGF. Likewise, inflammatory cytokines such as IL-1 α and IL-6 induce VEGF expression in different cell types and indicate that VEGF is a mediator for angiogenesis and permeability in inflammatory processes. Furthermore, mutation or amplification of Ras leads to an upregulation of VEGF.

16.2.4 Placental Growth Factor

Placental growth factor (PLGF) is expressed mainly in placenta, heart, and lung. As a homodimer it binds to VEGF-R1 and Nrp-1. Heterodimers with VEGF exist, and combined administration of PLGF and VEGF amplify the angiogenetic effect of VEGF (Park et al. 1994). Knockout mice do not have a discernible phenotype but show a declined recovery after experi-

mental myocardial infarction and a retardation in the formation of collateral vasculature (Carmeliet et al. 2001).

16.2.5 Vascular Endothelial Growth Factor-B

VEGF-B (synonyms: VEGF-related factor/VRF) is a ligand for VEGF-R1 and Nrp-1. Like PLGF it can form heterodimers with VEGF. There are also isoforms built through alternative splicing. The role of VEGF-B in vivo is currently unknown. It is expressed in skeletal muscles, heart, and brown fat. Phenotypical changes were specified for the heart (smaller heart, declined recovery after experimental myocardial ischemia) and the lung in relation to development of pulmonary hypertonia after hypoxia (Tammela et al. 2005).

16.2.6 Vascular Endothelial Growth Factor-C

In contrast to VEGF-A and VEGF-B, different forms of VEGF-C and VEGF-D are not built by alternative splicing but by proteolytic processing. VEGF-C is subjected to an intracellular conversion by PC5 and PC7 as well as to an extracellular proteolysis by plasmin. The final 21-kDa homodimeric protein binds to VEGF-R2 and VEGF-R3 with high affinity. It induces mitogenesis, migration, and survival of endothelial cells and is mainly expressed in regions in which lymph vessels are built. Expression stops in most tissues and remains only high in lymph nodes. Knockout mice cannot develop lymph vessels and die of tissue edema at embryonal stage E15.5–E17.5. It is not relevant for the development of blood vessels (Karkkainen et al. 2004). Several clinical trials showed a positive correlation between VEGF-C expression and lymphatic invasion, lymphatic metastasis, and survival (He et al. 2004; Tammela et al. 2005).

16.2.7 VEGF-D

Human VEGF-D is a mitogen for endothelial cells in vitro and it is angiogenetic as well as lymphangiogenetic in vivo (Saharinen et al. 2004). It binds to VEGF-R2 and VEGF-R3. In contrast, the murine VEGF-D binds only to VEGF-R3 (Baldwin et al. 2001), pointing to a divergent role in mice. It is expressed mainly in human tissue, most strongly during embryonal development in lung and skin. A relationship to lymphogenic metastasis could be shown in an experimental intrathoracic lung cancer model together with VEGF-C (Achen et al. 2001). Furthermore, a prognostic value for lymph vessel invasion and survival in certain cancer types could be shown (He et al. 2004; Tammela et al. 2005).

16.2.8 VEGF Receptors

VEGF binds to two related tyrosine kinase (TK) receptors, VEGF-R1 and VEGF-R2. They both show seven immunoglobulin-like domains in their extracellular portions, which are represented in Fig. 1 by circles. Similar to the growth factors, VEGF-R1 and VEGF-R2 are capable of building heterodimers that deploy stronger signaling than VEGF-R1 and VEGF-R2 homodimers (Carmeliet et al. 2001; Huang et al. 2001). Although the binding affinity of VEGF for VEGF-R2 is much lower than the binding affinity for VEGF-R1, it could be shown by selective activation of both receptors that VEGF-R2 is the primary receptor which transmits VEGF signals to endothelial cells (Tammela et al. 2005). On the other hand, it as assumed that VEGF-R1 functions as a decoy receptor. Thus, by competing for VEGF binding to VEGF-R2 it may cause a negative regulation of the VEGF impact on the endothelium (Park et al. 1994). This is in line with the occurrence of the extracellular domain of this receptor in a soluble form (sVEGF-R1) in mice and men. Nevertheless, VEGF-R1 knockout mice die in embryonal day (E)8.5 because of a disorganization of blood vessels and a much too strong growth of endothelial cells (Ferrara et al. 2003). VEGF-R2 is autoregulated, while VEGF, VEGF-C, and VEGF-D can stimulate its expression (Tammela et al. 2005).

VEGF-R3 is a member of the receptor-tyrosine kinase family (RTK) but does not function as a receptor for VEGF. Instead it binds to VEGF-C and VEGF-D. It possesses only six immunoglobulin-like domains, as the fifth domain is cleaved shortly after proteolytic biosynthesis. The resulting polypeptide chain, however, remains attached to the major molecule by a bisulfite bridge

(Tammela et al. 2005). Moreover, VEGF interacts with a family of coreceptors, the neuropilins (Nrp-1 and Nrp-2), which play a functional role in neuronal development and in immunological processes, but which cannot directly convey angiogenesis (Klagsbrun et al. 2002; Bagri and Tessier-Lavigne 2002; Tammela et al. 2005).

16.3 Angiogenesis Inhibitors in Clinical Trials

A compilation of antiangiogenetic agents now being tested in clinical trials can be found in Table 16.3 (Tandle et al. 2004). One has to differentiate between modified monoclonal antibodies, named "... abs," and synthesized inhibitors, named "..ibs," the latter representing so-called smart small drugs that inhibit growth factors or their receptor kinases by binding to essential structural domains. Most the antiangiogenetic agents aim at VEGF or its receptors.

Tyrosine Kinase Inhibitors

Semaxanib (Su5416) was the first specific synthesized inhibitor of VEGF-RTK activity and showed a growth inhibition in mouse xenotransplants of human tumors. In several phase II trials, results were disappointing, albeit providing a good security profile.

Table 16.3 Angiogenesis inhibitors in clinical trials (adapted from Tandle et al. 2004)

Drug	Target	Published trials
Bevacizumab (Avastin)	VEGF	Phase I, II, III
VEGF-Trap	VEGF	Recruiting
NM-3	VEGF	Recruiting
AE-941 (Neovastat)	VEGF, MMP	Phase I,II
IMC-1C11	VEGFR-2	Phase I
SU5416	VEGFR-2	Phase I, II
SU6668	VEGFR-2	Phase I
SU11248	VEGFR-1/2, PDGFR, KIT, FLT3	Phase I
PTK787/ZK222584	VEGFR-1/2	Phase I
ZD6474	VEGFR-2, EGFR	Recruiting
CP-547,632	VEGFR-2, FGFR-2, PDGFR	Recruiting
Endostatin	Various	Phase I
Angiostatin	Various	Phase I
TNP-470	Methionine aminopeptidase-2	Phase I
Thrombospondin-1 (ABT-510)	CD36	Recruiting
Vitaxin	αv/β3 integrin	Phase I
EMD 121974 (Cilengitide)	αv/β3, αv/β5	Phase I
Combretastatin A4	Endothelial tubulin	Phase I
ZD6126	Endothelial tubulin	Recruiting
2-Methoxy-estradiol (2-ME)	Microtubule	Recruiting
DMXAA	TNF-α induction	Phase I
Thalidomide	Various	Phase I, II, III
BMS-275291	MMP	Phase I
Celecoxib	COX-2	Phase I, II, III

SU6668 has a wide range of impact with inhibition of the RTK-activity of PDGFR, FGFR1, and VEGF-R2. It led to complete histological tumor regression of the xenografts by apoptosis of tumor microvessels. One phase I trial with 56 patients with different advanced tumors showed a good tolerance; however, only 1 patient showed a little response and 2 showed stabilization of the tumor illness.

SU011248 inhibits VEGF-R1, PDGFR, c-kit, and FGFR1 at high doses. In a mouse model, a synergetic effect in combination radiotherapy was observed. In a phase II trial with 63 patients with renal cancer, 21 cases showed a partial response (33%), and in 23 patients (37%) a stabilization was observed. The 1-year-survival rate was 65%.

ZD6474 is an orally administered inhibitor of VEGF-R2 and, to a lesser extent, also of EGFR. Moreover, it possesses the following activities against other RTKs: PDGFR>VEGF-R1>Tie-2>FGFR1. One phase I trial showed a secure clinical profile, but no response of the tumors. Further trials are in progress.

PTK787/ZK 222584 causes an inhibition of TK activity of VEGF-R1 and VEGF-R2 and can also be administered orally. Phase I studies showed that PTK/ZK has a good tolerance and effectiveness with different tumors. The following side effects were recorded: nausea (59%), fatigue (41%), vomitus (35%), vertigo (29%), and headaches (24%). A study of glioblastoma multiforme with 20 patients (of which 15 were evaluable) showed 4 stabilizations and 1 partial response. In advanced colorectal carcinemo, 35 patients received PTK/ZK in combinations with chemotherapy (oxaliplatin, 5-FU, folinic acid). No elevated oxaliplatin toxicity was observed. Of 28 evaluable patients, 1 showed a complete remission, 14 had a partial remission (50%), 9 were stabilized, and 4 had tumor progression. The median survival was 16.6 months. In a further study with 45 patients suffering from renal carcinoma, 37 cases could be evaluated; 7 responded to therapy (1 partial, 6 with little response). Mean progression time was 5.5 months, and 17 patients (46%) showed a stabilization and 5 (14%) a progression.

CBP-547,632 is an orally administered inhibitor with reduced RTK activity of VEGF-R2. It binds to EGFR and PDGFR-β. A phase I trial with 22 patients showed a good tolerance: 6 patients were stabilized over a period of 8 weeks, 1 patient over a period of more than 6 months.

16.3.1 Antibody-Based Antiangiogenesis Therapy

Bevacizumab (Avastin) is a humanized antibody against VEGF. Inhibition of tumor growth was shown in preclinical and early clinical trials. Tolerance was good. In the first decisive phase III study, Bevacizumab was administered as first-line therapy to 925 patients with metastasized colorectal carcinoma. They were randomized into two groups, one getting Avastin, the other a placebo in combination with irinotecan, 5-FU, and folinic acid (Hurwitz et al. 2004). Bevacizumab showed better data compared to the placebo as to median survival (20.3 months vs 15.6, $p = 0.00003$), progression-free survival (10.6 months vs 6.24, $p = 0.00001$), response-rate (45% vs 35%, $p = 0.0029$), and response duration (10.4 months vs 7.1, $p = 0.0014$).

The positive effect of anti-VEGF treatment in connection to chemotherapy may look like a paradox as it was supposed to lead to a reduced angiogenesis and blood circulation of the tumor and a worse availability of the chemotherapeutic agent on-site. There are two possible explanations for this. On the one hand, it could be shown that in tumor tissue there is an elevated interstitial pressure compared to normal tissue, which is the result of the enhanced permeability effect of VEGF. The blockade of VEGF leads to a decrease in pressure in the tumor tissue which results in a better blood circulation in the tumor. One the other hand, it is known that the tumor vasculature is inefficient and chaotic. Only 1% of the blood vessels are of functional importance. Anti-VEGF treatment normalizes the chaotic supply of vessels of the tumor, leading to a better circulation and effectiveness of chemotherapy (Ellis 2004).

The positive results of Avastin in combination with chemotherapy led to its authorization as the first antiangiogenesis drug approved for colorectal cancer by the FDA (in February 2004). It will be applicable for further cancer types such as non-small cell lung carcinoma or renal cell carcinoma (Muhsin et al. 2004).

IMC-IC11 is a chimeric IgG1 antibody against VEGF-R2, blocking the ligand receptor binding and preventing phosphorylation. A phase I trial with 14 patients suffering from metastasized colon carcinoma showed a prolonged stabilization. Of the cohort, 7 patients developed antibodies against the chimeric IgG1 molecule; 2 patients had neutralizing antibodies (Posey et al. 2003).

VEGF-Trap is a fusion protein connecting segments of human VEGF-R1 and of the extracellular domain of VEGF-R2 to a Fc-fragment of human IgG1. The effect is based on the binding and inactivation of circulating VEGF found in tissue. The strong affinity of VEGF-R1 to VEGF results in a binding of VEGF-Trap 100 times stronger than that of monoclonal antibodies to VEGF, the pharmacokinetics being the same in both cases. In contrast of humanized antibodies, it contains exclusively human amino acids, leading to a missing formation of anti-VEGF Trap antibodies. Moreover, only grade 1 toxicity was observed, e.g., fatigue, proteinuria, and constipation. Grade 3 and 4 toxicities as potential reasons for ending therapy early were not observed (Fayette et al. 2005).

16.3.2 Endogenous Angiogenesis Inhibitors

ABT-510 is a nanopeptide that is able to substitute the antiangiogenetic activity of natural angiogenesis inhibitor thrombospondin (TSP-1). A phase Ib trial with 36 patients—34 having been evaluated so far—receiving subcutaneous escalating doses of the agent showed an acceptable tolerance. One partial response as well as two stabilizations (8 and 16 weeks) were observed in soft-tissue sarcoma. One patient with a non-small cell lung carcinoma showed a prolonged stabilization of more than 24 weeks (Fayette et al. 2005).

16.4 Outlook

Meanwhile, the concept of tumor antiangiogenesis is finding its way into the clinic. The chances are good that it will not only revolutionize therapy but also the market for tumor pharmaceuticals. For bevacizumab, an annual turnover of more than US $3.5–4 billion is forecast (Muhsin et al. 2004). This is astonishing as Avastin is an indirect angiogenesis inhibitor, aiming at the tumor cells and its product VEGF, which can potentially evoke drug resistance.

Further promising therapy approaches aim at the signal pathways stimulated by VEGF and other angiogenesis stimulators, such as COX-2 inhibitors.

Attacking molecules of the extracellular matrix such as integrins or matrix metalloproteinase is also very interesting because not only angiogenesis but also motility and invasion of the tumor cells could be influenced (Serini et al. 2005; Rundhaug 2005). Gene therapeutical approaches can be considered especially for the activation of endogenous angiogenesis inhibitors (Tandle et al. 2004; Nyberg et al. 2005).

Even if many prophecies remain unfulfilled, these studies contribute to a better understanding of tumor angiogenesis in vivo and can help to explain the observed antiangiogenetic effect of some classical chemotherapeutics and other target-orientated growth factors and RTK inhibitors.

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17 Morphological Detection of Hormone and Growth Factor Receptors in Breast Cancer

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Abstract

In the multidisciplinary effort of today's clinical oncology, histopathology has to deal increasingly with the genetic and proteomic profiles of neoplastic cells obtained in ever smaller samples via various diagnostic routes. None of the numerous molecular markers available has surpassed hormone and growth factor receptors in their relevance for the selection of targeted therapies. In the following, morphologic concepts and histopathologic methods pertinent to the clinically most important receptors are discussed with a focus on tumors of the breast.

17.1 Molecular Pathology and Target Reconnaissance

The realm of the surgical pathologist in oncology is constantly expanding. Histopathological diagnoses once including only a tumor's name, stage, and grade are increasingly replete with information on metric details of resection margins, prognostic predictions, and molecular profiles of the neoplastic cells contained within the tissue resected, biopsied, or aspirated. Although a large number of single-gene molecular markers were proposed as medically relevant over the last three decades, comparatively few have established themselves in the clinic (Bast et al. 2001). Significantly, the most prominent ones—hormone and growth factor receptors—have proven histopathologically assessable and are now paramount to differential therapy thereby initiating the now pervasive medical trend toward targeted therapies.

Confidently making therapeutic decisions based on molecular pathology would not be possible without the judicious application of technological advances in immunohistology and in situ hybridization, and their careful interpretation within established diagnostic concepts. In this practice of integrating morphology and molecules the pathologists and their stains will continue to safeguard medicine in the future when diagnoses will mandate ever-more-detailed target reconnaissance.

In the following, morphologic concepts and histopathologic methods pertinent to hormone and growth factor receptors are discussed focusing on tumors of the breast in which their detection so far has had the greatest diagnostic and clinical impact.

17.2 Hormone Receptors

17.2.1 Estrogen Receptors

The human estrogen receptor gene ESR1 on chromosome 6q25.1 encodes a predominant 65-kDa product of 595 amino acids consisting of 4 domains: the N-terminal A/B, the DNA-binding domain C, the hinge domain D, and the ligand-binding domain E/F. While only about 10% of normal mammary epithelial cells express ESR1 gene products, these estrogen receptor proteins are immunohistologically detectable in 50%–85% of invasive breast cancers. The cause of this overexpression remains enigmatic, which is hardly surprising given that physiological regulatory influences on ESR1 have not been fully characterized.

During the last 30 years it has been firmly established that estrogen receptor (ER) positivity signifies a higher probability of responding to endocrine manipulations, thus necessitating reliable diagnostic assays for the selection of adjuvant therapies. The first biochemical or extraction assays, such as the radiolabeled ligand-binding assays (LBA) using dextran-coated charcoal (DCC), required the prospective collection of fresh tissue samples and quantitated the ER content of the tumor in femtomoles of ER protein per milligram of cytosol protein.

Probably mainly due to preanalytical influences, such as normal tissue dilutional effects, the ER content of breast cancers was found to range widely with the ER-richest tumors benefiting most from endocrine therapy.

In the 1990s the general availability of monoclonal antibodies and effective antigen retrieval protocols has made immunohistochemistry (IHC) the method of choice for determining hormone receptors. Its advantages include its relatively low cost, minimal tissue requirements, applicability to archival specimens, and most importantly the direct correlation with morphology. Comparing IHC with the biochemical radioactive ligand-binding assay (DCC), a good agreement has been noted with a discordance rate of 10%–30% for both ER and progesterone receptor (PR) status (Beck et al. 1994). Repeatedly the greater predictive and discriminatory power of IHC over extraction assays has been demonstrated (Harvey et al. 1999; Regan et al. 2006).

The 2005 International Expert Consensus on the Primary Therapy of Early Breast Cancer recognized that endocrine responsiveness of the primary tumor should be the first consideration for selecting adjuvant systemic therapies (Goldhirsch et al. 2005). Notwithstanding that ER IHC constitutes now routine pathology practice the world over, intra- and interlaboratory variation due to differences in fixation, antigen retrieval, and staining methods are still to be reckoned with. When the effect of formalin fixation time on ER staining was examined using only strongly ER^+ tumors, some of these were completely negative when fixed for only 3 h (Goldstein et al. 2003). Based on tests in 200 different clinical laboratories, false-negative rates between 30% and 60% (depending on the cut-off) for a low ER^+ tumor have been reported (Rhodes et al. 2000). Clearly the most discriminatory cut-off for calling a tumor ER-negative is still not definitively established—even using image analysis systems (Rhodes 2003)—as some proponents of compound IHC scores have suggested (Harvey et al. 1999).

Whereas in the extraction assays 10 fmol/mg or more of cytosol protein was taken as evidence for ER-positivity, the small numbers of ER-low tumors with between 1% and 9% of cells stained may mean that a gray area will persist for some time (Regan et al. 2006). The endocrine responsiveness of this albeit small (probably less than 3%) number of tumors remains to be clarified. It is therefore only prudent to recognize a category of "endocrine response uncertain" tumors and consider chemotherapeutic options depending on nodal and menopausal status (Goldhirsch et al. 2005).

However, 30%–40% of patients with unquestionably ER^+ tumors do not respond to hormonal therapy, suggesting that the only modest positive predictive value (30%–60%) of ER testing for response to single-agent hormonal therapies (Bonneterre et al. 2000; Mouridsen et al. 2001) may be rather biological than technical. A truly biological lack of response could result from mutational disruptions of the molecules' interactional functions not impacting on their immunohistological detectability. Although somatically corrupted binding sites are certainly occurring in breast cancers given the vast array of splicing variants, "missplicing" could well be the more important cause of discrepancies between IHC and biological response.

Similarly, the molecular mechanisms of true ER negativity are not fully elucidated, yet posttranscriptional and posttranslational mechanisms appear to be more likely than transcriptional blocks or promoter methylation.

17.2.1.1 Two Genes and Multiple Splices

The discovery of a second estrogen receptor gene, *ESR2*, on chromosome 14q encoding an estrogen receptor-β of predominantly 57 kDa that displays the greatest similarity to estrogen receptor-α in its DNA- and ligand-binding domains, has complicated the study of hormone receptor

expression even further (Mosselman et al. 1996). There is variability in the predominance of one form or the other in different organs. Whereas the breast, brain, bones, urogenital, and cardiovascular tract do express both genes, the liver and the gastrointestinal tract predominantly express ESR1 or ESR2, respectively.

In contrast to ESR1, ESR2 is apparently expressed in 80% of normal mammary and invasive cancer cells alike. There is, however, no consensus with regard to ESR2 expression during tumor progression, which is thought to be generally accompanied by rising ESR1 levels (Herynk and Fuqua 2004).

Looking at a small number of invasive breast cancers $ERα^-/ERβ^+$ tumor cells appear to be more proliferating yet not influenced by tamoxifen (TMX) (Jensen et al. 2001) and thus potentially requiring a different, more ERβ-specific therapy. However, such gene-selective antiestrogens have not reached the clinics. Equally the relevance of ERβ expression to breast cancer prognosis remains to clarified (Spiers et al. 1999; Nakopoulou et al. 2004). Consequently, ERβ testing cannot yet be recommended for inclusion in the diagnostic routine until the following questions have been sufficiently addressed:

- Can ERβ measurement be used to improve the prognostic value of ERα, or could it even be used as an independent prognostic indicator?
- What is the functional significance of ERβ splice variants, and can their presence be used to predict the likely response to hormonal therapy?
- Does ERβ represent a novel target in breast cancer and thus potentially allow the development of additional hormonal therapies that can add to the effectiveness of existing agents?

It has been proposed that the ESR1 to ESR2 transcript ratio may be more clinically relevant than individual levels so that a higher ratio compared to normal tissue may signify estrogen-dependent tumors whereas a lower ratio would be indicative of estrogen independency (Iwao et al. 2000). However, several naturally occurring isoforms due to alternative splicing have been described in steroid receptor genes, which in general complicates the ascertainment of transcriptional levels as an alternative to immunohistological evaluation (Herynk and Fuqua 2004). With this highly complex interplay in mind, it seems reasonable, at least for the time being, to ascribe the dominant role in the physiology and pathology of the breast to the ESR1 gene and its products.

17.2.1.2 Antibody Selection

Techniques to generate monoclonal antibodies against synthetic oligopeptides in rabbits (Spieker-Polet et al. 1995), a species known to produce high-affinity antibodies (RabMabs) against antigens nonimmunogenic for mice, has expanded the range of epitopes potentially available for interrogation by IHC. The first commercially available clone with antiestrogen receptor specificity, SP1, compares favorably with standard murine clones (Rossi et al. 2005), such as 6F11 and 1D5, on formalin-fixed, parafin-embedded (FFPE) tissue sections often without the need for heat-induced epitope retrieval (HIER). Combining monoclonals from different species on the same section allows for instructive multicolor immunofluorescence staining sufficiently robust to be used in diagnostic workup (Fig. 17.1).

The concurrent use of several antibodies with different epitopes could also shed light on the discrepancies between immunohistological reactivity and function caused by alternative splicing. For diagnostic routine work, preference should be given to those clones whose epitopes are least likely to be affected by polymorphic variation, somatic mutation, or missplicing.

There are now several different, partially epitope-mapped antiERβ-specific murine monoclonals commercially available, of which 14C8, 8D5-1, and PPG5/10 appear to be suited to FFPE samples (Skliris et al. 2002). Consequently the questions detailed in the previous section may be resolved in the not-so-distant future.

17.2.1.3 Scoring the Expression of ER

With the gradual replacement of the extraction tests, several IHC scoring protocols often combining the chromogenic staining intensity and the percentage of positive cells into semi-

quantitative immune reactivity scores have been proposed. However, over the years pathologists have increasingly realized that the intensity of chromogenic staining in IHC cannot be quantified with sufficient reliability as historically some advocates of compound scores using intensity grading have claimed.

Consequently, a number of current trials, such as those of the International Breast Cancer Study Group (IBCSG), use assessment protocols that do not integrate the staining intensity but include:

- Checks for expected immunostaining of non-neoplastic breast tissue (intense nuclear staining of at least a minor percentage of luminal epithelial cells) and lack of immunoreactivity of myoepithelial, stromal, and inflammatory cells
- Evaluation at low or intermediate magnification to take into account any significant heterogeneity of staining in different parts of the invasive tumor
- Assessment at higher magnification of the number of cells showing nuclear immunostaining compared with a sufficient number of randomly selected invasive tumor cells (minimum of 2,000 in homogeneous tumors; Regan et al. 2006)

At least until the claims (Harvey et al. 1999) of a positive correlation between semiquantitative IHC scores and the probability of benefiting from endocrine therapy have been independently tested, the controversy may persist.

Currently quantitative assays for ER gene products extracted from FFPE are under active development using different platforms. However, if these approaches will prove more than microscale reruns of the first generation—namely, extraction-based tests encumbered with all the problems of reliable normalization—remains to be seen.

17.2.1.4 Estrogen Receptors in Intraductal Carcinoma

In the relatively few studies conducted, around 80% of intraductal carcinomas have been found to express ER immunohistochemically mostly without any indication of the prognostic relevance (Perin et al. 1996; Cornfield et al. 2004) that has been established for age, size, grade, and distance to resection margins in ductal carcinoma in situ (DCIS). A single study has claimed a significantly higher risk for ipsilateral recurrence (Roka et al. 2004).

Source data from the NSABP-24 study suggest an approximate 60% risk reduction of future breast cancer events in those women whose DCIS was ER^+ and who were treated with TMX (Allred et al. 2002), while TMX appears to be ineffective in ER-DCIS. However, the extremely large intervals in these patients do not eliminate the possibility of a small yet clinically relevant effect.

Therefore definite recommendations regarding the reporting of intraductal ER expression, which should also include detailed scoring algorithms on the minimal number of intraductal tumor cells required for a reliable ER diagnosis, will have to await the results of the IBIS-II (http://www.ibis-trials.org/dcis/dcis.html) and NSABP B-35 trials (http://www.nsabp.pitt.edu).

17.2.2 Progesterone Receptors

In view of the substantial fraction of ER^+ patients failing to respond to hormone therapy, the tumoral expression of PR has always been an attractive candidate in the quest for the improved prediction of endocrine responsiveness. Assuming that PR is primarily regulated by ER, immunohistologically detectable PR should indicate a functionally intact estrogen response pathway.

As expected, several studies have demonstrated a more pronounced response to adjuvant endocrine therapy/hormonal intervention in ER^+/PR^+ tumors than in ER^+/PR^- tumors (Ferno et al. 2000 ; Ellis et al. 2001; Bardou et al. 2003).

However, ER^+/PR^- tumors appear to be larger, more aneuploid, more proliferative, and display a higher growth factor receptor activity than double positives. So the loss of PR in some of these tumors may well be due to increased ERBB2 activity rather than to disturbances in the ER signaling pathway (Arpino et al. 2005).

Although some uncertainties regarding the mechanism of hormonal resistance in these tumors remain, the evidence is sufficient to make

immunohistological detections of the PR expression mandatory in the routine workup of all invasive breast cancers.

The conceptual and methodological issues encountered with PR IHC are almost identical to those described above for ER. Yet of the two commercially available RabMab with anti-PR specificity, only Y85 compares favorably with standard murine clones such as, such as 1A6, on FFPE tissue sections. Now that dual-color immunofluorescence detection of ER and PR (Fig. 17.1) can be performed quite easily, an informative new window for the diagnosis of tumoral hormone receptor expression has opened up.

17.3 ERBB2

The ERBB2 gene (often referred to not by its official gene symbol but by historical names, such as *HER-2/neu*), encodes a 185-kDa transmembrane tyrosine kinase that belongs to a family of homologous receptors including ERBB1/EGFR, ERBB3, and ERBB4, which are all widely expressed throughout the body.

Upon ligand binding to their external domains these receptors form homo- or heterodimers, autophosphorylate some of their intracellular tyrosines, and thereby activate a complex intracellular signaling pathway.

Whereas the initial study linking overexpression of ERBB2 to breast cancer used DNA-, RNA-, and protein-based tests to demonstrate the underlying gene amplification (Slamon et al. 1987), subsequently IHC has prevailed in the vast number of often conflicting reports dealing with the more clinical aspects. However, consensus has been reached regarding the associations of ERBB2 overexpressing tumors with poor prognosis in node-positive patients, resistance to cyclophosphamide/MTX/5-FU, and sensitivity to anthracycline-containing protocols. In invasive breast cancer, most immunohistologi-

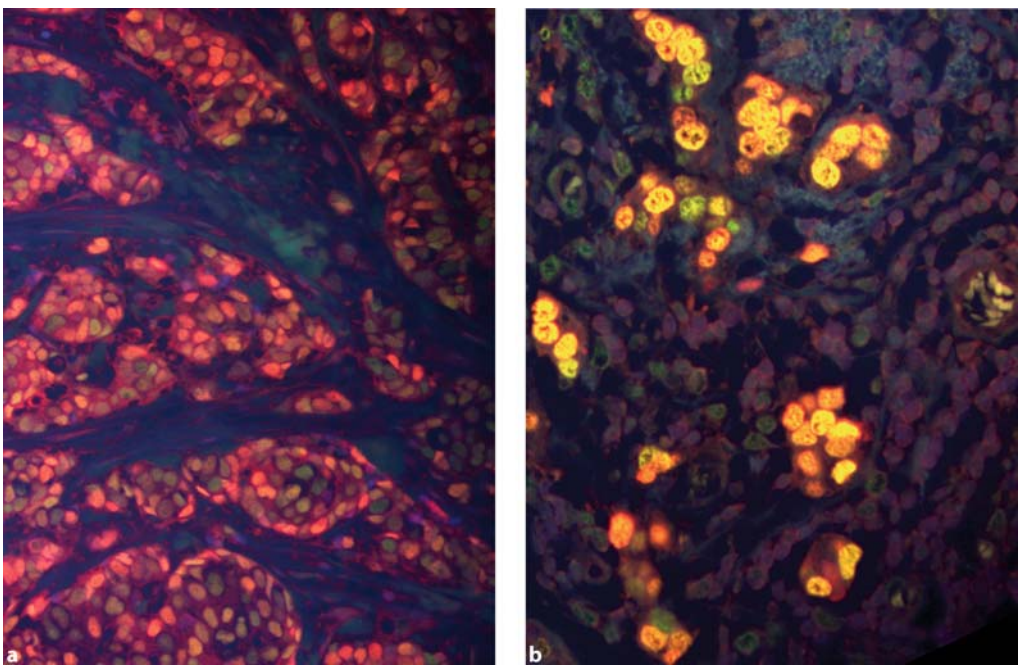

Fig. 17.1 Dual-color immunofluorescence staining of a hormone receptor-positive invasive ductal (**a**) and a hormone receptor-negative invasive lobular (**b**) breast cancer using 6F11-Cy2 (orange nuclear signals) and Y85-Cy3 (green nuclear signals) detection of ERα and PR, respectively

cal studies have found the ERBB2 gene product overexpressed in 10%–35%, with the lower end of this range being a more realistic estimate according to recent analyses using stringent criteria (Huang et al. 2005).

17.3.1 IHC Vs FISH: The Herceptin Controversy

The need for a reliable assay to assess ERBB2 overexpression has become dramatically apparent when chemotherapy combined with a humanized derivate (Tastuzumab) of the mouse antibody (4D5) was shown to prolong survival in metastatic breast cancers overexpressing ERBB2. The requirement for effective eligibility testing was officially confirmed in 1998 when trastuzumab/Herceptin was approved by the United States Food and Drug Administration (FDA) together with the DAKO HercepTest to diagnose overexpression in FFPE tissue sections immunohistochemically. The accompanying product literature described a 4-tiered scoring system based on assessing the proportion of cells with positive membrane staining and the color intensity of the chromogenic staining. This reliance on color-grading in the interpretation of expression levels has led to continuous and well-documented controversies (Hsu et al. 2002) culminating in the recognition of a "significant methodological problem" by a recent expert consensus statement (Goldhirsch et al. 2005).

Variability in tissue fixation, processing, and HIER between samples will be difficult to eradicate sufficiently to quantify immunohistochemical staining intensity objectively, even when aided by advanced image analysis systems.

To resolve the question of correspondence between ERBB2 amplification and overexpression, independent studies have tried to correlate ERBB2 status as determined by fluorescent in situ hybridization (FISH) and IHC on FFPE tissue sections with quantitative data obtained from frozen tissue samples (Bartlett et al. 2001; Press et al. 2002). Both found the FISH test more accurately predictive of the ERBB2 expression level in the frozen tissue than the IHC assay.

Acknowledging these observations, the FDA additionally approved a FISH-based system (Vysis PathVysion; Fig. 17.2.) to select patients for therapy with Herceptin. The dual-color (Spec-

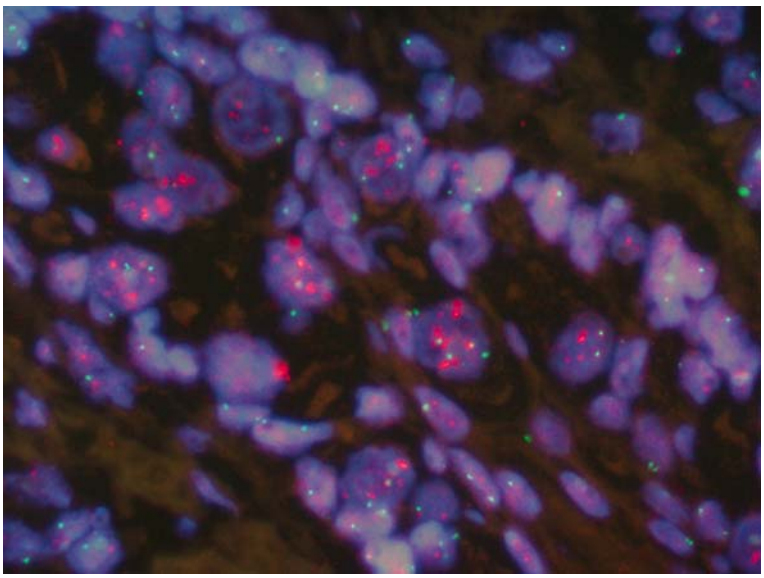

Fig. 17.2 Dual-color dual-probe ERBB2 CEP17 FISH showing intraductally spreading ERBB2-amplified tumor cells with multiple clustered orange signals often next to chromosome 17-centromeric green signals

trumOrange and SpectrumGreen)/dual-probe (ERBB2 and 17cen) format of this assay requires the straightforward enumeration of colored signals in tumor nuclei, thereby reducing interobserver errors to below 10% (Bartlett et al. 2001). Consequently, recommendations with regards to the preferable assay have changed considerably to the now prevailing view of FISH testing as the most reliable options to determine risk group or treatment choice (Goldhirsch et al. 2005).

The controversies over the reliability of semiquantitative IHC as highlighted by the problems over Herceptin eligibility testing has firmly established hybridization-based techniques in a rapidly growing number of pathology laboratories. This has happened to such a degree that FISH has been advocated as the primary test for ERBB2 estimation (Bofin et al. 2004; Press et al. 2005).

But until the logistics of first-line ERBB2 FISH have been sufficiently established, IHC will undoubtedly retain its current use as a prescreening tool (Bilous et al. 2003).

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Part III Prospects for Development

18 Antigen-Specific Cancer Vaccines

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Recent Results in Cancer Research, Vol. 176

Abstract

The past decade has seen a number of clinical vaccination trials investigating defined tumor antigens. These trials have taught us the immunologic consequences of various vaccination approaches, although the clinical efficacy of the vaccines has only reached the proof-of-concept level. Detailed and rigorous immune monitoring will be crucial for successful further development of cancer vaccines as well as defining patient populations likely to benefit. At a recent meeting of the Berlin Oncology Summer Seminar (BOSS) the clinical and translational cancer vaccine program ongoing at the Charité was presented, and this paper contains a summary of the presentation as well as the subsequent discussion.

18.1 T Cell Targets on Tumor Cells

Tumor cells present specific peptide epitopes in the context of human leukocyte antigen (HLA) class I molecules on the cell surface, which can selectively be detected by the T cell receptor (TcR) of CD 8$^+$ T lymphocytes. After recognition, activated CD8$^+$ T cells can exhibit cytotoxic effector function, leading to destruction of tumor cells. The first description of the tumor-associated antigen (TAA) MAGE-1 recognized by CD8$^+$ T cells more than a decade ago was a groundbreaking step in cancer immunology (van der Bruggen et al. 1991). During recent years, effective strategies to identify TAA recognized by specific T cells were developed and led to the characterization of various families of TAA, including the differentiation antigens, overexpressed antigens, cancer germline antigens, mutated antigens, and viral antigens (reviewed in Renkvist et al. 2001). These TAA have facilitated the development of immunotherapeutic approaches. The characterization of numerous MHC class I-binding epitopes of the TAA recognized by CD8$^+$ T cells simplified the development of synthetic vaccines. MHC class I-binding epitopes consisting of 9–12 amino acids can directly be injected for patient immunization. More recently, epitopes also derived from various TAA presented in association with MHC class II and recognized by CD4$^+$ T cells have been identified.

18.2 Vaccination Studies with Differentiation Antigens and Cancer-Testis Antigens

The first trials studying the immunogenicity and toxicity of peptide vaccination have been performed in patients with metastatic melanoma (Marchand et al. 1999; Jaeger et al. 1996; Cormier et al. 1996; Rosenberg et al. 1998; Scheibenbogen et al. 2000; summarized in Table 18.1). In general, objective tumor remissions were only occasionally seen and were usually restricted to melanoma patients with limited disease. For instance, in a MAGE-3 peptide trial reporting a 30% response rate the 7 patients showing an objective tumor remission all had metastatic disease limited to soft tissue or lymph nodes and 14 patients who were not included in the response evaluation had early progressive disease during vaccination (Marchand et al. 1999). Antigens tested in clinical studies were mostly cancer germline antigens or melanocyte differentiation

Table 18.1 Clinical studies of peptide vaccination in patients with metastatic melanoma

Antigen	Adjuvant	Patients (*n*)	Tumor response	T cell response (type of assay)	Reference
MAGE-3	None	39	7 CR/PR of 25	0/4 (Cr)	Marchand et al. 1999
Tyrosinase+MART-1+gp100	GM-CSF	3	3 CR/PR	3/3 (Cr)	Jaeger et al. 1996
MART-1	IFA	18	None	12/18 (Cr)	Cormier et al. 1996
Gp100	IFA	11	3 MxR	10/11 (ELISA)	Rosenberg et al. 1998
	IFA+IL-2	19	8 CR/PR, 3MxR, 3SD	3/19	
Tyrosinase	GM-CSF	18	1 MxR, 2 SD	4/15 (ELISPOT)	Scheibenbogen et al. 2000

Cr, chromium-release assay following in vitro sensitization; CR/PR, complete/partial remission; ELISA, cytokine-release assay following in vitro sensitization; MxR, mixed response; SD, stable disease

antigens either alone or combined with various adjuvants as outlined below.

Adjuvant vaccination of high-risk tumor patients following resection of the primary tumor or metastases or successful systemic therapy may have a much greater immunologic and therapeutic potential. There are several clinical phase I and II trials in patients with resected melanoma (Weber et al. 1999, 2003; Scheibenbogen et al. 2002a, 2003; Slingluff et al. 2001; Lee et al. 2001; Smith et al. 2003; Table 18.2). In recent studies, induction of specific T cells has been demonstrated in the majority of tumor-free patients by prolonged vaccination (8–13 cycles) with gp100 peptides emulsified in incomplete Freund's adjuvant (IFA) using direct tetramer staining (Weber et al. 2003; Lee et al. 2001; Smith et al. 2003). In a randomized trial, no differences in the frequency of peptide-specific T cells were observed between patients vaccinated every 2 weeks or every 3 weeks (Smith et al. 2003). Prolonged disease-free and overall survival of melanoma patients receiving peptide vaccination was observed compared to historical controls (Weber et al. 2003; Slingluff et al. 2001; Lee et al. 2001). Tyrosinase peptide vaccination resulted in long-term freedom from recurrence in several high-risk melanoma patients who had multiple cutaneous relapses prior to vaccination (Scheibenbogen et al. 2002a). However, no formal proof of the efficacy of adjuvant antigen-specific vaccination has been demonstrated in clinical phase III studies in melanoma patients so far. The ECOG (Eastern Cooperative Oncology Group) is currently performing a large multicenter study evaluating the effect of peptide vaccination and the role of GM-CSF in a randomized phase III trial in relapsed stage III and stage IV patients after resection of metastases.

18.3 „Essential" Tumor Antigens

The inherent limitation when using differentiation and cancer-testis antigens as a tumor target is based on the observation that many of these antigens are frequently lost during tumor progression, presumably as a result of either de-differentiation of tumor cell clones of under immunologic pressure. Thus an active search for tumor antigens that are essential for tumor cell proliferation has been undertaken by a number of research groups. Among these, proliferation-inducing transcription factors are very promising. The molecule that has received most attention in this respect is the transcription factor Wilms tumor gene 1 (WT1), which serves as a tumor suppressor gene in Wilms tumor, but as tumor promoter in most acute leukemias and a variety of carcinomas (Keilholz et al. 2002).

Table 18.2 Clinical studies of peptide vaccination in patients with resected melanoma

Antigen	Adjuvant	Patients (*n*)	T cell response	Reference
Tyrosinase+gp100	IFA+/−GM-CSF	48	34/39 (ELISA)	Weber et al. 2003
			37/42 (tetramer)	
MAGE-3	IFA+PADRE	18	5/14 (Cr)	Weber et al. 1999
Tyrosinase	None	9	3/9 (ELISPOT)	Scheibenbogen et al. 2003
	GM-CSF	9	4/9	
	KLH	10	0/10	
	GM-CSF+KLH	9	5/9	
Gp100	IFA or QS21+/−tetanustoxoid	22	3/21 (ELISPOT)	Slingluff et al. 2001
Tyrosinase+gp100	IFA+/−IL-12	48	33/38 (ELISA)	Lee et al. 2001
			37/42 (tetramer)	
gp100	IFA	30	28/29 (tetramer)	Smith et al. 2003

Cr, chromium-release assay

Overexpression of the WT1 in leukemic blasts of AML and ALL patients, and in myelodysplastic syndromes (MDS), is well documented, both at the RNA and protein levels (Cilloni and Saglio 2004). Physiologic hematopoietic stem cell compartments also express WT1 (Van Dijk et al. 2002). However, there is agreement that a „malignant" WT1 expression can be clearly distinguished from a physiologic expression based on quantitative detection methods such as semiquantitative RT-PCR, and real-time quantitative RT-PCR (Cilloni, Janssen). Several groups have already shown that human WT1-specific, HLA class I-restricted cytotoxic T lymphocytes (CTLs) can be established in vitro (Tsuboi et al. 2000; Scheibenbogen et al. 2002b; Gaiger et al. 2001) and that mice immunized with WT1 peptide or WT1 complementary DNA (cDNA) can induce WT1-specific CTLs and the resultant rejection of challenges by WT1-expressing tumor cells (Wu et al. 2005; Rosenfeld et al. 2003), indicating that WT1 can serve as a tumor rejection antigen. Furthermore, no damage to normal tissues was observed in the mice.

Clinical vaccination trials have recently been initiated in Japan and Germany. Preliminary results from an ongoing phase II trial performed at the Charité show immunologic and clinical efficacy of the vaccine. HLA-A2-positive patients received repeated WT1 126–134 peptide vaccination in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) and keyhole limpet hemocyanin as adjuvants. Induction of high frequency T cell responses was detected with up to 0.92%, 0.43%, and 0.42% in peripheral blood (PB) and up to 0.8% $CD3^+CD8^+$ T cells in bone marrow (BM) by tetramer analysis and intracellular cytokine cytometry. The first patient, who had second partial remission at study onset and progressed during the initial 4 weeks of vaccination with an increase of marrow blasts to 30%, subsequently achieved a very rewarding complete remission (CR) after 6 vaccinations (Mailaender et al. 2004). The results from a phase I clinical trial of WT1 peptide-based cancer immunotherapy in patients with acute leukemia, MDS, lung cancer, and breast cancer conducted at the Osaka University Clinic also revealed very encouraging results. The patients were intradermally injected with increasing doses of 0.3 mg, 1 mg, or 3 mg of an HLA-A*2402-restricted, 9-mer WT1 peptide emulsified with Montanide ISA 51 adjuvant (Oka et al. 2004).

18.4 Role of Immunological Vaccine Adjuvants

Following intradermal injection, TAA-derived peptides are thought to bind to empty MHC

class I molecules on dendritic cells, which then migrate to the draining lymph nodes where specific T cell activation occurs. Vaccination with TAA-derived peptides alone may be suboptimal to charge and activate dendritic cells, and elicit specific T cell responses; it thus requires simultaneous administration of adjuvants. GM-CSF has been used in various vaccine protocols, as it promotes local recruitment and migration of dendritic cells. Enhanced induction of $CD8^+$ peptide-specific T cells and objective tumor responses had initially been reported in three melanoma patients following the addition of GM-CSF to a multipeptide vaccine (Jaeger et al. 1996). In two consecutive small phase I trials performed in tumor-free melanoma patients, no difference in the induction or frequency of peptide-induced T cell responses analyzed by enzyme-linked immunosorbent spot (ELISPOT) assay was observed if tyrosinase peptides were applied alone or in combination with GM-CSF (Scheibenbogen et al. 2003). Weber et al. who vaccinated stage III patients after resection with gp100 and tyrosinase peptides emulsified in IFA with or without GM-CSF observed a trend of GM-CSF to moderately increase the frequency of specific T cells detected by tetramers (Weber et al. 2003). Another strategy to enhance T cell responses to MHC class I epitopes is the use of T helper antigens. Animal models show that the induction of $CD8^+$ T cell responses may require the presence of $CD4^+$ T helper cells, which provide stimulation of dendritic cells and cytokines. $CD4^+$ T helper cell responses may also be of importance for the long-term maintenance of $CD8^+$ T cells. Simultaneous induction of $CD8^+$ and $CD4^+$ T cell responses was either achieved using specific MHC class II epitopes or unspecific T helper antigens such as a pan-class II epitope (PADRE; Weber et al. 1999) or keyhole limpet hemocyanin (KLH; Scheibenbogen et al. 2003).

18.5 Immune Monitoring

Immune monitoring guides the rational development of cancer vaccines throughout their developmental stages, as reviewed in detail elsewhere (Keilholz et al. 2002). In early clinical trials immune monitoring should guide the selection of vaccine preparations and schedules for advanced clinical testing (vaccine potency assessment), and in phase III clinical trials immune monitoring should define surrogate markers for clinical efficacy that later on will allow investigators to perform bridging studies in case vaccine preparations or production methods are modified. However, there are a number of limitations that need to be acknowledged.

The first generation of assays detecting immune responses was developed several decades ago, and includes the proliferation assay in response to antigen exposure and the chromium release assay for assessment of cytotoxicity. Both assays assess the T cell response on the level of the entire cell population in the culture, thus requiring prior in vitro expansion. The second-generation ex vivo T cell assays dramatically improved our ability to reliably measure T cell response, since they are based on detecting single-cell events and thus provide quantitative results. The second generation flow cytometry assays detect T cells either by staining the T cell receptor with MHC-peptide tetramers, measuring the affinity of the TCR to a given epitope, or by measuring production of cytokines (most commonly interferon-γ) in response to antigens. ELISPOT and cytokine flow cytometry analyses provide functional information in contrast to tetramer staining; however, only the subset of specific T cells producing the respective cytokine is detected. The second generation assay technology and applicability was the major focus of the first International Society for Biological Therapy of Cancer (iSBTc) workshop, entitled „Immune Monitoring Workshop," held in Bethesda in 2001 (Keilholz et al. 2002). The recommendation of that workshop to perform monitoring preferentially with two of these second-generation assays has been largely followed since then for cancer vaccine trials.

The so-called third generation of T cell assays has been developed aiming at detailed analysis of the phenotype and functional repertoire of antigen-reactive T cells. In principle, these assays are based on the detection of specific T cells based on tetramers or cytokines and their further characterization by additional phenotypic or functional markers. This type of analysis can yield information on many facets of the nature of T cell responses, including T cell differentiation subsets,

cytokine production repertoire, proliferative and cytotoxic capacity, and migratory potential.

In current and future trials the contribution of individual aspects of immune cell functions and of the increasing numbers of defined T cell subsets for clinical vaccine efficacy is being addressed. Based on our current knowledge it can be assumed that immune protection is not a single-parameter or single-compartment endpoint, but is likely to be a balanced mosaic of immune response markers including both cellular and humoral effector functions. The capability of current flow cytometry to deliver multiparametric data is enabling a new biomarker-based approach for monitoring multiple markers of immune reactions, which will hopefully enhance our understanding of immune reactions associated with successful immunotherapy.

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19 Therapeutic Vaccination for the Treatment of Malignant Melanoma

Peter Walden

Recent Results in Cancer Research, Vol. 176

Abstract

With increasing knowledge of tumor-associated antigens and T cell epitopes, and the mechanisms of induction and regulation of T-cellular immune responses, therapeutic vaccination is increasingly being explored as a treatment option for cancer. Several clinical cancer vaccination trials, the majority of them with melanoma patients, have demonstrated efficient induction of tumor-specific cellular immune responses in patients. However, these immune responses, in most cases, do not translate into clinical responses. The clinical response rates in these trials are relatively low. The most likely causes for the lack of correlation of immunological and clinical responsiveness are loss of antigenicity and immune suppression. Nonetheless, many patients in the vaccination trials have experienced extended survival compared to clinical experience. Therapeutic vaccination thus appears suited for maintenance therapy where cure is not possible and is an interesting option for adjuvant therapy after surgical tumor resection. While the clinical efficacy of vaccination is expected to be better for early-stage cancer, advancement of the treatment of advanced-stage disease will require combination with other therapeutic principles.

19.1 Introduction

Early in the nineteenth century the successes of Jenner's smallpox vaccines seeded the idea that man can be immunized against any disease. Besides prophylaxis, therapeutic vaccination was attempted for infectious diseases and allergies as well as cancer (Kaufmann 1996).

Until very recently, the development of vaccines and vaccination schemes was largely empirical and geared toward the induction of antibody responses. Only with the identification of the first tumor-associated T cell epitopes and their source proteins some 15 years ago did T cells come into focus for vaccine development (Boon et al. 2006; Boon and van den Eynde 2003; Stuhler and Walden 2002). The design of T cell vaccines now incorporates the latest knowledge concerning the constitution of the immune system, the mechanisms of immune regulation, and tumor-associated antigens. $CD8^+$ T cells are the main effector cells of the immune system in its battle against tumor cells. These cells recognize antigens as peptides of usually 8–10 amino acids associated with major histocompatibility complex (MHC)—human leukocyte antigens (HLA) in man—class I molecules (van der Bruggen and van den Eynde 2006). The peptides are generated by limited proteolysis of cellular proteins and mirror the state of the proteome and the turnover of proteins in the cells. The immune system thereby gains insight into the physiology and pathophysiology of cells not accessible to antibodies.

The first tumor-associated T cell epitopes and antigens for human cancer were identified for melanoma. To date some 200 tumor-associated T cell epitopes derived from about 100 tumor-associated antigens are known, a large fraction for melanoma (Novellino et al. 2005). This knowledge has been translated into the design of new vaccines with defined composition and used for

monitoring the immunological effects of therapeutic vaccination, i.e., the responses of tumor-specific T cells in the cancer patients (Faries and Morton 2005; Stuhler and Walden 2002). The search for new tumor-associated antigens and T cell epitope is ongoing and new vaccines are in development, several already in clinical trials. These efforts are bound to lead to improved vaccine designs and vaccination regiments. In addition to this progression of tumor vaccinology, clinical experience to date allows us to draw a number of conclusions regarding the prospects for therapeutic vaccination as a treatment modality for cancer.

19.2 Tumor-Associated Antigens and T cell Epitopes

The first tumor-associated antigens and T cell epitopes were identified by expression cloning of complementary DNA (cDNA) prepared from tumor cells from a melanoma patient into COS cells that had been stably transfected with the genes for an HLA molecule, and probing these double-transfectants with T cells from peripheral blood of the same patient. After repeated subcloning, the gene of the tumor-associated antigen was isolated and sequenced for identification (Boon et al. 2006; van der Bruggen et al. 2002). It turned out to be a hitherto unknown protein expressed in tumor cells and testes only, and was dubbed MAGE. It was the first of the extended family of MAGE proteins and the first member of the group cancer-testis antigens. Since testis is an immune-privileged organ, these antigens are relatively tumor-specific and therefore preferred vaccine targets. No function of the tumor/testis-associated MAGE proteins has yet been determined.

Today, different approaches to antigen and T cell epitope discovery are employed in tumor immunology. These are mostly biochemical approaches (Demine et al. 2003; Demine and Walden 2005), i.e., direct extraction and sequencing by mass spectrometry of peptides presented by the MHC molecules of tumor cells and bioinformatic approaches (Rammensee et al. 1999; Bredenbeck et al. 2005; Filter et al. 2006), i.e., prediction of epitopes of known or presumed tumor-associated antigens with a number of different bioinformatic tools. The latter has proved to be the most productive strategy of T cell epitope determination. For clinical application as vaccine antigens, some of the tumor-associated T cell epitopes were modified to improve binding to the MHC molecules. Such modified epitopes often are more efficient inducers of T cell responses than their natural counterparts, not only because of their improved MHC binding but also because they can address a different T cell repertoire and, thereby, bypass cancer-induced anergy of the tumor-specific T cells (Trefzer et al. 2004, 2005). Such considerations have led to the concept of mimotope vaccination whereby the mimotopes, mimetics of natural T cell epitopes, are either generated by modifying known epitopes or designed de novo with combinatorial peptide libraries (Linnemann et al. 2000, 2001; Sherev et al. 2003). The potentials of such mimotopes have been demonstrated in animal models for melanoma as well as in clinical applications (Lustgarten et al. 2006).

With few exceptions, the tumor-associated antigens identified to date are normal self-antigens, and the T cell epitopes remain unaltered compared to the established genome-encoded sequences (Novellino et al. 2005; Table 19.1). The largest group of these antigens comprises differentiation antigens equally expressed by the tumor cells and the normal untransformed cells from which they originated. Examples for melanoma-associated antigens of this type are tyrosinase, tyrosinase-related proteins (TRP), MART-1/Melan A, and S100. For some tumors, embryonic antigens have been identified as tumor-associated antigens. Examples are α-fetoprotein (AFP) for hepatocellular carcinoma and the carcinoembryonic antigen (CEA) for colon carcinoma. For tumors with viral involvement, virus antigens may be targets for immune responses. The most relevant examples here are human papillomaviruses associated with cervical carcinoma. For melanoma, the latter two categories of tumor-associated antigens play no role. Tumor-specific neoantigens arising from mutations were believed to be the major focus of tumor-specific immune responses. So far, however, only a few such mutations have been found. Since they are not only specific for a particular tumor but also for the individual patient, they do not play any major role in tumor vaccine de-

Table 19.1 Tumor-associated antigens as sources of tumor-associated T cell epitopes

Class of antigen	Characteristics	Examples[a]
Differentiation antigens	Expressed by tumor cells and normal cells of the same histotype	Tyrosinase, tyrosinase-related protein, gp100, MIA
Cancer-testis antigen	Expressed by tumor cells and in testis only	MAGE family, CSAG
Embryonic antigens	Expressed by tumor cells and embryonic cells of the linage from which the tumor cells developed	CEA, AFP
Overexpressed or modified proteins	Proteins that are aberrantly highly expressed or proteins that gain antigenicity due to alterations in posttranslational modification	MUC, Her-2new
Mutated proteins	Protein with mutations leading to tumor-specific neoantigens, tumor-clone specific sequences of rearranged T cell receptors or immunoglobulins of T and B cell lymphomas	Mutated CDK-4, idiotype
Viral antigens	Virus genome-encoded proteins expressed in tumor cells transformed by oncogenic viruses	HPV E6 and E7

[a]See Novellino et al. (2005) for a comprehensive listing of tumor-associated antigens and T cell epitopes

velopment. The spectrum of tumor-associated antigens and T cell epitopes identified to date demonstrates that antitumor immune responses are autoimmune responses. Tumor-specific neoantigenicity seems to play no significant role.

19.3 Tumor-Specific T cell Responses

Several lines of evidence point at the key role of $CD8^+$ cytotoxic T cells in tumor immunity. A large number of lymphocyte depletion or reconstitution experiments in animal tumor models have proved their capacity to identify and kill tumor cells. Similarly, there are a number of in vitro experiments with human tumor and autologous $CD8^+$ T cells that prove the same point. Immunohistochemical analyses have demonstrated the presence of $CD8^+$ T cells in different tumors and, as in the case of melanoma, have correlated high frequencies of these cells in tumor infiltrates with better prognosis (Parmiani 2005; Rosenberg 2005a, b). Consequently, tumor vaccinology has, in contrast to previous efforts in vaccine development, focused on T cell vaccines. Tumor specificity of the T cells may be tested as follows. The receptor specificity can be directly visualized with fluorochrome-labeled tetramerized recombinant MHC molecules loaded with the tumor-associated T cell epitopes of interest, and then enumerated by flow cytometry. The second strategy is to trigger the cells with their cognate epitope, then permeabilize and stain them intracellularly for cytokines produced upon stimulation. The readout of numbers of specifically stained cells is done again by flow cytometry. The third technique is enzyme-linked immunosorbent spot (ELISpot), a cell-based ELISA for cytokines that allows counting of the numbers of spots on the microtiter assay plates and correlating them with the number of T cells that produce the indicator cytokine. All these techniques are in use for immune monitoring of the frequencies of tumor-specific T cells in cancer patients, and extensively for monitoring immune stimulation upon therapeutic vaccination (Whiteside 2000, 2004; Whiteside et al. 2003). Combinations of tetramer staining for T cell receptor specificity and functional assays for the capacity of the cells to respond to antigen allows, in addition to the enumeration of the tumor-specific T cells, the assessment of the frequencies of functionally intact versus anergic T cells (Trefzer et al. 2004).

19.4 Designs of Therapeutic Cancer Vaccines

T cell vaccines have to fulfill the following conditions for induction of $CD8^+$ T cell (Stuhler and Walden 1993, 1994, 2002). First, $CD8^+$ T cell induction is dependent on T cell help. Second, the

collaboration of cytotoxic precursor T cells and helper T cells has to be organized by antigen-presenting cells, most efficiently by dendritic cells (Schuler et al. 2003), that present epitopes for both T cell types on the respective MHC molecules, MHC class I for the $CD8^+$ and MHC class II for the $CD4^+$ helper T cells. Third, the antigen-presenting cells themselves need to be induced to express costimulatory molecules and immune-stimulatory cytokines (Schuler et al. 2003; Hemmi and Akira 2005). Figure 19.1 illustrates this three-cell interaction and the key molecules required for the productive induction of cytotoxic T cells. While the epitopes for the $CD8^+$ T cells are determined by the tumor cell antigenicity, epitopes or antigens for the $CD4^+$ T helper cells can be chosen freely as long as they can be presented by the same antigen-presenting cells as the epitopes for the effector cells (Stuhler and Walden 1994). Cognate interactions with T cells can already activate the antigen-presenting cells; this process can be substantially enhanced by independently triggering them via pattern recognition receptors, a group of receptors that binds generic microbial molecules and activates innate immune responses. Among the pattern recognition receptors, the so-called Toll-like receptors (TLR) have been particularly well studied (Hemmi and Akira 2005). Their ligands include CpG motifs of bacterial DNA, double-stranded viral RNA, lipopeptides, and lipoproteins that all have peculiar molecular features only found in microbes or viruses. Engagement of pattern recognition receptors by these molecules leads to activation of antigen-presenting cells involving expression of costimulatory molecules and cytokines. T cell vaccines thus should include components that trigger all the above cell types involved in induction of $CD8^+$ cytotoxic effector cells.

The early cancer vaccines often were incomplete in that only the epitopes for the effector cells were used. Lately, vaccines that comprise all three constituents have been developed. Numerous vaccine design platforms are being developed

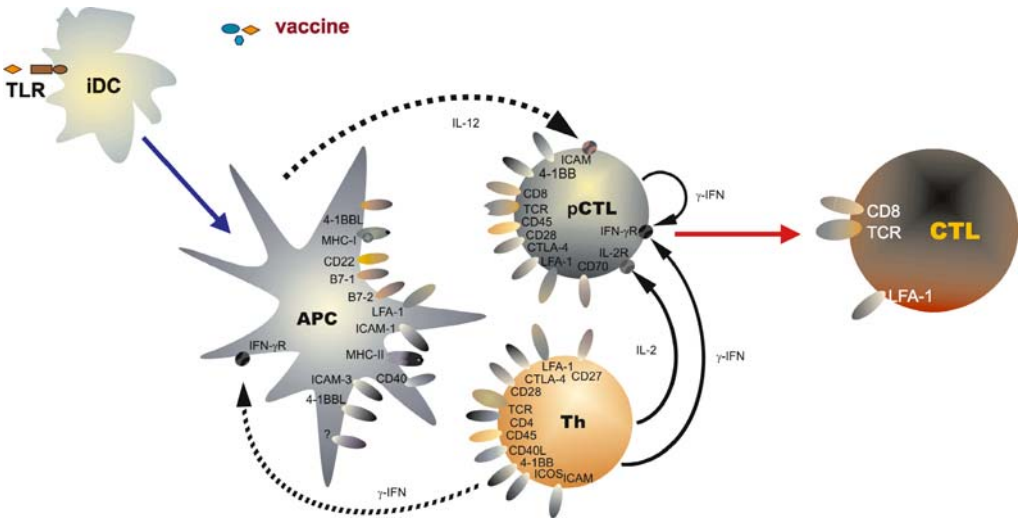

Fig. 19.1 The key cellular and molecular interaction involved in induction of cytotoxic $CD8^+$ T-cellular immune responses. The interacting cells are precursors of the cytotoxic T cells (*pCTL*), helper T cells (*Th*), and antigen-presenting cells (*APC*). The most important APC in this context are mature dendritic cells (*mDC*) that develop from immature DC (*iDC*) upon activation, e.g., through Toll-like receptors (*TLR*). The specificity of these three-cell interactions are mediated through the T cell receptors (*TCR*) of the $CD8^+$ pCTL and $CD4^+$ Th, and the presentation of their cognate epitopes by the MHC class I (*MHC-I*) and MHC class II (*MHC-II*) molecules, respectively, of the DC. The interactions of the cells and their activation are governed by arrays of adhesion, costimulatory molecules, and cytokines that can modulate the cellular responses. The products of these processes are cytotoxic effector cells (*CTL*) that can hunt for tumor cells and lyse them

and tested clinically (Walden and Sterry 2004). These platforms include mixtures of peptides and TLR agonists in free form or encapsulated in liposomes, virus-like particles and cellular formulations of various types such as allogenic tumor cells, tumor cells gene-technologically altered to express costimulatory molecules or immunestimulatory cytokines, dendritic cells loaded with tumor lysates, peptides, transfected with RNA or cDNA for tumor-associated antigens, and tumor cells fused with allogenic dendritic cells (Habib 2000; Kao et al. 2005; Parkhurst et al. 2003; Riker et al. 2006; Salcedo et al. 2005; Tumenjargal et al. 2003; Walden 2000; Whiteside and Odoux 2004). In the clinical trials conducted so far, dendritic cell-based vaccines appeared to have been most efficient in inducing tumor-specific T cell responses (Schuler et al. 2003). A major drawback of all cellular vaccines, however, is that they cannot be properly standardized. Future tumor vaccines therefore are bound to be improved formulations of synthetic vaccines (Table 2).

19.5 Lessons from Clinical Cancer Vaccination Trials

Vaccines of different designs have been tested in clinical trials accompanied by extensive monitoring of the immunological in addition to the clinical effects (Whiteside 2000). Despite the different designs, the outcomes of these trials are often very similar: the efficient induction of tumor-specific T cell responses is usually reported. In some patients, 20% and more of all circulating $CD8^+$ T cells were found to be specific for the vaccine antigens (Rosenberg et al. 2005; Trefzer et al. 2004). Several cases of clinical responses were reported. However, objective clinical responses, i.e., regression of the tumors by 50% and more, were the exceptions, and comparisons of the immunological and the clinical responses to therapeutic vaccination in cancer patients failed to demonstrate any correlation (Rosenberg et al. 2005; Trefzer et al. 2004). This conclusion can be illustrated with the results of a clinical trial with hybrid cell vaccines for the treatment of malignant melanoma at advanced stages conducted at the Department of Dermatology of the Charité (Trefzer et al. 2000, 2004, 2005; Trefzer and Walden 2003).

The hybrid cell vaccines tested in this trial were fusions of the patients' tumor cells with mature allogenic dendritic cells generated from peripheral white blood cells of healthy donors (Walden 2000). The use of mature dendritic cells that express the costimulatory molecules required for induction of cytotoxic $CD8^+$ T cells

Table 19.2 Design platforms for cancer vaccines

Platform	Examples
Cellular vaccines	Dendritic cells loaded with peptides, proteins, or tumor lysates
	Recombinant dendritic cells expressing defined antigens or whole tumor cell RNA
	Tumor cells (irradiated autologous or mixed allogenic tumor cells)
	Recombinant tumor cells expressing costimulatory molecules and/or cytokines
	Hybrid cells (fusion of autologous tumor cells and allogenic dendritic cells)
DNA/RNA vaccines	Plasmids with cDNA or defined tumor-associated antigens
	RNA of tumor-associated antigen
	Whole tumor cell RNA
Recombinant viruses	Retroviruses or adenoviruses carrying genes for tumor-associated antigens, maybe together with genes for cytokines or costimulatory molecules
Peptides/proteins	Synthetic peptides alone or together with antigens for helper T cells and/or Toll-like receptor agonists
	Tumor lysate

substitutes for TLR agonists. The allogenic MHC class II molecules are potent stimulators of T cell help, and the autologous tumor cells carry the entire range of tumor-associated antigens and T cell epitopes, some known and exploitable for immune monitoring, others unknown.

In this clinical trial with 17 patients with stage III and stage IV melanoma, 14 could be monitored for induction of tumor-specific T cell responses. Of these 14 patients, 11 responded with increased frequencies of $CD8^+$ T cells with specificity for known melanoma-associated T cell epitopes. In all these immune-responsive patients, T cells of various specificities responded, indicating that the complex antigenicity of the vaccines translated into a correspondingly complex pattern of T cell responses. Since the monitoring was done by intracellular cytokine staining and flow cytometry, the T cells detected in the peripheral blood of the patients were functionally competent cells. The frequencies of the cells with specificity for a single epitope ranged to above 4%. Despite this vigorous broad-range antitumor immune response, only 1 patient experienced a complete response which, however, is maintained even 5 years after vaccination therapy. In one case, a partial response was observed; and in 6 more, stable disease. Nine patients remained in progressive disease. There was no correlation of the clinical with the T cell responses; clinical responders—including complete response, partial remission, and stable disease patients—and progressive disease patients displayed similar immunologically responses with respect to the range of specificities and the increases in the frequencies of tumor-specific T cells upon vaccination.

The most likely cause for these discrepancies between the tumor-specific immune responses and the clinical responses is immune evasion with losses in tumor antigenicity. In fact, for all patients from whom tumors could be analyzed for antigen expression before vaccination and a progressing metastasis after vaccination, loss of several known melanoma-associated antigens—MHC class I or TAP expression, an essential component of the antigen-processing machinery—was found. Such an extent of immune escape suggests that indeed cytotoxic effector T cells with a broad range of specificities for tumor-associated antigens were induced to attack the tumor cells with the effect that only tumor cells with a broad range of antigenicity loss survived and expanded to dominate the tumors. While such an outcome testifies to the capacity of T-cellular immune responses to battle the tumor cells, it also illustrates the limits of antitumor immune responses. Notwithstanding the low rate of objective clinical responses, the median survival of the vaccinated patients was, at more than 20 months, two to three times as long as that expected from clinical experience. Such extended survival of late stage cancer patients was also reported from other trials (Tagawa et al. 2006). The side effects of therapeutic vaccination are largely restricted to slight inflammatory reactions at the sites of vaccine injection. This low degree of toxicity and the absence of severe adverse effects appear to be typical for vaccination therapies (Trefzer et al. 2005).

Since a large fraction of the melanoma-associated antigens are differentiation antigens expressed in the tumor cells and melanocytes alike, vaccine-induced autoimmunity is of concern (Chianese-Bullock et al. 2005; Ernstoff 2005). Among well over 2,000 patients that have been treated in different clinical trials worldwide with vaccines involving differentiation antigens, only very few cases of vitiligo, an indication of destruction of normal melanocytes, have been reported. In all but one case, these signs of autoimmunity were confined to small areas, sometimes linked to tumor sites but often not. Only one case of extensive vitiligo was reported, which reversed after termination of the immune treatment. So far no case of a self-perpetuating autoimmune disease was reported.

A second, equally severe mode of immune evasion is immune suppression (Whiteside 2006). For melanoma as for other tumors, immune suppression has been demonstrated to be mediated by immune-suppressive cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β (Javia and Rosenberg 2003; Ahmadzadeh and Rosenberg 2005a; Stuhler and Walden 2002). In addition, regulatory T cells are suspected to participate in silencing tumor-specific T cell responses (Ahmadzadeh and Rosen-

berg 2005b; Antony et al. 2005). In a clinical tumor-vaccination trial with mimotope vaccines, tumor cells were found to exist side-by-side with tumor-specific T cells, although they still expressed and presented the relevant antigens, and still were susceptible to cytolysis by cytotoxic T cells (Tumenjargal et al. 2003). Such cases clearly indicate a powerful disease-related immune suppression.

In summary, the outcomes of the clinical therapeutic tumor vaccination trials that were monitored for the clinical as well as immunological effects are similar in many cases:

- Different vaccine designs efficiently induce antitumor T cell responses.
- The toxicity of these vaccines is low.
- The clinical responses do not correlate with the immunological responses in that the clinical response rates are low, and responder and nonresponder patients respond similarly with enhanced frequencies of tumor-specific T cells.
- Immune evasion by losses in tumor antigenicity and/or tumor-related immune suppression are the most likely causes for discordant immune and clinical responses.

19.6 Prospects for Vaccination in Cancer Therapy

Future developments in cancer vaccinology have to take the above-discussed experiences into account. While there is ample room for improvements in vaccine designs and delivery schemes, the basic problem of immune evasion by antigen loss cannot be overcome by vaccination therapy. It needs to be defined, therefore, for which cancers, in which disease states, and possibly in which schemes of combination therapy therapeutic vaccination can best exert its potential against cancer.

Nearly all clinical trials for therapeutic cancer vaccine have been conducted with advanced-stage patients. These conditions are expected to be the most difficult to treat, and cure by therapeutic vaccination is bound to remain the exception. However, the extended survival of patients under vaccination therapy suggests its suitability for maintenance therapy where cure is not possible. Adjuvant therapy after surgery may be an important indication for tumor vaccination. A very extensive study with colon carcinoma patients who received vaccines after surgical resection of the tumors demonstrates a clear benefit for the vaccinated patients and lends strong support to the above notion.

Early stage cancers could be much more responsive to therapeutic vaccination. However, this option has not yet been tested in greater detail, and further conclusions have to await the outcomes of thorough clinical vaccination trials with early-stage cancer patients. Prophylactic vaccinations would be ideal and are in fact promising prospects for cancers with a microbial etiology. Vaccination against human papilloma viruses is currently being tested for prevention of cervical carcinoma. Although it will take many more years before the efficacy of such prophylaxis can be assessed properly, the initial investigations indicate a substantial reduction of precancerous conditions after vaccination.

For most cancers, prophylaxis is not possible and for most patients therapeutic vaccination is not a dependable option. Immune evasion by antigen loss or suppression too often quenches the antitumor effects of the vaccination. Therapy resistance, such as by antigen loss, is not restricted to immunotherapy; it is the key problem for clinical oncology in general. Given the genetic instability and heterogeneity of tumor cells as the likely basis for the selection of therapy-resistant tumor variants, future developments in oncology need to devise treatment regiments that can cope with the diversity of the tumor cells. Such new therapies could be combinations of different therapeutic principles that target the tumor cells at different pathogenically relevant sites. Therapeutic vaccination could be an important component of such combination therapies.

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20 Monoclonal and Recombinant Antibodies with T Cell Receptor-Like Reactivity

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Abstract

We will explain why major histocompatibility complex (MHC) molecules presenting peptides derived from tumour-associated antigens can be recognized not only by T cell receptors (TCR), but also by soluble proteins endowed with TCR-like reactivity. To understand how an antibody can display high affinity and specificity for a particular MHC:peptide complex, we have employed X-ray crystallography to determine the structure of a recombinant antibody, Hyb3, bound to human HLA-A1 molecules presenting the peptide EADPTGHSY that is derived from the tumour-associated antigen MAGE-A1. The results indicate that although Hyb3 recgonizes its target in a TCR-like diagonal binding mode, important differences between the two types of proteins exist that are probably due to the fact that TCR are part of a molecular assembly on the surface of effector cells, while antibodies such as Hyb3 have to carry out their function as individual molecules.

20.1 Introduction

20.1.1 Properties of an "Ideal" Tumour Target

One of the most severe problems in current tumour treatments is limiting their action to the malignant cells themselves. One advantage of immunotherapy is that it can be targeted to antigens (tumour-specific antigens, TSA) that are exclusively expressed by the malignant, but not by normal, cells. To be recognizable by cytotoxic effector cells or antibodies, TSA should be expressed on the surface of all tumour cells. Furthermore, the epitope on a given TSA should be structurally invariant to permit recognition unrestricted by polymorphisms. TSA must not necessarily be expressed at the cell surface as an intact protein, but could also be displayed in processed form, i.e. as a peptide bound to the highly polymorphic class I or II antigens of the major histocompatibility complex (MHC; the HLA complex in humans) (Horton et al. 2004). Even a peptide derived from a nuclear protein, e.g. a histone, can be presented by MHC molecules on the cell surface. The promiscuity of MHC antigens with regard to peptide binding leads to the presentation of hundreds or even thousands of different peptides by molecules of a given MHC type on a single cell. Besides peptides derived from normal cellular constituents, tumour cells can also display a variety of MHC:peptide complexes (pMHC) that are not found on any non-malignant cell (Renkvist et al. 2001).

Two major mechanisms generate TSA for presentation by MHC molecules (Boon et al. 2006): (1) point mutations occurring in tumour cells, which can lead either to protein fragments that are only presented in mutated form or to a change in an epitope of a previously presented peptide, and (2) de novo expression and proteolytic processing in tumour cells of a protein that is not expressed in normal antigen-presenting cells. Prototypical for the latter type are proteins that cannot be categorized as TSA but might be termed tumour-associated antigens and are encoded by so-called cancer-germline genes. Members of these gene families are expressed

exclusively in male germ cells and in a wide range of tumours. Since male germ cells reside within an immunologically privileged tissue in which there is no need for classical antigen presentation, they do not carry MHC molecules on their surface and therefore cannot present fragments of proteins encoded by cancer-germline genes. In contrast, tumour cells with an intact machinery of antigen presentation will display pMHC on their surface that include also peptides derived from these polypeptides. Therefore, such pMHC are very interesting targets for cancer immunotherapy studies, and have been employed in several clinical studies of therapeutic vaccinations.

Currently, the growing list of cancer-germline genes contains nearly 50 gene families with about 100 individual genes. Particularly prominent among these are the three MAGE (melanoma antigen-encoding gene) families that map to the X chromosome and comprise 21 related genes (*MAGE-A1–12*, *MAGE-B1–6*, *MAGE-C1–3*) (Chomez et al. 2001). The product of each of these genes may provide peptides that can be presented by several different HLA class I and II molecules. For example, MAGE-A1-derived peptides bind to at least 11 types of HLA-A, -B and -C and two types of HLA-DR molecules. The peptides originate from different parts of the MAGE-A1 protein: amino acids 161–169 represent the peptide (EADPTGHSY) binding to HLA-A1, while residues 230–238 provide the peptide (SAYGEPRKL) that binds to HLA-Cw3 and -Cw16. A particularly useful feature of HLA class I and II alleles is the fact that the maternal and paternal alleles are co-dominantly expressed, thereby increasing the chances of displaying a maximal number of different peptides on a given cell. In case of a tumour cell, this set of peptides will invariably lead to the expression of pMHC that can be classified as TSA. These cell surface antigens might be considered as "ideal" tumour targets.

20.1.2 Ligands for Tumour-Associated MHC: Peptide Complexes

The classical peptide-specific ligands for pMHC are TCR molecules on the surface of T cells. Tumour-specific T cells can be raised in the patients through vaccination, a strategy that so far has had only limited success (Boon et al. 2006). Another possibility is the adoptive transfer of tumour-specific T cells (Blattman and Greenberg 2004). However, the application of cellular reagents for therapy is complicated by a number of factors. The most serious drawback is that the use of a cytotoxic T lymphocyte (CTL) population is limited to the individual patient from whom it is derived; otherwise incompatibilities between the MHC types of the CTL preparation and the recipient as well as problems due to MHC restriction will arise. Furthermore, the expansion of CTL ex vivo is a difficult, time-consuming and expensive procedure.

To overcome these problems, a logical consequence would be to employ soluble TCR (sTCR), e.g. in a toxin-conjugated form, to target TSA on a tumour cell surface. Such molecules would be endowed with the same specificity as cell-bound TCR, but their use should not be individually restricted, as they could be administered to all patients whose tumour cells display the relevant pMHC. Unfortunately, the affinities of TCR are typically low, of the order of 10 μM, because T cells with TCR of higher affinity to self-MHC molecules are deleted during positive selection in the thymus (Starr et al. 2003; Rudolph et al. 2006). When a T lymphocyte recognizes its target cell, it is only the polymerization of several pMHC:TCR complexes, together with a variety of accessory molecules, that raises the avidity of the entire assembly to values allowing signal transmission into the effector cell. Therefore, in order to be useful in a soluble form, sTCR affinity has to be increased at least 100-fold. This necessitates cumbersome in vitro affinity maturation procedures of the cloned TCR α- and β-chains that may often be unsuccessful, although it has recently been demonstrated that such experiments can principally be carried out, using the 2C TCR that binds to two peptides presented by different rodent MHC class I molecules as a model reagent (Chlewicki et al. 2005).

Since antibodies react individually, mostly to unprocessed foreign antigens such as glycoproteins and polysaccharides, their affinity has to be much higher than that of TCR molecules: it is typically in the nanomolar range and may even

reach picomolar values (Winter and Milstein 1991). However, other than TCR, antibodies are not normally made to bind to pMHC, although this may occasionally happen (van Leeuwen et al. 1979). As antibodies with TCR-like reactivity should have a wide range of potentially useful applications, including direct visualization and quantification of pMHC as well as targeted delivery of toxins and drugs, considerable effort has been directed at producing such reagents. However, these attempts have largely been unsuccessful. Over the past 30 years, only a handful of monoclonal antibodies (mAb) have been selected that react with a variety of pMHC (see, e.g., Abastado et al. 1989; Aharoni et al. 1991; Murphy et al. 1992; Uchańska-Ziegler et al. 1993; Urban et al. 1994; Wang et al. 1994; Eastman et al. 1996; Porgador et al. 1997; Dadaglio et al. 1997; Rehm et al. 2000). To our knowledge, none of these mAb has found its way into the clinic so far.

20.1.3 Recombinant Antibodies Against pMHC

Recombinant antibodies (rAb) produced in vitro offer a possible solution to these difficulties. They are available in different formats, e.g. as Fab (V_H, C_H1 combined with V_L, C_L) or scFv (V_H covalently linked to V_L) and are originally part of large libraries with more than 10^8 different species of rAb that are mostly displayed either on the surface of bacteriophage (Smith 1985) or ribosomes (Hanes et al. 2000). There are various ways in which these libraries can be constructed, but they are usually semi-natural. The selection of specific rAb from a library involves several cycles of binding all components of the library to a target, e.g. a pMHC, eluting the bound reagents, followed by their amplification. Finally, a single or a small number of rAb emerge from the selection cycles; after cloning, individual rAb with the desired specificity can be obtained. This methodology is also very flexible: for example, reagents with an undesirable cross-reactivity can be eliminated already at an early stage using counter-selection steps.

Within the last few years, a considerable number of rAb have been produced against pMHC that carry fragments of TSA (Chames et al. 2000; Lev et al. 2002; Denkberg et al. 2002; Cohen et al. 2003; Held et al. 2004). The first of these, Fab-G8, exhibited specificity for the complex of HLA-A1 presenting a MAGE-A1-derived peptide (residues 161–169, EADPTGHSY), but only with moderate affinity (K_d=250 nM) (Chames et al. 2000). This reagent was subjected to a phage display-based affinity maturation process (reviewed by Wark and Hudson 2006) to identify clones with higher affinity but identical specificity. The resulting rAb, Fab-Hyb3 (Hyb3 in short), differs from Fab-G8 by only two amino acid substitutions in its heavy chain [both of them in complementarity-determining region 3 of the heavy chain (CDR-H3)], while the Fab-G8 light chain had been replaced with another by L chain shuffling, leading to the exchange of 22 residues within the V_L domain, more than half of them outside of the CDR loops (Chames et al. 2002). These differences between Fab-G8 and Hyb3 result in an 18-fold higher affinity (K_d=14 nM), while binding studies showed that Hyb3 retains its specificity for HLA-A1:MAGE-A1 complexes. The affinity of Hyb3 is about 1,000-fold higher than that of typical αβTCR.

The structural basis of MHC-restricted peptide specificity of αβTCR has already been illustrated by several examples of crystallized TCR fragments. A total of 24 pMHC:TCR structures are currently known, among them one structure of a γδTCR in complex with a non-classical rodent class I molecule (without a ligand in the binding groove; reviewed by Rudolph et al. 2006). However, no structure of a mAb or rAb in complex with a pMHC had been determined. It was therefore unclear how high affinity and specificity of the HLA-A1:MAGE-A1:Hyb3 interaction would be achieved. Furthermore, it was not known whether the binding mode of pMHC-restricted antibodies shares any structural features with those of natural ligands such as TCR, or pMHC ligands on natural killer (NK) cells.

Therefore, we determined the structure of the rAb Hyb3 bound to its pMHC target, the HLA-A1:MAGE-A1 complex, employing X-ray crystallography (Hülsmeyer et al. 2005). In the following we emphasize the similarities and differences in the pMHC binding modes exhibited by Hyb3, TCR and inhibitory receptors on NK cells.

20.2 Materials and Methods

All materials and experimental procedures have already been described (Hülsmeyer et al. 2005). Briefly, the HLA-A1 heavy chain and β_2-microglobulin were separately expressed as inclusion bodies in *Escherichia coli*, and the solubilized proteins were then mixed with the MAGE-A1 peptide (obtained by solid phase synthesis), followed by refolding of the ternary complex and its purification by size-exclusion chromatography. Hyb3 was also expressed in *E. coli* and purified as described for Fab-G8 (Chames et al. 2000). Purified aliquots of both protein preparations were then mixed (mass ratio pMHC:Hyb3 1:0.8) and incubated, and the resulting HLA-A1:MAGE-A1:Fab-Hyb3 (AMF) complex separated from uncomplexed protein by size-exclusion chromatography. After concentration to 15–20 mg/ml, the complexes were used for crystallizations.

Following optimization of crystallization conditions, crystals suitable for X-ray diffraction were obtained, and a native data set at 2.15 Å resolution was collected at the European Synchrotron Radiation Facility (Grenoble, France). All measurements were performed at 100 K. Processing of the data, structure determination by molecular replacement and preparation of the figures have been described (Hülsmeyer et al. 2005). The atomic co-ordinates and structure factors (code 1W72) are available from the Protein Data Bank (http://www.rcsb.org/).

20.3 Results and Discussion

20.3.1 Structural Properties of the HLA-A1: MAGE-A1 Complex

As expected, the overall topography of the HLA-A1:MAGE-A1 complex is very similar to those of previously determined pMHC structures (Fig. 20.1). The high resolution obtained (2.15 Å) permits the modelling of the antigenic peptide (EADPTGHSY, p1-p9) into the electron density without ambiguity. The middle section (p4–p7) is bulging slightly out of the peptide binding groove, which is usually, but not always, the case for nonameric peptides (Hülsmeyer et al. 2004; Fiorillo et al. 2005). Several water molecules contribute to the firm anchoring of the peptide to the α1- and α2-helices and the floor of the binding groove. There is only one side chain (p8Ser), which protrudes into the solvent. This is not the case for p4Pro and p7His, although both residues are clearly solvent-accessible.

20.3.2 Interactions Between HLA-A1: MAGE-A1 and Hyb3

The most remarkable feature regarding HLA-A1: MAGE-A1 recognition by Hyb3 is the unequal use of its CDR loops by the rAb. While nearly all TCR employ all of their six CDR for this purpose, only the CDR loops of the heavy chain and the CDR-L3 of Hyb3 contact the pMHC directly, through hydrogen bonds or van der Waals' contacts of less than 3.5 Å (Fig. 20.2). CDR-H1 recognizes exclusively residues of the α1-helix, and CDR-L3 interacts with both α-helices of the HLA-A1:MAGE-A1 molecule, whereas CDR-H2 and CDR-H3 exhibit an intermediate position: they contact the peptide as well as the α1- and, in case of CDR-H2, also the α2-helix. CDR-L1 and CDR-L2 contribute only marginally, as the distances to the pMHC are, in each case, larger than 3.8 Å.

Clearly, the most important interactions between Hyb3 and pMHC are mediated by CDR-H3. We have already given a detailed account of all relevant contacts (Hülsmeyer et al. 2005; see also Fig. 20.2) and will only briefly summarize the major findings. CDR-H3 contains many aromatic amino acids (^{98}FHYYYY^{100C}, Fig. 20.2) that together form a clamp-like structure over the α1-helix. Two amino acids of this loop (HisH99, TyrH100A) are located on the internal, while three (PheH98, TyrH100B and TyrH100C) are on the external side of the peptide-binding groove.

The absence of solvent molecules from this part of the structure indicates strict shape complementarity between the two molecules. HisH99 and TyrH100A are not only in contact with the α1-helix, but are also hydrogen-bonded to the peptide, either directly or indirectly, via buried water molecules. In addition, the conformation of the CDR-H3 loop is supported by an interaction between CDR-H1 and the beginning of the α1-helix (Fig. 20.2), because AspH30

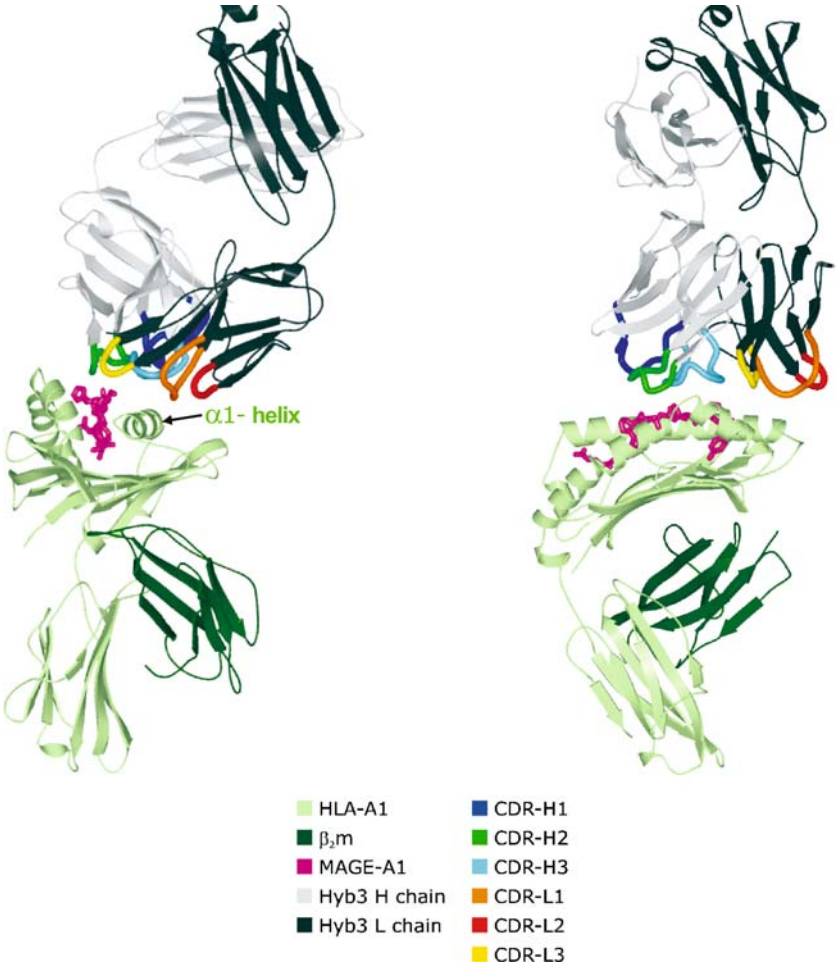


Fig. 20.1 Overall topography of the AMF complex. Views of the HLA-A1:MAGE-A1:Hyb3 complex viewed along the peptide axis, with the peptide C-terminus in front (*left*) or rotated by 90° (*right*; peptide N-terminus on the *left*). The colour code refers also to the complementarity determining regions of the heavy chain (CDR-H1,2,3) and of the light chain (CDR-L1,2,3) of Hyb3 (from Hülsmeyer et al., 2005)

and AspH31 form hydrogen bonds with Arg65 of HLA-A1, stabilizing it such that PheH98 within the CDR-H3 loop can pack hydrophobically against the aliphatic section of Arg65. These interactions bury more than 100 Å of Arg65 surface area, thereby providing a substantial contribution to the affinity of Hyb3 towards the HLA-A1:MAGE-A1 complex.

Another important contribution to the interaction between the two molecules comes from CDR-L3, which spans across the end of the binding groove, near the C-terminus of the peptide (Fig. 20.2). CDR-L3-mediated interactions also include a salt bridge from AspL95A to Lys146 of the HLA-A1 molecule, while the sole contact with the central portion of the α2-helix (Gln155) is formed by GlyH55 of the CDR-H2 loop. Only residues of CDR-H2, CDR-H3 and CDR-L2 contact the peptide, either directly or via interconnecting water molecules, and in just one case (GlyH55 to p7His) is a side chain of the peptide involved in the interaction. The relatively high

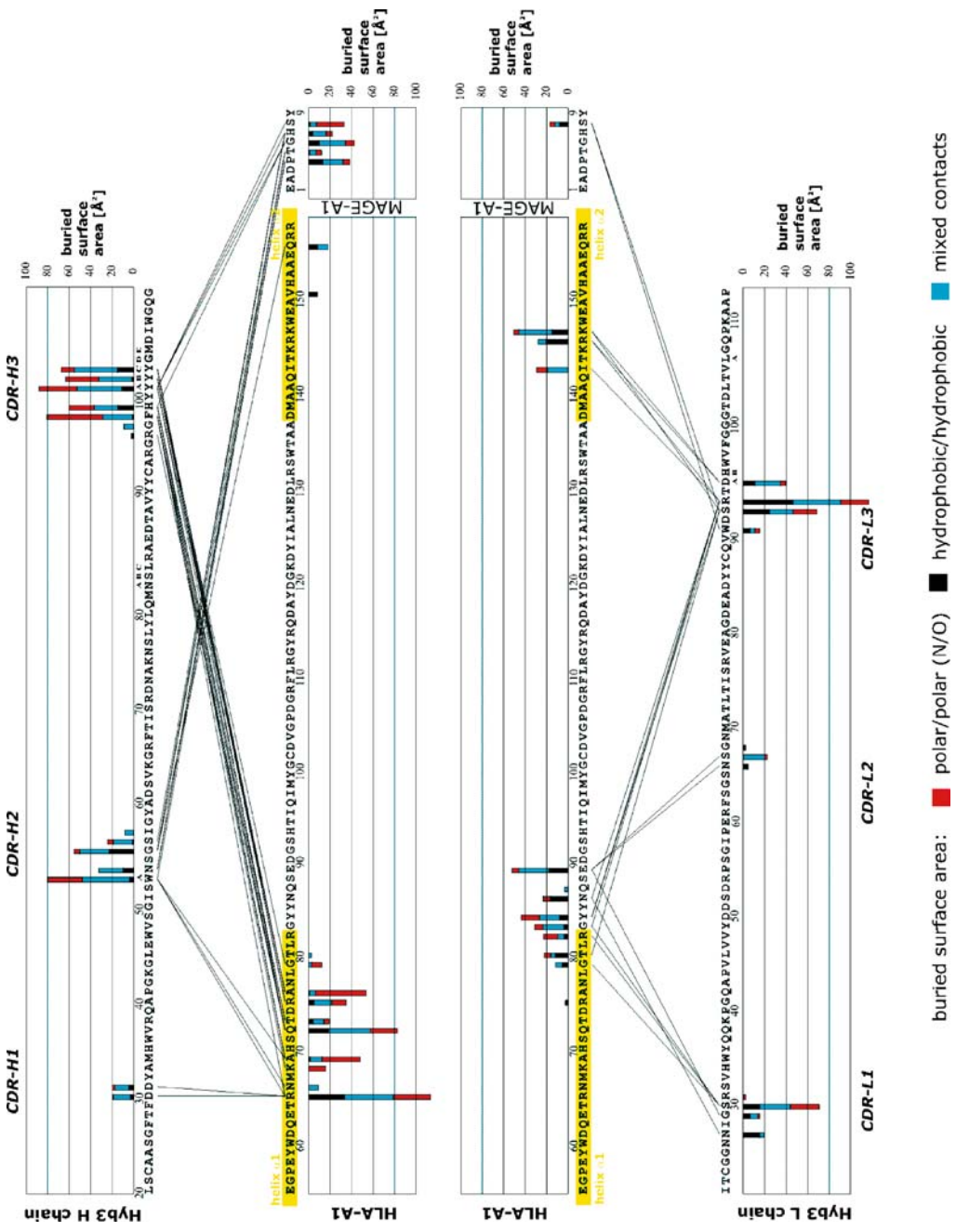


Fig. 20.2 Interaction of the HLA-A1:MAGE-A1 complex with Hyb3. The linkage plot shows the amount of surface area (in Å² per residue; in case of Hyb3, numbering follows the Kabat database) buried upon complex formation. Polar/polar contact areas (N/O) are shown as *red bars*, hydrophobic/hydrophobic contact areas (all other atoms) as *black bars* and mixed contact areas between polar atoms of one molecule and apolar atoms of the other as *blue bars*. Residues which occlude each other are connected by *straight lines*. The location of the CDR loops is indicated. α-Helices of the HLA-A1 molecule are coloured *yellow* (from Hülsmeyer et al., 2005)

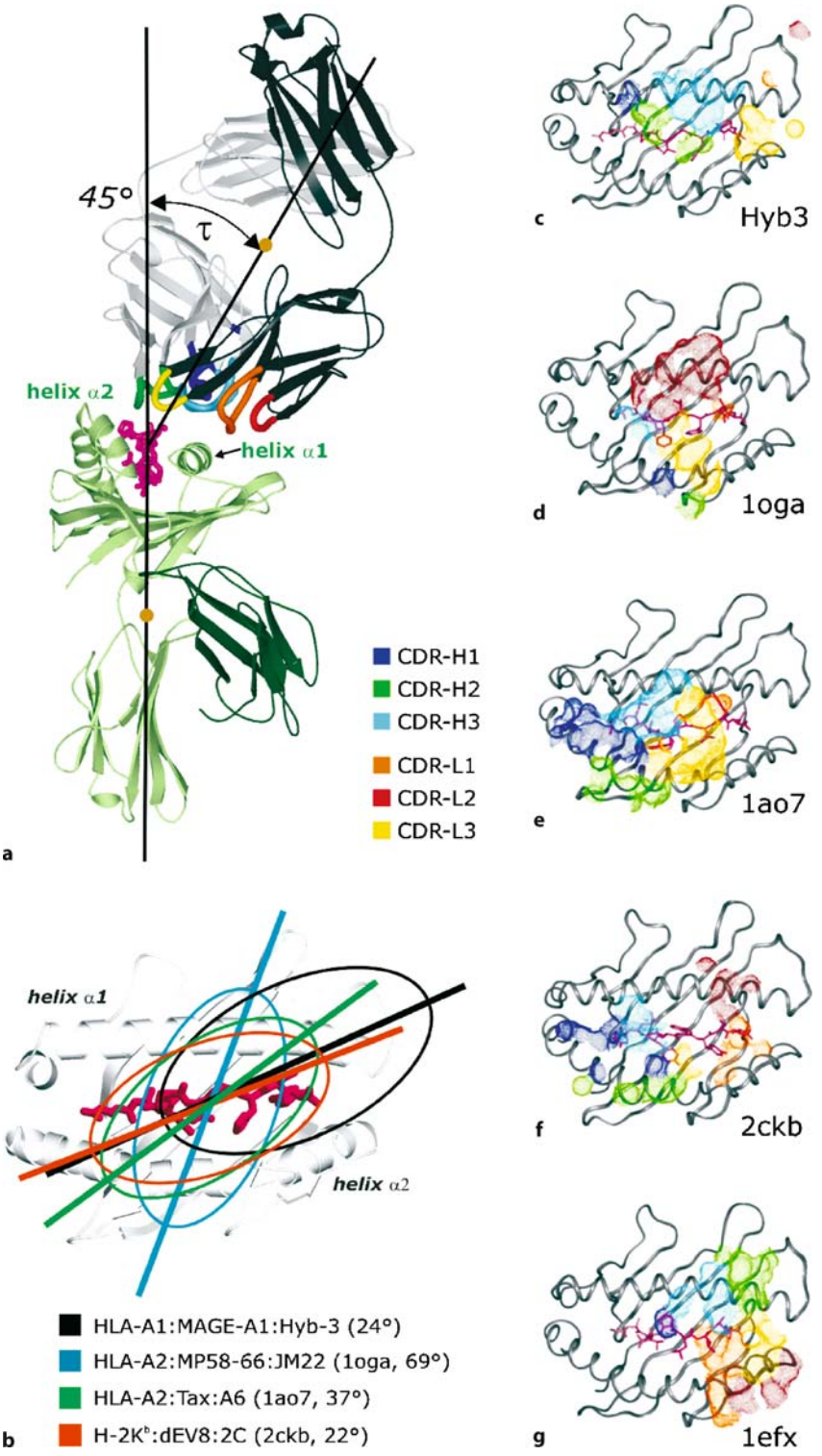
resolution of the AMF data set was sufficient to permit the localization of 653 water molecules. Six of these are completely buried in the AMF interface and contribute considerably to the high shape complementarity (see the following section).

20.3.3 Comparison of the Hyb3 Binding Mode with That of Natural pMHC Ligands

A detailed comparison with other pMHC:ligand complexes demonstrates that besides similarities, striking differences exist between the binding modes of natural ligands [TCR, killer inhibitory immunoglobulin-like receptors (KIR)] and Hyb3. For example, the calculation of a tilting angle (τ) between pMHC and TCR, as carried out by Hülsmeyer et al. (2005), demonstrates that the values for typical TCR are between 7° and 14°, indicating that the lines passing through the centres-of-mass of these TCR and the respective pMHC are nearly parallel to each other. In contrast, the first crystal structure of a γδTCR (G8) in complex with the non-classical MHC molecule T22 shows this TCR to recognize its target in a severely tilted orientation that has never been observed for any other TCR (Adams et al. 2005). For the AMF complex, the corresponding value is 45° (Fig. 20.3a), showing that the pMHC binding modes of Hyb3 and the TCR G8 are more similar to each other than to those of typical TCR. It is likely that this seemingly greater promiscuity in engagement of a pMHC is a reflection of two different facts. For one, the target of G8, the non-classical T22 molecule, is recognized by the TCR in peptide-devoid state, so that there is no reason to engage in a peptide-dependent binding mode. On the other hand, the recognition of HLA-A1:MAGE-A1 by Hyb3 is not restrained by the necessity to signal into the interior of an effector cell, without which pMHC:TCR interactions would not be meaningful. The large tilting angle τ between pMHC and rAb is probably a consequence of more interactions of Hyb3 with the α1- than with the α2-helix of this pMHC. However, it may well be that additional structures of antibodies with TCR-like specificity will reveal other modes of pMHC recognition.

Since the determination of the first structure of a pMHC:αβTCR complex had shown a diagonal binding of the TCR relative to the long axis of the binding groove on the pMHC target, it was thought that this diagonal positioning (range in most cases 22°–40°, although there are now several TCR with docking angles up to about 70° and two with a nearly orthogonal binding mode) constitutes a hallmark of pMHC recognition by these ligands (Fig. 20.3b; Rudolph et al. 2006). In nearly all cases, the TCR-Vα domain is located above the N-terminal portion of the bound peptide, while the TCR-Vβ domain occupies the area above the C-terminal half of the peptide (Fig. 20.3d–f). The diagonal TCR binding mode leads to a positioning of the two CDR3 loops above the peptide, while the CDR1 and CDR2 loops contact the α1- and α2-helices of the pMHC. However, it has recently been found that not all TCR engage in this pMHC docking mode. For example, the autoimmune Ob1A12 αβTCR recognizes the complex of HLA-DR2 and a self-peptide in an unconventional fashion, as the centre of its reactivity is shifted towards the N-terminal portion of the peptide, "neglecting" the C-terminal portion and the surrounding HLA-DRα- and β-chain residues. Furthermore, the binding orientation (84° angle) of Ob1A12 on the pMHC surface is nearly orthogonal (Hahn et al. 2005). The structure of G8 reveals even more unusual features, as this γδTCR recognizes its target in a highly unusual docking mode (87° angle). This leads to loss of nearly all contacts of the Vγ domain, whose CDR1 and CDR2 loops are not employed at all in target recognition. In contrast, Vδ of G8 engages in more than 100 contacts with the T22 molecule (Adams et al. 2005).

Like most TCR, Hyb3 recognizes its target in a diagonal binding mode as well (Fig. 20.3b). The 24° angle is nearly identical to those with which the murine 2C TCR recognizes the H-2K^{b}:dEV8 (22°), the H-2K^{b}:SIYR (23°) and the H-2K^{bm3}:dEV8 (23°) complexes (Rudolph et al. 2006). However, the Hyb3 footprint on the pMHC (Fig. 20.3c) differs from those of typical TCR (Fig. 20.3d–f) as it is severely shifted towards the α1-helix and that end of the binding groove which occupies the peptide C-terminus. The only two available structures of KIR in complex with a pMHC (both with HLA-C alleles) (Boyington et


45°
τ
helix α2
helix α1
CDR-H1
CDR-H2
CDR-H3
CDR-L1
CDR-L2
CDR-L3
a
c
Hyb3
d
1oga
e
1ao7
helix α1
helix α2
HLA-A1:MAGE-A1:Hyb-3 (24°)
HLA-A2:MP58-66:JM22 (1oga, 69°)
HLA-A2:Tax:A6 (1ao7, 37°)
H-2K^b:dEV8:2C (2ckb, 22°)
b
f
2ckb
g
1efx

◄ **Fig. 20.3a–g** Comparison of Hyb3, TCR and KIR binding modes with pMHCs. **a** The definition of the tilting angle τ of Hyb3 on top of the HLA-A1:MAGE-A1 complex. The lines passing through the centres-of-mass (*red circles*) of the two interaction partners and the centre of the peptide (C_α at p5) are used to calculate the angle τ between these two lines. Typically, small τ angles (7°–14°) are found in pMHC:TCR complexes (see text). **b** Comparison of the binding orientation of Hyb3 in the AMF complex with that of selected pMHC:TCR complexes. The angles are calculated from an axis between the disulphide bridges of the V_L and V_H domains of Hyb3 (or V_α and V_β of a TCR) and the axis spanning the N- and C-termini of the cognate peptide. The *ellipses* refer to the bodies of the Hyb3 or TCR molecule, respectively, schematically projected on the binding groove. TCR binding interactions are typically focussed on the centre of the peptide and the binding groove, while they are shifted towards the end of the α1-helix with Hyb3. **c–g** Footprints of the CDR loops of Hyb3, the V_α and V_β chains of three TCRs and of the interacting loops of a KIR on pMHCs. Surfaces of atoms within 4 Å distance to the pMHC are shown in colours corresponding to the respective CDR loops (see *legend*). The view is from Hyb3, the TCR or KIR onto the peptide-binding site of the pMHC. The MHC binding groove is shown as C_α ribbon (*grey*), the peptide in stick representation (*red*). **c** Hyb3 footprint, colour-coded according to the respective CDR loops and following Fig. 1. **d–f** TCR footprints shown for comparison: PDB ID 1oga (**d**), 1ao7 (**e**) and 2ckb (**f**). Colour coding according to the respective CDR loops of the TCR V_α- and V_β-chains as for the Hyb3 CDR-H and CDR-L loops. **g** Footprint of KIR2DL2 on HLA-Cw3 (PDB ID 1efx) with colouring in analogy to Hyb3 CDR loops: *dark blue* for residue 20–23, *green* for 43–46, *light blue* for 67–74, *orange* for 103–108, *red* for 130–135 and *yellow* for 182–184. Every type of ligand has found its individual solution for pMHC recognition (from Hülsmeyer et al., 2005)

al. 2000; Fan et al. 2001) show that these ligands recognize the area above the peptide C-terminus and the surrounding α1- and α2-helices in a roughly orthogonal binding mode (Fig. 20.3g).

The surface area buried by TCR upon contact with a pMHC varies over a wide range (1,239 Å–2,020 Å) and has no obvious connection with the number of CDR loops (nearly always six) that participate in the interaction (Rudolph et al. 2006). This is exemplified by the γδTCR G8 which buries 1,936 Å (Adams et al. 2005) and by Hyb3 which covers 1,930 Å (Hülsmeyer et al. 2005), although both ligands utilize only four of their six CDR loops for engaging with the respective target. The value for Hyb3 is considerably larger than those of typical protein:antibody interactions, which are about 1,500 Å (Jones and Thornton 1996). Clearly, a comparison of these values for TCR, KIR, antibodies in general, and Hyb3 in particular, reveals that the buried surface area is not necessarily a reflection of high affinity of the interaction between the two binding partners. This point is already evident from the interactions of three pMHC with the 2C TCR (buried surface area about 1,800–1,850 Å, K_d about 55–80 μM), the scBM3.3 TCR with H-2K^b:pBM1 (1,239 Å, K_d=2.6 μM) (Rudolph et al. 2006), and the AMF complex (1,930 Å, K_d=14 nM) (Hülsmeyer et al. 2005). With a value of around 1,500 Å, the surface area buried by a KIR is also within the range of that covered by typical TCR (Boyington et al. 2000; Fan et al. 2001).

Furthermore, when the median value of the shape complementarity statistic (S_c), a measure of the geometric match at protein–protein interfaces (Lawrence and Colman 1993), is considered, there is again no clear-cut relationship between the utilization of CDR loops, the affinity of the interaction, and the S_c value. The two structures of KIR complexed with HLA-C exhibit S_c values of 0.69 and 0.71, respectively (Boyington et al. 2000; Fan et al. 2001), in contrast to pMHC:TCR interactions for which widely varying S_c values (0.41–0.75) can be calculated (Rudolph et al. 2006). For example, the three different complexes of the 2C TCR with murine pMHC reveal S_c values of 0.41, 0.49 and 0.62, although in all cases the same TCR, twice the same peptide, and twice the same H-2K antigen are involved. For the T22:G8 interaction, the S_c value is 0.66 (Adams et al. 2005), and for the interaction between Hyb3 and HLA, this value is 0.69 excluding, and 0.72 including, water molecules in the interface (Hülsmeyer et al. 2005).

These comparisons between natural ligands and Hyb3 demonstrate that it seems impossible to identify a single factor—such as the tilt with regard to the main axes of the binding partners, the binding mode (diagonal or orthogonal), the buried surface or the shape complementarity—that is responsible for the vastly improved affinity of Hyb3. It is therefore particularly remarkable that many αβTCR directed against MHC class I molecules and Hyb3 share certain conserved fea-

tures regarding their pMHC contacts (Rudolph et al. 2006; Hülsmeyer et al. 2005). There are two prominent residues involved in αβTCR docking, at heavy chain position 65 (α1-helix) and 155 (α2-helix), which are also recognized by Hyb3 (Arg65 and Gln155 in case of HLA-A1). Two further residues at positions 69 and 72 on the α1-helix are overrepresented in pMHC class I: TCR interactions and are clearly involved in contacting Hyb3 residues as well (Ala69 and Gln72 in case of HLA-A1; Fig. 20.2). As neither T cells bearing these TCR nor Hyb3 are the results of immunization procedures, it appears likely that these solvent-exposed residues constitute particular "docking points" that provide the interaction between many pMHC and various ligands with the affinity needed to be productive. In the case of Hyb3, Arg65 and Gln72 are those pMHC residues that provide by far the highest amount of surface area that is buried upon contact with the ligand, and also Ala69 contributes considerably to the interaction (Fig. 20.2). A possible explanation for an involvement of these strategic residues also in pMHC:TCR interactions may be the hypothetical two-step mechanism of TCR docking, which involves an initial encounter of a TCR with the α1- and α2-helices, followed by recognition of peptide residues (Wu et al. 2002). Clearly, residues at the positions 65, 69, 72 and 155 of the MHC heavy chain could mediate the first step and might also be involved in positive selection of T cells within the cortical epithelium of the thymus (Rehm et al. 2000). As no comparable binding studies have so far been carried out with Hyb3 and the HLA-A1:MAGE-A1 complex, it is unknown whether similar considerations apply also in this case.

20.4 Conclusions

The purpose of our study was to understand the structural basis for the high degree of specificity and affinity of Hyb3 towards its pMHC target, and compare the binding mode of this pMHC-restricted rAb to those of natural ligands. Specificity is achieved by many close interactions to the peptide backbone that would be broken if another peptide were presented. For example, the closely related MAGE-A3 peptide (EVDPIGHLY, differing from MAGE-A1 only at positions p2, p5 and p8) is not recognized by Hyb3 in the context of HLA-A1. Binding assays with Hyb3 and the MAGE-A1 peptide with all possible amino acid substitutions at p2, p5 and p8 indicate that the positions p5 and p8, but not p2, are important for Hyb3 binding (B. Uchańska-Ziegler, unpublished observations). In case of p8, only amino acids with smaller side chains than the naturally occurring Ser allow high-affinity Hyb3 binding. These results are in excellent agreement with the structural findings, as p2 is not contacted by Hyb3, and p8 appears more important for the pMHC: Hyb3 interaction than p5 (Hülsmeyer et al. 2005). The HLA class I allele specificity of Hyb3 is not known in detail, but HLA-A1 is distinguished from nearly all other class I heavy chains by the presence of Ala76 (www.ebi.ac.uk/imgt/hla/). This residue participates markedly in the pMHC: Hyb3 interaction (Fig. 20.2) and might thus fulfil a role in assuring allele specificity.

The high affinity of the interaction is probably a consequence of several factors. In analogy to other affinity-improved antibodies (Li et al. 2003; Sundberg et al. 2003; Zahnd et al. 2004), it appears likely that burial of increasing amounts of apolar surface, in particular at the HLA-A1: MAGE-A1 and Hyb3 interface periphery, and improved shape complementarity must be the key structural elements that contribute to the higher affinity of Hyb3 in comparison to the original Fab-G8. Since a structure of the pMHC: Fab-G8 complex is not available, we cannot verify whether the in vitro maturation process in fact resulted in an increase of buried non-polar surface, although some mutations that distinguish Fab-G8 from Hyb3 occur in the periphery of the interface. The affinity increase caused by L chain mutations seems to derive mainly from indirect effects that stabilize the conformations of the CDR loops (Hülsmeyer et al. 2005).

Differences and similarities between the recognition of pMHC by TCR and antibodies have been noted previously (Stryhn et al. 1996; Messaoudi et al. 1999), and a systematic comparison of the binding of several phage display-derived rAb and TCR recognizing the Tax or M1 peptide in the context of HLA-A2 and a variety of heavy chain mutants has also been carried out (Biddison et al. 2003). The latter studies demonstrated that the binding of rAb as well as TCR is negatively affected by exchange of residues in the

vicinity of the N-terminal half of the peptide. Remarkably, mutations of the HLA-A2 heavy chain at Arg65 and Lys66 (Tax peptide) or Lys66 and Gln155 (M1 peptide) reduced or even abolished TCR and rAb reactivity, implying an overlap between the footprints of TCR and Fab (Biddison et al. 2003). We have already pointed out that the residues 65 and 155 of the MHC class I heavy chain play a crucial role in pMHC:TCR interactions as well as in the binding of Hyb3 to its target. The results from a study of two mAb directed against H-2K^b molecules presenting an ovalbumin-derived peptide indicate that the mAb and the α-chain of the single TCR analysed share parts of their footprints, but also that these footprints are similar to those of KIR on HLA-C:peptide complexes (Boyington et al. 2000; Fan et al. 2001). Since only the structure of the AMF complex has so far been determined, it is currently impossible to carry out detailed, structure-based comparisons with other MHC:antibody complexes to derive more general conclusions.

With regard to the issue of TSA and their recognition by truly tumour-specific ligands, we believe that mAb and in particular rAb are very promising reagents, specifically when they are compared with ex vivo cell-based procedures and sTCR. In contrast to cell-based therapies, their application is not limited to individual patients, and rAb can be genetically modified relatively easily to achieve the high affinity required for diagnostic and therapeutic interventions. The structure that we have determined provides a number of answers concerning rAb specificity and affinity, but it would be highly desirable to obtain more structures of rAb in complex with pMHC to evaluate to what extent our findings can be generalized. In addition, further library-derived ligands that do not belong to the immunoglobulin superfamily, such as ankyrin repeats (Binz et al. 2003), but exhibit high affinity and selectivity for tumour-specific pMHC as well, might be selected in the future.

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21 New Molecular Therapy Targets in Acute Myeloid Leukemia

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Abstract

Despite improvements to acute myelogenous leukemia (AML) therapy during the last 25 years, the majority of patients still succumb to the disease. Thus, there remains an urgent need for further improvements in this field. The present chapter focuses on exciting areas of research in the field of AML therapy, including promising results with regards to recent improvements in our understanding of angiogenesis, tyrosine kinase signaling, farnesylation, cell cycling, modulation of gene expression, protein degradation, modulation of intracellular proteins, apoptosis, metabolism, and the possible retargeting of oncogenic proteins.

21.1 Introduction

Acute myelogenous leukemia (AML) is an aggressive disorder that leads to rapid death due to bone marrow insufficiency if left untreated. With aggressive chemotherapy, sometimes followed by allogeneic hematopoietic stem cell therapy, long-term remission rates of up to 30%–40% can be achieved in younger patients. However, the median age of AML exceeds 60 years and treatment of elderly patients is associated with substantial toxicity and a poor final outcome. Thus, new therapeutic approaches are needed for the treatment of AML to improve both efficacy and the toxicity profile of AML therapy.

The efficacy of molecular targeted therapy in AML could be demonstrated by the therapy of the translocation (15;17)-positive acute promyelocytic leukemia (APL) with all-*trans* retinoic acid (ATRA) (Redner et al. 1999) and by the approval of gemtuzumab-ozogamicin (Mylotarg), a conjugate of an anti-CD33 antibody covalently linked to calicheamicin, for the treatment of relapsed AML (van der Velden et al. 2001). Other potential mechanisms that have been subjected to targeting in AML to date are: angiogenesis, tyrosine kinase signaling, farnesylation, cell cycling, modulation of gene expression, protein degradation, modulation of intracellular proteins, apoptosis, metabolism, and retargeting of oncogenic proteins. It has to be noted that due to the complexity of this issue, strategies involving the immune response in AML are not included; for a detailed description the reader is referred to other literature (Robin et al. 2005).

21.2 Inhibition of Angiogenesis

21.2.1 Introduction

The role of increased angiogenesis in AML has been elucidated since the beginning of this century (Hussong et al. 2000; Aguayo et al. 2000; Padro et al. 2000). Bone marrow infiltrated by AML blasts exhibits an increased microvessel density compared to normal bone marrow (Hussong et al. 2000; Padro et al. 2000) and microvessel density decreased in response to induction chemotherapy (Padro et al. 2000). Hypothetically, growth factors expressed by AML blasts stimulate the growth of vascular endothelial cells and vice versa. This hypothesis is supported by findings that the proangiogenic growth factor vas-

cular endothelial growth factor (VEGF) as well as the VEGF receptors 1 (VEGFR1/Flt-1) and 2 (VEGFR2/KDR) are expressed in AML cells, and human endothelial cells exhibit an increase in the expression of the myelogenous growth factor granulocyte-macrophage colony stimulating factor (GM-CSF) upon stimulation with VEGF (Fiedler et al. 1997).

21.2.2 Thalidomide

Besides other mechanisms of action, antiangiogenic effects have been shown for thalidomide in a mouse model (Kenyon et al. 1997; D'Amato et al. 1994). This antiangiogenic effect is probably mediated by an inhibition of endothelial cell proliferation (Moreira et al. 1999). We conducted a phase I/II-study with thalidomide in 20 patients with refractory AML or patients not eligible for intensive induction chemotherapy (Steins et al. 2002). Out of these 20 patients, 4 reached a partial remission (PR) and 1 patient a hematological improvement without fulfilling the criteria of PR. This response correlated with a decrease in bone marrow microvessel density and with the decrease of serum level of basic fibroblast growth factor (bFGF). Meanwhile, we observed 1 complete remission (CR) lasting for more than 20 months. However, no improvement of outcome could be shown when thalidomide was added to conventional induction chemotherapy in high-risk AML patients (Cortes et al. 2003). Another phase II study showed 3 responses out of 16 patients with advanced AML to thalidomide, with significant toxicity at doses of higher than 400 mg daily (Thomas et al. 2003). It is yet unknown whether analogs such as lenalidomide show a superior efficacy and toxicity profile than thalidomide in AML patients.

21.2.3 Bevacizumab

The humanized anti-VEGF antibody bevacizumab (Avastin) has promising efficacy in a variety of solid tumors in combination with cytotoxic chemotherapy (Glade-Bender et al. 2003). One study combined an induction therapy consisting of medium-dose cytarabine and mitoxantrone followed by the administration of Bevacizumab. In 48 high-risk AML patients, a CR was achieved in 33% of the patients (Karp et al. 2004). However, no comparison to a control group or a historical control was performed. In addition, a decrease in microvessel density after the administration of bevacizumab could be shown in 8 out of 11 evaluated patients, and VEGF serum levels decreased in 14 out of 15 patients evaluated after bevacizumab, showing a potential antiangiogenic effect of bevacizumab. However, controlled studies testing the efficacy of bevacizumab in AML are still missing.

21.2.4 VEGFR2 Inhibition

VEGF signaling is mediated by several tyrosine kinase receptors, among them VEGFR1 and VEGFR2 (Shinkaruk et al. 2003). VEGFR2, but not VEGFR1, is overexpressed in human untreated AML bone marrow samples and the expression correlates with the bone marrow microvessel density (Padro et al. 2002). Upon induction of a CR, VEGFR2 levels decreased to normal range. Stimulation of primary leukemic cells by VEGF led to an activation of growth and migration, and this activation was mediated through VEGFR2 (Dias et al. 2000). Therefore, blocking of VEGFR2-signaling by neutralizing antibodies or small inhibiting molecules might be a potential therapeutic approach.

Treatment of 42 advanced-stage AML patients with SU5416, an oral receptor tyrosine kinase inhibitor, showed a PR in 7 cases and 1 CR (Fiedler et al. 2003). In addition, we reported a prolonged therapeutic effect of this compound in another AML patient (Mesters et al. 2001). Even though this inhibitor is not specific for VEGFR2 but also inhibits VEGFR1, c-kit, and FMS-like tyrosine kinase 3 (Flt3), the following results indicated that at least in part this effect could be mediated through VEGFR2-signaling. First inhibition of VEGFR1 had no effect on the proliferation of AML blasts. Second, patients with overexpression of VEGF showed a significantly better response to treatment. Third, among 7 patients with low or no expression of VEGFR2 on leukemic blasts (less than 5% positive blasts determined by FACS), none responded to therapy, whereas 6 out of 16 patients with an expression of VEGFR2 on 5% or more blasts responded to therapy. Several

other small compounds with inhibitory effects on VEGFR2 have been identified, and studies with the effect on AML cells are awaited.

21.3 Receptor Tyrosine Kinase Signaling

21.3.1 Flt3 Kinase Inhibition

One of the most common genetic lesions in AML are activating mutations of Flt3, being present in approximately 30% of all AML. Two types of mutations occur in AML: internal tandem duplications (ITD) in approximately 25% of AML (Yokota et al. 1997; Kottaridis et al. 2003; Schnittger et al. 2002) and activating mutations in the tyrosine kinase domain (TKD) in ca. 5%–10% of AML cases (Yamamoto et al. 2001; Abu-Duhier et al. 2001; Jiang et al. 2004; Kindler et al. 2005). Both Flt3-ITD and Flt3-TKD exhibit a transforming potential (Yamamoto et al. 2001; Kindler et al. 2005; Mizuki et al. 2000), suggesting that activating Flt3 mutations play a pivotal role in leukemogenesis. In addition to the above-mentioned receptor tyrosine kinase inhibitor SU5416, which also inhibits Flt3 activity, several other inhibitors of Flt3 have been explored in clinical trials.

SU11248 (Sutent) has been identified as a potent inhibitor of the receptor tyrosine kinases platelet-derived growth factor receptors (PDGFR) A and B, VEGFR1, VEGFR2, and c-kit (Mendel et al. 2003). It also has specific inhibitory activity against wildtype Flt3, Flt3-ITD, and the Flt3-Asp835 mutation as a representative of Flt3-TKD mutations in vitro (O'Farrell et al. 2003a). A phase I feasibility study showed strong inhibition of Flt3-ITD phosphorylation in patients with AML carrying a Flt3-ITD mutation receiving a single dose with subsequent inhibition of downstream signaling. In AML patients with wildtype Flt3, inhibition of phosphorylation was weaker (O'Farrell et al. 2003b). Based on these results, a phase I trial with SU11248 was conducted with 15 AML patients refractory or not eligible for standard chemotherapy, 4 of them harboring a Flt3-ITD mutation and 11 with wildtype Flt3 (Fiedler et al. 2005). All of the patients with Flt3-ITD positive AML showed a morphological response or a PR of short duration; of the 10 evaluable patients with wildtype Flt3, 2 showed responses.

CEP-701 has been identified as a specific inhibitor of wildtype and mutant Flt3-ITD (Levis et al. 2002). Administration of CEP-701 in 14 AML patients with activating Flt3 mutations (13 with Flt3-ITD, 1 with Flt3-TKD) led to a measurable clinical response in 5 patients (35%), all of them harboring a Flt3-ITD (Smith et al. 2004). Preliminary results of a controlled study combining CEP-701 with salvage chemotherapy in relapsed AML patients with Flt3-ITD or Flt3-TKD mutations showed 10 responders out of 17 patients in the study group (5 CR, 3 near-CR—missing platelet recovery—and two PR) compared to 4 responses (2 CR, 2 near-CR) out of 17 patients evaluated in the control group treated with chemotherapy only (Levis et al. 2005). In addition, responders to therapy with chemotherapy and CEP-701 showed a marked inhibition of Flt3 activity and cytotoxic effects by CEP-701 in vitro.

PKC412 is another small molecule with a strong inhibitory effect on both mutant Flt3-ITD and Flt3-TKD proteins (Weisberg et al. 2002). It also exhibits inhibitory effects on VEGFR-2, c-kit, PDGFR-A, and PDGFR-B (Weisberg et al. 2002). In a study with 20 AML patients with Flt3 mutations (18 of them with Ftl3-ITD and 2 with Flt3-TKD), 1 patient showed a PR and 7 patients showed a significant clinical effect, as determined by a greater than 2-log reduction in peripheral blast count lasting for at least 4 weeks (Stone et al. 2005a). A follow-up study combined different dosage schedules of PKC412 with standard induction therapy in a total of 49 patients with newly diagnosed AML (Stone et al. 2005b). In this study, CR rates were above 70%, and patients with mutated Flt3 exhibited a significant higher remission rate than patients with wildtype Flt3 (91% vs 53%, $p = 0.03$).

Several other Flt3 inhibitors have been identified with in vitro inhibitory effects (Tse et al. 2002; Teller et al. 2002; Kelly et al. 2002), and their potential role in clinical settings requires further determination.

21.3.2 STI571 (Imatinib, Glivec)

One of the major advances in clinical hematology in the last several years was the introduction of STI571 (imatinib) in the treatment of chronic myelogenous leukemia (CML). STI571

represents one of the founding members of the new antitumor drug family, the tyrosine kinase inhibitors. STI571 is a selective inhibitor of the Abelson (*abl*) and the abl-related gene (*arg*) tyrosine kinases as well as an inhibitor of the receptor tyrosine kinases PDGFRA and -B and stem cell factor receptor (c-kit) (Pardanani and Tefferi 2004). In AML, several activating mutations of c-kit are frequent events in core binding factor (CBF) leukemias with alterations of the CBF transcription factor complex, either harboring the translocation t(8;21) or the inv(16) (Beghini et al. 1998; Care et al. 2003; Gari et al. 1999; Wang et al. 2005; Beghini et al. 2004). Mutations affecting the tyrosine kinase domain at D816 have been shown to be resistant to STI571 (Wang et al. 2005), whereas mutations in the extracellular or transmembranous domain are responsive to STI571 (Cammenga et al. 2005). Three patients with CBF leukemia and c-kit mutations are reported to have been treated with STI571 so far (Cairoli et al. 2005); 1 patient harboring an extracellular mutation of c-kit showed a rise in neutrophil count without decrease in the absolute blast count. In addition, the patient showed a transient regression of an extramedullary tumor. The other 2 patients harbored a D816 mutation. Not surprisingly, those patients were unresponsive to STI571 treatment. Two case reports with successful treatment of patients overexpressing c-kit with STI571 are reported in the literature (Kindler et al. 2003; Schittenhelm et al. 2003), but in these cases no information about the mutational status of c-kit is presented. In one study, 27 patients with AML and overexpressed wildtype c-kit either refractory to or not eligible for chemotherapy were treated with STI571. Observerd were 2 CR, 1 near-CR, and 2 minor responses (Kindler et al. 2004).

The presence of a Philadelphia chromosome with the expression of BCR-ABL is a rare event in AML; two patients with Philadelphia positive AML have been treated with imatinib in combination with chemotherapy so far, and both responded to STI571 with a molecular remission (Jentsch-Ullrich et al. 2004; Viniou et al. 2004).

Several novel tyrosine kinase inhibitors have been identified that show in vitro inhibition of D816 mutant c-kit: SU11652, SU11654, SU11655 (Liao et al. 2002), MLN518, PD180970 (Corbin et al. 2004), AP23464, and AP23848 (Corbin et al. 2005). A phase I study showed low toxicity of MLN518 in patients with relapsed and refractory AML (DeAngelo et al. 2003), but clinical studies about the efficacy of these compounds in D816-positive AML are missing.

21.4 Inhibition of Farnesylation

Farnesylation of the Ras proteins (v-Ras, n-Ras, and k-Ras) is the key reaction of the posttranslational modifications of Ras that are a prerequisite for Ras activation by ligand-bound growth factor receptors (Fig. 21.1). Upon activation of Ras, several growth stimulatory and antiapoptotic pathways are activated.

Activating Ras-mutations with constitutive activation in the GTP-bound form are present in ca. 30% of human AML (Reuter et al. 2000). The phenotype of cells transformed by v-Ras could sufficiently be reversed by farnesyl transferase inhibition (Kohl et al. 1993), and various hematopoietic cell lines are sensitive toward exposure to farnesyl transferase inhibition (Sepp-Lorenzino et al. 1995). A phase I trial treating high-risk leukemia patients with tipifarnib (R115777, Zarnestra), an orally applicable farnesyl transferase inhibitor (FTI), showed an overall response rate of 32%, with 2 CR and 6 PR out of 25 patients (Karp et al. 2001). In a phase II study recruiting 252 patients with refractory or relapsed AML, 18% of 138 evaluated patients showed a partial response with reduction of bone marrow blasts of more than 50% on treatment with tipifarnib, and 11 out of 169 treated patients showed a CR or near-CR (Harousseau et al. 2003).

21.5 Cell Cycle

21.5.1 CDK Inhibition

Almost all malignant processes show alterations of genes involved in the cell cycle (Sherr 2000). A critical point in the cell cycle control is the G1 to S transition. After passing this checkpoint, the cell is irreversibly committed to the next cell division (Krug et al. 2002). The key regulators of the G1 to S phase transition are complexes of cyclin D:cyclin-dependent kinase (CDK)4 or 6 and cyclin E:CDK2 (Krug et al. 2002), which lead to

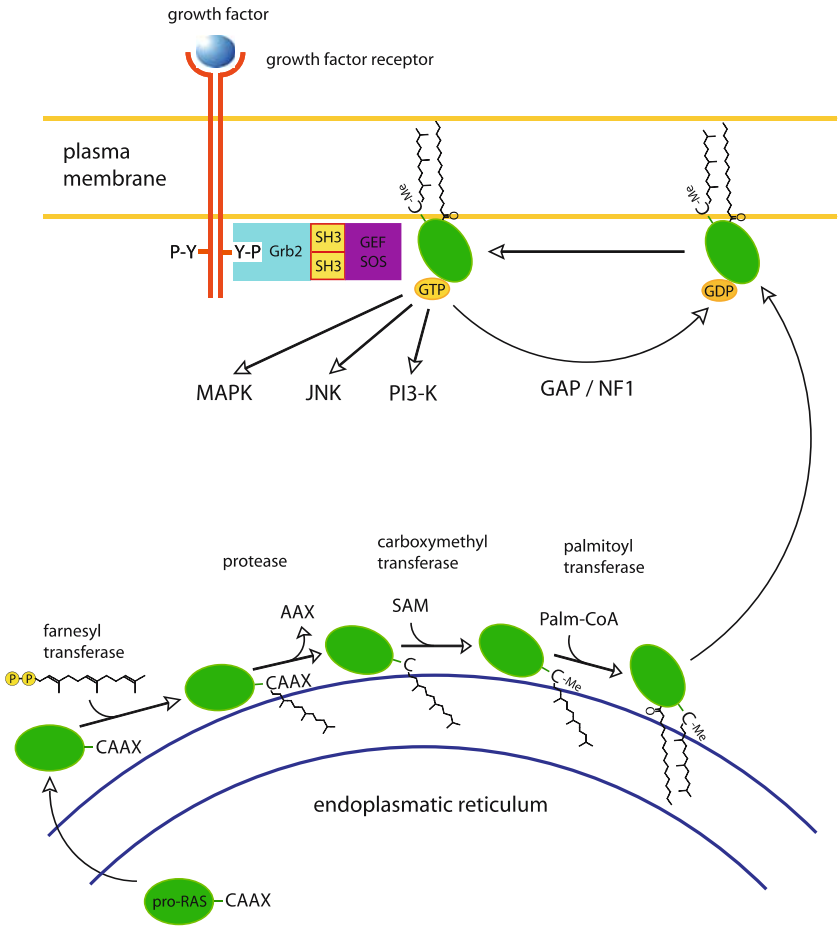

Fig. 21.1 The Ras pathway. Ras is synthesized as a propeptide (pro-Ras) in the endoplasmatic reticulum. In the key reaction of the posttranslational modifications, farnesyltransferase catalyzes the covalent binding of a hydrophobic farnesyl residue onto the terminal cysteine residue of Ras using farnesyl diphosphate as a substrate. In the subsequent reactions, hydrophobicity is further enhanced by cleavage of the terminal AAX residues by CAAX protease, carboxymethylation of the farnesyl-cysteine residue by carboxymethyltransferase using S-adenosyl-l-methionine (*SAM*) as a substrate and by adding a palmitoyl residue to a different cysteine residue catalyzed by palmitoyltransferase with palmitoyl-coenzyme A (*Palm-CoA*) as a substrate. The hydrophobic Ras protein then translocates to the inner plasma membrane binding to guanosine diphosphate (*GDP*). Upon binding of growth factors to corresponding growth factor receptors, Ras is activated by substituting guanosine triphosphate (*GTP*) fr GDP by guanine-nucleotide-exchange factors (*GEF*). Activated Ras then activates several effector pathways promoting growth and survival. Reversal of GTP-binding with the formation of inactive Ras-GDP is mediated by GTPase activator proteins [GTPase activator protein (*GAP*) and neurofibrin (*NFI*)]. *Grb-2*, growth factor receptor-binding protein; *JNK*, Jun amino-terminal kinase; *MAPK*, mitogen-activated protein kinase; *PI3K*, phosphoinositol-3-kinase; *SOS*, son-of-sevenless

the activation of transcription of S phase genes by releasing E2F transcription factors. The kinase activity of these cyclin:CDK complexes are inhibited by two cyclin dependent kinase inhibitor (CKI) families, the inhibitor of CDK4 (INK4) and the kinase inhibitor protein (CIP) family (Fig. 21.2). Inactivation of one CDK4 family member, p15INK4B, by promoter hypermethylation occurs in the majority of AML, as reviewed by Drexler (1998). The CIP family of CDK inhibitors, on the other hand, is activated by wild-type p53 upon DNA damage. Even though mu-

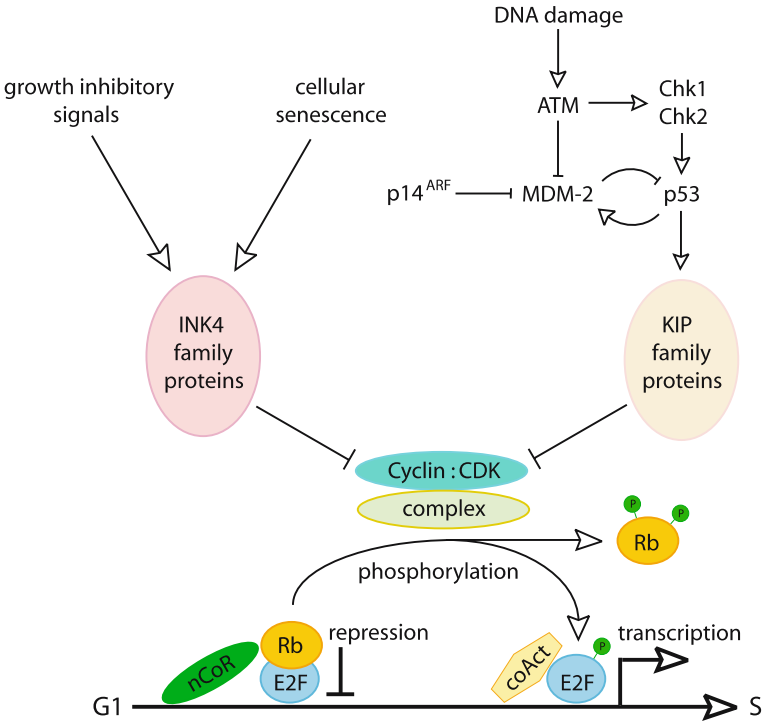

Fig. 21.2 Regulation of the G1 to S checkpoint. Hypophosphorylated proteins of the retinoblastoma (*Rb*) family (Rb, p107, p130) bind to elongation factor (*E2F*) transcription factors. These complexes recruit nuclear corepressors (*nCoR*) to promoter sequences of S phase-associated genes and repress the transcription of these genes. Complexes of cyclin D:CDK4/6 and cyclin E:CDK2 phosphorylate Rb family proteins and the E2F transcription factors. Upon phosphorylation, Rb dissociates from E2Fs. Phosphorylated E2Fs recruit coactivators (*coAct*) to the promoters, and transcription of S phase-related genes is activated. The activity of the cyclin:CDK complexes is negatively regulated by two protein families, the inhibitor of CDK 4 (*INK4*) and the kinase inhibitor protein (KIP) proteins. The INK4 protein family (i.e., p16, p15, p18, p19) are induced upon cellular senescence and growth inhibitory signals, i.e., transforming growth factor β (TGFβ). KIP family proteins (p21, p27, p57) are activated upon DNA damage by p53. DNA damage activates the ataxia telangiectasia mutated (ATM) protein, which in turn activates the checkpoint kinases (Chk) 1 and 2, and they activate p53 by phosphorylation. p53 itself is negatively regulated by murine double minute-2 (MDM-2) in a negative feedback mechanism, promoting its own degradation. However, activation of ATM also blocks MDM-2, thus preventing p53 from degradation upon DNA damage. An additional inhibitory effect on MDM-2 comes from $p14^{ARF}$, a protein sharing the same gene sequence with the INK4 protein p16 using an alternate reading frame (ARF). Taken together, upon DNA damage cell cycle progression is halted on this critical checkpoint by the p53 pathway

tations of p53 are rare events in hematological malignancies as compared to solid tumors, up to 30% of AML show a decreased expression of p53 (Schottelius et al. 1994). In addition, p53 regulators such as $p14^{ARF}$ are targeted in leukemias (Linggi et al. 2002) and low $p14^{ARF}$ expression is associated with poor survival (Müller-Tidow et al. 2004). With that in mind, inhibition of CDK activity might have a therapeutic role in the treatment of AML.

UCN-01, a nonspecific CDK inhibitor with additional inhibitory effects on protein kinase C (PKC), was already evaluated in AML. A study combining AraC with UCN-01 was conducted in 15 patients (Sampath et al. 2005). Only 1 response could be observed.

Several other CDK inhibitors, i.e., flavopiridol, roscovitine, BMS-387032 (Blagden and de Bono 2005) as well as the natural compound Indirubin and its analog meisoindigo (Xiao et al. 2002), have demonstrated antitumoral activity in several tumor entities. In addition, in vitro results suggest an antileukemic effect on AML blasts for flavopiridol (Arguello et al. 1998), roscovitine (Rosato et al. 2005), indirubin, and meisoindigo (Liu et al. 1996; Suzuki et al. 2005), making them promising compounds to be evaluated in clinical studies in AML.

21.5.2 DNA Synthesis

Troxacitabine is a novel cytosine analog with an L-conformation; it was initially designed as an antiretroviral drug (Kim et al. 1992). Troxacitabine exhibits strong antitumoral activity in a variety of tumors (Grove et al. 1995). Troxacitabine had clinical activity as a single dose in a dose-escalation study with complete responses in 3 out of 30 evaluable patients with advanced AML (Giles et al. 2001). This compound, in contrast to established D-nucleosides, cannot be inactivated by cytidine deaminase (CDA), a possible resistance mechanism in leukemic cells. However, a prospective randomized study showed inferior results of a troxacitabine containing induction therapy compared to standard induction therapy with idarubicin and cytarabine, and randomization into the troxacitabine-containing arms was terminated early (Giles et al. 2003).

Temozolomide is a small orally available alkylating prodrug with spontaneous conversion to the active drug monomethyl triazeno imidazole carboxamide (MTIC) (O'Reilly et al. 1993). Temozolomide induced apoptosis in several leukemic cell lines (Tentori et al. 1995). One phase I study reports of 1 CR and 2 near-CR in 16 patients with advanced-stage AML treated with temozolomide as a single agent (Seiter et al. 2002).

Clofarabine (2-chloro-2′-arabino-fluoro-2′-deoxyadenosine, CAFdA) is a fluorinated derivative of cladribine designed to exhibit both strong inhibition of DNA polymerases and of the ribonucleotide reductase (Faderl et al. 2005a). Clofarabine exhibits increased cytotoxicity toward several leukemic cell lines compared to fludarabine (Lotfi et al. 1999). A dose escalation study showed 1 CR and 1 hematological improvement in 16 advanced-disease AML patients (Kantarjian et al. 2003a). In a subsequent phase II study, 17 out of 31 patients responded to therapy (13 CR, 4 near-CR or PR) (Kantarjian et al. 2003b). Toxicity of cytarabine can be enhanced by combining it with prior administration of fludarabine or cladribine, acting through an accumulation of arabinoside-cytosine-triphosphate (ara-CTP), the product of cytidine kinase using cytarabine as a substrate. This effect is used in salvage therapies of relapsed acute leukemias (Gandhi et al. 1993). However, this approach was not reproducible with clinical relevance in a randomized phase III trial of the German AML Cooperative Group (German AML-CG, manuscript in preparation). Because clofarabine exhibits similar properties to fludarabine, this potential synergistic effect was used in a study treating 25 patients with advanced-stage AML with clofarabine and intermediate-dose cytarabine (Faderl et al. 2005b). Of the patients, 7 achieved a complete response and 3 a near-CR, showing a substantial clinical activity with this combination in patients with advanced AML.

21.6 Modulation of Gene Expression

21.6.1 Histone Deacetylation

The acetylation and deacetylation of amino acid residues in histones play an important role in the regulation of gene expression. Upon histone acetylation by histone acetyl transferases (HATs), histones undergo a conformational change, thereby enabling gene expression. Upon histone deacetylation by histone deacetylases (HDACs), the histone:DNA complex changes to a dense conformation and gene transcription is inhibited (Fig. 21.3). Not surprisingly, HATs are part of transactivation complexes, whereas HDACs are co-repressors of gene expression recruited by transcriptional repressors. Aberrant recruitment of HDACs is an important step in silencing gene expression in t(15;17) or t(11;15)-positive APL expressing the PML-RARα and the PLZF-RARαfusion proteins, respectively, or in t(8;21) or inv(16)-positive CBF leukemias expressing the AML-ETO and the CBFβ-MYH11 fusion

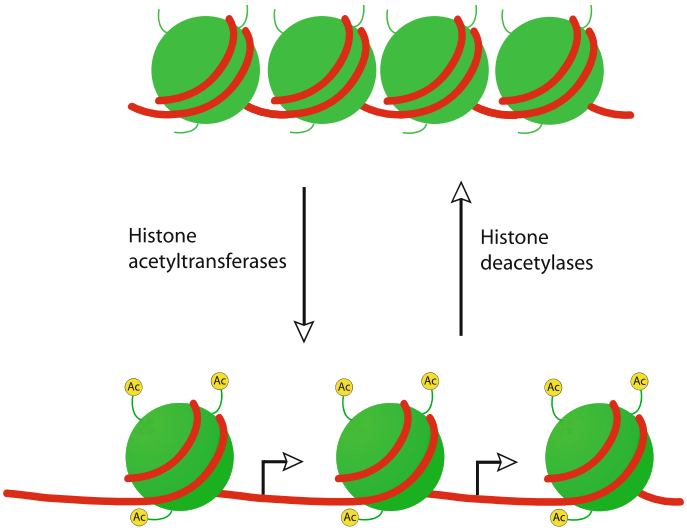

Fig. 21.3 Epigenetic regulation of gene transcription by histone acetylation. Histone acetyltransferases (HATs) catalyze the acetylation of N-terminal lysine residues of histones 2A, 2B, 3, and 4. Upon acetylation, the nucleosome undergoes a conformational change, enhancing the accessibility of target gene promoters for transcriptional activators. Histone deacetylases (HDAC) catalyze the reversal of this reaction, thus leading to a more dense formation of the nucleosome, and the transcription of gene promoters is inhibited. (Steffen et al. 2003, © 2003 National Academy of Science, USA)

protein, respectively (reviewed in Minucci et al. 2001).

Valproic acid is an established mood stabilizer and antiepileptic drug. Valproic acid is a potent HDAC inhibitor inducing hyperacetylation of the histones H3 and H4 (Gottlicher et al. 2001; Phiel et al. 2001). Treatment of primary leukemic cells with the HDAC inhibitor valproic acid led to marked differentiation of these cells, and this effect was potentiated by addition of retinoic acid (Gottlicher et al. 2001). Several phase II studies evaluating the addition of valproic acid with or without retinoic acid to induction therapy are ongoing. Preliminary results show 1 CR and 2 PR in 20 patients with advanced stage AML treated with valproic acid and ATRA (Craddock et al. 2005). One phase I/II trial combined valproic acid with the hypomethylating agent decitabine (see the following section), and 10 CR and 2 near-CR could be observed in 54 patients with advanced stage AML (Garcia-Manero et al. 2005).

Depsipeptide (FR901228) is another specific inhibitor of histone H3 and H4 acetylation (Nakajima et al. 1998) with differentiating effects in vitro (Klisovic et al. 2003). A phase I study with depsipeptide in 10 patients with AML showed progressive toxicity limiting the clinical use. In addition, no PR or CR were observed in the patients even though their blasts showed HDAC inhibition and histone acetylation in vitro (Byrd et al. 2005).

Phenylbutyrate is another HDAC inhibitor which entered clinical trials. Phenylbutyrate was combined with ATRA in patients with relapsed APL harboring a mutated, ATRA-resistant PML-RARα fusion protein (Zhou et al. 2002). Out of these 5 patients, 1 achieved a CR upon treatment.

21.6.2 Methylation

Another regulatory epigenetic mechanism of gene transcription that has been elucidated in recent years is the methylation of CpG islands in gene promoters (Robertson 2005). Upon methylation of certain CpG islands within the promoter of genes by DNA methyltransferases (Dnmt), expression of target genes is inhibited. Gene silenc-

ing by aberrant promoter hypermethylation is a frequent event in malignancies including AML (Claus et al. 2005). Two similar compounds with hypomethylating activity through inhibition of Dnmts have been identified: 5-aza-deoxycytidine (decitabine) and 5-aza-cytidine (azacitidine). Decitabine acts via inhibition of Dnmt1 with restoration of the expression of silenced tumor suppressor genes (Robert et al. 2003). In addition to hypomethylation by inhibition of Dnmts at low concentrations, both compounds exhibit cytotoxic activity by incorporation into DNA and inhibition of DNA synthesis at higher concentrations, mimicking the mechanism of action of other cytotoxic nucleosides (Kaminskas et al. 2005)

The first clinical studies with decitabine have been carried out in high decitabine concentrations, thus presumably acting at least in part by cytotoxic activity in patients. A pilot study with decitabine (Petti et al. 1993) showed the encouraging result of 3 CR and 1 PR out of 10 evaluable patients with decitabine as a first-line treatment in poor prognosis AML. In an approach to combine decitabine with standard cytotoxic agents, one European Organization for Research and Treatment of Cancer (EORTC) study combined decitabine with either idarubicin or amsacrine, two anthracycline-type agents, in patients with relapsed or refractory disease (Willemze et al. 1997). The results showed a CR in 23/63 patients (36.5%). On the other hand, hematological and nonhematological toxicities were prolonged compared to historical controls with standard chemotherapies and comparable with high-dose cytarabine containing salvage therapies, thus showing no superiority compared to established salvage therapies.

Subsequently, studies in patients with high-risk myelodysplastic syndrome (MDS) have been carried out with low-dose decitabine (Wijermans et al. 2005). The analysis of three phase II studies showed an encouraging response rate of 49% in 177 patients with advanced MDS (Wijermans et al. 2005). Based on these data, phase II studies with the treatment of elderly patients with AML not eligible for standard induction therapy with low-dose decitabine are ongoing.

The hypomethylating compound azacitidine has been approved for treatment of MDS (Kaminskas et al. 2005). Approval was based on a randomized study comparing treatment with low-dose azacitidine versus best supportive care in 191 patients with MDS (Silverman et al. 2002). In this study, 23% CR or PR and 37% hematological improvement could be detected in the study arm, versus no remissions and 5% improvement in the control arm. However, a small study with 11 patients with advanced-stage AML or not eligible for induction chemotherapy did not show significant responses to low-dose azacitidine (Lee et al. 1990).

21.7 Protein Degradation

Degradation of the majority of cellular proteins is mainly regulated by the proteasome. Cellular proteins designated for destruction are tagged with the small peptide ubiquitin and directed to degradation by the proteasome complex. Inhibition of the proteasome leads to an undirected accumulation of proteins with profound effects on the cells, resulting in apoptotic cell death in a dose-dependent manner. Malignant hematopoietic cells show a more profound sensitivity to proteasome inhibitors compared to normal cells (Soligo et al. 2001). Bortezomib (Velcade) is a proteasome inhibitor that exhibits clinical antitumoral activity against multiple myeloma (Jagannath et al. 2004; Richardson et al. 2003). One phase I dose escalation study with 15 patients with relapsed or refractory leukemia (11 AML, 1 ALL, and 3 MDS) has been conducted with bortezomib so far, with a maximum tolerated dose of 1.25 mg/m² (Cortes et al. 2004). In this dose escalation, proteasome activity decreased by an average of 66% after 1 h of administration. Of the 7 patients treated with 1.25 mg/m², 2 responded to therapy, one with a reduction of peripheral blasts from 65% to 5% and the other with the reduction of bone marrow blasts from 20% to 4%.

21.8 Modulation of Intracellular Proteins

Heat shock protein 90 (Hsp90) is a molecular chaperone induced under stress conditions. It

supports the proper folding, stability, and function of target proteins involved in cell growth and survival, i.e., protein kinases and hormone receptors (Neckers 2002). Hsp90 inhibitors cause the inactivation, destabilization, and eventual degradation of Hsp90 target proteins (Neckers 2002) and have thus reached attention as potential antitumoral drugs. In Flt3-ITD-positive AML, Flt3-ITD is a direct target of Hsp90 (Minami et al. 2002). Flt3-ITD transformed 32D cells, but not parenteral 32D cells, exhibited marked growth inhibition and apoptosis after treatment with Hsp90 inhibitors (Minami et al. 2002). Co-treatment of the Hsp90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17AAG) with either the FLT-3 kinase inhibitor PKC412 (George et al. 2004) or the histone deacetylase inhibitor LBH589 (George et al. 2005) had synergistic cytotoxic effects in human Flt-ITD-positive leukemic cells. In addition, 17AAG demonstrated synergistic cytotoxic effects with cytarabine in primary leukemia samples (Mesa et al. 2005). No information was given about the mutational status of Flt3 in these samples. The effects were also observed in the human AML cell lines HL-60 and ML1, which do express wildtype Flt3 (Mesa et al. 2005). It is of note that for 17AAG safety and efficacy was tested in a phase I clinical study with advanced malignancies (Banerji et al. 2005), and that this study showed an acceptable toxicity profile with effective target protein inhibition in vivo.

21.9 Apoptosis

AML cells exhibit a high resistance to apoptotic signals compared to normal bone marrow cells. Enhancing the apoptotic capacity of AML cells might therefore be a potential molecular target for the treatment of AML.

In AML, high expression of Bcl-2, an antiapoptotic regulator, correlated with a negative outcome after chemotherapy (Campos et al. 1993; Maung et al. 1994). Two clinical phase I studies with the Bcl-2 antisense oligonucleotide G3139 (Genasense) in AML have been conducted so far, both in combination with a cytarabine containing chemotherapy. In 17 patients with refractory or relapsed leukemia, the combination of G3139 with salvage chemotherapy containing high-dose cytarabine caused CR in 5 evaluable patients with toxicities comparable to chemotherapy alone (Marcucci et al. 2003). However, the decrease of Bcl-2 messenger RNA (mRNA) or protein level did not correlate with response to therapy. In a second study of previously untreated elderly patients, 29 patients received a standard induction chemotherapy combined with G3139, and, upon achievement of a CR, a consolidation therapy of high-dose cytarabine in combination with G3139 (Marcucci et al. 2005). Achieving CR were 14 patients, and they showed a significant decrease of Bcl-2 mRNA and protein levels 72 h after infusion of G3139, whereas nonresponders showed an increase of Bcl-2 mRNA and protein. This downregulation might be contributed to the therapy with G3139, but these results have to be taken with caution since cytarabine-induced apoptosis itself shows a degradation of Bcl-2 (Sreenivasan et al. 2003). Several other antiapoptotic members of the Bcl-2 protein family have been identified, with some showing an alteration in AML, but their potential clinical significance requires further studies.

Despite detectable expression of receptors for TNF-related apoptosis-inducing ligand (TRAIL), the majority of AML cells have shown resistance against TRAIL-mediated apoptosis (Wuchter et al. 2001). This might in part be due to the overexpression of the FLICE-like inhibitory protein (FLIP), an inhibitor of the extrinsic pathway (Irmler et al. 1997). Treatment with CDDO (oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid) and its C-28 methyl ester (CDDO-me) reduces FLIP protein levels in cells by enhancing degradation of FLIP, and incubation of AML cells with these compounds led to increased cell death at low concentrations with rapid depletion of cellular FLIP (Suh et al. 2003). In addition, CDDO induces differentiation of leukemic blasts in concentrations not sufficient for induction of apoptosis, making these compounds even more interesting for clinical use in AML (Koschmieder et al. 2005). So far, no clinical studies have been initiated with these compounds in AML.

A huge number of regulators of apoptosis exist, with a lot of them exhibiting a potential clinical use. For instance, inhibition of the apoptotic

regulators survivin and the X-linked inhibitor of apoptosis (XIAP) induced apoptosis in AML cell lines (Carter et al. 2003, 2005). The role of many other apoptotic regulators in AML still needs to be further evaluated.

Arsenic trioxide (As_2O_3) has shown substantial efficacy against APL in both de novo APL and APL relapsed after ATRA treatment (Niu et al. 1999). Various effects of As_2O_3 have been demonstrated, and to date it is not clear which effects contribute to the efficacy in APL. APL cells treated with As_2O_3 undergo apoptosis (Chen et al. 1996). Several possible mechanisms contribute to the induction of apoptosis. Expression of Bcl-2 is downregulated (Chen et al. 1996), the expression of several procaspases is enhanced and several caspases are activated (Soignet et al. 1998), telomerase activity is inhibited (Chou et al. 2001), and As_2O_3 generates intracellular hydrogen peroxide (Chen et al. 1998), thus destroying the mitochondrial inner membrane potential with subsequent release of cytochrome c into the cytoplasm (Kroemer and de The 1999). These are by all means not the only presumed mechanisms of action (see Gazitt and Akay 2005 for a review).

A combination of As_2O_3 with ATRA without chemotherapy in 108 APL patients has shown superior results compared to either single agent (Wang et al. 2004), making this chemotherapy-free combination a very interesting therapy modality in APL.

21.10 Metabolism

Statins are well-established inhibitors of the key enzyme of the cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase. Statins experience a broad clinical use in the treatment of hypercholesterolemia (Farmer and Torre-Amione 2000). In addition, statins exhibit proapoptotic properties, causing specific apoptosis of leukemic cells while leaving nonmalignant hematopoietic cells unaffected (Newman et al. 1994; Dimitroulakos et al. 1999). Apoptosis induced by lovastatin was abrogated by geranylgeranyl pyrophosphate (GGPP) (Xia et al. 2001), suggesting that inhibiting geranylgeranylation of target proteins is the key mechanism by which apoptosis is mediated. Geranylgeranylation occurs in ca. 1% of all intracellular proteins (Prendergast 2000), but Wu et al. could demonstrate that downregulation of the phosphorylation status of the extracellular signal-regulated kinase (ERK)1/2 within the Raf/MEK/ERK pathway contributes to lovastatin-induced apoptosis (Wu et al. 2004). In addition to these findings, co-treating leukemic cells with lovastatin and a MAP or ERK kinase (MEK)1 inhibitor had synergistic effects on apoptosis, showing a potential use of a combination therapy of statins with inhibitors of the Raf/MEK/ERK pathway in clinical studies.

21.11 Redirecting Oncogenic Signals

Several AML entities contain disease-specific fusion proteins, as described in Sect. 6.1, i.e., the AML-ETO, the PML-RARα, or the CBFβ-MYH11 fusion protein. One hallmark of these fusion proteins is the fusion of DNA-binding domains of transcription factors to repressor-complex binding domains, thus leading to a transcriptional repression of differentiation-associated genes (Redner and Liu 2005). One promising new concept of antileukemic therapy is the redirection of oncogenic fusion proteins to promoter regions of genes essential for cell survival. Since the oncogenic fusion protein is only present in the malignant clone, therapies against the oncogenic properties of these fusion proteins should be very specific and effective only against fusion protein-harboring tumor cells. The proof of principle of this concept was demonstrated by our group with the AML1-ETO fusion protein, being present in AML M2 leukemias harboring the chromosomal translocation t(8;21). A chimeric protein, designated M&M, was constructed with a fusion of the DNA-binding domain of myb, a transcription factor being crucial for cell survival, to the AML-binding domain of the myeloid Elf-like factor (MEF) (Steffen et al. 2003). Ideally, this protein should recruit repressor domains recruited by AML1-ETO to myb-sensitive promoter areas, thus leading to a repression of myb-dependent genes (Fig. 21.4). We could show that this fusion protein indeed effectively targeted AML1-ETO to myb target proteins. This targeting was followed by an induction of apop-

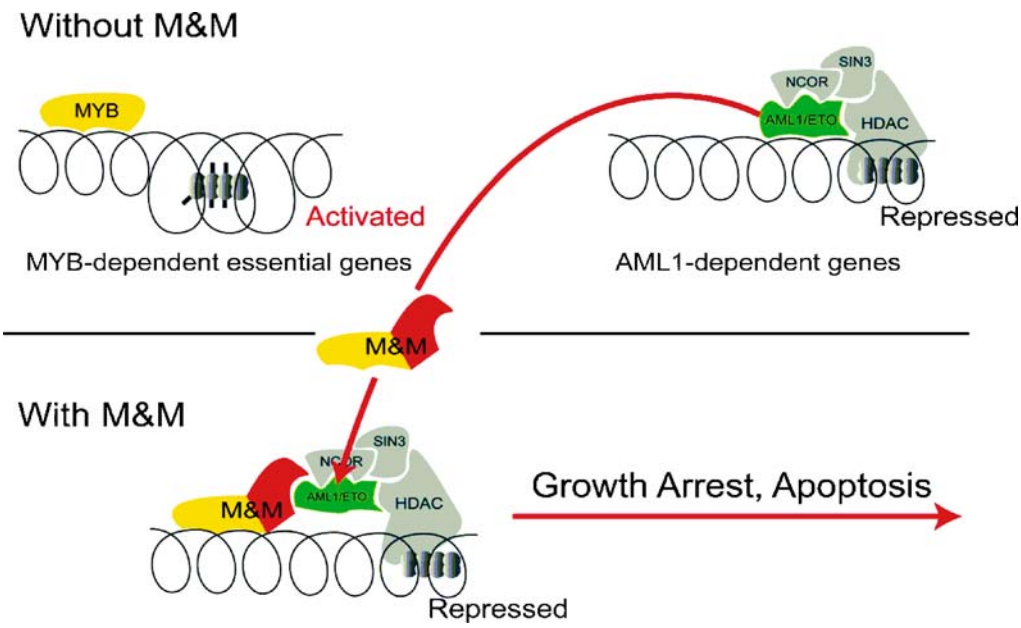

Fig. 21.4 Putative mechanism of the M&M protein. Binding of the AML1/ETO fusion protein in t(8;21)-positive AML cells to the MEF AML1 binding domain of the M&M chimeric protein recruits repressor complexes to promoters of MYB-dependent genes essential for cell growth and survival, inhibiting transcription of these genes and ultimately leading to growth arrest and apoptosis selectively in cells expressing the AML1/ETO fusion protein. Steffen et al 2003, © 2003 National Academy of Science, USA

tosis. This effect was highly restricted to AML1-ETO-positive cells, and no effect on transcription of myb-dependent genes could be detected in AML1-ETO-negative cells.

In contrast to our study, other possible ways of redirecting signal molecules could be e.g., the fusion of signaling molecules to apoptotic signals. The feasability of such an approach could be demonstrated by Howard et al. (2003). In this approach, the phosphotyrosine binding domain of Grb2 was fused to the death effector domain of Fadd, resulting in a linkage of the receptor tyrosine kinase ErbB2 with caspase-8 and resulting in profound cell death. Since this linkage may involve many different RTKs in normal tissues, antitumoral specificity of this fusion cannot be hypothesized, and it is not clear whether this approach will be suitable for clinical use. Furthermore, in contrast to our approach, this methodology does not interfere with the transcriptional machinery of a malignant cell.

One problem with the retargeting strategy for the treatment of AML so far is the delivery of the therapeutic fusion protein to the malignant cell. Even though highly efficient transfection rates can be achieved with retroviral transfection vectors at the moment, the transfection rates are still well below 100%, possibly allowing an escape mechanism for nontransfected leukemic cells. Thus, it may be crucial to use small molecules mimicking this strategy.

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