Virchows Arch (2006) 448: 744–755
DOI 10.1007/s00428-006-0189-2

REVIEW ARTICLE

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Personalized medicine and development of targeted therapies: the upcoming challenge for diagnostic molecular pathology. A review

Received: 4 July 2005 / Accepted: 20 September 2005 / Published online: 22 April 2006

Abstract Due to continuous technical developments and new insights into the high complexity of many diseases, molecular pathology is a rapidly growing field gaining center stage in the clinical management of tumors as well as in the pharmaceutical development of new anti-cancer drugs. The application of novel compounds in clinical trials has revealed promising results; however, the current diagnostic procedures available for determining which patients will primarily benefit from rational tumor therapy are insufficient. To read a patient's tissue as "deeply" as possible, in the future, gaining information on the morphology and on genetic, proteomic, and epigenetic alterations will be the upcoming task of surgical pathologists experienced in molecular diagnostics to provide the clinicians with information relevant for an individualized medicine. Among the different high-throughput technologies, DNA microarrays are now the first array approach close to enter routine diagnostics. Technically advanced and well-established microarray platforms can nowadays be evaluated by distinct bioinformatic tools capable of identifying both novel genes associated with disease development and clusters of genes predicting clinical outcome of an individual tumor. The automatic, highly parallel analysis of proteins and complex proteins lysates for early detection of cancers such as breast, prostate and ovary as proteomic patterns in the serum also appears at the horizon. In addition, an improved analysis of tumor samples via antibody or reverse-phase protein arrays is likely to provide the pathologist in the future with information about activated oncogenic signaling 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 incompatible with paraffin-embedded tissue samples, an investigation of DNA methylation using specialized high-throughput platforms has revealed the potential of being used in future diagnostics. Each of these approaches on its own might not suffice to extract all information required for an efficient individualized diagnostics. Therefore, a "multiplex approach" combining the different biological levels DNA, RNA, and protein, may be necessary to functionally classify malignant tumors. This appears to become a major challenge for diagnostic pathologists.

Keywords Review · Molecular pathology · Targeted therapy

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Introduction

Standard diagnostic procedures for human tumors are currently based on a combination of histopathology and immunohistology closely connected with clinical data. This strategy, in most cases, provides precise information on dignity, tissue origin, tumor type, stage, and grade as well as information on the completeness of surgical tumor removal. Up to now, these data comprise the most relevant information on a patient's prognosis and are a rational basis for therapy design. As the diversity of alterations in tumor cells is not completely mirrored by tumor cell morphology, pathologists and clinicians often observe that two patients harboring the "same" type of tumor in a seemingly "identical" stage show different clinical outcome with respect to survival and, in particular, with respect to therapy response. To improve this unsatisfactory situation, a patient-specific disease prediction based on tissue examination by pathologists would be extremely helpful.

During recent years, many molecular markers and marker patterns ("signatures") have been disclosed which, at least, partially predict prognosis and therapeutic

effectiveness. Considering the fact that cell function is controlled by a complex network of functionally active signaling pathways, it is unlikely that expression analysis of a single or a small number of proteins will precisely predict the clinical outcome of an individual tumor. Hanahan and Weinberg (2000) have described six essential alterations in cancer cells that are common to most human tumors: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. However, each of these alterations is achieved by different modifications in signal transduction, gene or protein expression, protein modification, and localization. Thus, a different and improved characterization of malignant tumors as compared to current standard diagnostics is required to reflect the multitude of genetic, proteomic, and metabolomic alterations typical for malignant cells. Several newly developed high-throughput technologies, such as DNA microarrays, protein and antibody arrays, proteome technologies such as mass spectroscopy MALDI-TOF, metabolomic analyses, methylation arrays, reverse-phase protein microarrays (RPAs), etc., provide the technical platforms for this approach and will gain more interest in the future. By integrating these technologies into standard diagnostic procedures, future molecular pathology will be able to dramatically increase the relevance of tissue-based examinations. Pathology will play a crucial role in therapy design and, by providing molecular diagnostic approaches, also in therapy adjustment and selection of effective drugs.

In this review, we will describe genomic, proteomic, and epigenomic approaches which have been shown recently to improve diagnostics and prognosis. We will focus on DNA microarrays as the most advanced technology in this field and suggest the development of an integrated approach for future diagnostic molecular pathology.

Genomics—DNA microarrays

Technology

The generation of gene expression profiles from cultured cells and tissue samples is nowadays a well-established method. In the majority of experiments, DNA fragments specific for individual genes on activated glass surfaces are used [13, 62]. RNA is prepared from the biological material to be analyzed, reverse transcribed into cDNA, labeled with fluorescent dyes, and hybridized to the array. The hybridization signals are detected by a laser scanner, the images are normalized in various ways, and the relative expression levels obtained for individual genes are further clustered into groups of genes with similar or identical expression patterns.

DNA microarrays consist of thousands of DNA elements (10,000–80,000 or even more) robotically tethered to a solid surface, e.g., coated glass slide, silicon, or nylon. The DNA sequences are either oligonucleotides or cDNA and represent different genes of interest. Each gene is usually represented by more than one feature to increase the specificity of the analysis. The cDNA clones are often selected from public databases, amplified from cDNA libraries, and purified before spotting. Oligonucleotides (20–80 bp) are alternatively chemically synthesized and spotted or may be directly synthesized on the array surface. In addition to sequences representing known genes, sequences representing expressed sequence tags (EST) coding for genes of unknown function can also be spotted on the solid surface, offering the potential of investigating new marker genes.

The target DNA usually is a cDNA derived from tumor cell mRNA which is amplified and simultaneously labeled by reverse transcriptase-polymerase chain reaction (PCR). The RNA is prepared from rapidly processed cell cultures or fresh frozen tissue. Novel fixation techniques and preparation methods are currently tested which might also allow in the future the use of RNA prepared from paraffin-embedded tissues to be utilized in diverse microarray experiments [28, 30, 54]. Target DNA, of course, can also be prepared from genomic DNA fragments. For labeling, fluorescent dyes are used nowadays. 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 to correct for spot integrity and for 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 overexpression, underexpression, no change, or complete absence for each gene in the tissue samples to be compared to each other (for review, see [52]).

Bioinformatics

The enormous amount of data obtained not only from each high-throughput experiment but also from data documentation, data processing, and data interpretation is a unique challenge requiring close cooperation 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. cDNA microarrays today 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. [72] 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 predefined 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.

Several methods for data analysis and interpretation have been developed, which can be separated into two classes: supervised and unsupervised methods [11]. A supervised approach means that some external information such as tumor grading or patients' survival define the relevant classes before the analysis; this method is used for "class prediction" and "class comparison" studies.

In contrast, for "class discovery" studies, an unsupervised approach is used, which defines different groups of tumors exclusively according to their gene expression profiles without using external information. An unsupervised classification detects similarities in gene expression profiles. For this purpose, the hierarchical clustering [24] combined with a graphic presentation is an excellent tool (Fig. 1). K-means or self-organizing maps are further examples of unsupervised methods frequently used in microarray analyses [79, 82]. It is important to note that cluster analysis should not be used for class prediction and class comparison studies.

Especially for "class prediction" studies, supervised approaches should be applied. The supervised algorithm establishes a model on the training set of samples, which assigns gene sets with a defined expression profile to predefined groups of tumor samples. After the class prediction algorithm has been trained, it can be used to predict the class of new samples. To assess the error rate (e.g., percentage of incorrectly classified tumors), a crossvalidation approach is often used. In this approach, the available data sets are split repeatedly into a training set and a test set. The algorithm is then trained on the training set and applied to the test set, and correct and false predictions are counted. A special type of cross-validation is the leave-one-out strategy, where just one sample is omitted from the training set (training on $n-1$). This one sample is then used as the test set. This procedure is repeated n times, each time using another sample as a test set. This approach achieves maximal efficiency of training yet requires high computational resources.

Such sample classification can be helpful to identify the set of genes that discriminates between predefined groups of patients with good or poor survival, e.g., estrogen receptor (ER) positivity or ER negativity. This approach has been described to discriminate, e.g., between tumor entities like different solid tumors [78], subgroups of acute myeloid leukemia and acute lymphocytic leukaemia [31], diffuse large B-cell lymphomas, between early- and late-stage ovarian carcinomas [52], and breast carcinomas with or without the potency to metastasize [85].

In a critical commentary, Simon, Radmacher, Dobbin, and McShane [72] already worried that "many investigators are not experienced in the analytical steps needed to convert thousands of noisy data points into reliable and

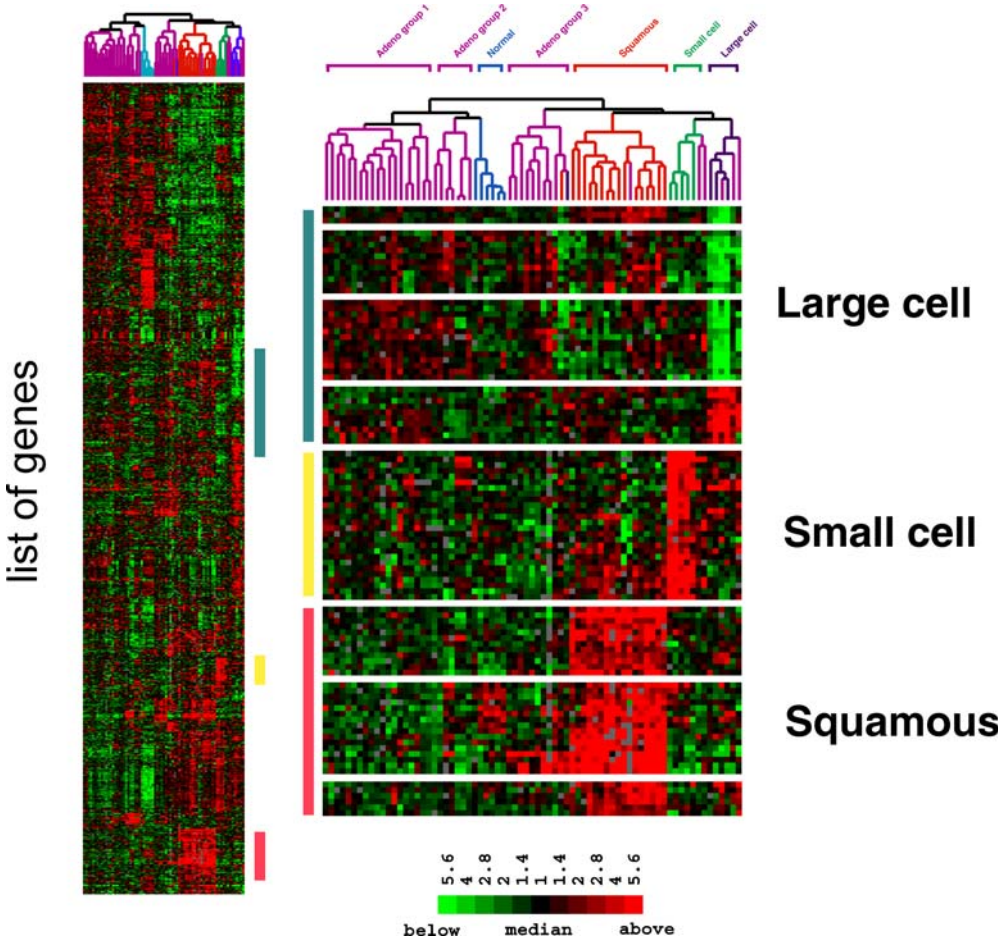

Fig. 1 Hierarchical clustering analysis exemplified for Topotecan-resistant (*R*) and Topotecan-sensitive (*S*) cell lines. All cell lines resistant to Topotecan (*left panel*) and all cell lines sensitive to Topotecan (*right panel*) express a unique set of genes. Each *row* in the cluster indicates the expression profile of a specific gene across all 19 cell lines. Each *column* indicates the individual cell line in which the gene is expressed. *Red*, *green*, and *black squares* indicate that expression of the gene is greater than, less than, or equal to the median level of expression across all cell lines, respectively. The *scale bar* reflects the fold increase (*red*) or decrease (*green*) for any given gene relative to the median level of expression across all samples

interpretable biological information". Furthermore, the molecular profiles are often relatively unstable due to tumour heterogeneity and platform specificity. The authors listed a number of prerequisites necessary to avoid misinterpretation of the data. In particular, the issue of class prediction is discussed as it includes derivation of classifiers, which predict prognosis, response to therapy, potential to metastasize, and many more. In a very recent publication by Michiels et al. [53], several of the most prominent classifiers were doubted. The authors concluded that the list of genes included in a molecular signature largely depends on the constitution and size of the training sets used and even claimed that a majority of the published classifiers are not significant. A comprehensive overview is also given by Scharpf et al. [67], suggesting several methodological approaches and illustrating the associated software to generate interpretable, robust, and less platform-dependent results.

A relatively new unsupervised approach to analyze array data is provided by linear independent component analysis (ICA) [37, 48, 49]. In contrast to cluster analysis methods, which assume that each gene belongs to just one cluster, ICA is based on the idea of a combinatorial control which is more realistic from a biological point of view. Gene expression levels are described as linear functions of common hidden variables which are (in the ideal case) related to distinct biological causes of variation such as transcriptional regulators or responses to treatments. Thus, ICA-based results show increased biological relevance and might describe new biological links previously undetected by clustering methods. An interesting study was conducted on endometrial carcinomas and benign endometrial samples [66]. The authors compared the power of ICA to other established methods and thereby detected (a) an improved separation of benign and malignant groups, (b) a characteristic expression pattern corresponding to the histological classification, and (c) a novel pattern of coregulated genes related to fatty acid metabolism which are clinically associated with endometrial carcinoma. Thus, a careful examination of histopathologically well-characterized tumor material via high-throughput methods and sophisticated bioinformatic approaches might even unravel novel disease mechanisms during such advanced diagnostic schemes.

Application of DNA microarrays

The application of high-throughput cDNA and oligonucleotide array technologies and the establishment of gene expression profiles has already revolutionized experimental tumor diagnostics in the last couple of years. With the use of these tools, otherwise indistinguishable tumor subgroups have been identified [2]. In addition, the development of gene signatures enabled prognostic conclusions which could not be drawn using standard histopathological and immunohistochemical methods [35, 84, 85], opening the door for a more comprehensive tumor analysis by diagnostic pathology. In the following, some examples of microarray applications will be described to demonstrate the wide range of applications where this technique could support and even improve the predictive power of histopathological diagnostics.

Molecular classification and prognosis

Based on hundreds of international studies including thousands of patients, an international classification system for malignant tumors based on conventional histopathology supported by immunohistochemistry has been established, the UICC–TNM system [73]. In general, the TNM system helps to estimate the prognosis of groups of tumors. However, this classical approach with conventional histopathology, analyzing morphological parameters, is not completely sufficient when the prognosis of an individual case shall be predicted. Therefore, the DNA microarray technique is currently under investigation to improve the current classification system by correlating array results with histomorphology and clinical behavior of tumors.

The first step towards a molecular classification was done by Khan et al. [39] who defined expression signatures from cancer cell lines which were indicative for the organ type of origin. A landmark study including gene expression profiling with DNA microarrays as an extension of histopathological classification has shown the possibility to differentiate between AML and ALL and to improve the precision of prognosis prediction [31].

Tumors with similar histological appearance but with different clinical behavior, such as small blue round tumors of childhood, are difficult to classify on a routine microscopical basis. Genetic profiling proved to correctly identify the four subgroups neuroblastoma, rhabdomyosarcoma, Ewings sarcoma, and special types of non-Hodgkin's lymphoma [40] and, thus, can be a valuable help in tumor classification.

The WHO grading system of oligodendrogliomas is somewhat limited due to subjectivity of histopathological evaluation. Gene expression profiling now unraveled two molecularly distinct subgroups which correspond to the morphological grading and, thus, can be applied to provide a more objective tool for grading and prediction of prognosis [89]. This approach may be extended towards other brain tumors where reproducible histological assessment is often difficult, if not impossible.

In a group of 55 breast cancer patients with balanced clinicopathological features, three subclasses of tumors with different 5-year survival could be identified by analyzing mRNA expression of only ≈1,000 candidate genes [7, 8]. In a population-based study, Sotiriou et al. [75] were able to suggest distinct aspects of breast cancer classification and prognosis based on gene signatures. The molecular analysis of pre-invasive vs invasive breast tumors revealed extensive similarities of the genetic alterations [51], suggesting that, in the early stage, the potential for invasive growth is already almost fully developed—an insight which might change the current strategy of early breast cancer treatment.

Further genetic subclassifications of tumor entities with distinct clinical features have been described for breast cancer [60, 74], cutaneous malignant melanoma [10, 86], large B-cell lymphoma [2], and B-cell lymphoma [14], pediatric ALL [92] and acute leukemia with MLL translocation [3], and adenocarcinoma of the lung [9, 26], ovary [57, 69], colon [25], and prostate [21].

Metastatic potential

Up to now, it is impossible to determine precisely whether a tumor which has not developed clinically detectable metastases at the time of diagnosis will metastasize during the following years. This information, however, would be of utmost importance to decide, e.g., whether an adjuvant therapy is necessary or not. The first steps to elucidate the metastasizing potency of an individual tumor have been published by van't Veer et al. [85]. In breast carcinomas with T1/T2, NO at time of diagnosis, the authors defined 70 "predictor genes" whose signature predicts with approximately 80% sensitivity and specificity the chance to set lymph node metastases. A recent study by Wang et al. [88] confirmed these data. This provides a rational to treat patients with adjuvant therapy or not.

Tissue assignment, primary and occult metastases

In clinical practice, it may happen that a metastasis is found while the primary tumor is unknown or it is unclear whether a certain metastasis is derived from a known primary tumor. Most often, histopathology, in addition to morphological immunophenotyping, can disclose the tissue origin. Nonetheless, gene expression profiling may be of additional help to classify cancer cells according to their tissue of origin [29, 39, 63, 78]. It was shown that the molecular signature of the primary tumor is preserved in its metastases and, thus, allows a clear assignment which gives the necessary information for an adequate treatment. Furthermore, it could be shown that DNA arrays are able to distinguish between lung adenocarcinomas and metastases of extrapulmonary origin [9]. The origin of cancer of unknown primary can be derived from characteristic expression patterns as the tumors often harbor distinct gene expression profiles characteristic for the organ type of origin [83].

Therapy response and drug resistance

Drug resistance remains a major problem during therapy for systemic cancer disease. Due to the high potency to adapt to therapeutical intervention, malignant tumor cells frequently develop escape mechanisms in response to radiation or cytostatic drugs. Although many attempts have been made [45, 46], up to now, reliable and practically applicable techniques to predict a tumor's reaction to drugs or radiation do not exist. The reason for this are multiple cellular mechanisms such as increased DNA repair, elevated levels of drug transporters, overexpression of detoxifying enzymes, and decreased rates of apoptosis which are often involved in the development of drug resistance. To monitor multiple alterations occurring in tumors being drug insensitive, highly parallel analyses, such as the DNA microarray technique and also proteomic analysis, are required. This opportunity opens new dimensions to predict therapy resistance and sensitivity.

A recent National Institutes of Health study [68] investigated 60 tumor cell lines (NCI60) which have been treated independently with more than 70,000 different agents, one at a time. Among other results, the study linked bioinformatics and chemoinformatics by correlating cellular drug response with transcriptomic information derived from DNA microarrays. The aim was to associate clusters of related drugs with clusters of gene alterations and, thereby, to define drug–gene relationships. This approach may contribute to the establishment of a defined expression database on which an individualized molecular pharmacology of tumor drug response can be established.

By comparing wild-type cell lines with derivatives resistant for thymidilate synthase (TS) inhibitors, Wang et al. [87] were able to identify certain patterns of genetic alterations correlating with TS resistance. The associated gene expression profile was partly tissue dependent, e.g., *YES1* was overexpressed in the epithelial cell lines while it was not upregulated in a lymphoblast cell line. Cisplatinum (cDDP) resistance was shown by microarray analysis to be accompanied with altered expression of genes coding for membrane proteins and a glycoprotein hormone subunit [33] not previously known to play any role in cDDP resistance.

In an excellent study by Zembutsu et al. [94], 85 human cancer xenografts were tested with regard to characteristic expression profiles in response to nine anticancer drugs often used in clinical therapy. More than 1,500 genes were identified whose expression profile has correlated in some way with chemosensitivity. The authors identified sets of genes which could partly be associated with chemosensitivity of particular tumor types (colon, breast, non-small cell lung cancer, etc.) to the different drugs applied. To predict the efficacy of adjuvant therapy in esophageal tumors, the DNA microarray technique was applied to 20 cancer specimens with clinically known responses [41]. There were 52 genes identified which were likely to correlate with patients' outcome and possibly with chemosensitivity and chemoresistance. This approach shows some potential to determine drug response in advance. In addition, bone marrow samples from 19 patients with acute lymphoblastic leukemia were investigated with regard to resistance to an ABL tyrosine kinase inhibitor [34]. On the basis of 95 differentially expressed genes, it appeared to be possible to distinguish responder from non-responder. Raponi et al. [65] showed the possibility to detect the pathways modulated by inhibitory drugs in AML elucidating the mechanisms of drug action. Thus, gene expression profiling can be helpful for the pretreatment assessment of anti-cancer therapy. Although a

vast number of experiments still have to be conducted, it might become possible to predict chemoresistance (Fig. 2) and to avoid non-effective drugs and unnecessary side effects for the patients. The discrimination between responders and non-responders before therapy will further stimulate the development of an individualized therapeutic strategy with a personalized combination of drugs.

Pitfalls of DNA microarray analyses

Numerous studies have shown characteristic expression patterns related to certain aspects of tumor biology. Dozens of genes were associated with prognosis, drug resistance, potential to metastasize, etc.; however, the clinical confirmation of the results is so far only on the way. The clinical application of DNA microarrays will require a high level of reproducibility and reliability of technology, of sample processing, and of analysis. A major obstacle is the still low inter-laboratory and inter-platform reproducibility of microarray data. Several of the reasons underlying this problem were described in 2003 by Tan et al. [80]. A comparison of the three most widely used commercially available platforms revealed significant discordance, which could in part be attributed not only to differences between the types of array but also to the different algorithms used for data evaluation. In 2001, the MIAME standard was already suggested, which includes a predefined set of information for a certain microarray experiment which allows for an independent evaluation of the results and for a conclusion to be drawn [12]. In a more recent publication by the Toxicogenomics Research Consortium, the impact of array type, data handling, image analysis, and experimental protocol onto the reproducibility of microarray analysis between different laboratories was investigated [5]. This study revealed that, like in the earlier reports, the type of microarray used has by far the largest influence onto the comparability of microarray data. More importantly, the authors conclude that, upon application of a common, commercial microarray platform, standardized experimental image analysis and data processing approaches correlation coefficients up to 0.9 can be achieved between experiments performed in different laboratories.

Thus, careful experimental design and a strict standardization of the whole procedure will be of utmost importance in the future for the reproducibility of microarray experiments. Furthermore, one should keep in mind the fundamental challenges, which have frequently obstructed cancer research during the last decades. These include inter- and intratumor heterogeneity, specimens either extracted, stored, or diagnosed inappropriately, and unexpected changes of cell line properties. These complications are still relevant and may be even accelerated by high-throughput techniques.

Proteomics

The rapid development of proteome technology has lifted tumor diagnostics to a new level. Despite the high relevance of DNA- and RNA-based information discussed above, nucleic acids are several layers of abstraction away from the physiological events that determine disease characteristics. Proteins govern metabolic processes, protein interaction, and posttranslational modifications and are, therefore, the major targets to be included in future molecular pathological approaches (see also review by Hanash [32]). Three fields of application are beginning to emerge: (a) highly sensitive mass spectroscopy coupled to an array-based separation of complex protein lysates (surface enhanced laser desorption time-of-flight, SELDI-TOF) or microdissected tumor samples are used for early

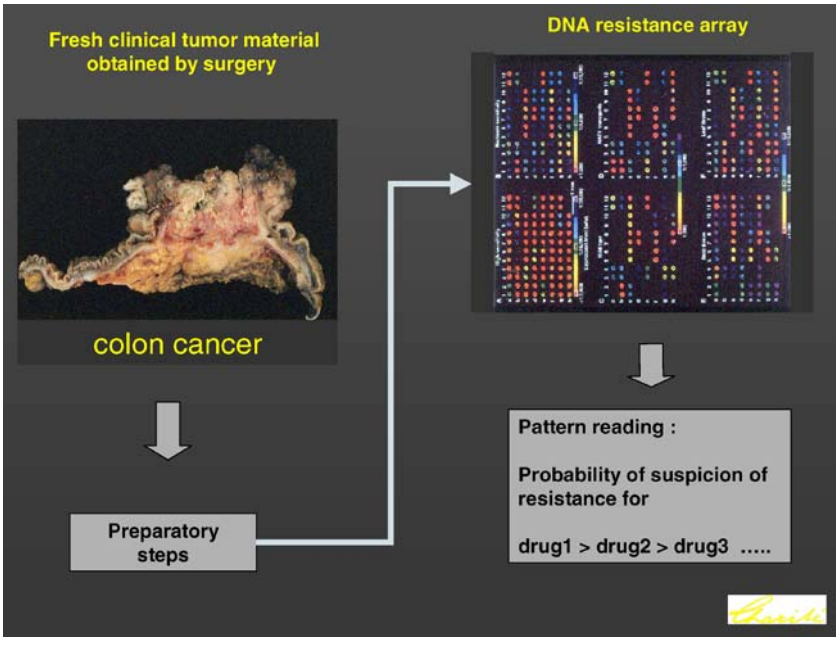

Fig. 2 A possible approach to predict chemosensitivity and/or chemoresistance of clinically relevant tumor samples. The low-density DNA arrays are specially designed to indicate the altered expression profile of sensitivity/resistance associated genes or EST. Different patterns appear to be correlated with sensitivity or resistance of malignant tumors for certain cytostatic drugs

detection and identification of new biomarkers for human tumors [59, 61], (b) a direct mass spectroscopic analysis and the establishment of protein profiles of tumor and normal tissue [14, 76, 91], and (c) the application of protein and antibody arrays for the specific identification of certain proteins and their activated forms in tumors [50, 55, 77].

Although technically advanced screening methods have improved early cancer detection, in some cancer types, such as ovarian carcinoma, new methods which allow easy and routine screening are urgently needed to ameliorate the currently frustrating clinical outcomes. For this purpose, the highly sensitive SELDI-TOF approach appeared as the most promising technological development during recent years. Petricoin et al. [61] reported the identification of serum proteomic patterns using SELDI-TOF and developed a bioinformatic tool to distinguish neoplastic ovarian disease from the normal status. By applying the serum spectra from 50 healthy women and 50 ovarian carcinoma patients as training sets, they proposed a proteomic signature which segregated cancer from non-cancer with a sensitivity of 100% and a specificity of 95%. Yet, these results were severely criticized and the expectations were smoothened in recent publications [4, 17, 96]. Furthermore, this provoked an intense debate about future processes of establishing and proving the reliability of novel technologies [22, 23, 64]. Thus, despite being an exciting new approach, K. Coombes et al. [17] recently stated that the "current state of the art in serum proteome profiling allows considerable room for improvement".

While serum proteomic analysis is currently tested in the identification of new and early cancer markers, SELDI-TOF has been successfully applied to the characterization of distinct tumor areas carefully dissected by the help of laser capture microdissection. This approach was used to identify markers differentially expressed in prostate cancer cells as compared to prostatic prostate intraepithelial neoplasms (PIN) lesions and normal prostate epithelium [16, 97]. Similar experimental schemes were also developed for a proteomic evaluation of bladder carcinoma [43], colon carcinoma [47], and breast carcinoma [19].

In addition to the direct identification of proteins using mass spectroscopy, antibody arrays and so-called reverse phase protein array approaches have also been developed and applied for the identification of tumor-associated proteins and also their activated phosphorylated derivatives. Nowadays still in a developmental stage, both types

Table 1 Examples of targeted therapy approaches

Drug	Tumor type	Target	Known action	Detection method	Remarks
Trastuzumab	Metastatic breast cancer	HER-2/neu	Rc blocking	IH, FISH, ISH	1
Cetuximab + Irinotecan	Metastatic colorectal cancer	EGFR	Rc blocking/immunologic response	IH, FISH	1
Imatinib Mesylate (Gleevec)	CML, GIST with activated c-kit receptor tyrosine kinase, other sarcomas	Bcr/abl, c-Kit, PDGF-R	Tyrosine kinase inhibitor	IH	1
Bevacizumab (Avastin) (+5FU)	Colorectal cancer	VEGF	Rc blocking	IH	2
G3139 (Genta, Berkley)	Hematologic malignancies and malignant melanoma	Antiapoptotic gene *bcl*-2 in	Bcl-2 antisense oligonucleotidecreasing Bcl-2 mRNA	Immunophenotyping by IH	2
Bortezomib, Epoxomicin	Multiple myeloma	Proteasome	Proteasome inhibitor		
Gefitinib (Iressa)	Non-small cell lung cancer	Mutated EGFR	Kinase inhibitor	Mutational analyses, immunoblotting	4
Erlotinib (Tarceva)	Non-small cell lung cancer	Mutated EGFR	Kinase inhibitor	Mutational analyses, immunoblotting	4
Rituximab (+ CHOP), Y90-Ibritumomab, I131-Tositumomab	Non-Hodgkin lymphoma	CD20	Lympholytic	Immunophenotyping IH	3
Gemtuzumab-Ozogamicin (calicheamycin)	AML (>60 years)	CD33	Anti-CD33 guided cytotoxic antibiotic, reduction of P-glycoprotein	Immunophenotyping by IH	3
Alemtuzumab (Campath)	B-CLL, T-NHL, osteogenic tumors[a]	CD52	Lympholytic	Immunophenotyping by IH	3
Rapamycin RAD001	Breast, prostate, renal cancer	TOR	Kinase inhibitor		4
BMS 354825	GIST	Kit	Tyrosine kinase inhibitor	IH	4
BAY43-9006	Melanoma	RAF kinase	Kinase inibitor		4

1 FDA-approved drugs requiring a pretherapeutic diagnostic eligibility test, *2* FDA-approved drugs targeting a specific pathway—no tests available, *3* FDA-approved antibody-targeted therapies for hematologic maliognancies guided by immunophenotyping, *4* under development, *Rc* receptor, *IH* immunohistochemistry, *ISH* in situ hybridization, *FISH* fluorescence in situ hybridization

[a]Patent no. EP:03029464.9, V. Krenn, Institute of Pathology (submitted)

of arrays might become valuable alternatives to cDNA or oligoarrays and also for immunohistochemical analyses during daily pathological routine. A major obstacle, among others, for these applications is the broad range of antibody specificity and affinity which complicates detection of defined antibody–antigen interactions [44]. Several studies which used antibody arrays to profile protein patterns in tumor samples derived from heptocellular carcinomas [81] or breast carcinomas [36, 42] applied verification strategies such as standard immunohistochemistry on tissue microarrays to demonstrate specificity of their platform. An interesting derivative of antibody microarrays are the RPAs. In this case, instead of antibodies, whole protein lysates are spotted onto glass surfaces and distinct proteins are detected via antibodies [58]. This procedure can be combined with laser capture microdissection and each patient set can be applied in miniature dilution curves to improve quantification accuracy and to enlarge the dynamic range. RPAs are generated from frozen tissue samples and, therefore, allow the use of detection antibodies specific for phospho-proteins. This technology was consequently described to compare survival pathway activation as measured by phosporylated Akt and Erk in normal prostate epithelium, in PIN, and in invasive prostate cancer [58]. In addition, it has been used in pathology for the investigation of the relative expression levels of Bcl-2 and Akt family members in follicular lymphomas [95]. One can anticipate the future implication of these methods in the elucidation of intracellular signaling networks downstream of tyrosine kinase receptors such as HER2 and also emerging from intracellular oncogenes such as RAS during cancer therapy. Many of the substances in the new generation of cancer drugs are designed to interfere with specific molecular targets, which are believed to have a critical role in tumor growth by regulating key signaling pathways (Table 1; Fig. 3). Yet, this requires the development of optimized protocols and superior detection techniques [27] and the use of reference standards as described recently by Sheehan et al. [70].

Epigenomics

Epigenetic modification of DNA by the addition of a methyl group to cytosine residues in CpG dinucleotides is one of the most important mechanisms of tumor suppressor gene inactivation known today [6, 38]. A DNA-based methylation analysis has several advantages when compared to the RNA-based gene expression analysis. The preparation of DNA suitable for such experiments can be successfully obtained from both frozen and paraffin-embedded tissues. Furthermore, according to current knowledge, methylation of genes is a rather stable modification not prone to produce as many artifacts due to RNA decay as in mRNA expression analysis. Methylation arrays are now consequently designed for the high-throughput analysis of DNA methylation at distinct CpG positions. Furthermore, a real-time PCR-based assay for gene- and methylation-specific detection has recently been developed [18]. For methylation arrays, genomic DNA from the tissue under investigation is treated with sodium bisulphite, resulting in the conversion of unmethylated cytosine residues into uracil [56]. Following this procedure, specific fragments from the regulatory, CpG-containing regions of genes are PCR-amplified using fluorescent-labeled primers. The uracil residues (UpG) are replaced by tyrosine residues (TpG) during PCR amplification. These fragments are then hybridized to their complementary oligonucleotides spotted onto a microarray, which are specific for the originally non-methylated CpG dinucleotide.

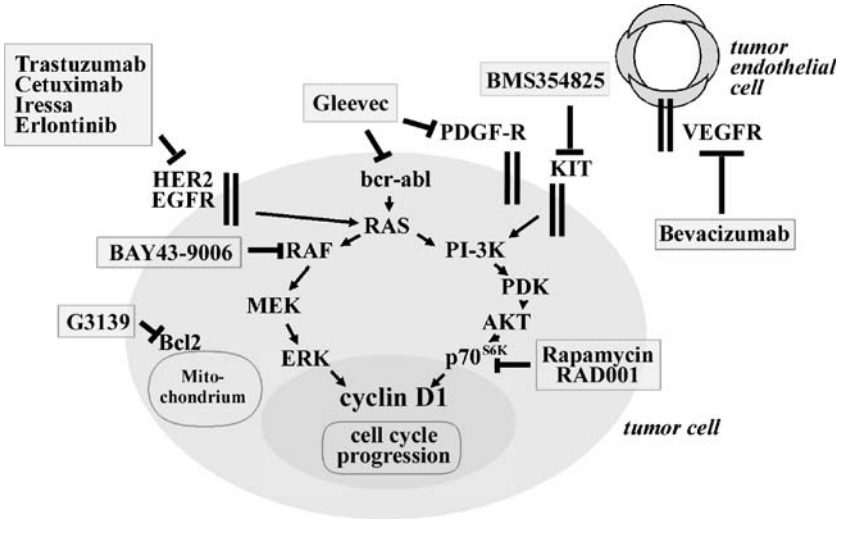

Fig. 3 Novel cancer drugs targeting signaling pathways. Several growth-stimulatory signaling pathways are activated in tumors due to tyrosine kinase receptor overexpression (*HER2* and *EGFR*) and mutation (*EGFR* and *KIT*). Inhibition of tumor cell proliferation is expected to occur via inhibition of receptor tyrosine kinases on tumor cells or on endothelial cells (*VEGFR*). Furthermore, intracellular protein kinases such as bcr/abl and RAF and phosphatidylinositol-related kinases such as mTOR can be targeted by specific inhibitors. Proteins like the antiapoptotic Bcl-2 can also be inhibited to support chemotherapy

The hybridization conditions are designed in a such way that a single mismatch between TpG and CpG variants yields a specific detection signal. Such applications have been used for the identification of CpG island methylation correlated with tumor progression [1] and to evaluate a potential significance for disease-free survival in ovarian carcinoma patients after chemotherapy [90]. In addition, sophisticated technologies (methylation target array) have enabled the detection of CpG island methylation of known tumor suppressor genes such as *WT1*, *BRCA1*, *p16INK4A*, and others in hundreds of tumor samples at the same time [15]. An interesting further development is the combined use of expression and methylation arrays, even in conjunction with an analysis of histone acetylation [71] using the same tissue samples. Such an approach allows the correlation of methylation data with expression data and the identification of functionally relevant genes harboring hypermethylated CpG islands with a higher accuracy [93]. Although a multitude of publications on high-throughput analysis of DNA methylation in tumor tissues already exist, it is evident that this technology has to be further developed to enter routine clinical diagnostic procedures. Nevertheless, it becomes clear that, in the future, a combination of transcriptomic, proteomic, and epigenomic analysis is required to identify the tumor-specific alterations with the highest relevance for prognosis and therapy.

Outlook—an integrated approach in future diagnostics

In recent years, the efforts devoted to unraveling the connection between activated receptor tyrosine kinases, intracellular oncogenes, and distinct intracellular signaling pathways and their role in growth control have uncovered a large number of potential therapeutic target structures. Herceptin and Gleevec targeting the *HER2/neu*, and *BCR/ABL*, *c-KIT*, and *PDGF-R oncoproteins*, respectively, are the first designer drugs which are already used with considerable success in the clinic. In the meantime, other drugs which act more or less specifically against a broad range of receptors and signaling components have entered clinical trials (Table 1) [20]. This development is intimately connected with the expectation that tumor therapy can 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 insitu hybridization) 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 tumors before and during therapy have to be developed and adapted to routine diagnostic pathology. This will clearly play an increasing role in the future tasks of pathological institutes.

Before transmission and establishment of such an integrated approach into daily routine can occur, extensive research has to be performed to allow a firm prediction on the activation of a certain pathway in clinical material. In this case, a strategy consisting of a parallel analysis of gene expression and methylation combined with a proteomic analysis of activated signaling pathways and overexpressed proteins will be the method of choice.

The implementation of bioinformatic procedures, which will undergo further standardization together with highly parallel tissue analyses, opens the door for a broad profiling of human cancer tissue, thus improving the possibility to predict the biological behavior of single tumors. Such a "multiplex approach" may help to functionally classify malignant tumors, provided that a "diagnostic algorithm", beginning with conventional histopathology in combination with immunohistochemistry, provides the basis. This basic evaluation will then be supplemented by specialized disease-specific analyses such as different kinds of micorarrays to predict tumor-associated features including tumor progression, drug response, and metastatic potential, which will have an important impact on tumor prognosis and adequate individual therapy.

The recent technical developments also indicate changing demands for routine pathological diagnostics within the next years. Only a consequent integration of the methods described within this review into pathological diagnostics will guarantee that, in the future, pathology is also the discipline holding the main body of knowledge about disease etiology and disease mechanisms.

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. Only when well-designed clinical studies prove the progress an implementation of molecular tumor profiling with individualized therapeutic strategies will become accepted to be routinely integrated in diagnosis and treatment of cancer. One major prerequisite for an advantageous way is the interdisciplinary cooperation of basis scientists, clinically oriented physicians, diagnostic pathologists, and clinicians which all must 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.

Acknowledgements The comments on the manuscript by Nils Bluethgen, Institute for Theoretical Biology, Humboldt University, Berlin are gratefully acknowledged. We appreciate the help of Balacz Gyorffy and we are especially grateful for the secretarial work of Mrs. von Bogen. This work was supported by Oligene GmbH, Berlin.

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