CANCER GENOMICS & PROTEOMICS *2*: 255-264 (2005)

Review

Application of Microarrays for the Prediction of Therapy Response in Breast Cancer

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Abstract. *Single genes, which can be used to predict response to therapy in breast cancer, including estrogen receptor (ER), HER-2, metallothionein and the ABC transporters are discussed. With the exception of the ER status, no single tumor marker has been shown to possess 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. With the advent of high-throughput quantification of gene expression, simultaneous assessment of thousands of genes is now possible, which allows identification of expression patterns in different breast cancers that might correlate with and, thereby, predict survival or response to treatment. Recent studies using microarrays to investigate survival prediction, chemotherapy resistance and therapy response are discussed. In vivo and in vitro experiments are discussed. Particular interest is given to anthracycline treatment, where in vitro drug resistance data may be useful for patient prognosis prediction. However, different microarray platforms can provide different results for the same experiment. A recommended statistical pathway is still not yet accepted. These problems have to be solved before future diagnostic applications using cDNA microarrays can be developed. In the near future, it can be expected that various microarray studies will be available to analyze hundreds to thousands of patients, selecting and validating predictive gene expression signatures.*

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Key Words: Breast cancer, cDNA microarrays, drug resistance, differential gene expression, response prediction, review.

The lifetime risk of being diagnosed with breast cancer is currently up to 1 in 8 women (1). Several factors, including early age of menarche, late age of menopause, pregnancy, obesity, serum estrogen concentrations and the use of estrogenic hormone replacement therapy or oral contraceptives, have been associated with an increased risk of developing breast cancer (2). Most patients whose cancer has not spread to the lymph nodes are cured by surgery and tamoxifen, but a small minority will go on to develop distant metastases and die. Chemotherapy is given as adjuvant treatment to reduce this risk, preventing distant metastases or as palliation to treat patients with metastatic disease. However, chemotherapy has frequent severe side-effects, including heart failure, leukemia and life-threatening infections.

Chemotherapy or hormonal therapy reduces the risk of distant metastases by approximately one-third (3). There are multiple combinations of cytotoxic drugs currently accepted as standard care. They are applied empirically despite the observation that all regimens are not equally effective across a population of patients with a particular type of breast cancer.

Estrogen receptor biology

A significant overall survival benefit was demonstrated with the administration of anti-estrogens in some breast cancer patients (4). Up to 75% of breast tumors expressing both estrogen receptor (ER) and progesterone receptor (PR) respond to tamoxifen.

The mechanism of action for these observations was investigated in *in vivo* and *in vitro*. *In vitro,* the MCF-7 human breast cancer cell line, selected for the ability to grow in the absence of estrogens, has been extensively studied (5). It has been demonstrated that anti-estrogens compete with estrogens for binding to ER. Anti-estrogens

include tamoxifen (6), toremifene, raloxifene and fulvestrant. Both ER and PR exist in two main forms. For the ER, these are known as ERα and ERβ, the products of distinct genes (7). The two forms of PR, termed PR-A and PR-B, are transcribed from a single gene under the control of separate promoters (8).

Interestingly, in preventive medicine, depending on the timing of exposure, increased estrogenic exposure can be associated with a reduced risk of breast caner (9).

Multidrug resistance

Cancer cells exposed to antitumor drugs may be directly induced to express genes that could confer drug resistance, thus allowing some cells to escape killing and form the relapsed resistant tumor. Alternatively, some cancer cells may express genes that could confer intrinsic resistance, and exposure to cytotoxic drugs select for survival these cells that form the relapsed tumor.

Multidrug resistance (MDR) is the simultaneous resistance of neoplastic cells to a variety of anticancer drugs of different chemical structures and different molecular mechanisms of action. In many MDR tumor cells, the drug-resistant phenotype is linked to drug efflux mediated by members of the superfamily of adenosine triphosphate binding cassette (ABC)-transporters (10), *e.g.* the "classic" membrane-embedded drug extrusion transporter *MDR1/P-glycoprotein (MDR1/P-gp)*, the product of the *MDR1* gene (11). Another member of the ABC-transporters, the MDR protein 1 (*MRP1*) gene was cloned from a multidrug-resistant lung cancer cell line (12). A third transporter was termed the breast cancer resistance protein (*BCRP*), because of its identification in multidrug-resistant MCF-7/AdrVp human breast carcinoma cells (13). All these membrane-embedded proteins act as drug efflux pumps, preventing cytotoxic agents from reaching lethal levels within the cells. Due to their tissue localization in the placenta, bile canaliculi, colon, small bowel and brain microvessel endothelium, these transporters may play physiological roles in protecting the organism from potentially harmful xenobiotics. To date, at least 12 human ABC-transporters have been associated with drug resistance and drug transport in cancer cells (14). At least 4 of them, MDR1/P-gp, MRP1, MRP2 and BCRP, can be expressed in specimens of breast cencer tissues (15). The spectrum of anticancer drugs effluxed by ABC-transporters includes the anthracyclines (*e.g.* doxorubicin), vinca alkaloids (*e.g.* vincristine), epipodophylotoxins (*e.g.* etoposide) and taxanes (*e.g.* paclitaxel) for *MDR1/P-gp* (16), anthracyclines, epipodophylotoxins, methothrexate, etoposide and vincristine for *MRP1*, and mitoxantrone, anthracylines, topotecan, irinotecan, methothrexate and flavopiridol for *BCRP* (14).

Potent and specific inhibitors of these ABC-transporters have been developed. For example, the reduction of *BCRP*-mediated MDR has been associated with the *MDR1/P-gp* inhibitor GF120918, mycotoxins Fumitremorgin C (FTC) (17) and Tryprostatin A (TPA) (18), or novobiocin, a coumermycin antibiotic which overcomes drug resistance by competitive inhibition (19).

Prediction of response

Only a proportion of patients will respond to a particular treatment. In breast cancer, hormonal therapy can achieve a 30% response, while the fluorouracil-doxorubicin-cyclophosphamide (FAC) combination achieves a 50-80% response (20). For optimum patient management, it is desirable to know, in advance, the likelihood that a tumor will respond to the therapy under consideration.

Predictive markers are factors that are associated with response or resistance to a particular therapy. The prototype predictive tests in oncology are ER and PR, which are used to select patients with breast cancer likely to respond to hormone therapy. Currently, most investigators use immunohistochemistry (IHC) to measure ER and PR, which can be carried out on small tumors, including core needle biopsy material. Patients with as few as 1-10% of cells staining for ER respond to hormone therapy (21).

The *HER-2* protein (*c-erbB-2, neu*) is a tyrosine kinase receptor without a known ligand (22). After heterodimerization, *HER-2* initiates intracellular signaling *via* the *MAPK*, phohphatidylinositol 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* (23). Down-modulation of *HER-2 in vitro* was associated with inhibition of growth, cell cycle progression – as a result of *p27* inactivation –, angiogenesis, and induction of immune response (24). Two main types of assay exist, *i.e.,* IHC and fluorescent *in situ* hybridization (25).

Although many different anticancer drugs appear to mediate tumor regression by inducing apoptosis, there is currently no consistent evidence that any of the molecules implicated in this process can be used as predictive markers (26). The *p53* tumor suppressor gene encodes a transcription factor, which is commonly mutated in human cancers (27). *p53* controls the expression of many genes that can be divided into categories of cell cycle inhibition, promotion of apoptosis, control of genome stability and inhibition of angiogenesis (28). Conflicting findings exist on the relationship between *p53* and response to chemotherapy (29). Similarly, other proteins involved in apoptosis, such as *bcl-2, bax, CD95,* or specific caspases, cannot currently be used for determining sensitivity or resistance to anticancer treatments (30).

The predictive value of ABC-transporters in breast cancer chemotherapy prediction was assayed in several studies. Although there was a trend, the relationship between pretreatment *MDR1/P-gp* concentrations and response to therapy was not significant ($p=0.088$) (31). Thus, *MDR1/P-gp* expression could merely be a measure of malignancy or advanced disease, rather than an indicator of chemotherapy resistance (32). It must be noted that no uniform measurement methodology for ABC-transporters has yet been established. Although, from a functional point of view, it appears most logical to interpret a reactivity associated with the cytoplasmatic membrane as specific, *MDR1/P-gp* in the cytoplasm can also contribute to the MDR in multiple myeloma cells (33). IHC studies identified more *MDR1/P-gp* expression than RNA-based methods. Using IHC in breast carcinoma cells, *MDR1/P-gp* was not even detectable in 88 tumor samples before or after chemotherapy exposure. Due to the high detection threshold, it was suggested that IHC should not be used for *MDR1/P-gp* detection in breast carcinoma cells (34). However, for some factors it was demonstrated that they can be involved in the regulation of *MDR1/P-gp* expression in breast cancer, *e.g.* the DNA-binding protein *YB-1* (35), or the enzyme cyclooxygenase 2 (*COX-2*) (36). Thus, detection of these factors may be useful to improve the analysis of the *MDR1/P-gp*-dependent drug resistance status of breast carcinoma.

Elevated expression of the low molecular weight *metallothionein (MT)* proteins can typically be found in breast cancer cases with less favorable prognosis. The *MT* gene has been described to be potentially down-regulated by estrogen receptor alpha. The predictive value of *MT* expression to predict response to tamoxifen treatment in breast cancer in relation to steroid receptor status was examined. The results demonstrated that elevated MT carried an ER status-independent unfavorable predictive value as far as the results of tamoxifen treatment were concerned (37).

Many additional clinical studies have correlated alterations in the expression of individual genes with breast cancer disease outcome, often with contradictory results. Examples include *cyclin D1* (38), *UPA, PAI-1* (39) and *c-myc* (40).

In summary, with the exception of the ER status, no single tumor marker has been shown to possess 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.

cDNA microarrays and breast cancer

With the advent of high-throughput quantification of gene expression, simultaneous assessment of thousands of genes is now possible, which allows the identification of expression patterns in different breast cancers that might correlate with and, thereby, predict survival or response to treatment. Because drug resistance or response almost certainly depends on the interplay of multiple genes, it is likely that the investigation of multiple markers will produce reliable predictive tests (41).

In breast cancer, pioneering studies have yielded the first expression patterns (42, 43). They have, in particular, addressed the important issue of molecular differences in hormone-responsive and non-responsive breast tumors. In these studies, cell lines were mainly used, while tumor samples were rarely tested and then generally in small numbers. Yang *et al.* (44) and Hoch *et al.* (45) compared the expression profiles of breast carcinoma cell lines known to represent the above categories and identified a few genes with differential expression.

The first study with an analysis of correlation with survival was published in 2000 (46). There, the extensive transcriptional heterogeneity of breast tumor at the transcriptional level was verified by studying the quantitative mRNA expression levels of 176 candidate genes in 34 primary breast carcinomas.

Classification. The major distinctions in breast cancer are based on ER and *HER-2* status rather than histopathological tumor type. The gene expression pattern, corresponding broadly to ER-positive and -negative tumors, have been named luminal and basal, respectively (47). By investigating patients with large operable or locally advanced breast cancers on Affymetrix HGU133A microarrays, it is possible to divide mammary tumor cells into three groups based on steroid receptor activity: luminal (ER+ and androgen receptor+), basal (ER– AR–) and molecular apocrine (ER– AR+) (48). Thus, using gene expression signatures, it was possible to identify a third group where androgen signaling replaces estrogen signaling as a major determinant of the steroid-related expression profile of the cells. The presence and prevalence of the molecular apocrine tumor was verified in four previously published data sets. In two studies, the molecular apocrine profile was associated with poor long-term survival (48).

Inflammatory breast cancer (IBC) is a rare but aggressive form of breast cancer. The tumor is often of high histological grade, negative for hormone receptors and highly angiogenic and invasive (49). Bertucci *et al.* investigated 81 patients and identified a 109-gene set, the expression of which discriminated IBC from non-IBC samples (50).

Survival prediction. A pathology examination and molecular markers broadly hint at whether a tumor may metastasize. However, individual patients with the same stage of the disease can have markedly different treatment responses and overall outcome. Van't Veer *et al.* used microarray

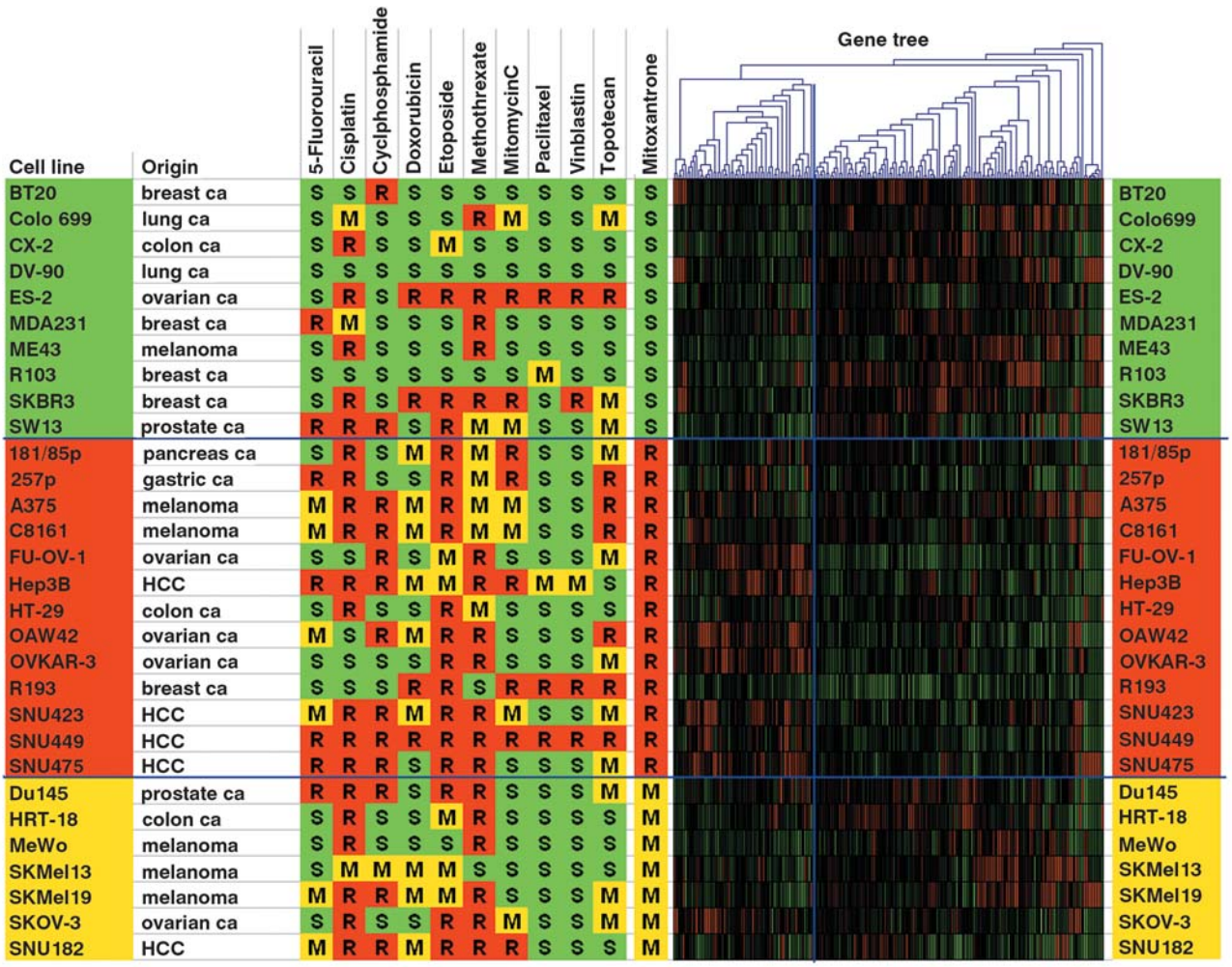

Figure 1. *Resistance signature meets gene expression signature. On the left side, the resistance characteristics of 30 cancer cell lines against 11 anticancer drugs are depicted (R: resistant, S: sensitive, M: intermediate). On the right side, the gene tree of the genes associated with mitoxantron resistance is shown. The picture was constructed using data published in (68).*

analysis on primary breast tumors of 117 young patients, and identified gene expression signatures predictive for short interval to distant metastases in patients without tumor cells in local lymph nodes at diagnosis (51). Their gene expression profiling of the primary tumor was reported to outperform all clinical parameters in predicting disease outcome. Interestingly, none of the previously identified individual genes were present in the predictive gene set of 70 genes published by van't Veer *et al.*.

The biggest flaw in their study lies in the incorporation of the test group – the group they derived the 70 genes from – into the validation group, potentially inflating the results. Thus, new validations are needed. TransBIG, a network involving about 40 partners in 21 countries, will complete a retrospective analysis to determine whether van't Veer *et al.'s* results are reproducible in a more diverse population. If the data is consistent with the original findings, TransBIG will launch a 5,000 patient prospective randomized trial. The results of this latter study will not be available before 2010, given the 5-year follow-up required for the data to mature.

Response prediction. The test to predict survival has a major limitation: it does not really identify which patients are likely to benefit from chemotherapy. Although it is assumed that patients most at risk of metastases would benefit most from chemotherapy, predictive tests to select responsiveness or resistance to a specific treatment must to be constructed. Patients with features of poor prognosis would be candidates for treatment other than standard chemotherapy, avoiding loss of time and toxicities related to first-line chemotherapy.

Early studies on the use of microarrays for predicting anticancer drug response focused on cell lines (52, 53). To

date, only a few preliminary studies have been published on the use of microarrays for predicting clinical response. Gillet *et al.* developed a low-density DNA array, which contains 38 genes of the ABC-transporter gene family (54). Their tool has been validated with three different multidrug-resistant cell lines known to overexpress either *MDR1*/*P-gp*, *MRP1* or *BCRP*. Chang *et al.* investigated core biopsy samples from primary breast tumors in 24 patients before treatment and then assessed tumor response to neoadjuvant docetaxel using Affymetrix HGU95-Av2 chips (55). The taxanes, docetaxel and paclitaxel, can be more effective than anthracyclines (56, 57), but only a small subset of patients benefit from the additional treatment (58). Chang *et al.* identified a 92-gene predictor, which could allow a test for docetaxel sensitivity. Two years later, they published a new follow-up analysis of docetaxel treatment response in these patients (59).

In our recent study (60), we contrasted the mRNA expression profiles using cDNA arrays with 43,000 cDNA clones of 13 different human tumor cell lines derived from gastric (EPG85-257), pancreatic (EPP85-181), colon (HT29) and breast cancer (MCF7 and MDA-MB-231) with drug-resistant sublines. The drug-resistant sublines either exhibited a "classic" *MDR1*/*P-gp*-dependent MDR phenotype or an "atypical" *MDR1*/*P-gp*-independent MDR phenotype. Although absolute changes in gene expression varied in the individual paired samples, a group of genes has been identified characterizing major common transcriptional changes exerted by the development of chemoresistance. The top 79 genes best correlated with anthracycline resistance and the top 70 genes with mitoxantrone resistance were identified. In an independent classification experiment, our model of resistance was applied to predict the sensitivity of 44 previously characterized breast cancer samples (61). The patient group, characterized by the gene expression profile as similar to those of anthracycline-sensitive cell lines, exhibited more than 50% longer survival than the drug-resistant group. The application of gene expression signatures derived from doxorubicin-resistant and -sensitive cell lines allowed the effective prediction of clinical survival after anthracycline monotherapy. Our approach demonstrated the ability to apply the results of *in vitro* experiments for actual patient prediction.

Doxorubicin treatment. Exposure of cancer cells to increasing concentrations of the cytotoxic anthracycline doxorubicin – which targets topoisomerase IIA blocking the G2-M transition – *in vitro* leads to development of cells with the MDR phenotype (62). The development of a fully resistant phenotype can be associated with many mechanisms including increased extrusion from the cell, metabolism, phase II conjugation and DNA damage repair. Gene expression signatures associated with doxorubicin resistance have been extensively studied, as it is one of the major anticancer drugs applied for breast cancer. Turton *et al.* analyzed 4224 genes for association with intrinsic or acquired doxorubicin resistance (63). Kudoh *et al.* investigated DNA microarrays containing 3800 genes to monitor the gene expression profiles of doxorubicin-induced and doxorubicin-resistant cells (64).

Recently, the priority of research has shifted to genome-wide microarrays. Ayers *et al.* have developed a multigene predictor of pathological complete response to sequential weekly paclitaxel and fluorouracil + doxorubicin + cyclophospahmide (T/FAC) neoadjuvant chemotherapy regimen for breast cancer using 42 patients' clinical results and cDNA arrays containing ~31k genes (65). Kang *et al.* identified the top 74 doxorubicin resistance-associated genes in drug-resistant gastric cancer cells using Affyemtrix HGU133A microarrays (66). Troester *et al.* identified *in vitro* and *in vivo* changes in gene expression induced by doxorubicin and 5-fluorouracil (67). The dominant expression response in each of the cell lines was a general stress response. They verified the *in vitro* responses with expression responses in breast tumors sampled before and after treatment.

Cell lines are as unique as the tumors from which they are derived. Thus, common response patterns only become identifiable when examining multiple cell lines in concert. 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 (68). A resistance index was determined to designate the cell lines as sensitive or resistant, then the subset of resistant *versus* sensitive cell lines for each drug was compared by interrogating Affymetrix U133A arrays. An individual prediction profile for the resistance to each chemotherapy agent was constructed, containing 42 to 297 genes (see Figure 1 for mitoxantrone). The study focused on the resistance at *in vivo* concentrations, making future clinical cancer response prediction feasible.

Biomarkers reflecting the short-term tumor response to doxorubicin can function as sensitive surrogates of long-term outcome. Modlich *et al.* identified genes undergoing expressional changes shortly after the beginning of neoadjuvant epirubicin/cyclophosphamide or epirubicin/taxol treatment (69). They took biopsies from patients with primary breast cancer prior to any treatment and 24 hours after the beginning of neoadjuvant chemotherapy. Sotiriou *et al.* evaluated the correlation between the expression profiles from fine-needle aspirations (FNA) performed on breast carcinomas and subsequent clinical responses after adjuvant chemotherapy. Expression profiles of ten samples before chemotherapy and 21 days after the first cycle of doxorubicin and cyclophosphamide therapy were compared.

They discovered that good responders exhibited more changes in their gene expression profiles after therapy than did the poor responders (70). They also demonstrated the suitability of FNA-derived cDNA microarray expression profiling of breast cancers as a comprehensive genomic approach for studying the mechanism of drug resistance.

Array problems

However, the application of current microarray technologies shows many flaws which have to be addressed before future diagnostic applications can be developed. These problems include the difference between array platforms and different statistical approaches.

Currently, several different microarray platforms are in use, including commercially available *in situ* synthesized Affymetrix and oligonucleotide arrays spotted on glass slides. Many university facilities produce their own spotted chips using PCR-amplified probes. A very important issue is to correlate the results obtained on one particular microarray platform to another. In the study of Modlich *et al.,* the results from the Clontech platform were compared with those of the Affymetrix platform. This direct comparison revealed a limited agreement between the two different array models. The lack of agreement between gene expression measurements from different commercial microarray platforms have been reported previously (71). In general, real-time RT-PCR confirmed the results on the Affymetrix system more often than those from spotted cDNA microarrays (72). Another example to illustrate these problems is a MDR gastric carcinoma model system consisting of the parental drug-sensitive cell line EPG85-257P and two different MDR variants, EPG85-257RDB and EPG85-257RNOV (73). The mRNA expression profiles were analyzed by using spotted cDNA arrays from Clontech (74), by the Stanford platform (60), by the Affymetrix technology (68) and by the Agilent approach (75). Each technology platform revealed different data that were in part identical, but in other parts contradictory. Furthermore, not all of these technology platforms were able to detect well-known differences in gene expression in these cell lines that had been demonstrated by "classic" blotting procedures previously, *e.g. BCRP* (76), *TAP* (77), or *GPC3* (78).

A second major impediment to the evaluation of microarray data is the problem of multiple testing. Even if there is no real change, the traditional $p=0.05$ can cause 5% of the investigated tests to be reported significant. On a microarray containing 10,000 genes, this will result in 500 false-significant differentially-regulated genes. Multiple testing corrections have been developed to solve this problem. For genome-wide studies, the calculation of the false discovery rate is a new method in which the p value is substituted by the q value, which also shows the level of significance (79). The q value of a measurement is the proportion of false-positive measurements when we accept it as significant.

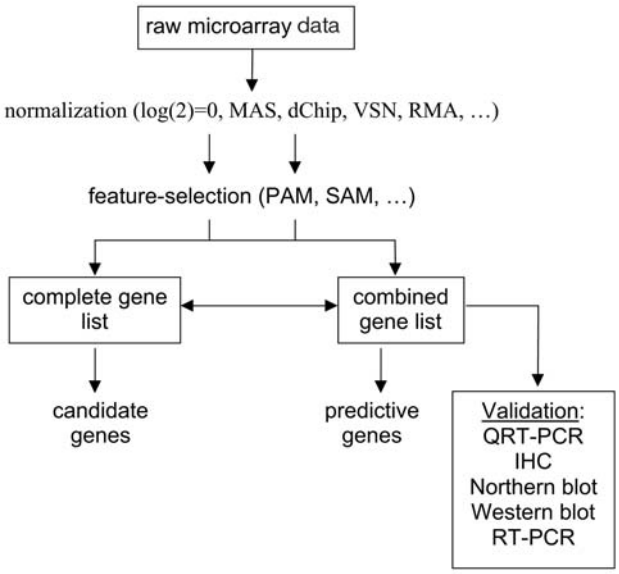

Figure 2. *Recommended statistical analysis pathway to achieve reproducible and comparable results in different microarray studies.*

The MIAME (minimum information about a microarray experiment) criteria have been accepted to ensure the availability of data about physical hybridization, scanning and *in silico* pre-processing steps (80). A final, perhaps most important, problem is the application of many different statistical algorithms for the same classification problem. The in-depth comparison of the different statistical methods is out of the scope of this review; for detailed discussion, please refer to the corresponding literature (see publications of the Microarray Gene Expression Data Society - http://www.mged.org/).

In Figure 2, a pathway for statistical analysis of microarray data is recommended. This includes at least two different normalization methods and a feature selection, which produces a q value. The validation should focus on genes which were significant in at least two different statistical calculations.

Outlook

A major impediment to the study of predictors of effectiveness of adjuvant treatment is the absence of surrogate markers for survival and, consequently, large numbers of patients and long-term follow-ups are needed. Although 'leave one out' cross validation approaches suggested that the selected gene lists could correctly classify most patients, the applied numbers are small and the

results, therefore, should be considered exploratory. Based on the characteristics of learning-based predictors, we can expect that second-generation predictors trained on larger training sets of cases will have improved precision, and larger training sets will improve the estimation of predictive accuracy. In the near future, we can expect microarray studies investigating hundreds to thousands of patients to select predictive genes which could be used as clinical diagnostic devices.

Acknowledgements

Own studies in the field were supported by the European Union (MERG-CT-2004-012454 and HPMD-CT-2000-00001), by the Berliner Krebsgesellschaft e.V., by Oligene GmbH, Berlin, Germany, and by the National Office for Research and Technology, Hungary.

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Received August 22, 2005
Accepted August 31, 2005