Clinical Impact of Gene Expression Profiling on Oncology Diagnosis, Prognosis, and Treatment

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Abstract: Genomics has enabled the examination of the totality of disease at the transcriptome level. Dependent upon a myriad of genetic aberrations for its pathogenesis, cancer has been the focus of gene expression profiling studies that have highlighted the potential clinical applications of this technology. This type of molecular profiling has the potential to enhance the ability of pathologists and oncologists to correctly classify tumors, not just into existing subgroups which may or may not have clear prognostic implications, but into new groups which carry predictable correlations with outcomes. Ultimately, these outcome predictions can be tied to specific treatment regimens, allowing clinicians to predict at the time of diagnosis to which therapy a given patient may best respond. Although this ultimate goal of personalized therapy remains in the future, the numerous studies to date have clearly demonstrated the overall feasibility of this approach. This review will showcase a few of these studies in several key tumor types with the goal of demonstrating which type of studies have been conducted and what types of results are currently possible.

Keywords: gene expression profiling, microarray, cancer classification, cancer prognosis, cancer treatment, hierarchical clustering, learning algorithms, responders.

INTRODUCTION

Genomics, the exploration of complex patterns of gene expression, has clearly impacted cancer basic research, shedding light upon tumorigenesis and progression, pathway elucidation, target identification, and the genes involved in drug resistance. In the past, much of this work has been conducted in immortalized cancer cell lines. However, through the use of primary patient samples, genomics also has the potential to impact the management of cancer in several ways. These include the assessment of the overall lifetime risk of cancer, screening for the presence of cancer, classification of the cancer and how this impacts prognosis, determination of appropriate therapy (weighing both efficacy and safety), monitoring the efficacy of therapy, and monitoring the disease (assessment of relapse/remission/minimal residual disease) [1]. Several of these applications fall most neatly into the realm of biomarker analysis, where specific biological markers indicate the risk or presence of disease. However, the classification, prognostication, and selection of treatment regimens for cancer are much more complex assessments of actual tumor specimens. Given the genetic pathogenesis of cancer and the numerous transcriptional manifestations of each individual genetic alteration, these assessments are likely to be multivariate in nature. Thus, the ability to examine the totality of the disease in order to impact disease management is a truly genomic endeavor.

This review will focus on the clinical impact of gene expression profiling in oncology diagnosis, prognosis, and treatment. Given the wealth of publications in this field over the last 4 years, this review can in no way be a comprehensive review of the literature. Nor is it the purpose of this review to engage in a detailed discussion of the specific genes identified as part of the relevant signatures in these publications. Rather, the intent is to highlight several key cancer types for which there has been significant progress with emphasis upon the methodologies and the types of information which can be gleaned from them. To truly impact clinical management of cancer, several sequential steps must be followed in the development of expression profiling for clinical use. These are not unlike those required for the development of any type of clinical assay and include: 1) preclinical exploratory research, 2) clinical assay evaluated for the ability to detect established disease or known parameters, 3) retrospective longitudinal studies to define if the assay parameter detects disease before it is clinically evident and define a positive screening result, 4) prospective screening to test the ability of the screen to detect the extent/characteristics of disease and determine the false positive rate, and 5) the determination of the impact of screening on reducing the burden of disease through modifications in disease management/treatment [2]. As yet, there have been no published prospective studies in the field of oncology which assess the ability of a gene expression profiling test to screen effectively and thereby impact the course of disease management in a personalized fashion (i.e. points 4 and 5). However, in light of the recent progress on the first three steps of development, it seems appropriate to review the field and anticipate the future applications in cancer management.

Review of Key Methodologies

In cancer research, genomic methods have been developed with the goal of examining the entire transcriptome through the use of gene expression profiling using microarrays or other methods which examine large sets of genes at a time.

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Numerous reviews on microarrays have already been published [3-5]. Key information which must be assessed in any array experiment include: sample information, array design and hybridization procedures, and experimental design and data analysis. Table **1** summarizes several of the key definitions which will be employed throughout this review. For detailed description of the terms, the reader is referred to the many microarray reviews.

SELECTED TUMOR TYPES

NON-HODGKIN'S LYMPHOMA (NHL)

Background

Non-Hodgkin's lymphoma (NHL) is the most common hematological malignancy, with an estimated incidence of 53,400 in 2003 in the US, and 23,400 deaths [6-7]. Lymphomas appear to develop at discrete stages of lymphocyte differentiation secondary to critical molecular alterations, especially chromosomal translocations. Thus, the advent of genotyping has dramatically aided in the discrimination of the many classes of lymphoma found in the World Health Organization (WHO) classification scheme [8-9]. Yet, despite these advances, considerable inter- and intra-observer variability renders pure histological diagnoses imprecise, and within a given histological type, prognosis varies widely with clinical characteristics [10]. Significantly, treatment protocols are generally based upon stage or prognosis rather than upon histological subtype of lymphoma with a few notable exceptions [11]. Thus, the International Prognostic Index (IPI) is dependent upon not only the classification of the lymphoma, but on five pretreatment clinical characteristics as well: age, tumor stage,

Table 1. Terms and Definitions

Sample Sets	
Training Set	The set of samples with known classification for which a gene set is identified which best classifies all the samples into their known classification groups in a retrospective sense. This set allows the computer to "learn" how to classify the samples.
Test Set/Validation Set	The independent set of samples with blinded classification against which the classifier gene set is tested for the ability to correctly predict the classification status in a retrospective sense.
Unknown Set	The independent set of samples with unknown classification for which the classifier gene set is used to classify the samples in a prospective sense.
Types of Arrays	
cDNA	These arrays are constructed by spotting cDNAs (usually from PCR amplification of particular libraries) onto a surface. Sample RNA can be radioactively labeled as it is amplified or modified such that quantitative expression can be measured. Alternatively the sample can be mixed with RNA from a control sample, each labeled with a different colored dye (typically Cy3 and Cy5), and the ratio of the fluorescent intensities of the dyes measured, determining the expression of the sample relative to that of the control.
Oligonucleotide	These arrays are constructed by several methods that generally involve the covalent synthesis of oligonucleotides upon the array. The sequence of the oligonucleotides can be selected from the most specific region of the target gene, or multiple short oligonucleotides can be used to represent a single gene. Affymetrix chips are synthesized by photolithography and generally hybridized to samples labeled with biotin. The hybridized arrays are then stained with fluorescently tagged streptavidin. Rosetta/Agilent arrays are synthesized by Inkjet technology and are typically run as ratio experiments with test sample and control sample labeled with different colored dyes.
Types of Analysis	
Cluster Analysis	Clustering is a multivariate procedure for identifying natural groupings in the data when neither the number of nor the elements in the groups are known.
Unsupervised (two-dimensional) Clustering	In this method, both samples and genes are clustered without any knowledge of the groupings within either set. Hierarchical clustering begins by finding the two most similar pairs of either samples or genes (i.e. two samples for which the gene expression patterns are most similar, or two genes for which their expression across all the samples are most similar), and clustering them. The procedure then finds the next most similar sample or gene to the first cluster, and so forth until all the data is included in one overall cluster. The relationships between the individual samples or genes are expressed in a dendrogram.
Supervised Learning	Clustering is conducted in a supervised manner in which one axis has known groupings. This allows the computer to "learn" how to distinguish those known groups.
Leave-One-Out Cross-Validation	A single sample from a set is removed and the remaining samples clustered to identify those genes most correlated with the class distinction. The removed sample is classified based upon those classifier genes. This sequence is repeated for each sample in the set, and the resultant gene sets used to build the predictor model.
k-Nearest Neighbor	A single test sample is compared to a training set. The k nearest samples in the training set are identified, and the test set is labeled according to the most common class of those k nearest neighbors. This is can be conducted as part of the leave-one-out cross-validation.
Principle Component Analysis	Multidimensional data is simplified into a limited number of dimensions by grouping patterns of data together to allow its easy visualization. Simplified gene expression data has been labeled "supergenes" or "metagenes."
Multidimensional Scaling	Data is visualized in three-dimensional space to allow visual grouping of samples. The position of each sample is determined by its gene expression pattern (often simplified by principle component analysis to three dimensions).
Support Vector Machine	This is a supervised classification method by which the computer is trained to classify groups correctly.
T-Test	This test determines whether the mean of a variable for samples in one group are significantly different from that of another group. This test assumes a normal distribution of samples in a group. Other methods such as the Mann-Whitney or the commercial Infoscore are more distribution-free.
Discriminant Analysis with Variance	This procedure identifies a linear combination of quantitative predictor variables that best characterize the differences between samples in two or more known groups.
Neighbor Analysis	This method creates an idealized gene expression pattern that maximally discriminates between classes and then searches for genes that behave near to the ideal rather than in random patterns.
Self-Organizing Maps	These allow the user to partition the data into a designated number of clusters.

the number of extranodal sites of disease, performance status, and serum LDH level [10,12]. However, even those patients with poor IPI scores can have significant long term overall survival (OAS) and failure free survival (FFS) [10]. A recent study focusing specifically upon diffuse large B-cell lymphomas (DLBCL), the most common subtype of NHL, also found a five year FFS of 73% for IPI scores of 0-2, but 37% with IPI scores of 3-4 when all patients were treated with regimens of CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) [13]. Moreover, patients with identical IPI scores but histologically different tumors can have dramatically different outcomes. Thus, in the case of many of the lymphomas, the medical need is not necessarily for added classifications of disease per se, but rather for classifications that carry prognostic implications which can impact treatment selection. Low risk patients may do very well with standard therapy, and thereby avoid the adverse effects of the more experimental regimens. Alternatively, for a high risk patient unlikely to benefit from the standard of treatment, more intensive or experimental therapies may be worthwhile.

Array Studies

Core studies in this area from both a Stanford-based group and a Harvard-based group have focused on the subclassifications of DLBCL and their association with patient outcomes.

Classification and Outcomes

In 2000, Alizadeh *et al.* (Stanford group) published their initial work using gene expression profiling to identify new subclassifications of DLBCL with the prognostic implications of that classification refinement. This study compared 96 normal and malignant purified lymphocyte samples at diagnosis (treatment naive) on their Lymphochip microarray against a pool of 9 lymphoma cell lines [14]. This array contains 17,856 specially selected cDNA clones from libraries largely derived from lymphocytes/lymphomas [15]. Two-dimensional unsupervised hierarchical clustering resulted in the clean segregation of the different histological classes of malignancies; i.e. the DLBCLs clustered together as did the chronic lymphocytic leukemias (CLLs) and the follicular lymphomas (FLs). The various states of activation of B and T cells likewise clustered together. Interestingly, the more indolent CLL and FL samples clustered near resting B cells while the more aggressive DLBCL samples where characterized by a strong signature of proliferation genes. Reclustering of those DLBCL samples against only those genes characterizing germinal center (GC) B cells resulted in the formation of two large subgroups distinguished by 100 genes (Fig. **1A**). One group showed an increase in the expression of GC genes and was therefore designated germinal center B-like DLBCL. The second group, termed activated B-like, showed little to no expression of those genes. Interestingly, the GC B-like patients appeared to have a better overall survival.

This initial study is remarkable in its ability to tease out clinically relevant information without any type of supervised learning algorithms or specific methods to identify discriminating genes. To further examine the prognostic value of the classification, the authors studied an expanded group of 240 chemonaive patients, 160 of whom served as a second training set, and 80 patients who served as a test set [16]. All patients were subsequently treated with CHOP and followed for 2-8 years. As before [14], unsupervised two-dimensional hierarchical clustering of the new training set segregated the samples into the GC B-like subgroup, the activated B-like subgroup, and a heterogeneous group of DLBCL termed diffuse. The overall survival of the three subgroups did significantly differ after CHOP therapy ($P < 0.001$) with a 60% 5-year OAS for the GC B-like group, a 35% OAS for the activated B-like group, and a 39% OAS for the diffuse type. However, there was no strict correlation found between the histological subclassifications of DLBCL, the molecular profile classification, or the IPI risk groups ($P = 0.44$).

To further refine the discrimination of risk groups, the authors used a Cox proportional hazards model to identify the 17 most discriminating genes which correlated directly with the clinical outcome of the training set ($P < 0.01$). Based on this profile, both the training set ($P < 0.001$) and the test set of 80 cases ($P < 0.001$) were assigned scores which correlated with overall survival. Within both the IPI-based risk groups (IPI 0-1, 2-3, 4-5) and the three profile-based subgroups of DLBCL (GC B-like, activated B-like, and diffuse), the molecular predictors were able to further distinguish high and low risk groups, indicating the predictor's prognostic value above and beyond those of the IPI or the molecular classifications (Fig. **1B**).

Shipp *et al.* (Harvard group) went directly to the supervised learning algorithms both to recreate histological classifications (FL vs. DLCBL) and to identify those DLBCL patients likely to be cured by conventional CHOP chemotherapy and those likely to be refractory [17]. These researchers began with a supervised binary clustering algorithm to identify genes on an Affymetrix oligonucleotide array (6817 genes) which distinguish the two classes of tumors. In addition to segregating DLBCL from FL samples, the authors assigned 58 DLBCL primary samples (not purified lymphocytes) to one of two groups, those from patients who achieved a complete response (CR) on CHOP (n = 32) and those from patients who were refractory or progressed on CHOP (n = 26). Leave-one-out cross-validation method successfully identified a 13-gene model which predicted outcome with the greatest accuracy, segregating the patients into one group with a 70% 5-year OAS, and one with only a 12% 5-year OAS ($P = 0.00004$) (Fig. **2**). Other classification algorithms such as support vector machines and k-nearest neighbors resulted in similar levels of discrimination. As in the case with the Stanford studies, the 13-gene molecular predictor provided independent risk assessment beyond that provided by the conventional IPI.

Similar studies have been conducted recently which further subdivide the DLBCLs into cases derived secondarily from follicular cell lymphomas [18], and mediastinal B cell lymphomas [19-20].

Discussion

Interestingly, both sets of studies arrived at similar conclusions despite significant differences in array platforms and the genes represented on them, patient selection and

types of sample used, and analysis methodology. While the Stanford group used a cDNA array with genes biased towards lymphocyte/lymphoma gene libraries, the Harvard group used the Affymetrix oligonucleotide HU6800 chip. Thus the former required ratio-based experiments, while the latter are single channel experiments. The Stanford group used purified lymphocytes from their patients while the Harvard group used solid lymph node specimens. In fact, several of the genes characterizing the FL signature in the latter study were specific to T-cells or dendritic cells, demonstrating how the stromal elements in the tumor microenvironment can characterize the signature. Lastly, the Stanford group relied heavily on unsupervised hierarchical clustering methods, using a Cox proportional-hazards analysis to identify discriminating genes. Their 17-gene predictor model was defined by those genes showing the greatest variance. By contrast, the Harvard group used several supervised learning algorithms to identify their optimal 13-gene predictor model. In both the initial Stanford study and the Harvard study, only a training set was used, although the Harvard group did perform cross-validation testing. The 2002 Stanford study, however, used both training and test sets to validate their conclusions with large numbers of samples per set.

Despite these differences, there was sufficient overlap in the genes represented on the two different array platforms to conduct comparative studies. Shipp *et al.* used a 90 gene classifier derived from the Alizadeh *et al.* 100 gene set to subdivide their DLBCL samples into GC B-like and activated B-like classifications. However, in their study, this classification did not correlate with outcome. There was also no overlap between their 13-gene predictor model and the 17-gene predictor model discovered by Rosenwald *et al.* This is hardly surprising given the profound differences in the methods by which these models were identified. By contrast, Wright *et al.* have also used the Stanford gene sets to segregate the two DLBCL subgroups in the Shipp *et al.* data set [21]. By using only those genes which most highly discriminated between GC B-like and activated B-like subgroups and were present on the Affymetrix arrays used by Shipp *et al.*, the authors did find significant differences in 5-year survival rates after chemotherapy ($P = 0.0051$). Thus, the use of molecular profiles to predict clinical outcomes in DLBCL has been confirmed by both groups. These predictors will need to be consolidated and validated in prospective studies to identify the sensitivity and specificity of these assays, and then examined prospectively in the context of the poor outcome patients to see whether early, more aggressive therapy truly achieves a better outcome for these patients.

While the use of DNA microarrays is currently impractical for a typical pathology laboratory, immunohistochemistry (IHC) is commonly used. Tissue microarrays permit the examination of many tissue samples by IHC simultaneously [22]. Hans *et al.* expanded the practical application of the DLBCL work through tissue microarrays comprised of 142 samples and stained for several of the markers identified as characterizing the DLBCL subtypes [23]. CD10 and bcl-6 expression were associated with CG B-like cases and better survival ($P = 0.019$ and $P < 0.001$ respectively), and MUM1 was associated with activated B-like cases and worse survival ($P = 0.009$). Using expression of these three markers by IHC, the authors classified the cases as either GC B-like or activated B-like. The 5-year overall survival for these two groups were 76% and 34% respectively, ($P < 0.001$), similar to the survival correlation seen by cDNA microarrays. Thus, in the future gene expression profiling findings can be brought to bear clinically through the expanded use of DNA microarrays or through more common techniques such as IHC when a limited number of classifying markers can be identified.

ACUTE LEUKEMIA

Background

The leukemias account for 30,600 new cases of cancer in the US and 21,900 deaths each year with the acute leukemias representing greater than half of these cases [6]. Due to the high prevalence of specific chromosomal abnormalities, particularly chromosomal rearrangements and translocations, the leukemias represent a class of hematological malignancies for which the advent of molecular biological techniques has had an enormous impact [6,24]. In acute myeloid leukemia (AML), specific chromosomal aberrations are associated with disease in about 40-55% of patients [24-25]. Examples include t(8;21) AML1-ETO, inv(16) CBFβ-MYH11X, t(15,17) PML-RARα, 11q23 abnormalities associated with mixed lineage leukemia (MLL), as well as monosomies and trisomies [24,26]. Although ALL can be broken down into precursor B-cell, precursor T-cell, and Burkitt cell subgroups, especially within the precursor B subgroup cytogenetic abnormalities play important prognostic roles. These aberrations include the t(9;22) BCR-ABL, 11q23 MLL, t(1;19) E2A-PBX1, and t(12;21) ETV-CBFα [24,26]. In the acute leukemias, the morphology, immunohistochemistry, flow cytometry, and cytogenetics are all currently required for the diagnostic and prognostic assessment of the tumor. In fact, the karyotype is the single most important prognostic factor in AML [27], while ploidy in ALL also carries a prognostic burden. Clinical features and patient characteristics such as age are also important prognostic factors.

Today, the drugs used in induction chemotherapy for AML are based on the cornerstone of cytarabine which is also used in consolidation therapy. By contrast, ALL has been classically treated with multi-agent induction regimens of prednisone, vincristine, an anthracycline, cyclophosphamide, and/or L-asparaginase although numerous other regimens are also used [24]. This is generally followed by consolidation therapy and continuation treatment with bone marrow transplants (BMTs) in relapsed/refractory ALL [28-29]. The success of these therapies has hinged upon the tailoring of the regiment to the individuals risk of relapse [28-29]. In general, the CR rates for either disease in the adult ranges from 60-90%, but 5-year OAS is poor [24]. Clear differences in the induction regimens necessitate the correct diagnosis between AML and ALL. And, while therapy is risk-based, even the best current prognostic factors account for only about 4% of the variability in prognosis [30]. Thus, for the acute leukemias, accurate risk assessment is critical in fine tuning the chemotherapy regimens and treating the higher risk patients with BMTs at an earlier stage.

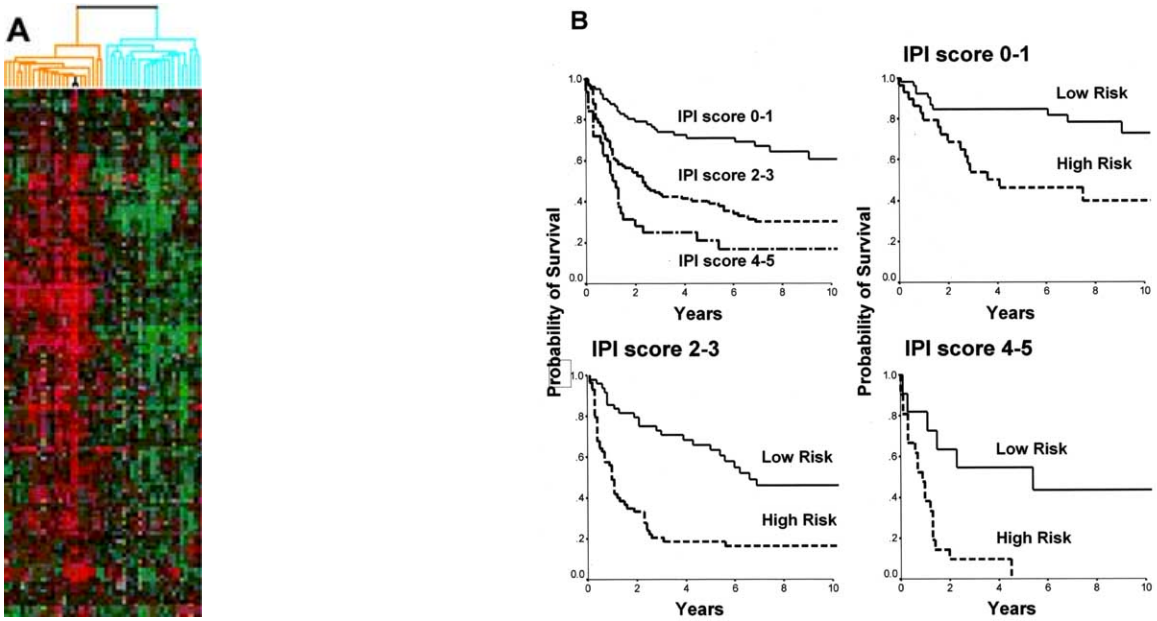

Fig. (1). Subclassification of DLBCLs into germinal center B-like and activated B-like. A, Hierarchical clustering of samples against genes characterizing germinal center B-cells, segregating GC B-like (orange) from activated B-like (blue) cases. Reprinted with permission from reference [14]. B, Kaplan-Meier plots demonstrating the overall survival among all patients (training and validation sets) broken down by IPI risk groups. Within the IPI score groups, patients are segregated based upon their gene expression-based predictor scores as high or low risk. Redrawn from reference [16].

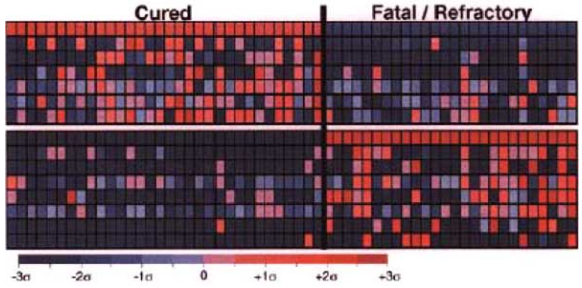

Fig. (2). Outcome predictor gene set for DLBCLs. Samples are represented as columns, genes as rows. The color scale at the bottom indicates relative expression in standard deviations from the mean. The genes in the top section, therefore represent those expressed at higher levels in the 32 cured DLBCLs, while those at the bottom represent genes expressed at higher levels in the 26 fatal/refractory tumors. Reprinted with permission from reference [17].

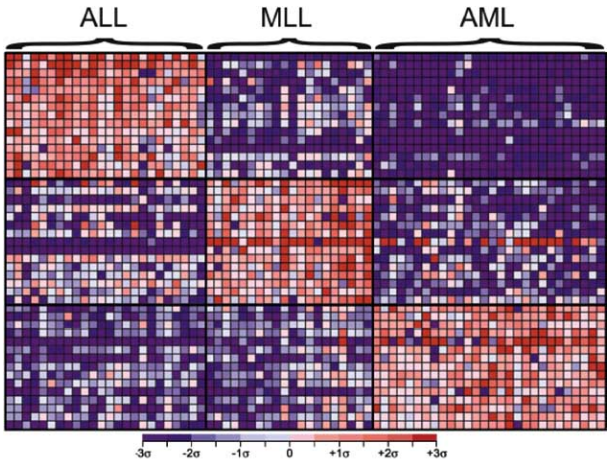

Fig. (3). Classifier gene sets in acute leukemias. Top 15 genes which characterize the three groups, ALL, AML, and MLL, are shown. Samples are represented as columns, genes as rows. The color scale at the bottom indicates relative expression in standard deviations from the mean. Reprinted with permission from reference [32].

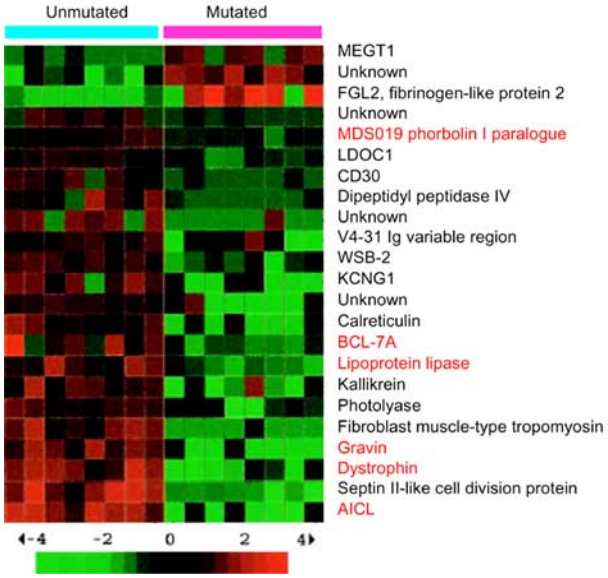

Fig. (4). Identification of classifier gene sets for CLL which discriminate between unmutated and mutated Ig V_H genes through hierarchical clustering. Samples are represented as columns, genes as rows. The unmutated cases are highlighted by a cyan bar, the mutated by a magenta bar. The color scale at the bottom indicates relative level of gene expression. Genes highlighted in red indicate genes identified in common in references [44] and [45]. Reprinted with permission from reference [45].

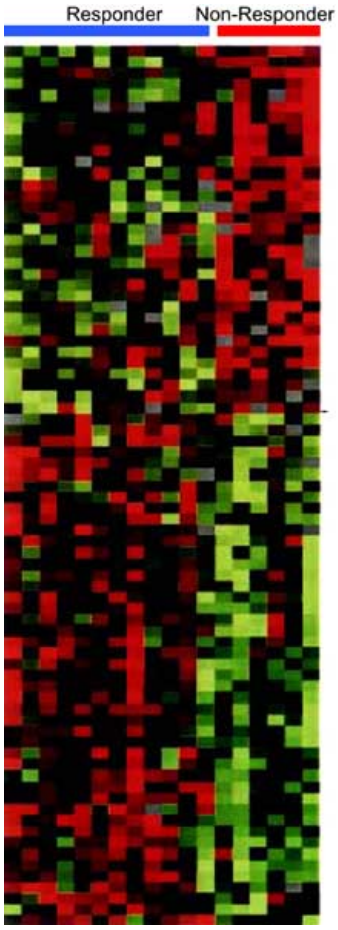

Fig. (5). Identification of predictor gene sets which discriminate between CML responders and nonresponders to Gleevec. Samples are represented as columns, genes as rows. The color scale is as follows: red represents overexpression, green underexpression, black unchanged expression, and gray little to no expression. Reprinted with permission from reference [43].

Array Studies

Numerous studies have been conducted on the molecular classification of the acute leukemias, particularly ALL. Initial studies on ALL focused on the ability to enhance classification of subgroups of ALL through class prediction and new class discovery [31]. As the field progressed, the knowledge of poor prognosis subgroups naturally led to attempts to associate these molecular classifications with clinical outcomes, in particular the risk of relapse, and the mechanisms thereof.

ALL and AML Classification

Golub *et al.* focused on the discovery of molecular classifiers to differentiate the two acute leukemias [31]. Isolated mononuclear cells from bone marrow samples of 27 ALL and 11 AML chemonaive patients were profiled on Affymetrix oligonucleotide arrays (6817 genes). Employing a neighbor analysis, the authors were able to identify the top 50 genes which correlated with the distinction between AML and ALL. This predictor gene set was used in cross-validation tests to assign 36 out of 38 samples correctly. An independent test set of 34 samples was also examined, resulting in 29 strong predictions which were 100% accurate with 5 ambiguous predictions. The predictions were equally accurate when predictor models of 10 and 200 genes were used. Self-organizing maps (SOM) were the used to demonstrate the identification of classes when the "correct" number of classifications is unknown. A designation of two clusters recapitulated the AML-ALL distinction. A second 4 cluster assignment further subdivided the ALL cases into T-ALL and two groups of B-ALLs, thereby illustrating that SOMs can indeed be used for class discovery.

Interestingly, Golub *et al.* was unable to find any correlation between molecular profiles and the outcomes. In subsequent work by that group, Armstrong *et al.* therefore examined the distinctions between ALLs, AMLs, and mixed lineage leukemias (MLLs) as the latter of these classes are known to carry particularly poor prognoses with early relapses after chemotherapy [32]. Twenty ALL, 20 AML, and 17 MLL samples were arrayed on Affymetrix chips bearing ~12,600 genes. Unsupervised principal component analysis successfully segregated all three groups. Using these same 57 samples, leave-one-out cross-validation was conducted, resulting in the accurate classification of 95% of the samples. A 100-gene predictor model was selected which best correlated with the three-class distinction and used to classify 10 test samples with 90% accuracy (Fig. **3**). Although no direct prognostic prediction was attempted, the enhanced ability to identify MLL patients carries prognostic implications. In addition, examination of the genes which distinguish MLL from traditional ALL imply that MLL might develop from earlier progenitor B cells than any other ALL, possibly explaining the lack of efficacy of standard ALL chemotherapeutics. The identification of a separate MLL signature in both adult and pediatric ALL has recently been corroborated by Kohlmann *et al.* [33].

ALL Outcomes

In a third paper by this same group, Ferrando *et al.* arrived at prognostic signatures for the T-cell subset of ALLs, which are characterized by overexpression of particular oncogenes of the basic helix-loop-helix family: LYL, HOX11, and TAL1 [34]. Using the overexpression of these genes as the definition of the clinical classes, 72 genes were found to best distinguish the three groups within 39 T-ALL samples profiles using neighbor analysis. Not surprisingly, the Kaplan-Meier plot of the oncogene groups was recapitulated in the plot of the clusters with the HOX11+ patients enjoying excellent OAS while the TAL1+ and LYL1+ patients faired less well. Hierarchical clustering of the samples using these 72 genes also found a fourth minor branch. Interestingly, the samples of this group all carried a MLL-ENL fusion gene, and their corresponding patients all survived during the course of the published follow-up (although there were only 3 patients in this group), suggestive of a new good prognosis subgroup of T-ALLs.

Moos *et al.* posed a more specific clinical question about risk of relapse of ALL in their work [30]. A single institution prospective design, the study identified 13 standard risk ALL patients and 10 high-risk ALL patients by National Cancer Institute criteria at diagnosis and tried to identify molecular predictors which correlated to the clinical risk assessment. Using a cDNA array of 4608 genes, a 20-gene model was defined, but both the t-test and Infoscore methods only resulted in 50-77% correlation with the clinical designation. However upon follow-up, three of the "standard risk" patient whom the predictor model classified as high risk in fact had slow marrow responses to therapy and there was one death. Conversely, the four "high risk" which were predicted to be standard risk by their profiles in fact responded rapidly to therapy. Although the numbers are quite small and prediction value is merely anecdotal, this is the only prospective study in this series. However, here a retrospective study which identified signatures that correlated to outcomes might have been preferable initially, prior to the application of those signatures in a prospective study. Clearly a larger and more rigorous study would be needed to determine if the molecular predictors indeed provide superior prognostic value than the conventional methods of risk assessment in ALL.

Table 2. Sensitivity and Specificity Calculated for the 112 Acute Leukemias

Subgroups	Test Set (n = 112)	
	Sensitivity	Specificity
T-ALL (n = 15)	100%	100%
E2A-PBX1 (n = 9)	100%	100%
TEL-AML1 (n = 27)	100%	98%
BCR-ABL (n = 6)	83%	98%
MLL rearrangement (n = 6)	100%	100%
Hyperdiploid >50 (n = 22)	100%	93%

In the largest study on ALL, Yeoh *et al.* also examined not only the subclassification of ALLs but also the risk of

relapse and the characteristics of secondary AML [35]. A total of 327 diagnostic bone marrow samples (215 for a training set, 112 for a test set) were examined on the Affymetrix HG_U95Av2 platform of over 12,600 genes. Through unsupervised hierarchical clustering of the training set, 7 major groups were identified which could be distinguished in the test set with 95% accuracy (Table **2**). These groups corresponded to T-ALL, hyperdiploid (>50 chromosomes), BCR-ABL, E2A-PBX1, TEL-AML1, and MLL rearrangements and one additional cluster which could not be identified with a specific cytogenetic abnormality.

The authors next examined four groups of samples to assess risk of relapse. They obtained 201 diagnostic samples from patients who remained in continuous complete remission, 32 from patients who developed hematological relapses, 16 from those who subsequently developed therapy-induced AML, and 25 samples from patients at the time of their relapse. Using discriminant analysis with variance (DAV), distinct profiles were identified for each category. However, despite the use of several supervised learning algorithms, no single signature predicted relapse across all cytogenetic subgroups. However, within both the T-ALL and hyperdiploid (>50 chromosomes) subgroups, profiling could predict relapse with 97% and 100% accuracy, respectively. Relapse predictors were also obtained from TEL-AML1, MLL, and the seventh class without specific cytogenetic abnormalities, but these predictors were not statistically significant. While again no single set of genes could predict the development of secondary AML across the various subgroups of ALL, within the TEL-AML1 subgroup a 20-gene predictor model was identified which predicted AML with 100% accuracy in cross-validation with statistical significance. Although there were no independent test sets to assess these outcome predictors nor have any prospective experiments been done to date with these predictors, this study illustrates the future potential of molecular profiling to aid in risk assessment in ALL, a critical step in selecting appropriately tailored treatment regimens.

ALL Treatment

While the ALL outcome studies mentioned above were not controlled for the type of chemotherapy used, Hoffman *et al.* has specifically examined profiles which indicate resistance to Gleevec, an Abl tyrosine kinase inhibitor, in those patients with t(9;22) [36]. Unlike in CML (*vide infra*), Gleevec efficacy in ALL is patchy and resistance rapidly develops. The authors studied 17 bone marrow samples obtained prior to treatment with Gleevec on the Affymetrix HuGeneFl platform of 5600 genes. Of those 17, 10 responders went on to achieve complete peripheral hematological remission and while 7 were nonresponders (either no response or a partial response). In addition, 8 bone marrow samples were also obtained during Gleevec therapy. Using hierarchical clustering and class membership prediction analysis, genes were identified which predicted the sensitivity of leukemias to Gleevec. Of those genes, a set of 50 discriminated between the 10 responders and 7 nonresponders, a set of 50 discriminated between the 10 responders and 8 treated cases which were resistant, and a set of 25 characterizing those that developed resistance while on the drug. In addition 56 genes identified those samples which developed secondary resistance to Gleevec. Although this study was small and lacked statistical power, nonetheless it points to the potential of gene expression profiling to impact the selection of appropriate drugs for ALL patients.

AML Classification

As was demonstrated by the numerous ALL studies, AML researchers have also sought to find molecular predictors of specific chromosomal abnormalities. Depending on the type of aberration, this search has met with variable success. Virtaneva *et al.* explored the poor prognostic class of AMLs bearing trisomy 8 using bone marrow samples from 10 chemonaive patients with trisomy 8, 10 chemonaive patients with normal cytogenetics, and 7 normal samples arrayed on Affymetrix HuGeneFL (6,606 genes) [25]. While unsupervised hierarchical clustering did successfully distinguish the normal samples from the AML samples, no segregation of the trisomy 8 vs. normal cytogenetics was achieved, despite the use of several class prediction methods.

Interestingly both the trisomy 8 and the cytogenetically normal subgroups of AML encompass a wide range of morphological heterogeneity. This is in marked contrast to other defined subgroups of AML in which specific cytogenetic aberrations dominate and there is a corresponding distinctive morphological phenotype. Of these latter types, 37 untreated AML patients with one of three translocations were examined by Schoch *et al.* in 2002 [27]. The translocations studied on Affymetrix arrays were t(8;21) AML1-ETO, inv(16) CBFβ-MYH11X, and t(15,17) PML-RARα. Through principle component analysis, 13 genes were sufficient to classify the three groups in the 32-member training set in leave-one-out cross-validation. A test set of 5 samples was also profiled and classified with 100% accuracy. Using a second method, the multiple-tree classifier method, a 29-gene model was identified which performed with 100% accuracy on both the training and test sets. Overlap in the 13-gene and 29-gene models was limited to only 6 genes. As has been seen before when differing analysis methods are applied, the overall strength of the prediction can be similar without the classifiers being the same. This work was expanded in subsequent studies to the identification of signatures unique to a wide range of specific AML and ALL cytogenetic aberrations using a set of 90 samples [37].

Signatures have likewise been identified that distinguish between de novo AML and AML arising secondarily from myelodysplastic syndrome (MDS) [38]. Since the patients with MDS-derived AML carry a worse prognosis than de novo AML patients (M2 subtype), this enhanced classification has ramifications in patient outcomes as well. In this small study by Oshima *et al.*, purified stem cells from a training set of 20 patient bone marrow aspirates were used to identify the 57-gene classifier model from Affymetrix U95Av2 arrays (12,625 genes) which upon leave-one-out cross-validation segregated the samples with 85% accuracy.

AML Treatment

Since the samples examined by Oshima *et al.* were all pretreatment samples, the authors also identified 37 genes

which discriminated between the 8 patients with complete responses to standard induction chemotherapy, and the 12 patients who did not respond completely [38]. Again, leave-one-out cross-validation accurately predicted the correct clinical outcome of 15 of the 20 patients, with 2 incorrect and 3 indeterminate predictions. Recognizing the need to move to a smaller number of markers for common clinical use, the authors also attempted to identify individual genes which could predict treatment failure.

Okutsu *et al.* studied 76 pretreatment patients with AML (excluding only the M3 subgroup) to identify predictors of good and poor responders to induction chemotherapy using the same regimen of cytarabine and idarubicin [39]. A training set of 21 good responders who achieved complete remission (CR) after a single course and 12 poor responders who failed to achieve CR after 2 courses was used to identify those genes whose median expression in the good responders group differed significantly from the median expression level in the poor responders group. In this way a 28-gene predictor was identified and a drug response score (DRS) for each patient ascribed. All 33 members of the training set were accurately segregated using this model. In addition, a test set of 20 new samples was examined (Table 3). Of the 10 patients predicted to be good responders by their drug response scores, 9 achieved CR after one course of induction chemotherapy. Of the 10 patients predicted to be poor responders, 8 failed to achieve CR after 2 cycles. Given the current 20-30% failure rate of induction chemotherapy in AML, this response prediction model has the potential to identify patients who have an 80-90% chance of failure and hopefully direct those individuals to alternative therapy and/or minimize their exposure to adverse events associated with standard induction chemotherapy.

Table 3. Sensitivity and Specificity of AML Drug Response. Score

Test Set		Remission Achieved (+)	Remission Achieved (-)	Total	Sensitivity	Specificity
DRS	(+)	9	1	10		
	(-)	2	8	10		
Total		11	9	20	82%	89%

Discussion

Thus, several groups have independently reported the use of molecular classifiers to categorize ALL and AML subgroups. This is especially effective in cases of specific cytogenetic aberrations which characterize individual subgroups. Moreover Yeoh *et al.* as well as Moos *et al.* have begun the arduous task of applying gene expression profiling to ALL outcome prediction. As in the case of DLBCL, although there was little/no overlap in the actual gene sets identified as classifiers or predictors, the power of the use of pattern recognition was corroborated through these multiple studies. In addition, the use of molecular profiles to identify patients unlikely to respond to a given chemotherapy regimen has been demonstrated for both ALL and AML, allowing the early selection of alternative therapies.

CHRONIC LEUKEMIAS

Background

The chronic leukemias account for just under half of the total leukemias, with CLL affecting 7,300 individuals each year in the US and CML affecting 4,300 [6]. As in the case with the acute leukemias, there is a high prevalence of specific chromosomal abnormalities, particularly chromosomal rearrangements and translocations [6,24]. However, the different cytogenetic backgrounds for the most part do not correlate well with survival. Instead, risk assessment in CLL is largely based upon stage (the Rai or Binet systems) which is based largely upon clinical features such as the extent of lymphocytosis, lymphadenopathy, hepatosplenomegaly, and hemoglobin and platelets counts. However, recently it has been discovered that the presence or absence of immunoglobulin (Ig) hypermutation in the B-cell CLLs does appear to have prognostic significance, with the unmutated Ig genes associated with worse prognosis [40]. Although risk assessment does play a role in CML, most patients undergo a classic disease course of a number of years in the chronic phase, with eventually transition into an accelerated phase followed by blast crisis with a fairly constant 25% probability of conversion to blast crisis for each year in the chronic phase [24].

Treatment of CLL has undergone a recent revolution due to the discovery of new therapeutics such as fludarabine, 2-chlorodeoxyadenosine, and pentostatin [41]. However, much of treatment remains largely palliative due to the age of the patients and the indolent nature of the disease. In fact, patients with the mutated Ig V_H gene may require no treatment at all. The treatment of CML has likewise undergone a revolution with the advent of Gleevec. Complete hematological responses can be achieved with Gleevec in approximately 98% of CML cases in the chronic phase (54% complete cytogenetic response) [42]. Resistance can eventually arise to Gleevec, but not at the rate at which it arises in t(9;22) positive ALL cases where resistance develops rapidly in nearly patients [36]. By contrast, Gleevec is much less effective during blast crisis. The only cure has historically been an allo-SCT, but this procedure is associated with considerable morbidity and mortality and is limited to the few patients with suitable donors [43,24]. The assessment of the stage of CML in patients and the sensitivity of those patient tumors to Gleevec has bearing on whether the patient can be treated chronically with Gleevec or other agents, or if the patient needs a stem cell transplant (SCT).

Array Studies

CLL Classification and Outcomes

In 2001, two studies were published simultaneously which addressed the issue of Ig V_H mutational status in B-cell CLLs. Rosenwald *et al.* used the familiar Lymphochip cDNA array, referenced against a pool of nine lymphoma cell lines, to profile 28 peripheral blood samples from chemonaive CLL patients (16 unmutated, 10 highly mutated, and 2 minimally mutated) [44]. Using an Affymetrix U95A oligonucleotide array (~12,000 genes), Klein *et al.* examined 34 CLL cases, 18 mutated, 16 unmutated [45]. In both cases, an unsupervised two-

dimensional hierarchical clustering of the CLL cases intermingled indiscriminately the mutated and unmutated cases. Both sets of authors then turned to supervised learning algorithms with training sets of clearly mutated and unmutated cases to identify their predictor models. Rosenwald *et al.*, using a 56-gene model correctly predicted 9 of 10 test samples while Klein *et al.*, with a 23-gene model, achieved accurate classification of 12 of 14 test samples (Fig. **4**). Interestingly, ZAP-70 was one of the most highly discriminatory genes in the Rosenwald *et al.* predictor model although it was not found in the Klein *et al.*, model.

Discussion

By themselves, these studies would be unlikely to affect the actual clinical management of CLL since the cost and effort involved in the microarray work exceeds even that of sequencing a patient's tumor to determine the mutational status, the obvious gold standard in this case. However, in 2003, Crespo *et al.* examined the expression of ZAP-70 alone in 56 patients with CLL by flow cytometry as well as Western Blotting and immunohistochemistry [46]. Ig V_H was unmutated in all cases in which >20% of leukemic cells were positive for ZAP-70, whereas it was mutated in 21/24 cases in which <20% were positive ($P < 0.001$). Not surprisingly, the ZAP-70 status therefore was found to also correlate with survival. Through the intermediacy of gene expression profiling, a single marker was identified which can quickly and inexpensively classify a subset of CLL patients who are likely to enjoy a good overall prognosis and require little to no therapy. An earlier study by Stratowa *et al.* also correlated specific gene expression patterns with B-cell CLL survival, albeit without the rigor of a prediction model demonstrated in training and test sets [47].

CML Classification, Outcomes, and Treatment

Dominated by a single translocation and following a well-defined course of disease, CML has afforded fewer opportunities for gene expression profiling-based classifications and outcome predictors. Some studies have been conducted looking at CML, nonetheless. A study by Ohime *et al.* published in 2001 examined purified hematopoietic stem cells from 13 samples of various stages of CML (chronic phase, accelerated phase, blast crisis) and compared those to the pooled stem cells from normal individuals using Affymetrix oligonucleotide arrays [48]. Although the results were somewhat complicated by the inclusion of both IFN-α treated and untreated patients, genes were discovered which distinguished the chronic phase from the other two phases, shedding light upon the basic mechanisms of stage progression. However, clinically this distinction is easily made without the use of profiling methods, and the potential application of these predictors is limited.

By contrast, the use of molecular profiling to help manage the appropriate use and timing of Gleevec with other treatment options such as SCT could have far wider utility. This was the focus of a study by Kaneta *et al.* published in 2002 which examined by cDNA arrays representing 23,040 genes (reference was a pool of mononuclear cells from 11 healthy volunteers) 22 samples of peripheral blood mononuclear cells from chemonaive CML patients prior to their treatment with Gleevec [43]. A training set of 18 samples was selected: 16 patients in the chronic phase to be treated with Gleevec 400 mg/d and 2 patients in blast crisis to be treated with 600 mg/d. After 5 months of treatment, the authors assigned each case as either a responder (n=12, <35% cells positive for the t(9;22)), or a nonresponder (n=6, >65% cells positive for the t(9;22)). For each gene, the median expression level for the responders and nonresponders were calculated and 79 genes for which there was a significant difference were selected as the molecular predictor (Fig. **5**). These genes were then rank ordered and leave-one-out cross-validation was conducted iteratively using the top 5 genes, then the top 10, 15, 20…75, 79 genes for all 18 samples in the training set. As a result, the 15-gene and 30-gene models provided 100% accurate assignments of both the 18 member training set, as well as a 4 case test set (2 chronic phase and 2 accelerated phase). Interestingly, even an unsupervised hierarchical clustering using these genes also neatly segregated the responders from the nonresponders.

Discussion

These results have the potential to greatly impact the appropriate clinical use of Gleevec. Although Gleevec has a very high hematologic complete response rate, its cytogenetic complete response rate is only about 54% [42]. The rapid identification of these cytogenetic nonresponders before they have been treated might lead to the early consideration of SCT in these patients and minimize drug costs.

BREAST CANCER

Background

Breast cancer is the most common malignancy affecting women with an estimated overall incidence of 212,600 in the United States in 2003 [6]. Causing an estimated 40,200 deaths in 2003, breast cancer is also the second most common cause of cancer deaths in women [6]. Risk factors include age, family history of breast cancer, history of endogenous or exogenous estrogen exposure, personal history of benign proliferative breast disease, history of radiation exposure, and the presence of inherited genetic susceptibility such as BRCA1 or BRCA2. The latter two genes carry a lifetime risk of developing breast cancer of 50-85% [49]. Although there are several histological classifications of breast cancer, with invasive ductal carcinoma the most common, the most important prognostic factor which guides treatment decision-making in breast cancer is the clinical TNM stage of the tumor [49]. By St. Gallen criteria, other ancillary considerations in the decision whether or not to give adjuvant treatment include age, race, hormone receptor status, tumor grade, tumor type, and mitotic rate [50]. However, studies have demonstrated that tumor grade concordance among three pathologists is only 43% [51]. Moreover, there is still a disconnect between the determination of prognosis and the direct selection of appropriate treatment. For example, a positive estrogen receptor (ER) and progesterone receptor (PR) status is

indicative of a good prognosis regardless of whether or not hormonal treatment is utilized, and one of the most important prognostic factors, the extent of lymph node extension, does not predict responsiveness to the various treatment regimens.

Stage I, II, or III breast cancer is treated with the intent to cure, with initial treatment comprised of surgical removal of the primary, either by breast conserving surgery or mastectomy, and lymph node assessment [49]. Adjuvant radiation treatment is considered the standard of care for most patients, and, for the roughly two-thirds of patients with ER and/or PR positive tumors, 5 years of tamoxifen is recommended with or without ovarian suppression [49-50]. Despite the success of multivariate risk assessment, recurrences do occur in all the stages of breast cancer, roughly 80% occurring prior to 5 years [52]. Overall, up to one third of node-negative patients will recur within 10 years, demonstrating the remaining limitations of the risk assessment criteria [53-54]. Adjuvant chemotherapy, especially anthracycline-based multi-agent regimens, has also been shown to improve clinical outcomes for all patients with breast cancer [49-50,55]. However, the relative benefit can be minimal depending upon the overall prognosis for the tumor. In those women with more favorable prognosis (small, node-negative, hormone responsive tumors), adjuvant chemotherapy may improve OAS by only 1-4% [49]. Thus, in theory the decision whether or not to use adjuvant treatment depends upon the prognosis [50,56]. In practice, many cancer patients will accept significant side effects to even marginally lower their risk of recurrence. Neoadjuvant chemotherapy also affords similar long-term survival benefit to patients as adjuvant chemotherapy, with those patients achieving complete responses having significantly improved outcomes than those who do not. Typically, however, neoadjuvant treatment is used only for patients with bulky tumors who still desire breast conserving surgery to decrease the tumor burden prior to surgery [49]. Unfortunately, for those patients with Stage IIIB or IV tumors, treatment is largely palliative. However, there are now innumerable regimens of multi-agent chemotherapy for breast cancer. Clinicians therefore look forward to the day when patients likely to respond to a given regimen can be identified in advance of treatment so that the appropriate chemotherapy can be selected when warranted.

Array Studies

As a solid tumor, breast cancer presents different challenges from the lymphomas and leukemias previously discussed. In many of the liquid tumor studies, peripheral blood or bone marrow samples were obtained, from which suspensions the purification of the tumor cells can be easily achieved. In addition, despite the significant discomfort of bone marrow aspirations, the acquisition of the samples does not require a major surgical procedure to obtain quantities of material sufficient for array studies. These challenges have been addressed in numerous fashions in solid tumor research. Profiling of cell lines representing stromal elements such as endothelial cells, lymphocytes, and adipocytes have been used to tease out the tumor specific signature from a homogenized sampling of the tumor [57]. Alternatively, laser capture microdissection can isolate >98% homogeneous populations of cells from frozen sections or paraffin blocks [58-59]. However, the vast majority of studies have simply used the heterogeneous populations of cells as is and have been able to tease out the signatures of various stromal elements from the tumor cells [60]. As in the case of the DLBCL study by Shipp *et al.* [17], the use of intact specimens can identify key tumor-microenvironment interactions which are critical to characterizing the tumor signature and behavior. In addition, several of the studies have demonstrated the ability to obtain reliable gene expression profile information from small diagnostic samples such as fine needle aspirates (FNAs), core biopsies, and even formalin-fixed paraffin embedded tissues (FPET). This has greatly facilitated the ability to conduct these types of studies on solid tumors.

Classification

Several groups have examined the correspondence between gene expression profiles and ER status. In a series of publications, Perou *et al.* [57] and Sorlie *et al.* [61] using cDNA arrays (8,102 genes) identified ER+ and ER- subsets

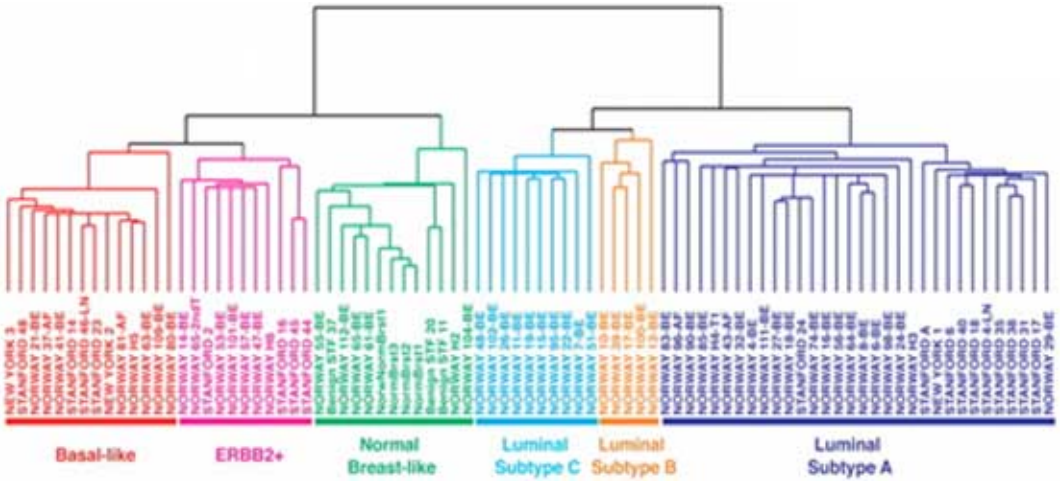

Fig. (6). Dendrogram resulting from the hierarchical clustering of 85 samples of invasive breast cancer, revealing 5 or 6 subtypes. Reprinted with permission from reference [61].

of invasive breast cancer by unsupervised hierarchical clustering (Fig. **6**). The ER+ group was characterized by a gene signature which expressed high levels of genes associated with breast luminal cells, and low levels of *Erb-B2*. This group contained 2 to 3 subtypes (A, B, and C) distinguished by the level of the ER positivity. The ER- group could be segregated into two subgroups, one which displayed high expressions of keratins 5/6 and 17, indicative of basal cells. The second subset of the ER- group overexpressed *Erb-B2*.

Bertucci *et al.* and van't Veer *et al.* also used an unsupervised approach to identify different subgroups of breast cancers which correlated roughly with ER status [62,63]. The former group used a cDNA array of only 176 genes to identify again two groups which were only roughly associated with LN or ER status. Group A (n = 15 including normal breast) tumors were 72% LN+ and 50% ER+ while group B (n = 20) tumors were 25% LN+ and 80% ER+. Thus, the groups did not segregate by ER status as neatly as did the Perou *et al.* samples. There was no correlation with age, tumor size, or histological grade. Using an oligonucleotide array of ~25,000 genes designed by Rosetta, van't Veer *et al.* profiled 98 invasive breast cancer tumors against a pool of all the tumors to highlight maximally those genes which characterized individual tumors. Beginning with a two-dimensional unsupervised hierarchical clustering, the authors identified again two groups of samples which roughly correlated with ER status as well as BRCA1 status as determined by IHC (Table **4**). In addition, these groups correlated roughly with the presence of a lymphocytic infiltrate and risk of metastasis, but not angioinvasion or histological grade.

Table 4. Characteristics of Patient Breast Tumors in Groups A and B Determined by Hierarchical Clustering. Data from reference [63].

Characteristic	Group A (n = 62)	Group B (n = 36)
ER	92% (57)	6% (2)
BRCA	3% (2)	44% (16)
Grade 3	53% (33)	97% (35)
Lymphocytic infiltration	8% (5)	50% (18)
Angioinvasion	31% (19)	22% (8)
Metastases	39% (24)	83% (30)

Gruvberger *et al.* applied a supervised principle component analysis to iteratively identify those genes which best segregated the known classification of ER status [64]. Using an initial training set of 47 primary breast cancers from node-negative patients (23 ER+, 24 ER-), the authors profiled the samples on cDNA arrays of ~4000 genes (with the cell line BT-474 mRNA as a reference). Using the top 100 discriminating genes, 100% of the 47 training samples were correctly classified as were 11 blinded test samples. This suggests that profiles can assess the expression of numerous genes in the ER pathway beyond that of ER alone, thereby providing more information than the single marker.

West *et al.* also used a supervised learning method to examine 49 samples of invasive ductal carcinoma, almost evenly divided upon the four classifications: ER+/LN+, ER-/LN+, ER+/LN-, and ER-/LN- (where LN- indicates zero positive LNs, and LN+ indicates ≥ 3 positive LNs) [65]. The top 100 discriminating genes were selected which discriminated on the basis of ER status 38 training samples. Nine additional samples were independently tested using the classifier genes, but due to either tumor heterogeneity or the difficulties of using either IHC or Western blotting as the "gold standard," the profiles in 5 of the cases correlated with only one of the gold standard measures and not the other. Leave-one-out cross-validation of the original training set was therefore performed, resulting in the accurate classification of all cases, with only 4/38 cases classified without full confidence. Lymph node status was also examined by similar methods. However, in cross-validation, the subsets demonstrated far greater overlap. However, the results suggest that ultimately the assessment of LN status, the most important clinical prognostic factor, might be conducted without surgery.

Discussion

These several studies were able to identify gene expression signatures which correlated with ER status. Despite the use of differing platforms, patient cohorts, tumor histologies, and analysis methods, nonetheless the same general conclusions were drawn. The lists of gene classifiers do in fact reveal some overlap, further validating these methods (Table **5**). As was shown by West *et al*., the IHC gold standard, which can be easily performed in any pathology department, is complicated by tumor heterogeneity, leaving open the possibility that better methods might exist for assessing ER or ER pathway status for prognostic purposes. Related studies have been conducted, identifying profiles associated with BRCA1 status [63,66].

Prognosis

Sorlie *et al.* carried their analysis further to explore the correlation of their ER+/ER- groups and subgroups with survival data generated as part of a prospective study involving 49 patients receiving neoadjuvant doxorubicin as a single agent for T3/T4 and/or N2 (M0) tumors with adjuvant tamoxifen if ER/PR+ [61]. When Kaplan-Meier plots were generated, breaking down the cohort by their molecular signatures, the 5 major tumor subclasses (merging luminal subtypes B and C) revealed significant differences in both OAS and recurrence free survival (RFS) ($P < 0.01$). As anticipated, the basal/ER- as well as the *Erb-B2*+/ER- subclasses were associated with the poorest survivals while the luminal groups fared better. Within the luminal/ER+ class, those within the A subgroup, generally but not consistently expressing high levels of ER, had longer OAS and RFS than those in the B and C subgroups. In this study, no comparison was made between these molecular prognostic classifications and other prognostic descriptors. However, the basal/ER- subclassification can be identified through its expression of cytokeratins 17 and 5 which can be assessed via IHC [51]. The correlation between this

Table 5. Genes Found in More Than One ER Status Prediction Study

Gene	Perou *et al.* [57]	West *et al.* [65]	Gruvberger *et al.* [64]	Bertucci *et al.* [62]
estrogen receptor	correlated	correlated	correlated	correlated
GATA binding protein 3	correlated	correlated	correlated	correlated
trefoil factor 3		correlated	correlated	
S100 binding protein		anticorrelated	anticorrelated	
serine or cysteine proteinase inhibitor, clade I		anticorrelated	correlated	
fructose-1,6-bisphosphatase		correlated	correlated	
hepsin	correlated	correlated		
cytochrome p450, IIB	correlated	correlated		
LIV-1 protein	correlated	correlated		
hepatocyte nuclear factor 3 alpha	correlated	correlated		
X-box binding protein 1	correlated	correlated		correlated
insulin-like growth factor 2			correlated	correlated
epidermal growth factor receptor	anticorrelated		anticorrelated	anticorrelated
glutathione *S*-transferase pi		anticorrelated		anticorrelated
keratin 7	anticorrelated	anticorrelated		
cadherin 3, type 1, P-cadherin (placental)	anticorrelated	anticorrelated		

phenotype and poor survival was corroborated using a stain for cytokeratins 17 and 5 expression [67,22].

A 23-gene predictor set which correlates with survival was identified by Bertucci *et al.* using a combination of supervised and unsupervised methods to assess by cDNA arrays (of only 176 or ~1000 genes) the correlation to survival amongst 55 poor prognosis patients (LN+ or LN- with >1 of the following characteristics: age <40, tumor > 2 cm, ER-, grade 3) who were treated with primary resection followed by adjuvant doxorubicin [62,68-69]. However from their unsupervised two-dimensional hierarchical clustering, these 23 prognostic genes fell into two different clusters, one which contained ER, and another which contained cell cycle and apoptosis genes. A combination of these larger gene sets was used. This allowed segregation of the samples into 3 to 4 groups with statistically significant differences in OAS ($P < 0.005$) and metastasis free survival (MFS) ($P < 0.05$). These groups were independent of other prognostic factors such as age, menopausal status, and histological type.

Table 6. Odds Ratio Determined for Selected Prognostic Markers in Breast Cancer. Data from reference [63].

Prognostic Factor	Odds Ratio (OR)	*P* Value
Predictor model	28	1.0 x 10-8
High grade	6.4	0.0008
Tumor size >2 cm	4.4	0.0028
Angioinvasion	4.2	0.01
Age ≤ 40	3.7	0.02
ER-	2.4	0.13

Roche *et al.* and Huang *et al.* used supervised learning algorithms to address specifically the risk of recurrence, a key problem in early stage breast cancers, rather than OAS [70-71]. Using a proprietary cDNA array from Millennium comprised of ~40,000 transcripts, Roche *et al.* identified 20 genes which discriminated 25 good outcome (>5 years DFS) and 18 poor outcome (<3 years DFS) breast cancers in stage T1 or 2 and N0 M0. Huang *et al.*, on Affymetrix U95Av2 arrays of >12,000 genes, identified metagenes which predicted recurrence at 3 years after surgical resection of the primary tumors on 52 tumor samples of heterogeneous clinical characteristics. Cross-validation achieved 90% accuracy of the assignments. The authors also identified metagenes which discriminated the 37 samples on the basis of risk of LN metastases with again 90% accuracy of prediction in cross-validation.

As in the Roche *et al.* work, van't Veer *et al.* used supervised learning methods to look specifically at the risk of metastases at 5 years [63]. Using tumors from 78 node-negative patients as a training set (34 patients developed distant metastases by 5 years, and 44 did not), the authors identified 231 discriminatory genes. Iterative leave-one-out cross-validation for each of 78 samples and genes sets of the top 5, 10, 15,…231 genes was conducted to identify an optimal 70-gene predictor model. Using this model, 65 of the 78 patients (83%) were accurately assigned to their

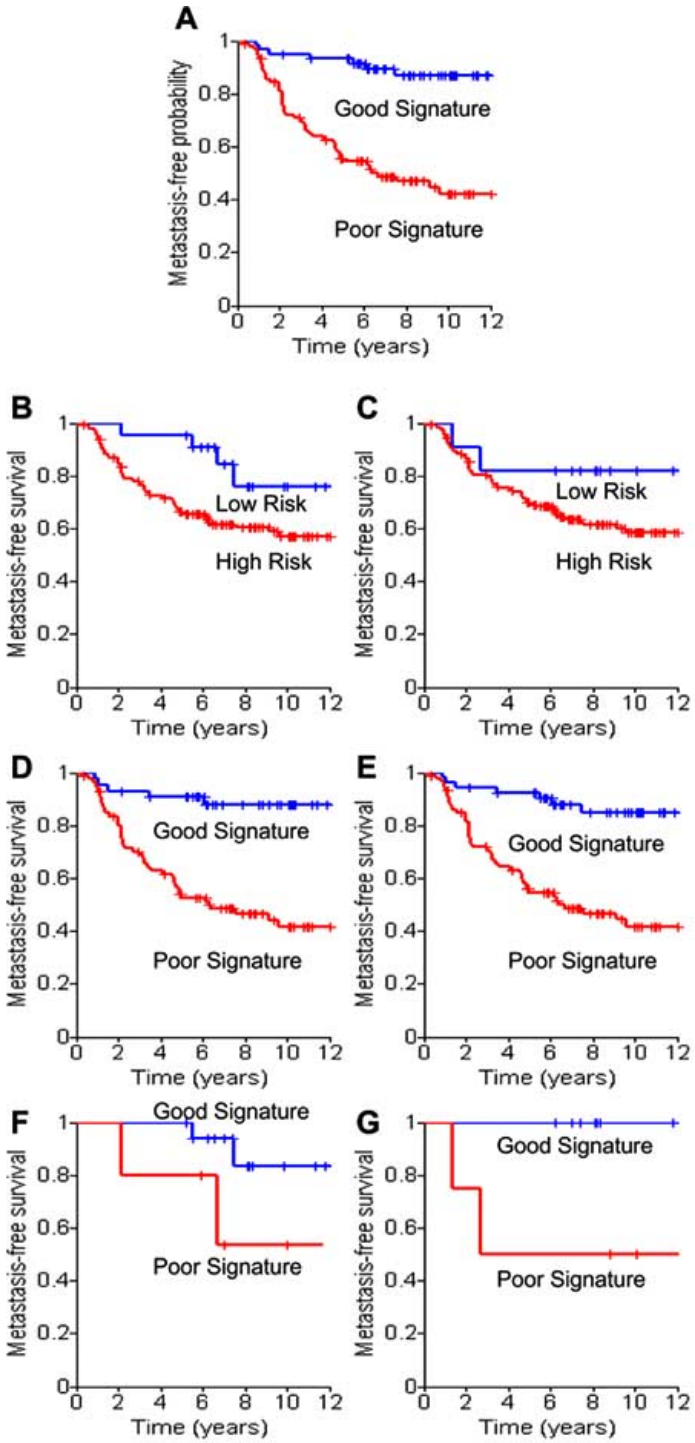

Fig. (7). Kaplan-Meier plots. A, Risk assessment based upon gene expression profiling signatures. B, Risk assessment based upon the St. Gallen criteria for low and high risk groups. C, Risk assessment based upon the NIH Consensus criteria for low and high risk groups. D, The high risk patients based upon St. Gallen criteria segregated by profiling signatures. E, The high risk patients based upon NIH criteria segregated by profiling signatures. F, The low risk patients based upon St. Gallen criteria segregated by profiling signatures. G, The low risk patients based upon NIH criteria segregated by profiling signatures. Reprinted with permission from reference [72].

respective prognostic groups. The predictor model was further validated using a test set of 19 patients, 17 of which were correctly classified. Multivariate analysis indicated that this predictor model was an independent factor in predicting metastases outcomes, and in fact carried an odds ratio (OR) of 28 fold, far exceeding that of other standard prognostic factors such as grade, tumor size, presence of angioinvasion, age, and ER status (Table **6**). The authors therefore suggest that their model be used to minimize the number of patients who receive unnecessary adjuvant treatment by identifying only those patients who are truly at high risk of developing further disease.

This analysis was further developed in a subsequent paper by van der Vijver *et al.* which demonstrated the utility of their predictor model on a much larger cohort of patients and compared the results with those of standard prognostic measures such as St. Gallen and NIH models [72]. Although the previous study included only LN- patients, on this cohort of 295 patients, the model was shown to still apply to the LN+ patients as well. Based upon the results of profiling, patients were segregated into good and poor prognosis groups which were independent of lymph node status. In addition, superior discrimination in poor and good MFS groups could be achieved by use of the prognosis signature within individual St. Gallen or NIH high or low risk groups (Fig. **7**). As this study was conducted retrospectively, the adjuvant treatment was not controlled, and no analysis was conducted addressing the differences between those patients who received adjuvant therapy and those that did not.

Two recent studies were presented at the 2003 ASCO meeting by Genomic Health, Inc, which also addressed the use of profiling to search for genes correlated with disease free survival (DFS) [73-74]. Rather than using cDNA or oligonucleotide microarrays, the authors demonstrated the used of RT-PCR of only 185 cancer-related genes on samples obtained from formalin fixed paraffin-embedded tumor tissue (FPET). By carefully selecting genes taken from the cancer biology literature, gene lists generated from other microarray studies of tumors, genomic databases, and basic molecular biology, they limited the number of genes which they needed to amplify by RT-PCR. Significantly, their RNA isolation method obviates the need for large fresh tumor samples, using instead FPET, the ubiquitous form of tissue storage in pathology laboratories. In this manner, the authors examined both a range of breast cancers at diagnosis as well as specifically high risk patients. In the former study [73], 146 cases were studied, 119 LN- and 27 LN+. At the time of the study, 105 patients were still alive after resection of the primary without recurrence of the tumor. By univariate Cox survival analysis, 20 genes ($P < 0.01$) were identified which correlated with DFS, and multivariate analysis demonstrated that predictor models with multiple genes carried more predictive power than did any single

Table 7. List of Genes Found in at Least 2 Breast Cancer Studies with Associated Prognostic Implication Indicated where Appropriate. Discrepancies between studies are boxed. "Not Available" indicates that the correlation to prognosis is not available. "Not Tested" indicates that the gene in question was not on the array in that study. No designation indicates that the gene was not selected as a discriminatory gene in that study. Reproduced in part from reference [76]. Data from references [61-63].

Gene	van't Veer *et al.* [63]	Bertucci *et al.* [62]	Sorlie *et al.* [61]
erb-B2		poor	poor
estrogen receptor		good	good
ceruloplasmin	poor	not tested	poor
3-oxoacid CoA transferase	poor	not tested	poor
Ser-Thr protein kinase	poor	not tested	not available
preferentially expressed antigen in melanoma	poor	not tested	poor
cellular retinoic acid binding protein 2		good	good
GATA binding protein 3		good	good
insulin-like growth factor 2		poor	good
mucin 1		good	good
myeloblastosis viral oncogene homolog-like 2		poor	poor
myelocytomatosis viral oncogene homolog		poor	poor
plasminogen activator, tissue		good	good
prolactin receptor		poor	good
SRY-box 9		poor	poor
transferrin receptor	poor		poor
X-box binding protein 1		good	good
B-cell CLL/lymphoma 2		good	good
epidermal growth factor receptor		poor	poor
keratin 18	good		good

gene. In the high risk study [74], 79 patients with large tumors and greater than 10 positive nodes were studied. At the time of the study 77% had suffered recurrence and/or death. While multivariate analysis revealed that a model including age, tumor size, nodes, grade, adjuvant hormone therapy, and chemotherapy accounted for 13% of the variance in DFS times, the addition of a 5-gene predictor to the other factors increased the variance accountability to 45%. Although no test sets or further validation was conducted as part of these communications, nonetheless they represent a tantalizing advance in bringing the use of profiling to the level of clinical utility.

Discussion

Thus, several groups have constructed predictor models of OAS and MFS or DFS. Although the studies differ widely in the design, execution, and analysis of the experiments, nonetheless predictor models can be constructed from profiling data, and these models can enhance our ability to predict outcomes of breast cancer patients beyond that of more traditional prognostic factors. This was elegantly demonstrated by the van der Vijver *et al.* study [72]. Sorlie *et al.* have compared their classification scheme (ER+/luminal A-C, ER-/basal, ER/*Erb-B2*+, normal) with those prognostic groups identified by van't Veer *et al.* and West *et al.* [75]. In both cases, using as many of the intrinsic genes which distinguish the Sorlie *et al.* subgroups as could be found on the microarrays used by the other two groups, the authors found that in both cases the good prognosis ER+/luminal A group could be distinguished most clearly. The poor prognosis *BRCA1*-expressing tumors from the van't Veer *et al.* study all fell within the poor prognosis ER-/basal group in the Sorlie *et al.* nomenclature. In addition, the Kaplan-Meier plot of the Sorlie *et al.* subgroups generated from the van't Veer *et al.* cohort data demonstrated similar patient stratification with respect to MFS as did the OAS and DFS plots of the Sorlie *et al.* cohorts. By contrast, using the 231-gene predictor model of van't Veer *et al.* on the Sorlie *et al.* data achieved only 47% accuracy in predicting recurrences within 5 years. Numerous differences in the studies could account for the discrepancies. Even in the ratio experiment-based studies, the use of different references could highlight different genes. Significantly, the van't Veer *et al.* study used a pool of all the tumors as a reference, rather than a pool of cell lines, thereby limiting the number of genes which show significance to those which emphasize characteristics of the individual tumors. Different gene sets were also represented on the various arrays. Clinical patient selection also differed between the studies, from locally advanced tumors (T3/T3 or N2 tumors treated with neoadjuvant and adjuvant therapy [61]) to early stage tumors (T1/T2, LN- and uncontrolled for adjuvant treatment [63]). Nonetheless, in a comparison conducted by Bertucci *et al.*, 26 predictor genes were found which were identified in at least two prognostic studies. This reinforced the conclusions of these studies that indeed prognostic information can be obtained from gene expression profiling that can help guide which patients should be given adjuvant treatment aggressively, and which have a good prognosis and are not likely to benefit from additional therapy (Table **7**) [76].

Treatment

In their neoadjuvant doxorubicin study, Perou *et al.* noted an early indication that profiling could be used to determine the efficacy of treatment [57]. In this study, profiles derived from pre- and post- treatment sample pairs were most similar to each other except in 3/20 cases in which the post-treatment profile clustered with the normal breast samples. Intriguingly, these breast tumors had responded to doxorubicin, suggesting that the responders had reverted to the normal profile. Of course, this response information is typically obtained clinically. However, Sotiriou *et al.* also found that comparison of pre- and post-neoadjuvant AC treatment (doxorubicin and cyclophosphamide) profiles could distinguish responders, but that this could be determined after only a single course of chemotherapy [77]. Using serial breast FNAs from 10 patients profiled on cDNA arrays with 7600 features, the authors found that the number of genes which significantly changed after a single course of AC were more than 10 fold greater in samples from those patients whose tumors responded than those that did not. Nonresponders can therefore be identified earlier and presumably switched to alternate therapies. In addition, with the clinical assignment of responders (CR and MRD patients, n = 5) and nonresponders (SD and PR, n = 5), the authors employed a t-test analysis to identify 37 genes from the pre-treatment profiles which most discriminated this binary designation. Leave-one-out cross-validation achieved 100% accuracy in the assigning the 10 samples to their appropriate response category ($P < 0.009$). Despite the small sample size and lack of a test set, this study suggests that within the pre-treatment profiles lies information of whether or not a given tumor will respond to a specific therapeutic regimen.

Subsequent to this study, several other groups have also begun identifying the profile of responders to various standard chemotherapies. Chang *et al.* employed Affymetrix U95Av2 arrays to profile the pre-treatment breast tumors (core biopsies) of 24 patients prior to the administration of neoadjuvant docetaxel [78]. This training set underwent t-test analysis to select 92 genes which discriminated between the responders, defined as those with minimal residual disease (MRD) < 25% (n = 11), and nonresponders with MRD > 25% (n = 13) ($P = 0.0015$). Leave-one-out cross-validation identified correctly 10 of 11 sensitive tumors and 11 of 13 resistant tumors for a positive predictive value (PPV) of 83% and a negative predictive value (NPV) of 92% (Table **8A)**. This response model also correctly predicted the responses of all 6 patients in a small test set.

At the 2003 ASCO meeting, Lajos Pusztai presented the findings of M.D. Anderson and Millenium, Inc., on the identification of responders to neoadjuvant paclitaxel + FAC (5-fluorouracil, doxorubicin, cyclophosphamide) [79]. Again using pre-treatment FNAs from breast cancer patients, the group arrayed these samples on a proprietary cDNA array from Millennium representing ~19,000 genes. The patient characteristics encompassed a mix of tumor stages, LN status, ER status, and HER2 status. Of the initial training set of 24 patients, 6 achieved a pathologic complete response (pCR). Based on the binary classifier of whether or not the patient achieved pCR, a supervised learning algorithm was applied to identify discriminating genes. In cross-validation

Table 8A. Accuracy of the Profile-Based Models in Predicting Breast Cancer Response to Chemotherapy. Results from the cross-validation experiments examining responses to neoadjuvant decetaxel monotherapy. Tabulated from reference [78].

A Cross-Validation [78]		Clinical Response (+)	Clinical Response (-)	Total	Sensitivity	Specificity	PPV	NPV
Profile-based Prediction	(+)	10	2	12				
	(-)	1	11	12				
Total		11	13	24	91%	85%	83%	92%

Table 8B. Accuracy of the Profile-Based Models in Predicting Breast Cancer Response to Chemotherapy. Results from the test set of 18 patients from experiments examining responses to neoadjuvant paclitaxel+FAC. Redrawn from reference [79]

B Test Set [79]		Clinical Response (+)	Clinical Response (-)	Total	Sensitivity	Specificity	PPV	NPV
Profile-based Prediction	(+)	3	0	3				
	(-)	4	11	15				
Total		7	11	18	43%	100%	100%	73%

k-nearest neighbor analysis, the response model was optimized as a 74-gene model (P = 0.09). On an independent test set of 18 new cases, a PPV of 100% and NPV of 73% were achieved (Table **8B)**. Despite these encouraging numbers, the sensitivity was only 43% and 95% confidence intervals were large. Future prospective studies are planned using the response models to dictate treatment decisions.

An FPET-based study on responses to tamoxifen was presented by Soonmyung Paik at the 2003 San Antonio Breast Cancer Symposium [51]. Representing the NSABP and Genomic Health, Inc., Dr. Paik described their work to understand which patients respond to tamoxifen adjuvant treatment of node-negative, ER+ tumors. Using the data from previous studies [73-74], the authors selected 21 genes from which a weighted recurrence score algorithm was developed. This was applied to the NSABP-14 prospective clinical trial of tamoxifen-treated patients. Of the 668 evaluable patients encompassing a range of ages and tumor sizes, 51% afforded low recurrence scores, 22% intermediate, and 27% high. In fact, the 10 year recurrence rate for those tumors with low scores was only 6.8%, compared to 30.5% for the high risk group (P < 0.00001 with small 95% confidence intervals). The profile-based score outperformed the prognostic factors of age or tumor size, affording an OR of 3.21 (P < 0.00001). Future studies plan to study which patients benefit when chemotherapy is added to adjuvant tamoxifen, and to compare tamoxifen-treated profiles with placebo.

Discussion

These treatment studies aim to identify a responders profile for each chemotherapy regimen so that individual tumors can be profiled at diagnosis, allowing the early selection of the regimen with the highest probability of success. Years of prospective studies are required before this ideal of personalized medicine can be realized, but the initial work clearly points to the theoretical feasibility of such a goal.

OTHER TUMOR TYPES

While it would be possible to discuss all studies done in other tumor types at the same level of detail, for the purposes of this review, many of the main points would be redundant. Thus a survey of the main findings of a few additional studies will be mentioned only briefly, with emphasis upon those studies which improve upon known prognostic assessments which therefore have the potential to impact clinical management.

NON-SMALL CELL LUNG CANCER

Background

Lung cancer is the most common cause of cancer deaths for both men and women in the United States, with an estimated 157,200 deaths in 2003 [6]. Roughly 70-80% of lung tumors fall under the loose category of non-small cell lung cancer (NSCLC) which encompasses the histological classes of squamous cell carcinoma (SCC), adenocarcinoma (AC), and large cell lung cancer (LCLC). These tumors are largely chemoresistant, and even when complete resection of the primary tumor is possible, 50% of these patients die from metastatic disease. It is currently not possible to identify those high risk patients, however, and stage I patients, who are often treated with surgery alone, also relapse at a rate of 35-50% [80]. By contrast, small cell lung cancer (SCLC) responds well to chemotherapy initially, but the disease course typically progresses to recurrence, resistance, and death.

Array Studies

Classification

Three groups, Garber *et al.*, Battarcharjee *et al.*, and Kikuchi *et al.*, found that unsupervised hierarchical

clustering directly segregated the several different groups of both NSCLC and SCLC lung cancer by histology with reasonable concordance between the discriminating gene sets [81-83]. Amongst the adenocarcinomas, the largest subset of NSCLC, several different subclasses were found which roughly correlated with tumor differentiation. These results were corroborated by Beer *et al.* who also noted that unsupervised hierarchical clustering of AC NSCLC afforded clusters which roughly correlated to grade of differentiation [80]. Kikuchi *et al.* also employed supervised learning methods to identify genes which segregated tumors with and without lymph node metastases, achieving 75% classification accuracy on a test set of only 4 samples [83].

Prognosis

Garber *et al.* noted that Kaplan-Meier plots of their three subclassifications of AC differed significantly in survival ($P = 0.002$) [81]. A correlation with survival was also noted by Bhattarcharjee *et al.* [82]. Through unsupervised hierarchical clustering, Wigle *et al.* identified two major groups which correlated with DFS by Kaplan-Meier plot ($P = 0.0022$) [84]. Unlike in the other NSCLC studies, these latter subgroups did not correlate to histology, perhaps because their patient cohort was not as heavily biased towards AC over SCC. Beer *et al.* performed additional levels of analysis, using supervised clustering to discriminate between 43 AC training samples based upon survival [80]. A 50-gene classifier model was chosen as optimal by leave-one-out cross-validation. When the high and low risk discriminating profiles were superimposed upon a test set of 43 samples analyzed on Kaplan-Meier plots by stage, high and low risk subgroups were identified within the stage I designation, demonstrating that gene expression profiling again provided significant enhancement of the conventional risk assessment by stage ($P = 0.003$). This was validated with an independent cohort of 84 cases. This enhancement of risk assessment in AC NSCLCs could point clinicians towards therapy beyond surgical resection for those high risk Stage I patients.

Treatment

Some research has pointed to the potential of gene expression profiling in characterizing lung tumor chemosensitivity [83]. Using tumor samples obtained *via* LCMD, the authors used a collagen gel droplet embedded culture drug sensitivity method to test the samples *ex vivo* for their responses to various chemotherapeutics. Expression levels of 7 genes were found to be associated with chemosensitivity of both AC and SCC NSCLC tumors to any of the 6 common drugs tested. Similar types of *ex vivo* drug testing for a wide variety of tumor types have been developed by several laboratories including commercial ventures [85].

MELANOMA

Background

Melanoma is the most deadly of the skin cancers, claiming 7,600 lives each year with an incidence of 54,200 in the US [6]. Despite its prevalence, very little is understood about the genetic mechanisms of progression in melanoma, and no markers define subsets of the disease [86-87]. In fact, the most reliable prognostic factor is simply the thickness of the initial lesion, but aggressive behavior can be seen even in early melanoma lesions regardless of size or thickness [87]. Although the tumors themselves are largely refractory to chemotherapy, a minority of patients will benefit from high dose IL-2 therapy, but currently there are no criteria to identify those patients who might respond [86].

Array Studies

Prognosis

Using gene expression profiling, Bittner *et al.* were able to identify a subclass of cutaneous melanoma which showed less aggressive behavior [88]. Comparing 31 melanomas (5 were primary biopsy samples, the rest tumor cell cultures) and 7 normal controls against a reference cell line by cDNA arrays (6,971 genes), the authors were able to employ unsupervised clustering methods to identify a single major cluster of tumors. This cluster was not associated with any of the clinical variables of age, sex, Breslow thickness, Clark's level, or survival in a statistically significant manner. However, when compared to profiles of highly aggressive uveal melanomas which were characterized by genes involved in motility and invasion, the profiles of the cutaneous subgroup showed counter expression of those genes. When tested *in vitro* for invasiveness, these tumors indeed had reduced motility ($P = 0.0063$) and invasive ability ($P = 0.005$). Although with small sample numbers and limited available survival data the correlation to survival could not be made with this subgroup, anecdotally only 3 deaths of 10 patients occurred in this subgroup compared to 4 out of 5 deaths in the remaining tumor samples ($P = 0.135$).

Treatment

Wang *et al.* attempted to identify molecular determinants of IL-2 responsiveness in their study of serial FNAs from 25 patients undergoing immunotherapy [89]. Samples were hybridized to cDNA arrays representing 6,108 genes with a NHEM cell line reference. Supervised comparison of pre- and post-treatment profile pairs revealed 17 genes ($P < 0.001$) which were correlated with clinical regression of the tumors after one course of IL-2 treatment. Examining only the pre-treatment samples, the authors next conducted a biased search for genes which discriminated between the 13 clinical responders and 11 nonresponders, identifying 33 predictive genes. Although no further validation or testing was performed with this gene set, this study again demonstrates the ability of molecular profiling to address critical questions about cancer management.

HEAD AND NECK

Background

Head and neck squamous cell carcinomas (HNSCC) have an estimated incidence of 27,700 cases in the US each year [6]. Associated with tobacco and ethanol use, HNSCC is

most commonly found in the mouth, pharynx, tongue, and larynx. Treatment is dependent upon the stage at diagnosis, but the neoplasm recurs in as many as 50% of patients following resection. Therefore, several different chemotherapy regimens are administered to high risk patients.

Array Studies

Prognosis

Although a few studies have been conducted on tumor samples to identify markers of tumor progression, markers of tumor location, novel tumor markers, and potential targets for therapy [90-91], one early study used unsupervised hierarchical clustering to identify two subgroups of HNSCCs which are distinguished by their survival [92]. In this study, Belbin *et al.* found 38% patients in the first subgroup died as a direct result of their cancers whereas none of the patients in the second group died of their cancers. The numbers of patients in each group was quite small (n = 8 each) and no further testing of this distinction was done. Additional studies by Cromer *et al.* and Ginos *et al.* identified gene sets which discriminate metastatic (within 3 years) or locally recurrent tumors versus nonaggressive tumors [93-94]. Although these studies were slightly larger, they too lacked power and were conducted without further validation through test sets of prospective analysis. Nonetheless, these studies suggest that DFS and OAS in HNSCC may be predicted through molecular profiling, leading to the early identification of those patients who might require more aggressive therapy.

Treatment

To address who might respond to specific chemotherapeutics from amongst the several used in the treatment of HNSCC, Kihara *et al.* examined the outcomes of 26 post-resection patients uniformly receiving adjuvant 5-fluorouracil and cisplatin [95]. All patients were SCC stage III/IV. The 20 cases which were used as a training set were segregated based upon survival into three roughly equal groups. A 52-gene predictor was developed using a supervised learning algorithm which distinguished between groups 1 and 3, allowing the computation of a drug response score which correlated with survival (Fig. **8**). This prognostic value was seen despite the fact that the tumors in groups 1 and 3 were similar by other common measures of risk (TNM stage, histological grade, lymph node dissection, and amount of post-surgical residual tumor). When used to study 6 test cases, this drug response score did in fact correlate with their prognosis.

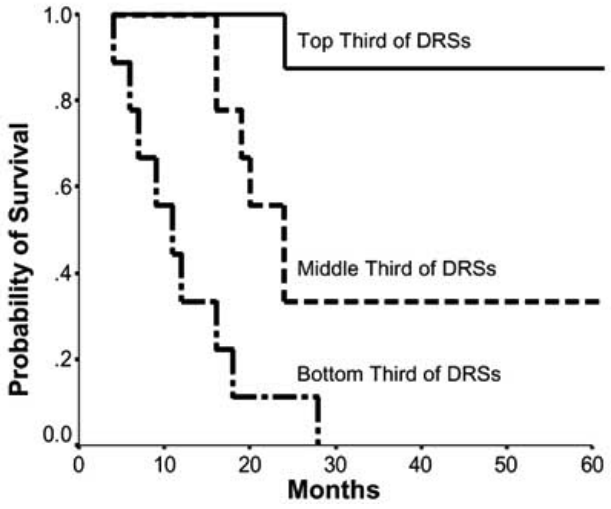

Fig. (8). Kaplan-Meier plot of the HNSCC patient survival segregated by drug response scores (in thirds). Plotted from data in reference [95].

PROSTATE CANCER

Standard measures of risk assessment in prostate cancer include stage, Gleason score (measure of tumor differentiation), and serum PSA. However, Singh *et al.* identified a 5-gene predictor model which classified cases as either PSA failures or those with RFS of >4 years [96]. Interestingly, the two groups had equal representation of other markers of risk. In fact, when Kaplan-Meier plots of the three conventional risk predictors were compared to that of the gene predictor model, the profile-based model demonstrated superior discrimination of high and low risk patients. Since approximately 30% of men undergoing radical prostatectomy will relapse, this type of assessment should aid clinicians in identifying high risk patients who may require treatment beyond surgical resection alone.

MEDULLOBLASTOMA

Gene expression profiling also was able to improve the risk assessment for children with medulloblastoma, a disease against which chemoradiotherapy has variable efficacy. Unfortunately, the only prognosticator for this cancer is stage, and it is imperfect. Pomeroy *et al.* demonstrated by supervised learning methods that an 8-gene predictor model could correctly classify 47 of 60 training samples (P = 0.0002) [97]. Predicted survivors by this model were found by Kaplan-Meier analysis to have an 80% 5 year OAS compared to only 17% for the predicted failures (P = 0.000003). This model also improved upon the stage-based risk assessments, distinguishing poor and good prognosis patients from within a single M0 category of medulloblastomas.

GLIOMA

Nutt *et al.* examined 50 glioblastomas and anaplastic oligodendrogliomas by Affymetrix U95Av2 arrays, demonstrating the ability of gene expression profiling to distinguish between these classes and therefore aid in prognosis and treatment assessments [98]. Although the classic histologies of these tumors are easily distinguishable, the nonclassic presentations can be easily confused, and there is only 69% concordance amongst neuropathologists [99]. Using a training set of only the classic presentations in a supervised learning algorithm, the authors identified genes which discriminated the two classes of tumors. Cross-validation optimized the gene set to a 20-gene model which correctly assigned 18 of 21 members of the training set.

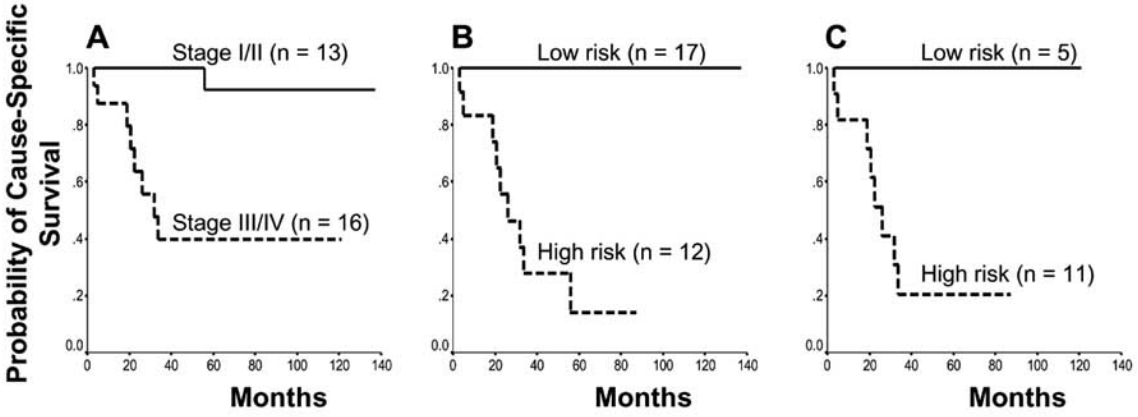

Fig. (9). Kaplan-Meier survival analysis of survival. A, Segregation of patients based upon stage. B, Segregation of patients based upon high or low risk determined by molecular predictors. C, Segregation of stage III/IV patients based upon high or low risk determined by molecular predictors. Redrawn from reference [100].

Since one of the misclassified tumors had a survival more in accord with its profile-based assignment, Kaplan-Meier comparison of the profile-based and the histology-based classifications was conducted, demonstrating the superiority of the profile based method (P = 0.031 versus P = 0.21, respectively). This superiority was validated with a test set of 29 nonclassic gliomas when Kaplan-Meier analysis was applied.

RENAL CELL CARCINOMA (RCC)

Using gene expression profiling of renal cell carcinomas (RCCs), Takahashi *et al.* improved upon stage-based risk assessment for this class of tumors [100]. Of the 70% of patients who present with localized tumors, 30% of those will later relapse and die of their disease. Currently the best means of assessing the risk of the patient are stage and grade. However, Takahashi *et al.* found that even unsupervised clustering led to the identification of two groups which were characterized by different 5-year survival rates. Supervised learning algorithms therefore readily identified predictor gene sets which distinguished the two groups, where as supervised learning algorithms based on the segregation of stage I/II from stage III/IV tumors did not identify significant predictor genes. Leave-one-out cross-validation correctly classified 25 of 26 tumors from the training set, resulting in 26 predictor gene sets in which 95% of genes were conserved. Kaplan-Meier plots using this profile-based classification subdivided stage III/IV tumors into high and low risk groups, demonstrating the enhancement of risk assessment possible through profiling (Fig. **9**).

OTHER METASTASIS STUDIES

Other studies have addressed distinctions between differences in profiles between tumors which metastasize and those that remain localized, suggesting fundamental changes that occur within the primary tumor which might predict the potential for metastasis [101-103]. In addition, a wealth of research on hepatocellular carcinoma has identified genes which segregate tumors on the basis of HBV or HCV infection [104], or genes which correlate to p53 status or vascular invasion [105].

Discussion

Although gene expression profiling experiments have played a significant role in cancer basic research for several years, over the past 4 to 5 years the use of profiling to study primary tumor samples to extract additional clinical information has come to the forefront. As has been illustrated by the many studies reviewed here, this technology has in fact made advances in our understanding of oncology diagnosis and treatment. This has come through the enhanced classification of tumors, the correlation of those classifications to patient prognosis, and the use of profiling to begin to predict which patients might benefit from specific chemotherapeutic regimens. In DLBCL, acute leukemias, breast cancer, NSCLC, and melanoma, new subclassifications of disease have been identified which in some cases appear to carry distinct prognostic value. New specifically prognostic classifications have also been identified in many of these tumor types, enhancing the risk assessment for these cancers beyond that afforded by standard prognostic markers. Profile-based predictor models in DLBCL have independent prognostic value beyond that of the IPI; in breast cancer profiles can provide predictions beyond those of the St. Gallen or NIH assessment models; and typical staging/risk assessment in NSCLC, prostate cancer, and RCC can also be enhanced by profiling. In addition, responder profiles have been identified for specific treatment regimens such as Gleevec (ALL, CML), induction cytarabine/idarubicin (AML), neoadjuvant doxorubicin (breast), neoadjuvant AC (breast), neoadjuvant docetaxel (breast), neoadjuvant paclitaxel+FAC (breast), adjuvant tamoxifen (breast), IL-2 (melanoma), and 5-fluorouracil/cisplatin (HNSCC).

These promising results in many cases have been corroborated by multiple studies conducted by different groups, suggesting that despite the numerous differences in the ways in which the studies were conducted, common end results, if not identical gene sets, can be obtained. Nonetheless, for these profile-based models to be applied in the clinical setting to truly affect the management of patients, they must become refined and standardized. Thus there will be a need to control for the genes represented on the arrays and in the platforms used. The latter encompasses

discrepancies in cDNA versus oligonucleotides, oligonucleotide length and number, ratio versus single channel, control samples, hybridization conditions, signal to noise, dynamic and linear ranges, sensitivity, reproducibility associated with biological variation, random noise, and measurement uncertainty. In addition, the cost, availability, and complexity of these experiments currently prohibit the widespread use of arrays or other molecular profiling methods (such as FPET PCR studies).

The shear size of the data sets demands advanced data handling methods that must also be made cost effective and available. In addition, for physicians to be able to compare the tumor profile of their patient to known prognostic classifier or responder/ nonresponder patterns, there must be an open database established for the sharing of data. Various groups have begun to collate this type of data for limited series of patients [106], but there is still a need for increased efforts in this area.

Although some studies have suggested that the heterogeneity of a tumor does not prohibit the use of array work for profiling, tumor sampling issues remain without a concrete answer as do the issues of obtaining adequate tissue. Studies on biopsy samples (FNAs and core biopsies) as well as the FPET work suggest that the lower limitations on material requirements are slowly being pushed back and entering the realm of the clinically feasible. However, with new HIPAA regulations, the acquisition of tumor samples for future studies has become more challenging, pushing researchers to more prospective studies which take significantly longer to complete.

Lastly, any new profile, such as those described in this review, must be further validated to prove that the use of this information can be replicated and used to affect patient management. For instance, this requires numerous prospective studies to demonstrate that patients with a responder or good prognosis profile do in fact respond to a given treatment at higher rates than an unscreened patient cohort. In addition, it must be shown that predicted nonresponders or poor prognosis patients do benefit more by early intervention with intensive or experimental therapy than by standard treatment. With the plethora of chemotherapy regimens available for each tumor type, these types of studies must be performed for each regimen before the goal of matching therapy to patient can be achieved.

Nonetheless, the goal of individualized therapy for cancer patients remains enticing, and the recent progress in gene expression profiling has demonstrated its overall feasibility although not its imminent fruition. Since each tumor in each patient is, in many ways, a unique disease, optimal treatment of cancer patients may ultimately depend upon their individualized treatment. Gene expression profiling provides a means to examine the entire transcriptional biology of a tumor, enabling the future individualized management of cancer patients.

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