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Gene Expression Profiling In Lymphoma Diagnosis and Research

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Abstract Gene expression profiling in the past 5 years has generated a large amount of data on a variety of malignancies. Unique gene expression signatures have been identified for the more common types of non-Hodgkin lymphoma (NHL), including clinically and biologically important subsets that have not been defined before. In addition, molecularly defined prognosticators have also been constructed for the major types of NHL and these prognosticators provide added value to the widely used International Prognostic Index. The new information should be included in our evaluation of NHL patients, especially when conducting clinical trials. Studies are ongoing to validate and refine these diagnostic and prognostic signatures and to develop platforms that are suitable for routine clinical applications. Similar studies will be performed on the less common types of NHL to complete the molecular classification of NHLs. It is also anticipated that gene expression profiling studies will lead to the identification of novel targets for the development of new therapeutic agents for NHL.

Diagnosis and classification of non-Hodgkin lymphoma (NHL) have been a challenging task and multiple classification schemes have emerged in the last 40 years. Rappaport (1) established a widely used, morphology based lymphoma classification system as detailed in the Armed Forces Institute of Pathology (AFIP) fascicle on Tumors of the Hematopoietic System in 1966. The Lukes and Collins classification and the Kiel classification (2-4) were proposed later, based on the conceptual advances in immunology and the ability to immunophenotype the neoplastic cells. Unfortunately, different approaches with their individual nomenclature and diagnostic criteria were not readily translatable and often led to confusion. The Working Formulation (5) was an attempt to provide a translation between a number of widely used classification systems. The most recent approaches to lymphoma diagnosis and classification, the Revised European American Lymphoma Classification (REAL) (6) and the World Health Organization (WHO) classification (7), have broader consensus and emphasize the delineation of disease entities by including histological and clinical findings, as well as cytogenetics, immunophenotyping, and molecular genetic information. However, further refinement of the current classification system is needed, as response to treatment and survival within each specific lymphoma category still show significant variability indicating further heterogeneity (8).

Development of a tumor is typically initiated by a genetic alteration in a cell, and this change predisposes the cell to undergo further genetic alterations (9). After a variable number of additional acquired genetic abnormalities, a malignant clone eventually arises, which has a growth and/or survival advantage compared to the normal counterpart. Rarely, the genetic alteration initiating carcinogenesis is inherited (10). The initiating

event often consists of chromosomal translocations, which have a propensity to occur in lymphocytes, especially B-lymphocytes. This is reflected by the incidence of lymphoma with the vast majority arising from the B cell, a small percentage from the T cell lineage, and rare tumors from natural killer (NK) cells and dendritic cells. During the maturation and development in both B and T cells, the physiological rearrangement of the antigen receptor genes introduces double strand DNA breaks that predispose to the occurrence of abnormal translocation events. Additionally, in B-lymphocytes, the germinal center (GC) reaction with somatic hypermutation and isotype switch provides additional opportunities for chromosomal translocations. Subsequent secondary abnormalities, including mutations, amplifications, deletions, and additional translocations occur with the development of a malignant tumor. These cumulative genetic abnormalities alter the composition of transcripts in the neoplastic cells. Thus, each malignant clone or tumor has its own molecular profile or signature of mRNA that can be determined experimentally by microarray analysis. We can postulate that the characteristics of a tumor and its clinical behavior are determined by the unique set of genetic lesions in the tumor cells and the functional effects are reflected by the unique gene expression signature of the tumor. Therefore, gene expression profiling can be exploited to improve not only the diagnosis and classification but also the prognostication and treatment of lymphomas. In the past five to six years, the microarray technology has been used extensively to investigate various kinds of human tumors including lymphomas that will be the principal focus of discussion in this paper.

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Principles of Microarray Analysis

Microarray assays are based on the specific nature of complementary DNA double helix formation during hybridization. The hybridization between DNA probes bound on a solid surface and their labeled complementary counterparts in the sample is performed in miniaturized volumes. The DNA probes attached onto a solid surface such as glass, can be synthesized *in situ* or spotted with cDNAs or oligonucleotides prepared off-line. The commercial company, Affymetrix, synthesizes oligonucleotides directly onto the surface by a photolithographic process. Each cDNA is probed by a set of 25 base pairs long oligonucleotides and each array can complement over 40,000 different cDNAs. Spotted microarrays are custom made by a number of commercial companies or core facilities at individual institutions. A robot is used to spot PCR amplified cDNA or synthetic oligonucleotides in the range of 50-70 bp onto the surface of glass slides covering over 10,000 different transcripts per microarray. For spotted microarrays, the sample of interest (test samples) is generally measured against a reference standard to obtain relative expression levels that can be compared across experiments. RNA extracted from the test sample and the reference is reversely transcribed into cDNA and each pool is labeled with different fluorescent markers, most often Cy3 or Cy5. The two cDNAs are then combined and hybridized to the microarray. After washing, scanning, and image processing, the microarray can be analyzed to provide a unique gene expression profile of the specific test sample. A single DNA microarray experiment can measure the expression of thousands of genes simultaneously with good reproducibility in carefully performed experiments. Comparison of data across different array platforms is still a difficult undertaking and represents an impediment in correlating results from different laboratories. However, whereas comparing the expression of individual genes often gives discrepant results, the comparison of large groups of genes serving specific functions is much more robust.

Data Management and Analysis

Each microarray experiment generates thousands of measurements that need to be processed and analyzed. Image processing includes the accurate measurement of fluorescence from a very small surface area, proper background subtraction, and data normalization. After image processing, a massive amount of information has to be analyzed. A number of analytical tools are currently available for detecting structures in the data set, for model fitting, class prediction/assignment, and class discovery (11-15). There is no single best tool for all purposes and the appropriate tools for an experiment depend on the experimental design, the data obtained, and the questions being addressed. It is not possible to have detailed discussions of analytical methods here and only a brief outline of one of the most commonly used clustering program will be presented – agglome-

rative hierarchical clustering (12). Gene expression data are most frequently presented in the form of a matrix with the list of genes on one axis and tumor samples on the other axis. Hierarchical clustering start by associating pairs of genes with the most similar pattern of expression across samples and then successively combining these initial clusters into larger clusters until all the genes are grouped together in to a single dendrogram. Tumor samples can be similarly clustered according to their overall similarity in gene expression profiles. Clustering can be performed in either a supervised or unsupervised format. In unsupervised clustering, the samples and genes are arranged according to a predefined computational algorithm. In supervised clustering, certain investigator-defined parameters are used to guide the clustering. These parameters may be clinical data, sets of genes with known biologic functions, or pathologic features.

While gene expression profiling is a powerful technique, one needs to be aware of certain pitfalls and limitations. Genes that are expressed at low levels are generally not reliably and reproducibly measured by current microarray technology. The cyanine dyes commonly used may not give equivalent direct labeling of cDNAs, but this problem can be overcome by using indirect labeling. Thousands of parameters are being measured in each tumor, whereas the number of tumor samples is often quite limited, making statistical assessment difficult. Although it is not possible to have a sufficiently large number of cases desirable for confident statistical conclusions, the number of cases included should be as large as possible and it is practically impossible to draw meaningful conclusions from a few cases. Because of this discrepancy in dimensionality, validation of the analytical results becomes very important. Different analytical tools may be applied to assess the validity of the conclusions from one analytical method. The reproducibility of the data structures may be tested by introducing random Gaussian noise to each data point and the perturbed data re-analyzed to determine the robustness of the structures. Another frequently used approach is to divide the patient samples into a test set and a validation set. The validity of the conclusions drawn from the test set will then be examined on the validation set. Another useful approach is to look for clinical or biological correlates of conclusions drawn from microarray analysis. We will use examples from microarray experiments performed on lymphomas in the past few years to illustrate approaches that may be taken for analysis and validation.

Gene Expression Profiling in Lymphoma: Illustrative Examples

There are ongoing and published gene expression profiling studies on many different types of human malignancies. One of the major themes in these studies is to explore the potential of gene expression analysis in class prediction (classifying tumors into currently defined categories) and class discovery (finding new tumor types that are biologically meaningful). The promises and challenges gleaned from the early studies are discussed below, using illustrative examples.

Alizadeh et al (16) studied three types of B-cell malignancies: diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and B-chronic lymphocytic leukemia (CLL), using a microarray enriched in genes known to be involved in cancer biology and immunobiology. Using unsupervised hierarchical clustering, these three categories of lymphoid malignancies were separated broadly into three corresponding clusters according to their overall gene expression pattern (Fig. 1). This suggests that distinct groups of lymphoma defined by traditional parameters have sufficiently different patterns of gene expression that they can be separated by the set of genes examined on the array (class prediction).

Some important confounding variables should be noted. One important variable is that different types of specimens were used for the different diseases. The DLBCLs were submitted as frozen tumor samples, whereas many of the FL and CLL samples were comprised of enriched tumor cells; therefore, there was differential expression of large sets of genes associated with stromal elements and infiltrating T-cells/macrophages in the tumors. In addition, DLBCL generally has a higher proliferation rate than FL and CLL and, hence, exhibits up-regulation of many genes associated with cell proliferation. The common expression of these large sets of genes may move cases into the same cluster despite the presence of important biologic differences.

The investigators noticed that there was a set of genes preferentially expressed by normal

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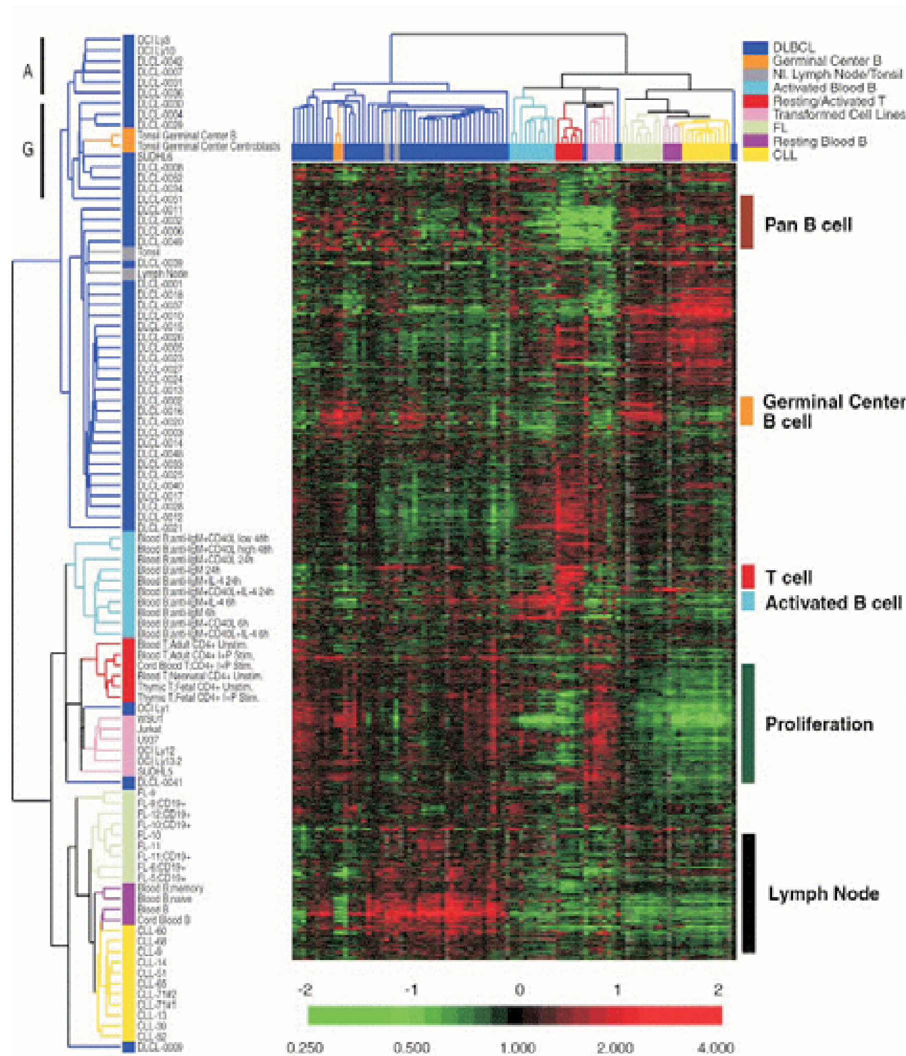

Figure 1. An example of unsupervised clustering of microarray analysis of normal lymphoid tissues, cell lines, and lymphoid malignancies. The dendrogram on the left is an enlargement of the one on top of the expression data matrix. Note that the dendrogram cluster cases according to their similarity of gene expression pattern and the B-chronic lymphocytic leukemia (CLL), follicular lymphoma (FL), and diffuse large B-cell lymphoma (DLBCL) cases are largely in three different clusters. Also note the expression data matrix with the red and green color, indicating over- and under-expressed genes, respectively, compared with the standard RNA pool (15).

germinal center (GC) B cells but not by peripheral blood B cells activated by a number of stimuli. When the set of GC-B cell associated genes was used to cluster the DLBCL cases, two broad groups were delineated. One group expressed many of the genes in the GC-B-cell associated profile, whereas the other expressed few of the GC-B-cell associated genes but, instead, expressed many of the genes on the activated B-cell profile. Hence, two subgroups of DLBCL that appear to be biologically distinctive can be defined by their gene expression profile (class discovery) (Fig. 2).

When apparently new tumor categories are discovered on analyzing gene expression data, the finding requires careful validation. Aside from reanalyzing the data using various tools, one can examine these new classes for biological and clinical relevance using parameters unrelated to gene expression profiling. Alizadeh et al (16) examined the clinical behavior of the two new classes of

DLBCL. A significantly better overall survival was associated with the group of lymphoma with the GC-B cell-like profile and this association appeared to hold even when cases with low clinical risk factors (IPI of <3) were examined. The clinical data thus provided independent support for the validity of the class discovery.

The validity of the class discovery can also be queried by independent biologic parameters. One cardinal feature of GC-B cells is the presence of ongoing somatic hypermutation of the immunoglobulin heavy chain variable region (VH) genes. The group of DLBCL with high expression of the GC-B cell signature would be expected to exhibit this characteristic, whereas the other group should not. This hypothesis was tested in 14 of the cases previously studied by Alizadeh et al (16). All 7 cases with the GC-B-like gene expression profile showed ongoing somatic hypermutation of their VH genes, whereas only 2 of 7 of the cases with the activated B-cell-like pattern showed ongoing mutations, but at a lower level compared with the

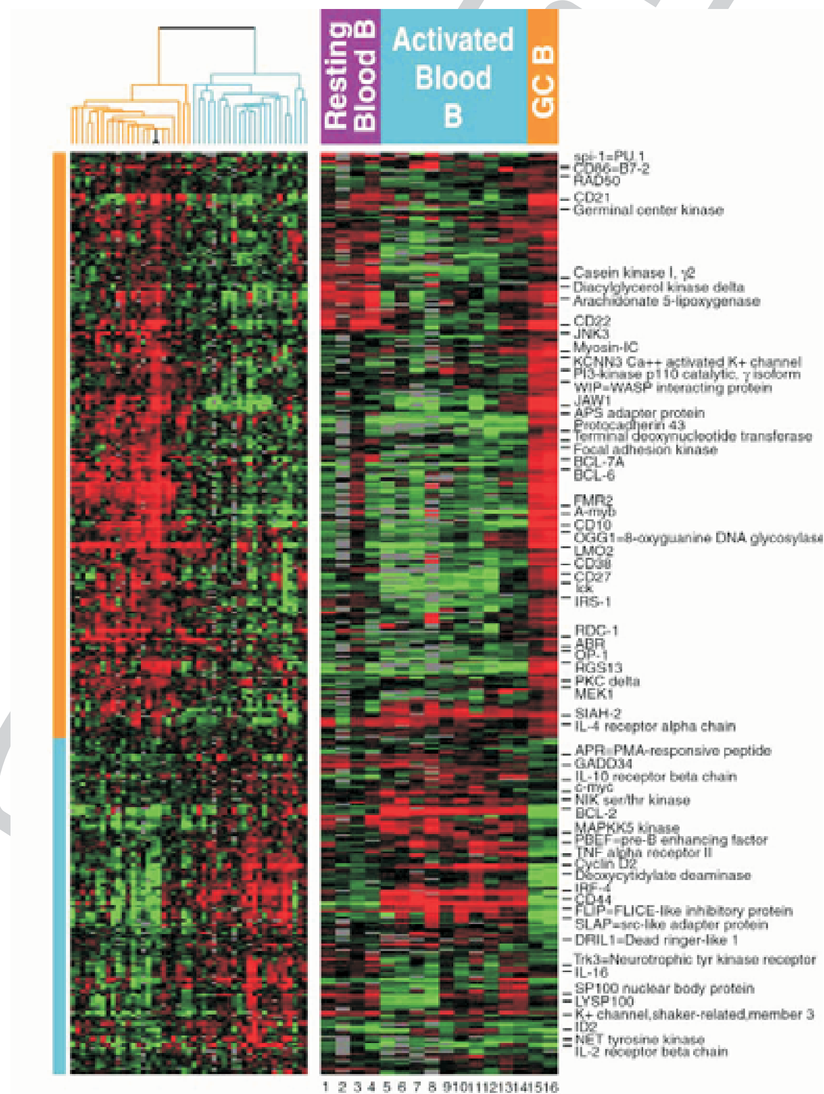

Figure 2. Diffuse large B cell lymphoma can be divided into two major subsets by gene expression profiling. The germinal center B-cell (GCB) subset highly expresses a group of genes that are also highly expressed in normal germinal center B-cells. The activated B-cell subset does not express this germinal center B-cell signature but highly expresses genes that are unregulated in peripheral blood B-cells activated by various mitogenic stimuli (15). The display is a heat map, ranging from bright green (underexpressed) to bright red (overexpressed).

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previous group (17). These two cases may have overlapping biologic characteristics with the GCB group or they may be mis-classified. Classification by hierachical clustering may not be ideal and more sophisticated approach may give rise to more accurate classification (18). The study by Alizadeh et al (16) contains only 40 patients with DLBCL and the findings should be confirmed in a new study with a larger number of patients. A subsequent larger study with 240 cases of DLBCL do indeed confirm the validity of this subclassification (19).

There is an unusual type of DLBCL that presents in the anterior mediastinum (PMBL) and tends to occur in young female patients. The tumor also tends to have prominent stromal fibrosis, with tumor cells typically having moderately abundant pale cytoplasm. Immunoglobulin expression tends to be undetectable in this tumor. There has been a long debate as to whether this represents a unique type of DLBCL. Two recent studies have demonstrated that this tumor shows a distinct gene expression profile that can differentiate it from the GCB and ABC type of DLBCL and hence other DLBCL that happens to occur in the anterior mediastinum (20,21) (Fig. 3). Interestingly, there is a substantial similarity of the gene expression profile of PMBL with Hodgkin lymphoma, suggesting that there may be some shared biological properties between these two tumor types (20,21). A re-examination of the series of DLBCL published previously (19) found 12 cases among the GCB subgroup that showed the gene expression signature of PMBL. It is interesting that not all the cases are primary mediastinal lymphomas, but the cases tend to occur in thoracic structures above the diaphragm.

Since the unique gene expression profile of a tumor is determined by the intricate interaction of the genetic abnormalities present, it is anticipated that some of the genetic abnormalities, especially those with a major influence on the biology of the tumor, will segregate with tumor subsets defined by gene expression profiling. Some genetic abnormalities may influence or determine the subsequent genetic evolution of a tumor and hence may be associated with certain unique gene expression patterns. The t(14;18)(q32;q21) is a

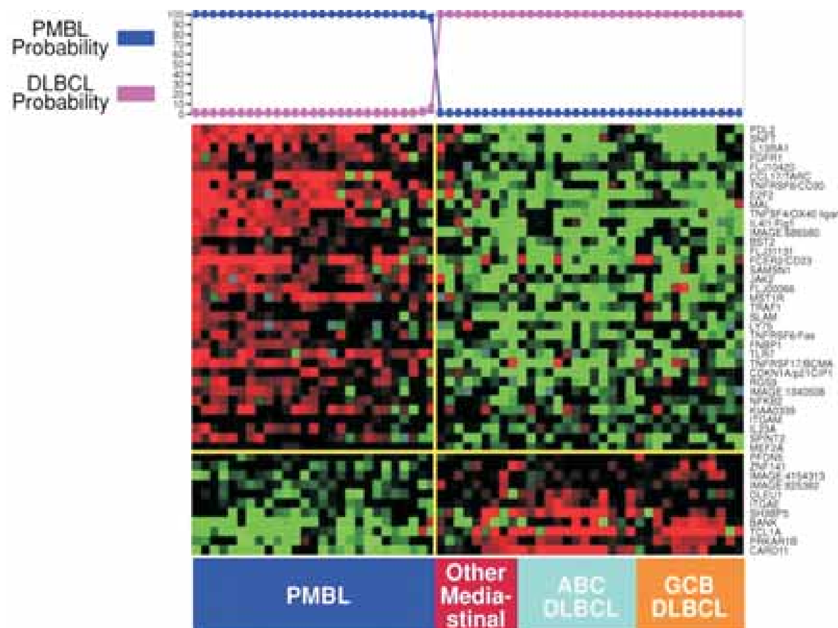

Figure 3. Primary mediastinal large cell lymphoma (PMBL) has a distinct gene expression signature that can distinguish it from other diffuse large B-cell lymphomas (DLBCLs) (19). Red areas indicate increased expression, and green areas decreased expression. Each column represents a single diffuse large-B-cell lymphoma, and each row represents a single gene. The gene expression signature characteristic of PMBL is indicated. It clearly distinguishes PMBL from other types of DLBCL. ABC – activated B-cells; GCB – germinal center B-cell.

hallmark of FL but is also detectable in 20-30% of cases of *de novo* DLBCL. If this translocation is an initiating event for these DLBCL as for FL, one would expect that the precursor cells of these large cell lymphomas would also start their journey in the GC. Different secondary events lead to alternative pathways resulting in either FL or DLBCL (22). It is likely, therefore, that DLBCL with t(14;18) would exhibit the GC-B cell-like expression profile. A recent study demonstrated that this is indeed the case (22), and a subsequent larger study again confirms this finding (23). Similarly, other genetic abnormalities are highly associated with subtypes of DLBCL, for example 9p24 gains and 2p14-16 amplification are frequently associated with PMBL (20), whereas identification of 18q21-22 and 6q21-23 losses are often associated with ABC-DLBCL (24).

A study of over 90 cases of well-characterized mantle cell lymphomas that are cyclin D1 positive has demonstrated a unique gene expression profile associated with this type of lymphoma (25). In this study, there were a few cases that were cyclin D1 negative and were initially excluded from the analysis but on re-examination, some of the cases expressed the mantle cell lymphoma signature. These cases were also studied by fluorescent in situ hybridization (FISH) and found to be t(11;14) negative. It is now possible to identify these cyclinD1 negative lymphomas for further evaluation of their biological and clinical features and their relationship with typical mantle cell lymphomas. These examples illustrate the class prediction and class discovery potentials of gene expression profiling in malignant lymphoma. A substantial amount of effort has also been directed at investigating the feasibility of using gene expression profiling on the original lymphoma biopsy to predict response to therapy and survival. This will be briefly discussed in the next section.

Construction of Predictors for Survival

In the construction of prognosticators, clinical data are used to supervise the analysis. Typically, the patients are divided into a training and a validation set and the training set is used to identify genes or signatures that should be included in the predictor. An outcome predictor score using the Cox proportional hazard model will then be constructed and tested on the validation set. In the series of DLBCL reported by Rosenwald et al (19), four gene expression signatures were found to predict survival. Each of these signatures consists of hundreds of genes but can be easily represented by only several genes. Thus, the GCB cell signature can be represented by three genes, the MHC class II signature by four genes, the lymph node signature by six genes and the proliferations signature by three genes (Table 1). Shipp et al, using a different approach, have identified a 13-gene predictor for their series of DLBCL (26). Because different arrays were used in these studies, it is difficult to directly compare the results. However, two of the important predictor genes in the latter study: PKC β2 and PDE4B are typically expressed by the ABC-DLBCL and their high expression would be expected to be predictive of poorer outcome.

Table 1. Survival predictors for diffuse large B-cell lymphoma

Signature	Representative genes in outcome predictor	Outcome prediction
Germinal center B cell	BCL-6	good
	centerin	
	M17	
MHC class II	DP alpha	good
	DQ alpha	
	DR alpha	
	DR beta	
Lymph node	alpha-actinin	good
	collagen type III α1	
	connective tissue growth factor	
	fibronectin	
	kIAA0233	
	plasminogen activator, urokinase	
Proliferation	c-myc	bad
	E21G3	
	nucleophosmin/nucleoplasmin 3	
Other	BMP-6	bad

For mantle cell lymphoma, a group of genes that are associated with cell proliferation appeared to be the major determinant of prognosis (25). Patients with tumors that had a high expression of this set of genes had short survival (highest quartile with median survival of 0.8 year), whereas patients with tumors having a low proliferation signature average had much longer survival (lowest quartile with median survival of 6.7 years) (Fig. 4). This proliferation signature appears to represent an integrator of a number of different signals that contribute to short survival in mantle cell lymphoma, including the deletion of the INK4/ARF locus and high levels of cyclin D1 expression.

Gene expression profiling has also been applied to study survival in follicular lymphoma (27). One hundred and ninety-one samples were

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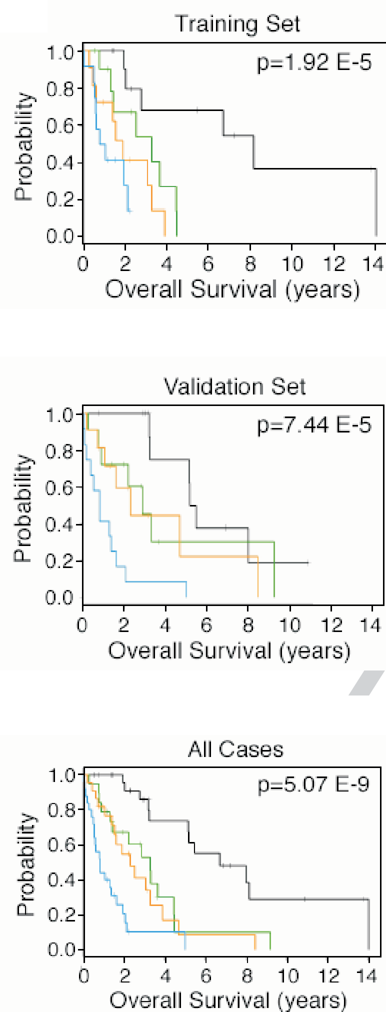

Figure 4. The proliferation signature average is a major determinant of survival in mantle cell lymphoma. The mantle cell lymphoma cases are divided into quartiles according to the proliferation signature average; there are significant survival differences among the quartiles (24). Black line – 1st quartile; green line – 2nd quartile; orange line – 3rd quartile; blue line – 4th quartile.

studied and a number of gene expression signatures were found to be predictive of survival. However, after multivariate analysis, two signatures were noted to have a strong synergy in predicting survival (Fig. 5). These two signatures were combined to form a molecular predictor for follicular lymphoma. The immune response-1 signature contains several genes that are known T-cell transcripts but it does not merely reflect the presence of tumor infiltrating T-cells and high expression of these genes is associated with better prognosis. The immune response-2 signature contains many transcripts expressed in myeloid and monocytic cells and high expression of these transcripts is associated with poor survival. Thus, these signatures appear to represent the composition and function of tumor-infiltrating immune cells and points to an important interplay between the host tumor response and the neoplastic cells in follicular lymphoma. The molecular predictor is independent of the International Prognostic Index and is helpful in identifying the group of patients with poor survival that requires special attention in management.

The determinants of survival differ significantly in the three types of lymphoma studied. Interestingly, in both DLBCL and follicular lymphoma, significant components of the prognosticators reflect tumor/host interaction. This observation indicates that it is essential to obtain the stromal signature in tumors in constructing outcome predictors. These studies also indicate that gene expression profiling can indeed provide a new and more biologically relevant approach to predicting survival.

Perspective

In the past five years, it has been demonstrated quite clearly that gene expression profiling is able to lead to the discovery of unique gene expression signatures for the major groups of B-cell lymphomas including some novel subtypes, the construction of molecular prognosticators, and insight into the molecular mechanisms that determine the behavior of a tumor (28,29).

Reliable diagnostic gene expression signatures have been discovered for the majority of B-cell lymphoma accounting for approximately 85% of all cases. The remaining lymphoma classes are uncommon, many representing 1% or less of all lymphomas. To study these tumors, a well coordinated international effort will be necessary to obtain the required samples for study. Molecular prognosticators have been constructed for DLBCL, follicular lymphoma, and mantle cell lymphoma. Whereas this represents a major advance in outcome prediction, the current prognosticators need to be further validated and refined. There are likely to be determinants of prognosis that are not included due either to current technical or analytical limitations. It is also likely that certain biological variables are not readily measured by gene expression profiling alone. Our analysis of genetic abnor-

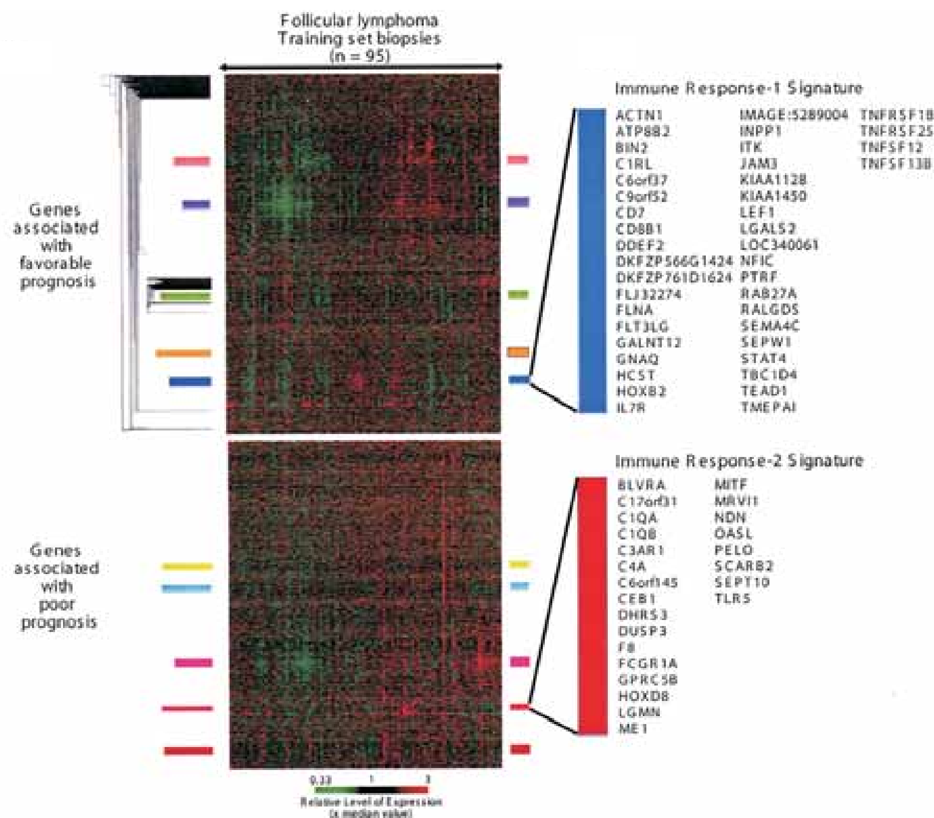

Figure 5. This figure shows the gene expression profile of the training set of follicular lymphomas. A number of gene expression signatures associated with survival can be identified. Multivariate analysis shows that two of these signatures are synergistic in predicting patient survival. Both of the signatures are associated with host/tumor interactions, indicating that this interaction is a major determinant of survival (26).

malities in DLBCL suggests that some of these abnormalities may add to the current gene expression based predictors. Future genetic studies utilizing more accurate and high resolution techniques such as multicolor karyotyping including SKY and M-FISH (30,31) and array based comparative genomic hybridization (32) or FISH analysis of specific loci will provide additional valuable information. Abnormalities in specific genes such as mutations and methylation may also be determined by a number of molecular assays. This information may complement our current gene expression model to provide a better model for predicting survival. In addition, this integrated information may help us understand the mechanisms underlying the difference in clinical and biologic behavior.

At this stage, few genes or pathways have been identified by gene expression profiling studies to be essential components in defining the clinical and/or biologic characteristics of lymphomas. In general, there are hundreds or more genes that are differentially expressed between even closely related categories of tumors. To identify the important versus the secondary or accompanying events is a major challenge. Gene expression data should not be interpreted in isolation. All ancillary information including various tumor and clinical characteristics could be helpful in their interpretation. It is also recognized that the examination of alterations in genetic/metabolic pathways provide much more reliable, robust information than the examination of single genes. Careful and detailed annotation of pathways is therefore extremely important in the future analysis of microarray data. A few interesting findings have been described. The RhoC gene has been implicated as a key determinant of metastatic potential and tumor invasion in melanoma cells (33). Constitutive activation of NFkB may be of crucial importance in

the activated B-cell (ABC) type of DLBCL (28). Down-regulation of c-MYC and IL-6 expression has been shown in myeloma cells exposed to thalidomide, and these are thus believed to be important target genes for the drug (34). The possible involvement of the p38 MAPK pathway in transformed follicular lymphoma have been suggested (35). There will likely be an exponential increase in reports on candidate genes/pathways in the next few years, and the painstaking task of confirming their importance and delineating the mechanisms of action will have to follow.

What is the best way of exploiting the information that we have obtained at this stage? It is possible at this stage to include the new molecularly-defined subtypes of lymphoma in our diagnosis. It is also desirable to include the measurement of the currently defined molecular predictors for DLBCL, mantle cell lymphoma and follicular lymphoma especially in a clinical trial setting. The number of transcripts that needs to be measured for these purposes is reasonably small and can be put into a miniarray format for expression profiling. However, this platform will require the availability of fresh or fresh frozen tissues to obtain high quality RNA. This will require a widespread acceptance of submitting tissue biopsies in the fresh state with a representative sample reserved for array analysis. It may be difficult to change the traditional pattern of tissue handling and there are situations where it is desirable to study a large series of archival cases for validation of diagnostic/prognostic signatures or other related retrospective investigations. It is, therefore, important to investigate the adaptation of the array platform to a platform suitable for archival tissues. We believe that the number of parameters that need to be measured can be substantially reduced with minimal loss of information (36,37). A quantitative RT-PCR or immunohistochemical platform can then be developed to apply the knowledge obtained from microarray experiments to the study of archival materials.

While it is useful to stratify patients to the most appropriate therapeutic regimens based on their individual risk factors, the choice of therapy and the understanding of the biologic basis underlying these risk factors are still limited. Gene expression profiling of cancer has shown promise in delineating the molecular mechanisms and the key genetic components determining biologic and clinical behaviors in malignant neoplasms. It is hoped that novel agents will be developed based on the molecular targets identified from microarray experiments. When novel, mechanism-based therapies become available, it will be essential to have the relevant molecular information on each tumor. One can envision the development of diagnostic microarrays containing all the essential genes that have been selected, based on knowledge gained from prior gene expression profiling studies. Every tumor could be examined by the relevant microarray at diagnosis, and the results would help to determine the appropriate therapeutic interventions. Comprehensive molecular tumor diagnostics and individualized treatment may become a reality in the not-too-distant future.

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