Diffuse large B-cell lymphoma: From biomarkers to novel mechanisms and new therapies

Izidore S. Lossos
University of Miami/Sylvester Cancer Center, Miami, FL

Diffuse large B-cell lymphoma (DLBCL) is the most common adult Non-Hodgkin’s lymphoma with an annual incidence of more than 25,000 cases in the United States. Although DLBCL has characteristic morphology, marked immunophenotypic, cytogenetic and molecular heterogeneity underlies the variable clinical outcome of DLBCL patients. Consequently, it is expected that clinical surrogates, such as the international prognostic index (IPI), while highly useful, do not adequately capture the molecular and cellular variability that underlies clinical behavior of DLBCL. Biologic mechanisms underlying DLBCL pathogenesis are complex and involve intricate relationships between multiple genes, signaling pathways and regulatory processes. Elucidation of DLBCL pathogenesis is necessary to allow recognition of new molecular therapeutic targets, discovery of DLBCL subgroups with distinct clinical outcome and identification of molecular prognostic markers that may more accurately predict DLBCL outcomes. Accomplishment of these goals can be of paramount importance and may form the basis for future risk-adapted treatments. Classically, attempts to elucidate DLBCL pathogenesis or identify new prognostic markers used a single gene approach. However the latter cannot account for the complex multigene processes underlying DLBCL pathogenesis and thus do not accurately reflect the complex changes observed in these tumors. Consequently, new investigational tools enabling simultaneous evaluation of multiple components of these biologic processes might further advance our understanding of DLBCL and potentially lead to specific molecularly targeted and patient tailored therapies.

DNA microarrays are a new technology used to measure the expression of tens of thousands of genes simultaneously, enabling a more comprehensive evaluation of gene expression. This technique allows the comprehensive analysis of messenger RNA (mRNA) expression in tumor samples. The clinical characteristics and behavior of a tumor are determined by the specific genetic changes present in the tumor cells that are reflected in their pattern of mRNA expression creating a “molecular signature” or “fingerprint” for the tumor. The full potential of microarrays has not yet been realized, however they may a) identify previously unrecognized disease entities with distinct biological and clinical features; b) elucidate the key genetic profiles and lesions that define each of these new nosologic entities; c) discover new molecular targets for future therapeutic intervention; d) identify genes that play a potential role in determining prognosis; e) discover previously unknown genes of major clinical relevance from numerous EST clones present on the arrays, and f) identify gene expression signatures correlated with response to specific therapeutic agent.

Less than half of patients with DLBCL will be cured with conventional chemotherapy regimens. Improvement in disease-free and overall survival may be obtained with the addition of monoclonal antibodies, such as rituximab. While standard pathologic
techniques do not reliably predict sensitivity to chemotherapy or outcome for individual patients, gene expression profiling has provided important insights into the biology of DLBCL, allowing a better molecular classification of tumors that are more homogeneous in pathogenesis and clinical behavior.

**Moving from microarray studies to marker panels**

The pivotal microarray study was performed by Alizadeh et al with the use of a cDNA Lymphochip array.\(^5\) The evaluation of tumors from 42 DLBCL patients treated with anthracycline-based chemotherapy led to the identification of two distinct subgroups based on the expression of genes characteristic of germinal center B cells (GC) or *in vitro* activated peripheral blood cells (ABC). Patients with GC subtype had a significantly better overall 5-year survival (76% versus 16%, \(P < 0.01\)), independent of the IPI score. These findings were further confirmed by the larger Lymphoma and Leukemia Molecular Profile Project (LLMPP) study.\(^7\) Using similar cDNA Lymphochip array platform, analysis of tumor samples from 240 DLBCL patients treated with anthracycline-based chemotherapy demonstrated a significant difference in the 5-year overall survival between the GC-like and ABC-like subgroups (60% versus 35% respectively). Although the early microarray expression profile studies were able to identify the presence of biologically distinct subgroups of DLBCL, they were unable to identify the relative contribution of individual gene, therefore making difficult to build clinically useful prognostic models based on a relatively small number of genes. To address this question, both Rosenwald\(^7\) and Shipp groups \(^8\) applied supervised analytical methodologies to the Lymphochip and Affymetrix-derived gene expression profiles of 240 and 58 DLBCL patients, respectively. This approach led to construction of outcome predictors based on expression of 17 and 13 genes, respectively. However, there was no overlap between the lists of genes comprising these two outcome prediction models. This disparity between large genome-scale expression profile models has been attributed to patient selection, technical differences, arrays composition and variable analytical approaches. Wright et al designed a method based on Bayes’ rule that could be used to translate experimental results across different microarray platforms.\(^9\) Expression data from 14 genes identified by the LLMPP\(^7\) and analyzed by Shipp\(^8\) was able to subdivide patients into GC-like and ABC-like, with significant different outcomes. Nevertheless, despite the positive results, this model may not be clinically useful because of complex manipulation with shifting and scaling of gene expression from Affymetrix data to match the mean and variance of the corresponding expression values in the cDNA microarray dataset.

In an attempt to devise a technically simple method that could be applicable for routine clinical use, we evaluated the mRNA expression of 36 genes previously reported to predict survival\(^10\) in tumor specimens from 66 DLCBL patients treated with anthracycline-based therapy. The top six genes ranked according to their predictive power on univariate analysis were used to construct a model based on their relative individual contribution into a multivariate analysis. Among the selected genes, *LMO2*, *BCL-6* and *FNI* predicted longer survival whereas *CCND2*, *SCYA3*, and *BCL-2* predicted shorter survival. Based on the expression of these 6 genes, patients could be subdivided into IPI-independent low, intermediate, and high-risk groups with significantly different 5-year survival ranging
from 65% in the low-risk to 15% in the high-risk subgroups. This model was subsequently validated in the data sets available from previously reported studies\(^7\),\(^8\).

Gene expression arrays are not widely available, require fresh tumor specimens, and are labor-intensive and expensive. Therefore, researchers have tried to use the information derived from RNA profiling studies to create prediction models based on more amenable technique such as immunohistochemistry (IHC). However, multiple IHC studies lead to contradictory results\(^9\),\(^10\) suggesting the lack of an ideal set of IHC markers for outcome prediction in DLBCL. Hans et al, complimented cDNA microarrays with immunohistochemistry (IHC) staining\(^11\). They proposed an IHC model based on 3 markers: CD10, BCL6 and MUM1 for determination of GC-like and ABC-like DLBCL subtypes. This model was shown to have positive predictive values of 87 and 73% for correctly identifying GC-like and ABC-like DLBCL subtypes and could predict patients’ survival: 76% of IHC-defined GC-like DLBCL survived at 5-year compared to 34% of non-GC patients. However, comparison of this IHC model with the gold-standard gene expression profiling revealed a 20% misclassification rate, suggesting the need for incorporating of additional IHC markers in an attempt to improve the predictive value of this model. In deed- recent study demonstrated that Han’s model cannot predict outcome in a large cohort of DLBCL patients (Natkunam-JCO in press). Since antibodies are not available for many of the GC-specific genes, novel monoclonal antibodies directed to newly identified RNA-based prognostic biomarkers need to be generated and assessed in the future IHC based prediction models\(^12\),\(^13\). Furthermore, although IHC is used routinely in diagnostic laboratories, its applicability for outcome prediction requires standard methods for tissue fixation, antigen retrieval protocols and staining methodologies, a uniform use of the same antibodies directed to specific epitope on the target protein and application of identical pre-determined thresholds used to define positivity for specific antibodies. This information, however, is currently unavailable.

Alternatively, it is possible to construct predictive models based on RNA-based gene expression profiling in formalin-fixed paraffin-embedded tissues, which are used routinely for IHC and thus are widely available. Unfortunately, the process of formalin fixation may contribute to RNA degradation and modification that limits the extractability of high-quality RNA by routine methods. Recent improvements in RNA extraction protocols have allowed the extraction of short informative RNA fragments from paraffin blocks, with potential use in RNA quantification\(^14\). We have recently developed an optimized methodology for RNA extraction from formalin fixed, paraffin-embedded lymphoid tissues\(^15\). Applicability of this new methodology in DLBCL patients is being currently investigated in ongoing studies and preliminary studies will be presented.

**Identification of therapeutic targets**

In addition, it is important to recognize that the usefulness of prognostic factors or models may depend on the specific clinical setting and therapeutic approach. Almost all of the previous studies were performed in newly diagnosed DLBCL patients before initiation of anthracyclin-based regimens in the pre-rituximab era. Improved survival
with the addition of rituximab to chemotherapy might be associated with a change in the predictive value of clinical and/or biological markers resulting in the loss of prognostic power of previously established markers or the discovery of new, previously unidentified predictors\textsuperscript{18,19}. Therefore, the predictive value of the previously established risk factors needs to be re-evaluated and new factors identified for patients treated with R-chemotherapy.

Array studies may also be used to discover genes of clinical relevance, among the multiple expressed sequence tags (ESTs) present on the arrays that play a role in determining prognosis and in the pathophysiology of lymphoma. An example of such discovery- cloning and identification of function of HGAL gene will be presented.

Identification of crucial signaling pathways for lymphoma cells may provide further insight into the mechanisms of lymphomagenesis and detect potential targets for gene-specific therapeutic developments. Hierarchical clustering of global gene expression has demonstrated that groups of genes abnormally activated or suppressed in the same pathway generate recognizable aberrant expression patterns. Using these distinct patterns, it is possible to generate hypotheses about the activity of signaling pathways in lymphoma cells, which require further direct experimental verification.

High levels of expression of NF-κB target genes have been observed in ABC-like DLBCL but not in GC-like DLBCL samples. The NF-κB family comprises 5 members (p50, p52, p65. c-rel and RelB) that form homo- and heterodimers and function as transcriptional factors. The NF-κB family members mediate variety of proliferation, apoptosis, inflammatory and immune responses and are critical for normal B-cell development and survival through a characteristic set of inducible genes. In most cells, NF-κB is retained in an inactive form in the cytoplasm, by binding to members of the IκB family of proteins. In response to signaling through diverse pathways, members of IκB family are phosphorylated by IκB kinase complex (IKK) and subsequently degraded by the ubiquitin-proteasome pathway. This leads to release of NF-κB family members that than translocate into the nucleus and activate transcription. To assess the mechanism and functional significance of NF-κB target genes in ABC-like DLBCL specimens, the activity of IKK was studied in cell line models of ABC-like DLBCL and GC-like DLBCL. The ABC-like DLBCL cell lines that demonstrated high expression of NF-κB target genes had constitutive activity of IKK that was absent in the GC-like DLBCL cell lines. Inhibition of IKK by dominant negative forms of IKKβ was cytotoxic to ABC-like but not to GC-like DLBCL cell lines. DNA content analysis showed that NF-κB inhibition caused both cell death and G1-phase growth arrest. Consequently, this study demonstrated that the NF-κB pathway is a potential therapeutic target in ABC-like DLBCL.

Translating these findings to the bedside, Goy et al. conducted a Phase II trial of bortezomib, a NF-κB inhibitor, in relapsed or refractory lymphoma. Of 12 patients with DLBCL, only one had a response. The lymphomas were not chosen based on their gene array subtype. A larger trial conducted by Cornell University in which patients with DLBCL are treated with R-CHOP with bortezomib will be presented.
PDE4B is a cyclic AMP (cAMP) phosphodiesterase highly expressed in ABC-like DLBCL and is associated with a poor clinical outcome. By inactivating cAMP PDE4B modulates several signaling pathways and induces cell cycle arrest and apoptosis of B cells. Stimulation of cAMP pathway in GC-like DLBCL, which expresses low levels of PDE4B, was associated with decreased phosphorylation and activity of AKT leading to mitochondrial membrane depolarization, dephosphorylation of BAD and marked apoptosis. In contrast, stimulation of cAMP did not affect the PDE4B high expressing ABC-like DLBCL. These observations suggest that PDE4B inhibitors and agents that target the survival pathway controlled by AKT might be used as potential therapeutic tools.

The findings that at least two markers of the GC-like phenotype, BCL-6 and HGAL, are IL-4 target genes whose expression correlates independently with better OS raised the hypothesis that endogenous or exogenous IL-4 might differently affect DLBCL subtypes. IL-4 is a pleotropic cytokine that regulates lymphocyte differentiation, proliferation and apoptosis. Analysis of DLBCL gene expression data revealed increased expression in GC-like DLBCL of multiple components of the IL-4 pathway suggesting its activation: IL-4Rα, insulin receptor substrate (IRS), phosphatidylinositol 3’-kinase p110 catalytic subunit, and protein kinase C delta. The effects of IL-4 on signaling in GC-like and ABC-like DLBCL were recently evaluated. IL-4 demonstrated qualitatively different effects on ABC-like and GC-like DLBCL cell-lines. In the ABC-like DLBCL, IL-4 induced activation of the AKT pathway, decreased cell proliferation, caused cell cycle arrest and lead to an aberrant and short-lived activation of the STAT6 signaling. In contrast, in the GC-like DLBCL IL-4 induced increase in cell proliferation and normally activated the STAT6 signaling. The differences in the IL-4-induced STAT6 signaling between the GC-like and ABC-like DLBCL stem from different expression profiles of protein phosphatases that regulate STAT6 dephosphorylation. These observations suggest that DLBCL subtypes may respond differently in vivo to the cytokine milieu of the tumor. Manipulation of the different responses of DLBCL subtypes to cytokine stimulation might have therapeutic applications.

Conclusion

Microarrays are powerful tools for discovery and hypothesis generation, allowing researchers to obtain an unbiased survey of gene expression in lymphoma samples. These studies allowed sub-classification of DLBCL into distinct subtypes with different pathogenesis and prognosis. Further, these studies enabled identification of new prognostic biomarkers and models in these tumors. However, the “prime-time” for their incorporation into routine clinical practice has not arrived yet. Continuous research will address the remaining hurdles to allow in the future the routine use of prognostic biomarkers in daily oncology practice. These advances will have significant implications for design of clinical trials, development of new therapeutic approaches and the planning of patients’ treatment.
REFERENCES