The search for novel immunophenotypic markers in aggressive B-cell lymphoma

Yaso Natkunam
Stanford University School of Medicine, Stanford, California

Introduction
Diffuse large B-cell lymphoma (DLBCL), the most common adult non-Hodgkin lymphoma, is well recognized as a heterogeneous disease. Although clinical indicators such as the International Prognostic Index (IPI) are used to define prognostic subgroups of DLBCL, less than half of the patients with this disease are cured by currently available therapy. At present, a consensus approach for predicting DLBCL prognosis and risk-adapted management of this lymphoma has not been achieved. Therefore, identification and characterization of new markers that distinguish different subtypes of DLBCL and better stratify patients into risk groups is highly desirable.

General approaches to finding and validating new immunophenotypic markers
Recent advances in genome-wide discovery tools such as gene expression profiling have vastly increased the number of new markers that can be detected by immunophenotypic methods in tumor tissue. The pathology literature contains hundreds of papers each year that report associations between immunohistologic tumor markers and clinical outcome. However, only a small fraction of these markers have helped improve the crusade of predicting prognosis or modifying therapy. We have previously suggested that a possible explanation for this discrepancy is that most immunohistologic studies try to find a characteristic within the tumor (such as differences in molecular features or protein expression) that correlates with clinical behavior. This exercise has the risk of yielding weak associations (less than two-fold differences) between putative prognostic factors and tumor subtypes that are often difficult to reproduce in subsequent studies. The epidemiological literature is fraught with examples of non-causal, weak associations (which are, however, statistically significant) between risk factors and disease frequency, and illustrates the ensuing confusion. Studies of prognostic markers in DLBCL are a particularly good example of this problem: over 40 molecular markers have been described to predict prognosis in DLBCL, but many have not been confirmed in a second independent cohort of DLBCL patients. Others have yielded conflicting results in subsequent studies: the germinal center-associated marker BCL6 correlates with overall survival in DLBCL patients in some studies but not in others. Other examples include BCL2, Ki-67 and FOXP1 where increased expression in some studies was found to be associated with improved survival whereas other studies found no difference or report the opposite association. Although differences in test platforms, data collection, staining techniques, thresholds used for designating positivity and statistical methodology are often raised as the cause of such discrepancies, and are valid considerations, they disqualify candidate markers from being adopted into routine clinical use. However, markers that have been linked to distinct causal mechanisms (such as a mutagenic agent leading to repeated DNA damage and dysplasia resulting in transformation) provide plausible candidates that are strongly correlated with clinical behavior. Thus, in searching for novel markers predicting outcome, it is important to keep in mind that markers of genuine prognostic value are often those related to an acquired genetic abnormality.
Causal genetic alterations can also raise important questions about the diagnostic criteria and classification of tumors. For example, the presence of ALK-1 expression in a subset of T-cell lymphomas confers a better outcome, and therefore, raises the question of whether ALK-1 expression should be a requisite criterion for the diagnosis of that type of T-cell lymphoma. As causal mechanisms become known, it is foreseeable that revisions in the classification will be necessary to refine diagnostic and prognostic categories that are clinically meaningful.

An important stride in the recognition of markers that impact clinical behavior has been provided by gene expression studies in lymphoma that link the cell of origin of the tumor with its clinical behavior. These studies have provided a compelling argument that lymphoid neoplasia retain at least some features of their cells of origin. For example, altered patterns of gene expression (gene expression signatures) in DLBCL that are associated with origin from either germinal center B-cells (GCB) or activated peripheral blood B-cells (ABC) were shown to confer differences in survival. However, further evaluation of individual markers and the expression of their cognate proteins have also shown that there is significant overlap in their expression in GCB and ABC subtypes, particularly at the protein level. Many newly characterized germinal center markers such as HGAL, LMO2, and JAW1/LRMP are also expressed in a significant number of DLBCL of the ABC subtype raising the possibility that the cell-of-origin-based subdivision, although a compelling framework for conceptualizing lymphoid malignancies, may not accurately predict clinical behavior. These results also raise the important consideration that single or small numbers of immunophenotypic markers may not be sufficient to segregate DLBCL into distinct prognostic groups.

Gene expression profiling studies have also uncovered DLBCL subtypes related to pathogenetic mechanisms: expression signatures specific to B-cell receptor signaling and the cell cycle, mitochondrial function and oxidative phosphorylation, and host inflammatory and immune response, have been defined and found to correlate with clinical outcome. These studies provide a wealth of markers that need testing and validation in DLBCL. One example is provided by the study of Farinha and colleagues which shows that tumor-infiltrating host inflammatory cells modulate clinical behavior in DLBCL.

It can be argued that immunohistologic studies could follow the lead of gene expression studies and be used to screen large numbers of markers on large numbers of lymphoma samples. Although creating a compendium of markers and reagents available for interrogating targets by immunophenotypic means would be a valuable resource (and several such compilations are already available through commercial as well as academic sources), high throughput screening by immunophenotypic techniques for markers would not be a practical or cost-effective approach.

Once a new marker has been identified, reagents to probe its expression in tumor samples are needed. Antibodies directed at markers discovered by gene expression methods are not always available, and therefore, significant amounts of time, effort and resources are needed to generate and amass these reagents. Once an antibody to a particular protein
becomes available, surveying its expression by immunophenotypic methods such as immunohistochemistry, flow cytometry and immunofluorescence microscopy can finally begin. In the characterization of protein expression patterns it is important to keep the following parameters in mind: specificity of the antibody for recognizing its target (Western/immunoblotting), expression in specific cell types (tumor cells versus host inflammatory cells, stroma), cellular localization (nuclear, cytoplasmic, cell membrane), tissue distribution (non-hematopoietic versus hematopoietic), expression patterns in normal hematopoietic tissue and cell types (tonsil, lymph node, thymus, spleen, bone marrow), expression in neoplastic hematopoietic tumors (lymphoma subtypes, leukemia, etc), and expression in special niches (intraepithelial lymphocytes, tumor vasculature, etc). In the past several years the use of tissue microarrays (TMA) for high-throughput characterization of protein expression has been in vogue and will be discussed in further detail.

The study of prognostic markers should also take into consideration the advances in treatment strategies that may render its use obsolete. For example, the addition of the anti-CD20 monoclonal antibody rituximab to anthracycline-based chemotherapy (R-CHOP) was recently shown to improve the survival of DLBCL patients. Additional studies have shown that the expression of BCL2 and BCL6 proteins no longer impact prognosis in DLBCL patients treated with R-CHOP. The clinical applicability of a prognostic factor may depend on a specific therapy and the pathway it impedes, and therefore, its usefulness should be reassessed when therapies change.

Under certain circumstances, immunophenotypic methods may not be sensitive enough for the detection of subtle differences in the expression of receptors and signaling molecules. Newly emerging technologies such as phosphospecific reagents for flow cytometry, proteomics and automated imaging and quantitation promise more sophisticated tools in the pathologist’s armamentarium to finesse diagnostic and prognostic capability in the future.

An overview of the Stanford tissue microarray database

Tissue microarrays (TMA) are a highly efficient method for studying protein and RNA expression, enabling rapid survey of hundreds of patient samples in a single experiment. A TMA is constructed from 0.6 – 2.0 mm cores taken from paraffin blocks of patient tumor samples that are then incorporated into a recipient paraffin block using a tissue arrayer. Its use enables simultaneous probing of hundreds of human samples by either antibodies to detect protein expression or in situ hybridization to detect gene expression.

The Stanford Tissue Microarray Database (TMAD) is a web-based resource that is freely available to the public (http://tma.stanford.edu), and provides investigators with tools to design, annotate, score and archive TMA data. Its main objective is to disseminate annotated high resolution light and fluorescence microscopic images of tissue cores with associated protein and RNA expression data such that collaborators worldwide can retrieve, share and analyze information of interest. To accommodate high throughput data from TMAs, TMAD offers a robust system by integrating commercially available hardware and software together with custom-designed new software tools from gene microarray analysis platforms that have been adapted for
analyzing protein and RNA expression data from TMAs. The output from these programs is in a format that is amenable for statistical analysis. The BLISS microscope-imaging system (Bacus Laboratories, Inc., Slide Scanner (BLISS), Lombard, IL) or an Ariol brightfield/fluorescence microscope (Applied Imaging, Hampshire, UK) is used for digital image collection and storage and allows for easy access of images for comparisons to be made across multiple stains and TMAs. As of July 2007, the Stanford TMAD contains over 200,000 digitized images generated from approximately 1500 stained slides of TMAs. Pathologists can access and review raw images of stained cores from a variety of tissue types. TMAD also incorporates the NCI thesaurus of oncology such that specific search parameters can be readily used to access cancer tissues or diagnoses. The high-resolution images provide excellent cytologic detail and subcellular localization of the probe for subsequent scoring and analysis by pathologists from remote locations, and provide a valuable tool for training, classification and standardization of data obtained from immunohistologic and in situ hybridization methodologies. This comprehensive system also allows for interpretative data to be accrued on an on-going basis such that staining results of novel markers can be analyzed and incorporated as they become available. Additional advantages include the ease of importing images and metadata such that the transport of samples and slides and the requisite permissions required for human tissue research is avoided. We have also created a novel method to make TMAs from suspension cells such that low numbers of cells from bone marrow and fine needle aspirates and cultured cells can be subjected to large-scale protein expression studies. Some TMA-based resources of interest are listed in Table 1.

Table 1: Tissue microarray-based resources

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<th>Resource</th>
<th>Description</th>
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<tr>
<td>The Stanford Tissue Microarray Database (TMAD) <a href="http://tma.stanford.edu">http://tma.stanford.edu</a></td>
<td>Information on standard probes as well as novel and emerging markers.</td>
<td>26</td>
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<tr>
<td>The Human Protein Atlas project <a href="http://www.proteinatlas.org/">http://www.proteinatlas.org/</a></td>
<td>Information on 48 normal human tissues and 20 cancers. Includes over 400,000 images corresponding to over 700 antibodies.</td>
<td>31</td>
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<tr>
<td>The Nordic Immunohistochemical Quality Control organization <a href="http://www.nordiqc.org">http://www.nordiqc.org</a></td>
<td>Stained images of thousands of clinically important protein targets. Participation from &gt;100 laboratories. In-depth information on antibodies and protocols</td>
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Novel markers in the stratification of prognostic subtypes in diffuse large B-cell lymphoma

Predictive models of survival in DLBCL using clinical outcome data to supervise the discovery of genes, resulted in the identification of non-overlapping candidate genes by several groups. These differences, attributed to differences in the types of gene arrays and the analysis platforms used in the investigations, underscore the complexity of outcome prediction in an extremely heterogeneous disease. Gene expression profiling studies require fresh tissue, are technically difficult and expensive; these considerations limit their widespread use. Therefore, considerable effort has focused on the analysis of protein expression of select markers by immunohistologic studies to define protein expression profiles that better identify risk groups. Protein expression studies in DLBCL, however, have also yielded conflicting results. In a study of 128 patients with de novo DLBCL, differentiation profiles were found to be associated with particular clinicopathological features but were not predictive of outcome with the exception of BCL2 which maintained predictive power. Another study of 177 nodal DLBCL concludes that sequential addition of BCL2 expression and GC phenotype (defined by expression of BCL6 and CD10 proteins) into the IPI, significantly improves risk stratification in DLBCL. Saez and colleagues report improvement of the predictive power of the IPI when combined with a biological score derived from immunohistologic analysis of 52 markers in 152 patients with DLBCL allowing stratification into different risk categories. A study comparing immunohistochemical data from 142 DLBCL previously analyzed by gene expression profiling found that BCL6 or CD10 protein expression conferred a superior survival similar to data from cDNA microarrays. Hans and colleagues proposed an immunohistologic algorithm based on the expression of three proteins – CD10, BCL6 and MUM1 – for determination of GCB and ABC subtypes of DLBCL. In comparison to gene expression profiling, this model was able to accurately classify 80% of DLBCL cases into their respective ‘cell-of-origin’ category. The high rate of misclassification suggested that additional markers are necessary to improve the robustness of this algorithm. We recently showed that in an independent cohort of 181 DLBCL patients treated with CHOP chemotherapy, this algorithm does not predict outcome. In addition, this algorithm does not impact the outcome of patients treated with R-CHOP, which is the new standard therapy for DLBCL.

Several newly identified GC markers lack commercially available paraffin-reactive antibodies. Thus, there is a need for antibodies to be developed such that novel markers can be characterized in routine biopsy samples of lymphoma. LMO2 was shown to be the strongest single predictor of superior outcome in DLBCL patients in a multivariate model based on the expression of six genes. This gene was of relevance in lymphoid and myeloid leukemias resulting from the deregulated expression of LMO2 as caused by chromosomal translocations and insertional mutations. LMO2 was also shown to be over-expressed in the GCB subtype of DLBCL in gene expression studies. To study its expression in tissue, we generated a monoclonal anti-LMO2 antibody and showed that LMO2 protein is expressed in GC-derived B-cell lymphomas, normal human bone marrow hematopoietic lineages and in leukemias. To date, no acquired genetic abnormalities are known that account for the over-expression of LMO2 in DLBCL; its expression is likely a reflection of the cell of origin or may be associated with a specific
function of LMO2 that is unknown. The prognostic value of LMO2 protein expression was investigated in TMAs containing diagnostic biopsies from 263 DLBCL patients who were treated with CHOP-like regimens, and was found to correlate with improved outcome in an IPI-independent manner. In addition, LMO2 protein expression was tested in 80 DLBCL patients treated with R-CHOP and was found to correlate with improved survival in that cohort. Among the markers tested (LMO2, BCL6, CD10 and MUM1) in R-CHOP patients, LMO2 was the only marker that remained predictive of overall and event-free survival which is indicative of its utility as a prognostic marker in the immunochemotherapy era.\textsuperscript{35}

**Immunoprofiling for risk prediction in aggressive B-cell lymphoma in the immunochemotherapy era**

As previously mentioned, multiple studies have shown that the addition of rituximab to CHOP chemotherapy improves the overall and event-free survival in all age groups of patients with DLBCL.\textsuperscript{16-19} The specific mechanism of action of rituximab is unknown, and therefore, whether rituximab therapy has biological specificity for subtypes of DLBCL or other lymphomas is as yet unclear. Studies have also shown that the adverse prognostic effect of BCL2\textsuperscript{20,21} and the favorable prognostic impact of BCL6\textsuperscript{5} on survival is abrogated in patients treated with R-CHOP. In addition, the ‘cell of origin’ determination by the immunophenotypic algorithm was not found to be predictive of clinical outcome in R-CHOP-treated patients.\textsuperscript{35,41} From studies in R-CHOP-treated patients it appears that patients with BCL2-negative (and perhaps BCL6-positive) DLBCL derive the greatest benefit from rituximab therapy. Whether this result is due to a specific effect of rituximab on pathways active in non-GCB lymphomas is currently unknown and requires further investigation.

Efforts to generate new reagents and characterize markers of potential diagnostic and prognostic impact are necessary for the success of novel targeted and patient-specific therapy development. Multi-institutional initiatives to bring together large groups of uniformly treated and clinically well-characterized patient samples are vitally needed and are imperative for validating prognostic markers that can improve clinical management.

**Conclusions**

Recent technologic advances have provided unprecedented opportunities for biomarker discovery. It is clear that the challenge is not the want of markers but the inability to keep up with confirmation and validation studies that facilitate swift translation of new knowledge into clinical practice. A few key guidelines should be followed in the selection of markers and TMAs have proved to be effective tools for studying protein expression in routine biopsies. Methodology should be standardized, robust and amenable for widespread clinical use. Validating a new marker in at least one other independent study should become a benchmark before it is incorporated into clinical studies. Given the heterogeneity of DLBCL it is important to consider using a broad enough panel of markers to best capture disease diversity. Changing therapies will bring with it new challenges as will the inevitable difficulties of designing clinical trials as patient-specific treatment strategies are increasingly chosen.
References


