New strategies in diffuse large B-cell lymphoma: Translating findings from gene expression analyses into clinical practice

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Abstract

Gene expression profiling has had a major impact on our understanding of the biology and heterogeneity of diffuse large B-cell lymphoma. Using this technology, biological subgroups of DLBCL can be identified that have unique targets for rational therapeutic intervention. This review summarizes these potential targets, and updates the progress of clinical development of exciting novel agents for the treatment of diffuse large B-cell lymphoma. Based upon these ongoing studies, it is likely that in the near future, we will utilize gene expression profiling, or an accurate surrogate, to define the best therapeutic approach for an individual patient with DLBCL.
Background
Diffuse large B-cell non-Hodgkin lymphoma (DLBCL) is the most commonly occurring lymphoma in the United States.(1) With standard chemotherapy, DLBCL, even when it presents in advanced stages, is a curable disease.(2) Over the past decade, significant survival improvements have been reported in the treatment of DLBCL, due largely to the routine incorporation of rituximab in standard doxorubicin-containing chemotherapy regimens.(3, 4) In a population based registry from Vancouver, British Columbia, the observed improvement in outcome following the introduction of rituximab was substantial: the 2-year progression-free survival for patients with DLBCL increased from 51% to 69% in the postrituximab era. Similarly, the 2-year overall survival estimate increased from 52% to 78% in the postrituximab era in this registry.(5) Therefore, despite improvements observed with rituximab, approximately one third of patients with advanced stage DLBCL are still refractory to therapy or will ultimately relapse, and the vast majority of patients with relapsed disease die of lymphoma. Therefore, novel therapeutic approaches beyond rituximab are needed.

Historically, clinicians and investigators have relied on prognostic schemes incorporating clinical risk factors to predict patients with DLBCL who are at high risk of disease progression, relapse, and death. In the 1990’s, the international prognostic index (IPI) for lymphomas was developed, and has remained the most robust clinical prognostic index for aggressive lymphomas since.(6) In this pooled analysis, adults with aggressive non-Hodgkin's lymphoma (mainly DLBCL) who were treated with doxorubicin containing chemotherapy were evaluated for clinical features predictive of outcome. The derived model had 5 features: age, tumor stage, serum lactate dehydrogenase concentration, performance status, and number of extranodal disease sites, and identified four risk groups with predicted five-year overall survival rates of 73 percent, 51 percent, 43 percent, and 26 percent. Although the IPI was derived from data before routine use of rituximab, a recent analysis of German clinical trials in the R-CHOP era confirms that the IPI remained prognostic in determining event-free, progression-free, and overall survival, and that the high risk group still has a relatively poor outcome with 3 year progression-free survival of 55% and 3 year overall survival of 59%.(7) These findings emphasize the heterogeneous clinical behavior of DLBCL in the rituximab era.
Gene expression profiling is a powerful genomics technique which utilizes DNA microarrays to measure the expression of thousands of genes simultaneously, resulting in a molecular profile of RNA in biopsy specimen. Using nonsupervised (“pattern recognition” algorithm) gene expression profiling comparing DLBCL samples to potential normal counterparts, cell of origin studies from the leukemia/lymphoma molecular profiling project indicate that at least three distinct subtypes of DLBCL exist: activated B-cell, germinal center B-cell and primary mediastinal. Two common oncogenic events in diffuse large-B-cell lymphoma, bcl-2 translocation and c-rel amplification, were detected only in the germinal-center B-cell-like subgroup. Activation of the nuclear factor-κB signaling pathway is a key feature of the activated B-cell–like subgroup but not the other subgroups. Many cases of primary mediastinal lymphoma contain a highly expressed gene fusion involving the major histocompatibility complex (MHC) class II transactivator CIITA, which impacts survival in this subtype of DLBCL. In further support of the concept that these subtypes are really different diseases, the activated B-cell lymphomas have inferior prognosis, which appears to be an even more powerful predictor of outcome than the IPI, in patients treated with CHOP-like regimens.

Gene expression profiling also provides prognostic information in the rituximab era. Currently, an intergroup trial coordinated by CALGB is randomizing patients to R-CHOP vs. R-EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin) and has incorporated gene expression analysis to validate outcome differences between germinal center and activated B-cell DLBCL. In an updated analysis from the aforementioned lymphoma/leukemia molecular profiling project, which included tumor biopsies from over two hundred patients with DLBCL treated with R-CHOP, a multivariate model demonstrated the importance of the microenvironment on outcome. The prognostically-favorable “stromal-1” signature reflected extracellular matrix and histiocytic infiltration. The less favorable “stromal-2” signature appears to be an angiogenic switch, in which development of lymphoma is accompanied by angiogenesis, perhaps induced in part by surrounding macrophages.

Shipp and colleagues have also reported a gene expression analysis in diagnostic tumor specimens for patients with DLBCL treated with CHOP-based therapy. They applied...
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a supervised learning prediction method to identify cured patients from patients with fatal or refractory disease. In this series, genes implicated in poor DLBCL outcome (fatal/refractory) included *NOR1, PDE4B* and *PKC-beta*, which regulate apoptotic responses to antigen receptor engagement.

Taken together, these gene expression studies have revolutionized the way we think about this most common adult lymphoma. Observed clinical heterogeneity can now be explained by dramatic differences in biology, despite a similar morphological appearance. These differences not only provide robust prognostic information, but have yielded several important novel targets for therapeutic intervention.(15) The remainder of this review discusses clinical progress of some of these targeted approaches, and looks forward to an era where specific subgroups of patients with DLBCL are approached with rationally targeted therapy.

**On the Horizon**

*Development of novel agents targeting findings from gene expression analysis*

**Nuclear factor kappa-B:** As previously mentioned, constitutive activation of the nuclear factor kappa-b (NFκB) pathway has been observed in the activated B-cell subset of DLBCL, which appears to confer an inferior prognosis. There are several inhibitors of NFκB and IκBalpha under development, with a goal of impacting this group of patients. Bortezomib is a proteasome inhibitor, which blocks degradation of phosphorylated IκBalpha, subsequently inhibiting NFκB activity. This agent is approved for treatment of multiple myeloma, and relapsed/refractory mantle cell non-Hodgkin lymphoma, and patients with elevated NF-kappaB measured by immunohistochemistry of mantle cell lymphoma had a trend toward better outcome when treated with this agent.(16, 17) As a single agent bortezomib confers very limited activity in DLBCL.(18) However, Dunleavy and colleagues have conducted an intriguing trial where patients with relapsed DLBCL were initially treated with bortezomib alone, followed by a combination of bortezomib and doxorubicin-containing chemotherapy (R-EPOCH).(19) In this study, single agent bortezomib also had no activity in DLBCL, but when combined with chemotherapy, it demonstrated a significantly higher response (83% vs 13%; P < .001) and median overall survival (10.8 vs 3.4 months; P = .003) in activated B-cell subtypes of DLBCL compared with germinal center subtype, respectively, as determined by gene expression
analysis. As the NF-κB pathway is a potent inhibitor of apoptosis induced by chemotherapeutic agents, it appeared in this trial that adding bortezomib to chemotherapy had synergy in the activated B-cell subgroup.

Additional studies have confirmed the safety and potential efficacy of combining bortezomib with R-CHOP chemotherapy. In a phase 2 study of R-CHOP and bortezomib, the overall response rate was 88%, and the 2-year progression-free survival was 64%. No differences were noted in outcome between germinal center and non-germinal center subtypes of DLBCL using immunohistochemistry to define the gene expression profile. Given these promising preliminary results, a randomized phase II trial is ongoing comparing standard R-CHOP to R-CHOP with bortezomib in patients with non germinal center DLBCL. This represents the largest study in de novo DLBCL limiting enrollment to a particular subtype defined by gene expression data.

Additional strategies are in development that may improve upon these results. For example, combinations of bortezomib, and other proteasome inhibitors such as carfilzomib, with histone deacetylase inhibitors appear to have particular promise in the laboratory against DLBCL of activated B-cell subtype, and currently are in phase I trials. In addition, small molecule Bcl-2 antagonists appear to promote bortezomib-mediated mitochondrial injury and lethality in DLBCL cells of both germinal center and activated B-cell subtypes in vitro, and could represent a future rational therapeutic combination.

**BCR signaling:** The B-cell receptor is present on both normal and most malignant B-cells, including DLBCL. Engagement of the B-cell receptor (BCR) provides important survival signals, and interruption of the B-cell survival signal can lead to B-cell death. Elegant studies performed with small interfering RNA to inhibit BCR expression have shown that constitutive signaling by BCR is critical for survival and proliferation of human B cell lymphomas. The primary role of BCR signaling in these cells appeared to be activation of spleen tyrosine kinase (Syk), which in turn leads to several downstream events promoting cell survival, including activation of Bruton's tyrosine kinase, phosphatidylinositol 3 kinase (PI-3K) and Akt, as shown in figure 1. A subset of DLBCL which can be identified using gene expression analysis which appears particularly dependent upon BCR survival signals, even in the absence of antigen engagement, referred to as tonic signaling. Pharmacological inhibition of
syk has demonstrated activity against this “BCR-dependent” subset of DLBCL in vitro(29). Additional rationale for inhibiting the BCR signal as a therapeutic modality comes from the data that activated B-cell type of lymphoma utilizes the signaling adaptor CARD11 for constitutive NF-kappaB pathway activity and survival. (30) Moreover, approximately one fifth of activated B-cell DLBCL have a mutated residue of CD79B which results in increased surface BCR expression and attenuated Lyn kinase, a feedback inhibitor of BCR signaling. This pathway, therefore, appears to have particular importance in subsets of DLBCL.

Fostamatinib disodium is an orally available Syk inhibitor under development for rheumatoid arthritis.(31) A phase I/II trial of this agent in a variety of lymphoma subtypes has been completed, and clinical responses were seen in over 20% of heavily pretreated, refractory DLBCL patients.(32) Gene expression analyses were not performed in this trial, but in the preclinical evaluation of syk inhibition, only the subset DLBCL cell lines and primary tumors with absent surface IgG or IgM or lower levels of cell-surface IgG had ineffective BCR signaling, and did not respond to this agent. Therefore, in the future, it may be possible to utilize IgM or IgG expression as a surrogate of BCR dependency in choosing patients for this therapeutic approach.

Other potential targets downstream of the BCR include Bruton’s tyrosine kinase (BTK) and PI3kinase. PCI 32765 is an orally available inhibitor of BTK, and a phase I study in various lymphoma subtypes has recently been completed. Twenty nine percent of patients with DLBCL have responded to treatment with this agent at various dose levels, and phase II trials of single agent, as well as combinations with rituximab and chemotherapy, are planned.(33) Gene expression analysis was not utilized to evaluate patients in this trial. CAL-101 is an inhibitor of phosphatidylinositol 3 kinase, also orally available and under development for various lymphoma subtypes.(34) Although there are no responses yet in DLBCL, preclinical studies of combinations involving this drug appear promising, and it is likely the same subset of patients responding to inhibition of Syk and BTK may be sensitive to this agent as well.(35) Given the low toxicity profile suggesting ease of combining with standard chemotherapy, as well as early single agent efficacy signals, members of this pathway represent an extremely promising target in DLBCL, and combinations of these agents may yield the best results in appropriate subsets of patients defined by gene expression analyses.(36)
**Protein kinase C beta:** As previously mentioned, gene expression analysis has suggested that the protein kinase C beta (PKC) gene is almost uniquely overexpressed in fatal/refractory DLBCL compared with cured DLBCL. PKCβ expression was also associated with poor outcome and shortened survival in a large independent series of primary DLBCL patient samples. PKCβ is downstream of multiple signaling pathways, including the BCR and NFκB as shown in figure 1. PKCβ appears to also have an effect on tumor angiogenesis through vascular endothelial growth factor signaling, which was associated with the poor prognosis stromal-2 gene expression signature. Therefore, inhibition of PKCβ has the potential to favorably impact multiple subgroups of high risk patients defined by gene expression profiling. Enzastaurin is a selective inhibitor of PKCβ, which has significant activity against DLBCL in vitro. A phase II study of single agent enzastaurin in relapsed DLBCL has been completed, which demonstrated a subset of patients who responded, and enjoyed very prolonged progression-free survival. Importantly, there was minimal toxicity even in the setting of prolonged exposure to the drug. No gene expression studies were performed as part of this study, but immunohistochemistry studies did suggest that PKCβ was expressed in virtually all patients on study. It was suggested that future studies incorporate an evaluation of PKCβ activity, as well as PKCβ protein expression as correlative studies in patients treated with enzastaurin. Based on these favorable results, a randomized phase III trial comparing enzastaurin to placebo as first remission maintenance treatment in high clinical risk DLBCL has been completed and is awaiting analysis. In addition, preliminary results from a randomized phase II study comparing R-CHOP to R-CHOP with enzastaurin were recently presented. There was a suggestion in improvement in progression-free survival in high clinical risk subgroup of patients treated with enzastaurin. Preclinical studies have suggested activity of combining enzastaurin with other targeted therapies, including bortezomib. It is hoped that adequate tissue samples will be available from these and future clinical studies to determine whether a high risk subgroup defined by gene expression profiling, rather than the IPI, may selectively benefit from this promising agent.

**Angiogenesis and the microenvironment:** As previously mentioned, gene expression analysis in tumor biopsies from patients treated with rituximab and chemotherapy has revealed the importance of the microenvironment. In particular, the “stromal-2” signature suggests that the presence of tumor-associated angiogenesis and macrophages
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DLBCL gene expression profiling

portends a poor prognosis. Additional data in support of this finding includes the observation that a high pretreatment serum vascular endothelial growth factor (VEGF) concentration is associated with poor outcome in non-Hodgkin's lymphoma, even when R-CHOP is utilized as initial therapy. (43, 44)  Bevacizumab is a recombinant, humanized, monoclonal antibody that recognizes all isoforms of VEGF. The Southwest Oncology Group (SWOG) conducted a trial of single agent bevacizumab in relapsed/refractory DLBCL. Although there was limited single agent activity, baseline urine VEGF and plasma vascular cell adhesion molecule-1 (VCAM) levels correlated with survival, and a small number of patients had prolonged stability of disease. (45)  It is not known whether or not these patients had the stromal 2 signature. A small phase 1 trial of bevacizumab with R-CHOP suggested safety, and a marginal positive correlation of VEGF level and response. (46)  Based upon these results, the SWOG subsequently performed a multicenter phase 2 trial of R-CHOP with bevacizumab in patients with newly diagnosed DLBCL. The study did not meet its prespecified endpoint, and there appeared to be increased toxicity from bevacizumab (cardiac dysfunction and gastrointestinal perforation) compared with historical controls of patients treated with R-CHOP alone. (47)  A subsequent phase 3 trial comparing R-CHOP to R-CHOP with bevacizumab was also stopped prematurely for toxicity concerns. Gene expression profiling was not incorporated in either of these studies.

A more promising agent that may favorably impact the microenvironment and target angiogenesis in DLBCL is lenalidomide. This immunomodulatory agent has pleiotropic effects in the treatment of lymphoma, including antiangiogenic effects. (48)  A phase II trial of lenalidomide monotherapy in relapsed/refractory aggressive lymphomas (more than half of patients had DLBCL) demonstrated significant clinical activity in a subset of patients with an overall response rate of 35% and minimal non hematological toxicities. (49)  No gene expression profiling was performed in this trial, but a subsequent study which retrospectively evaluated clinical outcomes of patients with germinal center B-cell-like versus nongerminal center B-cell-like DLBCL treated with salvage lenalidomide at four academic institutions has been published. (50)  Interestingly, in this retrospective analysis, a significant difference in clinical response to lenalidomide was observed in nongerminal center B-cell-like (52% response rate) versus germinal center B-cell-like (9% response rate) patients. Numerous trials are exploring lenalidomide in combination with R-CHOP therapy as upfront treatment, and as a single agent or with rituximab as maintenance treatment for patients with newly diagnosed DLBCL. For
example, a phase I/II study has determined that lenalidomide can be safely given at a
dose of 25 mg daily on days 1-10 of R-CHOP therapy, with high complete response
rates observed.(51) Hopefully, these trials will incorporate analysis of gene expression-
defined subgroups of patients, to determine the impact of this therapy on subsets of
DLBCL.

Conclusions
DLBCL remains a highly curable disease, but a substantial number of patients still
succumb. Recent biological insights from gene expression studies have confirmed that
biological heterogeneity explains this highly variable clinical outcome. More importantly,
using this promising technology, subgroups of patients can be identified who may benefit
from specific novel rationally targeted therapies (table 1). It is imperative that future
clinical trials in this disease utilize this technology as correlative studies to define these
subgroups of patients, and the impact of novel treatment approaches. Although
immunohistochemistry algorithms exist which have some correlation to gene expression
profiling, at the present time all of these algorithms are imperfect, and in the setting of
discovery it is necessary to utilize gene expression profiling to accurately determine
DLBCL subtype.(52) Evolving technology will soon allow these assays to be performed
on routinely obtained paraffin embedded tissues at reasonable cost.(53, 54) Particularly
in an age of novel therapies that have intriguing signals of activity, I expect we will utilize
gene expression profiling, or an accurate surrogate, to define the best therapeutic
approach for an individual patient in the clinic in the near future.

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Table 1: Novel rationally targeted agents for DLBCL derived from gene expression analyses

<table>
<thead>
<tr>
<th>Drug</th>
<th>Gene expression subgroup (ref)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib</td>
<td>ABC (9)</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>Enzastaurin</td>
<td>Fatal/refractory (14) ABC (9)</td>
<td>Protein kinase C beta</td>
</tr>
<tr>
<td></td>
<td>ABC (9)</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>Stromal 2 (13)</td>
<td>VEGF; angiogenesis</td>
</tr>
<tr>
<td>Lenalidomide</td>
<td>Stromal 2 (13) ABC (9)</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td></td>
<td>ABC (9)</td>
<td>Pleotropic targets</td>
</tr>
<tr>
<td>Fostamatinib</td>
<td>BCR-dependent (28) ABC (9)</td>
<td>Syk; BCR signaling</td>
</tr>
<tr>
<td>PCI 32765</td>
<td>BCR-dependent (28) ABC (9)</td>
<td>BTK; BCR signaling</td>
</tr>
</tbody>
</table>

Abbreviations: ABC: Activated B-cell; BCR: B-cell receptor; Syk: spleen tyrosine kinase; BTK: Bruton's tyrosine kinase.
Figure 1: B-cell receptor (BCR) pathway members and Protein Kinase C as potential targets in treatment of DLBCL. Abbreviations: Syk: spleen tyrosine kinase; BTK: Bruton’s tyrosine kinase; PI3K: phosphatidylinositol 3 kinase; PKC: protein kinase C beta; GSK3: glycogen synthase kinase.
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