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**New approaches to diagnosing and classifying
aggressive B-cell lymphomas**

Moderators

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Opening remarks and tribute to David Mason

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**Insights from expression arrays and genomic profiling of aggressive B-cell
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Panel discussion:

**What testing should we doing in diagnosis and stratification of aggressive B-cell
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Insights from Expression Arrays and Genomic Profiling of Aggressive B-Cell Lymphoma

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Introduction

Non-Hodgkin's lymphomas (NHL) are a heterogeneous group of diseases that are diagnosed and classified according to an integration of morphological, phenotypic, genetic, molecular and clinical features. This multidisciplinary approach has been very successful in recognizing a large number of distinctive entities with different pathogenetic mechanisms, biological behavior and management requirements. However, some categories in the current WHO classification remain biologically and clinically heterogeneous and probably include different entities. In addition, the diagnostic criteria in some lymphomas are not well defined rendering a low reproducibility even among expert pathologists.¹ Paradigmatic examples of these situations are B-cell lymphomas with aggressive behavior such as diffuse large B-cell lymphomas (DLBCL) and the tumors with overlapping features between DLBCL and Burkitt lymphoma (BL) that have been recognized with terms such as Burkitt-like lymphoma, atypical Burkitt, small non-cleaved cell lymphoma, and DLBCL with high grade features among others.²

Gene expression profiling using DNA microarrays represents a major technological advance that is increasing our knowledge of neoplastic disorders and particularly malignant lymphomas. Different platforms have been developed, including cDNA and oligonucleotides of short or longer length, that require slightly different technical approaches but the information generated is relatively similar and robust. The systematic application of this technology in the study of malignant lymphomas is providing relevant information on the expression signatures of different lymphomas helping to refine diagnostic criteria and identify potential new entities and tumor subtypes. Gene expression studies have been also very successful in defining strong predictors of survival relevant to stratify patients according to their biological risk and develop more tailored therapeutic strategies. Analysis of these expression profiles is also generating new insights into the molecular pathogenetic mechanisms of the tumors that may allow the identification of new therapeutic targets. Recognition of these signatures may improve our current schemes of lymphoma classification and provide new information that may benefit the management of the patients.

Diffuse large B-cell lymphoma

Identification of new molecular subtypes: Gene expression profiling has identified three major subgroups of DLBCL, termed germinal center B cell-like diffuse large B cell lymphoma (GCB DLBCL), activated B cell-like diffuse large B cell lymphoma (ABC DLBCL), and primary mediastinal DLBCL (PMBCL) (Table 1).³⁻⁶ The GCB tumors are characterized by the expression of genes related to germinal center cells including CD10,

bcl-6, HGAL, and centerin whereas ABC tumors have an expression pattern related to mitogenically activated B-cells close to cells with a secretory function including genes such as IRF4, and XBP1. Interestingly, ABC but not GCB DLBCL carry inactivating mutations of PRDM1/BLIM1 that functions as a mediator of plasmacytic differentiation.^{7,8} ABC and PMBCL but not GCB DLBCL have a constitutive activation of the NF-κB pathway that they require for survival and therefore may be an interesting target for therapy.^{4,9} The activation of the NFκB pathway in ABC tumors seems to occur through CARD11 signaling, an element that interacts with MALT1 and BCL10.⁹ A subset of ABC DLBCL seems to have an activation of the STAT3 pathway that may depend on JAK2 signaling since inhibition of this molecule induces blocks STAT3 function and induces apoptosis suggesting that this pathway may also be a potential therapeutic target in these tumors.¹⁰

Although PMBCL was not recognized in the initial microarray studies of DLBCL further investigations clearly defined a distinctive gene signature of this lymphoma. These tumors overexpress previously identified genes such as FIG1 and MAL but also a series of genes related to NFκB activation, Il13 receptors, JAK and PDL2 among others. Interestingly, PMBCL share a common group of genes with Hodgkin lymphoma cell lines raising the possibility of a common cell of origin of pathway of development.^{5,6}

Table 1: Genetic, Molecular and clinical characteristics of the DLBCL molecular variants recognized by expression profiling

Characteristic*	ABC	GCB	PMBCL
Genetic			
t(14;18)	No	35	No
3q gain/amplification	26	0	5
9p gain/amplification	6	0	37
12q12	5	21	5
Molecular			
Ongoing Ig Mutations	No	Yes	No
Bcl-2 rearrangement	0	26	0
Rel amplification	0	17	0
SOCS1 inactivation			45
PRDM1 inactivation	25	no	
NFκB activation	Yes	No	Yes
Clinical			
5-year survival	30%	59%	64%
Disease hallmarks	-	Late relapses	Predominantly Woman < 35 yrs Mediastinal

Numbers represent percentage of cases; ABC: activated B cell-like; GCB: germinal centre B cell-like; PMBCL = primary mediastinal large B-cell lymphoma.

In addition to these molecular differences, the three subtypes of DLBCL differ in genetic aspects (Table 1). Thus GCB DLBCL are characterized by frequent *REL* amplifications, *BCL2* translocations, and ongoing somatic hypermutation of the immunoglobulin genes.^{4,11} In addition, GCB DLBCL are characterized by frequent gains of chromosome 12q whereas ABC DLBCL carry frequent trisomy 3, gains and amplifications of 3q, and 18q21-and losses of 6q21-22. PMBCL recognized by microarrays expression profile have high number of gains and amplification of 9p21-ter and 2p14-16.¹²

The genetic and molecular differences between these subtypes of DLBCL strongly suggest that they correspond to different

clinical entities. In fact, patients with these tumors differ in the clinical outcome with 5-year survival rates of 59%, 30%, and 64% in GCB, ABC DLBCL, and PMBCL patients,

respectively.⁴ In addition, PMBCL occur predominantly in young women and the peculiar clinical phenomenon of late relapses seem to occur mainly in lymphomas with a GC profile.¹³

Other expression profiling studies have identified alternative subgroups of DLBCL characterized by expression signatures related to potential pathogenetic mechanisms. In particular, one subgroup was characterized by high expression of genes associated with oxidative phosphorylation (OxPhos subgroup) and mitochondrial function such as genes of the *BCL2* family. The “BCR/Proliferation” subgroup was enriched for B-cell receptor signalling and cell-cycle regulatory genes whereas the “Host Response” (HR) subgroup had increased expression of genes related to an inflammatory/immune response signature.¹⁴ These three subgroups did not differ in the outcome of the patients but may be of interest for the identification of new therapeutic strategies.

Identification of new prognostic models: Microarray studies in malignant lymphomas are providing new prognostic models that improve the current schemes mainly based on clinical criteria (International Prognostic Index, IPI). Interestingly, the expression signatures associated with prognosis are different for each disease entity, indicating that the biology of each lymphoma is regulated by different mechanisms. Thus, in DLBCL the prognostic model is based on the expression of 4 signatures: the germinal center related expression profile, the lymph node signature, proliferation, and the expression of MHC class II genes. The high expression of the first two signatures is associated with good prognostic whereas a high proliferation and loss of MHC gene expression confer a bad prognosis to the patients. These signatures are combined in a model that allows the classification of the patients in different risk groups with a 5-year survival of more than 70%, 34% and 15%, respectively. This model is independent and improves the IPI stratification.⁴ Interestingly, genetic studies have shown that certain chromosomal aberrations, particularly gains in chromosome 3, are associated with worst prognosis and the combination of these alterations with the expression based model improve the risk prediction in DLBL.¹²

The prognostic parameters in lymphomas are dependent on the management strategies of the patients. Thus, the introduction of Rituximab in the therapeutic protocols of DLBCL has improved significantly the survival of the patients and the new protocols have overcome the predictive value of bcl-2 expression in these tumors.¹⁵ Interestingly, a recent study by the LLMPP consortium has shown that GCB and ABC DLBCL still have different outcome in patients treated with R-CHOP. Similarly, the prognostic signatures maintain their predictive value in this cohort of patients.

Burkitt lymphoma (BL)

BL is a well defined entity characterized by a monotonous proliferation of medium-sized cells with a germinal center phenotype, negative or very weak bcl-2 expression, high proliferation (Ki67>90%) and the common presence of the t(8;14) translocation with c-myc rearrangement. Some cases may have more atypical morphology and some variations in the phenotype or in the genetic features. On the other hand, DLBCL may

have high proliferation and may also carry myc rearrangements that make the differential diagnosis with DLBCL difficult. This distinction is very relevant since the treatment for these lymphomas is different and the cure rate of properly treated BL patients is very high.

Two recent studies using microarray technology have described the gene expression profile of BL that refine the differential diagnosis with DLBCL.^{16,17} This BL signature was composed of a group of genes with high expression of myc targets genes and genes related to germinal center cells and low expression of NFkB target genes and MHC class I genes.¹⁶ Intriguingly, although there was a good correlation between the pathology and molecular diagnosis, the discordances were also important. Thus, 15-30% of the cases expressing the molecular signature of BL (mBL) had been diagnosed by expert pathologists as DLBCL or high-grade B-cell lymphomas. On the contrary, 0.5-4% of the cases without the molecular signature of BL had been called BL and around 3-8% of the cases diagnosed as DLBCL or high-grade lymphoma had a BL signature.¹⁸ The correlation of the molecular signature with the genetic aspects of the tumors revealed that most mBL have a Ig-myc rearrangement with very low grade of genomic complexity whereas cases of DLBCL that carry myc translocations are usually associated with very complex karyotypes. In addition, myc rearrangements with other partners were detected in lymphomas with an intermediate molecular signature between BL and DLBCL. Curiously, both studies identify occasional cases of molecular BL that lacked the rearrangement of myc gene.

The retrospective design of these microarray studies did not allow them to properly address the clinical significance of the molecular diagnosis. However, the data available in both studies highly suggest that tumors with mBL had a clinical presentation and better outcome that differ from the cases with a non-mBL signature and therefore may be useful for the clinical practice.

One important aspect revealed also in both studies is that the molecular distinction between BL and DLBCL in some cases is not very sharp. Hummel et al identified a subset of tumors with an expression signature intermediate between BL and DLBCL. Most of these cases were diagnosed as DLBCL by the pathologists, had bcl-2 expression and a Ki67 lower than 95%. Myc rearrangements were observed in 54% of these cases and in half of them was associated with rearrangements of bcl-2, bcl-6 or both corresponding to the so called “double hit” tumors in the literature.¹⁹ In the LLMPP study, 9 cases that were considered discrepant between the presence of a molecular Burkitt signature and the pathology diagnosis as DLBCL or High grade lymphoma were also peculiar. All of them had the t(8;14) translocation but 3 had an additional bcl-2 rearrangement, and 5 were positive for bcl-2 staining. Interestingly, these cases had a more complex karyotype, presented in older patients and had a worst outcome than cases in which both the molecular and pathology diagnosis were in agreement. These observations suggest that some aggressive lymphomas may have molecular and pathological features intermediate between BL and DLBCL. The 4rd edition of the WHO classification (2008) recognizes this category, not as a specific entity but as a biological heterogeneous category that is useful in allowing the classification of cases not meeting

criteria for classical BL or DLBCL. Probably, these cases may represent transformed FL, progressed DLBCL activating particular molecular pathways and perhaps some specific entity not well recognized yet. However, independently of the possible biological significance, these patients should be recognized and studied separately.

How to transfer genomic knowledge into the clinics?

Gene expression profiling studies in lymphomas is providing important information that has helped to define new molecular subtypes and refine the diagnosis in difficult areas. The recognition of the GCB and ABC DLBCL and the molecular distinction between BL and DLBCL are paradigmatic examples of this new approach. The prognostic models derived for expression profiling analysis stratify very precisely the patients according to their risk based on quantitative models that also reflect the biology of the disease. In addition, the information provided may help to apply more tailored therapies according to the molecular characteristics of the tumors. The new perspectives provided by the increasing number of molecularly oriented therapies will require microarray studies performed in well designed clinical trials to evaluate the performance and response of the patients. Those aspects are very relevant for the future management of the patients. However, the application in the daily practice of the current microarray technology and information is an important challenge that has to find its pathway. Aspects as the complexity of some lymphoid neoplasms and practical issues as the limited amount of material, presence of necrosis or partial involvement by the tumor may be difficult to overcome but similar problems are also faced by conventional pathology. The requirement of fresh samples is a logistic challenge but depending on the relevance for the patient of the information generated it will be necessary to design strategies to obtain this material. This will not be new in the practice of pathology. There were times in which the fresh material for breast cancer or lymphoma immunophenotyping was considered mandatory for treatment and obtained. Major challenges are the interpretation and validation of the many results generated by microarray studies, the design of friendly algorithms to translate into the daily practice this information, and the design of standard and reproducible protocols. Advances in bioinformatics tools will improve the analysis. Microarray technology is already very reproducible and robust. Some of the information generated by these studies may be translated by the use of different platforms such as quantitative PCR, other mRNA detection techniques or immunophenotyping. These approaches may be useful for small number of genes but may not perform well when algorithms using a high number of genes may be needed. In addition, these alternatives also face similar challenges as the microarray technologies such as the need for validation, standardization and reproducibility.^{20,21} All these new perspective and challenges in the new diagnostic era are overwhelming for individual groups and require the collaborative efforts of large consortiums and a high dose of imagination that will result in an improvement for the management of the patients.

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

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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.¹ 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.² 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 studies³⁻⁵ but not in others.^{6,7} 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.² 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.⁸⁻¹⁰ 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.⁸ 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,¹¹ LMO2¹² and JAW1/LRMP¹³ 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.¹⁴ 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.¹⁵

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.¹⁶⁻¹⁹ Additional studies have shown that the expression of BCL2 and BCL6 proteins no longer impact prognosis in DLBCL patients treated with R-CHOP.^{5,20,21} 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,²² proteomics²³ and automated imaging and quantitation²⁴ 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.²⁵ 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.²⁶ 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.^{27,28} 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.²⁶ 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.²⁶ 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.^{29,30} Some TMA-based resources of interest are listed in Table 1.

Table 1: Tissue microarray-based resources

Resource	Description	Ref
The Stanford Tissue Microarray Database (TMAD) http://tma.stanford.edu	Information on standard probes as well as novel and emerging markers.	26
The Human Protein Atlas project http://www.proteinatlas.org/	Information on 48 normal human tissues and 20 cancers. Includes over 400,000 images corresponding to over 700 antibodies.	31
The Nordic Immunohistochemical Quality Control organization http://www.nordiqc.org	Stained images of thousands of clinically important protein targets. Participation from >100 laboratories. In-depth information on antibodies and protocols	32

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.¹ 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.³³ 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.⁶ 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.³⁴ 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.³ 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.³ 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.³⁵ 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.³⁶ 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.³⁷⁻⁴⁰ *LMO2* was also shown to be over-expressed in the GCB subtype of DLBCL in gene expression studies.⁸ 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.¹² 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.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.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*^{5,20,21} and the favorable prognostic impact of *BCL6*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.^{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.

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

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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 States¹. 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)², 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 processes³. 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^{4,5}. Improvement in disease-free and overall survival may be obtained with the addition of monoclonal antibodies, such as rituximab⁵. 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.⁶ 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 Rosenwald⁷ and Shipp groups⁸ 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 LLMPP⁷ and analyzed by Shipp⁸ 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 survival¹⁰ in tumor specimens from 66 DLBCL 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^{11,12} suggesting the lack of an ideal set of IHC markers for outcome prediction in DLBCL. Hans et al, complimented cDNA microarrays with immunohistochemistry (IHC) staining¹³. 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^{14,15}. 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 quantification¹⁶. We have recently developed an optimized methodology for RNA extraction from formalin fixed, paraffin-embedded lymphoid tissues¹⁷. 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^{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- κ B target genes have been observed in ABC-like DLBCL but not in GC-like DLBCL samples. The NF- κ B family comprises 5 members (p50, p52, p65, c-rel and RelB) that form homo- and heterodimers and function as transcriptional factors. The NF- κ B 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- κ B is retained in an inactive form in the cytoplasm, by binding to members of the I- κ B family of proteins. In response to signaling through diverse pathways, members of I- κ B family are phosphorylated by I- κ B kinase complex (IKK) and subsequently degraded by the ubiquitin-proteasome pathway. This leads to release of NF- κ B family members that then translocate into the nucleus and activate transcription. To assess the mechanism and functional significance of NF- κ B 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- κ B 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 IKK β was cytotoxic to ABC-like but not to GC-like DLBCL cell lines. DNA content analysis showed that NF- κ B inhibition caused both cell death and G1-phase growth arrest. Consequently, this study demonstrated that the NF- κ B 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- κ B 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 pleiotropic 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-4R α , 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.

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Tumor and host genetics in diagnosis and risk stratification of aggressive B-cell lymphoma

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Lymphomas are neoplasms caused by clonal proliferation of lymphoid cells. The neoplastic clone acquires survival advantage and escapes normal regulatory mechanism as a consequence of gained genetic changes. These include mutations and translocations affecting the genome of the neoplastic cell and, sometimes, additional genetic material introduced by infectious agents such as viruses. These genetic changes and their phenotypic consequences have been one of the important determinants of current lymphoma classifications and risk stratification in B-cell lymphoma. (1, 2)

Host genetics

In recent years, it has become apparent that the biological properties, including the genetic make-up of the tumor cells, can not fully explain the pathogenesis, clinical behavior and outcome of B-cell lymphoma and host factors also play a role. The evidence for this comes from a number of sources. It has been long recognized that individuals carrying germline mutations in the ataxia telangiectasia, mutated (*ATM*), and Nijmegen breakage syndrome (*NBS1*) genes have highly increased risk of developing lymphoma suggesting other, less well-defined, host genetic polymorphisms may also play a role. Epidemiologic studies suggest that the patients with a family history of lymphoma or other hematological malignancies have an increased risk of developing lymphoma. (3, 4) There is a well established link between the presence of chronic inflammation and infections and development of lymphoma, perhaps best demonstrated by the association between *H. pylori* infection and gastric lymphoma. (5, 6) More recently, gene expression profiling have identified that the signals coming from the microenvironment, therefore belonging to the host rather than to the tumor, not only define the biological features of a neoplasm but also may be better predictors of outcome than the tumor characteristics. (7, 8) Furthermore, there is no doubt that the impact of host factors on clinical outcome will increase as new biological treatment modalities utilizing host immune mechanism to kill the tumor cells are introduced into routine practice.(9)

In line with these observations, the epidemiologists have started looking at ways identifying the host genetic factors that may account for the biological and clinical heterogeneity. This task has been greatly helped by the completion of human genome project. As a result, we now know that human genome contains 2.5 million single nucleotide polymorphisms (SNPs). These are non-randomly distributed and 93% of genes contain at least a SNP. Some SNPs are very common and some have functional and biological consequences. Initial epidemiological studies have focused on identifying genetic polymorphisms associated with increased risk of development of non-Hodgkin lymphoma (NHL) and have shown that polymorphisms involving a number of genes involving different biological pathways may play a development of NHL. Some examples of the pathways reported to be associated with increased risk of development of

NHL include oxidative stress pathway (*NOS2A*, *MPO*), energy regulation (*LEP*, *LEPR*), detoxification genes (*GSTM1*, *GSTT1*), DNA repair and methylation (*RAG1*), one-carbon (folate) metabolism (*CBS*, *FPGS*), hormone production (*CYP17A1*), cytokines and receptors genes (*IL10*, *TNF*, *BLYS*), innate immunity (*TLR4*) and oncogenes (*BCL6*, *Cyclin D1*). (See review by Skibola(10) for details) In particular, given the association between inflammatory/immune response and the development, biology and clinical outcome of NHL, many studies have focused on immune genes such as cytokines and cytokine receptors. One of the largest studies has been performed by the InterLymph Consortium and analyzed 3586 NHL cases, 4018 controls from 7 centers using a PCR based methodology to detect 12 SNPs in 9 genes that have important roles in proinflammatory or anti-inflammatory pathways (*IL1A*, *IL1RN*, *IL1B*, *IL2*, *IL6*, *IL10*, *TNF*, *LTA*, and *CARD15*). (11) The study reported that common polymorphisms in *TNF* and *IL10*, key cytokines for the inflammatory response and Th1/Th2 balance, could be susceptibility loci for non-Hodgkin lymphoma. Similar findings were observed by the study performed by Cerhan and colleagues at Mayo Clinic using high-throughput array based genotyping methodologies. (12) The study analysed 458 patients with NHL and 484 controls and 9412 SNPs from 1253 genes. The genes were selected based on biological criteria. The study indicated that genetic variation in immune response (*TRAF1*, *RIPK3*, *BAT2*, and *TLR6*), *MAPK* signaling (*MAP3K5*, *DUSP2*, and *CREB1*), lymphocyte traffic (*B3GNT3*, *SELPLG*, and *LSP1*), coagulation pathways (*FGG* and *ITGB3*) may be important in the etiology of NHL. Although these findings are yet to be validated by different methodologies and larger cohorts of patients, they strongly suggest a link between host polymorphisms in immune genes and risk of NHL.

Perhaps clinically more important observations have been the association between immune gene polymorphisms and clinical outcome of NHL after treatment. These include *TNF* polymorphisms and diffuse large B-cell lymphoma (DLBCL) (13), HLA Class II and *TNF* polymorphisms and NHL (14), *IL10* polymorphisms and DLBCL (15), *IL10* polymorphisms and T-cell lymphoma(16), cytokine gene polymorphisms (*IL8*, *IL2*, *IL12B*, *IL1RN*) and follicular lymphoma (17). This last study analysed 73 SNPs from 44 immunity and inflammation associated genes in 248 cases of follicular lymphoma with a median follow-up 59 months. The study reported that SNPs in *IL8*, *IL2*, *IL12B*, *IL1RN* were the most robust predictors of survival and a risk score that combined the 4 SNPs with the clinical factors was even more strongly associated with survival. A similar finding was also observed for DLBCL (Habermann, unpublished). These results are very encouraging and suggest that risk stratification of NHL may be significantly improved by identification of high risk host genotypes in addition to tumor and clinical parameters.

Tumor genetics

The most common aggressive B-cell lymphoma is DLBCL and the following discussion will focus on DLBCL. The genetics of tumor cells in DLBCL is often complex and differs significantly from case to case.(18) Nevertheless a number of recurrent genetic abnormalities are seen. Although these are not specific for DLBCL and can be also seen in a number of indolent or aggressive lymphomas, in the context of DLBCL they provide valuable biological information and have potential applications as clinical

risk predictors. The most significant genetic changes include translocations involving *BCL2*, *BCL6* and *MYC* genes, amplifications involving oncogenes such as *BCL2* and *REL* and deletions/mutations involving tumor suppressor genes *p53* and *p16* genes.

The clinical significance of these changes has been mostly investigated in DLBCL patients treated with CHOP before the establishment of RCHOP as the main stay of treatment. As certain adverse risk predictors such as *Bcl2* expression established in the pre-RCHOP, appear not to be applicable in the RCHOP era, the relevance of outcome data for genetic data summarized below as risk predictors remains questionable.

Translocation (14;18), juxtaposing *BCL2* gene next to the *IGH* promoter and leading to *Bcl2* protein expression is seen approximately 10-25% of DLBCL. Several retrospective suggests that t(14;18) translocated cases trend towards a worse prognosis but not every study could demonstrate a statistical significance.(19, 20). Interestingly, most cases of DLBCL carrying the t(14;18) fall within the germinal center B-cell-like (GCB) subgroup as identified by gene expression profiling. (21) In contrast, activated B-cell-like (ABC) DLBCL subgroup mostly lack t(11;18) but show genomic amplification in the 18q21 region which also includes *BCL2* gene. In this group, *BCL2* expression has a significant adverse effect on overall survival which is associated with 18q21 amplification.(22)

Rearrangements of *BCL6* gene are seen in 15-40% of DLBCL. In approximately, half of these cases the translocation partner is identified as *IGH*. In the remaining half, a number of different genes are juxtaposed next to the *BCL6* locus. The clinical significance of *BCL6* rearrangements has not been well-established. Some studies have shown no adverse impact on clinical outcome(23) however others have indicated a worse prognosis in the presence of *BCL6* rearrangement. This association appears to be particularly strong in cases where the rearrangement involves a non-*IGH* partner.(24) *BCL6* rearrangements, in contrast to t(14,18), tend to be seen in cases with ABC phenotype.(21, 25)

MYC gene rearrangements seen in a small percentage of DLBCL (5-10%) but, when present, are predictors of aggressive behavior. (26, 27) This clinical outcome is even worse if *MYC* rearrangement is accompanied by *BCL2* gene rearrangements.(28) Deletions and/or mutations involving the tumor suppressor *p53* and *p16* genes are associated with adverse outcome in DLBCL.(29-31) Mutations of the *p53* gene are seen approximately in a quarter of DLBCL and the mutations involving the DNA-binding codons appear to be adverse risk predictors independent of other well-established clinical and biological parameters.(30)

Overall the relationship between genetic alterations and phenotypic risk determinants such GCB versus ABC phenotype remains unclear. Further studies, involving large case cohorts treated with current management strategies and multi-parameter approaches will be required to answer these questions.

Conclusion

The clinical behavior of B-cell lymphoma is determined by a complex interplay between the host and tumor factors and is altered by medical intervention. Given that clinical

outcome varies significantly within the DLBCL cases even with the current management strategies, development of robust, reproducible and cost effective outcome predictors that would impact clinical practice is a necessity.

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