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REVIEW

Biomarkers of diffuse large B-cell lymphoma: impact on diagnosis, treatment, and prognosis

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Abstract: Introduction of immunochemotherapy as frontline treatment for diffuse large B-cell lymphoma (DLBCL) has significantly increased survival. However, patients refractory to rituximab-containing regimens have a very poor survival. These differences in clinical behavior might lie behind the biological heterogeneity well recognized in this disease. Advanced molecular research has helped us to define DLBCL subgroups which harbor distinct oncogenic events and response to immunochemotherapy. The field of biomarker discovery in DLBCL has become more complex over the last decade and a broad up-to-date review on this topic is lacking. The aim for this review was to offer the hematology community a comprehensive overview of clinical and biological markers which have a diagnostic and prognostic potential and that might be amenable to therapeutic targeting. Some well known markers are reassessed in light of recent findings.
Keywords: DLBCL, immunochemotherapy, rituximab, biomarkers

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin's lymphoma. Immunochemotherapy has significantly increased complete remission (CR) rates,[1–4] leading to improved survival.[5] However, cure rates reach only around 60%.[6] Patients refractory to rituximab-containing regimens have a poor survival, even with subsequent high-dose chemotherapy and autologous stem-cell transplantation.[7,8] Primary refractory patients have a dismal outcome. Many new treatment strategies are being explored, some involving targeted molecules.

Genetic research has shown that DLBCL constitutes a heterogeneous group of lymphoid malignancies that could not be unraveled by morphology and immunophenotype. DLBCL molecular subgroups utilize mutually exclusive oncogenic pathways and exhibit distinct epigenetic profiles,[9] supporting their distinct pathogenesis. Most importantly, these distinct subgroups have a different outcome. Only now is molecular profiling being brought to the diagnostic and prognostic arena.

A myriad of biological parameters have been described to help identify poor-risk patients. However, this field is constantly becoming more complex, and few markers have been validated in independent studies and have moved to controlled clinical trials. Treatment stratification according to risk, which is the only definitive method to prove the prognostic impact of a particular marker, is only now becoming a reality in this disease.

The aim of this study was to comprehensively review the biomarkers in DLBCL that have been shown to provide diagnostic and prognostic impact, and potentially

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offer a window for therapeutic intervention. The authors hope this data will provide lymphoma scientists and clinicians a broad perspective on the huge field of biomarker development in this disease.

Methodology

A PubMed search was performed using the terms "diffuse large B-cell lymphoma" and "biomarkers," "prognosis," "outcome," and "survival." Priority was given to papers reporting results from larger studies, most of them treated in the R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone) era. Exceptions were made when the problem addressed was considered of major biological impact or when coherent validation had been performed.

Studies on specific subtypes of DLBCL such as CNS lymphoma or viral-associated DLBCL were excluded. Finally, since primary mediastinal B-cell lymphoma has been demonstrated to represent a quite distinct lymphoid malignancy with a particular clinical presentation, molecular signature, and treatment approach, this is not covered in this review.

Establishing the diagnosis of DLBCL in 2013

A tissue-based histopathology examination remains the standard to establish a diagnosis of DLBCL. However, the World Health Organization classification reviewed in 2008 highlighted that only an integrative analysis involving clinical data, morphology, and molecular studies is able to subdivide DLBCL into its different sub-entities.[10]

Gene expression profiling (GEP) of DLBCL revolutionized diagnostic criteria and is the best prognostic tool for DLBCL

DNA microarrays have been used to explore the transcriptomic landscape of normal lymphocytes in a multitude of differentiation and activation states[11] and also of the different lymphoid malignancies. This technique allowed authors to abandon prognostic analysis of individual genes.

Through the use of the Lymphochip array, Alizadeh et al[12] were the first to devise the two molecularly distinct forms of DLBCL, which assume GEPs typical of distinct stages of B-cell differentiation: the germinal centre B-like (GCB)-DLBCL and the activated B-like (ABC)-DLBCL. GCB-DLBCLs exhibit immunoglobulin gene (Ig) ongoing somatic hypermutation.[13] ABC-DLBCLs have a high mutation burden in the IgH genes,[14] but did not go through class-switch recombination. These lymphomas express genes characteristic of plasma cells,[15] but are blocked in their differentiation potential, due to *BLIMP1* inactivation.[16] The two entities are very distinct in their genetic changes,[16–19] signaling pathway deregulation, and subsequent phenotype, and studies are trying to pinpoint which facets explain outcome differences that could be used as markers for selected therapy.

Importantly, it has been shown that patients with GCB-DLBCLs have an improved overall survival (OS) compared with ABC-DLBCLs.[15,20,21] Shipp et al[22] were not able to reproduce this data, most likely due to technical reasons, but designed a model incorporating 13 genes implicated in B-cell-receptor (BCR) signaling and apoptosis, highlighting that many processes can be involved simultaneously in patient outcome differences. Overall, these data support that the molecular classification is the most robust prognostic tool for DLBCL.

Immunohistochemistry is the widest technique used to predict the molecular stratification

Lossos et al[23] built a real-time polymerase chain reaction-based six-gene model for outcome prediction of DLBCL, which was robustly validated, is independent of clinical factors, and is valid in the rituximab era.[24] The depicted genes were selected from the previously defined GCB/ABC signatures. This translates an interest to identify simplified molecular methods to serve as surrogate for GEP results. Many groups have validated GEP data and prognostic impact from samples obtained from diagnostic formalin-fixed paraffin embedded (FFPE) tissue, which indicates that it will soon be possible to apply this stratification in the clinical setting.[25–27]

Immunohistochemistry (IHC) was the first technique to be explored as a means to identify surrogate markers for the molecular classification in DLBCL. Hans and coworkers established the first IHC algorithm that incorporated CD10, BCL6, and MUM1/IRF4.[28] This method had high sensitivity for GEP classification and confirmed that GCB-DLBCLs exhibit a significantly better OS. Since then, many other authors have developed different IHC algorithms, trying to improve sensitivity and specificity for GEP, as well as survival discrimination.[29–31] High rates of concordance with GEP classification have been reported by all groups. The Hans algorithm is by far the widest used.[32–47] However, many investigators have questioned its clinical applicability

due to discrepant results. The present authors have applied all IHC algorithms published to an independent dataset of DLBCL patients and have demonstrated that the agreement in patient classification among the different algorithms was extremely low (unpublished). The present authors believe these differences rely on population selection biases as well as technical questions regarding IHC staining and interpretation, problems which have already been highlighted.[48] Taking their own data and the data of others into consideration, the present authors do not believe it is time to use IHC-based methods to stratify patients in clinical trials. Collaborative studies involving expert hematopathologists should be run to address these problems and propose a standardized IHC methodology for clinical application.

Clinical prognostic markers in DLBCL

Although molecular studies provide robust prognostic data, they are still not available in all clinical settings, and in addition, new clinical factors continue to be identified that have prognostic significance in randomized controlled trials in DLBCL.

The International Prognostic Index (IPI), based on five prognostic factors reflecting patients' clinical and biological characteristics (age, Ann Arbor stage, serum lactate dehydrogenase, performance status, and number of extranodal sites) was originally developed as a prognostic model for intermediate-grade lymphoma.[49] The IPI is the most widely used score to predict outcome in DLBCL and is still valid after the introduction of chemoimmunotherapy as first-line approach.[50,51] However, many authors acknowledge that patients belonging to the same prognostic groups can still exhibit clear differences in survival, which might reflect different biological backgrounds. Moreover, a number of papers reinforce that some biomarkers have an IPI-independent prognostic impact. This data reflects that although IPI should still be used as an outcome predictor model in DLBCL, there is opportunity to improve upon it with new biomarkers.

Other clinical parameters have also been stated as independent prognostic markers. Age continues to be a significant predictor of outcome, either because treatment with chemoimmunotherapy is suboptimally offered to old patients, or because aging is a determinant of lymphoma biology, as has recently been suggested by a large molecular study.[52]

Male sex has been associated with worse outcome in independent large studies in DLBCL.[53] It is suggested that males have faster rituximab clearance so that the standard dose used is suboptimal.[54]

The maximum tumor diameter has an adverse prognostic impact on event-free survival (EFS) in R-CHOP-treated patients,[55] and patients with bulky tumor have been shown to have worse prognosis in other studies.[56]

Bone marrow involvement is associated with a poor prognosis, independently of the IPI. Those patients who have DLBCL, rather than discordant low-grade bone marrow infiltration appear to have a particularly bad outcome, with reported 10% OS at 5 years.[57–59]

Primary involvement of the Waldeyer ring appears to confer a better outcome.[59,60] It was suggested that rituximab has no impact on CR rates in primary extranodal DLBCL.[61]

Fluorodeoxyglucose (FDG)-positron emission tomography (PET) scanning provides diagnostic and prognostic impact in DLBCL

FDG-PET scanning was introduced in the revised International Working Group response criteria for DLBCL[62] for staging at diagnosis and for response assessment after treatment completion. A negative PET at the end of treatment is an excellent predictor of good outcome. The role of interim FDG-PET scan in DLBCL is being actively explored. Recent reports show an increase in progression-free survival (PFS) (and OS in some studies) in patients with a negative PET-computed tomography (CT) after two or four cycles of R-CHOP.[42,63,64] However, other studies reported contradictory results,[65,66] which can be explained by the absence of strict scoring criteria or best standard scoring method[67] and a high inter-observer variability.[68] Using PET-CT to guide optimal treatment is still not a reality in DLBCL.

Morphology and immunophenotype based prognostic clues

Although morphology has been disregarded as a method to obtain prognostic information, there are still suggestions that it is important to obtain a thorough pathological report at diagnosis. T-cell/histiocyte-rich B-cell lymphoma is an uncommon aggressive subtype of DLBCL with abundant T-cell infiltration and microenvironment inflammatory reaction.[69] Most cases carry a GEP characterized by a host immune response and have a very poor prognosis.[70]

A recent analysis from a large cohort of patients enrolled in the Ricover-60 trial showed that the immunoblastic morphology is an adverse prognostic factor at diagnosis.[40] A plasmablastic phenotype was associated with shorter survival in R-CHOP-treated patients.[71]

DLBCL cells express CD5 in 10% of cases. These cases have a distinct genomic and transcriptomic profile and have a poorer outcome,[72–74] which could be improved with R-CHOP.[75] The intensity of CD20 expression is heterogeneous in patients with DLBCL. In cases with decreased CD20 expression, survival is decreased independently of the IPI.[76,77] However, antigen intensity was assessed by flow cytometry, and it is difficult to reproduce this by IHC.

GCB/ABC-specific molecules as independent prognostic biomarkers

Since the publication of GEP results and the validation of the differentiation stage of the B-cell as a robust prognostic marker, many authors have been developing antibodies to identify the expression of several GCB and ABC-specific markers with an intent to develop simpler methods to assess outcome. However, careful analysis of these results should be taken due to the difficulties in the standardization as discussed above for IHC techniques.

CD10 is a marker for GC derivation detected in up to 30% of patients and was associated with improved OS in combination with IPI by many groups.[28,39,41,78] Biasoli et al[79] found that CD10 helps to segregate a group of low-IPI patients with a particularly better outcome. However, two papers have shown no prognostic impact for CD10 expression.[40,80]

IRF4/MUM1 was introduced in the first IHC algorithm for prediction of the molecular stratification as a post-GC marker. It was associated with worse OS[28,81] in some studies, whereas it showed no survival impact in others.[39,40]

FOXP1 is a transcription factor which has been detected at high levels in cases lacking GCB markers and expressing BCL6 and MUM1.[82] FOXP1-positive patients exhibit lower PFS, disease-specific survival, and OS, independently of the IPI.[46,83,84] *FOXP1* gains, found in 12% of cases, showed no correlation with protein levels and outcome.[84]

HGAL/GCET2 protein, a recently characterized GCB-specific marker, is an interleukin (IL)-4-induced gene involved in lymphoma cell motility.[85] GCET2 expression, either by mRNA or protein, is associated with a favorable outcome in patients with DLBCL, independently of the IPI.[86,87]

LMO2 emerged as a strong prognostic marker in DLBCL in GEP.[23] LMO2 protein expression in lymph nodes is restricted to the nucleus of normal GCB-cells[88] and a subset of GCB-DLBCLs and proved to have a positive impact on patient survival,[89] even after the introduction of rituximab.

The *PKCB* gene was identified in GEP data as a robust prognostic marker in DLBCL, being expressed at higher levels in ABC subtypes. DLBCLs expressing the protein kinase C-β have an inferior outcome, either with chemotherapy alone[90–92] or after addition of rituximab.[93]

Molecular prognostic markers

Single genetic aberrations rival with the GEP-based model for the prognostic scenario in DLBCL

MYC

MYC rearrangements are detected in 5%–10% of DLBCL cases and are usually associated with complex kariotypes.[94–96] *MYC* is the target of somatic hypermutation in some cases. These genetic aberrations lead to MYC overexpression and activation of a proliferative phenotype.

In patients included in two prospective clinical trials, the presence of *MYC* aberrations was associated with poorer OS and EFS, independently of the IPI and the GCB/ABC classification.[97] Other studies confirmed this data, correlating *MYC* aberrations with poor clinical characteristics and worse survival.[98–103] The presence of MYC staining by IHC was correlated with *MYC* rearrangements in two independent studies.[104,105]

In a quarter of cases with *MYC* rearrangements, a second hit chromosomal aberration involving *BCL2* or *BCL6* can be found. Large studies in these double-hit lymphomas[101,106,107] suggest a poor prognosis which cannot be solely explained by the presence of a *MYC* breakpoint and hence suggest a synergism between these genetic events. An IHC double-hit score using BCL2 and MYC was validated recently.[107] Patients staining positively for both proteins had a significantly lower CR rate, OS, and PFS, independently of the IPI and molecular subgroup. This data is supported by others.[105]

BCL2

BCL2 is an oncogene commonly targeted in DLBCL, activating an anti-apoptotic program in the malignant cells. Forty-five percent of GCB-DLBCLs are associated with t(14;18) translocations and consequently have *BCL2* overexpression. This oncogenic event was divergently correlated with outcome.[108–110] *BCL2* mutations were described in GCB-DLBCLs and correlated with *BCL2* translocations.[111] No impact on survival was detected in these cases.

The majority of ABC-DLBCLs have *BCL2* overexpression, due to transcriptional deregulation.[15,20] Studies have reported a negative outcome impact in these cases.[112,113] BCL2 protein expression has been associated with poor prognosis,[39,41,44,47,114,115] mainly in ABC cases and in the pre-rituximab era.[112] Some researchers were however unable

to reproduce this data.[40] It was suggested that the negative prognostic impact of BCL2 expression is modulated by rituximab,[116,117] but Iqbal et al[110] suggested that the negative prognostic impact is kept for GCB-DLBCLs.

BCL6

BCL6 is a transcriptional repressor molecule essential for the formation of the GC reaction. Most genetic aberrations involving *BCL6* lead to its overexpression. In consequence, B-cells cannot differentiate into plasma cells but continue to divide and proliferate.

GCB-DLBCLs harbor mutations within the *BCL6* autoregulatory domain,[118,119] whereas ABC-DLBCLs exhibit translocations deregulating *BCL6*.[11,118] *BCL6* rearrangements were associated with adverse clinical parameters and survival[41,120] by some groups but not by others.[118,121] The role of BCL6 protein expression as an independent prognostic variable is also controversial.[28,39,115,118,122,123] It was suggested that rituximab improved outcome only in BCL6 negative patients.[124]

TP53

TP53 encodes for the tumor-suppressor protein p53. Loss-of-function mutations in *TP53* are common in cancer and impair regulation of many biological processes controlled by p53: cell cycle, apoptosis, cell differentiation, DNA repair, angiogenesis, and genomic stability.

Mutations in the *TP53* gene are found in up to 20% of DLBCLs, with no differences in incidence between ABC and GCB subsets. *TP53* mutations have been associated with worse CR rates and survival in DLBCL.[125–131] It was suggested that DNA-binding mutations have a higher impact on OS than other genetic changes, and this finding might help to stratify GCB patients into different prognostic subgroups.[129,130]

One study indicated a strong association of *TP53* deletions and plasmablastic morphology, poor response to chemotherapy and short survival.[132] Another recent study has associated *TP53* deletions with shorter survival in R-CHOP treated cases.[133]

The use of IHC to predict *TP53* mutations has been explored. In one study, p53(+)/p21(−) IHC results correlated with gene status and were associated with a lower survival rate when compared with a p53(−) or p53(+)/p21(+) phenotype.[127] However, others found no correlation between IHC results and outcome.

CDKN2A

CDKN2A deletions are detected in up to 35% of patients with DLBCL. These cases show transcriptional deregulation of both p14ARF and p16INK4a, tumor-suppressor proteins involved in cell cycle control. Jardin et al have comprehensively shown that deletions of *CDKN2A* have a direct negative impact on patient survival. Moreover, these cases could be identified by specific GEP within an ABC transcriptome.[133]

C-REL

Sixteen percent of GCB-DLBCLs have amplification of the *C-REL* locus on chromosome $2p^{20}$, which encodes for the c-REL transcription factor. Positive c-REL nuclear expression is a surrogate marker for activation of the nuclear factor (NF)-κB pathway and was associated with better outcome in GCB-DLBCLs in a single study.[45]

IgH/IRF4 translocations

By screening for novel translocations involving the immunoglobulin genes, Salaverria et al[134] identified a new recurrent chromosomal translocation involving *IRF4* and *IgH* in DLBCL. Patients were predominantly GCB-type but exhibited a specific GEP and presented a favorable outcome.

TBL1XR1/TP63 gene fusions

Using novel algorithms for analysis of RNA sequencing data, Scott et al discovered a recurrent somatic gene fusion, which is present in 5% of the cases.[135] Interestingly, this genetic aberration was always detected by fluorescence in situ hybridization analysis and was restricted to GCB-derived cases.

MicroRNAs (miRNAs) in DLBCL

In recent years, different groups have unraveled the miRNA repertoire in DLBCL. Some miRNAs appear to be restricted to either GCB or ABC-DLBCLs. In two large cohorts of DLBCL treated with chemoimmunotherapy, the expression of certain miRNA was associated with prognosis. Alencar et al[136] reported that the expression of miR-18a, miR-181a, and miR-222, together with the IPI and a molecular score, were predictors of survival. Montes-Moreno et al[137] built a predictor model that incorporated expression of nine miRNA (miR-221, miR-222, miR-331, miR-451, miR-28, miR-151, and miR-148a, miR-93, and miR-491), the IPI, and the molecular classification. This model identified a subset of high-risk patients.

Other apoptosis and cell cycle-related molecules

Markovic et al[138] built an "apoptotic score" incorporating two members (Survivin and XIAP) of the inhibitor of apoptosis family of proteins and the death receptor CD95. They claim

that this score, together with the IPI, is an independent prognostic predictor for CR rate and OS. Positivity for survivin[138,139] and XIAP[138,140] was reported as an unfavorable feature by independent groups. Expression of cFLIP, a dual-function regulator for caspase-8 activation and apoptosis, on the other hand, was associated with better OS.[141]

The prognostic role of cyclin protein expression has been assessed by different groups. Saez et al[142] and others[143] reported that cyclin E overexpression constitutes a relevant adverse prognostic marker. A logistic regression model including cyclin E and other cell cycle regulators was able to divide patients into four prognostically distinct groups. p21 expression was reported as an independent predictor of good outcome after adjustment for IPI in R-CHOP-treated patients.[144] *P14 ARF* and *CDKN2B* inactivation were associated with poorer outcome in another study.[145]

Ki67 is a nuclear protein expressed by cells going through division. Ki67 positivity in tissues reflects the proportion of proliferating cells. Its expression was reported as an independent prognostic factor.[56] Using tissue microarrays from 1514 patients, Salles et al[51] built a prognostic model for rituximab-treated patients. Four risk groups were identified using BCL2, Ki67, and IPI, with improved discrimination of low-risk patients. However, results are contradictory,[114,124,142] and it is highly recognized that scoring Ki67 staining is subject to a very high inter-observer variability.

Other prognostic markers

There are innumerous other potential prognostic markers published in the literature. Most, however, were explored by a single group and have not yet been properly validated.

The BACH2 transcriptional repressor plays important roles in coordinating transcription activation and repression by *MAFK* and *BCL2* in cases with t(14;18). BACH2 expression levels in a large DLBCL cohort were associated with outcome, with patients with lower expression having a better OS and disease-specific survival.[146]

Sirtuin-1, a member of the intracellular regulatory proteins with mono-ADP-ribosyltransferase activity, has been studied by IHC. Its expression in non-GCB DLBCLs is associated with shorter OS.[147]

In the molecularly defined ABC-signature, the *BMI1* oncogene has been associated with a poor outcome, but BMI1 protein expression does not correlate with the ABC subtype as defined by IHC.[148]

Serum free-light chain (FLC) levels were tested in two US trials in DLBCL.[149] High FLC serum levels were the strongest parameter predicting worse outcome.

Genes in the glutathione (GSH) and ATP-dependent transporter (ABC) families were analyzed in two independent GEP datasets.[150] The glutathione peroxidase 1 gene has the most significant adverse effect on survival, after adjustment for the molecular subgroup and IPI. The expression of genes encoding for antioxidant defense enzymes and redox proteins were also explored.[151] DLBCLs with the worst prognosis have combined decrease in expression of catalase, glutathione peroxidase, manganese superoxide dismutase, and VDUP1.

The microenvironment provides strong prognostic information in DLBCL

GEP suggests that host inflammatory/immune response plays a role in the biology of DLBCL

Using unsupervised GEP analysis, Alizadeh et al[12] demonstrated that the tumor transcriptome reflects not only the differentiation state and the rate of proliferation of the malignant B-cell, but also the host response to the tumor. A "lymph-node" (LN) signature enriched for markers of macrophages, natural-killer (NK) cells, and matrix remodeling genes was present in normal lymph nodes and in most DLBCLs, but not in other B-cell malignancies. Rosenwald et al depicted an outcome predictor model that incorporated 16 genes and the expression of the *BMP6* gene and helped to score patients into divergent outcomes independently of the IPI and the molecular subgroup.[20] The LN genes introduced in the model were associated with a better outcome. Another GEP signature, the "MHC class-II" was also correlated with a good outcome. This supports that MHC class-II expression, known to be crucial for antigen presentation to the immune system has a role in treatment efficacy.

Monti et al[70] developed a GEP analysis method to segregate robust subsets of DLBCL, although this had no impact on survival. The "host response" cluster had high expression of components of the T-cell receptor, T/NK-cell activation, interferon-induced genes, cytokine receptors and tumor necrosis factor (TNF) ligands/receptors, as well as abundant macrophage, dendritic cell, and extracellular matrix components. This cluster was enriched for genes from the previously described LN signature, which adds strength to this perspective of a subset of patients having a strong immune response against the tumor, which might not be completely competent, but is partially explaining an improved outcome.

Lenz et al[21] used three diagnostic DLBCL lymph node samples for sorting malignant B-cells and remaining

non-malignant cells in order to perform GEP. Transcripts that had a differential signal value in either the CD19(+) or CD19(–) cells were used to build multivariate survival models that were validated in whole GEP data from almost 400 patients. In these survival models, a GCB-signature derived from the CD19(+) cell subset, together with two others derived from the CD19(–) cells were able to predict OS and PFS in the R-CHOP validation sets. Additionally, the IPI and the GEP-based model added to the predictive power of each other, suggesting a shared role for clinical and biological features contributing to patient outcome. The "stromal-1" signature was enriched for genes derived from macrophages and extra-cellular matrix components and was predictive of a good outcome. The "stromal-2" signature was enriched for genes involved in angiogenesis and conferred an adverse outcome. The relative expression ratio of each of the stromal signatures in an individual sample is what is most predictive of the length of survival. Finally, these authors have explored how previously described signatures performed in the rituximab treatment era. The LN signature, which shares a large amount of transcripts with the "stromal-1" signature, and the "proliferation" and GCB signatures remained survival predictors, whereas the "MHC class-II" signature lost prognostic impact.

The top 86 genes discriminating good and bad DLBCL anthracycline responders were enriched for transcripts from the microenvironment, especially involved in degradation and remodeling of the stromal matrix.[152] A French group developed a model comprising four genes of the GCB/ABC signature and two genes related to immune response (*APOBEC3G* and *RAB33A*), which showed to be predictive of outcome in patients receiving immunochemotherapy.[153]

Two of the genes included in the six-gene model of Lossos et al,[23] *SCYA3*, a chemokine, and *FN1* (fibronectine-1), reflect the tumor microenvironment. *FN1* has been shown to be expressed at very low levels by B-cells, supporting that the transcript is being translated from the LN accessory cells. It has been associated with a better outcome, which again highlights the good prognostic impact of a stromal response in DLBCL.

Alizadeh et al built a bivariate survival predictor incorporating *LMO2* and a second gene more highly expressed in nonmalignant cells, *TNFRSF9/CD137*.[154] This bivariate model synergizes with the IPI for predicting outcome in DLBCL. *TNFRSF9* expression was restricted to a minority of infiltrating T-cells. Using co-culture systems, the authors show that resting peripheral blood T-cells can start to express CD137 after contact with tumor cells, which could be potentiated by rituximab.[155]

Overall a substantial body of work consistently highlights that a biological facet of DLBCL is derived from the stromal microenvironment. In contrast to what is generally found in solid tumors and other lymphoid malignancies, in DLBCL the expression of genes derived from cells of the mononuclear phagocyte system and extra-cellular matrix components of the malignant LNs confer an improved outcome.

Polymorphisms in genes involved in immune and inflammatory responses have an impact on outcome

A large study showed that an *IL10* haplotype and single nucleotide polymorphisms in *IL8* receptor β, *IL1α*, *TNF*, and *IL4* receptor were strong predictors of OS, independently of clinical factors.[156] Lech-Maranda et al[157] reported that the IL-10–1082 genotype influenced clinical outcome in patients with DLBCL, but other authors failed to demonstrate this.[158] Other studies provided survival correlations in DLBCL with *IL6*, vascular endothelial growth factor (VEGF) receptor,[159] *IL4* receptor,[160] and lymphotoxin α[161] polymorphisms. Correlations between gene variants in immune function-related genes and outcome in lymphoma has never been properly explored functionally.

Malignant DLBCL cells found mechanisms that allow them to escape T-cell immune surveillance

Loss of MHC expression is an attractive mechanism of evading T-cell recognition that appears to be utilized by a subset of poor prognostic DLBCL cases.[25,37,162,163] GEP data consistently showed that the overexpression of MHC class-II genes correlates with better survival. Rimsza et al[164] found that OS survival is higher proportional to the degree HLA-DRA expression in tumors. Moreover, these authors found that the number of CD8(+) T-cells was significantly higher in MHC class-II(+) cases, which suggests that loss of HLA expression is partially responsible for less effective T-cell recruitment to tumors. Additionally, a poor host tumor-infiltrating T-cell response is seen in HLA-I/II negative cases.[164,165]

Challa Malladi et al have shown that 30% of patients have inactivating mutations in the β2-microglobulin (*B2M*) gene, which impair formation of the HLA class-I complex.[166] Analogous lesions were found in the *CD58* gene, which encodes a molecule involved in CD2 receptor ligation in T and NK cells. Overall, more than 60% of DLBCL exhibited aberrant expression of HLA-I and CD58.

An inflammatory cytokine profile was associated with worse prognosis in DLBCL

Several groups have explored pre-treatment serum cytokine levels in patients with DLBCL and correlated it with outcome. Patients with detectable levels of IL-10 had a more aggressive presentation and worse survival.[167,168] In other series, high levels of IL-18[169] and IL-2 receptor[170,171] were associated with poorer PFS and OS in the rituximab era.

Peripheral blood counts have impact on patients outcome

Many authors have implicated peripheral blood cell counts with outcome in DLBCL. A low absolute lymphocyte count (ALC) at diagnosis was correlated with adverse prognostic factors and strongly predicted response to R-CHOP.[172] A score incorporating the ALC and R-IPI was a better prognostic discriminator. A meta-analysis involving 1206 subjects has shown that the hazard ratios of low ALC for OS and EFS were 2.78 and 2.56 in the population that received R-CHOP.[173] Other authors suggested that a low absolute number of NK cells and not total lymphocytes relates to treatment response and EFS.[174]

Using large datasets, Porrata et al demonstrated that elevated monocyte counts and relative lymphopenia are adverse prognostic factors.[175] An absolute monocyte and lymphocyte count score predict PFS and OS in multivariate analysis,[176] together with the molecular classification and IPI. The prognostic impact of monocytosis was confirmed by another group.[177]

The number of regulatory T-cells (Tregs) is increased in the peripheral blood and tumors of patients with lymphoma, and this correlates with disease stage and serum LDH.[178]

Immunohistochemistry studies exploring the immune microenvironment in DLBCL

Most authors have focused on the use of IHC to enumerate and functionally characterize the microenvironment in DLBCL. An extensive number of suitable antibodies are available, as well as fairly standardized IHC staining methods. This should allow IHC to be extended to clinical practice, but as discussed above, the use of standardized criteria and proper validation is required.

Macrophages/stromal markers

Many authors have described macrophage activation states as a binary system, but this has clear limitations in characterizing the multitude of macrophage functions that can be achieved in different conditions.[179] Moreover, this model is based on mouse studies, and there are crucial interspecies differences in the macrophage gene transcriptional landscape between mice and humans.[180]

M1/"classically activated" macrophages, respond to interferon-γ or lypopolysacharide by producing proinflammatory cytokines, upregulating MHC molecules, and increasing phagocytic capacity.[181] This cell behavior has been extensively validated in vivo in infection and tumor models.

M2/"alternatively activated" macrophages are specialized at resolving inflammation by tissue remodeling[182,183] and immunosuppression.[184,185] They have high levels of dead cell scavenging receptors.[186] This phenotype helps cancer progression. In-vitro stimulation with different cytokines,[187–192] immune-complexes, steroid hormones, Toll-like receptors, or IL-1 receptor agonists[193–195] can induce an M2 phenotype.

The extent of macrophage infiltration as measured by CD68 staining has been correlated with different outcomes in DLBCL.[196–200] Some authors used co-staining methods to characterize macrophages in non-Hodgkin's lymphoma.[199] However, this is intrinsically difficult to standardize and analyze. The inconsistency of this data reflects the difficulties in scoring CD68 but also the complexity of macrophage functions that can hardly be mirrored using a low number of markers.

Macrophages in tumors are known to promote angiogenesis. Angiogenesis and related markers such as VEGF have been explored by several groups, with a suggestion that this is associated with a worse outcome.[201–203] The use of microvessel density as a marker of angiogenesis might bring conflicting results due to technical issues. Unexpectedly, Evens et al[204] reported that HIF-1α, a transcription factor highly involved in angiogenesis triggering, is expressed in several DLBCL patients and correlates with significantly improved PFS and OS.

The secreted protein, acidic and rich in cysteine (SPARC) is a glycoprotein that is promiscuously expressed in tissues and is involved in matrix remodeling, integrin activity, adhesion, growth factor signaling, and apoptosis. Lenz et al[21] and Meyer et al[205] have demonstrated that a subset of macrophages express SPARC in DLBCL, which was linked to a favorable prognosis. A model incorporating the GCB/ABC classification, SPARC, and microvessel density was highly predictive of OS and EFS in multivariate analysis after adjusting for the IPI.[206]

T-cells

The prognostic role of the total number of T-cells as well as CD4(+) and CD8(+) subsets as determined by IHC has never been properly explored. The impact of cytotoxic T-cells in

DLBCL is still unclear.[163,207,208] Some authors reported that a higher density of activated cytotoxic T-cells is a strong indicator for an unfavorable outcome. However, methodology and statistical approaches used were different, making comparisons difficult. On the contrary, Chang and colleagues reported that the presence of an intense infiltrate was associated with a favorable clinical outcome.[163] The clinical impact of forkhead box protein 3 (FOXP3)(+) Treg infiltration in DLBCL is still not clear, with different groups reporting different results.[208–211] Finally, the immunosuppressive marker PD-L1 was mainly expressed in the tumor-infiltrating T-cells, but was also found on the malignant cells in a subset of DLBCL.[212] PD-L1 might trigger PD-1 in T-cells, which could constitute a mechanism of immune escape. However functional data is lacking.

Other cells

Two groups have looked at the mast cell density in biopsies of DLBCL. A more dense infiltration of mast cells has been shown to improve outcome.[213]

Therapeutic issues

One of the most robust prognostic factors in DLBCL is refractoriness to immunochemotherapy. Recognizing rituximab refractory patients at diagnosis is a priority. Also of crucial importance is to bring to clinical practice different treatment strategies with a low toxicity profile, which would be amenable to be offered to the majority of our patients.

At the present time, only the IPI, and genomic methods to recognize double-hit lymphomas, have been used to select high-risk patients for more aggressive regimens, most of them incorporating consolidation with autologous stem-cell transplantation. However, these markers do not take into account biological differences that might be behind disease aggressiveness.

Many authors have been trying to explore the mechanisms behind rituximab-refractoriness. Polymorphisms in the activator *FcγRIIIa/CD16a* have been correlated with rituximab response[214,215] and inhibitory FcγRIIb on the B-cells and effector cells modulate rituximab activity.[216,217] FcγRII expression levels between different B-cell malignancies correlates with sensitivity to rituximab. There is increasing interest in devising mechanism to increase the affinity of therapeutic monoclonal antibodies (mAb) to the innate immune effector cell FcγRIIIa. As a result, new anti-CD20 mAb with engineered Fc-receptor with increased FcγRIII binding affinity are being investigated in ongoing clinical trials.

Results from clinical trials using signaling inhibitors are becoming available. Very promising responses are being reported in refractory DLBCLs with ibrutinib, a selective Bruton's tyrosine kinase. It has exclusive in-vitro and in-vivo cytotoxicity against ABC-DLBCLs, and it has recently been suggested that it might have synergistic activity with lenalidomide in blocking NF-κB pathway in this subset of DLBCLs.[218] This highlights the importance of carefully recognizing these patients at diagnosis.

Incorporation of molecular stratification into clinical trials is ongoing to offer bortezomib for ABC-DLBCLs both in Europe and in the US. NF-κB constitutional activation is a hallmark for the ABC-DLBCLs, and bortezomib is known to block this pathway by avoiding degradation of IκBα. It has been combined with chemotherapy and immunochemotherapy in Phase II trials. In accordance to its mechanism of action, bortezomib is more effective in ABC-DLBCLs, improving CR rates and OS.[32,219,220] Other methods of blocking the NF-κB pathway have been reported in pre-clinical models and have been shown to be more effective in ABC cell lines.

Bruton's tyrosine kinase inhibitors and other molecules are particularly effective in cases with chronically active BCR signaling.[221] Preclinical studies with Fostamatinib, a SYK inhibitor, showed that it is able to inhibit BCR signaling and induce cell-cycle arrest in a subset of patients, which can be identified either by a particular molecular cluster[222] or by PLCγ2 and AKT levels.[223] PKCβ inhibitors have been effective in preclinical studies restricted to a proportion of ABC-DLBCL cell lines with *CD79A/B* mutations.[224] PI3K inhibitors also have a prominent activity in ABC-DLBCLs with *CD79B* mutations.[225] Some of these compounds have shown significant activity in DLBCL in Phase II trials.[226,227]

In ABC-DLBCLs with *MYD88* mutations, IL-6 and IL-10 cytokines activate a JAK-family kinase and lead to expression of a STAT3-dependent gene program. STAT3 also potentially activates NF-κB signaling, as has been demonstrated by experiments using combinations of a JAK kinase inhibitor and an IKKβ inhibitor in ABC cell lines. Targeting JAK2/STAT3 is hence a potential approach for ABC-DLBCLs. This can be achieved by the use of JAK2 inhibitors,[228] HDAC inhibitors,[229] or IL-21.[230]

Targeting transcription factors that are oncogenically deregulated is another therapeutic opportunity. Small-molecule BH3 mimetics bind to the proapoptotic BCL2 family members and promote apoptosis. These molecules have shown activity in clinical trials including patients with relapsed/refractory lymphoma.[231]

BCL6 targeting might also be useful in either ABC or GCB-DLBCLs. In-vitro studies have shown that *BCL6* inhibition dampened BCR signaling by repression of *SYK*.[232] It is suggested that combined targeting of these two genes is a rational approach for these cases. Also, tandem targeting of the overlapping BCL6 and p53 might provide an effective therapeutic approach to lymphoma therapy.[233]

BCL6 disruption can be achieved either by using an inhibitor of the chaperone HSP90 or by targeting the *BCL6* BTB domain. The molecular pathogenesis together with the new targeted therapies have been elegantly reviewed by Shaffer et al.[234]

Immunomodulatory drugs act on many aspects of immune cell function[235–238] and angiogenesis, and lenalidomide has been shown to be effective as a single agent in highly treated patients with DLBCL. Lenalidomide has been tested in patients with relapse/refractory DLBCL,[239,240] either alone or in combination with rituximab,[241] with good results. Patients with non-GCB phenotype appear to respond better.[242] A Phase III trial is underway to clarify the role of lenalidomide in ABC-DLBCLs.

The Hedgehog (HH) signaling pathway has recently been implicated in DLBCL pathogenesis. HH signaling inhibition induces cell-cycle arrest in GCB-DLBCLs and apoptosis in ABC-DLBCLs.[243] Authors suggested that targeting *ABCG2*

Table 1 Biomarkers with prognostic potential in DLBCL

	Markers	Comments
Clinical	IPI, Age, Sex Maximum tumor diameter Concordant BM involvement Extranodal (Waldeyer ring)	The role of the clinical parameters reviewed has been assessed in rituximab treated patients.
Imaging	FDG-PET scanning	A negative PET at the end of treatment is an excellent predictor of good outcome. The role of PET-CT to guide optimal treatment is still unclear.
Pathology/ immunophenotype	Immunoblastic morphology CD5 expression CD20 expression intensity	Pathology results exhibit a high interobserver variability. The assessment of CD20 expression by IHC is not reliable.
GCB/ABC specific markers	CD10, GCET2, LMO2, PKCβ MUM-1, FOXP1 IHC algorithms	IHC results are contradictory, most likely due to patient selection and the use of different methodology and analysis.
Genetic abnormalities	*MYC* aberrations, Double-hit (*MYC*/*BCL2*) *BCL2* aberrations *BCL6* aberrations *TP53* deletions/mutations *CDKN2A* deletions *IgH*/*IRF4* translocations microRNA expression profile	MYC staining by IHC was correlated with MYC rearrangements. An IHC double-hit score using BCL2 and MYC was validated recently. The role of single *BCL2* and *BCL6* aberrations is debated. BCL-2 protein overexpression might have a negative prognostic impact in GCB-DLBCLs. It is suggested that rituximab improved outcome only in BCL-6 (–) patients. The use of IHC to predict TP53 mutations is not recommended. Deletions of *CDKN2A* have a negative impact on survival. These cases can be identified by specific GEP within an ABC transcriptome. Patients with *IgH*/*IRF4* translocations have a favorable outcome. Some miRNAs appear to be restricted to either GCB or ABC-DLBCLs. Prognostic models incorporating miRNAs have been explored by two groups.
GEP-based models	GCB/ABC BCR/proliferation, Lymph node, MHC-II 13-gene model Stromal-1/stromal-2 6-gene model by Lossos et al 6-gene model by Jais et al LMO2/TNFRSF9	RNA profiling in DLBCL has shown that the tumor transcriptome reflects the differentiation state and the rate of proliferation of the malignant B-cell as well as the host response to the tumor. More work has to be done on functional validation, independent validation of the methodology, development of simplified methods for clinical application.
Microenvironment	Loss of MHC class-II expression Polymorphisms in immune-related genes Cytokine levels: IL-10, IL-6 PB lymphocyte and monocyte counts Immune cells/stromal markers: Macrophages, SPARC, microvessel density cytotoxic T-cells, regulatory T-cells	A substantial body of work consistently highlights that a biological facet of DLBCL is derived from the stromal microenvironment. DLBCL cells use mechanisms to evade T-cell immune surveillance. Correlations between gene variants in immune function related genes and outcome in lymphoma has never been properly explored functionally. PB counts is a simple and attractive method for prognostic assessment. The role of macrophages and stromal response in DLBCL is still unclear. IHC approaches are not adequate to develop a functional model.

Note: Only most important markers and relevant comments have been stated.
Please see references in the body of the text.

and HH signaling may have therapeutic value in overcoming stroma-induced chemoresistance in DLBCL.[244]

YM155, a survivin suppressant, was used in early phase trials[245] in DLBCL. In-vitro studies suggest that there is synergism with rituximab. Targeting PIM kinases, which are markers of progressive disease in ABC-DLBCLs, has also been shown to be effective in preclinical studies.[246]

Preliminary results with these new compounds could be improved if patient selection was refined by biomarkers that identify specific populations with particular sensitivity. Moreover, the relapse/refractory setting might not be the best scenario to test targeted therapies. Finally these new treatments are able to be offered to older patients due to their low toxicity profile.

Conclusion

Recent advances in genetic research have improved our knowledge of DLBCL pathogenesis. Recognizing patients with different molecular backgrounds, response to treatment, and survival is crucial for designing new drugs and clinical trials. However, at the present time, no simple and well standardized method is available for this purpose that can compete with the IPI. It is highly likely that a clinico-biological index will be more informative than single markers for prognostic prediction. This is, however, not yet available. Moreover, only now is molecular classification being used in clinical trials for treatment stratification.

Incorporation of biomarkers into clinical practice is not yet a reality. Most of the biomarkers reviewed here lack independent validation (see Table 1). Very few have been scrutinized in prospective studies. There is a lack of well standardized methodologies for pathology and molecular studies. It has been demonstrated that the use of different IHC techniques results in highly variable results and poor reproducibility. Again, only when specific biomarkers are incorporated into prospective studies will we be able to assess whether the methodology is sufficiently robust and reproducible for subsequent use in the clinic.

Disclosure

The authors report no conflicts of interest in this work.

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