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DIFFUSE LARGE B-CELL LYMPHOMA: BIOLOGICAL PROGNOSTIC FACTORS AND TREATMENT WITH ANTI-CD20 ANTIBODY

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ABSTRACT

Despite great progress has been made in diffuse large B-cell lymphoma treatment, especially with the combination of the anti-CD20 antibody (rituximab) with the standard chemotherapy, this disease's clinical and biological heterogeneity is responsible for unpredictable responses to treatment and difficult prognostic assessment. The biological prognostication of these patients has been a target of incessant investigation, and several markers emerged as survival predictors in rituximab's era. However, the methods and expression cut-offs used varied between studies, creating inconsistencies in the obtained results. The identification of reliable and easily assessed prognostic biomarkers is extremely important, because it will allow creation of new target therapies and individualization of treatment regimens.

KEYWORDS: LYMPHOMA, LARGE B-CELL, DIFFUSE, PROGNOSIS, BIOLOGICAL MARKERS, BIOLOGICAL THERAPY

LINFOMA DIFUSO DE GRANDES CÉLULAS B: MARCADORES BIOLÓGICOS DE PROGNÓSTICO E TRATAMENTO COM ANTICORPO ANTI-CD20

RESUMO

Apesar de grandes avanços no tratamento do linfoma difuso de grandes células B, principalmente com a introdução do anticorpo anti-CD20 (rituximab) à quimioterapia convencional, a heterogeneidade clínica e biológica desta doença é responsável por respostas imprevisíveis e de difícil avaliação prognóstica. O prognóstico biológico destes doentes tem sido alvo de incessante investigação, e vários marcadores surgiram como preditores da sobrevivência na era do rituximab. No entanto, os métodos e níveis de pontos de corte usados variaram entre os estudos, criando inconsistências nos resultados obtidos. A identificação de marcadores biológicos de prognóstico fiáveis e facilmente avaliados é extremamente importante, pois poderá permitir a criação de novas terapêuticas alvo e uma maior individualização do tratamento.

PALAVRAS-CHAVE: LINFOMA, GRANDES CÉLULAS B, DIFUSO, PROGNÓSTICO, MARCADORES BIOLÓGICOS, TERAPÊUTICA BIOLÓGICA

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy worldwide, accounting for approximately 30% of all lymphomas.[1] It is an heterogeneous group of non-Hodgkin lymphomas that vary in their clinical presentation, morphology, biology and genetics. Although the recent addition of the anti-CD20 monoclonal antibody to the standard chemotherapy improved the survival of DLBCL patients, responses to treatment are miscellaneous and results are often unpredictable.[2,3]

The International Prognostic Index (IPI) has been one of the best predictors of survival in these patients.[4] It includes independent parameters, such as age, stage of disease, serum lactate dehydrogenase level, performance status and number of extranodal disease sites, and, according to these, subdivides the DLBCL patients into four risk groups, with different 5-year overall survival: low risk, low-intermediate risk, high-intermediate risk and high risk. However, even within identical IPI risk groups, important variability in outcomes has been observed.[5] Thus, assessing prognosis in these patients has been an important challenge.

Insight into the biologic heterogeneity of DLBCL has led to the identification of several prognostic biomarkers. However, the importance of individual markers and the best methods and criteria to assess them are still subjects of numerous controversies.

The aim of this paper is to review the biological prognostic factors of DLBCL and their relevance in the era of rituximab.

CELL OF ORIGIN

Studies assessed the various morphologic, immunologic and cytogenetic features of DLBCL and showed a great level of molecular complexity. Gene-expression profiling (GEP) data revealed three specific gene-expression signatures related to the cell of origin of these tumors, which are associated with distinct genetic alterations and significantly different survival rates: germinal-center B-cell (GCB)-like DLBCL, activated B-cell (ABC)-like DLBCL and primary mediastinal large B-cell lymphoma (PMLBCL).[6-9] The GCB-like tumors were found to have a significantly better prognosis than the ABC DLBCLs in patients treated with anthracycline-based chemotherapy, and these results were independent of IPI score.[6,7] The PMLBCLs are characterized by different clinical presentation and features similar to classic Hodgkin's lymphoma. Yet, this subtype, like GCB tumors, has also a favorable prognosis.[8,9]

Since GEP requires fresh or frozen tissues and is not widely available, various researchers tried to develop immunohistochemical (IHC) algorithms for paraffin-embedded tissues to reproduce GEP findings. Generally, these algorithms recur to a combi-

nation of antibodies against GCB- and ABC-specific antigens to separate DLBCL subtypes. One of the most used is the Hans algorithm, which distinguishes the GCB-like DLBCLs from non-GCB tumors using three markers (CD10, BCL6 and MUM1).[10] The GCB subtype is characterized by CD10 and/or BCL6 expression, whereas ABC subtype is defined by the absence of germinal center markers and the presence of MUM1/IRF4 antigen. More recently, was developed the Choi algorithm, based on expression of five markers (GCET1, CD10, BCL6, MUM1 and FOXP1) and with a higher concordance with GEP results than the Hans algorithm.[11]

The usefulness of DLBCL subdivision to predict prognosis is controversial in rituximab's era. Regarding to the IHC algorithms, some studies found no difference in the outcomes between GCB and non-GCB subtypes with the combination of anti-CD20 antibodies with standard chemotherapy[12-15], whereas others reported a persistent difference. [16] However, the GEP division of DLBCL was a persistent predictor of these patients' prognosis in the accomplished studies.[17]

TUMOR MICROENVIRONMENT

A different analytic approach, based on hierarchical clustering, indentified four gene-expression signatures that predicted survival in patients treated with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) chemotherapy: the "germinal center B-cell" (GCB), the "lymph node", the "major histocompatibility complex (MHC) class II" and the "proliferation" signatures. The "GCB" signature was associated to a favorable prognosis and resembled the distinction between ABC-like and GCB-like DLBCL. The "proliferation" signature was associated with a poor prognosis and contained MYC and its target genes. The non-expression of the "MHC class II" signature was associated with a worst outcome. The "lymph node" signature, that predicted a favorable prognosis, included components of the extracellular matrix, reflecting the tumor microenvironment.[7] This work raised questions about a possible role of the tumor microenvironment in DLBCL patients' prognosis, and, on this basis, new studies were developed.

Tumor microenvironment of DLBCL was found to have two different presentations: stromal-1 signature, characterized by extracellular matrix deposition and histiocytes infiltration, and the stromal-2 signature, reflected by angiogenesis and blood vessel density in the tumor stroma, and they predicted a good and poor prognosis in patients treated with immunochemotherapy, respectively.[17]

More recently, the stromal-1 signature was reproduced using an antibody against secreted protein, acidic and rich in cysteine (SPARC). A high expression of SPARC in the tumor's stroma was associated with a longer survival.[18] On the other hand, stromal-2 signature simulation was attempted by measuring the microvessel density (MVD) in the tumor, and a high MVD predicted a poor prognosis.[19] These studies were also carried out in patients treated with anti-CD20 antibodies.

MicroRNA

MicroRNAs are a class of short noncoding RNA molecules that negatively regulate gene expression. They bind to complementary target sequences in mRNA, causing repression of translation. Numerous different mechanisms are regulated by these molecules, including cell differentiation and proliferation, apoptosis, hematopoiesis, organ development and developmental timing.[20] Interestingly, the majority of human microRNAs are located at genomic regions associated with carcinogenesis, and deregulated expression of microRNAs seems to be a common feature of cancer.[21,22] Recently, many microRNAs have been associated with malignancy, acting either as oncogenes or tumor suppressors.

The importance of microRNAs in DLBCL has been assessed and a distinct microRNA expression between DLBCL subtypes was found. miR-155, miR-21, miR-221 and miR-222 are more highly expressed in the ABC subtype than in the GCB tumors.[23,24] Nevertheless, the distinction between GCB and ABC-like subtypes of DLBCL defined by microRNA expression signatures didn't show prognostic value, because the currently known miRNAs are present in both malignant B lymphocytes and nonmalignant cells of the tumor microenvironment.[25]

In patients treated with standard chemotherapy, some researchers found a group of microRNAs associated with prognosis. Reduced expression of miR-19A, miR-21, miR-23A, miR-27A, miR-34A and miR-127 identified poor event free survival (EFS) and/or overall survival (OS). However, in a multivariate analysis, only miR-127 influenced both EFS and OS.[26]

When the anti-CD20 antibody is combined with chemotherapy, the results are different. A cohort of 176 patients with DLBCL treated with R-CHOP (rituximab plus CHOP) was evaluated, and three

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miRNAs emerged as possible prognostic indicators: increased expression of miR-18A and miR-222 was associated with worse OS and progression free survival (PFS), respectively, and increased expression of miR-181A was associated with improved PFS. These findings were independent of the IPI.[25] In other study, high expression of miR-222, characteristically expressed in ABC-like cell lines, was also associated with inferior OS and PFS, independently of IPI.[24]

GENOMIC ABERRATIONS

Numerous genomic aberrations were found in DLBCL, and some were more frequent in one tumor subtype.

A significant part of GCB-like DLBCLs has the translocation t(14;18)(q32;q21) that causes a dysregulation on the antiapoptotic BCL2 gene and results in its overexpression, but this abnormality is not detectable in the ABC subtype.[7] GCB-like DLBCLs are characterized by high expression of a transcriptional repressor, BCL6, that is an important regulator of the germinal center reaction.[27] BCL6 is responsible for downregulating inhibitors of cell cycle progression. Additionally, BCL6 protects cells from DNA-damaged induced apoptosis, since it suppresses TP53 expression and other proteins involved in damage response.[28] TP53 is also negatively regulated by MDM2, which is overexpressed (due to amplification) in more than 10% of GCB DLBCLs.[29] More recently, genetic anomalies affecting two related histone and non-histone acetyltransferases, CREBBP and EP300, were identified.[30] These proteins are responsible for acetylating BCL6 and TP53, and their mutation leads to constitutive activation of BCL6 and to decreased TP53 tumor suppressor activity. CREBBP mutations are present in 32% of GCB DLBCL cases, and only in 13% of ABC DLBCLs.[30] Another recurrent abnormality in the GCB subtype involves the tumor suppressor PTEN, resulting in constitutive activation of the phosphatidylinositol 3-kinase (PI3k)/AKT signaling pathway. [29] This leads to cell proliferation, survival and growth.[31] The mechanisms of PTEN downregulation include PTEN deletions (in approximately 15% of GCB DLBCLs) and PTEN suppression caused by miR-17-92 locus amplifications (almost 15% of GCB DLBCLs).[29] None of these genomic aberrations was found in ABC-like DLBCLs. Amplification of a locus in chromosome 2 (2p16) also characterizes GCB DLBCLs, appearing in 30% of these cases.[29] One of the target genes of this abnormality codifies a subunit (Rel) of the nuclear factor kappa B (NF-κB) transcription factor.[29] However, the role of this aberration in GCB DLBCLs isn't yet clarified.

ABC-like DLBCLs are characterized by expression of several genes that are normally expressed in plasma cells, but full differentiation seems to be blocked by various genetic aberrations involving Blimp-1, the main regulator of plasmacytic differentiation.[6,32] Many additional abnormalities are important to ABC DLBCLs molecular biology. The constitutive activation of the NF-κB signaling pathway is the genetic hallmark of the ABC subtype, occurring in virtually 100% of these cases.[33] Researchers have found different anomalies that lead to constitutive activation of this cascade. One mechanism involves three proteins - CARD11, BCL10 and MALT1 - that form a signaling complex, the CBM complex, which is transiently activated in normal lymphocytes after antigen stimulation.[34] However, it is constitutively activated in ABC DLBCLs by different genetic abnormalities, like activating mutations in the coiled-coil domain of CARD11 (10% of ABC DLBCLs) and aberrations in the CD79A and CD79B signaling molecules.[35,36] Other mechanism of NF-κB activation is the downregulation (caused by mutations/deletions) of A20, a genomic sequence that encodes a negative regulator of NF-κB signaling.[37] Activating mutations in the Toll/IL-1 receptor domain of MYD88 (an adaptor protein involved in toll-like receptor and IL-1 receptor signaling) are present in 30% of ABC DLBCLs, and are responsible for constitutive activation of the NF-κB signaling cascade, as well as the JAK/STAT3 pathway, that also mediates cell survival in ABC DLBCLs.[38] Finally, the ABC subtype is associated with trisomy 3 or gain/amplification of chromosome arm 3q, amplification of 18q and consecutive overexpression of the BCL2 gene, loss of 6q and deletions affecting the INK4α/ARF locus on chromosome 9q21, that encodes two tumor suppressor proteins, p16 (INK4 α) and p14 (ARF) (upstream regulators of the p53 pathway).[29]

Despite numerous genomic aberrations were found in DLBCL, only a few seem to be important for these patients' prognosis in rituximab's era. A retrospective multi-center study was developed to assess the impact of genomic abnormalities on clinical outcome of patients treated with R-CHOP.[39] Twenty recurrent genetic lesions had an impact on the clinical course of the disease. Among them, deletions in the short arm of chromosome 8, particularly at 8p23-1, were associated with the most significant negative impact in OS. This region was also more common among non responders.[39] Inte-

restingly, losses of 17p (TP53) and 15q (TP53BP1) were more frequent in association with loss of 8p, suggesting that the concomitant dysregulation of genes mapped on different chromosomes could be responsible for a poor outcome after R-CHOP-21.[39] Del(9p21.3) showed a negative impact on response to treatment, although an influence on survival was not observed. In another study, researchers concluded that gains on chromosome 7q delineated a group of DLBCLs with distinct biological and clinical characteristics: two-thirds of these patients were female, had a longer overall survival, didn't have blood marrow involvement and had considerably less extra-nodal sites affected.[40] A recent study of germinal center-derived B-cell lymphomas recognized the relevance of the t(6;14)(p25;q32) translocation in DLBCL prognosis. This genetic anomaly deregulates MUM1/IRF4 oncogene and is associated with younger age at diagnosis and a favorable outcome.[41] However, the patients included in this research weren't treated with anti-CD20 antibody. Thus, if this translocation has prognostic significance in patients treated with immunochemotherapy is yet to be cleared.[41]

SINGLE MOLECULAR PROGNOSTIC FACTORS

TP53

p53 is a tumor suppressor protein responsible for DNA damage control and maintenance of genomic stability by inducing G1 arrest or apoptosis, if DNA is not repaired. p53 dysfunction can result in abnormal cell growth, increased cell survival, genetic instability and, eventually, malignant transformation. Mutations in TP53 gene are present in 10-20% of B-cell lymphomas, and were described in 18-30% of DLBCL patients.[42] Exons 5-8 of TP53, which contain highly conserved domains, have been identified as TP53 mutational hotspots.[43]

TP53 mutations have been associated with poor prognosis in several hematological malignancies (mantle cell lymphoma, Burkitt lymphoma and chronic lymphocytic leukemia).[44-46] Numerous studies shown a poor overall survival in DLBCLs with these mutations. One research had a more specific result, showing that TP53 mutations were associated with a poor outcome particularly in low and low-intermediate risk groups of DLBCL.[47] Other studies found that the impact on survival was greater if the mutations occurred in the core domain of TP53 and in GCB-like DLBCLs.[43,48] However, these studies were conducted in patients treated with chemotherapy, but not with the anti-CD20 antibody.

A more recent study reported the results of TP53 mutational profiles in a cohort of 506 primary DLBCL patients treated with R-CHOP and reached interesting conclusions.[49] The incidence of TP53 mutations was 21% and there was no difference between GCB and ABC subgroups. The survival was significantly better for patients with wild-type TP53 compared with mutated TP53 for both subtypes. TP53 was an independent factor of worse prognosis and progression free survival, along with an IPI score of more than 2, the ABC subtype and B symptoms.

p21, a cyclin-dependent kinase inhibitor, negatively regulates cell cycle progression and cellular proliferation. Although it is a downstream effector of p53, its expression is also determined by p53-independent mechanisms. In lymphoid malignancies, decreased p21 expression has been associated with poor outcomes in acute lymphoblastic leukemia and with aggressive mantle cell lymphoma variants.[50] High p21 expression was correlated with better outcomes in DLBCL patients treated with R-CHOP.[51] Furthermore, in the same study was found that the advantage of anti-CD20 antibody's addition to therapy was more pronounced in p21-positive patients.[51]

Multiple studies used strong TP53 nuclear staining associated with absent TP21 staining as an immunohistochemical surrogate for mutated TP53. The addition of p21 staining seems to improve the prognostic value of p53 expression.[48,51]

BCL2

BCL2 is an antiapoptotic protein important for normal B-cell development and differentiation. The overexpression of this marker has been reported in approximately 40-60% of DLBCL cases. There are different mechanisms of BCL2 overexpression. In GCB subtype, the t(14;18) translocation is responsible for BCL2 up-regulation in most cases. However, this genetic aberration is not present in ABC-like DLBCL.[7,52] In this subtype, BCL2 is up-regulated due to gene amplification or the constitutive activation of the NF-κB pathway.[33] BCL2 overexpression provides a survival advantage to tumor cells, and is believed to play an important role in resistance to chemotherapy.

BCL2 has been extensively studied as a prognostic factor in DLBCL. In patients treated with chemotherapy, some investigators found an association between BCL2 overexpression and a worse outcome in ABC-like tumors, but not in the GCB subgroup.[53,54] In the rituximab's era, some studies concluded that immunochemotherapy overcame the adverse influence of BCL2 in DLBCL prognosis,[55,56]

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whereas other studies correlated a poor outcome to BCL2 mRNA[57] or protein expression in R-CHOP-treated cohorts.[58]

More recently, a study evaluated the prognostic significance of BCL2 within each DLBCL subtypes defined by GEP in patients treated with R-CHOP.[59] Researchers observed a significant association of BCL-2 expression with poor overall survival and event-free survival in GCB-DLBCL, but not in the ABC subgroup, concluding that BCL2-positive ABC subtype had greater benefit from the addition of anti-CD20 antibody to chemotherapy than BCL2-negative tumors. Thus, immunotherapy apparently narrowed the differences in survival in the ABC subgroup. However, the opposite was observed in GCB DLBCL, with BCL2-positive tumors benefiting less from immunotherapy than BCL2-negative tumors, resulting in a significant difference in patient survival.[59] This finding may be explained by the differential mechanisms of BCL2 overexpression in each subtype and the molecular effects of anti-CD20 antibody.[60] This therapeutic molecule downregulates the NF-κB pathway, which is the most prominent BCL2 upregulation mechanism in ABC tumors, contrarily to GCB DLBCL, in which the t(14;18) translocation has a dominant role in BCL2 expression. The decrease of BCL2 expression by anti-CD20 antibodies may improve tumor cells' susceptibility to chemotherapy.[59,60] Therefore, the mechanisms of BCL2 overexpression possibly determines its value as a prognostic and predictive biomarker.[61]

BCL6

As mentioned above, the BCL6 proto-oncogene is necessary for germinal center (GC) formation and for T-cell-dependent antibody response, and protects cells from DNA-damaged induced apoptosis by suppressing TP53 expression and other proteins involved in damage response.[27,28] It is one of the key genes of the GCB signature and it is expressed in non-Hodgkin lymphomas that arise from GC B-cells. However, not all BCL6-positive cases are assigned to GCB-like IHC category of DLBCL. BCL6 expression may be dysregulated by rearrangements or mutations affecting the promoter region or the 5'nontranslated regulatory region. These rearrangements were observed in 30-40% of DLBCLs, and somatic point mutations were detected in 50-70%. One of the most frequent translocations is the t(3;14)(q27;q32), involving the immunoglobulin (IG) heavy chain gene.

BCL6 has been identified as one of the strongest predictors of outcome in DLBCL. Various studies using IHC and polymerase chain reaction showed that BCL6 protein expression alone or in combination with other GC markers predicts a favorable outcome in DLBCL patients treated with CHOP.[62-64] However, when the anti-CD20 antibody is add to chemotherapy, BCL6 loses its prognostic value.[64] Some investigators suggest this finding may indicate that the outcome improvement of DLBCL is primarily due to the beneficial effect of anti-CD20 antibody in the BCL6-negative subset of DLBCL.[64]

MYC

MYC is a transcription factor responsible for controlling numerous genes involved in cell cycle regulation, metabolism, protein synthesis, stress response and DNA repair. Its function is exerted by dimerization with MAX and subsequent binding to specific DNA sequences called "E-Box".[65] Additionally, MYC is involved in micro-RNA expression regulation.[66] Curiously, MYC expression is lower in the germinal center than in naive and memory B cells, possibly diminishing MYC-induced genomic instability in GC cells.[67]

Genomic abnormalities of the MYC gene include chromosomal translocations, mutations in regulatory sequences and promoter regions, and gene amplifications. MYC overexpression promotes cellular growth and proliferation.[67] MYC dysregulation occurs most commonly in Burkitt lymphoma, resulting from rearrangement with the immunoglobulin heavy locus, and it is considered as the lymphoma-initiating event.[67] In DLBCL, MYC rearrangements are present in 5-10% cases with typical morphology, and have been associated with poorer outcome in patients treated with immunochemotherapy.[68,69] The WHO updated classification harbors a new category defined as "B-cell lymphomas, unclassified, with features intermediate between DLBCL and Burkitt lymphoma".[70] 35 to 50% of these tumors have a MYC translocation, and morphologically are characterized by a mixture of medium to large-sized cells and a high proliferation rate.[70]

In one study, which assessed the prognostic significance of several markers (including MYC, BCL2, BCL6, p53, p21 and FOXP1), MYC rearrangement was the only biologic parameter that retained prognostic impact in DLBCL treated with R-CHOP, and it was independent of the IPI.[68] In other research, MYC rearrangements were also associated with higher risk of central nervous system (CNS) relapse.[69]

Lymphomas with multiple activated oncogenes, one of them being MYC, are often referred as "double hit" (DH) lymphomas.[67] DH lymphomas that harbor both MYC and BCL2 translocations

(MYC+/BCL2+) are the most common and have received special attention from investigators. These lymphomas have a particular aggressive clinical behavior and often complex karyotypes. MYC+/BCL2+ lymphomas have an extremely poor outcome, with a median survival of less than 1 year.[67] Physicians have experienced some difficulties in classifying many DH cases, and since they comprise a subset of extremely aggressive lymphomas, it was considered that they should be separated from other lymphomas. Hence, they are now fitted in the "B-cell lymphomas, unclassified, with features intermediate between DLBCL and Burkitt lymphoma" category. Whether to include morphologically regular DLBCL with MYC+/BCL2+ DH also in this category is still in discussion.

The importance of MYC and BCL2 simultaneous deregulation has been evaluated in a cohort of DLBCL patients treated with R-CHOP.[71] Only 5% of cases had concurrent MYC and BCL2 translocations, and their clinical outcome was extremely poor, with a 5-year overall survival of 27% . All of these tumors expressed BCL2 protein, and 71% expressed MYC protein. Overall, MYC protein expression was found in 33% of patients, whereas MYC translocation was present in 12%, suggesting the existence of other mechanisms that result in MYC up-regulation. Surprisingly, high MYC protein expression, high MYC mRNA expression and presence of MYC translocation, were only associated with an inferior outcome when BCL2 protein was coexpressed. In fact, presence of MYC+/BCL2+ protein expression predicted significantly inferior overall survival and progression free survival, even after adjusting for IPI, cell of origin and presence of MYC and BCL2 concurrent translocations.

LMO2

LIM domain only 2 (LMO2) encodes a transcription factor involved in regulation of crucial events like erythropoiesis, angiogenesis and embryogenesis.[72,73] Experiences in mice suggest that LMO2 plays a role in the development of all bone marrow-derived hematopoietic lineages.[74] Chromosomal translocations and point mutations of this gene, causing deregulation of LMO2 expression, were found in lymphoid and myeloid leukemia.[75] In DLBCL, LMO2 overexpression has been associated with the GCB subtype, and it is considered a marker for GCB differentiation stage, although its function in these cells and genomic aberrations linked to its overexpression haven't yet been elucidated.[6,76]

In a multivariate model based on the expression of six genes, LMO2 mRNA expression emerged as the strongest single predictor of favorable outcome in DLBCL patients submitted to CHOP or CHOP-like regimens.[77] The addition of the anti-CD20 antibody to standard therapy apparently hasn't changed the prognostic value of LMO2 in these patients. Several studies showed a significant and independent role of LMO2 expression (assessed by IHC) in predicting a superior outcome in patients treated with R-CHOP.[78,79]

KI-67

Ki-67 is a nuclear antigen expressed by dividing cells, reflecting the proportion of cells that are actively proliferating. It has been considered an useful prognostic index in various malignancies, including non-Hodgkin lymphoma.[80] In DLBCL, the prognostic significance of Ki-67 was controversial in the pre-rituximab era.[81-83] Studies in cohorts of patients treated with R-CHOP showed an adverse effect of high Ki-67 expression in the overall survival and progression free survival.[80,84] However, a consensual cut-off to differentiate "high" versus "low" Ki-67 expression was not defined.

HIF-1α

Hypoxia-inducible factor 1α (HIF-1α) is an important regulator of gene transcription: it controls the expression of more than 200 genes in response to cellular hypoxia, affecting numerous cellular processes, like metabolism, proliferation, apoptosis, glucose transport, angiogenesis, vascular tone and erythropoiesis.[85] HIF-1α is an oxygen sensitive subunit that heterodimerizes with the HIF-β subunit (forming the HIF-1 transcription factor) to bind DNA. In non-hypoxic conditions, HIF-1α is continuously synthesized and degraded, preventing HIF-1 stabilization. When hypoxia occurs, HIF-1 stabilizes and enhances glucose uptake and angiogenesis.[86]

High HIF-1α expression has been associated with high tumor grade and poor prognosis in solid tumors.[87] A group of investigators found a constitutive stabilization of HIF-1α in many non-Hodgkin lymphoma cell lines, including a significant proportion of patients with DLBCL, indicating a possible role of HIF activation in this disease.[88] However, in a subsequent study, the same researchers found an improved outcome in DLBCL patients treated with R-CHOP that had an increased HIF-1α expression, but not in those treated with CHOP.[89] Interestingly, some of the HIF-1α target genes found in this study are part of the favorable stromal-1 gene signature defined in a previous research.[17] Previous studies concluded that CD20 and HIF-1α were both regu-

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lated by reactive oxygen species.[86,90] Thus, a possible association between CD20 and HIF-1α could explain the prognostic significance of this marker in rituximab's era, but this hypothesis needs further investigation.

MHC MOLECULES

The major histocompatibility complex (MHC) is an important component of the cellular immune response. Whereas MHC class I proteins (human leukocyte antigen-A [HLA-A], HLA-B and HLA-C) are expressed in all nucleated cells, MHC class II proteins are constitutively expressed only in professional antigen-presenting cells, such as B lymphocytes, monocytes and dendritic cells. Class II proteins include three classical (HLA-DR, HLA-DQ and HLA-DP) and two nonclassical molecules (HLA-DO and HLA-DM), and the respective genes have similar regulatory regions. Although these proteins have an important role in eliciting immune responses, they are only a part of a large pathway involving B- and T-cell interactions.[91]

As mentioned above ("Tumor microenvironment"), four gene-expression signatures categorize the most predictive genes found using DNA microarrays: the "proliferation", the "MHC class II", the "lymph node" and the "GCB" signatures. The "MHC class II" and the "lymph node" signatures suggested that the host response to lymphoma cells might be an essential determinant of survival.[7] The MHC class II signature, in particular, led to the hypothesis of a possible role of antigen's presentation to the immune system in therapeutic responses, since loss of MHC class II was significantly correlated with poor outcome in patients treated with chemotherapy.[7] More recently, investigators assessed the prognostic value of MHC class II expression in patients that received immunochemotherapy. Results were consistent with previous findings, suggesting a prognostic value of MHC class II molecules in such patients.[92] Some researchers think that the importance of lost MHC class II is related to loss of tumor immunosurveillance, suggested by decreased numbers of tumor-infiltrating T-cells.[91] In fact, deletions of MHC genes appear particularly frequent in DLBCL affecting immune-privileged sites, namely the testis and the CNS.[93] The lack of HLA class I and II proteins expression allows tumor cells to escape the immune attack and to proliferate.

MHC class II gene expression is controlled by several transcription factors, which interact with the class II transactivator (CIITA) to form an enhanceosome complex. CIITA has been considered the master regulator of MHC class II gene transcription, and downregulation of this protein is one of the mechanisms causing loss of MHC class II expression.[94]

In a genome-coding analysis, frequent inactivating mutations and deletions were found in the β2-microglobulin (B2M) gene in DLBCL cases.[95] B2M encodes a polypeptide that, together with an α heavy chain, forms MHC class I molecules on the surface of all nucleated cells. The non-expression of B2M leads to impaired recognition of the tumor cells by cytotoxic T lymphocytes.

CONCLUSION

Several biomarkers showed prognostic relevance in DLBCL patients treated with the anti-CD20 antibody, and some of them have deserved special attention by investigators: cell of origin and tumor microenvironment are two promising indicators for future prognostic models, with ABC subtype and stromal-2 signature being markers of poor prognosis; LMO2 seems a reliable indicator of good prognosis; BCL2 and MYC overexpression showed a reproducible negative effect on these patients' overall and progression free survival. The main limitations of most of these studies were small cohorts and lack of standardization of the methods and cutoffs used to quantify the biomarkers' expression. The discrepant results obtained and the difficulty to show consistent prognostic value of individual biomarkers can also be explained by the complexity of DLBCL molecular biology.

Although considerable progress has been made in molecular prognostication of DLBCL, many prognostic biological factors, especially those with a negative impact, need to be independently validated in prospective cohorts so that they can justify a different therapeutic approach. Various of these biomarkers and cellular pathways appear as new treatment opportunities. Therapies targeting the NF-κB pathway (bortezomib),[96] B-cell receptor signaling (enzastaurin)[97] and BCL2 (ABT-737, obatoclax)[98,99] are under investigation. Modulation of the tumor microenvironment is a less explored treatment opportunity, but holds great promise.[60] Early results of some of these new target therapies are impressive, and provide hope that DLBCL patients will have greater survival chances in the future by selection of individualized treatment regimens.

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