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Usefulness of Immunohistochemistry in Identification of Prognostically Important Subgroups (GCB and ABC) in a Heterogeneous Group of Diffuse Large B-cell Lymphomas – a Review Article

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Although diffuse large B cell lymphomas (DLBCL) are considered in the WHO classification a specific histopathological type, their diversity in the clinical features, morphology and molecular aberrations strongly suggest that these tumors represent a heterogeneous group of neoplasms rather than a single clinicopathological entity. There have been various approaches to differentiate between separate nosological entities within DLBCLs based on various methods, such as the microarray technique or immunohistochemistry. Although it has been proven that gene expression profiling using cDNA microarrays could identify prognostically important subgroup of DLBCL: germinal center B-cell (GCB)-like DLBCL and activated B-cell (ABC)-like DLBCL, this method is impractical as a clinical tool. Therefore, investigators have started using immunohistochemistry in their studies. Employing various immunohistochemical antibodies, such as CD10, CD138, anti-Bcl-2, anti-Bcl-6, MUM1 and anti-p53, several groups have aimed at subclassifying DLBCL into the GCB and ABC subgroups with comparable differences in clinical behavior. This review summarizes these data and indicates their impact on DLBCL classification.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is one the most common subtypes of non-Hodgkin's lymphomas of adults and accounts for approximately 40% of cases [14]. It is a clinically, morphologically and genetically heterogeneous group of tumors [14]. Because of their heterogeneity, there have been many attempts at subclassifying DLBCLs. The first, Kiel classification subdivided the DLBCL group

according to morphological features into centroblastic and immunoblastic lymphomas [36]. The second classification system, termed the Working Formulation, subdivided this group of neoplasms according to their biological behavior into intermediate-grade and high-grade categories [27]. Another system the "Revised European-American Lymphoma" (REAL) classification, made a distinction based on the presence of determined morphological, immunophenotypic, genetic, and clinical features [17]. This classification was largely adopted by the World Health Organization (WHO) in 2000 and has been in effect to this day [16]. WHO listed several clinical entities, such as primary mediastinal large B-cell lymphomas (PMBCL), intravascular large B-cell lymphomas, primary effusion lymphomas (PEL) and several morphological variants [16]. Although in the present classification, DLBCL is considered a specific histopathological type, its diversity in clinical features, morphology, genetic and molecular aberrations strongly suggests that these tumors represent a heterogeneous group of neoplasms rather than a single clinicopathological entity. Therefore, WHO plans to present suggestions for changes in the classification in 2008 and add other clinical subtypes (www.eahpweb.com).

The heterogeneity of DLBCL is well reflected by the clinical course of the disease. Forty per cent of patients with diffuse large B-cell lymphomas respond well to current therapy and show long-term survival, but at least 50% of these patients relapse after conventional therapy [28]. The most effective tool for predicting the prognosis of patients with DLBCL is the International Prognostic Index (IPI) [1], which identifies subgroups of patients with very poor or good outcome. Although the importance of IPI has been validated in many clinical trials [1, 6], it is the only clinical marker that does not rely on morphological features. The

index alone is insufficient to distinguish between patients who will be cured with conventional therapy and those who will have refractory or relapsing disease and need more aggressive, alternative methods of treatment. Differentiating between various categories of DLBCLs could help in identifying prognostically important groups of patients with a distinct clinical course of the disease and predictable outcome. It is possible that alternative therapeutic strategies may be effective for some of these patients. The ability to identify these patients is very important.

GCB-like and ABC-like DLBCL

During the last decade, there have been numerous studies, which have tried to define new clinicopathological categories of DLBCL on the basis of morphologic features, expression of proteins or molecular aberrations.

In a precursor study in 2000, Alizadeh et al. suggested that DLBCL could be divided into prognostically significant subgroups according to gene expression profiling by cDNA microarray [2]. Their study showed diversity in gene expression among DLBCLs, identified molecularly distinct forms of DLBCL, which had gene expression patterns indicative of different stages of B-cell differentiation. One type demonstrated gene expression characteristic for germinal center B-cell and was called germinal center B-cell (GCB)-like DLBCL, the other showed gene expression normally induced during *in vitro* activation of peripheral blood B-cells and was termed activated B-cell (ABC)-like DLBCL. Patients with GCB-like DLBCL demonstrated significantly better overall survival rates as compared to those with the ABC-like subgroup [2]. This prognostic significance was independent of IPI, which was further confirmed in other studies [2, 32, 40]. The type 3 gene expression profile (a group of unclassified cases), described by Alizadeh et al. was a heterogeneous and poorly described subtype [2]. This type was associated with a poor outcome similar to the ABC group [2, 32], and was later classified as the non-germinal center group [32].

The results of the study of Alizadeh et al. were confirmed by a few other investigations [32,34,40], which divided DLBCLs into two molecularly distinct subgroups: GCB and non-GCB (or curable and fatal/refractory) [34], and demonstrated that characteristic gene expression profiles could also predict the outcome in DLBCL patients after chemotherapy [32].

Different oncogenic mechanisms

Further studies showed that these two molecularly distinct subgroups (GCB-like and ABC-like) of DLBCL were associated with different underlying oncogenic mechanisms.

In the GCB-like subgroup, the most frequent molecular aberration is translocation with involvement of the Bcl-2 gene t(14;18)(q32;q21), which occurs almost exclusively in this subgroup. The Bcl-2 gene rearrangement was reported to occur within both groups (GCB-like and ABC-like), but with a varying incidence rate [18, 20, 32].

Activation of the nuclear factor kappa B (NF- κ B) pathway is involved in the oncogenic mechanism in the ABC-like subgroup [37]. One of the NF- κ B target genes is MUM1 [26] (described later). Inhibition of the NF- κ B pathway in cell lines with the ABC-like phenotype resulted in an increased sensitivity to chemotherapy [26], which indicates that subclassification of DLBCL could be also important for the selection of treatment.

Immunohistochemistry as a tool for classifying DLBCLs

Although it has been shown that gene expression studies using cDNA microarrays could identify prognostically important subgroups of DLBCL [2, 6, 32, 40], this technology is expensive, generally unavailable and impractical as a clinical tool. In an attempt at finding a simpler and more reproducible method of DLBCL subclassification, the investigators started using immunohistochemistry in their studies. This method is more available and subclassification of DLBCLs made on the basis of immunohistochemistry could have a much wider medical application.

Using various immunohistochemical antibodies, such as CD10, CD138, anti-Bcl-2, anti-Bcl-6, MUM1 and anti-p53, the investigators tried subclassifying DLBCLs into the GCB and ABC subgroups with comparable differences in clinical behavior [3, 4, 5, 7, 8, 15, 26]. In the majority of these studies, the authors used CD10 and Bcl-6 as the germinal center B-cell-like group markers, and MUM1 as the activated B-cell-like group marker [3, 7, 15].

Algorithm allowing for DLBCLs subclassification

Hans et al. [15] suggested an algorithm (Fig.1) based on CD10, Bcl-6 and MUM1 expression identifying the GCB and ABC subgroups of diffuse large B-cell lymphomas, which were previously categorized in these subtypes by the microarray expression analysis.

CD10 (common acute lymphoblastic leukemia antigen = CALLA) is a proteolytic enzyme expressed on the surface of various cells; however, in reactive lymphoid tissue, its expression is restricted to germinal center cells [9, 22]. It is expressed in approximately 30 to 40% of DLBCL cases [22, 29]. The staining is membranous and usually homogeneous throughout the tumor. CD10 immunopositivity does not depend on the anatomical site of the tumor and is

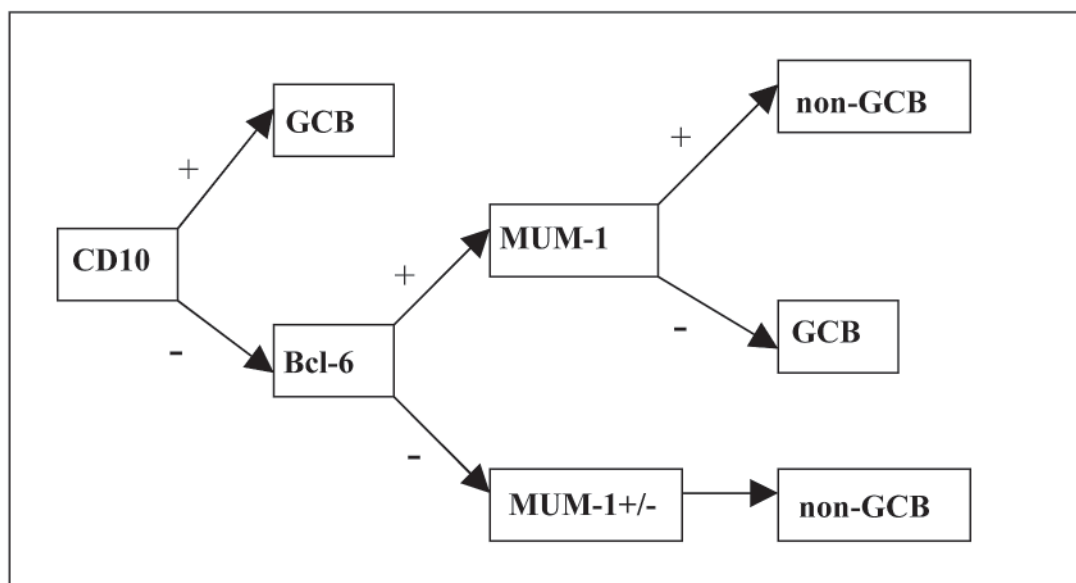


Fig. 1. The immunohistochemical algorithm for identification of prognostically important subgroups of DLBCL proposed by Hans et al.

associated with no specific clinical features [9]. Several authors have examined the prognostic significance of CD10 in DLBCLs with controversial results. Some of these studies reported an association between CD10 reactivity and a better prognosis [3, 4, 7, 15, 29]. In other investigations, no difference in the outcome between CD10+ and CD10- cases was found [8, 10]. These conflicting results and the variability in outcomes demonstrated in various studies suggest that using CD10 alone in predicting survival in DLBCLs is of dubious value.

Bcl-6 protein, used as the germinal center marker, acts as a transcriptional repressor and represses genes involved in lymphocyte activation and differentiation, cell cycle control and inflammation [33]. In normal lymphoid tissue it is expressed in a germinal center of B cells and in a small subset of CD4+ T cells [11]. Positive staining for Bcl-6 has been observed in the majority of DLBCLs, ranging from 50% to 70% of tumor cells, including both nodal and extranodal sites. Typically, the staining is strictly confined to the nucleus, sparing the nucleolus [30, 35]. Considering the fact that a variable number of tumor nuclei were positive, the investigators used a different cut off value (from 10% to 30%) for positive staining. The value of 10% was most commonly selected [4, 9] but there were studies, which showed that this level might be too low to allow for subdividing DLBCLs into appropriate and reproducible subgroups of patients [15]. The prognostic significance of Bcl-6 expression is still unclear and a limited number of immunohistochemical studies has been carried

out that examined the relationship between expression of this antigen in tumor cells and patient outcomes. The results of the investigations studies are conflicting. These differences could be explained by the differences in the cut off value and staining techniques and may be related to heterogeneity of the examined group of patients. In addition, there is also a suggestion that variability of biological consequences of Bcl-6 expression might depend on the presence or absence and the type of the underlying aberration of the Bcl-6 gene [9]. Interestingly, expression of Bcl-6 protein does not correlate with the presence or absence of the Bcl-6 gene rearrangement [30, 35].

It is preferred to associate Bcl-6 with other markers and use the three-marker model proposed by Hans et al. [15]. This model consists of CD10, Bcl-6 and MUM1 and is useful in identification of the GCB and ABC phenotypes with a significant prognostic value.

The ultimate of these markers - MUM1 (multiple myeloma oncogene 1) protein - is a lymphoid-specific member of the interferon regulatory factor (IRF) family of transcription factors, which play an important role in the regulation of gene expression in response to interferon and other signaling cytokines [12]. In normal lymphoid tissues, the MUM1 antibody shows nuclear staining in small subsets of Bcl-6 negative germinal center cells, in plasma cells and in some activated T cells [12, 38]. MUM1 is expressed only in lymphocytes. Its expression might denote the final step of intra-germinal center B-cell differentiation and following steps of B-cells maturation towards plasma cells [12, 38].

In DLBCLs, MUM1 was reported in 50% to 75% of cases [12]. The prognostic significance of MUM1 expression alone in DLBCL patients is limited. Hans et al. reported that expression of MUM1 in at least 30% of tumor cells was associated with a significantly worse outcome [15]. The results of other studies provided contrary information, since no correlation between MUM1 expression and overall survival was found [4, 8].

Varying results of many studies suggest that none of these markers alone constitutes a factor of a statistically important prognostic value, but determination of all these markers together (according to the algorithm proposed by Hans et al.) [15], provides a useful tool in identification of important subgroups of DLBCL.

According to many studies, this three-marker model, where Bcl-6 and CD10 are the germinal center markers and MUM1 is immunopositive in postgerminal center cells, is useful in identification of the non-GCB or ABC phenotype that is thought to have a strong prognostic significance for patients with DLBCL. However, there are studies, the results of which do not confirm this theory and whose authors did not find any significantly prognostic impact [8]. Thus, the usefulness of immunohistochemistry performed in formalin-fixed and paraffin-embedded tissue specimens in identification of the GCB and ABC subgroups by using only three markers: CD10, Bcl-6 and MUM1, is still under debate.

New immunohistochemical algorithm

A new algorithm (Fig.2) based on Bcl-2, CD10 and MUM1 expression, related to each subtype of DLBCL was proposed [26]. The authors of this study, Muris et al. [26], suggested that this algorithm had a stronger prognostic value.

Bcl-2 is an antiapoptotic protein, located on the outer membrane of mitochondrion. Bcl-2 is commonly expressed in normal lymphoid tissues, but does not occur in germinal center B-cells [9]. Its expression is found in 30-60% of DLBCL cases, more frequently in nodal rather than in extranodal tumors [31].

There are two different oncogenic mechanisms of the Bcl-2 gene overexpression in both DLBCL subgroups. In GCB-like DLBCLs, a translocation t(14;18)(q32;q21) is frequently detected. Its consequence is the replacement of the Bcl-2 gene under the control of the heavy-chain immunoglobulin gene, and overexpression of Bcl-2. The second mechanism, which occurs in 30% of DLBCLs (predominantly in the ABC-like subtype), is amplification of the BCL-2 gene [25, 31].

The prognostic value of Bcl-2 expression has been the subject of many studies with incompatible results. Several studies reported that high expression of Bcl-2 was a poor prognostic factor [3, 4, 21, 23], but there were reports, in which the investigators did not find any statistically sig-

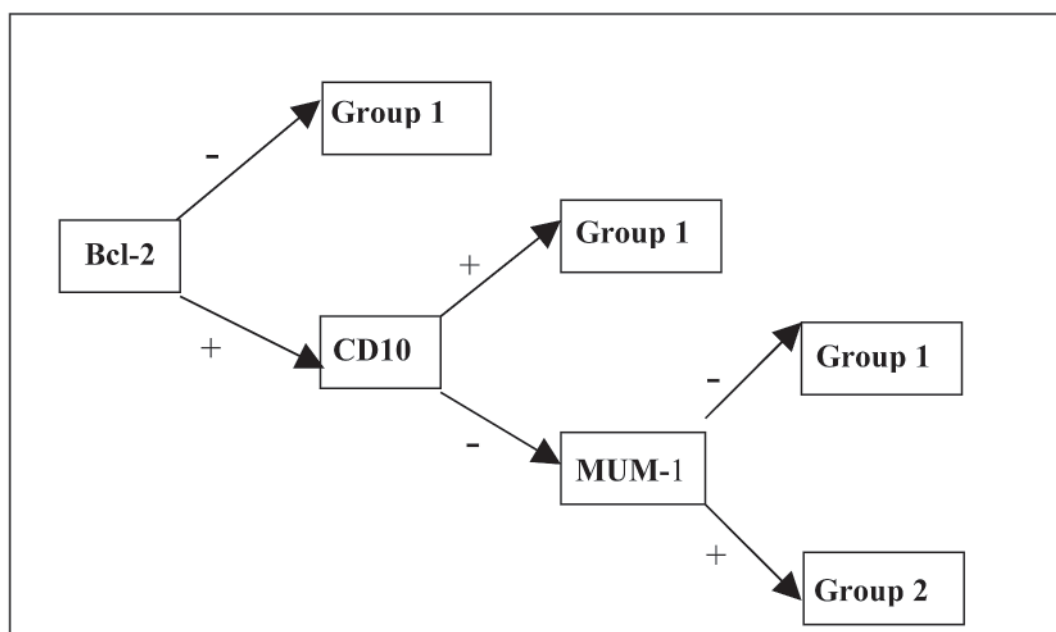


Fig. 2. The alternative algorithm based on expression of Bcl-2 proposed by Muris et al.

nificant differences in overall survival among cases with or without expression of Bcl-2 protein [23, 41]. These conflicting results could be a consequence of the absence of the determined cut-off value. The investigators did not use the same criteria to classify the Bcl-2-positive or negative cases and the fluctuations of the cut-off point ranged from 10% to 50%. Most studies indicated that higher cut-off value (>50% of positive tumor cells) increased the significance of marker expression. Higher expression was associated with a worse prognosis for the patient [9].

Other immunohistochemical markers

Because of the diversity of results in various studies, subclassification of DLBCL into prognostically important subgroups has proven to be a multifaceted problem. The inconsistency of diagnostic criteria has made it impossible to separate the subtypes of DLBCL in routine tests. Therefore, the investigators needed to find a panel of specific markers for both the GCB and ABC group, and define a diagnostic standard for pathologists. The ABC-like group has shown unique immunostaining for MUM1. The studies demonstrated that CD138 (syndecan-1) is another marker of post-germinal center differentiation, specific for later stages of this process. It is expressed mainly in normal and malignant plasma cells and in lymphoplasmacytoid cells. CD138 is a cell surface adhesion molecule that can bind a variety of cytokines and modulate their activity, as well as the activity of extracellular matrix components; it also participates in numerous processes, such as cell proliferation, cell migration, cell-matrix and cell-cell interactions [9]. This marker is not very common in DLBCL cases and the significance of CD138 expression in tumor cells has not been sufficiently examined and described yet. In a few studies, its expression was associated with worse patient survival [4].

Obviously, the most frequently mutated gene in human neoplasms and its product – p53 protein - were also subject to observations in DLBCL cases. Mutations in the TP53 gene were detected in 20% of DLBCL cases [24] and associated with drug resistance [39] and poor outcome [24]. Normally, immunohistochemical detection of p53 is impossible because of its short half-life (20 minutes), but in damaged cells, p53 undergoes post-transcriptional modifications, which may extend its half-life and render p53 protein detectable. Positivity for p53 is usually defined as > 5 – 15% of immunostained nuclei. The clinical significance of p53 expression in DLBCL cases is not clear. The investigators analyzed p53 expression with others markers. For example, Barrans et al. [3] examined p53 aberrations and bcl-2 expression in tumor cells. Their results showed that a combination of these markers was characteristic for patients with a very poor outcome [3].

All the studies have provided us with more information about DLBCL, but not enough to change the classification and divide this heterogeneous group of lymphomas into prognostically significant subtypes or even to define new diagnostic criteria.

Relationship between the GCB and ABC phenotype and morphological variants

Immunohistochemical markers have not been the only subjects of intensive studies aiming at creating a new subclassification of DLBCLs. The investigators tried to find a correlation between the GCB and ABC phenotype and morphological variants defined in the WHO classification. In the study carried out by Alizadeh et al, the investigators did not find any statistically significant relationship between different variants defined by the WHO classification and the GCB or ABC phenotypes identified by a distinct gene expression profile [2], but a certain tendency was observed.

The most frequent centroblastic variant is composed of large lymphoid cells with oval to round nuclei, 2-4 membrane-bound nucleoli and scanty amphophilic cytoplasm. This variant may have a monomorphic or polymorphic appearance [14]. In the centroblastic variant, the GCB phenotype was most frequently observed. The immunoblastic variant of DLBCL was associated with the ABC phenotype [32]. Further studies confirmed this tendency – the correlation between GCB immunophenotypic profile, centroblastic morphology and MUM1 immunopositive cases, characteristic for the ABC phenotype, and immunoblastic morphology [8].

Rituximab could mitigate changes between GCB and ABC

The conventional treatment of DLBCL is anthracycline-based (CHOP) chemotherapy. Recently, anti-CD20 monoclonal antibody (rituximab) has been added to this regimen and found to improve failure-free survival. The subclassification of DLBCL into the GCB type and ABC type using gene expression profiling or immunohistochemical staining was achieved made before the era of immunochemotherapy. It was reported that the GCB type of DLBCL showed a significantly better overall survival than the ABC type; however, the patients were treated with the CHOP regimen without rituximab. Recently, studies suggesting improvement in clinical outcome in non-germinal center type of DLBCL after addition of rituximab to the CHOP regimen have been published [13, 19]. With the well-accepted addition of rituximab to the typical large B-cell lymphoma chemotherapeutic regimen, a revalidation

of any survival differences between large B-cell lymphoma subtypes is necessary [19]. So far, only a few studies have been published and we need more investigations in this field.

Conclusions

Although DLBCL is considered a specific category in the WHO classification, the diversity of its clinical features, cell morphology, genetic and molecular aberrations shows that these tumors represent a heterogeneous group of neoplasms rather than a single clinicopathological entity. There have been various approaches to discriminate between nosological entities within DLBCLs based on different methods, such as the microarray technique or immunohistochemistry. Although it has been reported that gene expression profiling using cDNA microarrays could identify prognostically important subgroups of DLBCL, the method has proven to be impractical as a clinical tool in everyday practice. Thus, the investigators paid more attention to a cheaper, simpler and more available technique - immunohistochemistry. During the last decade, the authors of many studies have tried to suggest new categories of DLBCL on the basis of immunohistochemical staining using expression of numerous different proteins. Unfortunately, these studies have not helped to create a new classification of these neoplasms because of different findings and significant diversity of the results. These prognostic studies were performed in the material from the pre-rituximab treatment era. The recently published investigations suggest that the addition of rituximab to the typical CHOP regimen could mitigate advantages and disadvantages of DLBCL subclassification, thus a revalidation of any survival differences between large B-cell lymphoma subgroups is necessary. To date, we have not had at our disposal any clear criteria and reproducible diagnostic standards to change the classification and we need more studies to define new nosological entities and use this knowledge in daily practice for treatment. Despite the vast body of knowledge on this disease, we still have problems with a reliable and clinically useful classification of DLBCL. Further studies are needed, but we hope that the classification shall be developed soon thanks to the fast development of novel techniques and our increasingly growing knowledge of this disease.

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