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Chapter

DLBCL Subtypes and Prognosis Based on Immunophenotyping

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Abstract

DLBCL is the most common type of NHL diagnosed in the world. It is a highly heterogeneous disease with variable prognosis and is generally managed with standard chemo-immunotherapy and its variations. Immunohistochemistry has been found to be useful method to both sub-classify and to predict prognosis of this disease. IHC utilises various CD markers like CD10, BCL2 and IRF4 to divide DLBCL into GCB and non-GCB subtype. In clinical trials, GCB subtype has been shown to have a better prognosis and a response to treatment when compared to non-GCB subtype. Double hit/double expressor is a newer variant of DLBCL that stains positive for MYC and BCL2 or BCL6 and has been found to do better with more aggressive forms of therapy. Significance of various other CD markers is still largely unknown and further research is required in this area to better elucidate their clinical application.

Keywords: DLBCL, immunohistochemistry, gene expression profiling, CD markers, GCB DLBCL, non-GCB DLBCL, double hit DLBCL, double expressor DLBCL

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) represents an aggressive subtype of Non-Hodgkin’s lymphomas (NHL) that accounts for approximately 40% of such tumours [1]. DLBCL is a highly heterogeneous disease that has an estimated 5-year survival of 69% in some studies [2]. The treatment is generally centred around the CHOP (cyclophosphamide, doxorubicin, oncovin and prednisolone) chemotherapy and its various variations. This therapy has been augmented with the help of different intensification regimens, the addition of newer targeted agents, haematopoietic stem cell transplantation and other measures. With the use of these newer therapies, the prognosis of the disease has improved considerably over the previous decade. However, despite these advances, long-term survival remains poor and many patients relapse failing to respond to either primary or supplementary therapies.

Because of the inherent heterogeneity of the disease and the evolving treatment options, there is an unmet need for prognostic and prediction tools that can tell about the course of the disease and guide us towards appropriate therapy for different subgroups. This led to the use of IPI (International prognostic index) score [1]. This score was derived from various patient characteristics like age, Lugano stage, ECOG PS (Eastern cooperative oncology group physiological status) of the patient,
pre-treatment LDH (Lactate dehydrogenase) value and number of extra-nodal sites of disease. Each variable was given one point and the total score was calculated which helped to stratify the patient into various categories. These categories in turn were used to predict the 5-year survival rates for the patients. New versions of the score were further developed which incorporated the introduction of targeted agents to the chemotherapy regimens which drastically improved the survival of the patients treated. Despite all this, the score retains a degree of subjectivity and was not able to predict appropriate therapy for its “high risk” patients. Hence substantial efforts in research have been directed towards the ultimate improvement of this index and/or towards the definition of alternative methods, which may be used to evaluate risk and clinical outcomes.

To fill this gap many researchers have tried to file other biological, genetic and protein markers to better predict the prognosis and natural history of patients of DLBCL. The most extensive of these studies have analysed genome-wide RNA expression, utilising cDNA microarrays. Various array based studies have categorised DLBCL according to their unique expression profiles. These genetic expression signatures have been linked to ‘germinal centre (GC) cell’, ‘activated B cell (ABC) or non-germinal centre cell (NGC)’ or primary mediastinal lymphoma (PML) phenotypes [3]. These categories have been shown to predict prognosis and outcome of the patients depending on the inherent genetic makeup of the tumour cells. However, attaining intra-laboratory reproducibility, defining consensus of panels of prognostically significant biomarkers laboratory costs of these methods have proven to be a hindrance in its development.

Recently a number of proteomic based studies have tried to extrapolate and add to the findings of array based studies. Many of these have used immunohistochemical stains on tissue samples to identify specific protein antigens. The various algorithms used in clinical practice makes use of primarily three markers to stratify DLBCL into prognostic subgroups. These markers are: the neutral membrane metallo-endopeptidase: CD10, the cellular oncogene BCL6 and the B cell differentiation marker multiple myeloma oncogene MUM1/IRF4. These algorithms help us to predict tumour natural history and prognosis of the patient at the time of diagnosis.

The ultimate aim of these studies remains to discover novel markers and factors which may help us to better stratify DLBCL, which in turn may help to determine prognosis and response to chemotherapy. This will facilitate clinicians to define pre-therapy prognostic subgroups and accordingly alter therapy and improve overall patient outcomes.

2. Genomic expression profiling

As discussed above, researchers have been trying to build different methods and tools to sub-stratify DLBCL and to better understand its underlying pathobiology. Genetic expression profiling studies have shown that different patients have different genetic signatures in various subgroups. These profiles may be relevant to a patient’s response to chemotherapy and overall prognosis. The ultimate aim of genetic expression studies is to provide a unique molecular signature of the of tumour cells, which helps us to identify various subgroups which have a common cell of origin and similar methods of transformation which eventually translates into a common clinical profile. For example, in lymphoma patients GEP has been used to identify various oncogenic mechanism in form of signalling pathways that are active in various lymphoid
malignancies. These pathways serve as novel markers for future targeted therapies that may augment our understanding in the management of these patients.

Various genes have been identified that play an important role in the pathways involved in the proliferation of lymphocytes. It has been observed that lymphomas that are known to have a relatively indolent clinical course have been shown to express low levels of these genes, whereas they are highly expressed in tumours with an aggressive clinical course. In particular, gene expression profiling suggests that DLBCL comprises of at least two distinct diseases arising through distinct pathogenic mechanisms. Thus, GCB subtype of DLBCL shows high expression of genes that are involved in housekeeping function of a normal germinal centre B cell and resting B cell. Conversely, ABC type gene expression signature more commonly mimics activated B cell in peripheral blood. This subgroup has been seen to be commonly associated with BCL2 overexpression and constitutive expression of NF-KB.

Gene expression profiling studies have also shown that the clinical outcome in DLBCL is dictated by a discrete set of biological features that are reflected in tumour gene expression markers. It has been observed that increased expression of genes associated with GCB subtype are associated good response to chemotherapy and better clinical outcomes. In contrast, higher expression of genes linked with proliferation markers depicts a grim prognosis and a poor response to anthracycline based chemotherapy.

However, gene expression profiles are difficult to incorporate in routine diagnoses for various reasons. Firstly, GEP studies require fresh or frozen tissue with adequate amount of RNA for assessment. In the present era of minimally invasive surgeries and radiologically needle based biopsy samples this has indeed proved to be difficult. Secondly, the exorbitant costs for GEP have been a deterrent for its use in routine clinical practice. Lastly, unless a microdissection has been performed for the patient, there is a high chance that the tissue sample will also have a large amount of non-tumour parts. GEP analysis from such portions may not correctly predict the outcome. Therefore, the preferred approach would be to replace gene expression profiling with immunohistochemistry to identify the subtypes in DLBCL patients.

3. Immunophenotyping

Immunophenotyping is a technique used to study proteins expressed by cells. This technique is commonly used in basic scientific research and for purpose of diagnostic in laboratory settings.

Immunohistochemistry (IHC) uses antibodies targeted against certain antigens in specific tissues and helps to determine the type of cells and organ of origin. These antibodies are highly specific in nature and only combine to their corresponding protein in the tissue sample. There are numerous methods to identify the interaction between antigen and its corresponding antibody. The most commonly used method utilises a signalling pathway that gets activated by the antigen-antibody interaction. This pathway in turn cleaves a substrate to produces a chromogenic product which could be easily seen under a microscope. Alternatively, a fluorophore could be conjugated to the antibody and the resultant interaction could be visualised easily with a fluorescent microscopy. The basic method of IHC analysis is as follows.

- Fixing and embedding the tissue
• Cutting and mounting the section
• De-paraffinising and rehydrating the section
• Immunohistochemical staining
• Counterstaining (if desired)
• Dehydrating and stabilising with mounting medium
• Viewing the staining under the microscope.

Immunohistochemistry (IHC) is a very valuable tool that over the past three decades, has revolutionised the practice of diagnostic histopathology. It is widely used to better understand the characteristics of various tumours and to detect occult metastases, especially in the lymph nodes.

Applications in the field of lymphoma are mainly with providing information on the classification and sub-classification of lymphomas in diagnostic sample tissue. Other uses include identification of biological factors of prognostic importance in certain subtypes of non-Hodgkin's lymphoma (NHL) and a role in staging bone marrow (BM) biopsies after diagnosis is established on the primary tissue.

Various algorithms like Hans et al. [4], Choi et al. [5], Muris et al. [6], Nyman et al. [7] are used in clinical practice to classify DLBCL patients into GCB and non-GCB subtypes. Hans criteria is by far the most commonly used in clinical practice. It makes use of three markers namely CD10, BCL6 and IRF4 to classify the DLBCL subtypes. Patients who are CD10 positive are labelled as GCB subtype. If negative, the other markers are subsequently evaluated to further categorise them into GCB and non-GCB subtype. Recently the validity of HANS criteria in predicting the prognosis of the patients has been questioned as multiple variants of DLBCL (double positive triple negative) have been shown to have a different behaviour from their parent subtype.

CD markers or cluster of differentiation markers is a protocol used to identify and evaluate cell surface molecules that provides target for cellular immunophenotyping. Physiologically, CD molecules can function in several ways, often acting as receptors or ligands important to the cell. These CD molecules initiate a signalling cascade in the cell which alters the important physiological functions of the cell. Some CD proteins help in other important cellular functions such as cell adhesion, cell apoptosis and cell differentiation. Human CD markers are 371 in number.

CD10 is a membrane-bound protein that is expressed on the cells of variety of human tissues. Characteristically, it has a limited expression in the reactive lymphoid germinal centre cells [8]. Various small studies have demonstrated that CD 10 along with BCL2 expression in DLBCL is a predictor for inferior survival and poor clinical outcome [9, 10]. However, these studies included only a small number of patients with short clinical follow-up. Chang et al. conducted a study in a patient population in USA and demonstrated that DLBCL patients with a low expression of CD10 with flow cytometry or IHC along with less IPI score had a better prognosis as compared to the other DLBCL subgroups [11]. Similar study conducted by Mclure R F et al. did not find difference in outcome for patients with DLBCL that express CD10 [12]. However, in the study by Colomo et al. [13] the CD10+ cases were significantly more likely to have advanced disease, which may have negated any predictive value of CD10.
expression. Thus, in the current scenario, the retrospective nature of the studies and the contradictory nature of the results makes it very difficult to rely on CD10 alone to predict survival in DLBCL patients.

BCL6 is a zinc-finger protein that acts by downregulating the transcription in the cell and is generally expressed in germinal centre B cells and a subset of CD4 + T cells [14]. BCL6 related gene rearrangements have been detected in 16–37% of DLBCL, but most studies have found no difference in outcome [15]. One study found that BCL6 rearrangement predicted better overall survival, [16] whereas 2 other studies showed that it had a worse OS [17]. BCL6 rearrangement studies failed to identify all DLBCL patients that overexpress BCL6 protein as various other classes of mutations have also been identified that result in BCL6 overexpression [18]. The relationship between IHC positivity of BCL6 expression and clinical outcomes in DLBCL is still not well understood.

According to the Hans algorithm, BCL6 along with CD10 and IRF4 are used to determine the GCB phenotype. However, it was observed in some cases that BCL6 and IRF4 expression had a genetic signature similar to ABC subtype. This is a primary reason behind differences in clinical outcome utilising only BCL6 expression in DLBCL patients.

IRF4 is a lymphoid-specific member of the interferon regulatory factor family of transcription factors [19]. IRF4 is commonly expressed in plasma cells and a small subset of germinal centre cells, and has been reported in 50–77% of DLBCL patients [20]. Chang C C et al. conducted a study that demonstrated that IRF4 expression in at least 30% of tumour cell was associated with a poorer OS and EFS benefit after standard chemo-immunotherapy [21]. Others studies have also found IRF4 to be predictive of worse survival and response to chemotherapy [22]. The expression of IRF4 in tumour cells depicts the final step of germinal centre B-cell differentiation with subsequent maturation to antibody producing plasma cells [23]. Given this biological function, it seems likely that IRF4 has the potential to become a marker for the non-GCB phenotype. In accordance with many algorithms discussed earlier, IRF4 along with CD10 and BCL6, helps to identify the non-GCB subtype of DLBCL [4].

The significance of BCL2 expression in DLBCL is controversial and not well understood. By Southern blot analysis, the presence of BCL2 gene rearrangements did not seem to predict survival, [15, 24] with only one study reporting worse survival [25]. In fact, some studies suggest that patients with BCL2 gene rearrangements have better survival rates [26]. Several studies have shown that BCL2 expression is associated with a poor EFS [27]. In the cDNA study, [28] BCL2 mRNA has increased four-fold in the ABC group (71%) when compared with the GCB group (29%). However, mRNA expression does not always translate into protein expression. Other studies using an immunohistochemical panel that classified DLBCL as GCB and non-GCB groups have also found no difference in the BCL2 protein expression between these 2 groups [29].

CCND2(cyclin d2) protein regulates the difference checkpoints of the cell cycle. In certain research, it was reported that 27% of DLBCL patients express CCND2 [30]. In contrast, another study conducted on a small group of DLBCL patients did not find any evidence of genomic amplification of CCND2 in them [31]. To date no study has evaluated the prognostic significance of CCND2 expression in DLBCL.

A recent study used the co-expression of CD10 and BCL6 markers to determine the “GC phenotype,” and found that it was suggestive of better OS [29]. However, by using this more restrictive approach, some GCB’s with only CD10 and BCL6 expression will be misclassified as non-GCB. While it may be helpful to patients who will
perform better with current treatment, it may be more important to accurately identify patients who will perform poorly in order to provide more aggressive treatment at the time of diagnosis. To accurately sub-classify DLBCL, markers that predict GCB and non-GCB phenotypes should be used. Another study using an immunostaining panel of CD10, BCL6, IRF4, and CD138 was recently published, [13] but found no survival difference between the GCB and non-GCB patients.

The expression of MYC protein is commonly seen in patients of DLBCL, whereby some studies reporting the incidence of around 33% of all patients. Its high incidence suggests that MYC plays an important role in transformation of normal cells to tumour cells. A group of scientists from Denmark tried to evaluate a cohort of 193 cases of DLBCL treated with standard RCHOP regimen [32]. They evaluated the tissue samples with immunohistochemistry and found that 29% had an increased expression of MYC and BCL2. These patients specifically had an inferior OS, when controlled for clinical and molecular prognostic factors, specifically germinal-centre genotype vs. non–germinal centre genotype.

Johnson N A et al. evaluated cases of DLBCL with similar immunohistochemical stains to identify BCL2 and MYC [33]. In this study, it was found that simultaneous expression of MYC and BCL2 was positive in 21% of cases of DLBCL. Furthermore, increased MYC expression in conjunction with BCL2 portends a poor clinical outcome for the patient. These results were confirmed by a similar study after adjusting of clinical and high risk molecular factors. Taken together, protein positivity for MYC and BCL2 (termed double-expressor phenotype) was demonstrated to confer a poorer outcome after standard therapy.

The R-CHOP consortium tried to evaluate the genetic expression signature of 893 patients of DLBCL patients [34]. It was demonstrated that double expressor DLBCL patients had an inferior OS and PFS in both GCB and ABC subtypes of DLBCL. In this analysis, the poor prognosis of the ABC subtype was largely due to MYC expression, resulting in downregulation of genes encoding extracellular matrix proteins, those involving matrix deposition/remodelling and cell adhesion, and upregulation of proliferation-associated genes. Similarly, two randomised controlled trials in Germany failed to sub stratify prognostic subgroups based on cell of origin, whereas concomitant expression of both MYC and BCL2 was seen to have a poor overall survival after standard therapy.

Only a minority of DLBCL patients have basal MYC translocation. It was seen in various retrospective studies that such patients had a particularly poor outcome. Barrans et al. [36] reviewed 303 patients with previously untreated DLBCL, treated with standard R-CHOP chemotherapy. The two-year overall survival rate for the MYC translocation positive group was 35% as compared to 61% in the non-re-arranged group.

In a similar trial conducted in England, British Columbia Cancer Agency evaluated MYC rearranged (33%) DLBCL patients when compared with non MYC rearranged cases (72%) [37]. All patients were treated with RCHOP regimen. They found that 5-year survival in MYC rearranged DLBCL was significantly worse. Also, it was observed that concomitant BCL2 expression and MYC expression had a particularly poor outcome and patients did not achieve remission with the standard RCHOP therapy [38, 39].

Likewise, Vitolo U et al. conducted a trial in Germany using R-Mega CHOEP [rituximab, high dose cyclophosphamide, doxorubicin, vincristine, etoposide, prednisone] in patients of DLBCL. Similar to studies discussed above, the findings reiterated that MYC and BCL2 was associated with a poor overall survival either alone or in
combination [40]. Simultaneous translocations involving both MYC and BCL6 also appear to lead to poor prognosis in patients treated with R-CHOP [26].

Despite the poor prognosis of double expressor phenotype (MYC and BCL2 positive protein expression) being demonstrated in various studies as discussed earlier, definitive evidence does not exist that more intense therapies leads to better responses in these patients. Consequently, this phenotype has not been included in the latest WHO classification of haematolymphoid tumours (5th edition) [41]. The cause for the poor prognosis of this variant is still not known and could possibly be linked to its similarity to the ABC phenotype of DLBCL and the intrinsic poor prognosis of MYC expression in these patients. In future, more studies need to be conducted to better understand the genetic and molecular differences between double expressor and double hit DLBCL that could translate into therapeutic targets for this subgroup.

As discussed earlier, immunophenotyping has proved to be revolutionary in diagnosis and stratification of lymphoma patients. But some shortcomings are still present in these methods which needs to be improved with further research in this area. Firstly, we have already seen that IHC markers used are not specific for each subtype. Except the double hit and expressor phenotypes, literature on rest of the markers is still evolving. The significance of various CD markers is at best vague and informative. Secondly, in a developing country like India, the laboratory infrastructure and lab expertise required for IHC evaluation is still lacking in many centres. Thirdly, the algorithms used sub-classification of DLBCL requires further refinement as many subtypes have varied natural history.

4. Conclusion

DLBCL is the most common type of lymphoma in the world. There has been tremendous advancement in the field of diagnosis and therapy for these patients. But most of these treatments come at a cost of higher financial burden for the patient and more side effects that incur significant morbidity and even mortality. Hence this calls for a reliable tool for prognosis prediction that will help us identify these high-risk patients and guide us to consider them for these new and more aggressive therapies. IHC has proved to be revolutionary for diagnosis of cancer patients. Its role in lymphoma patients is even more important as many of these markers have been shown to have a prognostic significance for the patients. IHC is also proving to be a reliable indicator of prognosis for DLBCL patients and multiple algorithms are already in clinical use that are helping physicians to make decisions regarding therapy. Double hit and double expressor phenotypes are known to have a worse prognosis than other varieties of DLBCL. It has been demonstrated in various studies that patients with double hit phenotypes does better when treated with more aggressive forms of therapy. Other phenotypes like GCB and non-GCB have also shown to be predictive of prognosis although the evidence regarding this is weaker. Newer markers are also being investigated and requires more research for their incorporation in clinical practice.

IHC is proving to be an irreplaceable tool in the armamentarium of clinicians for the management of lymphoma patients. More markers and techniques are being discovered every day that will strengthen the utility of this test in the future. There are still some major gaps in our understanding of the pathophysiology and significance of these markers. This requires further definitive research in this area so that these markers and algorithms can better guide the physicians to choose from the existing therapy options which in turn will improve the prognosis of the disease.
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