RESEARCH COMMUNICATION

Clinicopathologic Evaluation of Subgroups of Diffuse Large B Cell Lymphoma by Immunohistochemistry

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Abstract

Diffuse large B cell lymphoma (DLBCL) has become an emerging epidemic in recent years. Striking heterogeneity in its clinical, biological and treatment responses prompted us to identify variation in our study group. The aim was to classify the DLBCL into prognosis-based subgroups according to the WHO classification and to evaluate their relation to clinical parameters (age, gender, anatomic location and B symptoms), as well as bcl 2 and Ki 67 status. Patients and Methods: A cross sectional study was carried out on 42 DLBCL patients, classified histologically and immunophenotypically into germinal center B cell like (GCB) or non-GCB type. Immunohistochemistry (IHC) was performed using antibodies against CD 10, MUM-1 and bcl 6; additionally anti-apoptotic protein bcl 2 and proliferative marker Ki 67 (using cutoff value of 70%) were also assayed by IHC. Results: Of the total 27/42 (64%) were males and 15/42 (36%) females, with a mean age of 44.1±15 years. 15/42 (36%) cases were of GCB type as compared to 27/42 (64%) of non GCB type. Extranodal involvement and B symptoms were seen in 18/27 (66.6%) and 20/27(74%) of the non GCB type, whereas bcl 2 protein expression and Ki 67 proliferative index (PI) <70% were each noted in 22/27 (81.4%). Conclusion: We document an astonishingly high number of non-GCB type DLBCL in our population. It is alarming to see such an aggressive tumor proliferating in our region. Significant association of non-GCB type with extranodal origin, B symptoms and low Ki 67 PI (<70%) is another concern.

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Introduction

DLBCL is the most commonly observed histopathologic type of B cell non-Hodgkin's lymphoma (B-NHL), accounting for 30% to 40% of all malignant lymphomas in adults (Bea et al., 2005) and 10% in children worldwide (Reiter et al., 2008). In Pakistan it has become an emerging epidemic in recent years (Abid et al., 2005).

DLBCL is an aggressive tumor but thought to be very chemosensitive; when used with Rituximab (a monoclonal antibody targeting CD20 on malignant cells), with significant considerable cure rates (Gisselbrecht., 2008). 40% of patients respond well with prolonged survival; but more than half of the patients who succumb to disease, increased the likelihood of variability in natural history and unrecognized molecular heterogeneity of DLBCL (Alizadeh et al., 2000).

Determination of the biologic behavior of disease, its extent and accurate assessment of responses of different therapeutic regimens; are necessary for appropriate management, disease outcome and further prevention of disease. The distinction of DLBCL according to cell of origin in germinal center (GC) and non-GC subgroups with their prognostic impact; was first time achieved by gene expression profiling (GEP) (Alizadeh et al., 2000; Rosenwald et al., 2002). The GC and non-GC phenotypes are said to be the predictors of outcome in DLBCL and can be used to stratify chemotherapy-treated patients into low- and high-risk groups (Nyman et al., 2007). The non-GC phenotypes of DLBC have inferior outcome with CHOP (cyclophosphamide/doxorubicin/vincristine/prednisone) based therapy (Shipp et al., 2002).

Due to the advancement in the field of IHC; the detection of different proteins can be achieved by modern immunohistochemical techniques (de-Jong et al., 2009). The fact that DLBCL subgroups can be classified immunohistochemically and can also predict survival similar to the cDNA microarray by using three markers; CD10, bcl 6 and MUM-1, has made this approach more applicable clinically (Hans et al., 2004). Though IPI predicts the outcome in DLBCL patients; a combination of clinical parameters along with biological markers; i.e. expression of bcl-6, bcl-2, CD10, MUM-1, major histocompatibility complex class II, and categorization as GCB type have also been described to predict the patient's response to therapy and survival (Veelken et al., 2007).

The varied response of DLBCL at the clinical, genetic, and molecular levels opens the research towards the better understanding of disease and targeted treatment. Numerous immunologic markers are now linked to different aspects of tumor biology and outcome, but a very deficient local data in Pakistan is available in this

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regard. Hence we conduct this study to evaluate different subgroups of DLBCL by IHC at our centre and to analyze the clinical parameters [age, gender, anatomic distribution (extranodal vs. nodal) and B symptoms] and status of anti-apoptotic protein bcl 2 and proliferative marker Ki 67, for optimal stratification of DLBCL patients related to these subgroups in our study group.

Materials and Methods

Case selection

The study was conducted at the Histopathology section of Dow Diagnostic Reference and Research laboratory (DDRRL) at the Dow University of Health Sciences (DUHS), which covers the major areas across Karachi. The laboratory also gets the referral cases from outside city for further refinement of diagnosis. A cross sectional study on 42 patients of DLBCL was carried out; which were previously diagnosed by IHC according to WHO classification within period of 2.8 years (October 2008 to June 2010). The required clinical information regarding age, gender, anatomic location and presence/absence of B symptoms (i.e. fever, weight loss; severe itching & drenching night sweats) was available with each case for evaluation. The patients who had one or more than one symptoms at the time of diagnosis; were declared as having B symptoms. The criteria to declare the primary anatomic location of the cases; international guidelines were used, which was described by others also (Lal et al., 2008). The case was considered as primary extranodal in origin; when no or only one/minor lymph node involvement was seen with predominant extranodal site. When predominantly nodal involvement (International Classification of Diseases of Oncology 3rd Revision; ICD-3, category C77) as well as spleen, thymus and Waldeyer's ring involvement (ICD-3; categories C42.2, C37.9, C14.2) was seen; the case was declared as primarily nodal in origin. Age of the patients was also divided into two groups, that is age less than 60 years (group 1) and equal to and more than 60 years (group 2) (Vose et al., 1988).

Haematoxylin and Eosin Staining

Cases were received in 10% neutral buffered formalin and routinely processed for 12 hours in an automated "Medite TPC 15" tissue processor to embed in paraffin wax. Representative tissue sections of 3 to 4 μm thickness were then cut on a microtome (SLEE 4062) of all the biopsied samples/paraffin embedded tissue blocks for H & E staining. When required, special stains were also used for better histological assessment.

Morphological subdivision of DLBCL was done according to World Health Organization (WHO) classification 2008 (Swerdlow eds.; 2008), as three common morphologic variants; centroblastic (when 90% or more of tumor cells were typical centroblasts), immunoblastic (when 90% or more tumor cells were immunoblasts) and anaplastic variant (when cells were round, oval and polygonal with large to very large in size having pleomorphic bizarre nuclei). The disagreement was resolved by committee meeting of the consultants on multihead microscope.

Immunohistochemistry

Following the procedural protocols, all the tissue sections were treated with primary antibodies against CD 10, bcl 6, MUM-1, bcl 2, and Ki 67 by Envision system with optimal dilutions. The antibodies were obtained from DAKO, Denmark (Abid et al., 2005). For quality assurance the positive and negative controls were run with each batch. The cases were categorized into GCB and non-GCB type according to WHO classification 2008 (Swerdlow eds.; 2008); previously seen by Hans et al (Hans et al., 2004) using three markers algorithm (CD 10, bcl 6 and MUM-1). Cases were assigned to the GCB group when CD10 alone was positive or both bcl 6 and CD10 were positive. When both bcl 6 and CD10 were negative, the case was assigned to the non-GCB subgroup. When bcl 6 was positive and CD10 was negative, the group was determined by the expression of MUM-1. When MUM-1 was negative, the case was assigned to the GCB group; when MUM-1 was positive, the case was assigned to the non-GCB group (WHO classification 2008). Slides were examined for the presence of nuclear/cytoplasmic/membranous staining (depending on the location of the positivity) within the tumor itself. Each case for IHC was evaluated by all authors using 4X, 10X and 40 X objectives (Nikon) separately. Disagreement was resolved by departmental consensus committee including associate and assistant professors of Department of Histopathology, DDRRL on multi-head microscope. Cases were considered positive if 30% or more of the tumor cells were stained with an antibody (Hans et al., 2004). Expression of Ki67 was assayed immunohistochemically in tissue samples by PI. A cut-off value of 70% was used (Broyde et al., 2009). The bcl2 was evaluated in a semiquantitative manner. The bcl2 was scored as follows: 0 (tumor cells negative), 1 (heterogeneous), 2 (positive) (Llanos et al., 2001).

Statistical analysis

The data were subjected to statistical software; Statistical Package for Social Sciences (SPSS) version 16. Results were expressed as mean±standard deviation, number and percentages. Categorical data were compared using Chi square test; p-value ≤ 0.05 was considered significant.

Results

Among 42 DLBCL cases; 27/42 (64%) were males and 15/42 (36%) females. The mean age for DLBCL was 44.1±15 years (age range=13 to 80 years). In males the mean age was 41.1 ± 14.4 years as compared to females with mean age of 49.6 ± 15.0 years.

Evaluation of DLBCL subgroups by IHC as well as morphology

Morphological studies demonstrated 27/42 (64.3%) cases of centroblastic variety, 14/42 (33.3%) were of immunoblastic in type and only one case of anaplastic variety (2.4%) was seen in this series.

The immunophenotypic categorization of DLBCL

Table 1. Association of Clinical Parameters, bcl 2 Protein Expression and Ki 67 PI with Immunophenotypic Subgroups of DLBCL

Clinical & molecular parameters	Subgroups of DLBC GCB type (n=15)	Non-GCB type (n=27)	Total n=42 (%)	P value (χ^2 test)
Age				
< 60 years	10	22	32 (76%)	0.280
≥ 60 years	5	5	10 (34%)	0.280
Gender				
Male	7	20	27 (64.2%)	0.076
Female	8	7	15 (35.8%)	0.076
Anatomic Site				
Nodal	11	9	20 (47.6%)	0.013
Extranodal	4	18	22 (52.4%)	0.013
B symptoms				
Present	3	20	23 (54.7%)	0.001
Absent	12	7	19 (45.3%)	0.001
Ki 67 PI				
< 70 %	6	22	28 (66.6%)	0.006
≥ 70 %	9	5	14 (33.4%)	0.006
Bcl 2 Protein Expression				
Positive	8	22	30 (71.4%)	0.053
Negative	7	5	12 (28.6%)	0.053

GCB, Germinal center B cell like; DLBCL, Diffuse large B cell lymphoma; P, probability value

Table 2. Specific Anatomic Distribution of DLBCL Subgroups

Specific anatomic location	Sub groups of DLBCL GCB	Non-GCB	Total n=42 (%)
Nodal n=20:			
Cervical Lymph node	2	7	9(21.4%)
Inguinal Lymph node	6	1	7(16.6%)
Axillary Lymph node	1	1	2(4.8%)
Supraclavicular Lymph node	2	0	2(4.8%)
Extranodal n=22:			
Head and neck	2	10	12(28.6%)
Gastro intestinal tract (GIT)	1	5	6(14.2%)
Bone	0	2	2(4.8%)
Buttock	0	1	1(2.4%)
Ovary	1	0	1(2.4%)

GCB, Germinal center B cell like; DLBCL, Diffuse large B cell lymphoma

showed 15/42 (36%) cases were of GCB type as compared to 27/42 (64%) cases of non-GCB type. The bcl 6 was positive in 13/15 (86.6%) cases of GCB type with most having uniform pattern; while 19/27 (70%) cases of non-GCB type showed non uniform pattern of bcl 6 positivity. CD 10 was positive in 9/15 (60%) cases of GCB type, while none of the case of non-GCB type showed positivity for CD 10. All the cases of non-GCB type showed MUM-1 positivity, on the contrary GCB type showed negative immunostaining for MUM-1. When compared immunophenotypic profile with morphological categories of DLBCL; no significant association (P=0.559, x2) was seen.

Immunophenotypic subgroups and clinical parameters, bcl 2 and Ki 67

The association of clinical parameters, bcl 2 status and Ki 67 PI depicted in Table 1. Specifically for GCB type the mean age was 46.4 ± 14 years and in non-GCB type the mean age was 42.8 ± 15.7 years; however age and gender showed no significant association in relation to DLBCL subgroups.

Significant association of non GCB type DLBCL was seen in relation to extranodal origin and occurrence of B symptoms. Ki 67 PI (a measure of the number of dividing/proliferating cells, positive for Ki 67 immunostaining with a total number of cells in a biopsy sample) was assessed with cutoff value of 70%, moreover the mean Ki 67 i.e. 48.2±24 was also noted (range=0-90). However significant association (P=0.006, x2) was seen with low Ki 67 PI (< 70%) in non-GCB type of DLBCL. Overall the bcl 2 protein expression was seen in higher number of cases, i.e. 30/42 (71.4%); when assessed in relation to subgroups of DLBCL, no significant association (P=0.053, x2 with expected count of 25%, when the minimum expected count should be 4.29%) was noted.

Subgroups of DLBCL with specific anatomic location

Table 2 shows the anatomic distribution of DLBCL subgroups. When these subgroups were compared to specific site; no significant association (P=0.018, x2 with 16 cells have expected count less than 5) was noted. However in extranodal involvement, head & neck region was most frequent site, while in nodal origin cervical lymph node was found to be commonest in non-GCB DLBCL.

Discussion

The determination of prognosis based subgroups in DLBCL have been reported variably (Chang et al., 2004; Brooks et al., 2008) by IHC. The great acceptance was achieved by Hans et al (Hans et al., 2004) using three markers algorithm; also described in WHO 2008 classification (Swerdlow eds., 2008) which we used in our study. The variability in response of treatment modalities (Alizadeh et al., 2000) in DLBCL patients also emphasized to explore this heterogeneous group of malignancy with clinical and biologic parameters in our setup. Pakistan is part of the lymphoma belt and increase incidence of DLBCL (Abid et al., 2005) in this part of the globe further enhanced the need of clinical evaluation of DLBCL patients with definite diagnosis of subtypes by modern diagnostic tools like IHC, not only to add more information for establishing the reliable statistics in our country but also to avoid treatment delays, complications and financial burden related to delayed/wrong diagnosis. Moreover a definite diagnosis is the key towards further prevention and control of this disease.

By determining the previously unrecognized categories of DLBCL; it has been advocated in recent years that patients with GCB type DLBCL had a significantly better prognosis (Rosenwald et al., 2002). We observed that in our set up the frequency of non-GCB type (64%) was much higher than GCB type (36%). This figure was quite different from Rosenwald et al (2002) with approximately 50% of the adult GCB type DLBCLs, while the results by Shiozawa et al (2007) showed 71% cases of non-GCB

type with further assessment of having higher frequency of non-GCB DLBCL in Asians. The proportions of GCB type with non-GCB between Western and Asian populations are still unclear and the discrepancy which was seen in existing body of information; may reflect the distinct genetic behavior/different study populations worldwide.

Apart from biologic distinction of DLBCL subgroups; the relevance of these immunohistochemical markers are of great value clinically. MUM-1 expression is found almost exclusively in non-GCB type in our study as described previously by Hans et al (2004) in association with non-GCB type and worse overall survival (OS) . Further analysis of immunohistochemical markers with survival data showed both CD10 (Ohshima et al., 2001) and bcl 6 (Lossos et al., 2001) as a good prognostic indicator and linked to GCB type DLBCL, this association of CD 10 and bcl 6 with GCB type is comparable with our findings. However the positivity of bcl 6 in non-GCB DLBCL in our series raises the question that whether the presence of this immunologic marker in non-GCB makes this tumor less aggressive in this region, if compared the survival of patients of our region with western literature. In our setup, because of the dearth of clinical based follow-up studies, there is dire need to generate data regarding survival, outcome and prognosis of patients in association with these markers. We could not predict patients outcome related to specific immunohistocemical marker because we had limitation of having follow-up data in our series; due to the study being performed in lab setting and most of the paraffin tissue blocks and biopsies received from outside Karachi; where patients come for a short duration, but our study is helpful in identifying the behavior of disease and prediction of patient's outcome at the time of diagnosis; when compared to previously described follow-up based studies worldwide (Lossos et al., 2001; Ohshima et al., 2001; Hans et al., 2004; Jerkeman et al., 2004).

When the data were analyzed for clinicopathologic review of DLBCL; comparable with western and local literature (Abid et al., 2005; Paepe et al., 2005), we also found male predominance with M/F ratio as 1.8:1, but in contrast with western literature; we observed slight early presentation of DLBCL in our set of series with mean age of 44.1±15 years. Luckily the early presentation of disease i.e. age less than 60 years; is associated with good prognosis in this part of world, previously seen by Lal et al (2008).

Significantly higher frequency of B symptoms (54.7%) and extranodal involvement (52.4%) was seen in patients of DLBCL in our series; which is comparable with other local series (Abid et al., 2005), but dissimilar with western literature as 20.4% of cases with B symptoms, while 26.3% with purely extranodal in origin (Park et al., 2007). Both the variables are predictor of poor survival (Park et al., 2007) and more than two extranodal sites involvement were related to worse OS and complete response rate to treatment (Colomo et al., 2003). Moreover in a local series it was predicted by univariate analysis that no or one extranodal site involvement; primarily of gastric or bone, with lack of B symptoms are factors associated with good prognosis (Lal et al., 2008). Although it is disappointing to have higher frequency of non-GCB type and its association with these two variables in this series; but prompt us to plan clinical based follow-up studies in future.

The prognostic role of Ki 67 especially in DLBCL is still unclear but low expression of Ki 67(<60%) with worse overall or failure free survival was documented by Jerkeman et al (2004). However in later series (Hasselblom et al., 2008) no correlation was observed between low Ki 67 PI and subgroups of DLBCL. We found significant association (P=0.006, x2) of low Ki 67 PI (<70%) with non-GCB type of DLBCL; again indicative of different biological and environmental factors in this part of the world.

The role of bcl 2 protein overexpression in DLBCL is controversial but in most western series its association was seen with worse OS and prognosis (Nyman., 2010; Muris et al., 2006) and it was advocated that bcl-2 is the strongest prognostic marker followed by CD10 and MUM-1. The higher frequency of bcl 2 positive DLBCL (71.4%) in our setup indicates the aggressive nature of disease in our distinct population but consistent to Neto et al (2010), no significant correlation was found between subgroups of DLBCL and bcl 2 protein overexpression in our set of series. It is worth noting that correlation of single immunohistochemical marker with patient's follow-up; can provide survival and prognostic data in our setup. Hence in this regard; whenever the immunohistochemical results will be compared with patient's follow-ups, future targeted approaches will be achieved. Our study is beneficial in provision of information to oncologists/ clinician related to single immunologic markers for determination of disease outcome at the time of diagnosis.

In conclusion, immunophenotypic subgroups of DLBCL display non-GCB type as more frequent in comparison to GCB type. It is alarming to see such an aggressive type with higher frequency in our setup. These subgroups with relation to gender and age show no significant association, however highly significant association of non-GCB type is seen with extranodal origin, occurrence of B symptoms and low Ki 67 PI (<70%). Although the frequency of bcl 2 protein expression was much higher in DLBCL patients but could not reach to statistical significance in relation to DLBCL subgroups. In conclusion further analysis of natural course of disease and distribution of DLBCL subgroups by IHC with patient's follow-up in this region; can help in identifying patient's outcome as well as provision of preventive measures against causative factors.

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