



Prediction of Survival in Diffuse Large B Cell Lymphoma According to Immunohistochemistry

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Abstract

BACKGROUND: The introduction of chemoimmunotherapy in the treatment of diffuse large B cell lymphoma (DLBCL) dramatically improved the outcomes compared to chemotherapy alone. However, a significant part of the patients become refractory and eventually relapse.

AIM: The purpose of this study is to analyze the survival differences between different groups of DLBCL according to Han's algorithm.

MATERIALS AND METHODS: We will study the medical records of 244 patients treated with RCHOP as first-line therapy who are diagnosed with *de novo* DLBCL in a cohort of 10 years in the University Clinical Center of Kosovo. According to immunohistochemical markers, the patients will be classified into two major groups, germinal center B cell-like (GCB) and non-germinal center B cell-like (non-GCB) subtypes, and five subgroups (Group 1, Group 2, Group 3 or double positive, Group 4, and Group 5 or triple negative [TN]).

RESULTS: The patients in the GCB group have better progression-free survival (PFS) and overall survival (OS) than the non-GCB group. Despite this, double positive (DP) (CD10⁺MUM1⁺) and TN (CD10⁻Bcl6⁺MUM1⁻) subgroups showed different PFS and OS to the same cell-of-origin group. The DP group showed similar OS and PFS with the non-GCB group, whereas the TN group showed similar OS and PFS with the GCB group.

CONCLUSION: These factors will provide valuable knowledge for predicting the prognosis and redirecting the choice of treatment for different groups of DLBCL.

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Introduction

Diffuse large B cell lymphoma (DLBCL) is the most common lymphoid malignancy and accounts for approximately 25% of all non-Hodgkin lymphomas [1], [2]. In the United States, the annual incidence of DLBCL is approximately 7 cases per 100,000 persons [2], and in Europe, 4.92 cases per 100,000 persons per year [3]. The annual incidence of DLBCL in Kosovo is nearly 1.24 new cases per 100,000 inhabitants.

DLBCL is a heterogeneous clinicopathologic entity that includes tumors derived from germinal center B cells (GCB) or post-germinal center B cells (also called activated B cells [ABC]).

GCB and ABC subtypes have very different clinical courses, with ABC having a much worse outcome [4], [5]. It has been noted that patients with these two different subtypes also respond differently to therapeutic medications, thinking that ABC and GCB can be like two different diseases. Because of these differences in response to therapy, having an assay to determine DLBCL subtypes is important in directing the

clinical approach to the use of current therapies, as well as in the development of new drugs.

The newest gold standard test for DLBCL typing is gene expression profiling (GEP) to determine the "cellular origin" and disease subtype. DLBCL can be divided into GCB group, ABC group, and type 3 (primary mediastinal B-cell lymphoma) by GEP study [6], [7]. The latter two are more aggressive than the GCB subtype.

Although this approach has some significant clinical and practical limitations as it is more expensive in routine clinical practice, not all DLBCL patients can be classified, and it is the subject of ongoing research.

Currently, immunohistochemical (IHC) analysis of lymphoma biopsy specimens appears to be a more widely applicable methodology in clinical practice because of its low cost to differentiate between subtypes of DLBCL. For this reason, IHC algorithms have been proposed to predict the GEP subtypes. Among the published IHC algorithms, Hans's algorithm was most widely used in routine practice. Hans's algorithm was created from three antibodies: CD10 a germinal center marker, Bcl6 associated with both

germinal and non-germinal center, and MUM1 as a post-germinal center marker [8]. Based on the combination of these three markers, Hans's algorithm has divided DLBCL into two main subtypes, GCB and non-GCB subtypes. GCB subtype is subdivided into three groups, Group 1 (CD10+BCL6+ MUM1-), Group 2 (CD10-BCL6+MUM1-), and Group 3 (CD10+BCL6+ MUM1+). The non-GCB subtype is subdivided into two groups, Group 4 (CD10-BCL6+-MUM1+ and Group 5 (CD10-BCL6-MUM1-).

Even though MUM1 is used as a post-germinal center marker, cases with co-expression of CD10 and MUM1 (CD10+MUM1+, double positive [DP]), which was classified as GCB subtype, Group 3 according to Hans algorithm, do exist.

DLBCL without any positive staining of these three markers (CD10-Bcl6-MUM1-, triple negative [TN]) was also noted. These cases, based on Hans's algorithm, are classified as non-GCB subtypes or Group 5. Little is known about the difference between different groups of GCB and non-GCB subtypes.

Material and Methods

Based on the data collected, the total number of patients diagnosed with *de novo* DLBCL at the Hematology Department of the University Clinical Center of Kosovo from September 2009 to November 2021 was 270. Cases of special variants, such as primary central nervous system lymphoma, primary mediastinal B-cell lymphoma, and HIV-positive DLBCL, are excluded from the whole cohort. Finally, a total of 224 cases were included in this study and were analyzed in a cohort of 12 years. The median follow-up time was 63 months (5–146 months). All the patients are treated with RCHOP as first-line therapy. Relapsed cases are treated with second or salvage therapy, such as RCHOEP, EPOCH, DHAP, and R/ICE.

Immunohistochemistry is performed in the Department of Pathology at the University Clinical Center of Kosovo. After optimal fixation for 24 h in neutral buffered formalin, tissue samples were processed in a tissue processor (Leica TP 1020), where they underwent an additional fixation procedure, dehydration, xylene cleansing, and immersion in liquid paraffin at 60°C. Subsequently, the labeled specimens were molded into paraffin blocks, sectioned at 3–4-micron thick sections, and applied on microscope glass slides. After the procedure of deparaffinization and gradual rehydration, tissue sections were stained with hematoxylin and eosin (HE). The HE-stained sections were examined in Olympus CX43 microscope. Then, the slides were incubated with primary antibodies against CD20, CD10, and MUM-1 for 30 min. In the next step, dextran polymer conjugated with peroxidase and secondary antibody

(EnVision+, DAKO, K534011) was applied for another 30 min. The visualization was carried out with DAB and chromogen. Complete membranous stain of neoplastic cells with CD20 and CD10 was considered positive. The nuclear stain of neoplastic cells with MUM-1 was considered positive. Threshold of >30% in CD10 negative tumors was considered MUM-1 positive.

The germinal center and non-germinal center classifications were determined by the Hans algorithm. If CD10 was positively stained, the sample was included in the germinal center phenotype. If CD10 and Bcl-6 both stained negatively, the sample was of the non-GC-phenotype. If CD10 was negative but Bcl-6 positive, the MUM-1 staining determined the phenotype. MUM-1-negative cases were GC-phenotype, and MUM-1-positive cases were non-GC phenotype. If the staining was positive for both CD10 and MUM1, cases were named DP or GCB phenotype, and cases negative for both CD10, Bcl6, and MUM1 were classified as TN or non-GCB phenotype.

All experimental protocols were approved by the Ethics Committee of the University Clinic Center of Kosovo and all patients provided informed consent in accordance with all needed requirements. Statistical analyses were performed with SPSS software, version 26. Progression-free survival (PFS) and overall survival (OS) were estimated by the Kaplan–Meier method and differences were compared with the log-rank test. PFS is evaluated from the date of diagnosis until relapse or death of any cause. OS is determined from the date of diagnosis until the last follow-up or death. OS and PFS were reported in months. A $p < 0.05$ is considered statistically significant. The study is a longitudinal retrospective cohort study. We analyzed PFS and OS between GCB and non-GCB subtypes, between Groups 1, 2, and 3, between DP (Group 3) group and other GCB subtypes (Group 1+2) or non-GCB subtype, between TN (Group 5) group and another non-GCB subtype (Group 4) or GCB subtype and between DP and TN group.

Results

A total of 224 patients were enrolled in the study. The median age of the patient at diagnosis is 64.0 years (range, 22–92 years), with 40.6% (90/224) of the patients being younger than 60 years. There was a slight male predominance, with 51% male and 49% female patients. The annual incidence of DLBCL in Kosovo from September 2009 to November 2021 was nearly 1.25 new cases per 100,000 inhabitants. The annual incidence is slightly higher in men compared to females, 50.9% versus 40.1%. The highest incidence was noted between 2016 and 2020, and the lowest incidence in 2010. The prevalence of DLBCL in Kosovo

in this study cohort is approximately 6.7 patients per 100,000 inhabitants.

According to Hans's algorithm, patients were divided into two major groups GCB and non-GCB subtype, and five subgroups. In the GCB subtype, we have 102 patients and in the non-GCB subtype, we have 122 patients. Group 1 (GCB subtype as CD10+BCL6+MUM1-) with 58 patients, Group 2 (GCB subtype as CD10-BCL6+MUM1-) with 23 patients, Group 3 (GCB subtype or DP as CD10+BCL6+MUM1+) with 21 patients, Group 4 (non-GCB subtype as CD10-BCL6+MUM1+) with 105 patients, and Group 5 (non-GCB subtype or TN as CD10-BCL6-MUM1-) with 17 patients.

Survival difference between GCB and non-GCB group

In the GCB group, we have 102 patients, and in the non-GCB group, we have 122 patients. The GCB patients showed a better PFS (median PFS: 98 vs. 54 months, $p < 0.0001$) (Figure 1a) and OS (median OS: 115 vs. 88 months, $p = 0.010$) (Figure 1b) than the non-GCB patients.

Survival differences between three subgroups of the GCB subtype

GCB group is divided into three subgroups. Group 1 or CD10+BCL6+MUM1- with 58 patients, Group 2 or CD10-BCL6+MUM1- with 23 patients, and Group 3 (DP) or CD10+BCL6+MUM1+ with 21 patients. According to the Kaplan–Meier curve, these three groups have shown a different median PFS of 117 months in Group 1, 96 months in Group 2, and 34 months in Group 3 (DP) with a significant $p < 0.0001$ (Figure 2a).

Furthermore, they have shown a median OS of 132 months in Group 1, 119 months in Group 2, and 46 months in Group 3, with a significant $p < 0.0001$ (Figure 2b).

We have analyzed the difference in PFS and OS between Group 1 and Group 2. Median PFS of

117 months in Group 1 and 96 months in Group 2 with no significant p-value ($p = 0.21$) (Figure 3a).

Median OS is 132 months in Group 1 and 119 months in Group 2 with a non-significant $p = 0.11$ (Figure 3b).

DP (Group 3) versus other GCG subtypes (Group 1+2)

The median PFS between the two groups is 109 in Group 1+2 versus 34 months in Group 3 or DP with a significant $p < 0.0001$ (Figure 4a).

The median OS between groups is 125 months in group 1+2 versus 46 months in Group 3 or DP with a significant $p < 0.001$ (Figure 4b).

DP (Group 3) versus non-GCB subtype

Median PFS of 54 months in the non-GCB group versus 34 months in the DP group without a significant p-value. ($p = 0.19$) (Figure 5a).

The median OS of 88 months in the non-GCB group versus 46 months in the DP group without a significant p-value ($p = 0.44$) (Figure 5b).

TN (Group 5) versus the other non-GCB subtype (Group 4)

Group 4 and the TN group are both part of the non-germinal group. The non-GCB group is divided into two groups, Group 4 (CD10-BCL6+MUM1+) with 105 patients and TN (CD10-BCL6-MUM1-) or Group 5 with 17 patients.

TN even if it is in the non-germinal group has shown different PFS and OS than the other non-GCB subtype.

TN has a median PFS of 80 versus 52 months of Group 4 with a significant $p = 0.013$ (Figure 6a).

The median OS of 100 months in the TN group versus 82 months in Group 4 with not a significant $p = 0.12$ (Figure 6b).

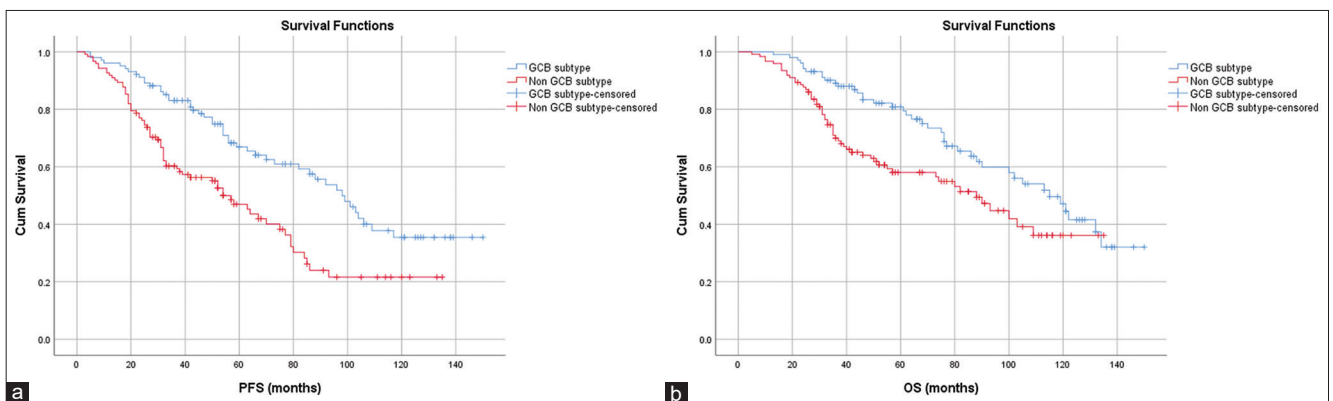


Figure 1: (a) Progression-free survival between germinal center B cell-like and non-germinal center B cell-like groups. (b) Overall survival between germinal center B cell-like and non-germinal center B cell-like groups

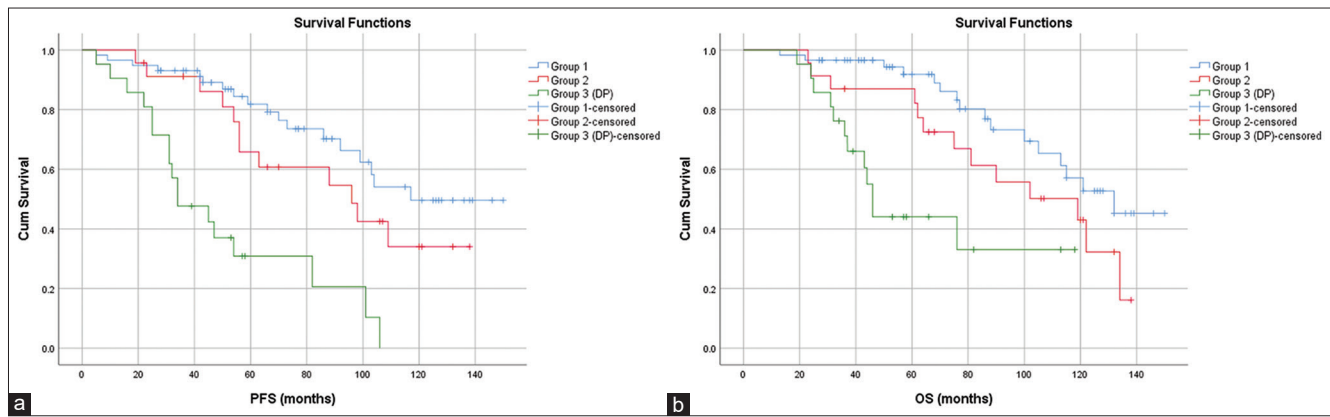


Figure 2: (a) Progression-free survival between three groups of germinal center B cell-like subtype. (b) Overall survival between three groups of germinal center B cell-like subtype

TN (group 5) versus GCB subtype

The median PFS between the two groups is 98 months in the GCB subtype versus 80 months in the TN group without a significant p-value ($p = 0.58$) (Figure 7a).

Median OS is 115 in the GCB subtype versus 100 months in the TN group, without a significant p-value ($p = 0.97$) (Figure 7b).

DP versus TN group

The TN group has shown a longer PFS than the DP group with a median PFS of 80 versus 34 months and a significant $p = 0.006$ (Figure 8a).

The median OS of the TN group is 100 months versus 46 months in the DP group, with a significant $p = 0.046$ (Figure 8b).

Discussion

The institution of chemoimmunotherapy in the treatment of DLBCL has significantly improved the

outcome of these patients compared with chemotherapy alone. The standard of care for the management of DLBCL is R-CHOP therapy which has provided long-term disease control in up to 90% of limited-stage patients and in up to 60% of advanced-stage patients [9]. At a median follow-up of 63 months in our study, 56.2% of patients survived and 68.7% (150/224) of the total patients had complete remission after first line RCHOP therapy.

DLBCLs are characterized by clinical, biological, genetic, and prognostic heterogeneity, requiring special consideration in their treatment. The international prognostic index has been used to predict the prognosis of aggressive NHL treated with doxorubicin-based therapies, findings confirmed even in the rituximab era [10], [11]. In addition, several studies have identified the utility of PET imaging and circulating tumor DNA for the prognosis of lymphoma patients [12]. Based on the cell of origin, with gene expression profile assay (GEP), we can identify three unique subtypes with different prognoses, GCB, ABC, and type 3 with the latter two having a much worse prognosis than the GCB type. According to molecular features, we have highly aggressive lymphomas, characterized by double-hit and triple-hit mutation (*c-myc*, *bcl2*, and/or *bcl6*) [13]. Based on genetic features, Schmitz *et al.* identified four distinct genetic subtypes of disease with MCD, BN2,

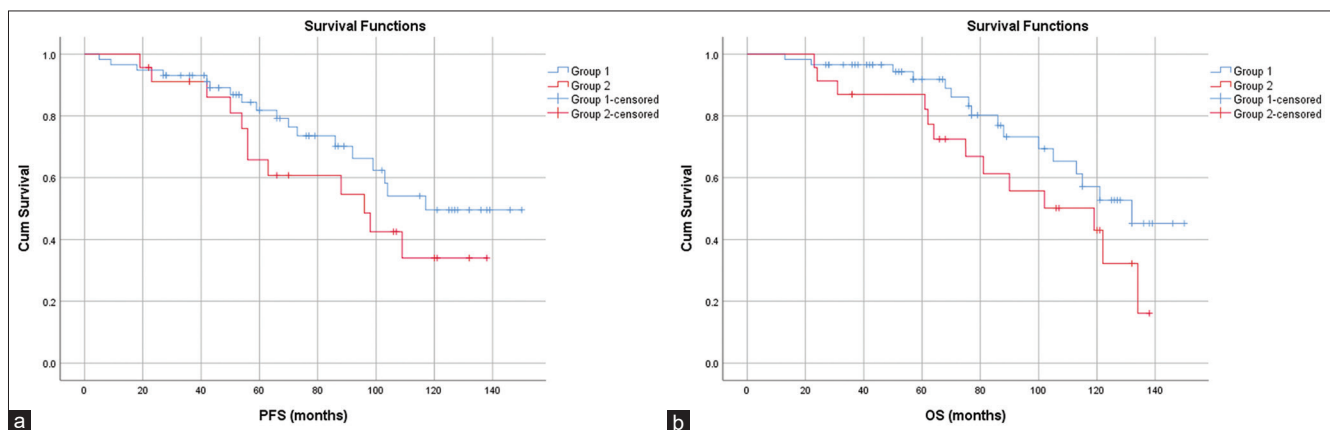


Figure 3: (a) Progression-free survival between Group 1 and Group 2. (b) Overall survival between Group 1 and Group 2

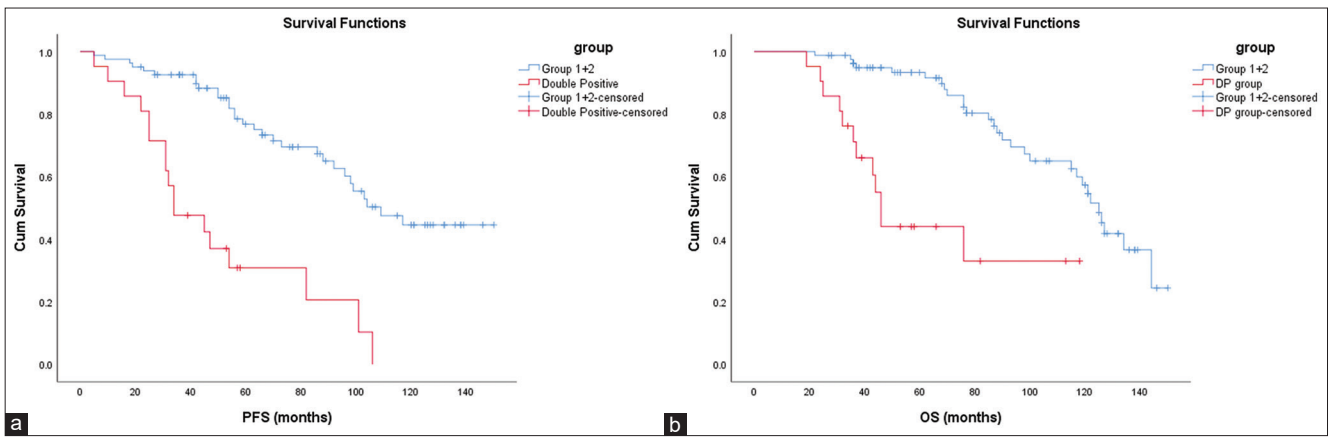


Figure 4: (a) Progression-free survival between Group 1+2 and Group 3 (double positive). (b) Overall survival between Group 1+2 and Group 3 (double positive)

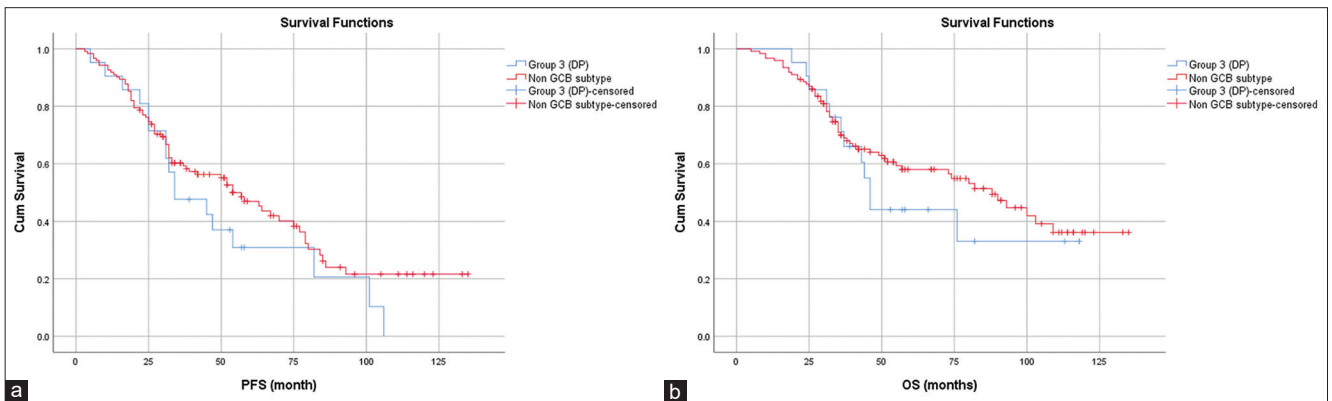


Figure 5: (a) Progression-free survival between Group 3 (double positive) and non-GCB subtype. (b) Overall survival between Group 3 (double positive) and non-germinal center B cell-like subtype

N1, and EZB mutation, respectively [14], with MCD and N1 conferring to poor prognosis.

Because gene GEP is expensive and impractical in the medical routine, IHC algorithms were developed as an alternative to this test. The majority of such algorithms were developed in the chemotherapy era and their predictive value in patients treated with chemoimmunotherapy was controversial [15], [16], [17], [18], [19], [20]. Among

these, the most studied one is Hans’s algorithm, which uses the IHC staining of CD10, Bcl6, and MUM1 to classify cases of DLBCL into germinal center B cell-like or non-germinal center B cell-like groups [8]. In some studies, the algorithm was consistent, but in others, it was not. The IHC algorithm derived by Hans *et al.* to assign DLBCL to GCB and non-GCB subgroups is considered imperfect and has a misclassification rate of 19.7% when compared with gene expression profiling

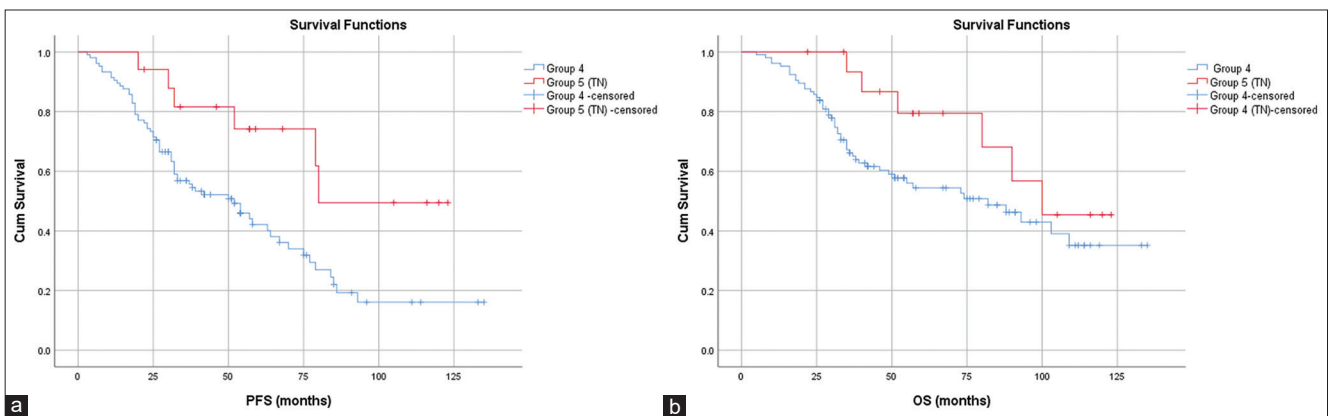


Figure 6: (a) Progression-free survival between Group 5 (triple negative) and Group 4. (b) Overall survival between Group 5 (triple negative) and Group 4

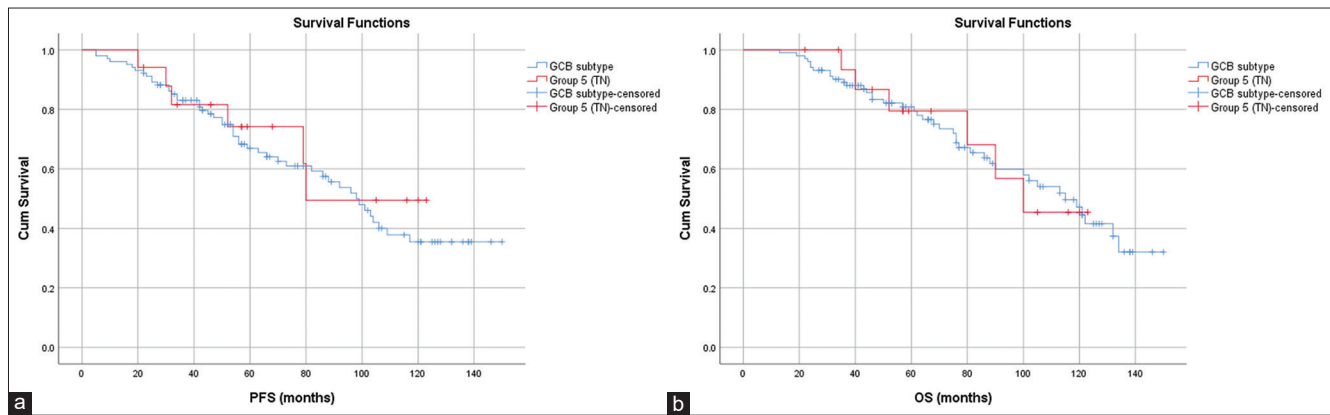


Figure 7: (a) Progression-free survival between Group 5 (triple negative) and germinal center B cell-like subtype. (b) Overall survival between Group 5 (triple negative) and germinal center B cell-like subtype

data. In this study, subtyping DLBCLs according to immunohistochemistry based on Han’s algorithm is considered. We assessed the prognostic value of 244 DLBCL patients treated with chemoimmunotherapy according to Hans’s algorithm. The results showed that GCB DLBCL patients had better PFS and OS than non-GCB DLBCL patients. Nyman *et al.* did not find a difference in survival between GCB and non-GCB subgroups in the post-rituximab era, which implies that the addition of rituximab eliminates the prognostic significance of the classification of DLBCL on the basis of the cell of origin [19]. Chaves *et al.*, in a study between 2000 and 2008, demonstrated that Han’s algorithm can predict the clinical outcome of patients with DLBCL undergoing front-line therapy with R-chemotherapy. Patients with non-GCB DLBCL while having a comparable initial complete response rate to R-CHOP had a shorter PFS and OS than GCB DLBCL patients [21]. Fu *et al.* in a study of 243 patients of DLBCL demonstrated that even if the addition of rituximab to CHOP therapy improved PFS and OS of GCB and non-GCB patients, still GCB subgroup had better PFS and OS than the non-GCB subgroup [22]. Lu *et al.* in a study of 306 patients still demonstrated that GCB patients had better PFS and OS than non-GCB patients [23]. Our study has shown that the survival of patients with GCB DLBCL is still superior

to the non-GCB DLBCL in the rituximab era which is consistent with the recent studies.

In a cohort of almost 12 years, 8.6% (21/244) CD10+MUM1+ (DP) and 7.6% (17/244) CD10–Bcl6–MUM1– (TN) DLBCLs patients were identified. The reported incidences of DP and TN were 2.3–14.3% and 5.5–19.1%, respectively [8], [24], [25].

However, TN DLBCLs, which should be classified as non-GCB subtypes according to Hans’s algorithm, were found to have different survival from the other non-GCB DLBCLs. The TN group was associated with better OS and PFS compared with other non-GCB patients. There was no difference in survival between the TN and GCB groups. These data raised the possibility that patients in the TN group have the same prognoses as patients in the GCB group. DP patients in this study demonstrated worse survival than other GCB patients, with lower PFS and OS than other GCB patients. DP group and non-GCB group did not differ in PFS and OS. These data demonstrated that the patients in the DP group have similar prognoses as patients in the non-GCB group. The importance of the DP and TN groups was demonstrated even in the study by Lu *et al.* where they had different prognoses to the same cell of origin group [23].

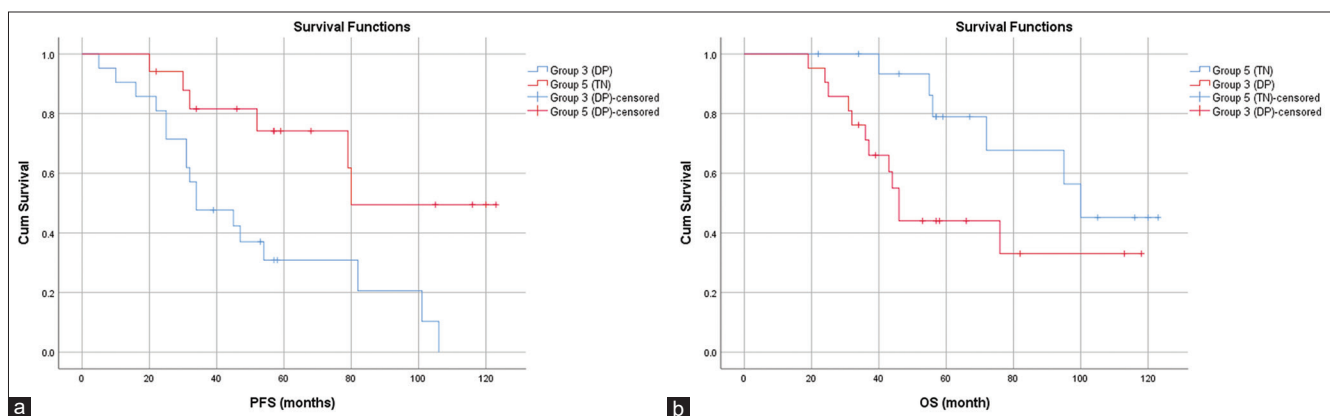


Figure 8: (a) Progression-free survival between Group 3 (double positive) and Group 5 (triple negative). (b) Overall survival between Group 3 (double positive) and Group 5 (triple negative)

Conclusion

In summary, although our study has proved that IHC algorithms retain prognostic significance in the chemoimmunotherapy era, controversy remains in the literature [18], [26], [27], [28], [29]. This is mostly due to differences in patient populations, antibodies, and protocols used, and in part due to the lack of homogenous or large cohorts. However, because our study is retrospective, prospective studies with a larger number of patients who are treated with rituximab plus standard chemotherapy are needed to confirm our findings. The presence of some special groups such as the DP and TN may affect the prognostic value of the Han's algorithm, which is generally neglected in other studies. Finally, the use of Han's algorithm in the absence of genetic methods in developing countries is an important method used in this study. Even if RCHOP therapy still remains the standard of treatment, the identification of these separate groups such as non-GCB and DP is important and may require different treatment approaches in the future.

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