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Prevalence and Prognostic Impact of MYC, BCL2, and BCL6 Rearrangements in Large B Cell Lymphoma Patients: A Multicenter Historical Cohort Study from Iran

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Abstract

Background: Diffuse large B cell lymphoma (DLBCL) is the most prevalent subtype of non-Hodgkin's lymphoma, characterized by remarkable molecular heterogeneity. This study evaluates the prevalence of MYC, BCL2, and BCL6 gene rearrangements among Iranian DLBCL patients.

Method: This historical cohort study encompassed 152 patients drawn from six reference hospitals who participated in the research. Interphase dual-color break-apart fluorescence in situ hybridization (FISH) was applied to formalin-fixed paraffin-embedded DLBCL specimens categorized as "not otherwise specified" alongside 20 normal controls. Survival data was analyzed using the Kaplan-Meier method and the Log-Rank test.

Results: Among the patients, 7 (4.8%), 4 (2.9%), and 15 (10.2%) exhibited MYC, BCL2, and BCL6 rearrangements, respectively. Additionally, 1.5% of the patients demonstrated double-hit (DH) characteristics with both MYC and BCL2 rearrangements, while no triple rearrangements were observed. The presence of rearrangements appeared to be independent of clinicopathological variables. Patients with rearrangements experienced reduced survival durations, with reductions of 26.6, 31.2, 9.1, and 34.2 months for MYC, BCL2, BCL6-rearranged, and DH tumors, respectively ($P > 0.05$). Adverse prognosis was associated with age, activated B-cell-like phenotype, disease stage, B symptoms, lactate dehydrogenase levels, and risk grouping according to the National Comprehensive Cancer Network (NCCN) International Prognostic Index.

Conclusion: DLBCL cases featuring MYC, BCL2, and/or BCL6 translocations are relatively rare. Patients harboring these rearrangements tend to exhibit aggressive disease progression with shortened overall survival. However, these differences did not reach statistical significance, necessitating further research to validate the incorporation of such tests into the routine workup of DLBCL patients.

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Introduction

Lymphomas are a heterogeneous group of hematological malignancies classified into two major categories: Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). The most common subtype of NHL is diffuse large B-cell lymphoma (DLBCL), which accounts for approximately 40 and 20% of lymphoma cases in adults and children, respectively.[1-3]

Most DLBCLs are diagnosed based on histopathologic examination and immunophenotyping by immunohistochemistry (IHC). What is more, as the World Health Organization (WHO) advocates, are IHC-based algorithms that further categorize DLBCLs into germinal center B-cell-like (GCB) and activated B-cell-like (ABC) groups based on their presumed cell of origin.[1, 3-4]

The 2017 update of the WHO classification of lymphoid neoplasms introduced a new entity, "high-grade B-cell lymphoma (HGBL), with MYC and BCL2 and/or BCL6 translocations" which encompasses those lymphomas that harbor either two or three of MYC, BCL2, or BCL6 gene rearrangements designated as double-hit (DHL) and triple-hit (THL) lymphomas, respectively. These types of lymphomas show resistance to standard treatments and are thus associated with an unfavorable clinical course.[5-7] Of note, MYC/BCL6-rearranged tumors bear high heterogeneity and distinct gene expression and mutational profiles from MYC/BCL2-rearranged lymphomas. That is why the upcoming 5th edition of the WHO classification of hematolymphoid tumors, which was released online in August 2022, classifies cases with dual MYC and BCL6 aberrations as a subtype of DLBCL or HGBL, not otherwise specified (NOS), and the International Consensus Classification (ICC) separates it into a provisional entity, "HGBL with MYC and BCL6 rearrangements.[8-10]

About 5%-25% of DLBCLs show MYC rearrangements.[5] MYC is an oncogene located on 8q24, which encodes a transcription factor involved in several biological processes mainly related to cell cycle regulation.[1, 3] Likewise, BCL2, located on 18q21, also acts in cell cycle control and is a well-known antiapoptotic protein. BCL2 was initially identified in the more indolent follicular lymphoma and is expressed by more than 50% of DLBCLs.[1, 3] Importantly, the rearrangements in MYC and BCL2 genes are gain-of-function aberrations that lead to overexpression of their corresponding proteins, which consequently cause cell proliferation and the intensification of the survival advantage of tumor cells to emerge. On the other hand, BCL6, located on 3q27, is a negative regulator of transcription, and its rearrangements mainly result in loss of function and subsequent upregulation of transcription, cell proliferation, and dysregulation of apoptosis.[1, 3, 11]

This study evaluated the prevalence of MYC, BCL2, and BCL6 rearrangements in DLBCL patients by fluorescence in situ hybridization (FISH) and their association with other prognostic factors and overall survival (OS).

To the best of knowledge, this is the first study of its kind from Iran.

Materials and Methods

Patient selection

This retrospective cohort study was conducted on 152 patients at six reference hospitals affiliated with the Shiraz University of Medical Sciences, all of whom had received a pathological diagnosis of DLBCL, NOS between 2008 and 2016.

Clinical data, encompassing age, gender, primary site of involvement, presence of B symptoms, nodal and/or extra-nodal involvement, lactic dehydrogenase (LDH) levels, Ann Arbor staging, Eastern Cooperative Oncology Group (ECOG) performance status, International Prognostic Index (IPI) score, and National Comprehensive Cancer Network-IPI (NCCN-IPI) risk categorization, were meticulously extracted through a comprehensive review of medical records. The classification of cell origin, whether Germinal Center (GC)-like or Activated B-cell (ABC)-like, had been determined using the HANS algorithm and was documented in the pathology reports.

All patients had undergone immunochemotherapy, precisely the R-CHOP regimen consisting of rituximab, cyclophosphamide, doxorubicin

hydrochloride, vincristine, and prednisolone.

After treatment, patients were diligently monitored through telephone contacts. The median follow-up duration was 43.5 months, ranging from 1 to 106 months. As of the latest follow-up assessment, 62 (40.8%) patients had deceased, while the remaining 90 (59.2%) were alive. This study received approval from the Shiraz University of Medical Sciences and its affiliated ethics committee (Approval No: 14070). Given the study's retrospective nature, the informed consent requirement was waived.

Interphase FISH

Tissue microarrays (TMA) were constructed employing cores from two representative tumor areas. The 1.0 mm diameter cores were spaced every 0.2 mm on each slide, with 30 patients on each TMA block.

FISH was performed on formalin-fixed paraffin-embedded tissue specimens using dual-color break-apart probes for MYC, BCL2, and BCL6 (The ZytoLight SPECT, ZytoVision GmbH, Bremerhaven, Germany).

In brief, 4-µm thick sections on positively charged slides were deparaffinized in a 70°C oven and two subsequent containers of xylene (each for 10 min) and rehydrated in consecutive baths of graded ethanol (100%, 100%, 90%, and 70% each for 5 min). Pretreatment and proteolysis were carried out by boiling the slides in pre-warmed Heat Pretreatment Solution Citric at 98°C for 15 min, followed by incubation with Pepsin Solution at 37°C for 10 min. After dehydration with graded ethanol (70%, 90%, and 100% each for 1 min), 10 µl of the probe was applied, and denaturation was performed at 75°C for 10 min. Next, the slides were transferred to a humidity chamber (Thermobrite System, Abbott, Illinois, USA) and hybridized overnight at 37°C. The following day, after washing extra probes in saline-sodium citrate (SSC) buffer and hydration in graded alcohol (70%, 90%, and 100% ethanol each for 1 min), 30 µl of DAPI/Antifade-Solution was administered onto the slides and the sections were incubated in the dark until evaluation.

Nikon E600 fluorescent microscope and Genesis software were employed to evaluate the signals and take representative images. In each case, 200 interphase nuclei were searched for break-apart signals. Standard controls, including ten reactive lymph nodes and ten tonsilar tissues, were all negative. Therefore, the technical cut-off was calculated as 3% by applying the inverse beta distribution (betainv) method. The clinical cut-off was set at 10% based on the previous reports.[12, 13]

Statistical analysis

The statistical analysis was carried out utilizing IBM SPSS Statistics 20.0 software. Chi-square, T-tests, and Mann-Whitney U tests were employed to facilitate comparisons. Survival data analysis was executed through the Kaplan-Meier method, complemented by the Log-Rank test. Survival curves were generated using GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). A significance level 0.05 was adopted as the threshold throughout the statistical analyses.

Results

The clinical information of the patients is summarized in table 1. The mean (±SD) age was 54 years (±16.2), ranging from 13 to 90 years. Cervical lymph nodes were the predominant site of involvement, accounting for 57% of patients with nodal disease. Axillary, inguinal, and mediastinal regions were affected in 16%, 13%, and 7% of cases, respectively. Extra-nodal disease was present in 48 (31.6%) patients, distributed as follows: 37% in the gastrointestinal tract, 35% in the head and neck area, 23% in skin and soft tissue, and 5% in the retroperitoneum and spleen.

7 (4.8%), 4 (2.9%), and 15 (10.2%) patients exhibited MYC, BCL2, and BCL6 rearrangements, respectively. Characteristic FISH findings for negative (no rearrangement) and positive (rearranged gene) results are depicted in figure 1. The mean (±SD) percentage of interphases harboring a break-apart signal was 38 (±18.2) for MYC, 30 (±14.1) for BCL2, and 36 (±21.5) for BCL6-rearranged DLBCLs, with ranges of 20-70, 20-50, and 15-80, respectively.

2 (1.5%) patients harbored DHL

rearrangements involving MYC and BCL2. Both DHL patients were male, presented with extra-nodal disease affecting the scalp and tonsils, had no B symptoms, normal LDH levels, low-stage disease (Stage I or II), and a ki67 proliferation index of 70%. However, one DHL patient was 81 years old with a GC-like tumor and a high intermediate IPI score, succumbing after 11 months, while the other DHL patient had an ABC-like lymphoma, a low IPI score, and remained alive for 50 months of follow-up. Notably, the percentage of positive cells for MYC/BCL2 was 40%/30% and 20%/50% for the former and latter patients, respectively.

No significant association was found between rearrangement status and age, sex, presence of B symptoms, IPI score, NCCN-IPI score, ECOG performance status, stage, and ki67 proliferation index (Table 1).

The mean OS time was 39.15 months, and it did not differ statistically between patients with rearrangements and those without any aberrations (Log-Rank test, $P > 0.05$). However, patients harboring rearrangements experienced markedly worse survival times (Table 2), with reductions of 26.6, 31.2, 9.1, and 34.2 months for CMYC, BCL2, BCL6-rearranged, and DHL tumors, respectively. Figure 2 illustrates the survival curves based on clinical, laboratory, and molecular parameters.

ABC-like and high-stage (Ann Arbor stage III/IV) DLBCLs exhibited worse outcomes, with approximately 19 months shorter OS times, though this did not reach statistical significance. Survival analysis based on ECOG performance status was inconclusive, as all cases with ECOG scores less than 5 were still alive.

Older age (>60 years) (OS, 95% confidence interval (CI): 50.0, 36.1-61.0 vs. 71.6, 62.2-80.9, $P = 0.034$), presence of B symptoms (OS, 95% CI: 43.7, 31.3-56.1 vs. 77.4, 67.3-87.4, $P < 0.001$), higher LDH levels (OS, 95% CI: 34.1, 23.3-44.8 vs. 79.2, 69.3-88.9, $P < 0.001$), and involvement of more than one extra-nodal site (OS, 95% CI: 46.2, 30.9-61.4 vs. 72.6, 62.9-82.3, $P = 0.022$) were significantly associated with an adverse prognosis.

Patients in low, low-intermediate, high-intermediate, and high NCCN-IPI risk groups demonstrated progressively worse outcomes (OS (95% CI): 63.3 (57.7-78.9), 54.8 (46.9-62.6), 28.9 (21.4-36.3), and 28.2 (18.5-37.8), respectively).

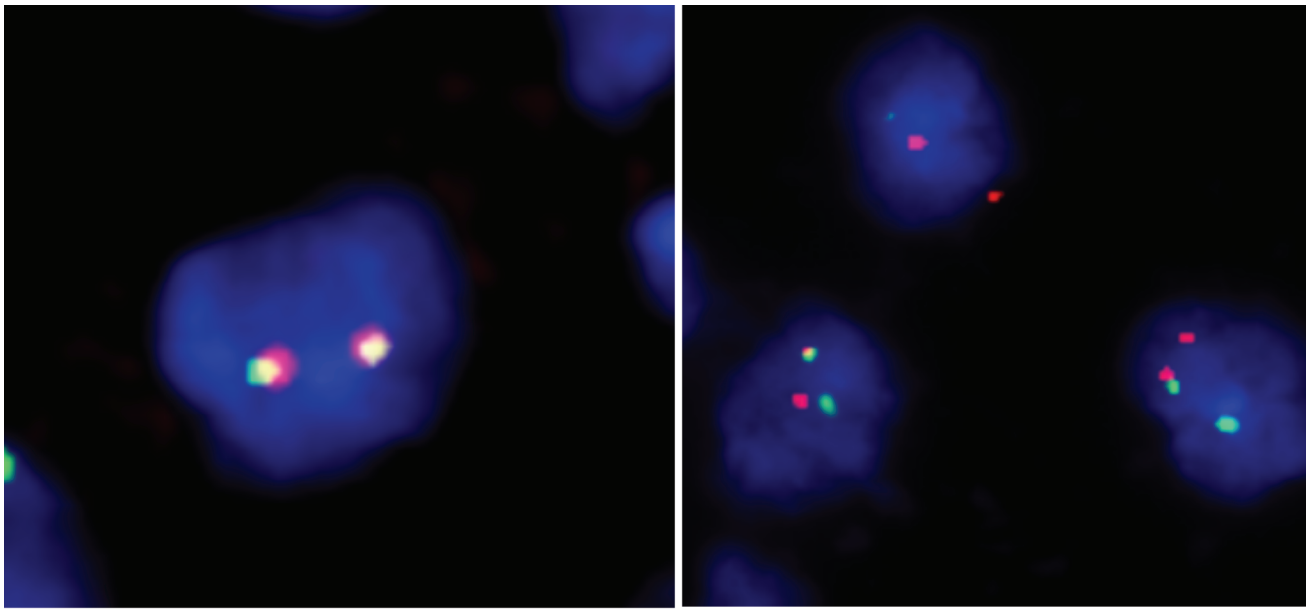

Figure 1. Fluorescent in situ hybridization utilizing a dual-color break-apart probe targeting the MYC gene is depicted. The red and green probes bind to the centromeric and telomeric segments of the MYC gene, respectively. Meanwhile, the background DNA within the nucleus is accentuated in blue using DAPI dye. Two fused red and green signals per nucleus denote the absence of the genetic rearrangement (left), whereas the presence of the rearrangement manifests as a configuration displaying one fused signal and two distinctly separated red and green signals (right).
DAPI: 4',6-diamidino-2-phenylindole

Table 1. Clinical features of the patients

	All patients (total: 152)		MYC rearranged (total: 7)		BCL2 rearranged (total: 4)		BCL6 rearranged (total:15)		Double-hit patients	
	N	%	N	%	N	%	N	%	N	%
Gender										
Male	85	55.9	5	71.4	2	50.0	11	73.3	2	100
Female	67	44.1	2	28.6	2	50.0	4	26.7	0	0
Nodal involvement										
Extranodal	55	36.4	4	57.1	4 ($P = 0.001$)	100.0	6	42.9	2	100
Nodal	96	63.6	3	42.9	0	0.0	8	57.1	0	0
B symptoms										
Absent	66	66.7	4	80.0	2	66.7	8	66.7	2	100
Present	33	33.3	1	20.0	1	33.3	4	33.3	0	0
LDH										
Normal	66	70.2	5	100.0	2	66.7	10	83.3	2	100
Elevated	28	29.8	0	0.0	1	33.3	2	16.7	0	0
Cell-of-origin based on HANS algorithm										
GC-like	43	31.2	3	42.9	2	50.0	7	50.0	1	50
ABC-like	95	68.8	4	57.1	2	50.0	7	50.0	1	50
Ann-Arbor staging										
1	81	53.3	4	57.1	2	50.0	4	26.7	1	50
II	39	25.7	2	28.6	2	50.0	8	53.3	1	50
III	20	13.2	1	14.3	0	0.0	3	20.0	0	0
IV	12	7.9	0	0.0	0	0.0	0 (P=0.040)	0.0	0	0
IPI Score										
Low	37	39.4	2	40.0	1	33.3	8	66.7	1	50
Low intermediate	13	13.8	0	0.0	0	0.0	1	8.3	0	0
High intermediate	21	22.3	3	60.0	2	66.7	1	8.3	1	50
High	23	24.5	0	0.0	0	0.0	2	16.7	0	0
ECOG performance score										
0	41	27.0	3	42.9	1	25.0	6	40.0	1	50
1	31	20.4	1	14.2	1	25.0	1	6.7	0	0
2	6	3.9	0	0.0	0	0.0	0	0.0	0	0
3	7	4.6	0	0.0	0	0.0	2	13.3	0	0
4	5	3.3	0	0.0	0	0.0	0	0.0	0	0
5	62	40.8	3	42.9	2	50.0	6	40.0	1	50
NCCN IPI										
Low	12	12.8	1	20.0	0	0.0	2	16.7	0	0
Low intermediate	32	34.0	1	20.0	1	33.3	7	58.3	1	50
High intermediate	26	27.7	2	40.0	1	33.3	1	8.3	0	0
High	24	25.5	1	20.0	1	33.3	2	16.7	1	50

ABC-like: Activated B cell-like; ECOG: Eastern Cooperative Oncology Group; GC-like: Germinal center-like; IPI: International Prognostic Index; LDH: Lactate dehydrogenase; N: Number; NCCN: National Comprehensive Cancer Network

The NCCN-IPI risk scoring combines the significant prognostic factors mentioned and logically correlates with OS ($P < 0.001$). A similar trend was observed for IPI (OS (95% CI): 29.0 (18.7-39.4), 26.4 (26.4-46.2), 27.5 (15.7-39.4), and 59.8 (52.9-66.7) in high, high-intermediate, low-intermediate, and low-risk groups, respectively; $P < 0.001$).

Discussion

In the present study, the investigation revolved around the rearrangements of MYC, BCL2, and BCL6 by FISH in Iranian DLBCL patients, evaluating their association with various clinico-pathologic characteristics, including OS. BCL6 emerged as the predominantly rearranged gene. Two double-hit patients were identified, with no triple-hit tumors observed. Additionally, it was found that, despite statistical non-significance,

these rearrangements adversely affected OS.

The prevalence of MYC, BCL2, and BCL6 rearrangements in the patient cohort was 4.8%, 2.9%, and 10.2%, respectively. These findings are comparable to those in a previous primary CNS DLBCLs (PCNSL) study, where the corresponding frequencies were 3.8%, 1.3%, and 12%, respectively.[13] They are also similar to the study conducted by Ting and colleagues.[14] However, others, particularly in recent years, generally have demonstrated higher positivity rates. The prevalence of MYC translocation has been reported as around 10% in the work of Cucco et al., up to 21.43% recorded by Ma et al., and regarding BCL2, Ma et al. and Abdul Salam et al. have described remarkably high frequencies of approximately 30%.[15-17] Similar high prevalences of 25%-40% are also on record for BCL6 rearrangements.[16-17]

The lower prevalence of MYC, BCL2, and BCL6 gene rearrangements in this series is likely attributed to the enrollment of patients with an expected better outcome, especially when considering the somewhat lower stage, lower LDH levels, and frequency of B symptoms compared to similar studies. Interestingly, Cucco et al. clearly state that their case selection was biased toward patients with a MYC translocation.[15] This underscores the potential consequences of clinical heterogeneity on the final results. Applying various FISH approaches (single versus multiple probes or break-apart versus fusion techniques) and distinct probe characteristics (such as length, location of binding, and type of dye) might be another reason for discrepant results. On the other hand, the

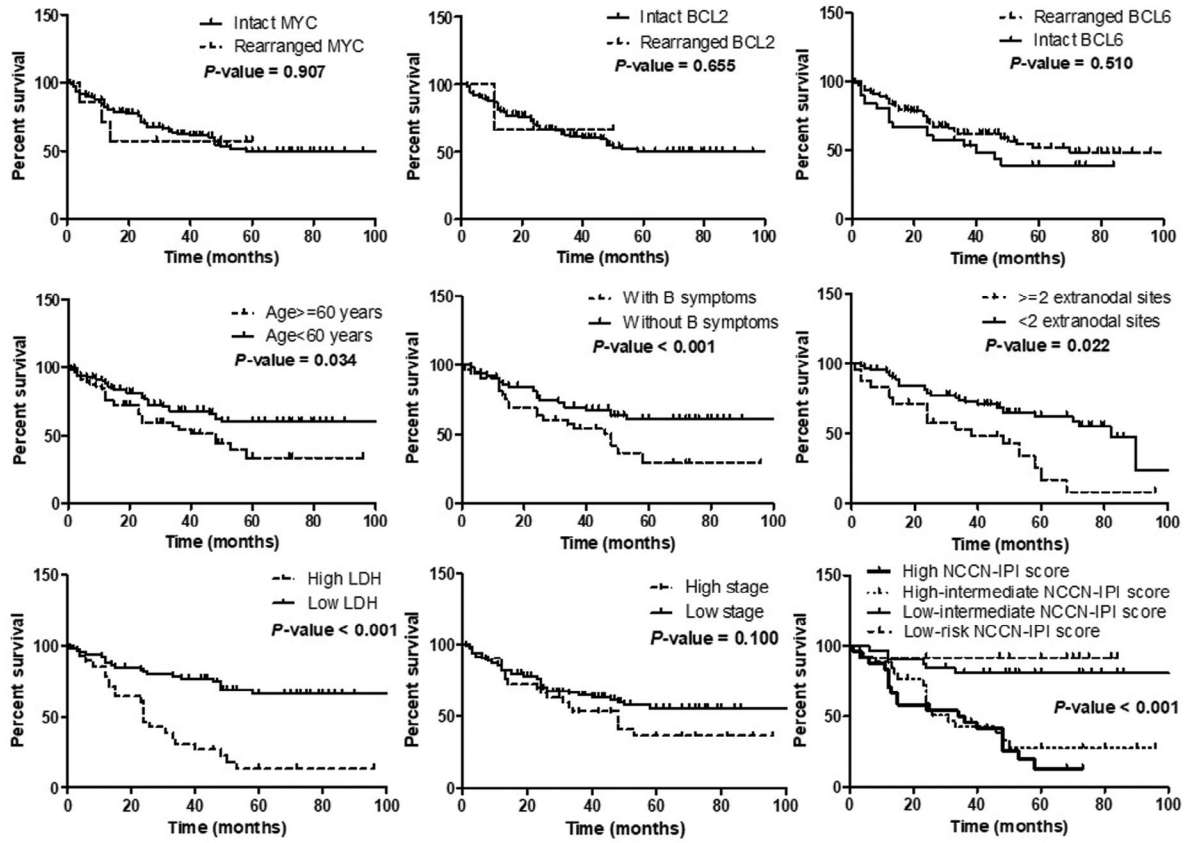

Figure 2. Kaplan-Meier curve analysis reveals that overall survival is not associated with MYC, BCL2, and BCL6 rearrangement status. Conversely, patients aged over 60 years, presenting B symptoms, elevated LDH levels, multiple extra-nodal site involvements, and a higher NCCN-IPI score exhibit a reduced overall survival. While an advanced stage demonstrates a connection to decreased survival time, this association does not attain statistical significance.

LDH: Lactate dehydrogenase; NCCN-IPI: National Comprehensive Cancer Network- International Prognostic Index

Table 2. Overall survival statistics based on MYC, BCL2, and BCL6 rearrangements status

Type of rearrangement	Mean OS (months)	Std. error	95% Confidence interval Lower	Upper	*P*-value (log-rank test)
C-MYC					
Rearranged (n=7)	38.4	9.47	19.9	57.0	0.907
Intact (n=140)	65.0	4.04	57.1	72.9	
BCL2					
Rearranged (n=4)	33.8	8.44	17.2	50.3	0.655
Intact (n=136)	65.0	4.08	57.0	73.0	
BCL6					
Rearranged (n=15)	54.0	6.87	40.5	67.5	0.510
Intact (n=128)	63.1	4.30	54.7	71.5	
Double-hit rearrangement					
Present (n=2)	30.5	13.79	3.5	57.5	0.785
Absent (n=133)	64.7	4.14	56.6	72.9	

n: Number; Std.: Standard; OS: Overall survival

differences in the prevalence of MYC, BCL2, and BCL6 gene aberrations may be a reflection of the striking molecular heterogeneity in DLBCL.[9, 18] Overall, a combination of case selection bias, technical aspects, and tumor heterogeneity contributes to the lower frequency of gene rearrangements in the cohort.

In the current study, DHLs (1.5% of all patients) had MYC and BCL2 rearrangements. According to multiple previous studies, coexisting rearrangements of MYC and BCL2 shape the most prevalent type of DHL.[11, 15-16] Similarly, in both the PCNSL series and the study by Ting et al., one patient (approximately 1.5%) had a DHL tumor.[13-14] DHLs are known to be associated with GCB-like status, and it has been postulated that they drive from BCL2-rearranged follicular lymphomas, which acquire a secondary MYC alteration, leading to aggressive behavior and poor response to standard treatments.[11, 15-16] Nevertheless, in this study, one DHL patient had GCB-like disease, and the other had ABC-like disease. Thus, the low DHL frequency prevented the discovery of such associations. The series did not include any triple rearrangement tumors, a rare finding in other studies. This study's results concerning double-hit and triple-hit tumors are relatively comparable to the available evidence.

In agreement with previous studies, it was also found that patients harboring any of these rearrangements are expected to experience a more aggressive clinical course and inferior outcome.[11, 14, 16, 19] However, the differences in OS failed to reach statistical significance, which may reflect the reasonably small sample size. Furthermore, it was demonstrated that both the IPI and the NCCN-IPI risk scores, along with their primary components, such as advanced age, the presence of B symptoms, elevated LDH levels, and involvement of multiple extra-nodal sites, all exert detrimental effects on the overall prognosis.[3, 16] Of note, The low IPI/NCCN-IPI score was associated with more than 30 months of longer outcomes compared to the high-risk IPI/NCCN-IPI group.

The present study is not without limitations. The retrospective nature makes it prone to the entry of possible errors in medical records into the study. Moreover, the relatively low sample size and the high censored rate (almost 60% alive subjects) lower the power of the statistical analysis, particularly the survival analysis, in finding significant differences. Furthermore, despite the present study not including comprehensive genetic profiling, the effects of the molecular heterogeneity of DLBCL in the study findings should not be overlooked.

Conclusion

DLBCL with MYC and BCL2 and/or BCL6 translocations is relatively uncommon. Despite these rearrangements, patients manifest an

aggressive form of the disease, resulting in diminished OS; however, these discrepancies lack statistical significance. Consequently, the current justification for incorporating these assessments into the routine evaluation of patients with DLBCL and NOS remains unsubstantiated. Further investigation is imperative to elucidate the clinical advantages of these tests, potentially by implementing more intensified treatment protocols tailored to tumors bearing such genetic rearrangements.

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Conflict of Interest

None declared

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