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ORIGINAL ARTICLE

BCL2 positive and BCL6 negative diffuse large B cell lymphoma patients benefit from R-CHOP therapy irrespective of germinal and non germinal center B cell like subtypes

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Summary

Purpose: *Despite major advances in the treatment of diffuse large B cell lymphoma (DLBCL), approximately one third of the patients progress or die, suggesting the existence of additional oncogenic events. The purpose of this study was to evaluate the prognostic value of the "Hans classifier", and BCL2 and MYC protein expression and gene alterations in DLBCL patients treated with CHOP or R-CHOP chemotherapy over a 5-year period. Furthermore, we tried to correlate these parameters with the International Prognostic Index (IPI).*

Methods: *The immunohistochemical (IHC) expression of CD10, BCL6, MUM1 and BCL2 on paraffin-embedded formalin-fixed tumor samples from 103 centroblastic DLBCLs was analyzed. IHC expression of MYC and fluorescence in situ hybridization (FISH) for MYC and BCL2 gene alterations was performed on 67 samples using the tissue microarray (TMA) method.*

Results: *The Hans algorithm was not predictive of survival in both therapy groups. No significant difference in BCL2 and MYC alterations or MYC protein expression in relation to complete response (CR), event-free survival (EFS) and overall survival (OS) was observed in our study. High IPI correlated significantly with poor outcome and it was identified as independent prognostic factor for OS and EFS (both p=0.000). The 5-year OS was 61% in the R-CHOP compared to 38% in the CHOP group (p=0.007). Rituximab significantly improved the OS in the BCL2 positive (60 vs 29%, p=0.008), and the BCL6 negative (73 vs 25%, p=0.001) cases.*

Conclusion: *IPI is an independent prognosticator for DLBCL patients and the addition of rituximab significantly improved survival. Furthermore, patients with BCL2+ and BCL6- DLBCL benefited from R-CHOP.*

Key words: *BCL2+, BCL6-, DLBCL, R-CHOP*

Introduction

DLBCL is the most common lymphoma with clinical, morphological, immunohistochemical, and molecular heterogeneity [1].

In the last two decades there has been a significant improvement in the outcome of these patients after the addition of rituximab to standard chemotherapy [2,3]. Despite this progress, there is still a significant number of patients with unfavorable course and disease outcome [4]. The accurate identification of patients with poor outcome and definition of a risk-adopted treatment strategy still remains a challenge. By now, only the IPI, based on 5 independent clinical and laboratory parameters, is being routinely used as a predictor of survival [5,6]. However, a substantial variability in outcomes has been observed in IPI

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subgroups.

Therefore, further attempts have been made to identify new prognostic parameters that might explain disease aggressiveness and tumor progression.

On the basis of the cell-of-origin (CoO) concept, gene expression profiling (GEP) has identified two major subtypes of DLBCL with different prognosis [7,8]. As GEP is not part of the current routine diagnostic work-up, different attempts have been made to find an IHC algorithm as a surrogate for GEP. Hans and colleagues proposed an algorithm based on CD10, BCL6 and MUM-1/IRF4 expression determined by IHC, to distinguish germinal center B cell (GCB) from non-GCB subtypes of DLBCL, with different outcomes [9]. However, the prognostic value of the Hans IHC algorithm as a surrogate for GEP is still controversial.

Recently, significant progress in molecular techniques has helped to better understand the tumor biology and consequently redefine the DLBCL risk groups [10-12].

MYC and BCL2 are oncogenes that are often deregulated in B-cell lymphomas [13]. BCL2 gene product is an anti-apoptotic protein that is important in normal B-cell development and differentiation [13], and plays an important role in the response of malignant cells to chemotherapy [13,14]. BCL2 protein overexpression has been reported in approximately 40-60% of patients with DLBCL, and has been associated with the t(14;18)(q32;q31), which is largely restricted to GCB-DLBCL [13-15], whereas in ABC-DLBCL the mechanism of BCL2 overexpression is associated with constitutive NF-kB activation [12-15] with or without 18q21 amplification [16]. The role of BCL2 as a predictor of survival in DLBCL is controversial [17,18].

MYC product is a transcriptional factor with a central role in cell proliferation, differentiation and metabolism [13]. MYC translocation has been reported to occur in DLBCLs with a frequency of 3 to 16% [19-24]. A recent study suggested that DLBCL with c-MYC gene translocation has a poorer prognosis and is poorly responsive even to rituximab plus CHOP therapy [19,22,23]. MYC protein overexpression has not been fully investigated in the new therapeutic era [24,25].

Finally, approximately 5% of DLBCLs are "double-hit" (DH) lymphomas [13,26]. The term DH is mostly used for the cases with common chromosomal breakpoints, affecting the MYC/8q24 locus in combination with a t(14;18)(q32;q21) involving BCL2 [26,27]. Patients with DH lymphomas are considered to constitute a poor prognostic group [26,27].

The aim of this study was to evaluate the prognostic value of "Hans classifier", and to compare BCL2, BCL6 and MYC protein expression and gene alterations in DLBCL patients treated with CHOP or R-CHOP chemotherapy.

Furthermore we tried to correlate these parameters with IPI and their significance was evaluated according to treatment response and OS and EFS.

Methods

A retrospective analysis was performed on 103 *de novo* DLBCL patients, diagnosed at the Clinic of Hematology, Clinical Center of Serbia, from January 2001 to December 2005. DLBCL patients treated with R-CHOP or CHOP protocols with or without adjuvant radiotherapy and/or surgery over a 5-year period. The response rates were analyzed according to the Cheson's criteria [28].

Only those cases considered to be DLBCL not otherwise specified (NOS), according to WHO classification, were included [1]. Cases of histologic transformation of indolent lymphoma to DLBCL, and those with central nervous system, intravascular or primary mediastinal lymphomas were not included. Only DLBCL with centroblastic morphology were selected to provide morphological homogeneity of the cases.

This study was performed on diagnostic tissue samples after approval by the Institutional Review Board of the Clinic for Hematology, Clinical Center of Serbia, according to the Helsinki Declaration and good clinical practice policy.

Histologic review and TMA construction

Standardized methods for tissue fixation and processing were used. IHC was performed on archival, paraffin-embedded tissue samples, on 4μm-thick tissue sections, using a manual method for CD20 (L26; Dako, Glostrup, Denmark), CD3 (Dako), CD10 (clone 56C6; Dako), Ki-67 (Dako), BCL6 (clone PG-B6p; Dako), MUM1 (clone MUM1p; Dako), BCL2 (clone 124; Dako) according to the manufacturer's instructions applying an avidin-biotin complex method (LSAB2 kit peroxidase, Dako) using 3-amino-9-ethylcarbazole (AEC) as high sensitivity substrate ready-to-use chromogen (Dako). Sections were counterstained with Mayer hematoxylin.

The patients were classified into germinal center B cell like (GCB) and non GCB subtypes according to the Hans algorithm [9].

The material necessary to construct a TMA was available in 67 of 103 patients. Three TMA blocks were constructed using duplicate 0.6-mm cores [9,29]. MYC IHC was done on TMA sections using the antibody

clone Y69 (ABCAM, Cambridge, UK) at a 1:50 dilution.

Cases were considered positive when at least 50% of tumor cells expressed BCL2 protein [9,30]. For MYC, a cutoff of more than 30% of tumor cells was used [31].

The diagnostic samples were evaluated by three hematopathologists (VCM, MPJ, TT), discrepant scores were reviewed and an agreement was reached.

Fluorescence in situ hybridization analysis

Three TMAs were studied with FISH for MYC and BCL2 [29], using commercial, directly labelled split probes BCL2 and C-MYC as centromeric probes for chromosomes 8 and 18 (Abbott Vysis, Des Plaines, Ill, USA) as previously described [32].

Slides were examined under a Leica DM 2500 fluorescence microscope (Leica, Wetzlar, Germany) equipped with single and triple band pass emission filters. Digital images were taken using a Leica DFC3000 G camera (Leica, Wetzlar, Germany) operated via the Leica CW 4000 software.

Clinical and laboratory data

In all patients standard clinical and laboratory data were collected [5,33]. The IPI was calculated according to the 5 high risk features: age >60 years, performance status (PS) >2, Ann Arbor tumor stage 3 and 4, LDH >460 U/L, and number of extranodal sites >1. Patients were divided into a low risk group (0 to 2 factors) and a high risk group (3 or 4 factors) [5,6].

Statistics

EFS was measured from the start of treatment to the date of primary treatment failure, relapse, or the date of last uneventful follow-up. OS was measured from the beginning of treatment to the time of last follow-up (censored patients) or time of death.

For univariate analysis, the x^2 and Fischer exact tests were used to assess the association between molecular and clinical and laboratory variables. Survival analysis was performed by the Kaplan-Meier method. The statistical significance of differences in EFS and OS between groups of patients was estimated by the log rank test. Statistical significance of prognostic variables was also evaluated by multivariate analysis using the Cox proportional hazard model. For nonparametric variables and analysis of factors influencing the treatment outcome, the nonparametric Mann-Whitney U test and Kruskall-Wallis test were applied.

All tests were two-sided, and p values less than 0.05 were considered statistically significant. All statistical analyses were performed by licensed Statistical Analysis Software (Stat Soft, Inc. Tulsa USA, 2013. STATISTICA data analysis software system, version 12; www.statsoft.com) [34].

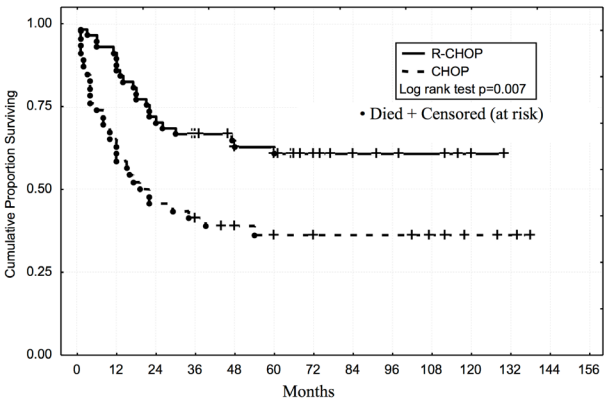


Figure 1. Overall survival rate according to treatment.

Results

Clinical characteristics

The laboratory and clinical features of 103 patients with DLBCL are listed in Table 1.

The majority of DLBCLs arose in the lymph nodes (N=45;43.68%). Other lymphatic organs primarily infiltrated were the spleen in 9 (8.73%) cases, and the tonsil in 2 (1.9%) cases. Five tumors involved exclusively the bone marrow. Forty two cases (40.77%) presented with primary extranodal DLBCL, the majority of them (26 cases) in the stomach but also in the epipharynx (4 cases), lung (3 cases), bone (3 cases), the small and large intestine (2 cases), the uterus, testis, thyroid gland and maxillary sinus (one case each).

Forty six (44.66%) patients received CHOP or CHOP-like therapy, and 57 (55.33%) patients received R-CHOP therapy. The 5-year OS was 61% in the rituximab group and 38% in the group treated with standard chemotherapy alone (p=0.007) (Figure 1). The 5-year EFS was 60 and 36% for the rituximab and chemotherapy-alone groups, respectively (p=0.007).

After the first-line therapy, CR was achieved in 75 (73%) patients. CR was observed more frequently in patients with low Ann Arbor stage, low IPI score, and normal LDH in the CHOP therapy group (p=0.07, p=0.001, p=0.001, respectively).

Univariate analysis showed that high IPI significantly influenced OS (75 vs 25%, p=0.000) for both therapy groups (Figure 2). In multivariate analysis, high IPI was identified as independent prognostic factor for OS and EFS (both p=0.000).

Unfavorable variables predicting OS were: high IPI, bulky disease, high serum LDH levels and B-symptoms in the CHOP group (p=0.000, p=0.02, p=0.000, p=0.05, respectively) and high IPI, advanced Ann Arbor stage (III-IV) and B-symptoms

Table 1. Clinical and immunohistochemical characteristics of DLBCL patients

Characteristics	*N*	*%*
Patients	103	100
Age, years, median (range)	56 (17-87)	
<60	59	57
≥60	44	43
Gender		
Male	55	53
Female	48	47
Ann Arbor stage		
I-II	30	29
III-IV	73	71
B-symptoms		
yes	80	78
High serum LDH		
yes	67	66
Bulky disease		
yes	26	25
Extranodal involvement yes	83	81
Bone marrow involvement yes	33	32
IPI risk group		
Low, 0-2	53	51
High, 3-5	50	49
Presentation		
Nodal	45	44
Other lymphatic organs	11	11
Extranodal	47	46
Subtype		
GCB	28	27
Non-GCB	75	73
Bcl-2 (IHH) positive	63	61
Bcl-6 (IHH) positive	58	56
Therapy		
CHOP	46	45
R CHOP	57	55

GCB: germinal center B cell

in the R-CHOP therapy group (p=0.002, p=0.01, and p=0.05, respectively).

Immunohistochemistry

According to the Hans algorithm, a GCB phenotype was observed in 28 (27%) and non-GCB in 75 (72%) cases. CD10 was expressed in 21% if the cases, BCL6 in 56% and MUM-1 in 64%.

CR was observed more frequently in patients with GCB DLBCL in the group of patients which received CHOP therapy (p=0.07) and significantly more frequently in the R-CHOP therapy group (p=0.05). Neither single antigen expression was able to predict CR except MUM-1 expression in the group of patients treated with CHOP (p=0.01).

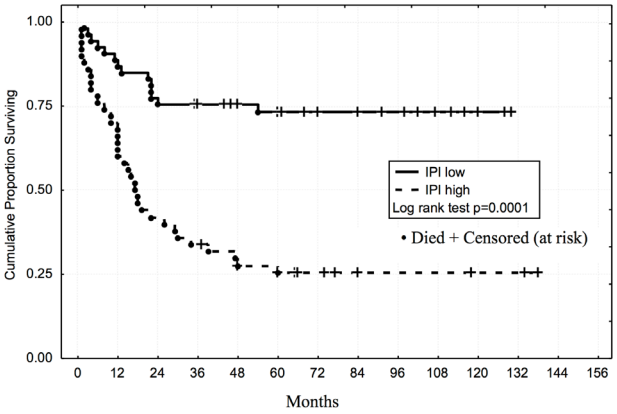


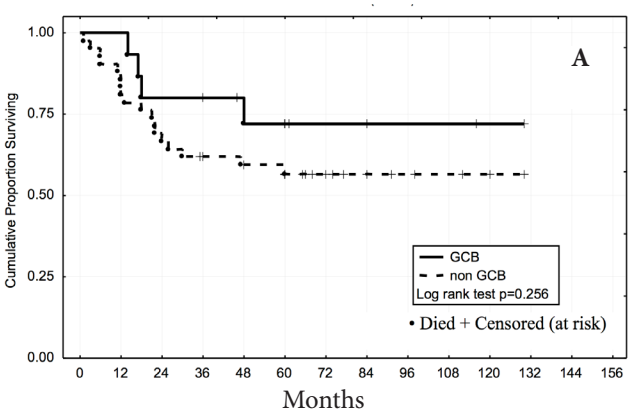
Figure 2. Overall survival rate according to International Prognostic Index.

Rituximab-treated patients in the GCB subgroup had better OS and EFS than those in the non-GCB subgroup (OS 72 vs 57%, p=0.256; EFS p=0.332), but the difference did not reach statistical significance (Figure 3A). Subclassification on the basis of the CoO was not predictive of survival in patients treated with standard chemotherapy alone (GCB vs non-GCB, p=0.7) (Figure 3B). Neither single antigen expression nor the differentiation profile assessed by IHC algorithms was able to predict OS significantly.

Patients in the GCB group who received R-CHOP had significantly better OS than those treated with standard therapy alone (5-year OS 72 vs 38%, p=0.031). Patients with non-GCB DLBCL subtype who received R-CHOP had significantly better OS than those treated with standard chemotherapy alone (5-year OS 57 vs 37%, p=0.05).

We also examined the importance of BCL6 expression in patients with DLBCL who were treated with standard chemotherapy alone, and in those treated with rituximab. In the group of patients who received R-CHOP, BCL6 negative cases had better OS than BCL6 positive cases (73 vs 51%, p=0.139). In the CHOP group, BCL6 positive cases had better OS than BCL6 negative ones (47 vs 25%, p=0.086).

The addition of rituximab to standard chemotherapy did not improve the OS of patients in the BCL6 positive group (51 vs 47%, p=0.568), but patients with BCL6 negative DLBCL had significantly better OS in the R-CHOP group than those treated with standard chemotherapy alone (73 vs



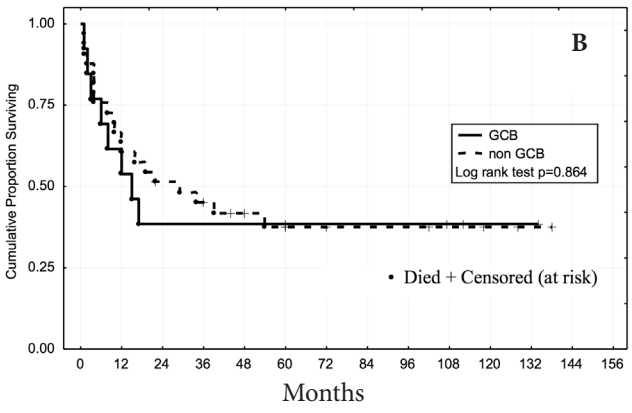
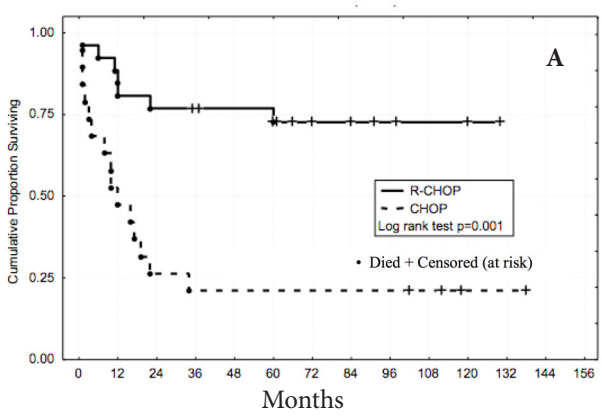


Figure 3. A: Overall survival rates in rituximab-treated patients according to cell of origin. **B:** Overall survival rates in non-rituximab treated patients according to cell of origin.



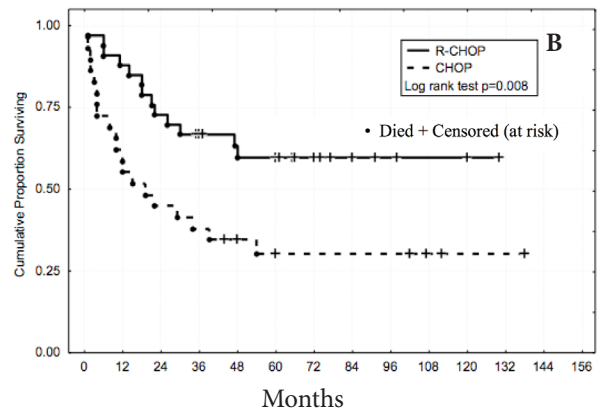


Figure 4. A: Overall survival rates of BCL6 negative patients according to treatment. **B:** Overall survival rates of BCL2 positive patients according to treatment.

25%, p=0.001) (Figure 4A).

BCL2 protein expression was detected in 63% of the patients (Figure 5A). Expression of BCL2 protein was not predictive of OS in either group treated with R-CHOP (p=0.901) or with CHOP (p=0.267). Addition of rituximab to standard chemotherapy significantly improved the OS of patients in the BCL2 positive group (60 vs 29%, p=0.008) (Figure 4B), but not in the BCL2 negative group (59 vs 53%, p=0.456).

BCL2 alterations were detected in 13 of 67 (19.40%) DLBCL (3 rearrangements and 10 amplifications) (Figure 5B). Nine of 13 cases with BCL2 alterations showed BCL2 positivity by IHC. BCL2 protein expression (45 of 65; 69.23%) and BCL2 amplification (10 of 10; 100%) were associated with the non-GCB subtype. BCL2 translocation (3 of 3, 100%) was associated with the GCB subtype.

MYC protein was overexpressed in 27 cases (40.29%) which showed ≥30% of tumor nuclei stained positive, while 20 cases (29.85%) showed MYC positive nuclear staining in 10-30% of tumor cells (Figure 5C). Twenty cases (29.85%) were negative. Among the MYC+ group, 40% of the cases were non-GCB subtype and 28% were GCB subtype.

In total, 13 of 67 (19.40%) cases of DLBCL had MYC alterations. MYC rearrangement and MYC amplification were detected in 5 (7.46%) of 67, and 8 (11.94%) of 67 patients, respectively (Figure 5D). The MYC rearrangement was the sole translocation in 4 of 67 (5.97%) tumor samples. One case with MYC rearrangement had BCL2 amplification. Two cases with MYC amplification had BCL2 amplification also.

Four cases (4 of 5; 80%) of DLBCL with a MYC rearrangement showed MYC protein overexpression, and only 2 out of 8 cases (25%) MYC amplifications. An additional 19 (19 of 54; 35%) cases without MYC gene alterations had increased MYC

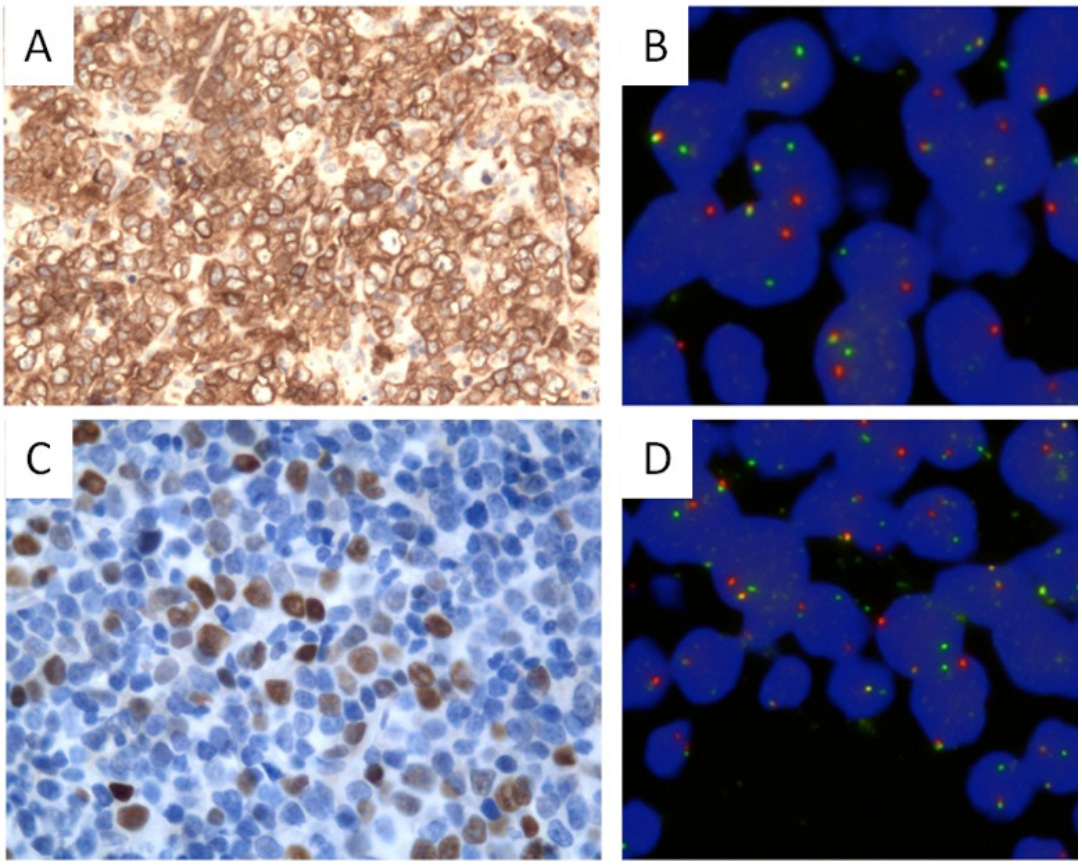


Figure 5. BCL2 and MYC IHC expression and FISH in DLBCL cases. **A:** BCL2 expression (immunohistochemistry x200). **B:** BCL2 rearrangement (FISH x1000). **C:** MYC expression (immunohistochemistry x400). **D:** MYC rearrangement (FISH x1000).

expression by IHC. Patients with MYC gene alterations more frequently had high-risk IPI scores than the other patients (74.1 vs 66.6%, p=0.529).

The median age of 3 male patients with dual MYC/BCL2 alterations was 57 years (range 39-70). Two patients had stage IV disease, high-risk IPI scores, and survival of 4 and 6 months, respectively. Non-GCB subtype, high MYC protein expression and BCL2 protein expression were detected in all DLBCL patients with dual MYC/BCL2 alterations. Concurrent protein expression of MYC and BCL2 was present in 12 (17.91%) of 67 patients.

Discussion

DLBCL is a disease with marked heterogeneity [1]. Despite major progress in treatment, the outcome is fatal in almost half of the patients with DLBCL [35,36].

Many recent reports have indicated that only IPI score has an important role as prognostic factor for survival of DLBCL patients [37]. In the present study, univariate analysis showed that high IPI significantly influenced OS in both therapy groups. Additionally, we identified IPI, Ann Arbor stage and LDH to be predictors of CR achievement in the CHOP, but not in the R-CHOP therapy group.

Since rituximab was added to CHOP a general improvement in survival rates has been achieved [2,37]. We confirmed that 5-year OS and EFS were significantly better in the rituximab group.

Despite major advances in treatment, approximately one third of the patients experience refractory disease or early relapse, suggesting the existence of additional oncogenic events. In this respect, recent studies focused on the evaluation of molecular and genetic markers associated with survival [10,12,18,25].

As GEP is not part of the current routine diagnostic work-up, several IHC algorithms have been proposed as surrogates for GEP, but with conflicting results and variable degrees of success [9,38,39]. The study of Garcia et al. showed that none of the 5 IHC algorithms was able to predict OS and progression-free survival (PFS) based on

GCB vs non-GCB subtypes [40]. In our study, GCB vs non-GCB phenotype according to Hans' algorithm had no prognostic impact on OS in either CHOP or R-CHOP therapy groups, which was in accordance with the current literature. However, our study revealed that CR achievement was significantly more frequent in patients with GCB DLBCL in the R-CHOP therapy group.

BCL6 protein overexpression in DLBCL has been associated with a better prognosis in CHOP-treated patients [9,10,17]. In the study of Winter et al. in BCL6 negative patients treated with R-CHOP, failure free survival (FFS) and OS were prolonged compared to CHOP alone [41].

Our findings demonstrated that in the group of patients treated with CHOP alone, BCL6 positive cases had better OS than BCL6 negative cases. On the contrary, in the group of patients who received R-CHOP, BCL6 negative cases had better OS than BCL6 positive cases. Patients with BCL6 negative DLBCL had a significantly better survival in the R-CHOP group than those treated with CHOP alone. The addition of rituximab benefits BCL6 negative but not BCL6 positive cases, which is consistent with the majority of studies [41-43].

The poor prognosis of CHOP-treated DLBCL patients with BCL2 overexpression is reported in some studies, but in patients treated with R-CHOP no correlation with survival was seen [44]. The addition of rituximab was reported to have eliminated the negative impact of the BCL2 overexpression [3,45]. Our study showed that expression of BCL2 protein was not predictive of OS in either group treated with rituximab, or the group treated with standard chemotherapy alone. Additionally, we confirmed that rituximab significantly benefited BCL2 positive but not BCL2 negative cases, which is in concordance with recently published data.

Most studies have shown that MYC high protein expression was associated with inferior outcome in the R-CHOP, but not in the CHOP-treated patients [13,46]. The small number of uniformly treated cases with gene alterations and MYC+ cases using IHC limited the statistical correlations with outcome in our study. The results of Green et al. and Johnson et al. showed that DLBCL patients with dual expression of MYC and BCL2 protein had a poor prognosis with immunochemotherapy [13,47]. Our findings revealed that survival of DLBCL patients with dual expression of MYC and BCL2 protein in the R-CHOP group was significantly better than in patients treated with CHOP.

The mechanism by which BCL2, BCL6 and MYC overexpression selectively influences the outcome of R-CHOP-treated patients in contrast to patients treated with CHOP only is unknown [45-47].

The incidence of the genetic changes and MYC and BCL2 protein expression in our study is similar to that found in other series and the presence of MYC rearrangement correlated with MYC protein expression [24,27,46,47].

An additional finding from the MYC protein expression studies is the identification of DLBCLs with MYC protein overexpression in the absence of MYC alterations. In our study we also identified 35% DLBCLs with high MYC protein expression in the absence of a MYC alteration. These data suggest that mechanisms other than gene alterations may cause overexpression of the MYC protein [46,47].

In conclusion, our results showed that Hans algorithm was not predictive of survival. We have confirmed that IPI is an independent prognosticator for DLBCL patients and that the addition of rituximab significantly improves survival. Furthermore, patients with BCL2+ and BCL6- DLBCL benefited from R-CHOP. These results should be investigated in larger series of patients.

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