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BCL2, BCL6, IGH, TP53, and MYC protein expression and gene rearrangements as prognostic markers in diffuse large B-cell lymphoma: a study of 44 Turkish patients

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The purpose of this study was to determine the frequency of BCL2, BCL6, IGH, TP53, and MYC protein expression and rearrangements of the respective genes in diffuse large B-cell lymphoma (DLBCL) patients and to assess their prognostic values. Samples from 44 patients with DLBCL were evaluated using fluorescence in situ hybridization and immunohistochemical analyses. *BCL6* was the most rearranged gene (63.6%), followed by *MYC* (31.8%), *TP53* (22.7%), and *BCL2* (18.2%). Multiple rearrangements were detected in 40.9% of the cases. BCL6 was the most expressed protein (78.6%), followed by TP53 (69.04%), BCL2 (59.5%) and MYC (14.3%). Expression of multiple proteins was detected in 67.4% of the cases. BCL2 ($P = .003$) expression had a significant negative influence on overall survival,whereas BCL6 ($P = .014$) expression had a significant positive influence. Our results with a different pattern of gene rearrangements and associated protein overexpression indicate the molecular genetic complexity of DLBCLs, which reflects the morphologic, biologic, and clinical heterogeneity of these lymphomas.

Keywords BCL2, BCL6, TP53, MYC, diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma, accounting for approximately 40% of all B-cell non-Hodgkin lymphomas (NHLs). DLBCLs form a highly heterogeneous type with different clinical, morphological, immunological, and cytogenetic characteristics, treatment responses, and prognoses (1,2). During the past decade, most studies about the heterogeneity of DLBCL have focused on genetic and molecular analyses (3). Gene expression profiling studies have identified two prognostically important subtypes of DLBCL—germinal center B-cell (GCB)–like DLBCL and activated B-cell (ABC)–like DLBCL—with a survival advantage in GCB-like DLBCL patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine sulfate, and prednisone (R-CHOP); patients with GCB-like DLBCL had higher overall survival (OS) and progression-free survival (PFS) rates than those with ABC-like DLBCL (4).

DLBCLs show considerable molecular genetic heterogeneity. The most frequently detected genetic alteration is rearrangement of *BCL6* at 3q27, occurring in ≤35% of cases (5). The reported prognostic impact of BCL6 protein expression and gene rearrangement in DLBCLs is controversial. A favorable prognostic impact of BCL6 protein expression by immunohistochemistry (6–8) and *BCL6* mRNA expression by real-time reverse transcription–PCR assay have been documented, whereas *BCL6* rearrangement was an adverse predictor of disease-free survival (9). *BCL2* rearrangement involving the t(14;18)(q32;q21) is

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expressed in 20–30% of DLBCL cases (10). Prior studies have reported varying results regarding the prognostic significance of BCL2 protein expression and gene rearrangement in DLBCLs. A number of studies have linked a negative relationship between survival and overexpression of the antiapoptotic BCL2 protein in patients with DLBCL (9,11–17). Barrans et al. and Tang et al. reported decreased survival in patients with a *BCL2* rearrangement (18,19), whereas other studies attributed better survival in *BCL2*-rearranged cases (20,21). Rearrangement of the *MYC* gene, frequently involving the t(8;14)(q24;q32), is characteristic of Burkitt lymphoma, but its presence has also been confirmed by Southern blot analysis or fluorescence in situ hybridization (FISH) analysis at a frequency of 5–15% in DLBCL patients, who had an unfavorable prognosis (11,12,22,23). Double-hit B-cell lymphomas, which are characterized by a *MYC* breakpoint in combination with *BCL2* breaks, as well as *BCL6* rearrangement, also run an aggressive clinical course (24). Secondary molecular lesions have also been implicated in the pathogenesis of DLBCL. The *TP53* gene plays a central role in cell-cycle arrest or cell death after DNA damage and prevents genetic instability (10). *TP53* deletion, as detected by FISH analysis, and protein expression have been identified as strong predictors of poor survival in patients with DLBCL (25–27). Additionally, coexpression of *TP53* with *MYC* and *BCL2* was associated with a poorer prognosis than expression of TP53 alone (28,29).

Taken together, we have two main purposes in this study: 1) to determine the frequency of BCL2, BCL6, IGH, TP53, and MYC protein expression and gene rearrangements and 2) to assess the prognostic impact of these markers in DLBCL patients.

Patients and methods

Patients

A total of 44 patients who had been diagnosed with DLBCL between 1996 and 2011 at Eskişehir Osmangazi University Hematology Department were included in this study. Patients were classified according to the WHO classification (30). The samples were prepared from the paraffin blocks of lymph nodes that were recovered from the archives of the Department of Pathology. Histological sections of 3–4 μm thickness taken from a representative tissue block were placed on charged slides for fluorescence in situ hybridization (FISH) and immunohistochemical analysis. Slides for FISH were referred to the Cancer Cytogenetics Section of the Medical Genetics Department. Approval was obtained from the Ethics Committee of our university for the study and the study was performed in accordance with ethical standards of the Helsinki Declaration.

FISH analysis

FISH analysis was performed using Vysis CEP 3 Single Color DNA Probe, Vysis LSI BCL6 Dual Color Break Apart Rearrangement DNA Probe, Vysis CEP 8 Single Color DNA Probe, Vysis MYC Dual Color Break Apart DNA Probe, Vysis LSI IGH/CCND1 Dual Color Dual Fusion Translocation DNA Probe, Vysis LSI IGH/BCL2 Dual Color Dual Fusion Translocation DNA Probe, LSI P53 (17p13.1) Single Color DNA Probe, and Vysis CEP 17 Single Color DNA Probe (Abbott Molecular, Abbott Park, IL) and were used to detect the IGH/BCL2, BCL6 translocations according to the manufacturer's protocols. The slides were analyzed using an Olympus BX61 fluorescence microscope (Olympus, Tokyo, Japan) and images were captured with a charge-coupled device camera using an image analysis system (Applied Imaging, Grand Rapids, MI). At least 50–100 nuclei were examined for each probe whenever possible. The signals derived from the target gene or chromosome centromere were calculated. The cutoff values for each probe were as follows: 3–5% for the LSI BCL6/CEP 3, MYC/CEP 8, and LSI P53 (17p13.1)/CEP 17 probe set and 1% for the LSI IGH/BCL2 t(14;18)(q32;q21) dual-color, dual-fusion translocation probe set and the LSI IGH/CCND1 t(11;14)(q13;q32) dual-color, dual-fusion translocation probe set.

Immunohistochemistry

Immunostaining for BCL2 (clone:100/D5) (Neomarkers, Fremont, CA), MYC (clone 9E10.3, working dilution 1:75) (Neomarkers), and TP53 (clone: D0-7) (Neomarkers) was performed using the labeled streptavidin–biotin–peroxidase complex method as described in the instructions provided by the manufacturer. BCL6 (clone bl6.02:same as PG-B6p, working dilution 1:20) (Neomarkers) staining was performed using a Lab Vision HRP polymer detection system (Thermo Fisher Scientific, Waltham, MA). Immunohistochemical results were evaluated semiquantitatively. A cutoff of 10% of immunoreactive neoplastic cells was used to consider a case positive (7,8,31). Staining showing nuclear localization for BCL6, MYC, and TP53 and cytoplasmic localization for BCL2 in degrees weak to strong were evaluated as positive. Tumors with any follicular pattern were excluded.

Statistical analysis

OS was calculated from diagnosis until death (all causes, events) or last follow-up. PFS was defined as the time from initial diagnosis to progressive disease under therapy, failure to achieve complete remission, additional therapy, relapse, or death from any cause. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. Multivariate analyses were performed with the use of a Cox regression model to determine hazard ratios of an evolving event. All statistical analyses were performed using the SPSS IBM Statistics 20 (IBM Corporation, Chicago). A P value of $<.05$ was considered significant.

Results

Clinical features

The demographic and clinical characteristics of the patients are shown in Table 1. Of the 44 patients, 24 were male and 20 were female, and the age at diagnosis ranged from 27 to 77 years (mean age, 56.6 y). Overall, advanced stage (III/IV) lymphoma was observed in 23 cases (52.3 %), 30 patients

Table 1 Clinical characteristics of DLBCL patients at diagnosis (n = 44)

Characteristic	No. of patients	% of patients
Age		
Mean (minimum−maximum), y	56.6 (27−77)	
Sex		
Male	24	55
Female	20	45
Ann Arbor Cancer Stage		
I	8	18.2
II	13	29.5
III	13	29.5
IV	10	22.8
IPI group		
Low risk	16	36.4
Low/intermediate risk	12	27.3
High/intermediate risk	12	27.3
High risk	1	2.2
Nonassessable	3	6.8

(68.2%) presented with high serum lactic acid dehydrogenase (LDH) levels, and 14 of the 32 patients with available data had high serum β2-microglobulin levels. According to the International Prognostic Index (IPI), 16 cases (36.4%) were classified as low risk, 12 cases (27.3%) were classified as low/intermediate risk, 12 cases (27.3%) were classified as high/intermediate risk, one case (2.2%) was classified as high risk, and three cases (6.8%) were nonassessable.

Rearrangements detected by FISH

FISH analysis was successfully performed for all patient samples. Rearrangement of *BCL6* was found in 28 patients (63.6%), *BCL2* rearrangement in eight (18.2%), *MYC* rearrangement in 14 (31.8%), and *TP53* rearrangement in 10 (22.7%) cases. Figure 1 shows representative examples of FISH analyses.

Multiple rearrangements were detected in 18 (40.9%) cases: *BCL6* and *MYC* (nine cases); *BCL6* and *TP53* (eight cases); *BCL6* and *BCL2* (four cases); *MYC* and *TP53* (four cases); *MYC* and *BCL2* (two cases); *BCL6* and *MYC* (one case); *BCL6, MYC,* and *TP53* (three cases); *BCL6, BCL2,* and *TP53* (two cases); and *BCL6, BCL2, MYC,* and *TP53* (one case).

Immunohistochemical results

Reliable immunohistochemistry analysis was possible in all patients except in two patients because of inadequacy of tumor sample for the analysis. Expression of BCL6 was found in 33 patients (78.6%), BCL2 in 25 (59.5%), MYC in six (14.3%) and TP53 in 29 (69.04%). Figure 2 shows representative examples of immunohistochemical stains.

Expression of multiple proteins were detected in 29 (67.4%) cases; BCL6 and TP53 (22 cases), BCL6 and BCL2 (21 cases), BCL2 and TP53 (17 cases), BCL6, BCL2 and TP53 (14 cases), BCL6, MYC and BCL2 (five cases), BCL6, MYC and TP53 (four cases) and BCL6, MYC, BCL2 and TP53 (three cases).

Correlation with clinical outcome

Univariate analysis showed that among the clinical parameters, an IPI score >2 ($P = .013$) and advanced stage (III or IV, $P = .025$) were significantly associated with worse OS. In a multivariate analysis incorporating IPI score, cancer stage, and *BCL2* and *BCL6* rearrangements, only *BCL2* ($P = .004$) and *BCL6* ($P = .039$) rearrangements remained as independent prognostic factors for OS, whereas the prognostic significance of IPI score and cancer stage disappeared. Only *BCL2* ($P = .004$) rearrangement was independently associated with PFS.

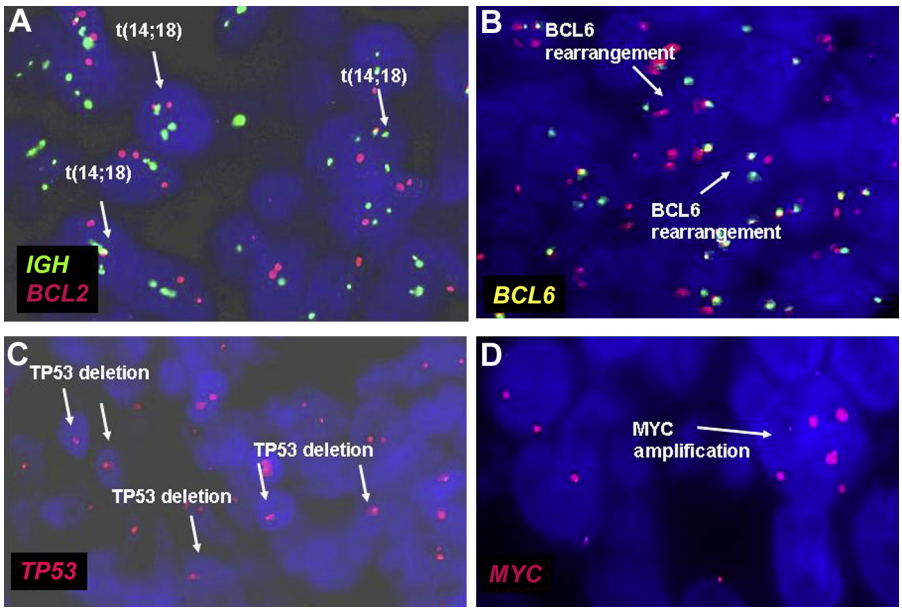


Figure 1 Representative FISH results showing rearrangements of (A) *BCL2*, (B) *BCL6*, (C) *TP53*, and (D) *MYC*.

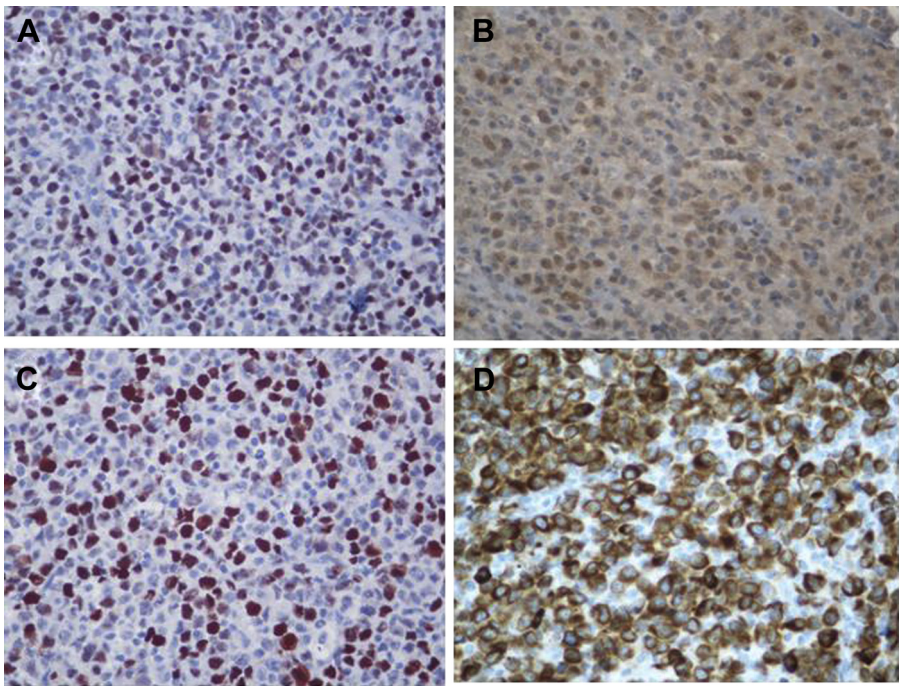


Figure 2 Representative examples of immunohistochemical stains. (A) BCL6 expression in a case with *BCL6* rearrangement. (B) C-MYC expression in a case with negative *C-MYC* rearrangement. (C) TP53 expression in a case without *TP53* deletion. (D) BCL2 expression in a case without *BCL2* rearrangement (immunoperoxidase, ×40).

Patients with rearrangements of *BCL6* ($P = .053$), *MYC* ($P = .483$), or *TP53* ($P = .877$) tended to have shorter OS, whereas patients with rearrangements of *BCL2* ($P = .302$) were in a trend for better OS (Figure 3). However, these trends did not reach statistical significance, because of the small sample size. Trends toward worse PFSs were observed for *BCL6* ($P = .091$), *BCL2* ($P = .357$), *C-MYC* ($P = .592$), and *TP53* ($P = .916$) rearrangements.

BCL2 ($P = .003$) expression had a significant negative impact on overall survival, whereas BCL6 ($P = .014$) expression had a significant positive impact. Although there was a trend toward worse survival with MYC ($P = .197$) expression and better survival with TP53 ($P = .85$) expression, these relationships failed to reach statistical significance. Patients with BCL2 ($P = .02$) expression had significantly shorter PFS, whereas a trend toward a better PFS was observed for patients with BCL6 ($P = .078$) and TP53 ($P = .843$) expression and a trend toward a worse PFS was observed for C-MYC ($P = .056$) expression.

Discussion

For more than a decade, the clinical factors described by the IPI have been used for risk stratification of patients with DLBCL. However, DLBCL patients with identical IPI risk groups show considerably variable outcomes, indicating the biological and clinical heterogeneity of this disease (32). Whereas gene expression profiling studies are uncovering biological heterogeneity with prognostic significance in DLBCL, the incorporation of this information into treatment algorithms awaits further investigation. This situation has motivated us to evaluate the clinical and prognostic significance of protein expression and rearrangements of *BCL2, BCL6, IGH, TP53,* and *MYC* in a series of representative patients with DLBCL.

Cytogenetic analysis is now a routine part of the diagnosis and management of various types of NHL. Traditional cytogenetic studies as well as FISH analyses utilizing locus-specific DNA probes are used to identify specific genetic abnormalities that provide insight into the pathogenesis of this complex disease and to define distinct subgroups with variable prognoses (33). Cytogenetic and molecular cytogenetics in DLBCL showed that tumor cells carry nonrandom chromosomal aberrations, most frequently chromosomal translocations, deletions, or amplifications, as well as gene alterations, including somatic hypermutations (34).

By FISH analysis, a gene-specific abnormality was identified in 88.6% of our series, and the most frequently involved gene of those we evaluated was *BCL6* (61.4% of all rearrangements). Furthermore, 18 patients (40.9%) showed rearrangements of more than one gene. Immunohistochemically, protein expression of the four genes studied were identified in 95.3% of our patients, with the highest expression of BCL6 (76.7% of the expression patterns identified). In an analysis of 74 cases of DLBCL using FISH with commercially available probes for split-signal targeting, *BCL2, BCL6, MYC, BCL10,* and *MALT-1* gene rearrangements were identified in 48 patients (65%). *BCL6* was the most frequently rearranged gene (45%), followed by *BCL2* (21%), *BCL10* (18%), and *MYC* (16%) (10). A larger series of 156 DLBCL patients was investigated for rearrangements of *BCL2, BCL6,* and *MYC* by Southern blot analysis. Structural alterations of *BCL2, BCL6,* and *MYC* were detected in 25 of 156, 36 of 116, and 10 of 151 patients, respectively (12). In a similar analysis of 137 patients with de novo DLBCL,

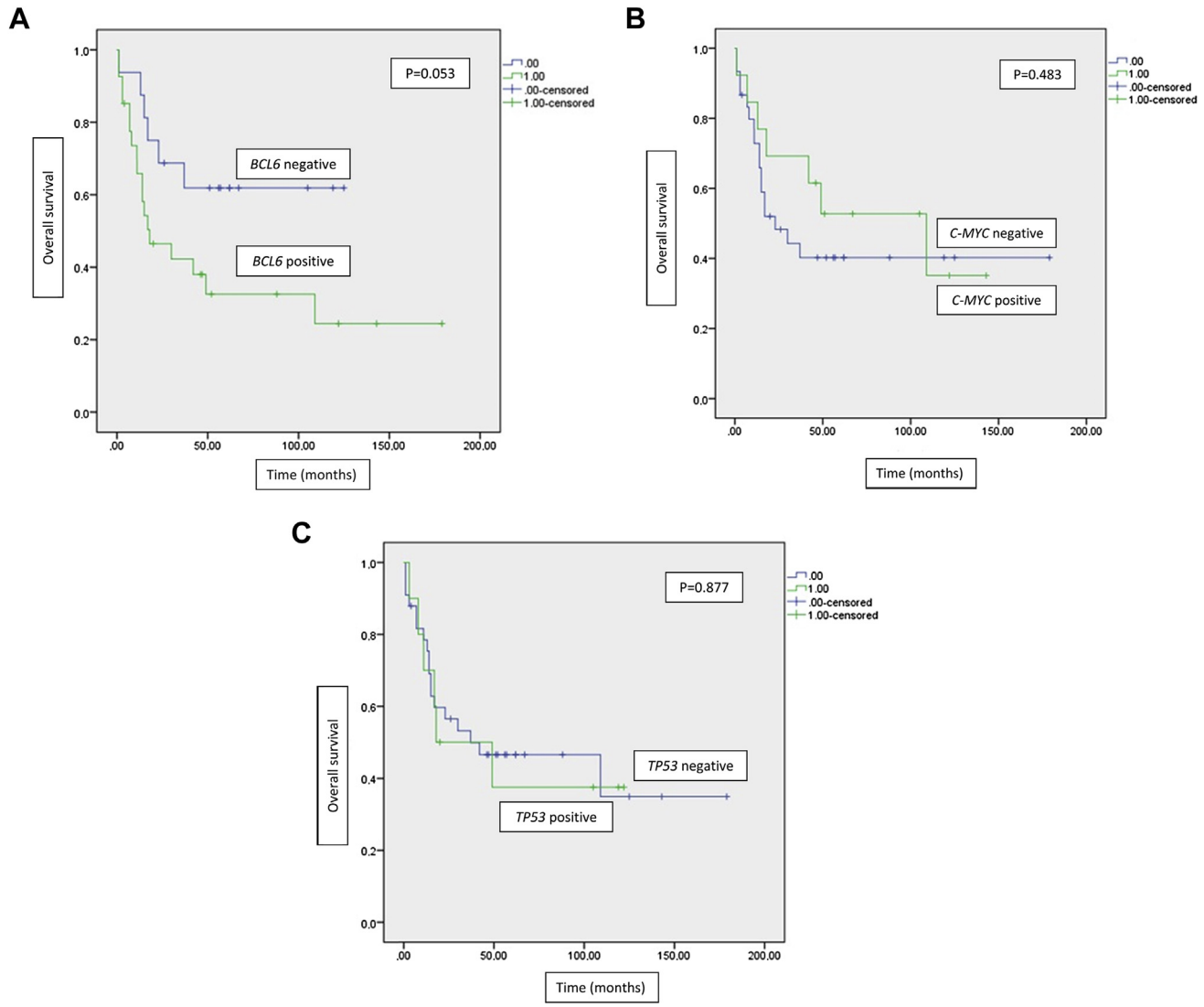


Figure 3 Overall survival of patients according to (A) *BCL6* rearrangement status, (B) *MYC* rearrangement status, and (C) *TP53* rearrangement status.

structural alterations of *BCL1, BCL2, BCL6,* and *MYC* were detected by Southern blot analysis in 15.3%, 5.8%, 16.1%, and 10.2% of patients, respectively (1). The pattern of cytogenetic abnormalities in our study is consistent with those of these previous reports. The presence of different genetic rearrangements in the same lymphoid tissue samples underline the complex nature of molecular events in DLBCLs, which is a reflection of the morphologic and clinical heterogeneity of this disease.

We found no concordance between gene rearrangements and protein overexpression. BCL6 protein expression was found in 19 of 25 patients with *BCL6* rearrangement, versus 14 of 16 patients without rearrangement; BCL2 protein expression was found in four of seven patients with *BCL2* rearrangement, versus 21 of 35 patients without rearrangement; MYC protein expression was not found in any of the patients with *MYC* rearrangement, versus six of 29 patients without rearrangement; and TP53 protein expression was found in six of 10 patients with *TP53* deletion, versus 23 of 33 patients without deletion. In their study, Monni et al. attributed BCL2 overexpression in cases that lack the t(14;18) translocation or *BCL2* gene amplification to additional mechanisms, such as point mutations or post-translational changes (35). Another explanation, by Kramer et al., of the fact that many DLBCL cases without detectable *BCL2* rearrangements show overexpression of the protein is DNA amplification instead of translocation (12). Moreover, the absence of BCL2 protein expression was detected in cases with a molecular rearrangement. This might be explained by mutations in the open reading frame of the translocated *BCL2* gene leading to absent or diminished protein production or genetic events developing during tumor progression that may abrogate the need for BCL2 (36). A study searching for a correlation between *TP53* mutation and TP53 expression documented that there was no significant difference in the frequency of TP53-positive and TP53-negative patients, with or without gene mutation, and no significant association between cytogenetic complexity and TP53 expression was found. However, cases with higher TP53 protein positivity (>25%) had complex cytogenetic

abnormalities. Non-missense mutations—premature stop codon, duplication, or deletion—were found to prevent TP53 expression in patients with B-cell NHL, as in lung, breast, and ovarian cancers (25). Villuendas et al. have also shown that the expression of TP53 was not always dependent on *TP53* mutations (37). Our results reinforced the absence of synonymy between gene rearrangement and protein overexpression. The question of what leads to the oncogene activation in cases without any rearrangement or amplification still remains to be answered.

In our cohort, patients with rearrangements of *BCL6, MYC,* and *TP53* tended to have shorter OS, whereas patients with rearrangements of *BCL2* were in a trend for better OS. BCL2 expression had a statistically significant negative influence on OS, whereas BCL6 expression had a statistically significant positive influence. Although there was also a trend toward worse OS with MYC expression, patients with TP53 expression tended to survive longer. The prognostic significance of *BCL2* and *BCL6* in DLBCLs is still controversial, probably reflecting the heterogeneity of the disease and different molecular techniques used (11,12,38–40). However, there is now a clear consensus that *MYC* translocations confer a worse prognosis in patients treated with CHOP, both in combination with and without rituximab (41,42). The poor clinical outcomes of patients with double-hit B-cell lymphomas treated with standard R-CHOP have been shown in publications of Johnson et al. and Green et al., suggesting the need for new therapeutic approaches in clinical trial settings in these patients (43,44). The adverse effect of *TP53* aberrations on the survival of DLBCL patients treated with R-CHOP has been documented in a recent study by Stefancikova et al. (45).

We conclude that 1) a different pattern of gene rearrangements and associated protein overexpression has shown the complex nature of molecular events in DLBCLs, which reflect the morphologic, biologic, and clinical heterogeneity of these lymphomas and 2) rearrangements of these genes and especially expression of these oncogenic proteins may serve as prognostic markers in DLBCL patients.

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