Determination of apoptosis, proliferation status and O^6-methylguanine DNA methyltransferase methylation profiles in different immunophenotypic profiles of diffuse large B-cell lymphoma

Diffüz büyük B-hücreli lenfomanın farklı immünofenotipik profillerinde apoptozis, proliferasyon durumu ve O^6-metilguanin DNA metiltransferaz metilasyon profillerinin tespiti

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Abstract

Objective: Our aim was to investigate the expression of apoptosis-associated proteins (bcl-2, bcl-xl, bax, bak, bid), apoptotic index (AI) and proliferation index (PI) in germinal center B-cell-like immunophenotypic profile (GCB) and non-GCB of diffuse large B-cell lymphoma (DLBCL).

Materials and Methods: The methylation status of the promoter region of O^6-methylguanine-DNA yerine O^6-methylguanine-DNA methyltransferase (MGMT) gene and its relation with immunophenotypic differentiation of DLBCLs were also investigated. 101 cases were classified as GCB (29 cases) or non-GCB (72 cases). Apoptosis-associated proteins and PI were determined by IHC, and TUNEL method was used to determine AI. MGMT methylation analysis was performed by real-time PCR.

Results: The PI was significantly higher in GCB compared with non-GCB ($p=0.011$). Percentage of cells stained with bcl-6 was positively correlated with the percentage of cells expressing bcl-2 ($p=0.023$), AI ($p=0.006$) and PI ($p<0.001$), while a significant negative correlation was observed with the percentage of cells expressing bax ($p=0.027$). The percentage of cells stained with MUM1 showed a significantly positive correlation with the percentage of cells expressing bcl-xl ($p=0.003$), bid ($p=0.002$), AI ($p<0.001$), and PI ($p=0.001$). MGMT methylation analysis was performed in 95 samples, and methylated profile was found in 31 cases (32.6%). GCB was found in 6 cases (22.2%) and non-GCB was determined in 25 cases (36.8%) out of 31 with MGMT methylated samples. There was no significant association between MGMT methylation status and immunophenotypic profiles ($p=0.173$).

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Conclusion: These results suggest that *bcl-6* protein expression may be responsible for the high PI in GCB. Additionally, we found that apoptosis-associated proteins were not significantly associated with immunophenotypic profiles. *(Turk J Hematol 2011; 28: 15-26)*
Key words: Lymphoma, B-cell, apoptosis, proliferation, methylation

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Özet

Amaç: Diffüz büyük B-hücreli lenfoma (DBBHL)'nın germinal merkez B-hücresi benzeri (GCB) ve non-GCB profillerinde apoptozis-ilişkili proteinlerin (*bcl-2, bcl-xl, bax, bak, bid*) ekspresyonunu, apoptotic indeksi (AI) ve proliferasyon indeksi (PI)'ni araştırmaktır. Ayrıca, O^6-methylguanine-DNA methyltransferase (MGMT) geninin promoter bölgesinin metilasyon durumunu ve onun DBBHL'nın immünofenotipik diferansiyasyonuyla ilişkisini araştırmaktır.
Yöntem ve Gereçler: 101 olgu GCB (29 olgu) ve non-GCB (72 olgu) olarak sınıflandırıldı. Apoptozis-ilişkili proteinler ve PI immünohistokimyasal olarak saptandı ve TUNEL yöntemi AI'i belirlemek için kullanıldı. MGMT metilasyon analizi, real-time PCR ile gerçekleştirildi.
Bulgular: PI, non-GCB ile karşılaştırıldığında GCB'de anlamlı şekilde yüksek saptandı (p=0.011). *Bcl-6* ile p: PI, non-GCB ile karşılaştırıldığında GCB'de anlamlı şekilde yüksek saptandı (p=0.011). *Bcl-6* ile pozitif boyanan hücrelerin yüzdesi *bcl-2* (p=0.023), AI (p=0.006), ve PI (p<0.001) eksprese eden hücrelerin yüzdesi ile pozitif şekilde korele iken, *bax* eksprese eden hücrelerin yüzdesi ile negatif korelasyon gözlendi (p=0.027). MUM1 ile boyanan hücrelerin yüzdesi *bcl-xl* (p=0.003), *bid* (p=0.002), AI (p<0.001) ve PI (p=0.001) eksprese eden hücrelerin yüzdesi ile anlamlı şekilde pozitif korelasyon gösterdi. MGMT metilasyon analizi 95 örneğe uygulandı ve metilasyon profili 31 olguda (%32.6) saptandı. 31 MGMT metile örnekten 6 olgunun (%22.2) GCB ve 25 olgunun (%36.8) non-GCB olduğu belirlendi. MGMT metilasyon durumu ve immünofenotipik profiler arasında anlamlı ilişki saptanmadı (p=0.173).
Sonuç: Bu bulgular, *bcl-6* protein ekspresyonunun GCB'de yüksek PI'inden sorumlu olabileceğini öne sürmektedir. Ek olarak, apoptozis-ilişkili proteinlerin immünofenotipik profilerle anlamlı ilişki göstermediğini saptadık. *(Turk J Hematol 2011; 28: 15-26)*
Anahtar kelimeler: Büyük B-hücreli lenfomalarda apoptosis ve proliferasyon

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Introduction

Diffuse large B-cell lymphoma (DLBCL) constitutes the largest group of aggressive lymphomas in adults and 30-40% of adult non-Hodgkin lymphomas (NHL) in western countries [1,2]. DLBCLs show diversity in clinical presentation, morphology and genetic and molecular properties, which suggests that these tumors represent a heterogeneous group of neoplasms rather than a single clinicopathologic entity [2,3]. Therefore, patients who are diagnosed as DLBCL could show remarkably different history, clinical behavior and outcome [1].

Different mechanisms such as deregulation of cell cycle and apoptotic pathways are involved in the pathogenesis of DLBCL. As for the molecular pathogenesis of DLBCL, distinct chromosomal translocations and aberrant somatic hypermutations as well as numerical chromosomal anomalies such as duplications and deletions, which are also common to other malignancies, have been reported [4-7].

DLBCL originates from germinal center (GC) and post-GC B-cells that normally have encountered with antigen [8,9]. Different methods are applied to determine B-cell differentiation antigens in DLBCL. In spite of the distinct advantages of cDNA and oligonucleotide microarray techniques, immunohistochemistry is a commonly used method to determine the GC B-cell-like (GCB) and non-GCB-like profiles because it is cheap and easily applied [10-12]. Hans *et al.* [11] reported that the classification of DLBCL into GCB and non-GCB profiles based on CD10/*bcl-6*/MUM1 immunophenotypic differentiation is prognostically relevant to the cDNA classification in 71% of GCB and in 88% of non-GCB. It is well known that the expression of *bcl-6* and CD10 are associated with increased apoptosis and proliferation in lymphoid malignancies [13-18]. Bai *et al.* [10] reported that increased expression of apoptotic index (AI) in DLBCL with GCB profile is associated with high expression of the pro-apoptotic proteins (*bax, bak, bid*) and low expression of the antiapoptotic protein (*bcl-xl*). However, data related to apop-

tosis and proliferation status in CD10/*bcl-6*/MUM1 immunophenotypic differentiation profiles is considerably limited. Although the immunohistochemical expression of the apoptosis-associated *bcl-2* family proteins, *bcl-2, bax, bak*, and *mcl1*, was reported in DLBCLs [19-28], the expression levels of *bcl-xl, bad, bid* proteins and their relations with the status of apoptosis and proliferation were not extensively analyzed in these lymphomas [25].

Altered DNA methylation profile has been comprehensively studied in the pathogenesis of cancer. Compared with normal cells, cancer cells frequently demonstrate genome-wide hypomethylation, hypermethylation of tumor suppressor gene, and loss of genomic imprinting [29]. In human cancers, the gene encoding the DNA-repair enzyme *O^6-methylguanine-DNA methyltransferase* (MGMT) is not commonly mutated or deleted. Loss of MGMT expression is mainly due to epigenetic changes, specifically methylation of the promoter region [1,30]. MGMT protects cells from the toxicity of environmental and therapeutic alkylating agents, which frequently target the O^6 position of guanine. Inactivation of the MGMT gene via hypermethylation of its promoter region increases sensitivity of cells to the genotoxic effect of alkylating agents both *in vitro* and *in vivo* [1,3]. Recent research has focused on the relationship between MGMT-methylation status and immunophenotypic differentiation in DLBCLs [30,31].

The aim of this study was to investigate the expression profiles of apoptosis-associated proteins (*bcl-2, bcl-xl, bax, bak, bid*), apoptotic index (AI), and proliferation index (PI) in GCB and non-GCB immunophenotypic profiles of DLBCL. In addition, the methylation status of the promoter region of the MGMT gene and its relation with immunophenotypic differentiation of DLBCLs were investigated.

Materials and Methods

Materials

A total of 101 cases of *de novo* DLBCLs, diagnosed according to the World Health Organization (WHO) classification [2], were obtained from the files of the Department of Pathology, Faculty of Medicine, Ege University. Clinicopathological parameters for all patients were obtained from the pathology records. Ethical committee approval was obtained for this study.

Immunohistochemistry

For immunohistochemical staining, sections of 5-μm-thickness were cut from formalin-fixed, paraffin-embedded tissue blocks and placed on electrostatic-charged, poly-L-lysine-coated slides (X-traTM, Surgipath Medical Industries, Richmond, IL, USA). Sections were dehydrated at 60ºC for a minimum of 2 hours (h). All immunostaining procedures including deparaffinization and antigen retrieval processes were performed on BenchMark XT® automated stainer (Venatana Medical Systems, USA). After counterstaining of the slides with hematoxylin in automated stainer, dehydration, incubation in xylene and mounting processes were performed manually, and immunostaining procedure was completed. *Bcl-6* (dilution: 1/20, clone: P1F6, Dako SA, Glostrup, Denmark), CD10 (dilution: 1/25, clone: 56C6, Spring Bioscience, Pleasanton, CA, USA), IRF4/MUM1 (dilution: 1/25, clone: MUM1p, Dako SA, Glostrup, Denmark), *bcl-2* (dilution: 1/40, clone: *bcl-2*/100/DS, Novocastra, Newcastle upon Tyne, UK), *bcl-xl* (dilution: 1/20, clone: 2H12, Zymed, South San Francisco, CA, USA), *bax* (dilution: 1/200, code: A3533, Dako SA, Glostrup, Denmark), *bak* (dilution: 1/100, code: A3538, Dako SA, Glostrup, Denmark), bid (dilution: 1/100, clone: NB110-40718, Novus, Littleton, CO, USA), and Ki-67 (dilution: 1/150, clone: MIB-1, Dako SA, Glostrup, Denmark) were used as primary antibodies.

Reactive lymph nodes and normal thymic tissue samples were used as positive controls. Negative controls were treated with the same immunohistochemical method by omitting the primary antibody.

At least 10 fields selected on the basis that they contained immunopositive cells were counted by using the 40x objective lens on the light microscope. The number of immunopositive cells was divided by the total number of the counted cells, and the expression was defined as the percentage of positive cells. CD10, *bcl-6*, and MUM1 proteins were considered positive when at least 25% of neoplastic cells were immunopositive according to the previously defined criteria [12]. The CD10/*bcl-6*/MUM1 immunophenotypes and their designation to GCB and non-GCB profiles were determined according to the classification by Hans *et al.* [11]. The expressions of *bcl-2, bcl-xl, bax, bak*, and *bid* proteins were considered positive when at least 10% of neoplastic cells were immunopositive [10]. PI with

Ki-67 was determined as the percentage of positive cells within the total number of the counted cells.

Tunel Method

The terminal deoxynucleotidyl-transferase (TdT)-mediated *in situ* labeling technique (TUNEL; *in situ* Cell Death Detection Kit, POD, Roche) was performed on the 5-μm-thick sections of formalin-fixed, paraffin-embedded tissue for demonstration of DNA fragmentation. Briefly, after deparaffinization and dehydration, slides were rinsed in phosphate-buffered saline (PBS) (pH 7.4). The peroxidase activity was blocked by incubation for 15 minutes (min) in 3% hydrogen peroxide in PBS at room temperature. Tissue sections were digested by incubation for 30 min with proteinase K (20 μg/ml) at 37°C, and then were rinsed in PBS. TUNEL reaction mixture was prepared according to the manufacturer's recommendations, and a mixture of 50 μL per slide was added. Slides were incubated for 1 h at 37°C in dark. One positive control and two negative controls were included in each set of experiments. Reactive lymph nodes were used as positive controls. Negative controls were treated similarly by omitting the TdT reaction step. Slides, once again, were rinsed in PBS. To examine the slides in light microscope, 50 μl Converter-peroxidase (POD) was added per slide and slides were incubated for 30 min at 37°C in humid and dark conditions. Slides were rinsed in PBS, and then were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB Substrate, Roche) for 10 min at room temperature. Slides were counterstained with Harris' hematoxylin and mounted. The evaluation of the results was performed as Bai *et al.* [32] had described previously. Morphologically intact TUNEL-positive cells were considered as positive and referred to as apoptotic cells. The number of apoptotic cells was recorded by using the 40X objective in at least 10 randomly selected fields. The AI was expressed as a percentage of the number of apoptotic cells within the total number of counted cells.

O^6- Methylguanine-DNA Methyltransferase Methylation Analysis

Genomic DNAs were extracted from four or five 5-μm-thick sections of formalin-fixed, paraffin-embedded tissue, using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Valencia, CA). Briefly, after deparaffinization with xylene, tissue samples were digested with proteinase K treatment. DNAs that emerged from cells were collected in the column through ethanol treatment. Cellular remnants and chemical agents were removed by washing buffer, and genomic DNA was eluted in DNAase-free buffer and stored at -20°C.

Commercial kit (EZ Methylation Gold-Kit, Zymo Research, Orange, CA) was used for bisulfite modification of isolated DNA. Briefly, DNA concentration was measured and arranged to make 500 ng/μL, and 130 μL conversion reagent was added to 20 μL DNA sample. Samples were incubated for 10 min at 98°C and then for 2.5 h at 64°C. Samples were transferred to column with 600 μL M-binding buffer. After homogenization, samples were centrifuged. Samples were rinsed with 100 μL M-wash buffer, and were then incubated in 200 μL M-desulfonation buffer for 20 min at room temperature. After incubation, samples were rinsed two times, and then 10 μL M-elution buffer was added. Sodium bisulfite-treated genomic DNA was stored at -20°C.

Primer and probe sequences of the promoter region of MGMT gene were used, which target the localization of 1067 -1149 bp amplicon, as described by Esteller *et al.* (33) (GenBank Accession Number: X61657). Final reaction volume for analysis of both methylated and unmethylated profiles was performed in 20 μL volume: 2 μl from each primer (final concentration: 0.5 μM), 2 μl TaqMan probe (final concentration: 0.2 μM), 4 μl LightCycler TaqMan Master mixture, 5 μl DNA sample, and 5 μl polymerase chain reaction (PCR)-grade water. The cycling conditions for methylation-specific PCR were: 10 min at 95°C for *Taq* activation, followed by 45 cycles of 95°C for 10 seconds (sec), 60°C for 20 sec, and 72°C for 1 sec for amplification. PCR products were run on a 3% agarose gel containing ethidium bromide.

Statistical Analysis

χ^2 test, Mann-Whitney test and analysis of variance were applied for statistical analysis. Mann-Whitney test was used to analyze the relationship of positivity of apoptosis-related proteins, PI (Ki-67) and AI with immunophenotypic differentiation profiles (CD10/bcl6/MUM1). Spearman correlation coefficient test was used to analyze any significant relationship between PI, AI and percentage of posi-

tive cells with apoptosis-related proteins, CD10, bcl-6, and MUM1, whether evaluated as positive or negative using cut-off values. The results were considered as statistically significant when $p<0.05$. All the statistics were calculated using the SPSS 11.0 program (SPSS 11.0 Inc., Chicago, IL, USA) for Windows.

Results

Patients [53 males (52.5%), 48 females (47.5%)] were aged between 19 and 84 (mean 55.53±14.12). Localization was nodal in 42 (41.6%) cases andextranodal in 58 (57.4%) cases, while one case was unknown.

Immunohistochemical expression of *bcl-6*, CD10, MUM1, *bcl-2, bcl-xl, bax, bak,* and *bid* proteins was found in 51/101 (50.5%), 20/101 (19.8%), 49/101 (48.5%), 32/101 (31.7%), 8/101 (7.9%), 67/101 (66.3%), 82/101 (81.2%), and 67/99 (66.3%) cases, respectively (Figure 1 A-E). The mean PI was 46.15% (±32.82) as assessed by Ki-67 staining. The mean AI was 1.94% (±2.68) as determined by the TUNEL method (Figure 1 F).

Two major immunophenotypic profiles were distinguished according to the pattern of differentiation described by Hans *et al.* [11]: (a) GCB immunophenotypic profile: 29 cases (CD10+: 20 cases, CD10- /*bcl-6*+ /MUM1-: 9 cases) and (b) non-GCB immunophenotypic profile: 72 cases (CD10- /*bcl-6*-: 47 cases, CD10- /*bcl-6*+ /MUM1+: 25 cases) (Table 1).

Mann-Whitney test was used to analyze the association of two immunophenotypic differentiation profiles in relation to the AI, the expression levels of apoptosis-related proteins and PI (Ki-67) (Table 2). Compared to the non-GCB profile, the GCB profile was significantly associated with a higher PI ($p=0.011$). However, no other significant correlations were determined between the two major differentiation immunophenotypic profiles regarding AI and the expression levels of apoptosis-related proteins *bcl-2, bcl-xl, bax, bak,* and *bid* ($p>0.3$).

The percentage of cells stained by CD10, *bcl-6* and MUM1 were analyzed in relation to apoptosis-related proteins, AI and PI by Spearman correlation coefficient test, regardless of previous evaluation with cut-off points to consider the results as positive or negative (Table 3). The expression of *bcl6* was positively correlated with the expression of *bcl2* ($r=0.226$, $p=0.023$), the AI ($r=0.272$, $p=0.006$) and the PI ($r=0.515$, $p<0.001$), but a significantly negative correlation was observed with the expression of bax ($r=-0.221$, $p=0.027$). The expression of MUM1 showed significant positive correlation with the expression of *bcl-xl* ($r=0.295$, $p=0.003$), *bid* ($r=0.313$, $p=0.002$), AI ($r=0.341$, $p<0.001$), and PI

Table 1. CD10/bcl-6/MUM1 immunophenotypic differentiation profiles (Total n=101)

Immunophenotypic differentiation profiles			n (%)
Germinal center B-cell-like profile			
CD10+	*bcl-6-*	MUM1-	1(1%)
		MUM1+	2 (2%)
	bcl-6+	MUM1-	13 (12.8%)
		MUM1+	4 (4%)
CD10-	*bcl-6+*	MUM1-	9 (8.9%)
TOTAL			29 (28.7%)
Non-germinal center B-cell-like profile			
CD10-	*bcl-6-*	MUM1+	18 (17.8%)
		MUM1-	29 (28.7%)
CD10-	*bcl-6+*	MUM1+	25 (24.8%)
TOTAL			72 (71.3%)

Table 2. The immunophenotypic differentiation profiles in relation to the apoptotic index (AI), the expression of apoptosis related proteins, and the proliferation index (PI) (Mann-Whitney test)

	Percentage of positive expression	Immunophenotypic differentiation profile	Mean rank	p values
AI	1.93	GCB profile	51.33	0.941
	1.91	non-GCB profile	50.87	
bcl-2	18.52	GCB profile	53.52	0.561
	15.69	non-GCB profile	49.99	
bcl-xl	1.86	GCB profile	46.95	0.341
	6.16	non-GCB profile	52.63	
bax	43.07	GCB profile	45.81	0.257
	50.87	non-GCB profile	53.09	
bak	58.34	GCB profile	51.36	0.937
	60.80	non-GCB profile	50.85	
bid	42.00	GCB profile	45.34	0.297
	51.81	non-GCB profile	51.93	
PI (Ki-67)	59.17	GCB profile	62.66	0.011*
	41.69	non-GCB profile	46.31	

GCB profile: Germinal center B-cell-like profile, *Non-GCB profile*: Non-germinal center B-cell-like profile

*indicates the statistically significant correlations ($p<0.05$)

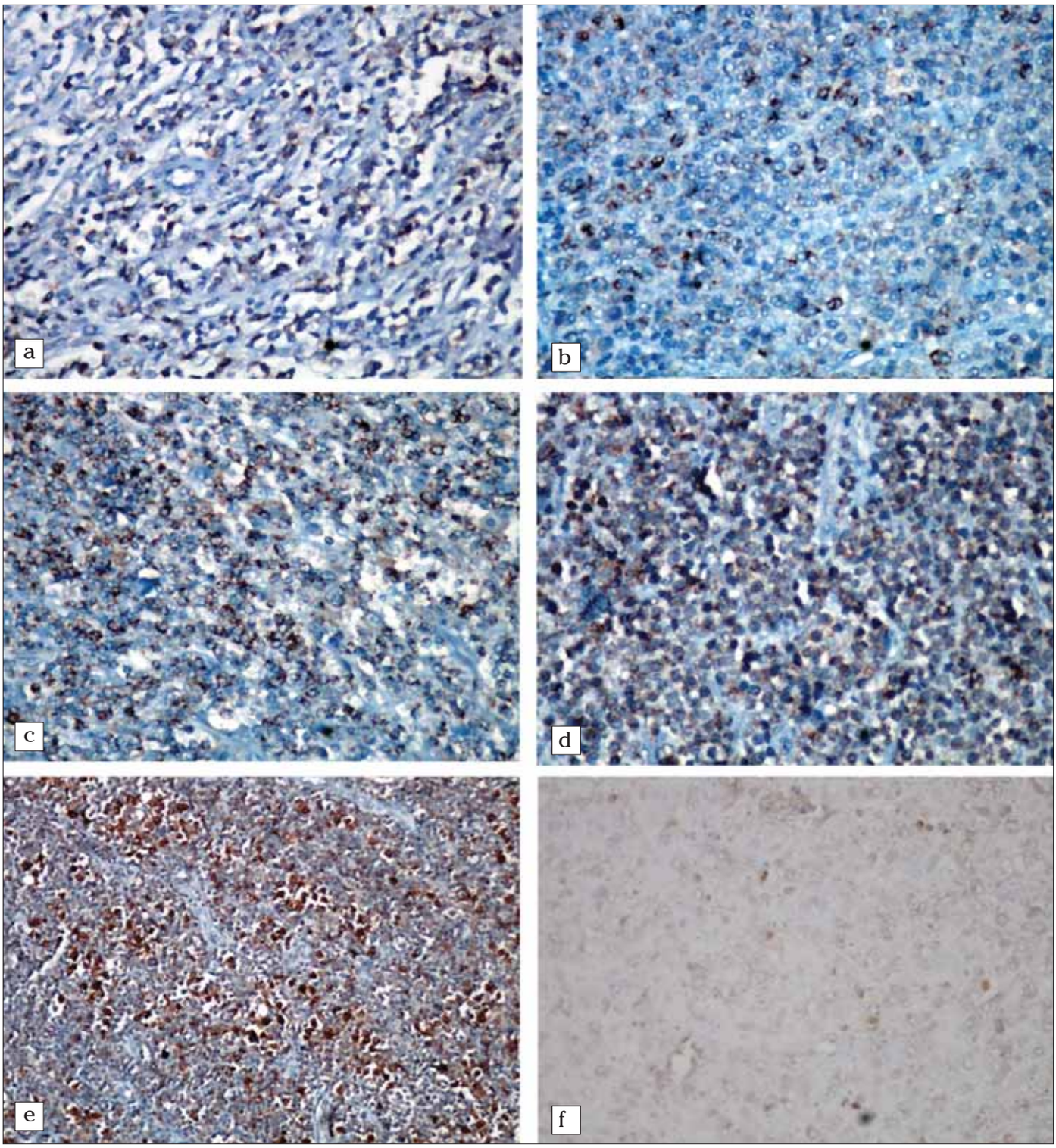

Figure 1. Immunohistochemical expression of antiapoptotic proteins (a) *bcl2* and (b) *bcl-xl* and apoptotic proteins (c) *bax*, (d) *bak* and (e) *bid* in neoplastic cells of diffuse large B-cell lymphomas (a-d, x400; e, x200). (f) Staining of apoptotic cells by the TUNEL method (x400)

(r=0.330, p=0.001). Similarly, the Spearman correlation coefficient test was used to analyze the relations between apoptosis-related proteins, AI and PI. The expression of *bcl-xl* protein showed significant positive correlation with the expression of *bak* (r=0.198, p=0.047) and *bid* (r=0.198, p=0.049) proteins. The expression of *bax* protein showed significant positive correlation with the expression of *bak* (r=0.229,

Table 3. Correlations between CD10, *bcl-6* and MUM1 proteins and the apoptosis related proteins, the apoptotic index (AI), and the proliferation index (PI) (Spearman's correlation test)

	bcl-2	*bcl-xl*	*bax*	*bak*	*bid*	AI	PI
CD10	r=0.092	r=0.050	r=0.055	r=-0.024	r=-0.001	r=0.130	r=0.148
	p=0.360	p=0.619	p=0.585	p=0.811	p=0.991	p=0.195	p=0.139
bcl-6	r=0.226	r=0.176	r=-0.221	r=0.123	r=0.114	r=0.272	r=0.515
	p=0.023*	p=0.078	p=0.027*	p=0.220	p=0.260	p=0.006*	p<0.001*
MUM1	r=0.122	r=0.295	r=0.009	r=0.152	r=0.313	r=0.341	r=0.330
	p=0.225	p=0.003*	p=0.932	p=0.129	p=0.002*	p<0.001*	p=0.001*

r, Spearman's correlation coefficient. The positive or negative sign of the Spearman's correlation coefficient r expresses significant (p<0.05) or nonsignificant (p=0.05 or p>0.05) positive or negative correlations between two continuous variables

*indicates the statistically significant correlations (p<0.05)

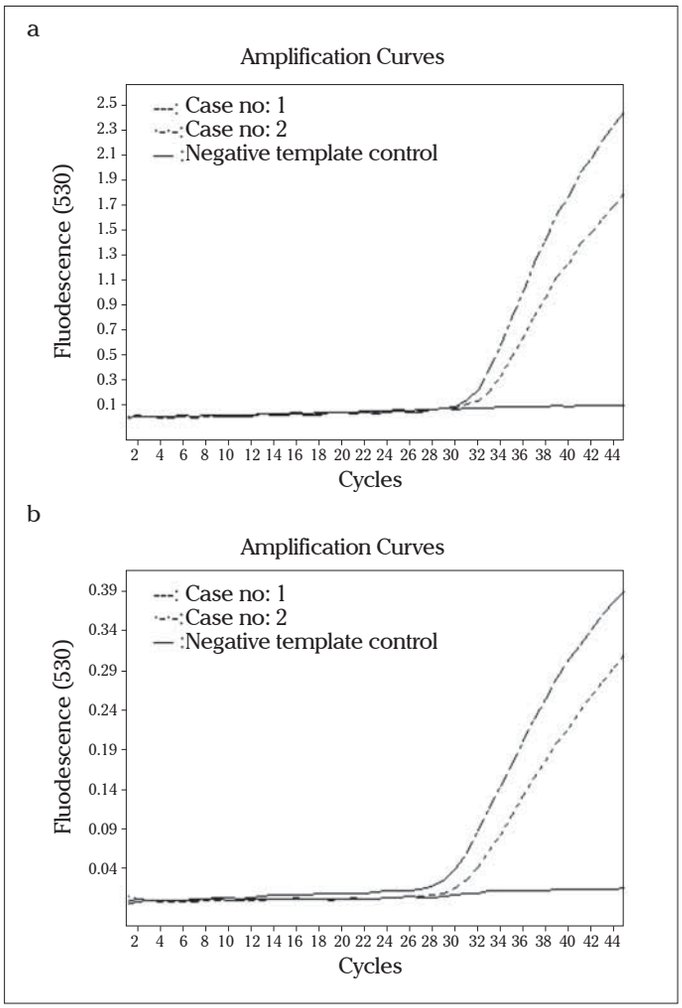

Figure 2. (a) Amplification curve after real-time PCR with methyleted specific primer and probe set. (b) Amplification curve after real-time PCR with unmethyleted specific primer and probe set

p=0.022) and *bid* (r=0.223, p=0.027) proteins. The expression of *bak* protein also showed significant positive correlation with the expression of *bid* (r=0.214, p=0.033) protein. The AI showed significant positive correlation with the PI (r=0.349, p<0.001).

MGMT promoter methylation analysis was performed in 95 patients with DLBCL (Figure 2). MGMT methylated profile was found in 31/95 (32.6%) samples while MGMT unmethylated profile was determined in 64/95 (67.4%) samples. The GCB profile was determined in 6 cases (22.2%) and non-GCB profile in 25 cases (36.8%) out of the 31 with MGMT methylated profile (Table 4). There was no significant association between MGMT promoter methylation status and the two immunophenotypic differentiation profiles (p=0.173) (Table 5). There was also no significant association between MGMT promoter methylation status and the expression levels of apoptosis-related proteins, the AI and the PI (p>0.06).

Discussion

In the present study, we found that in DLBCLs, the GCB profile was significantly associated with a higher PI compared to the non-GCB profile. In addition, the percentage of cells reacting with the GC B-cell-related *bcl-6* protein showed significant positive correlation with PI. The correlation between *bcl-6* and proliferation is not conclusive in the published data. A review of the literature indicates that *bcl-6* could have a role both as stimulator or inhibitor of cell cycle progression and proliferation [16,32-37]. Some *in vitro* studies showed that *bcl-6* expression was associated with delaying cell cycle progression and decreased proliferation [15,35]. Albagli *et al.* [16] demonstrated that *bcl-6* mediates growth suppression associated with impaired S phase progression in human U2OS osteosarcoma cells. Hosokawa *et al.* [35] established Ba/F3 pro-B cells carrying a human *bcl-6* transgene, and revealed that

Table 4. The immunophenotypic differentiation profiles in relation to the MGMT methylation status

	MGMT methylation status		Total number of
	Methylated	Unmethylated	cases
GCB	6 (22.2%)	21 (77.8%)	27
non-GCB	25 (36.8%)	43 (63.2%)	68
Total	31 (32.6%)	64 (67.4%)	95

MGMT, *O^6- methylguanine-DNA methyltransferase GCB profile*: Germinal center B-cell-like profile, *Non-GCB profile*: Non-germinal center B-cell-like profile

Table 5. Association between MGMT methylation status and expression of CD10/*bcl-6*/MUM1 proteins (χ^2 test)

Parameter		MGMT methylated (%)	p values*
CD10 expression	+	5 (26.3%)	0.514
	-	26 (34.2%)	
bcl-6 expression	+	14 (29.2%)	0.469
	-	17 (36.2%)	
MUM1 expression	+	12 (26.7%)	0.242
	-	19 (38.0%)	
CD10/bcl-6/MUM1 coexpression	CD10+/*bcl-6*-/MUM1-	0	0.729
	CD10+/*bcl-6*+/MUM1-	5 (38.5%)	
	CD10+/*bcl-6*-/MUM1+	0	
	CD10+/*bcl-6*+/MUM1+	0	
	CD10-/*bcl-6*-/MUM1+	4 (23.5%)	
	CD10-*bcl-6*-/MUM1-	13 (46.4%)	
	CD10-/*bcl-6*+/MUM1-	1 (12.5%)	
	CD10-/*bcl-6*+/MUM1+	8 (34.8%)	

MGMT, *O^6- methylguanine-DNA methyltransferase*
*indicates the statistically significant correlations ($p<0.05$)

induced *bcl-6* protein downregulates the expression of cyclin A2 and inhibits cell proliferation, though Shaffer *et al.* [34] demonstrated that *bcl-6* may induce cell cycle progression and maintain proliferation by blocking the expression of the cyclin-dependent kinase inhibitor p27 and by repressing blimp-1 expression, which decreases c-myc expression. Furthermore, Allman *et al.* [37] revealed that *bcl-6* protein expression was 34-fold higher in rapidly proliferating GC B-cells than in the resting B-cells. Bai *et al.* [18] suggested that the resistance to antiproliferative signals through the P19 (ARF) -p53 pathway and downregulation of the expression of cyclin-dependent kinase inhibitor p27 are at least partly responsible for the association between *bcl-6* and increased proliferation in DLBCL. Xu *et al.* [38] investigated the role of *bcl-6* gene rearrangement and *bcl-6* expression in subgroups of DLBCLs and showed that DLBCLs with *bcl-6* gene rearrangement had higher proliferative activity than those without *bcl-6* gene rearrangement. On the basis of the previously mentioned results, results of this study suggest that *bcl-6* protein expression could be responsible for high PI in the GCB profile of DLBCL.

Additionally, in this study, *bcl-6* expression showed significant positive correlation with the AI and anti-apoptotic protein *bcl-2* expression and negative correlation with pro-apoptotic protein bax expression. In association with the proliferation of *bcl-6*, the data in the literature indicates that *bcl-6* may have a role as stimulator or inhibitor of apoptosis [15,16,39-41]. Some studies showed that *bcl-6* may protect cells from apoptosis [39,41]. Kojima *et al.* [39] demonstrated that *bcl-6* may have a stabilizing role to protect spermatocytes from heat shock-induced apoptosis in *bcl-6*-deficient mice. Baron *et al.* [41] showed that the human programmed cell death-2 (PDCD2) gene is a target of *bcl-6* repression in Epstein-Barr virus-negative Burkitt lymphoma cell line expressing high levels of *bcl-6*. Furthermore, they immunohistochemically demonstrated the inverse relationship between *bcl-6* and PDCD2 expression in human tonsils [41]. Consequently, they proposed that *bcl-6* may downregulate apoptosis by means of its repressive effects on PDCD2. However, some other studies suggested that high expression of *bcl-6* may induce apoptosis [14-16,40]. Albagli *et al.* [16] used the human osteosarcoma cell line U2OS transfected with *bcl-6* and demonstrated that *bcl-6* mediates dose-dependent growth suppression, which is associated with impaired S phase progression and trigger of apoptosis. Yamochi *et al.* [14] showed that viability of CV-1 and HeLa cells infected with a recombinant adenovirus expressing *bcl-6* was markedly reduced due to apoptosis. Furthermore, induction of apoptosis by *bcl-6* overexpression was preceded by downregulation of apoptosis repressors *bcl-2* and *bcl-xl*, which suggests that *bcl-6* might also regulate the expression of these apoptosis repressors [14]. Bai *et al.* [18] found that high expression of *bcl-6* shows significant correlation with negative *bcl-2* expression. They suggested that the association between increased *bcl-6* expression and increased apoptosis in DLBCL might be due, at least to some extent, to the downregulation of *bcl-2*, which is induced by

bcl-6 overexpression [18]. In contrast to previous studies, in this study, we determined that *bcl-6* expression shows a significantly positive correlation with *bcl-2* expression. Moreover, we found that *bcl-6* expression shows significant negative correlation with pro-apoptotic protein bax expression. It is well known that apoptosis-related proteins *bcl-2* and *bcl-xl* show anti-apoptotic effect, whereas *bax*, *bak* and *bid* show pro-apoptotic function. These proteins, as members of the *bcl-2* family, exert their specific effects by dimerizing with themselves or with each other [25]. If the balance favors the presence of free *bcl-2*, apoptosis is inhibited, whereas when bax predominates, apoptosis is initiated [25]. Thus, the ratio of the anti-apoptotic to the pro-apoptotic proteins determines whether a given cell will respond to or ignore an apoptotic stimulus [25]. On the basis of these results, we suggest that pro-apoptotic function of *bcl-6* partly occurs independent of an anti-apoptotic effect of *bcl-2* and pro-apoptotic effect of *bax*.

In this study, no significant correlations were determined between two major immunophenotypic differentiation profiles regarding AI and the expression levels of apoptosis-related proteins *bcl-2, bcl-xl, bax, bak,* and *bid*. However the percentage of cells positive for MUM1, which is major marker of non-GCB immunophenotypic differentiation profile, showed a significantly positive correlation with the positivity percentage of anti-apoptotic protein *bcl-xl*, pro-apoptotic protein bid, AI, and PI. Bai *et al.* [10] determined that the expression of MUM1 showed significant negative correlation with the expression of *bax* and *bid* and significant positive correlation with the expression of *bcl-xl*. They proposed that this result may provide an explanation for the significant positive correlation between MUM1 and *bcl-xl* expression in their study, because the MUM1 (IRF4) gene is also a nuclear factor-Kappa B target. Nuclear factor-Kappa B, depending on the stimulus and the cellular context, can activate pro-apoptotic (e.g. CD95, CD95L, TRAIL receptors), anti-apoptotic (c-FLIP, *bcl-2, bcl-xl*, c-IAP1, c-IAP2) and cell cycle (cyclin D1, cyclin D2, c-myc) genes [32]. This status may explain how MUM1 expression shows significant positive correlation with pro-apoptotic protein *bid*, AI, PI, as was also shown in the results of Bai *et al.* [10].

In this study, we determined that DLBCLs frequently express *bcl-2* family member apoptosis-related proteins as *bcl-2* in 31.7%, *bcl-xl* in 7.9%, *bax in* 66.3%, *bak* in 81.2%, and *bid* in 66.3%. Bairey *et al.* [25] examined the role of *bcl-2* family proteins in aggressive NHLs and determined that of the entire group of 44 samples, 25 (57%) showed *bcl-2* staining, 11 (25%) showed *bcl-xl* staining, 17 (39%) showed *bax* staining, and 16 (36%) showed *bak* staining. Among these proteins, *bcl-2*, the most exclusively investigated, is a potent suppressor of apoptosis and is determined in unexpectedly high levels probably in all cancers of humans [50]. Simonian *et al.* [43] suggested that *bcl-2* and *bcl-xl* can downregulate or upregulate apoptosis. Sclaifer *et al.* [44] showed that all cases with a positive expression of bax expressed either *bcl-2* or *mcl-1* anti-apoptotic proteins, suggesting that the presence of bax in the tumor cells must be associated with apoptosis-inhibiting proteins, allowing malignant cell survival. Kiberu *et al.* [22] investigated the correlation between apoptosis, proliferation and *bcl-2* expression in NHLs, and they suggested that expression of *bcl-2* is not necessarily related to low levels of apoptosis, as some *bcl-2* positive high-grade tumors also had high levels of apoptosis. Nevertheless, they found that the majority of lymphomas expressing *bcl-2* had average levels of apoptosis [22]. These results suggested that *bcl-2* independent apoptosis is an important factor influencing cell death in many NHLs [22]. Overall results of previous studies [10,19,22-27] and our results indicate that the expressions of *bcl-2* family proteins are variable and heterogeneous in DLBCL. The dual effect of *bcl-2* family proteins may show individual variation in the pathogenesis and prognosis of DLBCLs.

In this study, we found that the AI showed significant positive correlation with the PI. This result is correlated with previously reported results [10,22,45]. However, we were unable to show any relation between apoptosis-related proteins and PI.

In this study, we determined that MGMT promoter methylation was detected in 32.6% of cases. MGMT methylation was reported at a frequency from 36% to 52% in Western populations in previous studies [1,3,30,46,47]. Higher rates (75.9%) were found in Middle Eastern populations [38]. This status may be explained by the differences in etiologic factors such as viral infections and exposure to environmental factors as well as differences in the genetic susceptibility. Furthermore, substantial ethnic differences existed with respect to molecular features of malignant tumors [31,48-50].

No significant association between MGMT promoter methylation status and the two immunophenotypic differentiation profiles was observed in this study. Al-Kuraya *et al.* [31] investigated the interrelationship between MGMT methylation status and immunohistochemical coexpression of CD10/*bcl-6*, and they did not show a significant association. Furthermore, Al-Kuraya *et al.* determined that their frequency of GCB profile DLBCL (13%) was somewhat lower than described in previous studies. They also found a much higher rate of MGMT methylation in their patients (75.9%) as compared with previous studies [1,3,30,31,46,47]. In our study, no significant association was found between MGMT promoter methylation status and the expression levels of apoptosis-related proteins, the AI and the PI. There is no similar previous study searching the relation of MGMT promoter methylation and expression levels of apoptosis-related proteins, the AI and the PI. Further studies could be done to elucidate the role of MGMT promote methylation in DLBCLs.

In summary, these results suggest that *bcl-6* protein expression may be responsible for the high PI in the GCB profile of DLBCLs. Additionally, we found that expression status of apoptosis-related *bcl-2* family proteins (*bcl-2, bcl-xl, bax, bak, bid*) was not significantly associated with the immunophenotypic differentiation profiles. We also determined that there was no significant association between MGMT promoter methylation status and the immunophenotypic differentiation profiles of DLBCLs.

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Conflict of interest statement

None of the authors of this paper has a conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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