

Diffuse large B-cell lymphomas with germinal center B-cell-like differentiation immunophenotypic profile are associated with high apoptotic index, high expression of the proapoptotic proteins bax, bak and bid and low expression of the antiapoptotic protein bcl-xl

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The aim of this study was to analyze the relations between differentiation immunophenotypes and the status of apoptosis and proliferation in diffuse large B-cell lymphomas. Therefore, the bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles were studied in relation to (a) the apoptotic index, (b) the apoptosis-associated bcl2 family proteins bcl2, bcl-xl, bax, bak, bad and bid, (c) the proliferation index (Ki67) and (d) the cell cycle proteins cyclin A, cyclin B1, cyclin D3, cyclin E, p53, Rb, p16 and p27 in 79 cases of diffuse large B-cell lymphomas. Two major differentiation immunophenotypic profiles were distinguished: the germinal center B-cell-like profile; 31 cases (bcl6+/CD10±/MUM1-/CD138-: 29 cases and bcl6-/CD10+/MUM1-/CD138-: two cases) and the nongerminal center B-cell-like profile (bcl6±/CD10-/MUM1+/CD138-); 48 cases. The expression of bax, bak and bid and the apoptotic index were significantly higher in the germinal center B-cell-like profile than in the nongerminal center B-cell-like profile ($P=0.045, 0.018, 0.003$ and 0.034 , respectively). In contrast, the expression of bcl-xl was significantly lower in the germinal center B-cell-like profile than in the nongerminal center B-cell-like profile ($P=0.026$). The expression of bcl6 and CD10 showed significant positive correlation with the expression of bax ($r=0.659, P<0.001$ and $r=0.240, P=0.033$, respectively), bak ($r=0.391, P<0.001$ and $r=0.233, P=0.039$, respectively) and bid ($r=0.652, P<0.001$ and $r=0.238, P=0.035$, respectively) and significant negative correlation with the expression of bcl-xl ($r=-0.536, P<0.001$ and $r=-0.250, P=0.029$, respectively). The expression of MUM1 showed significant negative correlation with the expression of bax ($r=-0.276, P=0.014$) and bid ($r=-0.266, P=0.018$) and significant positive correlation with the expression of bcl-xl ($r=0.238, P=0.037$). The above findings indicate that diffuse large B-cell lymphomas with germinal center B-cell-like immunophenotypic profile are associated with increased apoptosis status, high expression of the proapoptotic proteins bax, bak and bid and low expression of the antiapoptotic protein bcl-xl.

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Diffuse large B-cell lymphomas represent the most common type of non-Hodgkin's lymphomas in Western countries and are characterized by hetero-

geneous clinical, immunophenotypic and genetic features.^{1–7} There is accumulating evidence that diverse mechanisms resulting in the deregulation of cell cycle and apoptotic pathways are involved in the pathogenesis of diffuse large B-cell lymphomas.^{3–6}

Recently, the global gene expression profile of diffuse large B-cell lymphomas was analyzed by cDNA^{8–10} and oligonucleotide microarrays.¹¹ Alizadeh *et al*⁸ identified two molecularly distinct

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groups of diffuse large B-cell lymphomas, the germinal center B-cell-like and the activated B-cell-like diffuse large B-cell lymphomas. This was confirmed by Rosenwald *et al*⁹ who, in addition to the two gene expression groups, also described the type 3 diffuse large B-cell lymphomas. The germinal center B-cell-like diffuse large B-cell lymphomas were characterized by the expression of genes of the normal germinal center B cells (eg *bcl6*, *CD10*, *CD38*), the activated B-cell-like diffuse large B-cell lymphomas were characterized by the expression of genes that are normally induced during *in vitro* activation of peripheral blood B cells and the type 3 diffuse large B-cell lymphomas did not express either set of genes at a high level.^{8–10} The gene expression signature of the activated B-cell-like diffuse large B-cell lymphomas included a gene that is translocated in lymphoid malignancies, *IRF4* (*MUM1/LSIRF*), antiapoptotic genes such as *FLIP* and *bcl2* and a subset of the genes that are characteristic of plasma cells.^{8–10} The patients with germinal center B-cell-like diffuse large B-cell lymphomas had more favorable clinical outcome than those with activated B-cell-like or type 3 diffuse large B-cell lymphomas.^{8–10}

Since the cDNA and oligonucleotide microarrays technology is not generally available, many studies investigated the immunohistochemical expression of B-cell differentiation antigens (eg *bcl6*, *CD10*, *MUM1*, *CD138*) in diffuse large B-cell lymphomas and some of them correlated the differentiation immunophenotypes with clinical data.^{12–32} Importantly, in one of these studies, Hans *et al*,³² showed that the classification of diffuse large B-cell lymphomas into germinal center and nongermlinal center B-cell-like groups based on the *bcl6/CD10/MUM1* differentiation immunophenotypes is prognostically relevant and predicts the cDNA classification in 71% of germinal center B-cell-like and 88% of activated B-cell-like or type 3 diffuse large B-cell lymphomas. On the other hand, the expression of *bcl6* and *CD10* was associated with increased apoptosis and proliferation in cell lines and lymphoid malignancies.^{33–40} In this respect, we recently showed that increased expression of *bcl6* and *CD10* is associated with increased apoptosis and proliferation in diffuse large B-cell lymphomas.⁴¹ However, to the best of our knowledge, little is known about the relations between the *bcl6/CD10/MUM1/CD138* differentiation immunophenotypes and the status of apoptosis and proliferation in these lymphomas. In addition, although the immunohistochemical expression of the apoptosis-associated *bcl2* family proteins, *bcl2*, *bax*, *bak* and *mcl1* was reported in diffuse large B-cell lymphomas,^{16,18,20,21,27,42–50} the expression levels of *bcl-xl*, *bad* and *bid* proteins and their relations with the status of apoptosis and proliferation were not extensively analyzed in these lymphomas. Therefore, the *bcl6/CD10/MUM1/CD138* differentiation immunophenotypic profiles were studied in rela-

tion to (a) the apoptotic index, (b) the apoptosis-associated proteins *bcl2*, *bcl-xl*, *bax*, *bak*, *bad* and *bid*, (c) the proliferation index (*Ki67*) and (d) the cell cycle proteins *cyclin A*, *cyclin B1*, *cyclin D3*, *cyclin E*, *p53*, *Rb*, *p16* and *p27* in 79 cases of diffuse large B-cell lymphomas.

Materials and methods

Materials

In all, 79 cases of *de novo* diffuse large B-cell lymphomas (37 nodal and 42 extranodal) classified according to the WHO classification¹ were selected from the files of the Department of Pathology of the University of Ioannina on the basis that complete clinicopathological parameters were available.

Immunohistochemistry

Immunostainings were performed on formalin-fixed, paraffin-embedded tissue sections by the labelled streptavidin–avidin–biotin method using monoclonal antibodies directed against *CD138* (dilution 1:10, clone AM411-10 M, BioGenex, CA, USA), *bcl-xl* (dilution 1:25, clone 2H11, Zymed, South San Francisco CA, USA) and *bad* (dilution 1:40, clone sc-8044, Santa Cruz, CA, USA). In addition, the following polyclonal antibodies were used: *bax* (dilution 1:40, code A3533, Dako SA, Glostrup, Denmark), *bak* (dilution 1:40, code A3538, Dako SA, Glostrup, Denmark), *bid* (dilution 1:40, clone sc-11423, Santa Cruz, CA, USA) and *IRF-4/MUM1* (dilution 1:40, clone sc-6059, Santa Cruz, CA, USA). Pretreatment of the sections with 10 mM sodium citrate buffer (pH 6.0) in a microwave oven was performed. The monoclonal antibodies directed against *bcl6*, *CD10*, *bcl2*, *Ki67*, *cyclin A*, *cyclin B1*, *cyclin D3*, *cyclin E*, *p53*, *Rb1*, *p16* and *p27* proteins, the corresponding positive controls and the counting approach for the expression status of these proteins were reported in detail previously.^{41,51,52} The same approach was used for the counting of *MUM1*, *CD138*, *bax*, *bak*, *bad*, *bid* and *bcl-xl* immunopositive cells. Briefly, a continuous score system was adopted by using the $\times 40$ objective lens and counting at least 10 fields selected on the basis that they contained immunopositive cells. 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 in the total number of the counted cells. Positive expression for *MUM1* and *CD138* proteins was considered when at least 25% of neoplastic cells were immunopositive according to previous criteria.¹² The *bcl6/CD10/MUM1/CD138* immunophenotypes and their assignment to germinal center or nongermlinal center B-cell-like profiles were determined taking into consideration previous criteria.³² Positive expression for *bcl-xl*, *bax*, *bak*,

bad and bid proteins was considered when at least 10% of neoplastic cells were immunopositive. Reactive lymph nodes and normal thymuses from our previous studies were used as positive controls.^{41,51,52} Negative controls were included and consisted in the same immunohistochemical method with omission of the primary antibody.

Statistical Analysis

Mann–Whitney test, χ^2 tests, Spearman's correlation coefficient test and analysis of variance were used for statistical analysis. The results were considered as statistically significant when $P < 0.05$. The program SPSS for Windows Release 10 was used for statistical analysis.

Results

Immunohistochemical expression of MUM1, CD138, bax, bak, bad, bid and bcl-xl proteins was found in 48/79 (60%), 0/79 (0%), 73/79 (92%), 41/79 (52%), 60/79 (76%), 44/79 (56%) and 46/77 (60%) cases, respectively (Figure 1) (Table 1). High expression status of bax, bak, bad, bid and bcl-xl proteins (at least 25% of neoplastic cells immunopositive) was found in 36/79 (46%), 20/79 (26%), 25/79 (32%), 30/79 (38%) and 46/77 (60%) cases, respectively.

The immunohistochemical expression of bcl6, CD10, bcl2, cyclin A, cyclin B1, cyclin D3, cyclin E, p53, Rb, p16 and p27 proteins, the proliferation index (as assessed by the Ki67 staining) and the apoptotic index (as assessed by the TUNEL method) were reported in detail previously.^{41,51,52}

Differentiation Immunophenotypic Profiles

Two major immunophenotypic profiles were distinguished according to the pattern of differentiation: (a) the germinal center B-cell-like differentiation immunophenotypic profile; 31 cases (bcl6+/CD10+/MUM1-/CD138-: 26 cases, bcl6+/CD10-/MUM1-/CD138-: three cases and bcl6-/CD10+/MUM1-/CD138-: two cases) and (b) the nongerminal center B-cell-like differentiation immunophenotypic profile; 48 cases (bcl6+/CD10-/MUM1+/CD138-: 24 cases and bcl6-/CD10-/MUM1+/CD138-: 24 cases) (Table 2).

Relations between the Two Major Differentiation Immunophenotypic Profiles and Apoptotic Index, Proliferation Index, bcl2 Family Proteins and Cell Cycle Proteins

The Mann–Whitney test was used to analyze the two major bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles in relation to the apoptotic index, the proliferation index (Ki67) and the expression levels of bcl2 family proteins and cell cycle proteins (Table 3). The germinal center B-cell-like profile, compared to the nongerminal center B-cell-like profile, was significantly associated with higher apoptotic index ($P = 0.034$), higher expression of bax ($P = 0.045$), bak ($P = 0.018$) and bid ($P = 0.003$) proteins and lower expression of bcl-xl protein ($P = 0.026$) (Table 3). No significant correlations were found between the two major bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles and the expression levels of bcl2, bad,

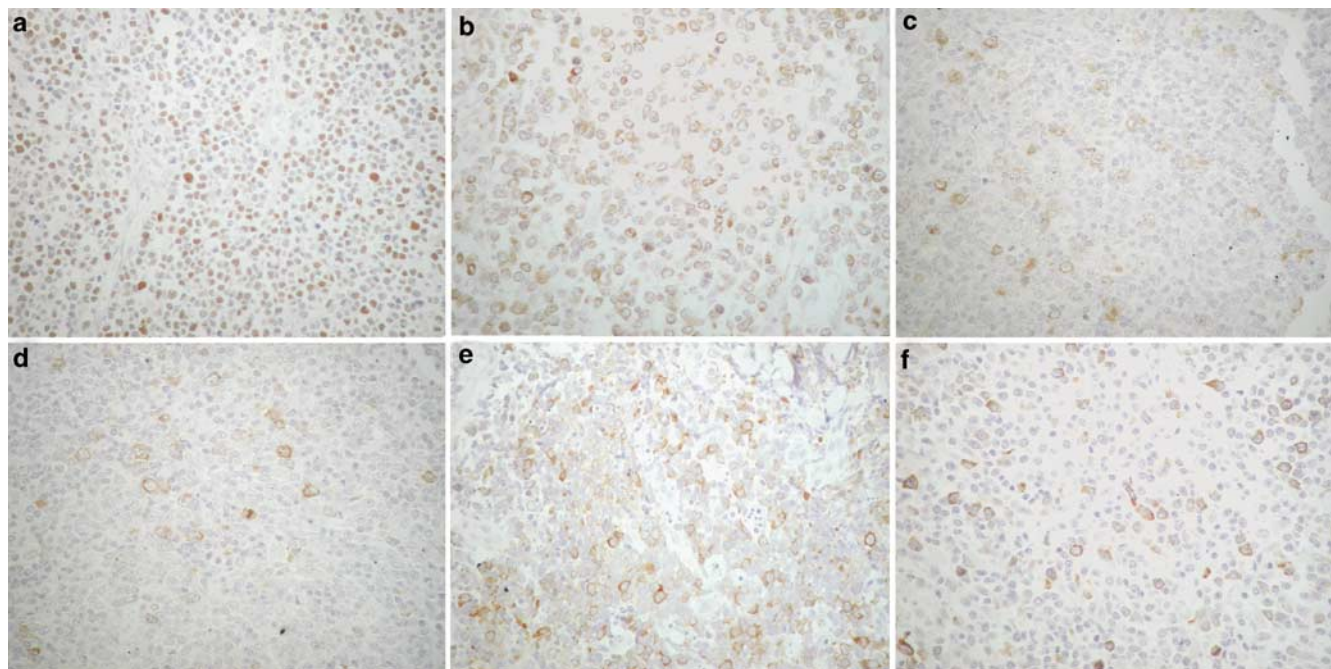


Figure 1 Immunohistochemical expression of (a) MUM1, (b) Bax, (c) Bak, (d) Bad, (e) Bid and (f) Bcl-xl in neoplastic cells of diffuse large B-cell lymphomas (magnification $\times 400$).

Table 1 Expression of MUM1, CD138, bax, bak, bad, bid and bcl-xl proteins

Proteins	Positive cases/total number of cases
MUM1	48/79 (60%)
CD138	0/79 (0%)
bax	73/79 (92%)
bak	41/79 (52%)
bad	60/79 (76%)
bid	44/79 (56%)
bcl-xl	46/77 (60%)

Positive expression for MUM1 and CD138 proteins was considered when at least 25% of neoplastic cells were immunopositive. Positive expression for bcl-xl, bax, bak, bad and bid proteins was considered when at least 10% of neoplastic cells were immunopositive.

Table 2 Bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles

Differentiation immunophenotypic profiles	Number of cases
<i>Germinal center B-cell-like profile</i>	
bcl6+/CD10+/MUM1-/CD138-	26 (33%)
bcl6+/CD10-/MUM1-/CD138-	3 (4%)
bcl6-/CD10+/MUM1-/CD138-	2 (3%)
Total cases	31/79 (40%)
<i>Nongerminal center B-cell-like profile</i>	
bcl6+/CD10-/MUM1+/CD138-	24 (30%)
bcl6-/CD10-/MUM1+/CD138-	24 (30%)
Total cases	48/79 (60%)

Ki67, cyclin A, cyclin B1, cyclin D3, cyclin E, p53, Rb, p16 and p27 proteins (Table 3). The basic statistical data (mean values, standard deviation, standard error, range) for the expression levels of the bcl2 family proteins and for the values of the apoptotic index in relation to the two major bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles are presented in Table 4. The relations between the expression of bcl2 family proteins (positive vs negative cases) and the two major bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles are presented in Table 5.

The χ^2 tests were used to analyze the two major bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles in relation to the expression status (high- vs low-expression status) of bcl2 family proteins, cell cycle proteins and the proliferation index (Ki67). The germinal center B-cell-like profile was significantly associated with high-expression status of bak ($P=0.036$) and bid ($P=0.001$) proteins and low expression status of bcl-xl protein ($P=0.031$) (Table 6). No significant correlations were found between the two major bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles and the expression status (high- vs low-expression status) of bcl2, bax, bad, Ki67, cyclin A,

Table 3 Differentiation immunophenotypic profiles in relation to the apoptotic index (AI), proliferation index (PI) and the expression of bcl2 family and cell cycle proteins (Mann-Whitney test)

	Differentiation profile	Number of cases	Mean rank	P-values
P53	1	31	35.16	0.129
	2	48	43.13	
Rb	1	31	42.17	0.396
	2	48	38.25	
P16	1	31	43.60	0.255
	2	48	37.28	
P27	1	31	41.02	0.711
	2	48	39.34	
PI (Ki67)	1	31	42.98	0.351
	2	48	38.07	
Cyclin A	1	31	37.19	0.381
	2	48	41.81	
Cyclin B1	1	31	43.48	0.277
	2	48	37.75	
Cyclin D3	1	31	38.10	0.548
	2	48	41.23	
Cyclin E	1	31	39.77	0.934
	2	48	40.15	
bcl2	1	30	37.03	0.709
	2	46	39.46	
bax	1	31	46.32	0.045*
	2	48	35.92	
bak	1	31	47.48	0.018*
	2	48	35.17	
bid	1	31	49.50	0.003*
	2	48	33.86	
bad	1	31	44.02	0.203
	2	48	37.41	
bcl-xl	1	30	32.02	0.026*
	2	47	43.46	
AI	1	26	37.21	0.034*
	2	36	27.38	

Differentiation profile 1: germinal center B-cell-like immunophenotypic profile (bcl6+/CD10+/MUM1-/CD138-: 26 cases, bcl6+/CD10-/MUM1-/CD138-: three cases and bcl6-/CD10+/MUM1-/CD138-: two cases; total 31 cases).

Differentiation profile 2: nongerminal center B-cell-like immunophenotypic differentiation profile (bcl6+/CD10-/MUM1+/CD138-: 24 cases and bcl6-/CD10-/MUM1+/CD138-: 24 cases; total 48 cases). *indicates the statistically significant correlations ($P<0.05$).

cyclin B1, cyclin D3, cyclin E, p53, Rb, p16 and p27 proteins (data not shown).

Relations between bcl6, CD10 and MUM1 Proteins and bcl2 Family Proteins

The Spearman correlation coefficient test (for assessment of correlation between two continuous variables) was used to analyze the relations of bcl6, CD10 and MUM1 proteins with bcl2 family proteins and the relations between MUM1, proliferation index and apoptotic index (Table 7). The relations between bcl6, CD10, proliferation index and apoptotic index were reported previously.⁴¹ The positive or negative sign of the Spearman correlation coefficient r in Table 7 expresses significant ($P<0.05$) or nonsignificant ($P=0.05$ or $P>0.05$) positive or

Table 4 Differentiation immunophenotypic profiles in relation to the apoptotic index (AI) and the expression levels of bcl2 family proteins (analysis of variance)

	Differentiation profile	N	Mean value	s.d.	s.e.	Minimum value	Maximum value	P-values
bax	1	31	27.2581	20.8901	3.7519	5.00	80.00	0.013*
	2	48	17.9583	11.5260	1.6636	5.00	70.00	
bak	1	31	17.4516	13.4680	2.4189	1.00	50.00	0.009*
	2	48	10.5417	9.4147	1.3588	1.00	50.00	
bad	1	31	14.7742	7.0318	1.2629	1.00	25.00	0.193
	2	48	12.5833	7.3740	1.0643	1.00	30.00	
bid	1	31	24.7419	17.5612	3.1541	1.00	60.00	0.001*
	2	48	12.8125	12.9740	1.8726	1.00	60.00	
bcl-xl	1	30	21.9667	27.3590	4.9950	0	80.00	0.020*
	2	47	38.1277	30.2587	4.4136	0	100.00	
bcl2	1	30	47.0000	43.7981	7.9964	0	100.00	0.938
	2	47	46.2128	42.2790	6.1670	0	100.00	
AI	1	26	3.4895	2.3971	0.4701	0.34	9.70	0.018*
	2	36	2.2983	1.4377	0.2396	0.26	6.13	

N, number of cases.

Differentiation profile 1: germinal center B-cell-like immunophenotypic profile (bcl6+/CD10+/MUM1-/CD138-: 26 cases, bcl6+/CD10-/MUM1-/CD138-: three cases and bcl6-/CD10+/MUM1-/CD138-: two cases; total 31 cases).

Differentiation profile 2: nongerminal center B-cell-like immunophenotypic differentiation profile (bcl6+/CD10-/MUM1+/CD138-: 24 cases and bcl6-/CD10-/MUM1+/CD138-: 24 cases; total 48 cases).

*indicates the statistically significant correlations ($P < 0.05$).

Table 5 Differentiation immunophenotypic profiles in relation to the expression of bcl2 family proteins (positive vs negative cases)

	Differentiation profile 1	Differentiation profile 2
bax positive	29	44
bax negative	2	4
bak positive	20	21
bak negative	11	27
bid positive	23	21
bid negative	8	27
bad positive	25	35
bad negative	6	13
bcl-xl positive	13	33
bcl-xl negative	17	14
bcl2 positive	18	29
bcl2 negative	12	18

Differentiation profile 1: germinal center B-cell-like immunophenotypic profile (bcl6+/CD10+/MUM1-/CD138-: 26 cases, bcl6+/CD10-/MUM1-/CD138-: three cases and bcl6-/CD10+/MUM1-/CD138-: two cases; total 31 cases).

Differentiation profile 2: nongerminal center B-cell-like immunophenotypic differentiation profile (bcl6+/CD10-/MUM1+/CD138-: 24 cases and bcl6-/CD10-/MUM1+/CD138-: 24 cases; total 48 cases).

Positive expression for bcl-xl, bcl2, bax, bak, bad and bid proteins was considered when at least 10% of neoplastic cells were immunopositive.

negative correlations between two continuous variables. The following significant correlations were found: (a) the expression of bcl6 protein showed significant positive correlation with the expression of bax ($r = 0.659$, $P < 0.001$), bak ($r = 0.391$, $P < 0.001$) and bid ($r = 0.652$, $P < 0.001$) proteins and significant negative correlation with the expression of bcl-xl protein ($r = -0.536$, $P < 0.001$), (b) the expression of CD10 protein showed significant positive correlation with the expression of bax ($r = 0.240$, $P = 0.033$), bak ($r = 0.233$, $P = 0.039$) and bid

Table 6 Differentiation immunophenotypic profiles in relation to the expression status of bcl2 family proteins (high vs low the expression status) (χ^2 tests)

	Differentiation profile 1	Differentiation profile 2	P-values
bcl2 high	18	29	0.881
bcl2 low	12	18	
bax high	17	19	0.184
bax low	14	29	
bak high	12	8	0.036*
bak low	19	40	
bid high	19	11	0.001*
bid low	12	37	
bad high	12	13	0.278
bad low	19	35	
bcl-xl high	13	33	0.031*
bcl-xl low	17	14	

Differentiation profile 1: germinal center B-cell-like immunophenotypic profile (bcl6+/CD10+/MUM1-/CD138-: 26 cases, bcl6+/CD10-/MUM1-/CD138-: three cases and bcl6-/CD10+/MUM1-/CD138-: two cases; total 31 cases).

Differentiation profile 2: nongerminal center B-cell-like immunophenotypic differentiation profile (bcl6+/CD10-/MUM1+/CD138-: 24 cases and bcl6-/CD10-/MUM1+/CD138-: 24 cases; total 48 cases).

High-expression status of bcl2 family proteins: at least 25% of neoplastic cells immunopositive.

Low-expression status of bcl2 family proteins: less than 25% of neoplastic cells immunopositive.

*indicates the statistically significant correlations ($P < 0.05$).

($r = 0.238$, $P = 0.035$) proteins and significant negative correlation with the expression of bcl-xl protein ($r = -0.250$, $P = 0.029$) and (c) the expression of MUM1 protein showed significant negative correlation with the expression of bax ($r = -0.276$, $P = 0.014$) and bid ($r = -0.266$, $P = 0.018$) proteins and significant positive correlation with the expression of bcl-xl protein ($r = 0.238$, $P = 0.037$) (Table 7).

Table 7 Correlations between bcl6, CD10 and MUM1 proteins and bcl2 family proteins (Spearman's correlation test)

	<i>bax</i>	<i>bak</i>	<i>bid</i>	<i>bad</i>	<i>bcl2</i>	<i>bcl-xl</i>
bcl6	$r=0.659$ $P<0.001^*$	$r=0.391$ $P<0.001^*$	$r=0.652$ $P<0.001^*$	$r=0.118$ $P=0.299$	$r=-0.207$ $P=0.070$	$r=-0.536$ $P<0.001^*$
CD10	$r=0.240$ $P=0.033^*$	$r=0.233$ $P=0.039^*$	$r=0.238$ $P=0.035^*$	$r=0.053$ $P=0.640$	$r=-0.056$ $P=0.627$	$r=-0.250$ $P=0.029^*$
MUM1	$r=-0.276$ $P=0.014^*$	$r=-0.193$ $P=0.089$	$r=-0.266$ $P=0.018^*$	$r=-0.115$ $P=0.312$	$r=0.050$ $P=0.667$	$r=0.238$ $P=0.037^*$

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 ($P<0.05$).

Table 8 Correlations between bcl2 family proteins, apoptotic index (AI) and proliferation index (PI) (Spearman's correlation test)

	<i>bax</i>	<i>bak</i>	<i>bid</i>	<i>bad</i>	<i>bcl2</i>	<i>bcl-xl</i>	AI	PI
<i>bax</i>		$r=0.311$ $P=0.005^*$	$r=0.364$ $P=0.001^*$	$r=0.224$ $P=0.047^*$	$r=-0.207$ $P=0.070$	$r=-0.437$ $P<0.001^*$	$r=0.146$ $P=0.258$	$r=0.207$ $P=0.068$
<i>bak</i>	$r=0.311$ $P=0.005^*$		$r=0.447$ $P<0.001^*$	$r=0.100$ $P=0.381$	$r=-0.230$ $P=0.044^*$	$r=-0.242$ $P=0.034^*$	$r=0.212$ $P=0.098$	$r=0.253$ $P=0.025^*$
<i>bid</i>	$r=0.364$ $P=0.001^*$	$r=0.447$ $P<0.001^*$		$r=0.181$ $P=0.110$	$r=-0.189$ $P=0.100$	$r=-0.331$ $P=0.003^*$	$r=0.149$ $P=0.248$	$r=0.116$ $P=0.307$
<i>bad</i>	$r=0.224$ $P=0.047^*$	$r=0.100$ $P=0.381$	$r=0.181$ $P=0.110$		$r=-0.005$ $P=0.962$	$r=-0.288$ $P=0.011^*$	$r=0.189$ $P=0.142$	$r=0.011$ $P=0.923$
<i>bcl2</i>	$r=-0.207$ $P=0.070$	$r=-0.230$ $P=0.044^*$	$r=-0.189$ $P=0.100$	$r=-0.005$ $P=0.962$		$r=0.237$ $P=0.039^*$	$r=-0.293$ $P=0.022^*$	$r=-0.101$ $P=0.382$
<i>bcl-xl</i>	$r=-0.437$ $P<0.001^*$	$r=-0.242$ $P=0.034^*$	$r=-0.331$ $P=0.003^*$	$r=-0.288$ $P=0.011^*$	$r=0.237$ $P=0.039^*$		$r=-0.115$ $P=0.376$	$r=-0.159$ $P=0.168$
AI	$r=0.146$ $P=0.258$	$r=0.212$ $P=0.098$	$r=0.149$ $P=0.248$	$r=0.189$ $P=0.142$	$r=-0.293$ $P=0.022^*$	$r=-0.115$ $P=0.376$		$r=0.501$ $P<0.001^*$
PI	$r=0.207$ $P=0.068$	$r=0.253$ $P=0.025^*$	$r=0.116$ $P=0.307$	$r=0.011$ $P=0.923$	$r=-0.101$ $P=0.382$	$r=-0.159$ $P=0.168$	$r=0.501$ $P<0.001^*$	

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 ($P<0.05$).

Relations between bcl2 Family Proteins, Apoptotic Index and Proliferation Index

The Spearman correlation coefficient test was used to analyze the relations between bcl2 family proteins, apoptotic index and proliferation index (Ki67) (Table 8). The positive or negative sign of the Spearman correlation coefficient *r* in Table 8 expresses significant ($P<0.05$) or nonsignificant ($P=0.05$ or $P>0.05$) positive or negative correlations between two continuous variables. The following significant correlations were found: (a) the expression of *bax* protein showed significant positive correlation with the expression of *bak* ($r=0.311$, $P=0.005$), *bid* ($r=0.364$, $P=0.001$) and *bad* ($r=0.224$, $P=0.047$) proteins, (b) the expression of *bak* protein showed significant positive correlation with the expression of *bid* protein ($r=0.447$, $P<0.001$) and the proliferation index (Ki67) ($r=0.253$, $P=0.025$) proteins and significant negative correlation with the expression of *bcl2* protein ($r=-0.230$, $P=0.044$), (c) the expression of *bcl-xl* protein showed significant negative correlation with the expression of *bax* ($r=-0.437$, $P<0.001$), *bak* ($r=-0.242$, $P=0.034$), *bid* ($r=-0.331$, $P=0.003$)

and *bad* ($r=-0.288$, $P=0.011$) proteins and significant positive correlation with the expression of *bcl2* protein ($r=0.237$, $P=0.017$) and (d) the expression of *bcl2* protein showed significant negative correlation with the apoptotic index ($r=-0.293$, $P=0.022$) (Table 8).

Correlation with Clinicopathological Parameters

Using χ^2 tests, no significant correlation was found between tumor localization (nodal vs extranodal) or tumor stage (I–IV) and the two major bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles (data not shown).

Discussion

In the present study, we analyzed the relations between the bcl6/CD10/MUM1/CD138 differentiation immunophenotypic profiles and the status of apoptosis and proliferation in diffuse large B-cell lymphomas. In addition, we analyzed the expression patterns of the bcl2 family proteins *bcl2*, *bcl-xl*,

bax, bak, bad and bid in relation to the apoptotic index and the proliferation index.

In the present study, we found that in diffuse large B-cell lymphomas the germinal center B-cell-like differentiation immunophenotypic profile was significantly correlated with high apoptotic index, high expression of the proapoptotic proteins bax, bak and bid and low expression of the antiapoptotic protein bcl-xl. In addition, the expression of the germinal center B-cell-related bcl6 and CD10 proteins showed significant positive correlation with bax, bak and bid expression and significant negative correlation with bcl-xl expression. Furthermore, we recently reported that the expression of bcl6 and CD10 proteins showed significant positive correlation with apoptotic index.⁴¹ The aforementioned results, taken together, may be related to findings in various cell lines and lymphoid malignancies suggesting that the expression of the proteins bcl6 and CD10 is associated with increased apoptosis.^{33–40} With respect to the relation between bcl6 and apoptosis, of particular interest are the *in vitro* studies of Yamochi *et al*³⁵ and Tang *et al*.³⁶ Indeed, Yamochi *et al*³⁵ showed that bcl6 overexpression induced apoptosis of CV1 and HeLa cells which was preceded by downregulation of the antiapoptotic genes *bcl2* and *bcl-xl*. In addition, Tang *et al*³⁶ showed that the forkhead transcription factor AFX activates apoptosis by induction of bcl6 which directly binds to and suppresses the promoter of *bcl-xl*. These findings suggest that the significantly lower expression of the antiapoptotic protein bcl-xl that we found in diffuse large B-cell lymphomas with germinal center B-cell like immunophenotypic profile may be due, at least in part, to downregulation of bcl-xl expression induced by bcl6 overexpression.³⁶ With respect to the relation between CD10 and apoptosis, it was suggested that CD10 might degrade cytokines that could play a protective role in B- and T-cell apoptosis.³⁸ This activity may be consistent with the capacity of CD10 to hydrolyze a variety of active peptides, including growth and chemotactic factors.³⁸ Thus, it is possible that CD10 participates in the process of selection in the germinal center and the thymus by increasing the threshold of cytokines required to prevent B- and T-cell apoptosis, respectively.³⁸

In the present study, we found that in diffuse large B-cell lymphomas the nongerminal center B-cell-like differentiation immunophenotypic profile was significantly associated with low apoptotic index, low expression of the proapoptotic proteins bax, bak and bid and high expression of the antiapoptotic protein bcl-xl. In addition, the expression of the MUM1 protein, which is a main feature of the nongerminal center B-cell-like immunophenotypic profile,^{12,32} showed significant negative correlation with bax and bid expression and significant positive correlation with bcl-xl expression. The aforementioned results, taken together, may be related to findings that activated (nongerminal) diffuse large

B-cell-like lymphomas are characterized by constitutive nuclear factor-Kappa B activity which may upregulate many antiapoptotic nuclear factor-Kappa B target genes such as bcl2, bcl-xl, A1, TRAF1, TRAF2, c-IAP1 and c-IAP2.^{33,53} These findings suggest that the significantly higher expression of the antiapoptotic protein bcl-xl that we found in diffuse large B-cell lymphomas with nongerminal center B-cell-like immunophenotypic profile may be due, at least in part, to upregulation of bcl-xl expression induced by the constitutive nuclear factor-Kappa B activity.³³ In addition, the *IRF4* (*MUM1/LSIRF*) gene is also a nuclear factor-Kappa B target^{33,53,54} and this may provide an explanation for the significant positive correlation between MUM1 and bcl-xl expression in the present study. IRF4, a transcription factor that is required for the proliferation of B and T lymphocytes, has been suggested to be a mediator of nuclear factor-Kappa B proliferative responses in activated (nongerminal center) B-cell-like diffuse large B-cell lymphomas.⁵⁴

In the present study, we found that diffuse large B-cell lymphomas frequently express bcl2 family proteins: bax in 92%, bak in 52%, bad in 76%, bid in 56% and bcl-xl in 60%. These results, taken together with previous findings^{16,18,20,21,27,41–50} showing that diffuse large B-cell lymphomas frequently express bcl2, mcl1, bax and bak proteins, suggest that apoptotic mechanisms mediated by bcl2 family proteins are likely to be involved in the pathogenesis of diffuse large B-cell lymphomas. Interestingly, the present and previous findings^{16,18,20,21,27,42–50} indicate that the expression of bcl2 family proteins is variable and heterogeneous in diffuse large B-cell lymphomas. This is likely to reflect differentially regulated expression of bcl2 family proteins, which may be related to abnormalities in gene structure and/or expression. It is known that variations of bcl2 expression in a part of diffuse large B-cell lymphomas may be ascribed to t(14;18) of bcl2 to IgH locus or to amplification of the bcl2 gene genomic locus.^{3–6,14,16,21} The variations in bax, bak and bcl-xl expression in diffuse large B-cell lymphomas may not be related to underlying gene mutations since they were rare in these tumors.^{50,55,56} Interestingly, bad-deficient mice develop, with aging, diffuse large B-cell lymphomas of germinal center origin⁵⁷ suggesting a putative role of bad in the pathogenesis of these neoplasias. Further studies are required to gain insight into the regulation of the expression of the bcl2 family proteins in diffuse large B-cell lymphomas.

In the present study, we found that the apoptotic index was positively correlated with the expression of the proapoptotic proteins bax, bak, bad and bid and negatively correlated with the expression of the antiapoptotic proteins bcl2 and bcl-xl. Thus, the differential expression of bcl2 family proteins may provide an explanation, at least in part, for the variations of the apoptotic index observed in diffuse

large B-cell lymphomas.⁴¹ In addition, the proliferation index (Ki67) was positively correlated with the apoptotic index and the expression of the proapoptotic proteins bax, bak, bad and bid and negatively correlated with the expression of the antiapoptotic proteins bcl2 and bcl-xl. These results are in keeping with previous findings that apoptotic and proliferative indices are positively correlated in B-cell lymphomas^{42,58} and with recent data that bcl-xl and bcl2 inhibit cell proliferation.^{59,60} Interestingly, the antiproliferative effects of bcl-xl and bcl2 were related to their ability to enhance G (0) arrest thereby contributing to cell cycle delay in G (0)–G (1) transition.⁵⁹ Thus, it was suggested that the cell cycle effects result from intrinsic functions of bcl-xl and bcl2.⁵⁹ Furthermore, other apoptosis-associated bcl2 family proteins such as mcl1 may be involved in the regulation of cell proliferation and differentiation by sustaining or inhibiting cell viability at critical points of the cell cycle (review in Craig⁶¹).

The relations between differentiation immunophenotypic profiles and apoptosis status may be relevant to the clinical behavior of diffuse large B-cell lymphomas. Indeed, recent studies reported that (a) the 5-year survival of diffuse large B-cell lymphomas with germinal center B-cell-like immunophenotypic profile was better than that of diffuse large B-cell lymphomas with nongerminal center B-cell-like immunophenotypic profile^{20,32} and (b) the immunexpression of bcl6 or CD10 proteins was associated with better overall survival whereas expression of MUM1 protein was associated with worse overall survival in diffuse large B-cell lymphomas.^{8,13,22,28,32} In addition, high bax immunexpression, which was associated with the germinal center B-cell-like immunophenotypic profile in the present study, was found to be related to better 5-year overall survival in diffuse large B-cell lymphomas.⁴⁸ On the basis of the above results, it could be hypothesized that germinal center B-cell-like diffuse large B-cell lymphoma may be more susceptible to apoptosis and, as a consequence, may be more sensitive to treatment.

In conclusion, the present study indicates that diffuse large B-cell lymphomas with germinal center B-cell-like immunophenotypic profile are associated with increased apoptosis status, high expression of the proapoptotic proteins bax, bak and bid and low expression of the antiapoptotic protein bcl-xl. In contrast, diffuse large B-cell lymphomas with nongerminal center B-cell-like immunophenotypic profile are associated with decreased apoptosis status, low expression of the proapoptotic proteins bax, bak and bid and high expression of the antiapoptotic protein bcl-xl. In view of previous results^{20,32} showing that the expression of the germinal center B-cell-like differentiation immunophenotypic profile is associated with favorable clinical outcome, it could be hypothesized that germinal center B-cell-like diffuse large B-cell lymphoma may be more

susceptible to apoptosis and, as a consequence, may be more sensitive to treatment.

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