Cluster Analysis of Apoptosis-Associated bcl2 Family Proteins in Diffuse Large B-cell Lymphomas. Relations with the Apoptotic Index, the Proliferation Profile and the B-cell Differentiation Immunophenotypes

MARIA BAI1, ANGELOS SKYRLAS1, NIKI JOHN AGNANTIS1, SEVASTI KAMINA1, PANAGIOTIS KITSOULIS2 and PANAGIOTIS KANAVAROS2

1Department of Pathology and 2Department of Anatomy-Histology-Embryology Medical Faculty, University of Ioannina, Ioannina, Greece

Abstract. Background: There is evidence that apoptotic mechanisms mediated by bcl2 family proteins are involved in the pathogenesis of diffuse large B-cell lymphomas (DLBCL). In order to gain further insight into the apoptosis profile of DLBCL, 79 cases were investigated to determine whether distinct clusters of the combined expression levels of bcl2 family proteins can be identified in these lymphomas. Materials and Methods: The combined immunohistochemical expression levels of the proteins bax, bak, bad, bid, bcl2 and bcl-xl were evaluated by cluster and discriminant analysis. The produced clusters were analyzed in relation to the apoptotic index, the proliferation profile and the B-cell differentiation immunophenotypes. Results: Cluster analysis produced: a) a low expression (69/79 cases) and a high expression pro-apoptotic cluster (10/79 cases) for the combined expression levels of the pro-apoptotic proteins bax, bak, bad and bid and b) a low expression (37/76 cases) and a high expression anti-apoptotic cluster (39/76 cases) for the combined expression levels of anti-apoptotic proteins bcl2 and bcl-xl. The decreasing order of discriminant power for the percentages of tumor cells expressing pro-apoptotic and anti-apoptotic proteins was % bax + cells > % bak + cells > % bid + cells > % bad + cells and % bcl2 + cells > % bcl-xl + cells, respectively. The high expression pro-apoptotic cluster was significantly associated with higher mean values of Ki67 (p=0.047) and cyclin A (p=0.033) expression. The high expression pro-apoptotic cluster was significantly associated with the germinal center B-cell bcl6/CD10/MUM1/CD138 differentiation immunophenotype (p=0.043). Conclusion: This study identified distinct clusters of DLBCL with respect to the combined expression levels of the apoptosis-associated bcl2 family proteins. These findings, taken together with our previous observations that distinct clusters with respect to the apoptotic index and the proliferation profile are identified in DLBCL, indicate that subgroups with distinct cellular kinetic properties can be defined in these lymphomas. The cluster analysis approach might be useful for the identification of subgroups of DLBCL with different clinical behavior since increased proliferation and apoptosis were reported to be associated with aggressive tumor behavior in these lymphomas.

Diffuse large B-cell lymphomas (DLBCL) represent the most common type of non-Hodgkin’s lymphomas in Western countries and are characterized by heterogeneous clinical, immunophenotypic and genetic features (1-6). There is accumulating evidence that diverse mechanisms resulting in the deregulation of cell cycle and apoptotic pathways are involved in the pathogenesis of B-cell lymphoid malignancies, including DLBCL (3, 4, 7-67). For example, cell cycle deregulation in B-cell lymphoid malignancies may result from alterations of the p53, Rb and p27 tumor suppressor pathways (7-20) and apoptosis deregulation may result from impairment of the pathways regulated by the bcl2 family members (21-38, 51, 52). Many of the aforementioned studies (7-67) described the expression patterns of various cell cycle, proliferation and apoptosis-associated proteins in DLBCL and some of them correlated the immunophenotypes with clinical data. Evidence was provided that increased proliferation and apoptosis is associated with increased tumor grade and aggressive tumor behavior in various B-cell lymphomas including DLBCL (2, 5, 53-62). Thus, it is important to analyze, in detail, the proliferation and apoptosis profiles of lymphomas and to determine whether distinct groups with low or high proliferation and/or apoptosis status can be identified in these tumors and whether these groups have clinical relevance. In
this regard, cluster analysis may define distinct groups with different proliferation or apoptosis profiles (53, 63, 66). Importantly, Leoncini et al., (53) defined distinct clusters of proliferation (by analyzing simultaneously the immunohistochemical expression levels of Ki67, cyclin A, cyclin B1 and cdk1 proteins and the mitotic index) in a series of 98 cases of diffusely growing B- and T-cell lymphomas of various histological types, including 29 cases of DLBCL. In addition, cluster analysis was used to identify groups of DLBCL on the basis of gene expression profiling by cDNA microarrays (68, 69) and flow cytometry data (70). Indeed, by cluster analysis of the global gene expression signature, three major molecularly distinct groups of DLBCL were identified according to the B-cell differentiation profiles: the germinal center (GC) B-cell-like, the activated B-cell-like and the type 3 DLBCL (68, 69).

The GC B-cell-like DLBCL were characterized by the expression of genes of normal GC B-cells (e.g. bcl6, CD10, CD38), the activated B-cell-like DLBCL were characterized by the expression of genes that are normally induced during in vitro activation of peripheral blood B-cells and the type 3 DLBCL did not express either set of genes at a high level (68, 69). Interestingly, the gene expression signature of the activated B-cell-like DLBCL included high expression of anti-apoptotic genes such as c-FLIP, bcl2, bcl-xl, A1, TRAF1, TRAF2, c-IAP1 and c-IAP2 (67-69). This latter finding indicates links between B-cell differentiation profiles and expression of apoptosis-related genes in DLBCL.

Since the cDNA microarrays technology is not generally available, many studies have successfully used immunohistochemical analysis of DLBCL for the identification of the expression profiles of proteins involved in B-cell differentiation, apoptosis and proliferation (reviewed in 1, 2, 5, 6). In this respect, we recently investigated the bcl6/CD10/MUM1/CD138 B-cell differentiation immuno-phenotypes and the proliferation and apoptosis profiles in 79 cases of DLBCL (49-52). In one of these studies, the GC bcl6/CD10/MUM1/CD138 differentiation immunophenotype was found to be associated with high apoptotic index (AI), high expression of the pro-apoptotic proteins bax, bak and bid and low expression of the anti-apoptotic protein bcl-xl (52). In another study we identified, by cluster, analysis clear-cut cluster solutions for the AI and the proliferation profile (combined immunohistochemical expression levels of the proteins Ki67, cyclin A and cyclin B1), thereby permitting separation of our series of DLBCL into distinct groups with low and high apoptosis status and into distinct groups with low, intermediate and high proliferation profiles (50). In the present study, we analyzed, by cluster and discriminant analysis, the combined immunohistochemical expression levels of the apoptosis-associated bcl2 family proteins bcl2, bcl-xl, bax, bak, bad and bid in the same series of DLBCL. The aims of the present study were: a) to determine by cluster analysis whether distinct clusters of the combined immunohistochemical expression levels of pro-apoptotic (bax, bak, bad, bid) and/or anti-apoptotic (bcl2, bcl-xl) bcl2 family proteins could be identified in DLBCL; b) to investigate, by discriminant analysis, which of the individual proteins is closest to the results of the cluster analysis in its capacity to distinguish between distinct pro-apoptotic or distinct anti-apoptotic protein clusters; and c) to analyze the relations between the clusters of bcl2 family proteins, the AI, the proliferation profile (expression levels of the proteins Ki67 and cyclins A, B1, D3 and E) and the bcl6/CD10/MUM1/CD138 B-cell differentiation immunophenotypes.

### Materials and Methods

**Materials.** Seventy-nine cases of DLBCL (37 nodal and 42 extranodal), classified according to the WHO classification (2) were included in the study. The results of the TdT (terminal deoxynucleotidyl-transferase)-mediated in situ labelling (TUNEL) method and the immunohistochemical results were reported in detail in our previous studies (49-52). Briefly, the AI, as assessed by the TUNEL method, could be evaluated in 62 cases (range of values 0.26-9.7%, mean value 2.79%, standard deviation 1.97%) (50). Immunohistochemical expression of the proteins bcl2, bax, bak, bad, bid, bcl-xl, Ki67 and cyclins A, B1, D3 and E was found in 47/76 (62%), 73/79 (92%), 41/79 (52%), 60/79 (76%), 44/79 (56%), 46/77 (60%), 79/79 (100%), 79/79 (100%), 79/79 (100%), 35/79 (43%) and 18/79 (22%) cases, respectively (49-52). On the basis of the bcl6/CD10/MUM1/CD138 patterns, two major differentiation immunophenotypes were identified: a) GC B-cell-like differentiation immunophenotype: (bcl6+/CD10+/-MUM1-/CD138-: 29 cases and bcl6-/CD10+/MUM1-/CD138-: 2 cases); total 31 cases; and b) non-GC B-cell-like differentiation immunophenotype: 48 cases) (bcl6±/CD10-/MUM1+/CD138-); total 48 cases (52).

### Table I. Clusters of the pro-apoptotic (bax, bak, bad and bid) and anti-apoptotic (bcl2 and bcl-xl) bcl2 family proteins.

<table>
<thead>
<tr>
<th>Pro-apoptotic proteins</th>
<th>Number of cases</th>
<th>Mean value± Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax 10 (HEC) 37 (LEC) 39 (HEC) 37 (LEC)</td>
<td>59.00%±14.68 4.78%±9.82 85.25%±16.46 38.56%±30.93</td>
<td></td>
</tr>
<tr>
<td>Bak 10 (HEC) 37 (LEC) 69 (LEC) 69 (LEC) 69 (LEC)</td>
<td>16.18%±6.66 10.18%±7.41 43.00%±14.94 13.88%±12.32 19.00%±3.16</td>
<td></td>
</tr>
<tr>
<td>Bid 10 (HEC) 69 (LEC) 69 (LEC) 69 (LEC) 69 (LEC) 35/79</td>
<td>19.00%±3.16 12.63%±7.36</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anti-apoptotic proteins</th>
<th>Number of cases</th>
<th>Mean value± Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcl2 39 (HEC) 37 (LEC) 69 (LEC) 69 (LEC)</td>
<td>85.25%±16.46 4.78%±9.82 38.56%±30.93 23.70%±27.17</td>
<td></td>
</tr>
<tr>
<td>bcl-xl 39 (HEC) 37 (LEC) 69 (LEC) 69 (LEC)</td>
<td>38.56%±30.93 23.70%±27.17</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HEC: high expression cluster, LEC: low expression cluster
Statistical analysis. The k-means cluster analysis, discriminant analysis, analysis of variance, multiple analysis of variance and Chi-square tests were applied for statistical analysis using the program SPSS for Windows Release 11. The results were considered as statistically significant when $p<0.05$.

Results

Cluster and discriminant analysis of the combined immunohistochemical expression levels of bcl2 family proteins. The combined entry of the values counted for the expression levels of the pro-apoptotic proteins bax, bak, bad and bid into a k-means cluster analysis separated the entire cohort into two clusters: a low expression pro-apoptotic cluster consisting of simultaneous low bax, bak, bad and bid expression (69/79 cases) and a high expression pro-apoptotic cluster consisting of simultaneous high bax, bak, bad and bid expression (10/79 cases) (Table I). In addition, the combined entry of the values counted for the expression levels of the anti-apoptotic proteins bcl2 and bcl-xl into a k-means cluster analysis separated the entire cohort into two clusters: a high expression anti-apoptotic cluster consisting of simultaneous high bcl2 and bcl-xl expression (37/76 cases) and a low expression anti-apoptotic cluster consisting of simultaneous low bcl2 and bcl-xl expression (39/76 cases) (Table I). Using analysis of variance, the two-cluster mean values were significantly different with respect to pro-apoptotic proteins bax (F-value=248.689, $p<0.001$), bak (F-value=81.874, $p<0.001$), bid (F-value=46.271, $p<0.001$) and bad (F-value=7.210, $p=0.009$) and with respect to the anti-apoptotic proteins bcl2 (F-value=660.491, $p<0.001$) and bcl-xl (F-value=4.931, $p=0.029$). Using multiple analysis of variance between the pro-apoptotic cluster (low vs. high expression pro-apoptotic cluster) as the independent variable and all four pro-apoptotic variables (bax, bak, bad and bid) simultaneously as the dependent vector, a Wilks’ lamda of 0.193 (F-value=77.547) was produced ($p<0.001$). Using multiple analysis of variance between the anti-apoptotic cluster (low vs. high expression anti-apoptotic cluster) as the independent variable and the two anti-apoptotic variables (bcl2 and bcl-xl) simultaneously as the dependent vector, a Wilks’ lamda of 0.100 (F-value=328.343) was produced ($p<0.001$).

Discriminant analysis was applied to determine which of the individual parameters (the pro-apoptotic bax, bak, bad and bid proteins or the anti-apoptotic bcl2 and bcl-xl proteins) is closest to the results of the cluster analysis in its capacity to distinguish between the pro-apoptotic clusters (low vs. high expression pro-apoptotic cluster) or between the anti-apoptotic clusters (low vs. high expression anti-apoptotic cluster). The decreasing order of discriminant power for the pro-apoptotic proteins was as follows: bax: Wilks’ lamda=0.236, F-value=248.689, $p<0.001$; bak: Wilks’ lamda=0.047, F-value=22.0336, $p<0.001$; bid: Wilks’ lamda=0.033, F-value=19.2396, $p<0.001$; and bad: Wilks’ lamda=0.009, F-value=7.210, $p<0.001$.

Table II. Clusters of the pro-apoptotic (bax, bak, bad and bid) bcl2 family proteins in relation to the expression levels of proliferation-associated proteins (Analysis of variances).

<table>
<thead>
<tr>
<th>Differentiation immunophenotype</th>
<th>Pro-apoptic clusters of cases</th>
<th>Mean value</th>
<th>Std. deviation</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>HEC 10</td>
<td>72.0000</td>
<td>20.8433</td>
<td>0.047*</td>
</tr>
<tr>
<td></td>
<td>LEC 69</td>
<td>57.0725</td>
<td>22.0336</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total 79</td>
<td>58.9620</td>
<td>22.3230</td>
<td></td>
</tr>
<tr>
<td>Cyclin A</td>
<td>HEC 10</td>
<td>44.0000</td>
<td>22.9492</td>
<td>0.033*</td>
</tr>
<tr>
<td></td>
<td>LEC 69</td>
<td>29.5072</td>
<td>19.2396</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total 79</td>
<td>31.3418</td>
<td>20.1741</td>
<td></td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>HEC 10</td>
<td>22.1000</td>
<td>12.5206</td>
<td>0.518</td>
</tr>
<tr>
<td></td>
<td>LEC 69</td>
<td>18.6087</td>
<td>16.2925</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total 79</td>
<td>19.0506</td>
<td>15.8388</td>
<td></td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>HEC 10</td>
<td>25.5000</td>
<td>30.9524</td>
<td>0.314</td>
</tr>
<tr>
<td></td>
<td>LEC 69</td>
<td>16.7536</td>
<td>24.6918</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total 79</td>
<td>17.8608</td>
<td>25.5075</td>
<td></td>
</tr>
<tr>
<td>Cyclin E</td>
<td>HEC 10</td>
<td>13.0000</td>
<td>15.4919</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>LEC 69</td>
<td>5.6667</td>
<td>11.1364</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total 79</td>
<td>6.5949</td>
<td>11.9094</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HEC: high expression cluster, LEC: low expression cluster.
*: indicates the statistically significant correlations ($p<0.05$).

Table III. Clusters of the pro-apoptotic (bax, bak, bad and bid) and anti-apoptotic (bcl2, bcl-xl) bcl2 family proteins in relation to the bcl6/CD10/MUM1/CD138 differentiation immunophenotypes (Chi-square tests).

<table>
<thead>
<tr>
<th>Differentiation immunophenotype</th>
<th>Clusters of pro-apoptotic proteins</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEC</td>
<td>LEC</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>45</td>
</tr>
<tr>
<td>Total cases</td>
<td>10</td>
<td>69</td>
</tr>
</tbody>
</table>

Chi-square test: $p=0.043^*$

Abbreviations: HEC: high expression cluster , LEC: low expression cluster.
Differentiation immunophenotype 1: germinal center B-cell-like immunophenotype; 31 cases).
Differentiation immunophenotype 2: non-germinal center B-cell-like immunophenotype; 48 cases).
*: indicates the statistically significant correlations ($p<0.05$).
Clusters of bcl2 family proteins in relation to the apoptotic index, the proliferation profile and the bcl6/CD10/MUM1/CD138 differentiation immunophenotypes. The pro-apoptotic and the anti-apoptotic clusters of Table I were analyzed in relation to the values of the AI (50). Using analysis of variance, no significant correlations (p>0.05) were revealed.

The pro-apoptotic and the anti-apoptotic clusters of Table I were analyzed in relation to the proliferation profile (expression levels of the proteins Ki67 and cyclins A, B1, D3 and E) (49-51). Using analysis of variance, no significant correlations were found between the expression of these proteins and the expression levels of the proliferation-associated proteins Ki67 and cyclins A, B1, D3 and E. Using Chi-square tests, no significant correlations (p>0.05) were revealed between the three major combined expression patterns of bcl2 family proteins and the expression levels of the proliferation-associated proteins Ki67 and cyclins A, B1, D3 and E. Using Chi-square tests, no significant correlations (p>0.05) were revealed between the three major combined expression patterns of bcl2 family proteins and the two major bcl6/CD10/MUM1/CD138 differentiation immunophenotypes.

Discussion

The bcl2 family of proteins represents a critical intracellular checkpoint in the apoptotic pathway and comprises both pro-apoptotic (e.g., bax, bak, bok, bad, bik, bim, bid) and anti-apoptotic (e.g., bcl2, bcl-xl and mcl1) members (71, 72). The functional balance of anti-apoptotic versus pro-apoptotic influences determines a cell's susceptibility to apoptosis. The mechanisms of action of some of these proteins, principally homodimerization and heterodimerization, have been extensively investigated (71, 72). Indeed, bax homodimers promote apoptosis, while bcl2 homodimers and bcl2/bax heterodimers inhibit apoptosis; similarly, bcl-xl inhibits apoptosis, while bcl-xS and bak inhibit the anti-apoptotic function of bcl-xl (71, 72).

Many studies have shown that DLBCL frequently express bcl2 family proteins, suggesting that apoptotic mechanisms mediated by bcl2 family proteins are likely to be involved in the pathogenesis of DLBCL (21-38, 52). The expression of bcl2 family proteins was variable and heterogeneous in DLBCL, indicating differentially regulated expression of bcl2 family proteins (21-38, 52). It was suggested that the differential expression of bcl2 family proteins may account, at least in part, for the variations of the apoptotic status observed in DLBCL (52). The differential expression of bcl2 family proteins might also be related to the variations of the low expression cluster of the anti-apoptotic proteins bcl2 and bcl-xl (6/76 cases; 8%); b) the anti-apoptotic pattern consisted of cases belonging simultaneously to the low expression cluster of bax, bak, bad and bid and the high expression cluster of bcl2 and bcl-xl (35/76 cases; 46%); and c) the mixed pattern with cases belonging neither to the pro-apoptotic nor to the anti-apoptotic pattern (35/76 cases; 46%).
proliferation profile in DLBCL since recent studies suggested that the anti-proliferative effects of bcl-xl and bcl2 result from intrinsic functions of these genes (73-75). In addition, other bcl2 family proteins, such as mcl1, may be involved in the regulation of cell proliferation by sustaining or inhibiting cell viability at critical points of the cell cycle (75).

In order to gain further insight into the apoptotic profile of DLBCL and its relations with the proliferation profile and the B-cell differentiation profile: a) we evaluated, by cluster and discriminant analysis, the combined expression levels of the apoptosis-associated bcl2 family proteins bax, bak, bad, bid, bcl2 and bcl-xl; and b) we analyzed the produced clusters in relation to the AI, the expression levels of the proliferation-associated proteins Ki67 and cyclins A, B1, D3 and E and the major bcl6/CD10/MUM1/CD138 B-cell differentiation immunophenotypes. Cluster analysis produced a two cluster solution for the pro-apoptotic bcl2 family members bax, bak, bad and bid (low and high expression pro-apoptotic cluster) and a two cluster solution for the anti-apoptotic bcl2 family members bcl2 and bcl-xl (low and high expression anti-apoptotic cluster). In addition, the high expression pro-apoptotic cluster was significantly associated with higher mean values of Ki67 and cyclin A. Furthermore, when the three distinct combined expression patterns (pro-apoptotic, anti-apoptotic and mixed pattern) of bcl2 family proteins (bax, bak, bad, bid, bcl2 and bcl-xl) were analyzed in relation to the AI, the proliferation profile and the major bcl6/CD10/MUM1/CD138 differentiation immunophenotypes, the apoptotic pattern was associated with higher mean values of the AI. The above findings, taken together, indicate an association between high expression levels of the pro-apoptotic proteins bax, bak, bad and bid, high AI and high expression levels of the proliferation-associated proteins Ki67 and cyclin A in DLBCL. This is in keeping with other observations showing a positive correlation between AI, mitotic index and immunohistochemical expression of proliferation-associated proteins (Ki67, cyclin A, cyclin B1) in B- and T-cell non-Hodgkin's lymphomas (22, 53) and in mucosa-associated lymphoid tissue lymphomas (56). Thus, the definition by cluster analysis of distinct groups with respect to the AI (SI) and the combined immunohistochemical expression patterns of apoptosis-associated and proliferation-associated proteins (50, 53, present data) indicates that subsets with distinct cellular kinetic properties can be defined in DLBCL. The identification by cluster analysis of distinct protein expression profiles using immunohistochemistry (50, 53, present data) may be paralleled to the identification by cluster analysis of distinct RNA expression profiles using cDNA microarrays (67-69). Therefore, since immunohistochemistry is a reliable routine diagnostic and research method, the present and previous studies (50, 53) add the information that cluster analysis of the immunohistochemical expression levels of apoptosis and proliferation-associated proteins can successfully determine distinct combined immunohistochemical expression profiles of these proteins in DLBCL and may be also applied in other types of lymphoid malignancies (76-81). Furthermore, this cluster analysis approach might be useful for the immunohistochemical identification of groups of DLBCL with different clinical behavior, since increased proliferation and apoptosis were reported to be associated with increased tumor grade and aggressive tumor behavior in B-cell lymphomas, including DLBCL (5, 53-62).

In the present study the GC B-cell bcl6/CD10/MUM1/CD138 differentiation immunophenotype in DLBCL, which is characterized by the expression of bcl6 and CD10 proteins, was significantly associated with the high expression cluster of the pro-apoptotic proteins bax, bak, bad and bid. This provides further support to the accumulating evidence that the expression of the GC-B-cell-associated bcl6 and CD10 proteins is associated with increased apoptosis status (51, 52, 82-87).

In summary, the present study identified distinct clusters of DLBCL with respect to the combined immunohistochemical expression patterns of the apoptosis-associated bcl2 family proteins. The present findings, taken together with our previous observation that distinct clusters with respect to the apoptotic index and the combined immunohistochemical expression patterns of proliferation-associated proteins are identified in DLBCL, indicate that groups with distinct cellular kinetic properties can be defined in these lymphomas. The cluster analysis approach might be useful for the immunohistochemical identification of groups of DLBCL with different clinical behavior, since increased proliferation and apoptosis were reported to be associated with aggressive tumor behavior in these lymphomas.

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