Differential Expression of BCL-2 Family Proteins in ALK-Positive and ALK-Negative Anaplastic Large Cell Lymphoma of T/Null-Cell Lineage

George Z. Rassidakis,*† Andreas H. Sarris,* Marco Herling,* Richard J. Ford,† Fernando Cabanillas,* Timothy J. McDonnell,‡ and L. Jeffrey Medeiros†

From the Departments of Lymphoma-Myeloma,* Molecular Pathology,† and Hematopathology,‡ University of Texas M.D. Anderson Cancer Center, Houston, Texas

Anaplastic large-cell lymphoma (ALCL) of T- or null-cell lineage, as defined in the revised European-American lymphoma classification, includes a subset of tumors that carry the t(2;5)(p23;q35) resulting in overexpression of anaplastic lymphoma kinase (ALK). Patients with ALK+ ALCL are reported to have a better prognosis than patients with ALK− ALCL. Because the mechanisms for this survival difference are unknown, we investigated the hypothesis that apoptotic pathways may be involved. We therefore assessed expression levels of the anti-apoptotic proteins BCL-2 and BCL-XL and the pro-apoptotic proteins BAX and BCL-XS in T/null-cell ALCL using immunohistochemical methods and correlated the findings with ALK expression and apoptotic rate (AR), the latter assessed by a modified Tdt-mediated dUTP nick-end labeling assay. ALK was detected in 21 of 66 (31.8%) ALCLs. BCL-2 was not detected in 21 ALK+ ALCLs but was present in 26 of 45 (57.8%) ALK− ALCLs (P < 0.0001). ALK+ and ALK− ALCLs also showed significant differences in expression of BCL-XL, BAX, and BCL-XS. ALK− tumors less commonly had a high level of BCL-XL (1 of 17 versus 14 of 35, P = 0.01), and more commonly had high levels of BAX (13 of 18 versus 15 of 36, P = 0.05), and BCL-XS (11 of 16 versus 12 of 31, P = 0.05) compared with ALK− tumors. ALK+ tumors also had a higher mean AR than ALK− tumors (3.4% versus 1.1%, P = 0.0002). Differential expression of BCL-2 family proteins may be responsible for the higher AR observed in ALK+ ALCL and provides a possible biological explanation for the better prognosis reported for patients with ALK+ ALCL. (Am J Pathol 2001, 159:527–535)

Accepted for publication April 13, 2001.

Address reprint requests to L. Jeffrey Medeiros, MD, Department of Hematopathology, Box 72, University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, Texas 77030. E-mail: jmedeirol@mail.mdanderson.org.
BCL-2 expression has been studied extensively in aggressive non-Hodgkin’s lymphomas, principally diffuse large B-cell lymphoma, and has been correlated with worse prognosis.28–31 In contrast, BAX, BCL-XL, and BCL-XS expression and their clinical significance, are less well studied with discordant results.32–34 Relatively little is known about expression of BCL-2 family member expression and apoptotic rate (AR), the latter evaluated by a modified Tdt-mediated dUTP nick-end labeling (TUNEL) assay. Our results indicate that there are striking differences in expression of BCL-2 family member proteins in aggressive non-Hodgkin’s lymphomas of T-cell lineage.35

In this study, we hypothesized that alterations of apoptotic pathways may be responsible for the different prognosis of patients with ALK+ and ALK− ALCL. We therefore assessed the expression levels of BCL-2, BAX, BCL-XL, and BCL-XS in a series of ALCL of T/null phenotype. We correlated these findings with ALK expression and apoptotic rate (AR), the latter evaluated by a modified Tdt-mediated dUTP nick-end labeling (TUNEL) assay. Our results indicate that there are striking differences in expression of BCL-2 family proteins between the ALK+ and ALK− tumors.

Materials and Methods

Study Group

This group included 66 cases of systemic ALCL of T- or null-cell lineage accessioned at The University of Texas MD Anderson Cancer Center between 1984 and 2000. No patients had received therapy before biopsy. The clinicopathological features of these patients are shown in Table 1. The median age of patients with ALK+ tumors was 35 years compared with 49 years for patients with ALK- tumors (P = 0.005). All other clinical parameters including Ann Arbor stage were comparable. The histopathological diagnosis of ALCL was based on both morphological and immunohistological criteria according to the revised European-American lymphoma and World Health Organization classifications and all specimens were reviewed at the time of immunohistochemical analysis. All cases were routinely processed, fixed in 10% buffered formalin, and embedded in paraffin. Immunohistochemically, all ALCL cases expressed CD30 and were negative for B-cell antigens (CD20 and/or CD79a). All T-cell tumors were positive for one or more T-cell antigens (CD3, CD5, CD43, or CD45RO). Tumors negative for CD3 and CD5 and positive for CD43 or CD45RO were considered to be of T-cell lineage in this study, although null-cell lineage cannot be excluded as CD43 and CD45RO react with histiocytes. Null-cell cases were negative for all T-cell antigens.

### Immunohistochemical Methods

Tissue sections (3 or 4 μm thick) were deparaffinized in xylene and rehydrated in a graded series of ethanol.

The panel of antibodies used included: ALK-1 (1:30) and BCL-2 (1:40) (DAKO, Carpinteria, CA); BAX (1:40) and BCL-XL (1:25) (Zymed, South San Francisco, CA); and BCL-XS (1:50) (Calbiochem, San Diego, CA) and MIB-1 (1:120) (Immunotech, Westbrook, ME). For all antibodies, heat-induced epitope retrieval was performed using a modification of the method described previously by Shi and colleagues.38 Tissue sections were placed in plastic Coplin jars containing preheated target retrieval solution (DAKO), heated in a household vegetable steamer (Sunbeam-Oster, Model Sunbeam 4713/5710, 900 W) for 35 minutes, and allowed to cool at room temperature for at least 15 minutes. Subsequent steps of the immunostaining procedure were performed using the DAKO Autostainer at room temperature, and included the following: 1) blocking of endogenous peroxidase in 3% H2O2 in phosphate buffered saline (PBS), pH 7.4, for 5 minutes; 2) blocking of nonspecific protein-binding sites using protein blocking solution (DAKO) for 5 minutes; 3) incubation with the primary antibody for 1 hour; and 4) detection using the streptavidin-biotin-peroxidase based LSAB+ kit (DAKO) for 2 × 15 minutes. We used 3,3’ diaminobenzidine/H2O2 (Biogenex, San Ramon, CA) or 3-amino-9-ethyl carbazol as the chromogen and hematoxylin as the counterstain.

We used two cell lines as positive controls for ALK, SU-DHL1 and KARPAS 299, known to express ALK as a result of the t(2;5)(p23;q35). These cell lines were grown in mid-logarithmic phase, fixed overnight in buffered for-

Table 1. Clinical Characteristics of Patients with ALK-Positive and ALK-Negative Anaplastic Large-Cell Lymphoma of T/Null Lineage

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ALK Positive</th>
<th>ALK Negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Median</td>
<td>35</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>3–60</td>
<td>17–79</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11 (52%)</td>
<td>33 (73%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Female</td>
<td>10 (48%)</td>
<td>12 (27%)</td>
<td></td>
</tr>
<tr>
<td>Skin involvement</td>
<td>4/21 (19%)</td>
<td>13/45 (29%)</td>
<td>0.5</td>
</tr>
<tr>
<td>Ann Arbor Stage:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>7 (33%)</td>
<td>20 (44%)</td>
<td>0.4</td>
</tr>
<tr>
<td>III–IV</td>
<td>14 (67%)</td>
<td>25 (56%)</td>
<td></td>
</tr>
<tr>
<td>Serum LDH &gt;1.5× normal</td>
<td>12/21 (57%)</td>
<td>21/45 (47%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Serum β₂-microglobulin &gt;2.5 mg/L</td>
<td>13/21 (62%)</td>
<td>25/45 (55%)</td>
<td>0.6</td>
</tr>
<tr>
<td>Albumin &lt;3.6 g/dL</td>
<td>5/21 (24%)</td>
<td>16/45 (33%)</td>
<td>0.7</td>
</tr>
<tr>
<td>WBC (mean ± SD)</td>
<td>12.1 ± 13.4</td>
<td>9.9 ± 4.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Anemia</td>
<td>11/21 (52%)</td>
<td>21/45 (47%)</td>
<td>0.8</td>
</tr>
</tbody>
</table>
malin, and embedded in paraffin. A case of follicular lymphoma bearing the t(14;18) served as positive control for BCL-2. Control slides from prostate tissue were used as positive controls for the BAX, BCL-XL, and BCL-XS as described elsewhere. In addition, reactive small lymphocytes in tissue sections served as internal positive controls. The K562 cell line was used as a negative control in all experiments. A lymph node with follicular hyperplasia was used as a positive control for MIB-1. Another internal control was the application of normal rabbit serum (DAKO) without primary antibody to the control slides, to exclude nonspecific cross-reactions with the primary antibody.

All immunostained slides were evaluated by two of us (GZR and LJM). Any cytoplasmic or nuclear staining of ALCL cells was considered positive, irrespective of intensity. Expression levels for BAX, BCL-XL, and BCL-XS were determined by counting at least 1000 neoplastic cells in each case. Based on the distribution of data (histograms), and for the purpose of statistical analysis, we considered BAX expression as high when the percentage of positive neoplastic cells was >50%. BCL-XL or BCL-XS expression was considered to be high when the percentage of BCL-XL or BCL-XS-positive lymphoma cells was >25%. No statistical cutoffs were used for the BCL-2 or ALK results as these were all-or-none phenomena. Proliferation index (PI), assessed in a subset of 10 ALK+ and 10 ALK− tumors, was determined by counting the percentage of MIB1-positive nuclei.

**Modified TUNEL Assay**

Tissue sections were mounted on coated slides, deparaffinized in a graded series of ethanol, rehydrated, and pretreated with proteinase K (20 μg/ml) for 25 minutes at 37°C. Slides were then incubated for 5 minutes in 3% H2O2 in PBS at pH 7.4, to block endogenous peroxidase activity. Terminal deoxynucleotidyl transferase (TdT) (New England Biolabs, Beverly, MA) was subsequently applied (15 U/slide) for 1 hour, at 37°C, in 20 mmol/L Tris-acetate, pH 7.9, 50 mmol/L potassium acetate, 10 mmol/L magnesium acetate, 1 mmol/L dithiothreitol, 0.25 mmol/L CoCl2, 24 μmol/L biotin-dATP (Life Technologies, Gaithersburg, MD). Terminal deoxynucleotidyl transferase catalyzes the addition of deoxynucleotidates to the 3’ hydroxy terminus of DNA molecules. The TUNEL assay was modified by substituting dUTP for dATP. For the detection of labeled termini, streptavidin-biotin-horse-radish peroxidase complex (LSAB+ kit) and 3,3’-diamino-benzidine (both from DAKO) were used, according to the manufacturer’s instructions. The slides were counterstained with hematoxylin.

After quenching endogenous peroxidase activity, positive control sections from K562 cells were incubated with DNase I (2.5 μg in 50 μl/slide in Tris-buffered saline containing 6 mmol/L MgCl2) for 30 minutes at 37°C. Tissue sections from the same cell line incubated with a reaction mixture lacking TdT served as negative controls in each experiment.

At least 2000 nuclei from ALCL cells were evaluated in each case and the percentage of positively stained nuclei was considered to be the AR.

**Statistical Analysis**

Statistical comparison of patient age between ALK+ and ALK− groups was based on the one-way analysis of variance test. The nonparametric Spearman’s rank correlation coefficient was applied to evaluate the strength of the relationship between BAX, BCL-XL, BCL-XS, AR, and PI. The chi-square and Fisher’s exact tests were used to compare the expression of all proteins as groups (positive versus negative, low versus high) with various clinicopathological parameters. The Mann-Whitney U test was chosen for the nonparametric correlation of BAX, BCL-XL, BCL-XS, AR, and PI between ALK+ and ALK− ALCL.

Progression-free survival (PFS) was chosen to evaluate the clinical outcome of the patients because various factors after relapse might impact overall survival of the patients. PFS was defined as time from initiation of therapy to last follow-up, primary treatment failure, or relapse. Analysis was based on the method of Kaplan and Meier with Mantel-Cox log-rank test. All computations were performed using StatView statistical program (SAS Institute, Cary, NC).

**Results**

**ALK Expression**

Twenty-one of 66 (31.8%) ALCL of T/null lineage were positive for ALK-1 with a nuclear and cytoplasmic pattern of staining (Figure 1). Seventeen of 21 ALK+ cases were reported to carry the t(2:5)(p23;q35) by reverse transcriptase-polymerase chain reaction (cases referred from other institutions) or long-range genomic DNA polymerase chain reaction, as reported previously. Seventeen (81%) of 21 ALK+ and 31 (68.9%) of 45 ALK− tumors expressed one or more T-cell antigens. Histological subtypes of the ALK+ cases included: 12 classical pleomorphic, 7 monomorphic, 1 sarcomatoid, and 1 lymphohistiocytic.

**Expression of BCL-2, BAX, BCL-XL, and BCL-XS**

BCL-2 was not detected in any of the 21 ALK+ ALCL, whereas 26 of 45 (57.8%) ALK− tumors expressed BCL-2 (P < 0.0001, Fisher’s exact test) (Figure 2). The percentage of BCL-2-positive cells ranged from 14.3 to 98%, but most cases expressed high levels of BCL-2 (mean, 78.1%; median, 88.5%). BAX immunoreactivity was observed in 43 of 54 (79.6%) ALCL assessed (Figure 3). The proportion of BAX-positive cells ranged from 4 to 93% (mean, 46.2%; median, 54%). Using a 50% cutoff, 13 of 18 (72.2%) ALK+ tumors had high BAX expression compared with 15 of 36 (41.7%) ALK− tumors (P = 0.05).
(Table 2). BCL-XL-positive cells were detected in 23 of 52 (44.2%) ALCL assessed. The percentage of BCL-XL-positive cells ranged from 2 to 96% (mean, 19.8%; median, 7%) (Figure 4). Using a 25% cutoff, 1 of 17 (5.9%) ALK+/H11001 tumors had high BCL-XL compared with 14 of 35 (40%) ALK-/H11002 tumors (P = 0.01) (Table 2). Twenty-eight (59.6%) of 47 ALCL assessed were BCL-XS-positive. The percentage of BCL-XS-positive cells ranged from 1 to 82% (mean, 26.8%; median, 20%) (Figure 5). Using a 25% cutoff, 11 of 16 (68.8%) ALK+/H11001 tumors had high BCL-XS compared with 12 of 31 (38.7%) ALK-/H11002 tumors (P = 0.05). All BCL-2 family proteins were immunolocalized principally in the cytoplasm of neoplastic cells.

**Apoptotic Rate**

The mean percentage of apoptotic neoplastic cells was 1.9 ± 1.8% for the entire study group, ranging from 0.1 to 7.9%. Staining was restricted to the nucleus of apoptotic cells (Figures 6 and 7). Fragmented nuclei and nuclear
debris near apoptotic cells were also positively stained. The mean AR was 3.4% in ALK+ tumors, threefold higher than ALK− tumors ($P = 0.0002$, Mann-Whitney $U$ test) (Table 2, Figure 8). AR also correlated with BCL-2 expression. For 53 tumors with both AR and BCL-2 results, the mean AR was 2.5% in BCL-2-negative cases compared with 0.9% for BCL-2-positive cases ($P = 0.0016$).

### Proliferation Index

Tumor PI, evaluated in a subset of 20 cases of ALCL, ranged from 20.4 to 94.6% (mean, 69.2 ± 18.9%; median, 75%). These results are similar to those reported in other types of diffuse aggressive non-Hodgkin’s lymphomas.37 No statistical difference in PI was observed between ALK+ and ALK− tumors (Table 2).

### Association between BCL-2 Family Proteins, AR, and PI

Box plots demonstrating the distribution of BCL-2, BAX, BCL-XL, and BCL-XS expression between the ALK+ and ALK− groups are shown in Figure 8. For the entire study group, correlation between the continuous variables BAX, BCL-XL, BCL-XS, AR, and PI showed a slight statistical association between PI and BAX ($r = 0.45$, $P = 0.07$) and between PI and BCL-XS ($r = 0.55$, $P = 0.04$) expression (Spearman’s rank $R$ correlation coefficient). The ratio BCL-XS/BCL-XL was also found to be significantly higher in ALK+ compared with ALK− tumors (mean, 6.5 versus 1.6). More specifically, a BCL-XS/Bxl-XL ratio >3 was observed in 56% of the ALK+ tumors compared with 17% of the ALK− tumors ($P = 0.03$, Fisher’s exact test).

### Clinical Outcome

BCL-2-positive cases of ALCL showed a statistical trend toward worse PFS although this did not reach statistical significance ($P = 0.13$, by log rank; Table 3). Expression of BAX, BCL-XL, and BCL-XS, did not correlate significantly with PFS (Table 3). Using a cutoff of 1.9% (median AR of the entire study group), AR did not significantly impact PFS ($P = 0.5$ by log rank). However, using the median AR (2.9%) of the ALK+ ALCL group as a cutoff, none of 10 patients with AR higher than 2.9% failed therapy with a median follow-up period of 39 months.

### Discussion

In this study we investigated the hypothesis that ALK expression may correlate with expression of BCL-2 family proteins in ALCL. Our series included 66 biopsy specimens obtained from untreated patients with ALCL of T- or null-cell lineage. Twenty-one tumors were ALK+ and 45 tumors were ALK−.

BCL-2 expression was not detected in any of the ALK+ tumors. Because BCL-2 immunoreactivity was found in ~60% of the ALK− cases in this series, it is possible that NPM-ALK down-regulates BCL-2 gene expression in vivo. Approximately 80% of ALCL cases were immunoreactive with BAX in our study. Notably, the majority of the ALK+ tumors showed high levels of BAX expression (Table 2). This imbalance between BAX and BCL-2 favors apoptosis, and in part may explain the susceptibility of ALK+ ALCL to chemotherapy.

In previous studies, BCL-2 and BAX have been shown to be expressed in diffuse large B-cell lymphoma, in levels comparable with those presented here for the ALK− ALCL subgroup. In addition, these previous studies have correlated BCL-2 immunoreactivity with poorer prognosis in diffuse large B-cell lymphoma.31–33 In the present study, patients with BCL-2-positive ALCL showed a statistical trend toward unfavorable prognosis, but this did not reach statistical significance ($P = 0.13$). BCL-2 and BAX expression also have been reported in T-cell lymphoid malignancies.36 However, detailed correlation between expression of BCL-2-related proteins and various types of T-cell lymphoma has not been reported.

The anti-apoptotic protein BCL-XL was not detected, or was expressed at lower levels, in ALK+ cases compared with the ALK− cases of ALCL. In contrast, the anti-apoptotic partner, BCL-XS, was more frequently detected in ALK+ ALCL (Table 2). In one previous study, Xeri and colleagues34 detected BCL-XL and BCL-XS in a variety of lymphoma types. However, the number of cases examined is limited.34 As far as we are aware, our study provides novel information concerning the expression of both spliced forms of the BCL-X gene in ALCL.
Figure 4. BCL-XL expression in ALCL of T/null lineage. A: Absence of BCL-XL expression in a case of ALK+ ALCL with skin involvement. Co-existing skin adnexal structures are weakly positive for BCL-XL. B: High expression levels of BCL-XL in an ALK− ALCL. A small number of reactive small lymphocytes show weak cytoplasmic immunoreactivity for BCL-XL (DAB, hematoxylin counterstain; original magnification, ×400).

Figure 5. BCL-XS expression in ALCL of T/null lineage. A: Many BCL-XS-positive tumor cells in an ALK+ ALCL. However, a small number of large anaplastic cells are BCL-XS-negative. B: Rare BCL-XS-positive tumor cells in a case of ALK− ALCL (DAB, hematoxylin counterstain; original magnification, ×400).

Figure 6. AR in ALCL of T/null lineage. A: An ALK+ ALCL case with AR of 6.5%. The positive signal is restricted to the nuclei of apoptotic cells (DAB, hematoxylin counterstain; original magnification, ×200). B: An ALK− ALCL with AR of 0.9%. Only one apoptotic tumor cell is found in this field (DAB, hematoxylin counterstain; original magnification, ×400).
The mitogenic effect of NPM-ALK has been shown by previous in vitro studies. Recently, Bai and colleagues also showed that NPM-ALK activates an anti-apoptotic signaling pathway via activation of phosphatidylinositol 3-kinase, which in turn activates the serine-threonine kinase Akt/PKB. The latter kinase is capable of phosphorylating the pro-apoptotic BCL-2 family protein BAD, thus preventing BAD from binding to and inhibiting the anti-apoptotic proteins BCL-2 and BCL-XL, resulting in tumor cell survival. The impact of this pathway in the molecular pathogenesis of ALK+ ALCL in vivo needs to be further investigated. It seems likely that this pathway has a limited role in ALK+ ALCL in vivo because levels of BCL-2 and BCL-XL are undetectable or low. However, our data do not allow an assessment of the functional status of these molecules (bound or unbound to BAD or other BCL-2 family proteins). Other mechanisms involving aberrant ALK tyrosine kinase signaling need to be further investigated at the molecular level in relation to cell proliferation and apoptosis in ALCL.

The AR, as determined by modified TUNEL assay, was found to be threefold higher in ALK+ compared with ALK− tumors (P = 0.0002 by Mann-Whitney U test). The undetectable or low levels of the anti-apoptotic proteins BCL-2 and BCL-XL, and the statistically higher expression of the pro-apoptotic homologues BAX and BCL-XS provide a biological explanation for the higher AR observed in ALK+ ALCL. Moreover, the high BCL-XL/BCL-XL ratio noticed in ALK+ cases strengthens these associations. It is also possible that down-regulation of BCL-XL might be associated with decreased cell survival in a manner independent of BCL-2 expression levels, similar to that shown in cell cultures derived from follicular lymphomas.

However, we do not mean to imply that differential expression of BCL-2 family proteins is the only explanation for the significant difference in AR between ALK+ and ALK− ALCL. Other potential explanations include the Fas-mediated apoptotic machinery. Previous in vitro studies using cell lines derived from systemic ALCL cases have provided evidence that t(2;5)-positive ALCL cells express CD95 and undergo CD95-induced apoptosis. Clusterin, recently reported to be specifically expressed in ALCL cell lines and tumors, also may be involved. Clusterin (also known as sulfated glycoprotein 2, human apolipoprotein A-I, J) is a highly conserved glycoprotein, which among its other functions, promotes cell death. Although Wellmann and colleagues reported that clusterin was not related to ALK expression, it remains unclear whether overexpression of this cell death promoter might contribute to increase AR in a subset of ALK+ ALCL. The NPM gene, the second partner of the NPM-ALK hybrid, also can be translocated to chromosomal regions other than 2p23. For instance, NPM-MLF1 is the chimeric product of t(3;5)(q25.1;q34), which is associated with myelodysplastic syndromes. In a recent paper by Yoneda-Kato and colleagues, overexpression of NPM-MLF-1 was capable of inducing apoptosis in NIH3T3 mouse fibroblasts. This induction required the presence of an intact NPM dimerization domain. Notably, co-expression of BCL-2 rescued the fibroblasts from NPM-MLF1-mediated cell death.

In summary, we have shown that expression of BCL-2 family proteins and AR differ significantly between ALK+
and ALK—ALCL of T/null lineage, and are not obviously related to PI. These findings further support the concept that ALK+ ALCL of T/null lineage is a clinicopathological entity, distinct from ALK− ALCL, as has been suggested by others. The absence or low levels of anti-apoptotic proteins, such as BCL-2 and BCL-XL, probably plays a role in the good response to chemotherapy observed in patients with ALK+ ALCL.

References


