Apoptosis and Proliferation in Subcutaneous Panniculitis-Like T-Cell Lymphoma

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Subcutaneous panniculitis-like T cell lymphoma (SPTCL), designated recently as a distinct clinicopathologic entity in the World Health Organization Classification, is a neoplasm composed of cytotoxic T-cells that preferentially involves subcutaneous adipose tissue. Histologically, SPTCL is characterized by extensive karyorrhectic debris and tumor necrosis suggesting that apoptotic mechanisms are involved in its pathogenesis. We assessed the apoptotic index (AI) and proliferation rate (PR) of 13 cases of SPTCL by TUNEL test and Ki-67 immunostaining, respectively. We also immunohistochemically assessed for expression of BCL-2 (anti-apoptosis), BAX (pro-apoptosis), and P53 and correlated the results with apoptosis and proliferation. We detected a high AI (median 8.1%) in 11 cases of SPTCL, and 12 cases had low BCL-2 and high BAX expression. BCL-2 expression inversely correlated with AI ($P < .001$) and BAX ($P < .001$). We found a low PR (cutoff ≥ 25%) in eight (61%) cases. There was an inverse correlation between AI and PR ($r = -.58$, $P = .04$). Ten cases were assessed for P53; immunostaining results were heterogeneous but P53 expression correlated with large cell cytologic features. Our findings demonstrate that SPTCLs have a high AI that may be explained by differential expression of BCL-2 and BAX in the neoplastic cells.

KEY WORDS: Apoptosis, Immunohistochemistry, Proliferation, Subcutaneous panniculitis-like T-cell lymphoma.

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Subcutaneous panniculitis-like T cell lymphoma (SPTCL) is a rare type of non-Hodgkin's lymphoma composed of cytotoxic T-cells, recently recognized as a distinct clinicopathological entity in the World Health Organization classification (1). Clinically, patients present with subcutaneous nodules, most commonly involving the extremities. The clinical course of patients with SPTCL is variable. Many patients have an aggressive clinical course that may respond to intensive chemotherapy regimens (2, 3). A subset of patients initially has an indolent clinical course (2, 4). Histologically, the neoplastic cells involve the subcutaneous tissue in a panniculitis-like pattern, infiltrating fibrous septae and rimming fat spaces (1, 2, 4). Marked karyorrhectic debris and tumor necrosis are frequent in SPTCL. These findings suggest that apoptotic mechanisms may be involved in pathogenesis.

Tumor growth depends on the balance between apoptosis and proliferation. One major regulator of apoptosis is the BCL-2 family of proteins. BCL-2, the first member of this family characterized, inhibits apoptosis (5). BAX, in contrast, is another member of this family that promotes apoptosis. The ratio of BCL-2 to BAX correlates with sensitivity to apoptosis in various non-Hodgkin's lymphomas (6, 7) but has not been reported previously in SPTCL. Another regulator of apoptosis is the p53 gene, which encodes for a protein that induces growth arrest and apoptosis after DNA damage (8). P53 also has been shown to down-regulate BCL-2 and up-regulate BAX expression *in vivo* and *in vitro* (9). Some tumor types appear to show coordinate regulation of proliferation and apoptosis by a common genetic mechanism (10). One example, in some B-cell tumors, is dysregulated activation of the MYC transcription factor that is a potent inducer of both cell proliferation and apoptosis (11). It is believed that MYC expression induces cell proliferation in a cytokine-dependent manner. Tumor cells undergo apoptosis when cytokines become limited (11).

Apoptosis and proliferation can now be assessed in fixed, paraffin-embedded tissue sections. Apoptotic index (AI) can be quantified using the terminal

deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay. Proliferation rate (PR) can be assessed using the MIB-1 antibody specific for Ki-67, a nuclear-associated proliferation antigen. Monoclonal antibodies for BCL-2, BAX, and p53 are also available.

The goal of this study was to apply these methods to a group of SPTCL cases. The results suggest that SPTCL is characterized by a high AI and that differential expression of BCL-2 and BAX may be involved in the susceptibility of SPTCL to apoptosis.

MATERIALS AND METHODS

Case Selection

Thirteen cases of SPTCL were included in this study. These cases were collected from the files of the Department of Hematopathology at our institution or were cases sent in consultation to one of us (LJM).

Immunohistochemical Methods

Most cases had been analyzed immunohistochemically to determine cell lineage using antibodies specific for CD3, CD4, CD8, CD20, and CD43. All cases were of T-cell lineage, positive for T-cell antigens and negative for CD20 (Table 1).

For this study, each case was analyzed using immunohistochemical methods and monoclonal antibodies for expression of BAX (1:40, Zymed, South San Francisco, CA), BCL-2 (1:40), p53 (1:50) and Ki-67 (1:20, DAKO, Carpinteria, CA).

All tissue sections (3 to 4 μm thick) were deparaffinized in xylene and rehydrated in a graded series of ethanols as described previously (12). For all antibodies, heat-induced epitope retrieval was performed using a modification of a method described previously (12). Tissue sections were placed in plastic Coplin jars containing preheated target retrieval solution (DAKO), heated in a household vegetable steamer (Sunbeam-Oster, Model 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 (DAKO) at room temperature and included the following: blocking of endogenous peroxidase in 3% H_2O_2 in phosphate buffered saline (PBS), pH 7.4, for 5 minutes, blocking of nonspecific protein binding sites using protein blocking solution (DAKO) for 5 minutes, incubation with the primary antibody for 1 hour, and detection using secondary anti-mouse antibody and the streptavidin-biotin-peroxidase–based LSAB+ kit (DAKO) for 15 minutes. We used 3,3' diaminobenzidine (3,3'-diaminobenzidine)/H_2O_2 (Biogenex, San Ramon, CA) or 3-amino-9-ethyl carbazol (AEC) as the chromogen and hematoxylin as the counterstain.

Any cytoplasmic (BCL-2, BAX) or nuclear (P53, Ki-67) staining of neoplastic cells was considered positive, irrespective of intensity. Expression levels for all antibodies were determined by counting $\geq$500 neoplastic cells in each case. The results of CD8 and Ki-67 immunostaining were useful for localizing the neoplastic cells. Based on visual inspection of the distribution of the data, low or high expression was determined using the following cutoffs: BCL-2, $\geq$10%; BAX, $\geq$10%; P53, $\geq$10%; and Ki-67, $\geq$25%.

Modified TUNEL Assay

Formalin-fixed, paraffin-embedded tissue sections were mounted on coated slides, deparaffinized, rehydrated in a series of graded alcohols, and pretreated with proteinase K (20 μg/mL) for 25 minutes at 37° C. Slides were then incubated for 5 minutes in 3% H_2O_2 in PBS at pH 7.4, to block endogenous peroxidase activity. Terminal deoxynucleotide transferase (TdT; New England Biolabs, Beverly, MA), in 20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 0.25 mM CoCl2, and 24 μM biotin-

TABLE 1. Summary of Clinical, Immunophenotypical, and Molecular Findings in SPTCL Cases

Case No.	Age (y)	Sex	Anatomical Site	CD3	CD8	CD4	CD43	TCR-GR
1	20	F	Inguinal region and arm	+	+	−	+	+
2	37	F	Upper and lower extremities	ND	+	−	+	+
3	57	M	Thigh	+	+	−	+	+
4	25	F	Cheek mass	+	+	−	+	+
5	56	F	Upper and lower extremities	+	+	−	ND	+
6	30	M	Abdomen and axilla	ND	+	−	+	+
7	20	F	Abdomen	+	+	−	+	+
8	UK	F	Upper extremity	+	+	−	+	+
9	41	F	Chest	+	+	−	+	+
10	76	F	Abdomen	+	ND	ND	+	ND
11	40	M	Orbit	+	+	−	ND	ND
12	10	M	Upper and lower extremities	+	+	−	+	+
13	24	M	Neck	+	+	−	ND	+

SPTCL, subcutaneous panniculitis-like T-cell lymphoma; ND, not done; UK, unknown; TCR-GR, T-cell receptor gene rearrangement.

dATP (Life Technologies, Gaithersburg, MD) was applied subsequently (15 U per slide), for 1 hour, at 37° C. Terminal deoxynucleotide transferase catalyzes the addition of deoxynucleotides to the 3' hydroxy terminus of DNA molecules, including protruding, recessed, or blunt-ended double- or single-stranded DNA fragments. The TUNEL assay was modified by substituting dATP for dUTP, as described elsewhere (13). For the detection of labeled termini, streptavidin-biotin-HRP complex (LSAB+ kit) and substrate solution (both from DAKO) were used according to the manufacturer's instructions. The slides were counterstained with hematoxylin.

After quenching endogenous peroxidase activity, tissue sections of a cell pellet of K562 cells were incubated with a reaction mixture lacking TdT, which served as a negative control for each experiment.

At least 500 apoptotic nuclei and nuclear fragments were evaluated in each case. The nuclear fragments appeared as single rounded cells in a clear space or halo surrounded by contiguous cells. When more than one apoptotic fragment was seen per halo, these fragments were considered to be derived from the same cell and were counted as one. The ratio of apoptotic cells to the total number of the tumor cells within the same microscopic fields was reported as the AI. Based on the distribution of the data, ≥5% was used as a cutoff to distinguish between low and high AI.

Gene Rearrangement Analysis

Statistical Analysis

Statistical comparisons of expression of BCL-2, BCL-2 and P53, AI, and PR were performed using the Spearman rank correlation coefficient test. Fisher's exact test was also used for parametric correlation of BCL-2, BAX, P53, AI, and PR categorical variables.

Eleven cases were analyzed for T-cell receptor (TCR) gene rearrangements; all cases for TCRγ and one case for TCRβ and TCRγ. DNA was extracted from either paraffin-embedded or frozen tissue using standard methods. In four cases, the TCRγ gene was assessed by a polymerase chain reaction (PCR) assay using consensus Vγ and Jγ primers and analyzed by denaturing gradient gel electrophoresis (14). In seven cases, the TCRγ gene was assessed using a four-color PCR assay in combination with an automated post-PCR capillary electrophoresis method, as described elsewhere (15). In this assay, each variable-region (Vγ) primer is 5' end-labeled with a different fluorescent dye, allowing determination of the Vγ family involved in each TCRγ rearrangement.

In one case, the TCRβ gene was assessed using Southern blot hybridization. DNA was digested with the *Eco*RI, *Hin*dIII, and *Bam*HI restriction enzymes and analyzed using a cDNA probe specific for the constant region of the TCRβ.

RESULTS

Clinical and Histologic Features

Patient age ranged from 10 to 76 years (median, 37 y). There were eight females and five males. The most commonly involved sites were upper and lower extremities in six, trunk in four, and head and neck region in three. Seven patients had multiple skin lesions, and six patients presented with a solitary skin tumor.

In each case, the subcutaneous tissue was infiltrated by a mixture of small to medium-sized atypical lymphoid cells, small reactive lymphocytes, and histiocytes. A predominance of large atypical lymphoid cells was present in six cases. The atypical lymphoid cells infiltrated the adipose tissue in a panniculitis-like pattern, localized in the septae between adipocytes. The neoplastic cells also rimmed fat spaces (Fig. 1). The epidermis was not involved in any case. The deep dermis was involved in two cases. Karyorrhectic debris was prominent in nine cases (Fig. 1, inset). Karyorrhectic bodies were diffusely observed in all areas, predominantly adjacent to the necrotic zones. Geographic zones of coagulation necrosis were seen in nine cases. The neoplastic infiltrate was seen adjacent to the blood vessels and infiltrated the blood vessels in some cases. However, none of these neoplasms was primarily angiocentric.

Immunophenotypic and Molecular Results

The immunophenotype of these cases is summarized in Table 1. All 13 cases had a T-cell immunophenotype, positive for CD3 and/or CD43. In all 12 cases assessed, the neoplastic cells were positive for CD8 (Fig. 2) and negative for CD4.

The results of studies to assess clonality are summarized in Table 1. All 11 cases assessed had clonal TCRγ and TCRβ gene rearrangements: all 11 had TCR γ, and 1 case had TCR β. Seven cases analyzed for specific Vγ family usage showed the following findings: four cases had a rearrangement that used VγII, one case had rearrangements that utilized VγII and VγIV, one case had three rearrangements that used VγI, VγII and VγIV, and one case had a rearrangement that used Vγ IV.

Apoptotic Index and Proliferation Rate

The AI in these cases ranged from 1.9 to 18.4% (median = 8.1; Table 2). Using a cutoff of ≥5%, 11 cases had a high AI and 2 cases had a low AI.

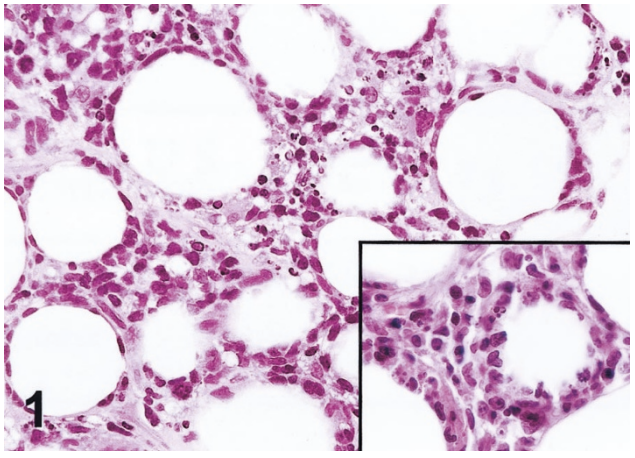

FIGURE 1. Mixed small and large atypical lymphocytes rimming fat spaces (200×) (***inset:*** numerous apoptotic cells and fragments; 400× both hematoxylin and eosin).

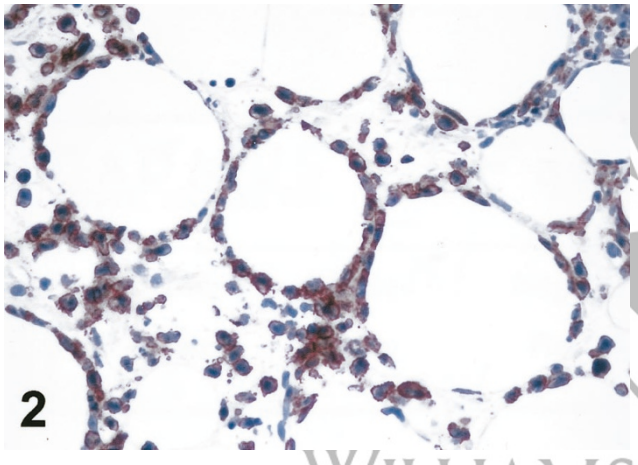

FIGURE 2. The neoplastic cells are positive for CD8 (immunoperoxidase staining, 400×)

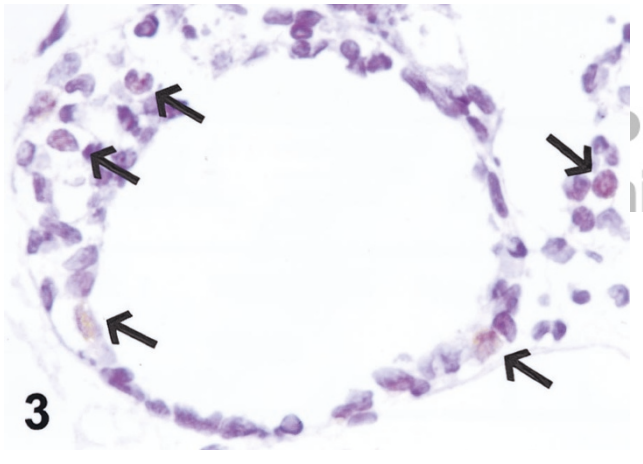

FIGURE 3. Rare reactive small lymphocytes expressing BCL-2 (***Arrows;*** immunoperoxidase staining, 1000×)

The PR (Ki-67 labeling index) ranged from 10 to 57.2% (median = 17.9; Table 2; Fig. 6). Using a cutoff value of ≥25%, 5 cases had high PR and 8 cases had low PR.

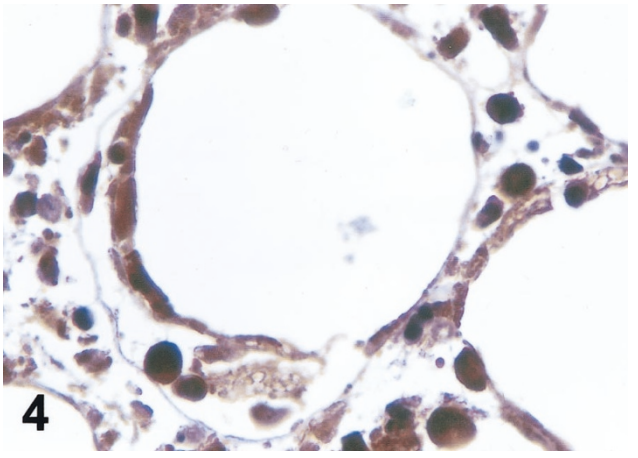

FIGURE 4. Strong intracytoplasmic expression of BAX in the cells rimming the fat spaces (immunoperoxidase staining, 1000×)

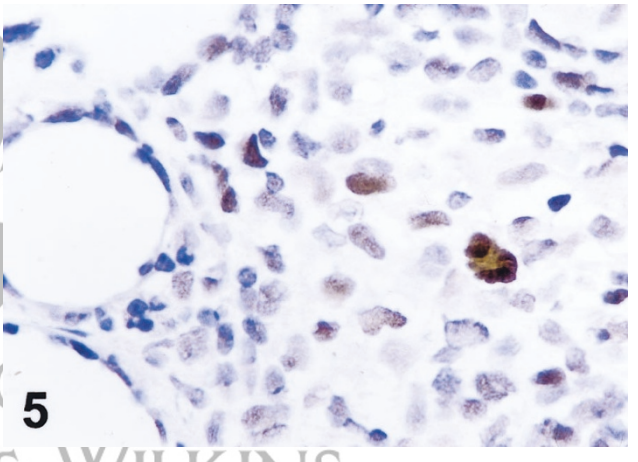

FIGURE 5. Strong P53 expression in the large neoplastic cells in comparison to weak staining in small lymphocytes (immunoperoxidase staining, 1000×)

BCL-2, BAX, and P53 Expression

Tumor cells in all 13 cases expressed cytoplasmic BCL-2 (Fig. 3) and BAX (Fig. 4). The expression of BCL-2 ranged from 2 to 15% positive cells (median, 2.8; Table 2). Using a cutoff of ≥10%, 12 cases had low and 1 case had high BCL-2 expression. BAX expression ranged from 5.3 to 90% positive cells (median, 35.0; Table 2). Using a cutoff of ≥10%, 12 cases had high BAX expression, and 1 case had low BAX expression.

P53 expression was assessed in 10 cases. The expression level of p53 ranged from 0.6 to 28.0% positive cells (median, 9.9). Using a cutoff of ≥ 10%, five cases had high and five cases had low p53 expression. Staining intensity for p53 protein showed significant variability between individual cases. Large neoplastic cells found in six cases in variable numbers were all strongly positive for p53 (Fig. 5).

TABLE 2. Summary of Apoptotic Index, Proliferation Rate, and Expression Levels of BCL-2, BAX, and p53 in SPTCL Cases

Case No.	Bcl-2 (%)	Bax (%)	AI (%)	PR (%)	P53 (%)
1	2.8	19.4	5.3	21.6	9.9
2	3.0	16.3	2.3	34.5	14.8
3	2.0	30.0	15.2	35.5	4.4
4	2.6	5.3	9.8	11.3	ND
5	2.3	35.0	9.4	36.0	25.1
6	6.0	59.6	10.0	17.9	11.3
7	2.6	36.6	18.4	10.0	0.6
8	2.8	54.5	8.0	15.5	19.5
9	5.0	50.0	16.8	10.4	0.6
10	6.0	15.0	1.9	57.2	ND
11	3.5	21.8	8.1	44.1	1.0
12	15.0	78.0	13.8	15.0	28.0
13	2.0	90.0	6.0	10.0	ND

SPTCL, subcutaneous panniculitis-like T-cell lymphoma; ND, not done.

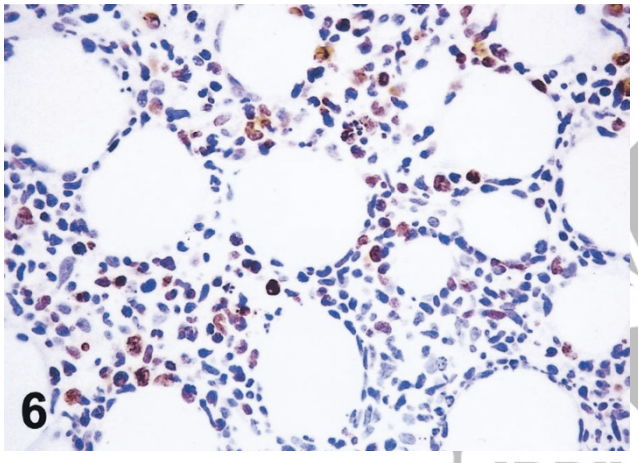

FIGURE 6. Nuclear staining with Ki-67 in virtually all neoplastic cells (immunoperoxidase staining, 200×)

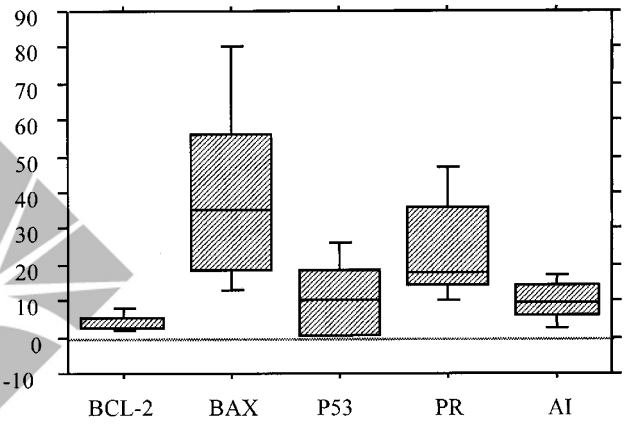

FIGURE 7. Box plot summarizing the distribution of ranges of BCL-2, BAX, P53 expression, proliferation rate (PR), and apoptotic index (AI).

Correlation of BCL-2, BAX, and P53 with the Apoptotic Index and Proliferation Rate

Box plots demonstrating the distribution of BCL-2, BAX, P53, AI, and PR are shown in Figure 7. A statistically significant inverse correlation between AI and PR ($r = -.58$, $P = .04$) was noted. No correlation between other variables was found using Spearman's correlation coefficient.

Using cutoffs chosen before statistical analysis, we used Fisher's exact test and found additional statistically significant correlations. BCL-2 and BAX expression were inversely correlated ($P < .0001$). Twelve cases with low BCL-2 expression showed high levels of BAX expression. BCL-2 expression also inversely correlated with AI ($P < .001$). Eleven cases with low BCL-2 expression had a high AI.

Apoptotic index also inversely correlated with PR ($P = .04$). Eight cases with high AI had low PR. Two cases with low AI had high PR and three cases had both high AI and PR. No other statistically significant correlations were identified.

DISCUSSION

Tumor growth depends on a balance between cell death via apoptosis and cell proliferation (16). Based on the frequent single cell necrosis seen in SPTCL, we and others hypothesized that increased susceptibility to apoptosis may be a common feature of this tumor type (17). Here, we assessed 13 cases of SPTCL for expression of apoptotic proteins and also determined AI and PR.

We found that SPTCL cases have a variable but generally high AI. In our study, the median AI was 8.1%, with a range up to 18.4%. This is significantly higher than has been reported in many other types of non-Hodgkin's lymphoma, as seen in Table 3 (12, 18–28). For example, nodal peripheral T-cell lymphomas are reported to have an AI that ranges from 0.3 to 1.8%, depending on histologic type. In general, peripheral T-cell lymphomas of large cell type have higher AI than peripheral T-cell lymphomas of small cell type (20, 27). Various types of small cell and large cell B-cell lymphomas of different types also show a much lower median or mean AI than in SPTCL. Low-grade tumors such as B-CLL and follicular lymphomas have an AI in the range of 0.09 to 0.2% (18, 19) and 0.05 to 0.76%, respectively (18, 19, 21). High-grade lymphomas such as diffuse large B-cell lymphoma and Burkitt lymphoma are reported to have an AI of 0.83 to 1.68% and 1.72%, respectively (1, 20, 24, 25).

TABLE 3. Summary of Apoptotic Index (AI) and Proliferation Rate (PR) in Various Types of B- and T-Cell Non-Hodgkin's Lymphomas

Histologic Type	AI (%)	PR (%)
B-Cell		
B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (18, 20)	0.09–0.2	8.89
Follicular lymphoma (18)		
Grade I	0.12	14.5
Grade II	0.47	15.4
Follicular lymphoma (NOS) (19, 21)	0.05–0.76	19
Low-grade B-cell MALT lymphoma (stomach) (22, 23)	1.1–5.3	9.1
Lymphoplasmacytoid lymphoma (20, 24)	0.49	12.5
Mantle cell lymphoma (20, 24)	0.10	17
Diffuse large B-cell lymphoma (20, 24, 25)	0.43–1.68	50–54.3
Burkitt lymphoma (1, 20)	1.72	>99
T-cell–rich B-cell lymphoma (26)	2.2	13.0
T-Cell		
Precursor T-lymphoblastic lymphoma (26)	6.8	43.4
Angioimmunoblastic T-cell lymphoma (27)	0.8	20.2
Peripheral T-cell lymphoma (NOS) (18, 20, 24, 26–28)	0.3–1.81	31.5–70
Lennert's lymphoma (26)	2.1	11.4
Anaplastic large cell lymphoma (12)		
ALK+	3.4	71.0
ALK−	1.1	67.5

MALT, mucosa-associated lymphoid tissue; NOS, not otherwise specified; ALK, anaplastic lymphoma kinase.

The low BCL-2 expression seen in SPTCL was strongly correlated with high BAX expression ($P < .001$). Moreover, BCL-2 expression ($P < .001$) correlated inversely with AI. In contrast, although BAX protein was highly expressed in SPTCL it did not correlate with AI, nor did BAX/BCL-2 ratio correlate with high AI. This would support the notion that low BCL-2 levels are the more important factor in explaining the high apoptotic rate of SPTCL. These results are in accord with those of others who have reported that BCL-2 expression correlates inversely with AI in various types of non-Hodgkin's lymphomas (29–31).

The PR in SPTCL cases in this study was variable, ranging from 10.0 to 57.2%, with a median of 17.9%. This rate is lower than that reported in other types of peripheral T-cell lymphoma. In one study, Chott and colleagues (28) assessed PR in a series of nodal peripheral T-cell lymphomas. The PR in the tumors in their series ranged from 30 to 70%. In general, high-grade lymphomas show high PR, with the highest rates observed in Burkitt's lymphoma, >95%, and diffuse large B-cell lymphoma and anaplastic large cell lymphoma, in the range of 55 to 71% (12). Others have shown that the PR of non-Hodgkin's lymphomas correlates well with AI. Leoncini *et al.* (30), for example, showed that the PR correlated with AI in a number of non-Hodgkin's lymphomas of T-cell and B-cell lineage. However, SPTCL appears to be an exception to this rule; we observed an inverse correlation between AI and the PR.

The tumor suppressor protein P53 is another important regulator of apoptosis. P53 has been shown to be a sequence specific activator of BAX and can down-regulate BCL-2 and up-regulate BAX expression in the murine leukemia cell line M1 (9). Mice deficient in P53 show increased BCL-2 and decreased BAX protein levels. We noted increased p53 expression in the large cells of six cases of SPTCL. It is likely that tumor cells showing strong p53 expression have mutant P53 protein, as wild-type p53 has a short half-life and is not usually detected using immunohistochemical methods. It is possible that dysregulated P53 may interfere with apoptosis, but we noted no correlation between P53 and either BAX or BCL-2 expression. As with other T-cell lymphomas, dysregulated P53 expression may be a late occurrence in SPTCL that correlates with histological transformation (32).

Although not the focus of this study, we showed that all 11 cases of SPTCL assessed had clonal TCRγ gene rearrangements. In seven cases, the TCRγ gene was assessed using a recently described PCR assay that allows one to determine the Vγ gene family used in rearrangement (15). The TCRγ gene is relatively simple, with 11 functional variable region segments that can be segregated into four homologous families (33). Our results in seven cases suggest that the VγII family is commonly used, as six of seven SPTCL had TCRγ rearrangements that used VγII. Others have assessed the TCRδ gene in SPTCL and suggested preferential use of the VδII gene (34).

One technical problem that we encountered in assessing BAX and BCL-2 expression in SPTCL was the difficulty in distinguishing the neoplastic population from admixed reactive lymphocytes. The morphology of SPTCL is remarkable for its heterogeneous mixture of reactive lymphocytes, histiocytes, and reactive stromal cells that can obscure the neoplastic cell population. The cytologic atypia of the neoplastic cells is variable and sometimes minimal. In analysis of BCL-2 and BAX expression in routinely stained tissue sections, distinguishing of neoplastic cells from reactive lymphocytes can be difficult. We found the results of the CD8 immunostain useful in highlighting the areas most suitable for assessing expression of apoptotic proteins and apoptotic index. Alternatively, the Ki-67 immunostain is also useful for this purpose. However, reactive lymphocytes may also be CD8 positive and in the G1-S phase of the cell cycle. Thus, both the CD8 and Ki-67 immunostains overestimate the number of neoplastic cells.

In summary, the results of this study suggest that SPTCL cases have a high AI and that the suscepti-

bility to apoptosis may be explained by differential expression of BCL-2 and BAX in the neoplastic cells.

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