

# Prognostic Significance of Apoptosis in Synovial Sarcoma: Correlation with Clinicopathologic Parameters, Cell Proliferative Activity, and Expression of Apoptosis-Related Proteins

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**bcl-2 overexpression in synovial sarcomas has been recently reported. Although it is widely known that bcl-2 suppresses apoptosis in various cells, there are no studies that have examined the significance of apoptosis in synovial sarcoma. In the present study, we visualized apoptotic tumor cells by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end-labeling (TUNEL) method in 49 cases of primary synovial sarcoma. The degree of apoptosis was analyzed in relation to several clinicopathologic parameters, cell proliferative activity, and immunohistochemical expression of apoptosis-related proteins, including bcl-2, bax, bcl-x, bak, p53, p21 (WAF1/CIP1), Fas, and Fas ligand. TUNEL index (TUNEL-I) significantly correlated with the mitotic index (MI) ( $\bar{n} = 0.60$ ,  $P < .0001$ ) and Ki-67 labeling index (MIB1-I) ( $\bar{n} = 0.52$ ,  $P = 0.0005$ ). There was a highly significant association between high TUNEL-I value ( $>.8\%$ ) and poor prognosis (log-rank test;  $P < .0001$ ). Many synovial sarcomas were diffusely positive for bcl-2 family proteins (bcl-2, bax, bcl-x, and bak) and were negative or only sporadically positive for Fas, Fas ligand, p53, and p21 (WAF1/CIP1) proteins. The results indicated that increased rate of apoptosis in primary synovial sarcoma was considered to be an indicator of poor prognosis. In addition, apoptosis in synovial sarcoma may be controlled by multiple**

**apoptosis-regulating mechanisms, including the bcl-2 family.**

**KEY WORDS: Apoptosis, bcl-2 family, Fas-Fas ligand, p53, Prognosis, Synovial sarcoma, TUNEL.**  
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Synovial sarcoma is a soft tissue sarcoma of unknown histogenesis, occurring most frequently in adolescents and young adults. It is classified into three subtypes: the biphasic type composed of both epithelial and spindle-cell components, the monophasic type composed of either epithelial or spindle-cell component, and the poorly differentiated type (1). Most synovial sarcomas are characterized by nonrandom chromosomal translocation, t(18;X)(p11.2;q11.2) (2). Reverse transcription-polymerase chain reaction (RT-PCR) for chimeric SYT-SSX fusion transcript originating from the specific translocation is now regarded as a diagnostic tool of this tumor (2). A number of investigators regard synovial sarcoma as "soft tissue carcinosarcoma" because it shares morphologic and immunologic properties of the epithelium as well as mesenchyme (3). The clinical behavior of synovial sarcoma has been described to correlate with some morphologic and biologic parameters (e.g., histologic subtype, percentage glandularity, tumor necrosis, and cell proliferative activity determined by mitotic rate or immunohistochemical staining of proliferating cell nuclear antigen) (4, 5).

Recent studies have shown overexpression of bcl-2 protein in synovial sarcomas (6-8). bcl-2 proto-oncogene was initially described in B-cell lymphomas (9), and the bcl-2 protein is known to

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prolong the survival of a variety of cells by blocking apoptosis (10). Apoptosis is a highly conserved, genetically mediated process of selective cell deletion that occurs during embryogenesis and in normal tissue homeostasis as well as in various diseases (11). Apoptosis is also a fundamental process in tumor cell kinetics, and susceptibility to apoptosis in untreated malignant tumors varies with tumor type (12). Although the precise mechanisms that regulate apoptosis of tumor cells are not completely understood, there are numerous endogenous and exogenous initiating factors (*e.g.*, growth factor deprivation, Fas and its ligand, viral infection, irradiation, cytotoxic chemotherapy) (11–13). In addition, apoptosis is controlled by a number of proto-oncogenes and tumor suppressor genes, including *bcl-2* (and its related genes), *p53*, *Rb*, and *c-myc* (11–14).

Because apoptosis in malignant tumors may account for spontaneous cell loss, its potential prognostic value and therapeutic application have been of great interest in recent years. In the present study, we evaluated the extent of apoptosis in primary synovial sarcomas with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end-labeling (TUNEL) method (15). The extent of apoptosis was analyzed in correlation with several clinicopathologic parameters, extent of cell proliferation, and immunohistochemical expression of various apoptosis-regulating factors (*e.g.*, *bcl-2* family (16), *p53* (13, 17), *p21*(WAF1/CIP1) (18), Fas (19), and Fas ligand (20) proteins). The aims of this study were (1) to assess the extent of apoptosis in primary synovial sarcomas; (2) to clarify the relationship among apoptosis, cell proliferative activity, and expression of apoptosis-related proteins; and (3) to elucidate the significance of apoptosis in the prognosis of synovial sarcoma.

## MATERIALS AND METHODS

Cases of primary synovial sarcoma, which had been procured from 1963 to 1996, were selected from three sources: medical records in the Second Department of Pathology Laboratory, Kyushu University; Department of Pathology, National Kyushu Medical Center; and Department of Pathology, National Kyushu Cancer Center. Formalin-fixed, paraffin-embedded tissue blocks were available for 49 patients. All patients had not had preoperative chemotherapy or radiotherapy at the time of initial surgery. All tumors were staged according to the American Joint Committee on Cancer (AJCC) staging of soft tissue sarcomas (21).

Synovial sarcomas were diagnosed using hematoxylin and eosin (H&E), periodic acid-Schiff with

and without diastase digestion, colloidal iron for mucin with and without hyaluronidase digestion, silver impregnation for reticulin, and/or immunohistochemical staining of cytokeratin and vimentin. RT-PCR for SYT-SSX fusion transcript was used for diagnostic confirmation in four tumors, including one from which a previously reported synovial sarcoma cell line, SN-SY-1, was established (22). All microscopic slides were reexamined to confirm the diagnosis, and the most representative one or two paraffin-embedded tissue blocks were used for subsequent studies.

### Histologic Study and Assessment of Mitosis

Necrosis was categorized as absent (“not”) and present (“any”) by examining H&E sections of each synovial sarcoma. Mitotic nuclei were counted on each representative H&E section. The mitotic index (MI) was determined by counting the number of mitoses in four separate sets of 10 high-power fields ( $\times 400$ ) and selection of the highest counts.

### TUNEL and Assessment of Apoptosis

Visualization of DNA fragmentation, a marker of apoptosis, was performed by the TUNEL method using the *In Situ* Apoptosis Detection Kit (Takara Biomedicals, Kusatsu, Japan) according to the instructions provided by the manufacturer. Briefly, formalin-fixed, paraffin-embedded synovial sarcoma tissue sections were dewaxed and rehydrated, and nucleic acids were stripped from proteins by treatment with 15  $\mu\text{g}/\text{mL}$  proteinase K (Sigma, St. Louis, MO) in 0.01 mol/L phosphate-buffered saline (PBS), pH 7.4, for 15 min at room temperature. Sections were reacted with terminal deoxynucleotidyl transferase (TdT; Takara Biomedicals) and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate (dUTP; Takara Biomedicals) for 60 min at 37° C in a humid chamber. Sections were then incubated with horseradish peroxidase (HRP)-conjugated murine anti-FITC antibody (Takara Biomedicals) for 30 min at 37° C. End labeling of fragmented DNA was visualized with hydrogen peroxide containing 3,3'-diaminobenzidine tetrahydrochloride (DAB)/PBS. Paraffin-embedded sections of normal human lymph nodes and gastric mucosa were used as positive controls. The presence of a clear nuclear staining was indicative of apoptotic cells.

In each section, at least 5000 synovial sarcoma cells were examined in the most evenly and distinctly labeled areas at high-power field ( $\times 400$ ), and the number of TUNEL-positive tumor cells was counted. In counting TUNEL-positive synovial sarcoma cells, viable tumor areas just around necrosis were avoided. The TUNEL index (TUNEL-I) was

calculated as percentage of TUNEL-positive tumor cells per examined tumor cells. Assessment of TUNEL-I was performed independently by three authors (SK, TF, YO), and the validity was statistically confirmed by the Friedman test using the StatView 4.02 software (Abacus Concepts, Inc., Berkeley, CA). TUNEL-I data assessed by the first author (SK) was used in the following study.

### Ki-67 Immunostaining and Assessment

Four- $\mu$ m-thick synovial sarcoma tissue sections were cut from formalin-fixed, paraffin-embedded tissue blocks. They were dewaxed in xylene, rehydrated through graded ethanol solutions, rinsed in PBS for 5 min, and then immersed in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. For antigen retrieval, sections were then microwaved in 0.01 mol/L sodium citrate-buffered saline (CBS), pH 6.0, for 30 min at 95° C using a Microwave Processor, model H2800 (Energy Beam Science, Inc., Washington, DC). After slides were rinsed in PBS for 5 min, they were blocked with a solution of 10% normal rabbit serum in PBS at room temperature for 10 min and incubated at 4° C overnight with the murine monoclonal anti-Ki-67 antibody (MIB1, Immunotech, Marseille, France) at a dilution of 1:100 in 1.0% bovine serum albumin (BSA)/PBS. Subsequent reaction was made by the streptavidin-biotin complex/horseradish peroxidase (SAB/HRP) method using a HISTOFINE SAB-PO (M) Immunohistochemical Staining Kit (Nichirei, Tokyo, Japan) according to the protocol provided by the manufacturer. Positive reactions were visualized with hydrogen peroxide containing DAB/PBS.

Positively stained tumor cell nuclei were counted in the most evenly and distinctly labeled areas. The Ki-67 labeling index (MIB1-I) was expressed as the percentage of positively stained nuclei based on the count of at least 2000 tumor cells.

### Immunostaining for Apoptosis-Related Proteins

Immunohistochemical stainings were performed with primary antibodies listed in Table 1. For antigen retrieval of bcl-2, bax, Fas, and Fas ligand proteins, dewaxed tumor tissue sections were incubated in 0.1% trypsin (Sigma)/PBS for 15 min (for bcl-2, Fas, and Fas ligand proteins) and 30 min (for bax protein) at 37° C. For antigen retrieval of bcl-x, bak, p53, and p21(WAF1/CIP1) proteins, dewaxed tumor tissue sections were microwaved in CBS for 30 min at 100° C using a H2800 Microwave Processor. The procedure of immunohistochemical staining was similar to that described in Ki-67 (MIB1) immunohistochemical staining, using a HISTOFINE SAB-PO (M) (for mouse primary antibodies),

**TABLE 1. Primary Antibodies**

Antigen	Source	Dilution	Clonality
bcl-2	DAKO, Glostrup, Denmark	1:100	M (124)
bax	Santa Cruz Biotechnology, Santa Cruz, CA	1:200	R (P-19)
bcl-x <sup>a</sup>	Santa Cruz Biotechnology	1:200	R (S-18)
bak	Santa Cruz Biotechnology	1:200	G (N-20)
Fas	Santa Cruz Biotechnology	1:100	R (C-20)
Fas ligand	Santa Cruz Biotechnology	1:100	R (N-20)
p53	Oncogene Science, Uniondale, NY	1:100	M (1801)
p21 (WAF1/CIP1)	Oncogene Science	1:100	M (EA-10)

M, murine monoclonal; R, rabbit polyclonal; G, goat polyclonal.

<sup>a</sup> Reactive for both bcl-xS protein (small molecular weight product) and bcl-xL protein (large molecular weight product).

SAB-PO (R) (for rabbit primary antibodies) or SAB-PO (G) (for goat primary antibody) Immunohistochemical Staining Kit (Nichirei). Positive reactions were visualized with hydrogen peroxide containing DAB/PBS.

The expression of apoptosis-related proteins was semiquantitatively evaluated as negative (–; no positively stained tumor cells were present), low expression (+; only a few [ $<10\%$ ] positively stained tumor cells were present), moderate expression (++), or extensive expression (+++; numerous [ $>50\%$ ] positively stained tumor cells were present).

### Statistical Analysis

Results were analyzed using the StatView 4.02 software. Correlation between TUNEL-I and categorized data of age of patients; gender; necrosis; tumor size; and expression of p53, p21 (WAF1/CIP1), bcl-x, or bak protein was analyzed by the Mann-Whitney *U* test. Correlation between TUNEL-I and histologic type, categorized expression of bcl-2 or bax protein, was analyzed by the Kruskal-Wallis test. Correlations among TUNEL-I, MI, and MIB1-I were examined by the Spearman rank correlation test and Mann-Whitney *U* test. Survival curves were constructed by the Kaplan-Meier method and compared by the log-rank test. Cox proportional hazards regression analysis with stepwise selection of variables was used to assess the relative importance of different factors on survival. A two-tailed *P* value of less than 0.05 was considered significant.

### Double Fluorescence Labeling with TUNEL and bcl-2 Immunohistochemistry

Double fluorescence labeling with TUNEL and bcl-2 immunohistochemistry was performed using formalin-fixed, paraffin-embedded tissues of five synovial sarcomas. After dewaxing and rehydration, sections were treated with 15  $\mu$ g/mL proteinase K for 5 to 7 min at room temperature. After sections were

washed with PBS, they were incubated with a murine monoclonal antibody to bcl-2 protein (124; DAKO, Glostrup, Denmark) at a dilution of 1:40 for 60 min at room temperature and detected with a tetramethylrhodamine isothiocyanate (rhodamine)-conjugated rabbit antimouse IgG antibody (DAKO) diluted at 1:40. After sections were washed with PBS, they were then incubated in a humid chamber with a solution containing TdT and FITC-dUTP for 60 min at 37° C. After sections were washed with PBS and mounted with Antifade (Oncor, Gaithersburg, MD), they were examined under an Axiophot Fluorescence Microscope (Carl Zeiss Japan, Tokyo, Japan).

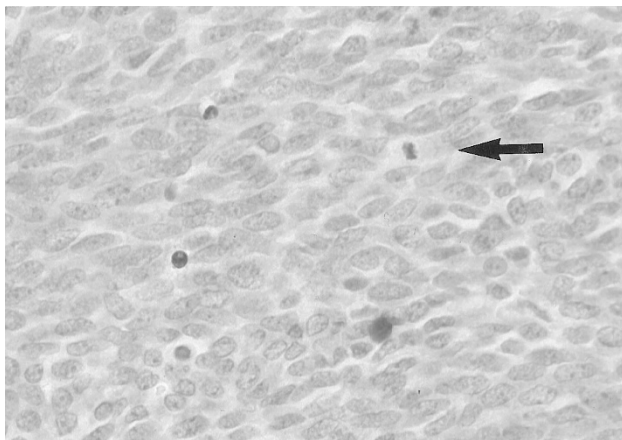
## RESULTS

### Clinicopathologic Features

Patients included 23 male and 26 females, with a mean age of 39.6 years (range, 9 to 70 years). Primary tumors were located in the lower extremities in 35 patients, upper extremities in 12 patients, and the trunk in 2 patients. Staging of the primary tumors was as follows: Stage I, 18 patients; Stage II, 25 patients; and Stage III, 6 patients. The length of follow-up period was calculated from the date of initial surgical treatment and ranged from 2 to 229 months (mean, 60.9 months). Histologically, 14 tumors were of the biphasic type, 34 were of the monophasic fibrous type, and 1 was a poorly differentiated tumor. At the conclusion of this study, 20 patients were still alive and 29 had died of the disease.

### Apoptosis

TUNEL-positive synovial sarcoma cells, many showing homogeneously stained condensed nuclei, were found scattered among viable tumor cells (Fig. 1). In general, the nuclear labeling of TUNEL reac-



**FIGURE 1.** Apoptotic tumor cells in monophasic fibrous synovial sarcoma (TUNEL-I = 1.78). Note the tumor cells with TUNEL-positive nuclei. Note the mitotic tumor cell without nuclear labeling by TUNEL (*small arrow*).

tion was distinct, making it easy to identify reactive nuclei. On occasion, TUNEL-positive tumor cells were more frequently observed in viable tumor areas around necrosis. In a small number of well-differentiated biphasic synovial sarcomas, TUNEL-positive tumor cells were more frequently observed in epithelial glandular areas than in fibrous areas.

TUNEL-I ranged from 0.1 to 2.7% (mean, 0.84%). In categorized data analysis to examine the correlation between TUNEL-I and clinicopathologic parameters, TUNEL-I of the large tumor group (>8 cm) was significantly higher than that of the small tumor group ( $\leq 8$  cm;  $P = .01$ ; Table 2).

Univariate survival analysis by the Kaplan-Meier method and log-rank test using a TUNEL-I cutoff value of 0.8% showed that the survival rate of patients with low ( $\leq 0.8\%$ ) TUNEL-I values was significantly higher than that with high ( $> 0.8\%$ ) TUNEL-I values ( $P < .0001$ ; Fig. 2). In survival analysis, we tentatively defined 0.6, 0.7, 0.8, 0.9, or 1.0% as a cutoff for high *versus* low TUNEL-I value; 0.8% had the most significant prognostic effect on survival and finally was selected as the cutoff value.

### Cell Proliferation

Cell proliferative activity of synovial sarcomas as determined by MIB1-I and MI demonstrated a considerable variability among cases. Immunohistochemical staining for Ki-67 antigen using MIB1 antibody was usually distinct in the nuclei of synovial sarcoma cells, making identification of Ki-67-positive tumor cells very easy (Fig. 3). In biphasic synovial sarcomas, the extent of Ki-67-positive tumor cells was similar in both epithelial glandular and spindle-cell fibrous areas.

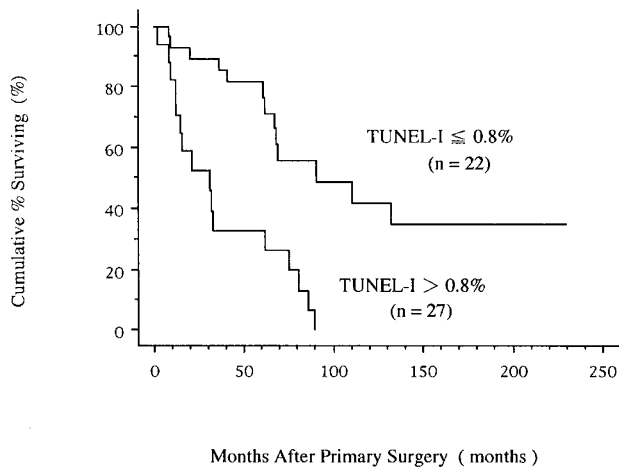
MIB1-I ranged from 0.2 to 23.8% (mean, 7.12%), and MI ranged from 1 to 25 (mean, 8.63). There was

**TABLE 2. Correlation Between Apoptosis (TUNEL-I) and Clinicopathologic Parameters**

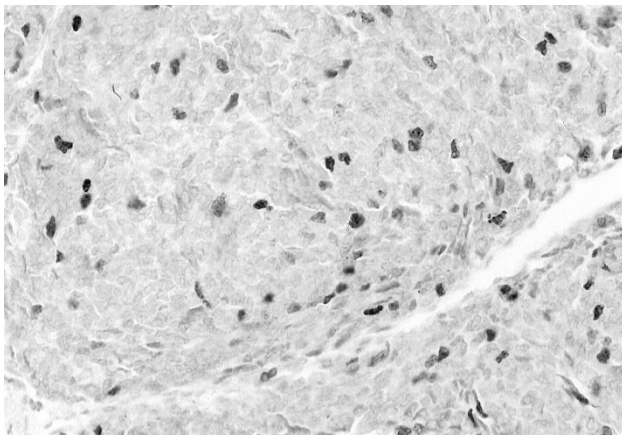
Clinicopathologic Parameters	n	Mean TUNEL-I $\pm$ SE (%)	P Value <sup>a</sup>
Age (y)			
$\leq 40$	22	0.614 $\pm$ 0.126	0.09
$> 40$	27	1.058 $\pm$ 0.162	
Gender			
Male	23	0.924 $\pm$ 0.170	0.52
Female	26	0.780 $\pm$ 0.140	
Histologic subtype			
Biphasic	14	0.846 $\pm$ 0.212	0.86
Monophasic fibrous	34	1.170 $\pm$ 0.362	
Poorly differentiated	1		
Necrosis			
Not	34	0.709 $\pm$ 0.118	0.074
Any	15	1.185 $\pm$ 0.217	
Tumor size			
$\leq 8$ cm	25	0.596 $\pm$ 0.119	0.01
$> 8$ cm	20	1.189 $\pm$ 0.186	

TUNEL-I, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end-labeling index; SE, Standard error of the mean.

<sup>a</sup> Mann-Whitney *U* test.



**FIGURE 2.** Univariate survival analysis with TUNEL-I cutoff value of 0.8% (Kaplan-Meier method and log-rank test;  $P < .0001$ ).



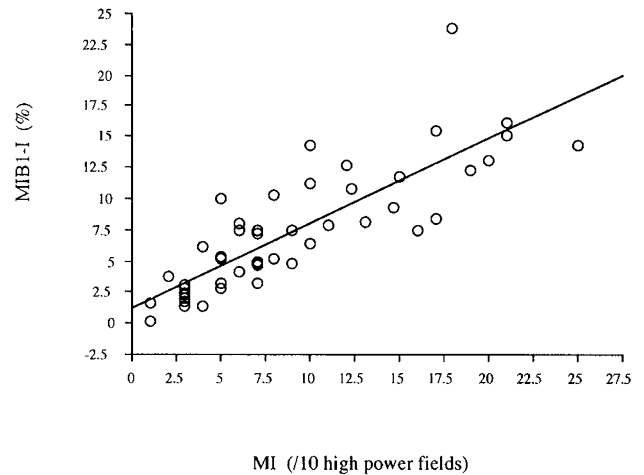
**FIGURE 3.** Ki-67 immunohistochemical staining of monophasic fibrous synovial sarcoma: Ki-67 immunostaining with MIB-1 antibody demonstrates distinct nuclear labeling of proliferating tumor cells.

a significant linear correlation between MI and MIB1-I (Spearman rank correlation test,  $\tilde{r} = .88$ ;  $P < .0001$ ; Fig. 4).

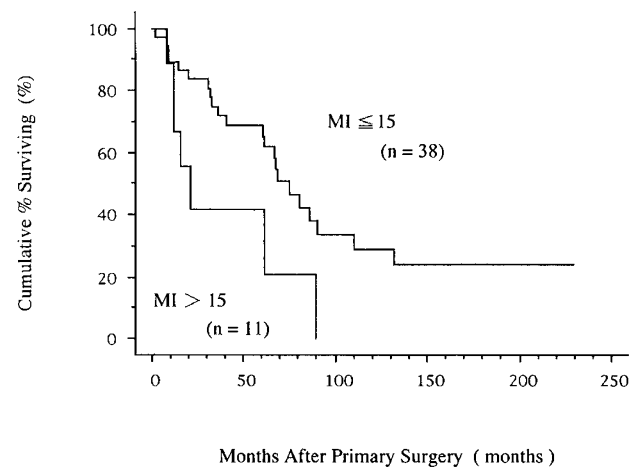
Univariate survival analysis using an MI cutoff value of 15 demonstrated that survival rate of patients with synovial sarcoma of low MI value ( $\leq 15$ ) was significantly higher than that of high MI value ( $> 15$ ) (log-rank test;  $P = .023$ ; Fig. 5). Univariate survival analysis using an MIB1-I cutoff value of 11% also showed that survival rate of patients with synovial sarcoma of low MIB1-I value ( $\leq 11\%$ ) was significantly higher than that of high MIB1-I value ( $> 11\%$ ; log-rank test;  $P = .0095$ ; Fig. 6).

#### Apoptosis and Cell Proliferation

With regard to apoptosis and cell proliferation, there were significant linear correlations between TUNEL-I and MI (Spearman rank correlation test,  $\tilde{r} = .60$ ;  $P < .0001$ ; Fig. 7) and between TUNEL-I



**FIGURE 4.** Correlation between mitotic index (MI) and MIB1-I (Ki-67 labeling index) (Spearman rank correlation test,  $\tilde{r} = .88$ ;  $P < .0001$ ).



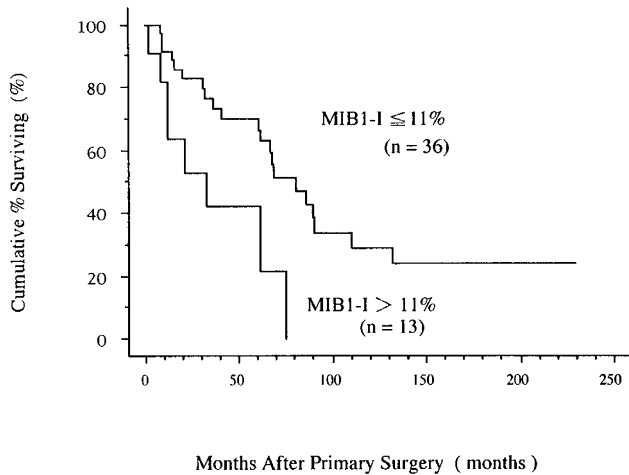
**FIGURE 5.** Univariate survival analysis with mitotic index (MI) cutoff value of 15 (Kaplan-Meier method and log-rank test,  $P = .023$ ).

and MIB1-I (Spearman rank correlation test,  $\tilde{r} = .52$ ;  $P = .0005$ ; Fig. 8).

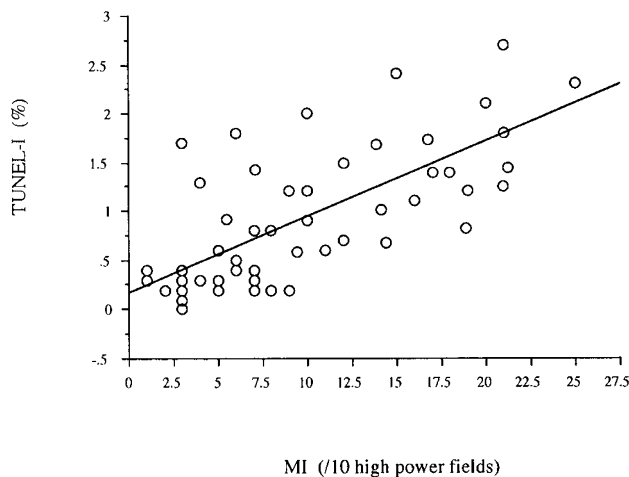
Mann-Whitney  $U$  test showed a significant difference of TUNEL-I values between the synovial sarcoma group of low MI value ( $\leq 15$ ) and that of high MI value ( $> 15$ ) ( $P = .0007$ ; Table 3). Mann-Whitney  $U$  test also exhibited a significant difference of TUNEL-I values between synovial sarcoma group of low MIB1-I value ( $\leq 11\%$ ) and that of high MIB1-I value ( $> 11\%$ ;  $P = .0057$ ; Table 3).

#### Apoptosis-Related Proteins

Immunohistochemically, bcl-2, bax, bcl-x, and bak proteins were positive in the majority of examined synovial sarcomas and showed diffuse perinuclear and/or cytoplasmic staining (Fig. 9). A proportion of biphasic synovial sarcomas exhibited a topographic immunostaining pattern for bcl-2 (Fig. 10) and bax proteins, and their intense staining was observed predominantly in fibroblastic spindle-cell



**FIGURE 6.** Univariate survival analysis with MIB1-I (Ki-67 labeling index) cutoff value of 11% (Kaplan-Meier method and log-rank test,  $P = .0095$ ).



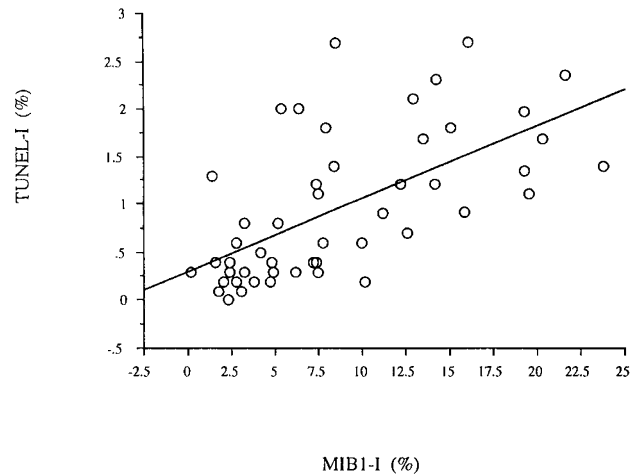
**FIGURE 7.** Correlation between TUNEL-I and mitotic index (MI) (Spearman rank correlation test,  $\bar{r} = .60$ ;  $P < .0001$ ).

areas. p53 and p21 (WAF1/CIP1) proteins were negative or sporadically positive in the nuclei of spindle-shaped fibroblastic or epithelial glandular tumor cells of a small number of monophasic and biphasic synovial sarcomas (Fig. 11). Both Fas and Fas ligand proteins were immunohistochemically negative in most synovial sarcomas and were sporadically positive in infiltrating inflammatory cells.

Synovial sarcoma groups that were categorized by the extent of immunohistochemical staining of bcl-2, bax, bcl-x, bak, p53, and p21 (WAF1/CIP1) proteins failed to show any statistical differences of the TUNEL-I values (Table 4) and had no significant prognostic effect on survival (data not shown).

#### Multivariate Survival Analysis

In addition to MI, MIB1-I, and TUNEL-I, univariate survival analysis by the Kaplan-Meier method and log-rank test revealed that advanced AJCC



**FIGURE 8.** Correlation between TUNEL-I and MIB1-I (Ki-67 labeling index) (Spearman rank correlation test,  $\bar{r} = .52$ ;  $P = .0005$ ).

**TABLE 3. Correlation Between Apoptosis (TUNEL-I) and Cell Proliferation**

Cell Proliferation Index	Mean TUNEL-I $\pm$ SE (%)	P Value <sup>a</sup>
MI (per 10 HPF)		
$\leq 15$ (n=38)	0.617 $\pm$ 0.092	0.0007
$> 15$ (n=11)	1.660 $\pm$ 0.237	
MIB1-I (%)		
$\leq 10$ (n=36)	0.638 $\pm$ 0.098	0.0057
$> 10$ (n=13)	1.425 $\pm$ 0.244	

TUNEL-I, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end-labeling index; SE, standard error of the mean; MI, mitotic index; MIB1-I, Ki-67 labeling index.

<sup>a</sup> Mann-Whitney *U* test.

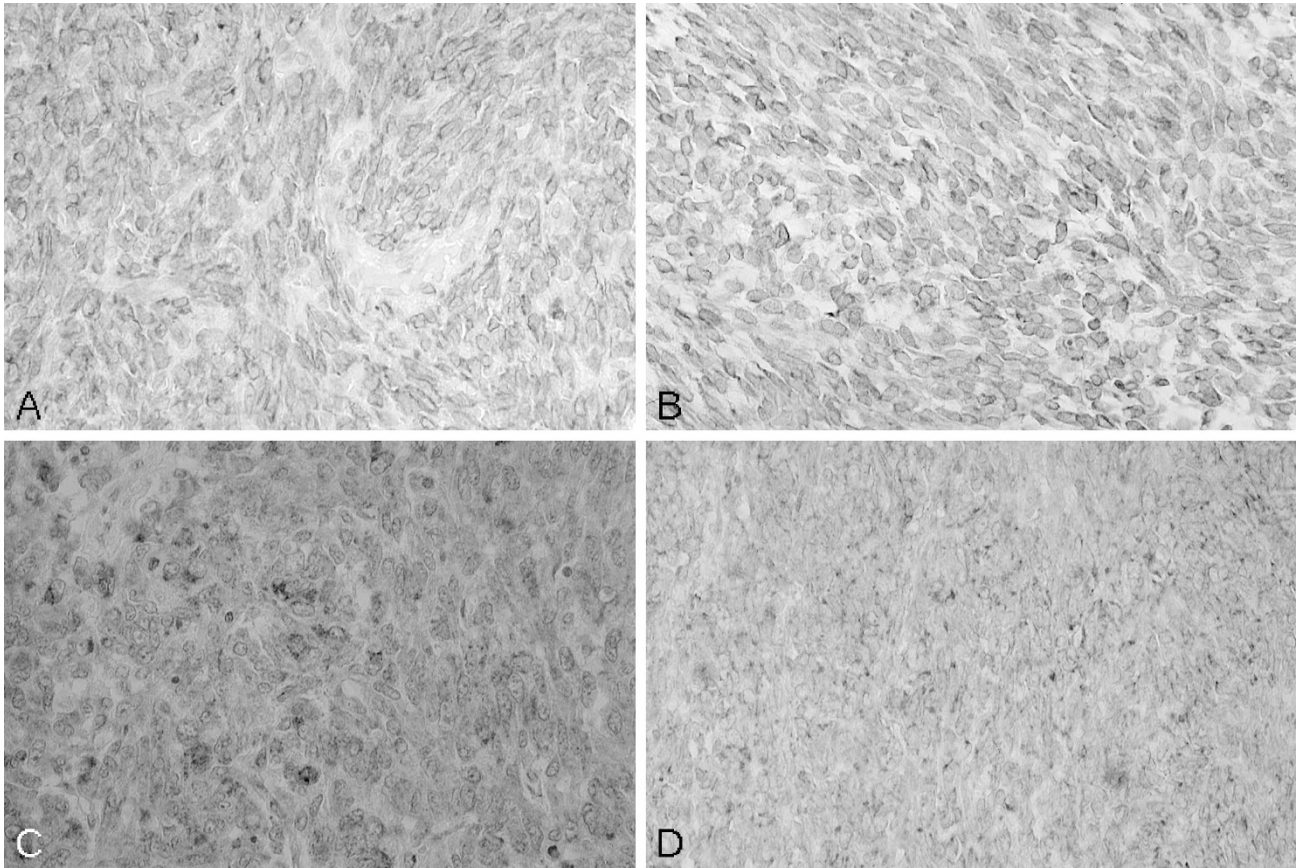
stage ( $P < .0001$ ), tumor necrosis ( $P = .014$ ), and tumor size of more than 5 cm ( $P = .014$ ) significantly correlated with poor survival. The six variables were entered into multivariate analysis by the Cox proportional hazards regression model with stepwise selection of variables, revealing that AJCC stage and TUNEL-I correlated significantly and independently with survival (Table 5).

#### Double Fluorescence Labeling with TUNEL and bcl-2 Immunohistochemistry

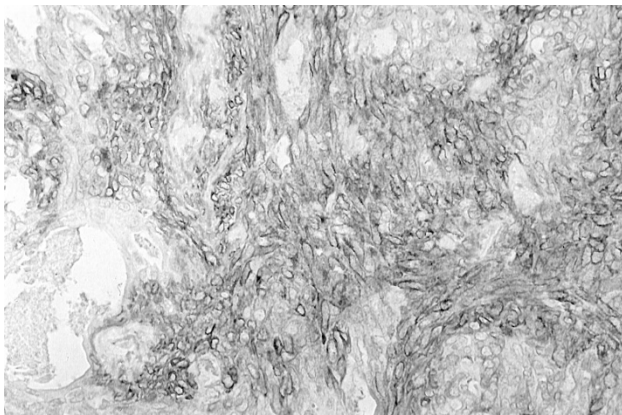
Double fluorescence labeling with TUNEL and bcl-2 immunostaining showed that a proportion of TUNEL-positive, apoptotic synovial sarcoma cells expressed bcl-2 protein (Fig. 12).

#### DISCUSSION

We established a new synovial sarcoma cell line, SN-SY-1, which showed diffuse perinuclear and cytoplasmic immunoreactivity for bcl-2 protein, and Southern blot analysis demonstrated no rearrangement of the *bcl-2* gene (22). In addition, ultrastructural localization of bcl-2 protein in cultured synovial sarcoma cells was similar to that previously



**FIGURE 9.** Immunohistochemical staining of bcl-2 (A), bax (B), bcl-x (C), and bak (D) proteins in monophasic fibrous synovial sarcoma. Most tumor cells show perinuclear and/or cytoplasmic staining.



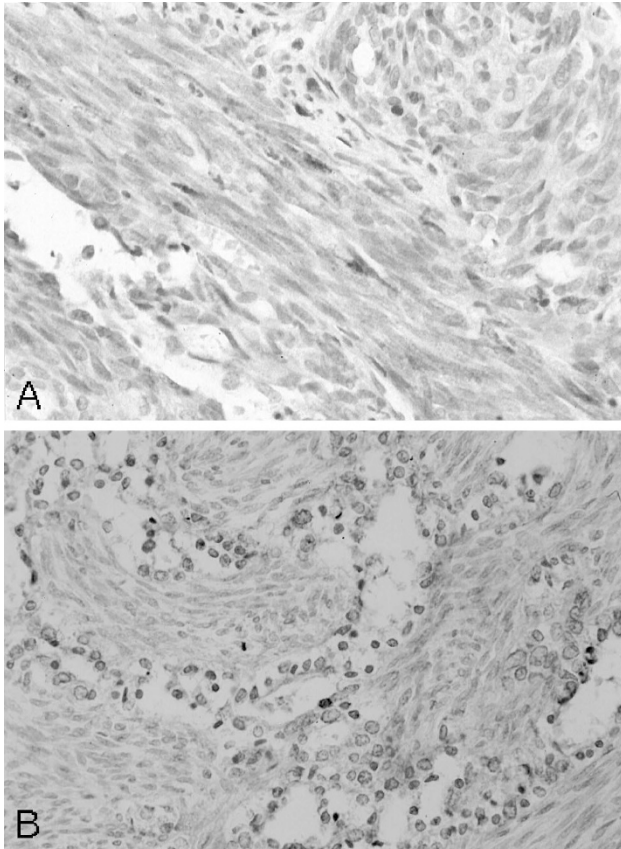
**FIGURE 10.** Immunohistochemical staining for bcl-2 protein in biphasic synovial sarcoma. Intense immunoreactivity for bcl-2 protein is observed in the spindle-cell component.

reported in benign and malignant lymphoid and epithelial cells (23). We thus speculated that the bcl-2 protein might play a role in the tumorigenesis of synovial sarcoma by suppressing apoptosis in a manner similar to that described for lymphomas (22, 23). The present study represents an extension of these early findings and was designed to examine the significance of apoptosis in synovial sarcoma.

Since the establishment of morphologic criteria of apoptosis, several studies have examined apo-

ptosis in various human tumors. Apoptotic cells can be recognized by nuclear shrinkage, compaction, segregation of chromatin against the nuclear envelope, and condensation of the cytoplasm (24). However, such recognition of apoptotic cells is not necessarily easy because of the variable degree of similarity between these cells and inflammatory cells or mitotic figures. In the present study, to facilitate the distinction of apoptotic synovial sarcoma cells, we used the TUNEL method (15), a standard technique for identifying fragmented nuclear DNA, which also enabled us to easily recognize apoptotic nuclear changes that were not always reliably identified on H&E sections.

Apoptosis in human cancers has been described in relation to high tumor grade, progression, and high mitotic rate (25–28). However, the prognostic value of the frequency of apoptotic tumor cells is still controversial (25). In the present study, the extent of apoptosis (TUNEL-I) in primary synovial sarcomas showed a significant linear correlation with the mitotic rate (MI) and cell proliferative activity determined by Ki-67 immunostaining (MIB1-I). Similar relationships between apoptosis and cell proliferation have been reported in other tumor types (26, 29, 30), suggesting a mechanistic link between apoptosis and cell proliferation. The rela-



**FIGURE 11.** Immunohistochemical staining for p53 and p21 (WAF1/CIP1) proteins in synovial sarcoma. **A**, sporadic immunoreactivity for p53 protein is observed in the spindle-shaped, fibroblastic tumor cells of monophasic fibrous synovial sarcoma; **B**, epithelial glandular tumor cells of biphasic synovial sarcoma are positive for p21 (WAF1/CIP1) protein.

tionship between apoptosis and cell proliferation may be explained in part by the fact that many proteins operating in the cell cycle checkpoints (e.g., p53; Rb; cyclins D1, A, and B) are also regulators and inducers of apoptosis (25).

In the present study, elevated TUNEL-I significantly correlated with poor prognosis in synovial sarcoma. In multivariate analysis, it was proved that TUNEL-I had independent prognostic value. A similar association between high apoptotic rate and poor prognosis has been described in other tumors (e.g., non-small cell lung carcinoma (31), breast carcinoma (32), and ovarian carcinoma (27)). Because it is widely known that elevated cell proliferation generally is associated with poor prognosis in many cancers, including synovial sarcoma (4, 5), the association between high apoptotic rate and poor prognosis in synovial sarcoma could be explained by the correlation between apoptosis and cell proliferation.

Hirakawa *et al.* (6) first examined the expression of bcl-2 protein in synovial sarcomas using formalin-fixed, paraffin-embedded tissues and reported that 15 of 19 cases (79%) were immunohis-

**TABLE 4. Correlation Between Apoptosis (TUNEL-I) and Apoptosis-Related Proteins**

Apoptosis-Related Proteins	n	Mean TUNEL-I $\pm$ SE (%)	P Value
bcl-2			
-	0		
+	5	0.680 $\pm$ 0.432	
++	13	0.746 $\pm$ 0.183	0.4024 <sup>a</sup>
+++	31	0.918 $\pm$ 0.140	
bax			
-	0		
+	11	1.064 $\pm$ 0.252	
++	13	0.682 $\pm$ 0.221	0.4085 <sup>a</sup>
+++	25	0.817 $\pm$ 0.141	
bcl-x			
-	0		
+	1		
++	10	0.922 $\pm$ 0.248	0.7323 <sup>b</sup>
+++	38	0.836 $\pm$ 0.124	
bak			
-	3	0.600 $\pm$ 0.173	
+	23	0.814 $\pm$ 0.157	0.8333 <sup>b</sup>
++	14	1.008 $\pm$ 0.249	
+++	9	0.750 $\pm$ 0.204	
Fas			
-	39	0.743 $\pm$ 0.111	0.0821 <sup>b</sup>
+	10	1.256 $\pm$ 0.284	
++ and +++	0		
Fas ligand			
-	44	0.851 $\pm$ 0.111	0.4745 <sup>b</sup>
+	5	0.733 $\pm$ 0.533	
++ and +++	0		
p53			
-	36	0.740 $\pm$ 0.121	0.0899 <sup>b</sup>
+	13	1.082 $\pm$ 0.224	
++ and +++	0		
p21 (WAF1/CIP1)			
-	40	0.767 $\pm$ 0.119	0.1987 <sup>b</sup>
+	9	1.020 $\pm$ 0.249	
++ and +++	0		

TUNEL-I, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end-labeling index; SE, standard error of the mean; -, no positively stained tumor cells are present; +, positively stained tumor cells are <10%; ++, positively stained tumor cells are 10 to 50%; +++, positively stained tumor cells are >50%.

<sup>a</sup> Kruskal-Wallis test.

<sup>b</sup> Mann-Whitney *U* test.

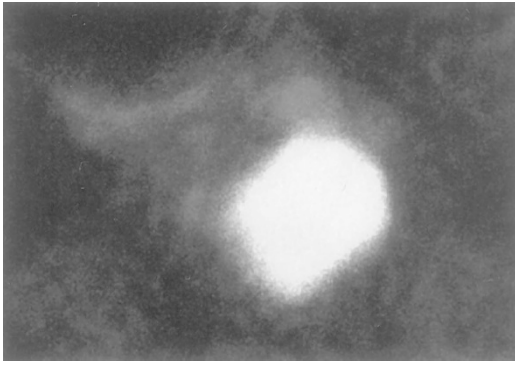
**TABLE 5. Prognostic Variables for Survival Selected by the Cox Proportional Hazards Regression Model with Stepwise Selection of Variables**

Variable	Coefficient	Exp (Coefficient)	P Value
TUNEL-I	0.735	2.085	0.0021
Stage	1.225	3.403	<0.0001

TUNEL-I, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end-labeling index.

tochemically positive. More recent, Suster *et al.* (8) examined 70 cases of synovial sarcoma and detected a positive immunostaining for bcl-2 protein in all cases. In the present immunohistochemical study using paraffin-embedded tissues, bcl-2 protein was positive in all synovial sarcomas and showed a moderate (++) to extensive (+++) staining in 44 of the 49 tumors (90%). The mean TUNEL-I of the examined synovial sarcomas was relatively low (0.84%), compared with that reported in colon carcinomas (1.9%) (33), small cell lung





**FIGURE 12.** Double fluorescence labeling with triphosphate *in situ* nick end-labeling and bcl-2 immunohistochemistry in synovial sarcoma. Note a synovial sarcoma cell with triphosphate *in situ* nick end-labeling-positive (fluorescein isothiocyanate-labeled) nuclei and immunopositive (rhodamine-labeled) cytoplasm for bcl-2 protein.

carcinomas (2.3%) (34), and ovarian carcinomas (1.3%) (27). It might be possible that the low TUNEL-I value in synovial sarcoma is due to the suppressive effect of overexpressed bcl-2 protein.

Recently, various forms of the bcl-2 family have been shown to be involved in the control of cell viability (16, 35, 36). Some members (*e.g.*, bcl-2, bcl-xL [large molecular weight product of *bcl-x* gene], and mcl-1 proteins) inhibit apoptosis, whereas others (*e.g.*, bax, bcl-xS [small molecular weight product of *bcl-x* gene], bak, and bad proteins) promote it, and the ratio of different family members (*e.g.*, bcl-2/bax) seems to determine the survival or death of cells after apoptotic stimuli (16). In the present study, immunohistochemical staining for bax, bcl-x (including both bcl-xL and bcl-xS), and bak proteins also exhibited a diffuse perinuclear and cytoplasmic positivity in most monophasic and biphasic synovial sarcomas as well as bcl-2 protein. Those members of the bcl-2 family were supposed to be involved in the regulation of apoptosis in synovial sarcoma. However, our present study failed to elucidate any significant correlation between the extent of apoptosis and immunohistochemical expression of bcl-2, bax, bcl-x, or bak protein.

Although synovial sarcomas have been shown to diffusely express bcl-2 protein (6–8), it is unknown whether apoptotic synovial sarcoma cells simultaneously express bcl-2 protein *in vivo*. In this study, double fluorescence staining with bcl-2 immunohistochemistry and TUNEL identified a considerable number of apoptotic synovial sarcoma cells that expressed bcl-2 protein. The finding indicates that apoptosis occurs even in synovial sarcoma cells that express bcl-2. Accordingly, it was assumed that some apoptosis-promoting factors (*e.g.*, bax, bak) may be involved in the promotion of apoptosis in such synovial sarcoma cells. Diffuse immunohistochemical expression of bax and bak proteins in

synovial sarcomas seemed to support this hypothesis; however, double fluorescence labeling with TUNEL and immunohistochemistry for bax or bak protein failed to successfully detect apoptotic synovial sarcoma cells that express bax or bak protein (data not shown). Proteolytic pretreatment of the TUNEL might damage epitopes or cellular structures where the proteins were present and, as a result, impair the immunoreactivity. We used formalin-fixed, paraffin-embedded synovial sarcoma tissue in the double fluorescence experiment; however, fresh-frozen tissue might be preferable.

Recent studies have shown that many tumor cells express both Fas and Fas ligand on their surface. The Fas-Fas ligand system is known as one of the major pathways for induction of apoptosis in cells and tissues (37). On contact with Fas ligand, Fas-bearing cells rapidly undergo apoptosis (19, 20). In the present immunohistochemical study, synovial sarcomas showed rare immunopositivity for both Fas and Fas ligand proteins. Accordingly, Fas-Fas ligand system was considered not likely to play an important role in the induction of apoptosis in primary synovial sarcomas.

Wild-type p53 prevents cells with damaged DNA from proliferation by arresting the cell division cycle until such damage is repaired or by pushing the damaged cell down an irreversible apoptotic pathway; however, mutant p53 is known to inhibit apoptosis (13, 17, 38). Immunohistochemical staining of p53 protein using a monoclonal antibody (*e.g.*, PAb 1801), which recognizes domains present in both the wild and mutated p53 proteins, was believed predominantly to detect mutated p53 protein because the wild-type p53 protein is rapidly degraded in routinely handled tissue (39). Whereas recent studies have shown that immunohistochemical p53 overexpression is not necessarily linked with *p53* gene mutation (40), p21 (WAF1/CIP1), a downstream *p53* effector gene, is activated by wild-type p53 protein and is involved in the regulation of cell cycle G1-S transition and apoptosis (18, 41, 42). In the current immunohistochemical study, p53 and p21 (WAF1/CIP1) proteins both were sporadically positive mainly in the tumor cells of a small number of monophasic and biphasic synovial sarcomas, and the expression seemed to have no remarkable association with the extent of apoptosis, clinicopathologic parameters, and expression of bcl-2 family proteins.

In summary, the present study demonstrated that extent of apoptosis in primary synovial sarcomas correlated with that of cell proliferative activity and increased apoptosis was an indicator of poor prognosis and survival. A number of apoptotic synovial sarcoma cells that were detected by TUNEL expressed bcl-2 protein. In addition, our immunohistochemical study concerning apoptosis-related

proteins revealed that multiple bcl-2 family proteins were expressed in synovial sarcomas and possibly involved in the regulation of apoptosis. Although the mechanism of apoptosis in primary synovial sarcoma is not completely clarified, increased rate of apoptosis is likely to be a useful marker for aggressive tumor growth and poor prognosis.

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## Book Review

***Damjanov I: Pathology for the Health-Related Professions, 2nd Ed, 545 pp, Philadelphia, W.B. Saunders, 2000 (\$52).***

With the publication of his second edition, Dr. Ivan Damjanov continues to offer an excellent book that is targeted to a group of students who are in desperate need of an appropriate level of text. The most difficult task for authors of books for the allied health professions is to give enough material that is readable and comprehensible, without inundating the student with extraneous information. Given the range of degree levels and specificity of specialty that falls under the “allied health” umbrella, Dr. Damjanov’s text can and should be considered as reading for any program.

The second edition most notably improves on the illustrations of the first, with color. Now, the book not only is user friendly in the readable sense but also is more pleasing to the eye. The allied health target audience usually does not have the luxury of a laboratory section to accompany the lecture component of pathophysiology courses. Therefore, the illustrations are a welcome and necessary improvement. In addition, the introductory “normal” sections were thankfully retained. I would like to see them perhaps enhanced a bit in future editions.

The clinicopathologic reviews have been replaced by comprehensive review questions at the end of each chapter. Although the 4-year hiatus between editions allowed for adequate and complete evaluation by colleague and student alike, I was not one of those in favor of eliminating the clinicopathologic review. I see the future of teaching as “case based,” and the reviews were a tendency toward that. Therefore, what is missing and what would truly set this book apart as the “gold standard” is the implementation of case studies with review questions. The clinicopathologic review came close, but. . .

The second edition of *Pathology for the Health-Related Professions* has retained its excellence while adding some improvements and corrections. The author continues to accommodate the needs of instructor and student alike by emphasizing basic pathophysiologic concepts and mechanisms *versus* pages of gross and microscopic descriptions. It is required reading in my course.

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