Journal of Surgical Oncology 2008;97:259–266

Prognostic Value of bax, bcl-2, and p53 Staining in Primary Osteosarcoma

MARIA-KYRIAKI A. KASETA, MD,[1]* LUBNA KHALDI, MD, PhD,[2] ILIAS P. GOMATOS, MD,[3] GEORGE P. TZAGARAKIS, MD,[1] LEONIDAS ALEVIZOS, MD,[3] EMMANUEL LEANDROS, MD, PhD,[3] PANAYIOTIS J. PAPAGELOPOULOS, MD, PhD,[1] AND PANAYIOTIS N. SOUCACOS, MD, PhD[1]

[1]First Department of Orthopaedic Surgery, KAT Hospital, University of Athens, Athens, Greece
[2]Department of Pathology, University Hospital of Larissa, Larissa, Greece
[3]First Department of Propaedeutic Surgery, Laboratory of Surgical Research, Hippokrateion Hospital, University of Athens, Athens, Greece

Background and Objectives: To investigate the immunohistochemical expression of three apoptosis-related genes (bax, bcl-2, and p53) and apoptosis (TUNEL) in patients with primary osteosarcoma, and examine potential correlations between gene expression and clinicopathological characteristics in these patients.

Materials and Methods: Thirty-five primary osteosarcoma specimens and 18 tissue specimens deriving from non-malignant osseous lesions were immunohistochemically stained for bax, bcl-2, and p53 proteins, while apoptosis was investigated by the TUNEL method. The results were statistically analyzed.

Results: P53, bax, and bcl-2 protein expression was observed in 22 (62.9%), 29 (82.9%), and 18 (51.4%) osteosarcoma patients, respectively. Non-specific positive TUNEL staining (±) was observed in two primary osteosarcoma cases (5.7%). None of the benign controls expressed any of the genes studied. None of the apoptosis-related genes studied was able to predict overall or disease-free survival in our group of patients. Nevertheless, increased bax/bcl-2 protein expression ratio was associated with a decreased 4-year survival and disease free survival ($P = 0.0229$ and $P = 0.0370$, respectively). Furthermore, all the patients who were bax(+)/bcl-2(−)/p53(+) relapsed within the 4-year follow-up period ($P = 0.0385$).

Conclusions: The increased apoptotic rate as determined by an elevated bax/bcl-2 protein expression ratio or by the bax(+)/bcl-2(−)/p53(+) protein expression pattern, appears to identify groups of osteosarcoma patients with unfavorable prognosis.

J. Surg. Oncol. 2008;97:259–266. © 2007 Wiley-Liss, Inc.

KEY WORDS: osteosarcoma; apoptosis; p53; bcl-2; bax; prognosis

INTRODUCTION

Osteosarcomas occur mainly in children and young adults. In patients older than 40 years, osteosarcomas are usually associated with preexisting conditions such as irradiated bones or Paget's disease [1]. Despite, advances in molecular biology there are no established molecules or molecular alterations in osseous tumor cells that may predict clinical outcome in osteosarcoma patients. Apoptosis (programmed cell death) plays an important role in the progression of malignant tumors [2]. Deregulation of the molecules controlling apoptosis may contribute to the process of tumorigenesis by reducing the rate of cell death, leading to the accumulation of genetic defects [3]. The most important known molecules regulating apoptotic cell death are bax, bcl-2, and p53.

Bcl-2 and p53 proteins have been assigned independent prognostic value in colorectal [4] and prostate carcinomas [5], and bax in patients with pancreatic adenocarcinomas [6], while in other malignancies, such as breast carcinoma [7], the prognostic role of the above molecules has not been delineated.

Currently, there is limited data on the clinical meaning of these molecules in primary osteosarcomas. Relevant studies report p53 mutations in 15–31% of osteosarcoma patients [2]. Few reports investigated p53 protein expression with respect to clinical outcome and most of them failed to reveal any prognostic significance [8]. Bcl-2 protein expression has only been examined in 19 metastatic osteosarcoma cases, with no data on post resection overall survival [9]. There are currently no reports on the predictive value of bax protein expression in patients with primary osteosarcoma.

Our study was designed to investigate the prognostic significance of bax, bcl-2, and p53 protein immunohistochemical expression in a consecutive series of surgically treated osteosarcoma patients. Furthermore we sought to: (1) determine apoptosis related coexpression patterns predictive of clinical outcome; (2) correlate immunohistochemical findings with the patients' clinicopathologic characteristics (such as presenting symptoms, tumor stage and histological grade); (3) determine bax, bcl-2, and p53 expression in non-neoplastic bone lesions.

MATERIALS AND METHODS

This retrospective, therapeutic study was designed in order to primary investigate the immunohistochemical expression of bax, bcl-2, and p53 molecules in classic osteosarcoma tissue specimen and correlate it with survival data. We obtained paraffin sections and retrospectively reviewed the medical records from 35 consecutive patients with primary osteosarcoma who were diagnosed and treated from January 1993 to June 1999. The inclusion criteria were patients with histologically confirmed (by incisional diagnostic biopsy) primary osteosarcoma who were operated with the intention to remove the entire primary tumor burden with wide resection margins. Three patients (8.6%) who denied operative treatment were excluded from the respective survival analysis. The follow-up was 7–119 months (mean, 35.5 months; median, 26 months), and was designated as

*Correspondence to: Maria-Kyriaki A. Kaseta, MD, 52 Pipinou Street, Athens 11251, Greece. Fax: 1130-210-6722259.
E-mail: labsures@med.uoa.gr

Received 8 March 2007; Accepted 11 September 2007
DOI 10.1002/jso.20913
Published online 27 December 2007 in Wiley InterScience (www.interscience.wiley.com).

the time between histological diagnosis of the primary tumor and the most recent postoperative evaluation of the patient alive. A favorable treatment outcome was freedom from relapse at the most recent follow-up. An unfavorable treatment outcome was tumor recurrence (local or distant) or death from the tumor. Eighteen tissue specimens originating from nonmalignant osseous lesions [4 osteoarthritis (22.2%); 6 osseous osteomas (33.3%); 1 osteoblastoma (5.5%); 7 fractures (38.8%)] were used as controls. All tissue specimens were immunohistochemically stained for bax, bcl-2, and p53 proteins, while apoptosis in malignant tissue specimens was further investigated by TUNEL staining.

The mean age at presentation was 30 years (±16.51 years) with a median of 21 years (range, 14–67 years). Nineteen patients (54.3%) were male and 16 patients (45.7%) were female (Table I). Localized pain for a median of 4 months was the main symptom in 32 patients (91.4%). A palpable mass was present for a median of 3 months in 21 patients (60%), and a soft tissue mass was present in 27 patients (77.1%) (Table I).

We used the Enneking staging system for evaluation. The evaluation of osteosarcoma was based on clinical examination, plain radiography, computerized tomography (CT), and magnetic resonance imaging (MRI) of the affected limb. All patients had complete preoperative staging including chest CT and Tc-99 bone scintigram. Four tumors were Stage IB (11.4%), three were Stage IIA (8.6%), 26 were Stage IIB (74.3%), and two were Stage III (5.7%). Only three of the osteosarcomas were considered intracompartmental (8.6%). Four patients presented with low grade lesions (11.4%), and 31 (88.6%) patients presented with high grade lesions. The mean tumor diameter was 11.3 cm.

The treatment protocol included surgery, preoperative chemotherapy, and adjuvant therapy according to the stage of the tumor. Six patients (17.1%) had marginal excision (0.5–1 cm surgical margin) of the primary tumor, 14 (40%) patients had a wide resection, and 12 patients (34.3%) had amputation of the affected limp. Twenty-one patients had chemotherapy (60%) after histological diagnosis until

TABLE I. Demographics and Clinicopathological Characteristics of 35 Patients With Primary Osteosarcoma

Characteristic	Frequency	Percentage
Age of disease presentation (years)	14–67 (mean: 30, median: 21)	
Gender		
Male	19	54.3
Female	16	45.7
Tumor size (cm)	1.5–45 cm (mean: 11.3, median: 10)	
Enneking stage		
IA	—	—
IB	4	11.4
IIA	3	8.6
IIB	26	74.3
IIIA	—	—
IIIB	2	5.7
Histologic type		
Osteoblastic	12	34.3
Chondroblastic	7	20
Paraosteal	4	11.4
Mixed type	4	11.4
Telangiectasic	3	8.6
Classic type	3	8.6
Fibroblastic	2	5.7
Histologic grade		
Low grade	4	11.4
High grade	31	88.6
Affected bone		
Femur	19	54.3
Tibia	6	17.1
Humerous	4	11.4
Ulnar	1	2.9
Ribs	1	2.9
Not reported	2	5.7
Osseous location		
Diaphysis	4	11.4
Metaphysis	31	88.6
Response to neoadjuvant chemotherapy[a]		
Good response	9	42.9
Poor response	12	57.1
Type of surgery		
Marginal excision	6	17.1
Wide resection	14	40
Amputation	12	34.3
Diagnostic biopsy alone	3	8.6
Adjuvant therapy		
Chemotherapy	26	74.3
Radiotherapy	2	5.7
Chemo/radiotherapy	6	17.1
None	1	2.9

[a]Histologic response to chemotherapy was graded as good if no viable tumor cells or 90% or more tumor necrosis was observed, and as poor in cases with less than 90% tumor necrosis (Picci et al. [11]).

4 weeks preoperatively. Adjuvant therapy was given in 34 patients (97.1%) and included chemotherapy alone in 26 patients (74.3%), radiotherapy alone in 2 patients (5.7%), or a combined chemotherapy/radiotherapy regimen in 6 patients (17.1%). One patient refused to enter the chemotherapy protocol. Four low grade patients received adjuvant chemotherapy based on their tumor size as well as their poor response to neoadjuvant treatment (which was administered due to their initial reluctance to undergo a surgical resection of their primary tumor). Adjuvant radiotherapy was administered in those patients who developed local recurrences at inaccessible sites or refused repeat surgery [10].

Preoperative chemotherapy consisted of one block of methotrexate 4-hr infusion (12 g/m^2) with leucovorine rescue (8 mg/m^2), followed by cisplatin (120 mg/m^2) and doxorubicine (75 mg/m^2). Cisplatin was delivered on day 7 during 48 hr as a continuous intravenous infusion and was followed by doxorubicine administered as a 24-hr continuous infusion. Postoperative chemotherapy was scheduled 7 days postoperatively with two cycles of doxorubicine (90 mg/m^2) and three cycles each of methotrexate (12 g/m^2) and cisplatin (120–150 mg/m^2 if tumor necrosis was >90% or <90% respectively) [11]. Three patients (9.4%) died before completion of treatment, while four patients (12.5%) did not complete their courses.

Immunohistochemical studies were performed on formalin fixed and paraffin embedded sections from surgical biopsy specimens using the streptavidin-biotin-peroxidase method (Novostain Super ABC, Novocastra Laboratories Ltd, Newcastle, UK) with monoclonal antibodies specific for bax (dilution 1:50, DAKO, Glostrup, Denmark), p53 (dilution 1:50, DAKO), and bcl-2 (dilution 1:50, DAKO).

Appropriate positive and negative controls were used for our staining technique [12], and all slides were evaluated by two independent reviewers blinded to the patients' outcomes. Cells were examined at more than 10 optical fields and at different magnifications (10×, 40×). The immunoreaction was positive (+) when more than 25% of the cells were stained. Tumors expressing one of the proteins in more than 50% of tumor cells were indicated as (++).

We performed TUNEL staining using the in situ Death Detection POD kit (Roche Diagnostic GmbH, Mannheim, Germany). We used two different pretreatment protocols after deparaffinization and rehydration. The conventional pretreatment protocol included incubation with proteinase K (20 g/ml in 10 Mm Tris/HCl, pH 7.4) followed by incubation in 0.1% Triton X-100 (Structure Probe, Inc., West Chester, PA) in 0.1% sodium citrate. The second (optimized) pretreatment protocol included microwave irradiation at acidic pH (pH 3) for 10 min followed by incubation with Proteinase K (20 g/ml) for 15 min at 37°C. Slides were rinsed with PBS and incubated with 3% H_2O_2 for 10 min at room temperature to block endogenous peroxidase activity. This was followed by PBS washing and incubation with a mixture of TdT solution and fluorescein isothiocyanate dUTP solution in a humidified chamber at 37°C for 60 min. We then washed again with PBS and incubated the slides with antifluorescein antibody Fab fragments conjugated with horseradish peroxidase (POD converter) in a humidified chamber at 37°C for 30 min. Diaminobenzide (DAB) was applied after washing with PBS, followed by light counterstain with hematoxylin. The presence of clear nuclear staining was indicative of apoptotic cells. We counted the number of TUNEL positive tumor cell nuclei. At least 1,000 tumor cells per specimen were examined in ten randomly selected fields by light microscopy (400×). The apoptotic index was the percentage of apoptotic cells in the tumor and was classified into three groups: less than 1% (−), 1–3% (+) and more than 3% (++).

Data were expressed as mean (± standard deviation [SD]) or median and range. Overall survival and disease free survival were calculated starting from the date of surgical excision of the primary tumor. Survival and disease free survival analyses were performed using the Kaplan Meier method with a log rank test. We used the Cox proportional hazards model to determine independent prognostic factors. Univariate analyses comparing bax, bcl-2, and p53 protein expression in the various subgroups of patients as determined by stage, grade, and other clinicopathological variables were performed with the chi square test (Pearson, Mantel-Haenzel test for linear association) using Yate's correction or Fisher's exact test whenever needed. Probability values of <0.05 were considered significant.

RESULTS

One co-expression pattern revealed prognostic significance with respect to overall survival and two with respect to postoperative recurrence rates. Twenty patients expressed bax protein in more than 50% of their tumor cells (bax[++]). In this group of patients, bcl-2 protein expression was associated with a favorable clinical outcome (63.64% vs. 11.11%, $P = 0.031$; Fig. 1) and a higher ($P = 0.0370$) disease free survival (45.45% vs. 11.11%), something which was also observed when low grade cases were excluded from the survival analysis ($P = 0.05$). This suggests that increased bax expression in combination with negative bcl-2 staining (increased bax/cl-2 expression ratio) is unfavorable with respect both to survival and tumor recurrence. Patients with bax(+)/bcl-2(−)/p53(+) tumors (n = 8) exhibited a higher ($P = 0.039$) treatment failure rate (100% vs. 62.5%; Fig. 2) and presented a lower ($P = 0.040$) recurrence free survival compared with the rest of the group (n = 24) and patients who were bax(−)/bcl-2(−)/p53(−) (n = 3) (0% vs. 66.67%) (Fig. 3), respectively. These results remained statistically significant when low grade cases were excluded from the survival analysis. Patients who were bax(+)/bcl-2(−)/p53(+) had a higher ($P = 0.015$) incidence of pathological fractures compared with those without (62.5% vs. 14.8%). The bax(+)/bcl-2(−)/p53(+) lesions were located at the metaphysis of long bones, had a diameter greater than 5 cm, and were associated with the development of earlier metastatic disease.

No correlation was found between overall survival and expression of only one of the three proteins. Overall survival after primary treatment was 6.5–118 months (median, 25.3 months). The 5-year survival rate for 27 bax positive patients who fulfilled the follow-up

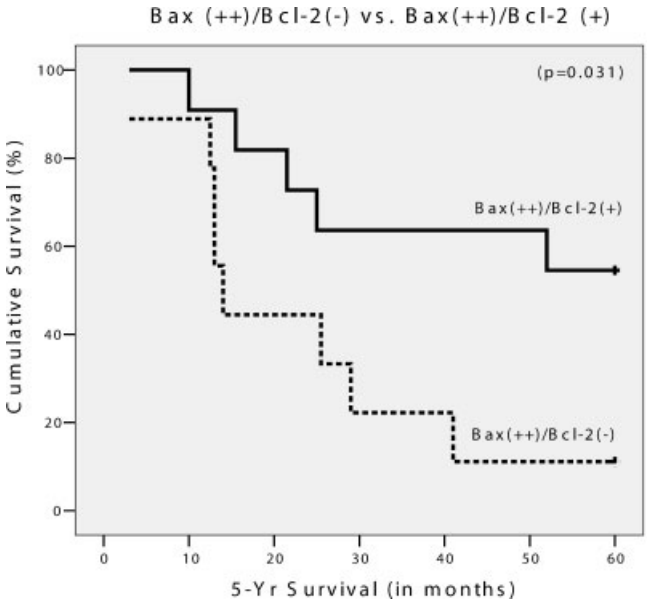

Fig. 1. A Kaplan–Meier survival analysis shows a decreased 5-year survival rate in bax(++)/bcl-2(−)osteosarcoma patients compared with bax(++)/bcl-2(+)osteosarcoma patients.

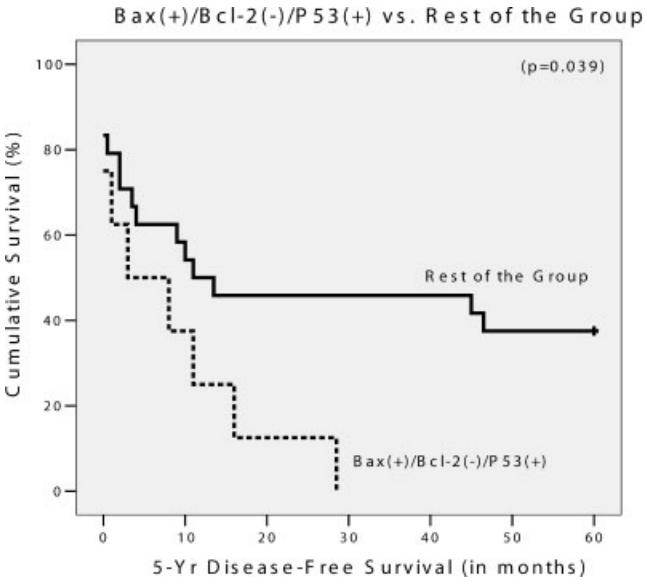

Fig. 2. A Kaplan–Meier survival analysis shows a decreased 5-year disease free survival in bax(+)/bcl-2(−)/p53(+) osteosarcoma patients compared with the rest of the group.

criteria (consented to the proposed operative treatment) was similar to the bax negative patients (29.6% vs. 60%). Patients who were bcl-2 positive (n = 16) had a similar survival rate compared with bcl-2 negative patients (37.5% vs. 31.3%). The overall survival rate was also similar for p53 positive patients and p53 negative patients (28.6% vs. 45.5%).

Bax, bcl-2, and p53 individually failed to predict 5-year disease free survival. Disease free survival was 0.5–117 months (median, 11 months). Tumors with bax, bcl-2, and p53 protein expression did not differ regarding 5-year disease free survival compared with their negative counterparts (22.2% vs. 60%) (31.25% vs. 25%) and (23.81% vs. 36.36%), respectively.

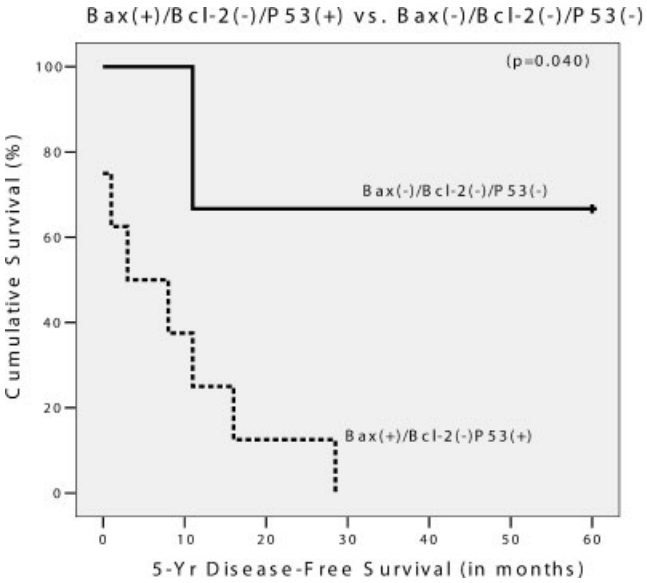

Fig. 3. A Kaplan–Meier survival analysis shows a decreased 5-year disease-free survival in bax(+)/bcl-2(−)/p53(+) osteosarcoma patients compared to patients with the bax(−)/bcl-2(−)/p53(−) protein expression pattern.

Bax, bcl-2, and p53 immunostaining were observed exclusively in patients with osteosarcoma, while all benign tissue specimens were negative for the three proteins studied. Twenty-two of 35 patients expressed p53 protein (62.9%) (Fig. 4). Bax protein expression was observed in 29 patients (82.9%) (Fig. 5) and bcl-2 protein expression in 18 (51.4%) (Fig. 6). TUNEL staining was negative in 94.3% of patients (33 of 35 patients), with the exception of one chondroblastic and one osteoblastic osteosarcoma (5.7%) where weak positive staining (±) was observed (Fig. 7).

None of the clinicopathological factors tested by univariate analysis (patient age, gender, tumor diameter, tumor stage, pathological grade, type of surgical treatment, response to neoadjuvant chemotherapy, and administration of adjuvant therapy) were associated with the clinical outcome of overall and relapse free survival (Table II), which was further confirmed by the subsequent multivariate analysis (Table III).

Immunohistochemistry results did not correlate with the stage or the grade of the underlying tumor. We observed a higher ($P = 0.003$) level of p53 protein expression in patients with a soft tissue mass on magnetic resonance imaging (MRI) than those without (44.4% vs. 12.5%). Expression of p53 positive cells was more often in extracompartmental than intracompartmental osteosarcomas (68.8% vs. 0%; $P = 0.044$), and in tumors with diameter more than 5 cm ($P = 0.05$).

DISCUSSION

Although surgery combined with preoperative chemotherapy has improved survival for patients with osteosarcoma, the search for new markers which may more accurately identify high risk patients for death or relapse of disease is still ongoing [13]. Apoptosis and/or its regulating proteins, including bax, bcl-2, and p53 proteins, have been correlated with the clinical outcome of various tumors treated by surgery and/or adjuvant chemoradiotherapy [5,7]. Significant results have been reached in this respect both from separate [9] and combined protein expression [4] analysis. In the current study, we addressed the prognostic value of combined as well as separate bax, bcl-2, and p53 protein expression in patients diagnosed with primary osteosarcoma. Our study group included 32 consecutive patients with primary osteosarcoma who had a potentially curative resection of their tumor. The low incidence of the disease as well as the fact that our work was retrospectively conducted in a single institution both account for the difficulty in compiling a larger group of patients with identical

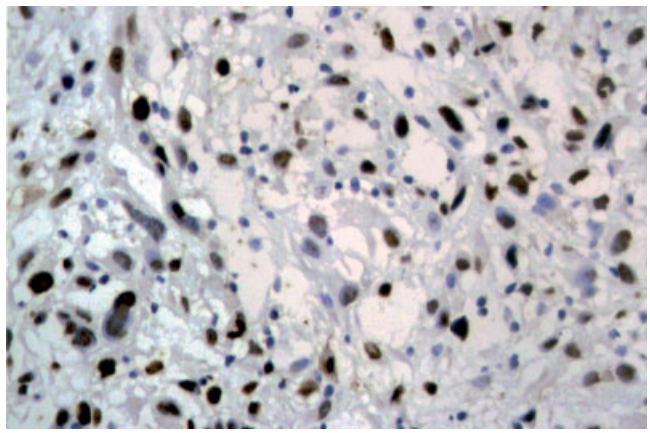

Fig. 4. A histological specimen shows positive nuclear staining for p53 protein in chondroblastic type osteosarcoma (Stain, TUNEL; original magnification, 40×). [Color figure can be viewed in the online issue, available at www.interscience.wiley.com.]

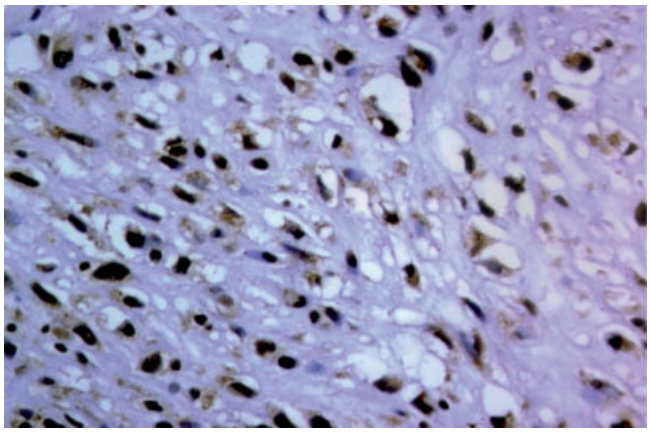

Fig. 5. A histological specimen shows positive intracytoplasmic staining for bax protein in chondroblastic type osteosarcoma (Stain, TUNEL; original magnification, 40×). [Color figure can be viewed in the online issue, available at www.interscience.wiley.com.]

clinicopathological characteristics. Patients were unselected for their stage and histological grade. To avoid possible confounding effects of preoperative chemotherapy on immunohistochemical results, all tissue specimens were from diagnostic surgical biopsies before the administration of any treatments.

Immunohistochemical detection of in situ apoptosis with TUNEL was ambiguous and lower than expected (5.7%) despite using two different pretreatment protocols. Because there is no previous evidence regarding the application of TUNEL in primary osteosarcoma tissue specimens, our findings can be attributed both to the inherently low sensitivity of the TUNEL technique when applied in archival tissue specimens [14], as well as the unique characteristics of the tissue specimens studied. On the other hand, immunohistochemical staining for bax, bcl-2, and p53 was routinely performed.

In the past, a number of different clinicopathological factors have been studied to provide information regarding the prognosis of patients with primary osteosarcoma. Osteolytic types of osteosarcoma, pathologic fractures, inadequate margins at the time of surgery, metastatic

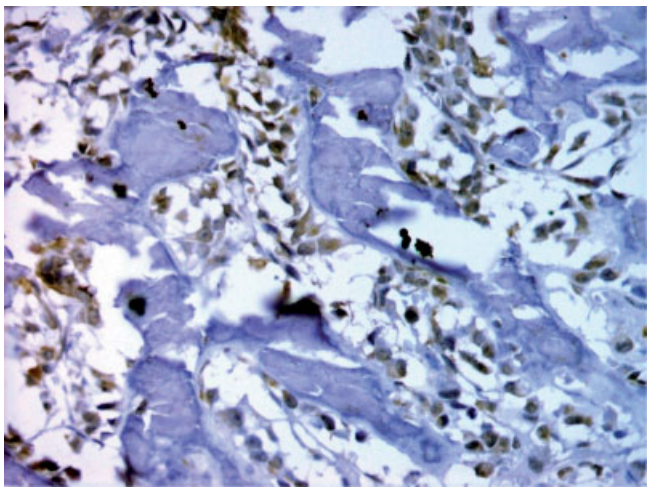

Fig. 6. A histological specimen shows positive intracytoplasmic staining for bcl-2 protein in osteoblasts and osteoclasts in Grade II osteosarcoma (Stain, TUNEL; original magnification, 40×). [Color figure can be viewed in the online issue, available at www.interscience.wiley.com.]

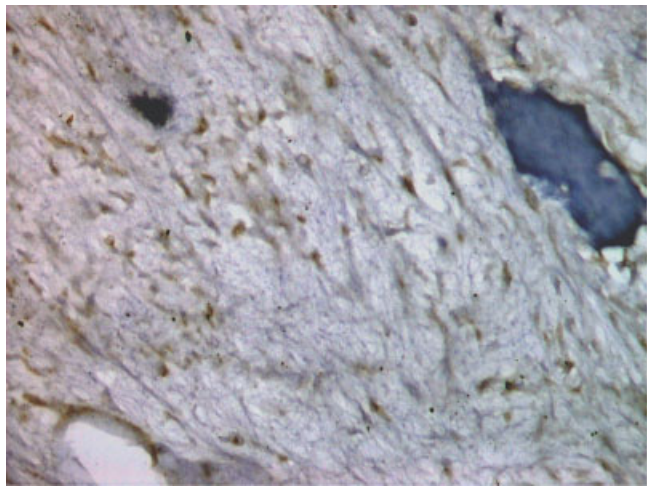

Fig. 7. A histological specimen shows weak TUNEL staining localized to a few fibroblasts in fibroblastic type osteosarcoma (Stain, TUNEL; original magnification, 40×). [Color figure can be viewed in the online issue, available at www.interscience.wiley.com.]

disease at presentation or later, and tumors localized to the trunk, pelvis, or femur have been associated with lower survival rates [1]. However, only absolute tumor volume and histologic response to adjuvant chemotherapy predict outcome in patients with primary osteosarcoma [15]. In our study, determinants such as age, gender, tumor diameter, tumor stage, histological grade, type of surgical treatment, and the administration of adjuvant chemotherapy failed to predict survival in the univariate and multivariate analyses. This was not the case with the combined expression patterns of bax, bcl-2, and p53 proteins.

P53 is one of the best known tumor suppressor genes. Mutant forms can act as dominant oncogenes when they can no longer induce apoptosis in response to DNA damage, and demonstrate a longer half life than the wild type p53 [12]. In this respect, overexpression of p53 is considered a surrogate of altered function. The reported amount of p53 positive tumor cells in patients with osteosarcoma is from 21% to 63% [16,17]. By using monoclonal antibody against p53, we detected p53 protein expression in 22 patients with primary osteosarcoma (62.9%), but could not predict overall and recurrence free survival. Most investigators examining the association between p53 expression and survival have documented a poor prognosis in patients with p53 (+) tumors [4,18] Our data are consistent with studies showing that p53 overexpression is not associated with clinical outcome and disease recurrence in patients with primary osteosarcoma [15], and head and neck sarcoma [18], (Table IV) indicating the unique characteristics of primary osteosarcoma. We did find a correlation between p53 overexpression and extracompartmentally expanding osteosarcomas, as well as with tumors measuring greater than 5 cm in diameter. Extracompartmental tumors, or tumors greater than 5 cm, indicate locally advanced disease and disrupt the anatomy of the affected limb. P53 protein expression in locally advanced tumors suggests a highly aggressive tumor phenotype [25]. However, this aggressive phenotype was not associated with a worse 5-year survival rate in p53(+) against p53(−) patients (Table II), possibly because of the wide resection margins achieved in all our cases.

The family of bcl-2 related proteins is one of the most biologically relevant classes of apoptosis regulators; some function as suppressors and others as promoters of apoptosis. The bcl-2 gene seems to encode an intercellular protein shown to block or delay apoptosis in several human neoplasms [26]. Eighteen of our patients (51.8%) demonstrated increased bcl-2 protein expression by their tumor cells but with no apparent association with outcome. Although a similar percentage

TABLE II. Five-Year Overall and Disease Free Survival According to Clinicopathological Variables in 32 Surgically Treated Patients With Primary Osteosarcoma

Variable (n)	5-year survival (95% CI)	Significance (*P*-value)	5-year disease free survival (95%CI)	Significance (*P*-value)
Age				
≤20 years [14]	35.7% (20.77–44.16%)	0.904	28.6% (12.02–38.19%)	0.822
>20 years [18]	33.3% (25.13–44.92%)		27.8% (10.57–34.21%)	
Gender				
Male [17]	29.4% (25.28–45.14%)	0.964	17.7% (10.58–32.12%)	0.468
Female [15]	40% (20.89–43.98%)		40% (11.98–40.22%)	
Tumor diameter				
≤5 cm [6]	50% (31.41–59.93%)	0.255	50% (20.19–59.31%)	0.161
>5 cm [26]	30.8% (22.81–39.57%)		23.1% (10.59–29.1%)	
Tumor stage[a]				
I [4]	0% (5.11–44.64%)	0.228	0% (0.8–10.2%)	0.185
II [26]	42.3% (27.34–44.54%)		34.6% (16.85–37.27%)	
III [2]	0% (0–55.88%)		0% (0–41.94%)	
Pathological grade				
Low grade [4]	0% (5.11–44.64%)	0.181	0% (0.8–10.2%)	0.104
High grade [28]	39.3% (27–61.43.39%)		32.1% (16.51–35.81%)	
Response toneoadjuvant chemotherapy				
Good response [9]	55.6% (27.09–55.68%)	0.164	33.33% (12.87–45.57%)	0.421
Poor response [12]	25% (18.29–42.87%)		25% (6.14–33.61%)	
Type of surgical treatment				
Marginal excision [6]	33.3% (17.11–53.06%)	0.998	33.3% (7.8–50.69%)	0.834
Wide resection [14]	35.7% (22.08–45.71%)		28.6% (6.24–33.19%)	
Amputation [12]	33.3% (21.64–45.02%)		25% (12.01–38.49%)	
Adjuvant therapy				
Yes [31]	32.3% (N[b])	0.294	25.8% (N[b])	0.249
No [1]	100% (N[b])		100% (N[b])	
Bax expression				
Bax(+) [27]	29.6% (23.96–40.3%)	0.239	22.2% (11.17–29.57%)	0.095
Bax(−) [5]	60% (25.67–61.34%)		60% (20.38–61.42%)	
Bcl-2 expression				
Bcl-2(+) [16]	37.5% (23.99–45.38%)	0.758	31.25% (14.39–39.86%)	0.561
Bcl-2(−) [16]	31.3% (22.41–43.84%)		25% (8.18–31.89%)	
P53 expression				
P53(+) [21]	28.6% (4.79–41.69%)	0.345	23.81% (10.62–31.38%)	0.465
P53(−) [11]	45.5% (24.43–49.57%)		36.36% (12.76–44.24%)	
Gene coexpression patterns				
Bax(++)/Bcl-2 (+) [11]	54.5% (32.13–55.87%)	**0.031**	45.5% (22.36–52.28%)	**0.037**
Bax(++)/Bcl-2 (−) [9]	11.1% (11.07–34.39%)		11.11% (0–24.62%)	
Bax(+)/Bcl-2(−)/p53(+) [8]	12.5% (0–35.87%)	0.153	0% (0–12.7%)	**0.039**
Rest of the group [24]	41.7% (27.17–61.60%)		37.5% (0–54.21%)	
Bax(+)/Bcl-2(−)/p53(+) [8]	12.5% (0–35.86%)	0.150	0% (N[b])	**0.040**
Bax(−)/Bcl-2(−)/p53(−) [3]	66.7% (20.99–69.01%)		66.7% (17.53–69.81%)	

SD, standard deviation; CI, confidence interval. Bold values indicate *P*-value that reach prognostic significance.
[a]Enneking staging system.
[b]Null value.

TABLE III. Multivariate Analysis in Patients With Primary Osteosarcoma

	5-year survival		5-year disease free survival	
Variable	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Age[a]	1.08 (0.42–2.76)	0.87	1.08 (0.43–2.68)	0.87
Gender	1.43 (0.52–3.94)	0.49	1.19 (0.45–3.10)	0.73
Tumor size[b]	2.33 (0.65–8.36)	0.19	1.87 (0.59–5.92)	0.29
Enneking stage	2.79 (0.52–15.13)	0.23	2.05 (0.39–10.59)	0.39
Histologic grade	0.14 (0.01–1.49)	0.10	0.17 (0.02–1.84)	0.15
Type of surgery	0.82 (0.41–1.62)	0.56	0.83 (0.44–1.57)	0.57

[a]20-year cutoff.
[b]5 cm cutoff; administration of adjuvant therapy was not included in the multivariate analysis, as only one patient did not receive any type of adjuvant therapy.

TABLE IV. Literature Review for Immunohistochemical bax, bcl-2, and p53 Staining on Archival Tissue Specimens From Patients With Primary Osteosarcoma

Study	Number of patients (design)	Primary treatment	Proteins studied (method)	Bax (%)	Bcl-2 (%)	P53 (%)	Followup time, clinical outcome endpoint	Prognostic significance
Ferrari et al. [9]	19 high grade osteosarcomas	Surgery + chemo	P53, bcl-2 (IHC)	—	10/19 (53%)	4/19 (21%)	NR—(only disease-free survival + postmetastasis survival studied)	Non-significant
Pakos et al. [8]	499 patients (meta-analysis of 16 reports)	Chemo	P53 (IHC + RT-PCR)	—	—	NR	24 months	Non-significant
Tsai et al. [19]	22 patients (retrospective)	Limp spacing surgery + chemo	P53 (IHC)	—	—	6/22 (27%)	Median, 711 days	Non-significant
Nakashima et al. [20]	26 patients (retrospective)	Chemo ± Surgery	P53 (IHC)	—	—	8/21 (38%)	3 years	NR
Junior et al. [18]	25 patients (head and neck osteosarcomas-retrospective)	Surgery ± chemo/radiotherapy	P53 (IHC)	—	—	13/25 (52%)	Mean, 6 years (1 month to 40 years)	NR
Kawaguchi et al. [21]	23 patients (retrospective)	—	P53 (IHC + PCR-SSCP)	—	—	5/23 (22%)	—	NR
Park et al. [22]	52 patients (retrospective)	Surgery ± chemo	P53 (IHC)	—	—	33/52 (63%)	Mean, 46 months 3-year survival studied	Non-significant
Papai et al. [23]	21 patients	Chemo + surgery	P53 (IHC)	—	—	6/21 (29%)	—	Non-significant
Posl et al. [24]	36 osteosarcoma patients	NA	Bcl-2 (IHC)	—	29/36 (81%)	—	NR	NR

IHC, immunohistochemistry; NR, not reported; RT-PCR, reverse transcriptase-polymerase chain reaction; SSCP, single strand conformation polymorphism; NA, not applicable; chemo, chemotherapy.

(53%) of bcl-2 positive tumor cells has been reported elsewhere [9], limited evidence exists for the prognostic value of bcl-2 protein in primary osteosarcoma patients (Table IV). Only Ferrari et al. [9] reported a nonsignificant difference in disease free survival between bcl-2(+) and bcl-2(−) tumors. However, these results derived from a more limited group of patients with no data regarding overall survival. Similar nonsignificant correlation between bcl-2 immunohistochemical staining and survival has been reported in patients with synovial sarcoma and gastrointestinal stromal/smooth muscle tumors [16,17]. Nonetheless, there are studies reporting either a strongly positive [6] or negative [22,27] clinical impact of bcl-2 overexpression in different malignancies, suggesting the prognostic significance of bcl-2 varies between tumors or the specific situation.

Bax is a bcl-2 associated protein with proapoptotic properties which can suppress the ability of bcl-2 to block apoptosis [26]. There are currently no reports on bax protein expression in primary osteosarcoma patients (Table IV). In our study, overall and disease free survival with respect to bax positive and bax negative patients did not differ. Increasing evidence indicate a positive [28] or negative [29] prognostic value of bax in several tumor types. Our results confirm those of Kawaguchi et al. [21] who found no correlation between survival and bax immunohistochemical expression in patient with synovial sarcomas. There are similar reports on non-Hodgkin gastric lymphoma and ovarian cancer [12,30].

Although bax, bcl-2, and p53 proteins alone failed to predict prognosis in patients with primary osteosarcoma, their combined expression patterns demonstrated an impact on the clinical outcome. We found that patients with increased bax protein expression levels (>50% of the tumor cells) but bcl-2 negative had decreased disease free survival and overall survival compared with patients with the reverse bcl-2 protein expression pattern (i.e., bax[++]/bcl-2[+]), results which were further confirmed when low grade osteosarcoma cases were excluded from the survival analysis. This suggests that a high bax/bcl-2 protein expression ratio is associated with an unfavorable outcome in patients with primary osteosarcoma. The reverse results have been reported in previous studies, where a high bcl-2/bax expression ratio correlated with a worse prognosis in patients with squamous cell carcinoma of the tongue [31]. However, Georgiou et al. [32] demonstrated that bax(+)/bcl-2(−) laryngeal tumors presented with a more aggressive clinical phenotype. This coexpression pattern probably counterbalances the accelerated proliferation status of the malignant cells, indirectly characterizing a more aggressive tumor.

It appears that bax, bcl-2, and p53 protein expression is absent in benign osseous lesions. Similar results regarding p53 immunoreactivity have been previously reported in benign osseous lesions [33], but bax and bcl-2 protein expression had not been analyzed.

CONCLUSIONS

Although each of these proteins examined alone failed to predict prognosis, decreased overall and disease free survival was seen in three combinations of bax, bcl-2, and p53 protein expression patterns. Their combined analysis amplifies prognostic accuracy and provides a better description of the biologic behavior of primary osteosarcomas that can be used to develop more aggressive treatment protocols. Although a larger series of patients will have to be analyzed to confirm these preliminary results, our findings indicate the unique characteristics of primary osteosarcoma and suggest an approach for subsequent prospective studies.

ACKNOWLEDGMENTS

We are indebted to the Athens University and to the Greek Ministry of Health and Welfare for sponsoring this study. We are also indebted to Dr E. Ch. Papadopoulos for reviewing our manuscript.

REFERENCES

1. Copeland MM, Sutow WW: Osteogenic sarcoma: The past, present, and future. Int Adv Surg Oncol 1979;2:177–200.
2. Gokgoz N, Wunder JS, Mousses S, et al.: Comparison of p53 mutations in patients with localized osteosarcoma and metastatic osteosarcoma. Cancer 2001;92:2181–2189.
3. Pruneri G, Pignataro L, Carboni N, et al.: Clinical relevance of p53 and bcl-2 protein over-expression in laryngeal squamous-cell carcinoma. Int J Cancer 1998;79:263–268.
4. Buglioni S, D'Agnano I, Cosimelli M, et al.: Evaluation of multiple bio-pathological factors in colorectal adenocarcinomas: Independent prognostic role of p53 and bcl-2. Int J Cancer 1999;84:545–552.
5. Bauer JJ, Sesterhenn IA, Mostofi FK, et al.: Elevated levels of apoptosis regulator proteins p53 and bcl-2 are independent prognostic biomarkers in surgically treated clinically localized prostate cancer. J Urol 1996;156:1511–1516.
6. Friess H, Lu Z, Graber HU, et al.: Bax, but not bcl-2, influences the prognosis of human pancreatic cancer. Gut 1998;43:414–421.
7. Dimitrakakis C, Konstadoulakis M, Messaris E, et al.: Molecular markers in breast cancer: Can we use c-erbB-2, p53, bcl-2 and bax gene expression as prognostic factors? Breast 2002;11:279–285.
8. Pakos EE, Kyzas PA, Ioannidis JP: Prognostic significance of TP53 tumor suppressor gene expression and mutations in human osteosarcoma: A meta-analysis. Clin Cancer Res 2004;10:6208–6214.
9. Ferrari S, Bertoni F, Zanella L, et al.: Evaluation of P-glycoprotein, HER-2/ErbB-2, p53, and Bcl-2 in primary tumor and metachronous lung metastases in patients with high-grade osteosarcoma. Cancer 2004;100:1936–1942.
10. Claude L, Rousmans S, Carrie C, et al.: Standards and Options for the use of radiation therapy in the management of patients with osteosarcoma. Bull Cancer 2005;92:891–906.
11. Picci P, Bacci G, Campanacci M, et al.: Histologic evaluation of necrosis in osteosarcoma induced by chemotherapy. Regional mapping of viable and nonviable tumor. Cancer 1985;56:1515–1521.
12. Skirnisdottir I, Seidal T, Gerdin E, et al.: The prognostic importance of p53, bcl-2, and bax in early stage epithelial ovarian carcinoma treated with adjuvant chemotherapy. Int J Gynecol Cancer 2002;12:265–276.
13. Davis AM, Bell RS, Goodwin PJ: Prognostic factors in osteosarcoma: A critical review. J Clin Oncol 1994;12:423–431.
14. Labat-Moleur F, Guillermet C, Lorimier P, et al.: TUNEL apoptotic cell detection in tissue sections: Critical evalua tion and improvement critical evaluation and improvement. J Histochem Cytochem 1998;46:327–334.
15. Bieling P, Rehan N, Winkler P, et al.: Tumor size and prognosis in aggressively treated osteosarcoma. J Clin Oncol 1996;14:848–858.
16. Kawauchi S, Fukuda T, Oda Y, et al.: Prognostic significance of apoptosis in synovial sarcoma: Correlation with clinicopathologic parameters, cell proliferative activity, and expression of apoptosis-related proteins. Mod Pathol 2000;13:755–765.
17. Mousses S, McAuley L, Bell RS, et al.: Molecular and immunohistochemical identification of p53 alterations in bone and soft tissue sarcomas. Mod Pathol 1996;9:1–6.
18. Junior AT, de Abreu Alves F, Pinto CA, et al.: Clinicopathological and immunohistochemical analysis of twenty-five head and neck osteosarcomas. Oral Oncol 2003;39:521–530.
19. Tsai JY, Aviv H, Benevenia J, et al.: HER-2/neu and p53 in osteosarcoma: An immunohistochemical and fluorescence in situ hybridization analysis. Cancer Invest 2004;22:16–24.
20. Nakashima H, Nishida Y, Sugiura H, et al.: Telomerase, p53 and PCNA activity in osteosarcoma. Eur J Surg Oncol 2003;29:564–567.
21. Kawaguchi K, Oda Y, Sakamoto A, et al.: Molecular analysis of p53, MDM2, and H-ras genes in osteosarcoma and malignant fibrous histiocytoma of bone in patients older than 40 years. Mod Pathol 2002;15:878–888.
22. Park YB, Kim HS, Oh JH, et al.: The co-expression of p53 protein and P-glycoprotein is correlated to a poor prognosis in osteosarcoma. Int Orthop 2001;24:307–310.
23. Papai Z, Feja CN, Hanna EN, et al.: P53 overexpression as an indicator of overall survival and response to treatment in osteosarcomas. Pathol Oncol Res 1997;3:15–19.
24. Posl M, Amling M, Werner M, et al.: Osteosarcoma—Apoptosis and proliferation. Study of bcl-2 expression. Pathologe 1994;15:337–344.
25. Cadwell C, Zambetti GP: The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth. Gene 2001;277:15–30.
26. Oltvai ZN, Milliman CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74:609–619.
27. Santini D, Tonini G, Vecchio FM, et al.: Prognostic value of Bax, Bcl-2, p53, and TUNEL staining in patients with radically resected ampullary carcinoma. J Clin Pathol 2005;58:159–165.
28. Harima Y, Nagata K, Harima K, et al.: Bax and Bcl-2 protein expression following radiation therapy versus radiation plus thermoradiotherapy in stage IIIB cervical carcinoma. Cancer 2000;88:132–138.
29. Tomazic A, Pegan V, Ferlan-Marolt K, et al.: Cyclin D1 and bax influence the prognosis after pancreatoduodenectomy for periampullary adenocarcinoma. Hepatogastroenterology 2004;51:1832–1837.
30. Pagnano KB, Silva MD, Vassallo J, et al.: Apoptosis-regulating proteins and prognosis in diffuse large B cell non-Hodgkin's lymphomas. Acta Haematol 2002;107:29–34.
31. Xie X, Clausen OP, De Angelis P, et al.: The prognostic value of spontaneous apoptosis, Bax, Bcl-2, and p53 in oral squamous cell carcinoma of the tongue. Cancer 1999;86:913–920.
32. Georgiou A, Gomatos IP, Ferekidis E, et al.: Prognostic significance of p53, bax and bcl-2 gene expression in patients with laryngeal carcinoma. Eur J Surg Oncol 2001;27:574–580.
33. Ueda Y, Dockhorn-Dworniczak B, Blasius S, et al.: Analysis of mutant P53 protein in osteosarcomas and other malignant and benign lesions of bone. J Cancer Res Clin Oncol 1993;119:172–178.