EXPRESSION OF P-GLYCOPROTEIN IN HIGH-GRADE OSTEOSARCOMAS IN RELATION TO CLINICAL OUTCOME

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Abstract *Background.* Increased levels of P-glycoprotein occur in some osteosarcomas. In this study we determined the relation between P-glycoprotein status and outcome in patients with high-grade osteosarcoma.

Methods. P-glycoprotein status was determined immunohistochemically in specimens of osteosarcoma of the extremities (stage II) from 92 patients who were treated with surgery and chemotherapy. The P-glycoprotein status was analyzed in relation to the length of event-free survival.

Results. The presence of increased levels of P-glycoprotein in the osteosarcoma was significantly associated with a decreased probability of remaining event-free after diagnosis ($P = 0.002$). In a multivariate analysis, P-glycoprotein status ($P = 0.001$) and the extent of tumor necrosis after preoperative chemotherapy ($P = 0.04$) were independent predictors of clinical outcome. The risk of adverse events was increased substantially (rate ratio, 3.37; 95 percent confidence interval, 1.60 to 7.10) among patients with increased levels of P-glycoprotein in tumor cells, as compared with patients who did not have increased levels of P-glycoprotein in tumor cells.

Conclusions. In patients with high-grade osteosarcoma treated with surgery and chemotherapy, the presence of increased levels of P-glycoprotein in tumor cells is associated with a significantly increased risk of adverse events and is independent of the extent of necrosis after preoperative chemotherapy. (N Engl J Med 1995;333: 1380-5.)

THE resistance of tumors to multiple drugs is a major problem in cancer chemotherapy. P-glycoprotein, a transmembrane ATP-dependent efflux pump encoded by the *MDR1* gene,[1] has a central role in multidrug resistance in vitro,[2] and its clinical relevance is under investigation.[3,4] Increased amounts of P-glycoprotein may confer multidrug resistance on cells by preventing the intracellular accumulation of a variety of cytotoxic drugs,[5] including doxorubicin, the most effective agent for the treatment of osteosarcomas.

Drug resistance may represent an important prognostic factor in osteosarcoma, the most frequent primary malignant bone tumor. Although the outcome of osteosarcoma has improved considerably since the addition of chemotherapy to regimens involving surgery, systemic relapses still occur in 40 to 50 percent of cases.[6] Preliminary observations have shown a wide range of P-glycoprotein levels in osteosarcomas,[7-9] and a trend toward a poorer outcome in patients whose tumors express high levels of the *MDR1* gene.[8,9] Moreover, as compared with the primary lesions, a significantly higher proportion of the metastases of osteosarcoma were resistant to multiple drugs.[9] The ability to predict the course of osteosarcoma by identifying patients who will not respond to conventional chemotherapy would help guide treatment, allowing the inclusion of agents capable of modulating multidrug resistance.[10-14]

We sought to determine whether the presence of increased levels of P-glycoprotein is a predictor of the clinical behavior of osteosarcoma. We studied 92 patients with high-grade osteosarcoma of the extremities who were treated with surgery and chemotherapy.

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METHODS

Patients and Tumors

Between September 1986 and December 1989, 319 patients with newly diagnosed osteosarcomas were seen at the Rizzoli Institute in Bologna, Italy. Of these, 67 did not have conventional high-grade osteosarcoma (26 had malignant fibrous histiocytoma of bone; 11, parosteal osteosarcoma; 7, periosteal osteosarcoma; 7, low-grade central osteosarcoma; 6, post-radiation osteosarcoma; 6, osteosarcoma as part of dedifferentiated chondrosarcoma; and 4, osteosarcoma arising in Paget's disease), 33 had evidence of metastases, 24 did not have osteosarcoma of the extremities, 7 were older than 40 years, and 1 had received treatment elsewhere. Of the remaining 187 patients who were considered eligible for a clinical study whose aim was to optimize chemotherapy for osteosarcoma,[15] 23 declined to participate and were treated with surgery followed by chemotherapy. The clinical and pathological characteristics of the remaining 164 patients have been described previously.[15]

The present study included 92 patients for whom tumor samples from the biopsy specimens (obtained before chemotherapy and preserved in archival paraffin-embedded tissue blocks) were available for immunohistochemical analysis. Biopsy specimens from the remaining 72 patients were inadequate for P-glycoprotein evaluation (47 patients underwent a needle biopsy and 25 patients had been given a diagnosis elsewhere). Table 1 summarizes the clinical and pathological characteristics of the 92 patients with adequate tissue for analysis and compares them with the entire group of 164 patients. There were no significant differences between the two groups, indicating that the subgroup of 92 patients whose tumors were analyzed for P-glycoprotein was representative of the group as a whole. All tumors were classified as stage II conventional high-grade osteosarcomas.[16,17] The tumors were classified as intracompartmental (stage IIA) in 2 patients and extracompartmental (stage IIB) in 90 patients.

Chemotherapy was given before and after surgery.[15] The preoperative regimen included two cycles of methotrexate (8 g per square meter of body-surface area) plus leucovorin calcium (180 mg), cisplatin (120 mg per square meter), and doxorubicin (60 mg per square meter). In each case, the surgical procedures took into account the location and extent of the tumor and the life expectancy of the patient. A limb-salvage procedure was performed in 83 patients (90 percent), whereas 9 (10 percent) underwent amputation. The surgical margins of the tumor specimens were histologically defined according to the system of Enneking et al.[17] The margins were radical in

Table 1. Clinical and Pathological Features of the Subgroup of 92 Patients with Osteosarcoma in Whom P-Glycoprotein Immunoreactivity Was Assessed and of the Entire Group of 164 Patients with Osteosarcoma.*

Variable	Subgroup (N = 92)	Total Group (N = 164)
	no. of patients (%)	
Age		
≤14 yr	43 (47)	75 (46)
>14 yr	49 (53)	89 (54)
Sex		
Male	45 (49)	89 (54)
Female	47 (51)	75 (46)
Anatomical site		
Femur	44 (48)	81 (49)
Tibia	25 (27)	49 (30)
Humerus	16 (17)	23 (14)
Pelvis	6 (7)	6 (4)
Other	1 (1)	5 (3)
Histologic grade†		
3	6 (7)	12 (7)
4	86 (93)	152 (93)
Histologic subtype		
Osteoblastic	63 (68)	105 (64)
Chondroblastic	13 (14)	23 (14)
Fibroblastic	7 (8)	12 (7)
Telangiectatic	9 (10)	22 (13)
Other	0	2 (1)
Response to chemotherapy‡		
Good	67 (73)	117 (71)
Poor	25 (27)	47 (29)

*Because of rounding, not all subgroups total 100 percent.

†The criteria of Dahlin and Unni[16] were used to establish the histologic grade.

‡The response to chemotherapy was considered good if the extent of tumor necrosis was 90 percent or greater and poor if it was less than 90 percent.

8 patients (9 percent), wide in 73 (79 percent), marginal in 5 (5 percent), and intralesional in 6 (7 percent). The extent of tumor necrosis was evaluated according to a previously described semiquantitative method.[18] The response to chemotherapy was considered good if the extent of tumor necrosis was 90 percent or greater and poor if it was less than 90 percent. Sixty-seven patients had a good response (73 percent), whereas 25 had a poor response (27 percent). Postoperative chemotherapy for those with a good response included a 21-week course of the same drugs used preoperatively, whereas patients with a poor response received a 30-week regimen that also contained ifosfamide (2 g per square meter) and etoposide (120 mg per square meter). During and after postoperative chemotherapy, the patients underwent clinical and radiographic evaluation of the operated-on limb and the chest every two months. After completing chemotherapy, the patients were seen every two months for the first two years, every four months during the third year, and every six months thereafter.

Adverse events were defined as a recurrence of the tumor at any site, local or systemic, or death during remission. Event-free survival was calculated from the date of the initial diagnosis. All patients were followed for a minimum of 63 months (median, 72). Results were updated in December 1994: 64 patients (70 percent) were free of disease, whereas 28 (30 percent) had pulmonary metastases.

Monoclonal Antibodies and Immunohistochemical Analysis

Monoclonal antibodies C219 (Centocor, Malvern, Pa.), MRK16 (Kamiya Biomedical, Thousand Oaks, Calif.), and JSB-1 (Sanbio, Uden, the Netherlands) recognize different, mutually exclusive epitopes of P-glycoprotein.[19-21] Optimal conditions for P-glycoprotein immunostaining were established on sections obtained from the drug-sensitive U-2 OS human osteosarcoma cell line and its multidrug-resistant derivative,[22] U-2 OS/DX[30]. The drug resistance of U-2 OS/DX[30] is 15 times higher than that of its parent; this is a low level of resistance that is representative of that generally present in clinical settings.[23] For P-glycoprotein immunostaining, the cells were pelleted, fixed with buffered formalin for 24 hours, and embedded in paraffin. Tissue sections from normal kidney were also used because the reactivity of proximal tubules differs from that of glomeruli, so that the results of P-glycoprotein immunostaining are positive in proximal tubules and negative in glomeruli.[24,25] The optimal dilutions were found to be 1:3 for C219, 1:80 for MRK16, and 1:20 for JSB-1. Only JSB-1 and MRK16 were used in the tumor samples because these produced the least variation in the results of immunostaining in different experiments and produced uniform results in tissue sections.

Five-micrometer sections from undecalcified, formalin-fixed, paraffin-embedded tissue samples were placed on poly-L-lysine–coated slides (Sigma, St. Louis). The avidin–biotin–peroxidase procedure was used for immunostaining.[26] After deparaffinization and rehydration, the sections were treated with 0.3 percent methanol–hydrogen peroxide to block endogenous peroxidase activity, incubated with normal horse serum (Vector, Burlingame, Calif.) for 30 minutes at 37°C, and incubated with primary antibody overnight in a moist chamber at 4°C. The following day, the tissue sections were incubated with secondary biotinylated horse antimouse antibody and with avidin–biotin–peroxidase complex (Vector). The final reaction product was revealed by exposure to 0.03 percent diaminobenzidine (Sigma), and the nuclei were counterstained with Gill's hematoxylin. For each specimen, a negative control was obtained by staining the sample with the secondary antibody only, and a positive control was obtained by using a monoclonal antibody for vimentin (V-9, Boehringer, Mannheim, Germany; 1:100 dilution) as a primary antibody. In each experiment, normal kidney tissue was also included as a control.

The results of P-glycoprotein immunostaining were independently interpreted by three observers who had no previous knowledge of the clinical outcome in each patient. The results were expressed according to a semiquantitative scale. Tumor samples were graded as zero when there was a complete absence of staining for P-glycoprotein. The P-glycoprotein–positive tumor samples were graded from 1 to 3 according to the distribution of positivity and the degree of immunostaining of the plasma membrane and the Golgi region, as follows: a score of 1, scattered positive cells (involvement of less than 10 percent of the specimen) and weak immunostaining; a score of 2, diffuse positivity (more than 10 percent of the specimen) and weak immunostaining; and a score of 3, diffuse positivity (more than 10 percent of the specimen) and strong immunostaining (Fig. 1). The highest degree of positivity found in any area of the section was recorded. On the basis of the findings obtained in osteosarcoma cell lines, increased levels of P-glycoprotein were inferred in the samples graded either 2 or 3.

Statistical Analysis

Fisher's exact test[27,28] was used to evaluate the association between two dichotomous variables. Kaplan–Meier plots[29] and the log-rank test[30] were used to evaluate the association of the percentage of necrosis and the expression of P-glycoprotein with event-free survival. Cox proportional-hazards regression analysis with forward selection of variables[31] was performed to estimate the rate ratios for possible risk factors for the occurrence of adverse events. Data analysis was performed with the SAS/PC statistical package, version 6.04 (SAS Institute, Cary, N.C.).

Results

Of the 92 osteosarcomas we examined, 56 did not show any immunoreactivity for P-glycoprotein and 8 had only scattered positive cells. These 64 tumors (70 percent) were considered P-glycoprotein–negative. Of the remaining 28 tumors, P-glycoprotein immunostaining was diffuse, although weak in intensity, in 12, and diffuse and strong in 16. These 28 tumors (30 percent) were considered to have increased levels of P-glycoprotein. In these specimens, both membrane and cy-

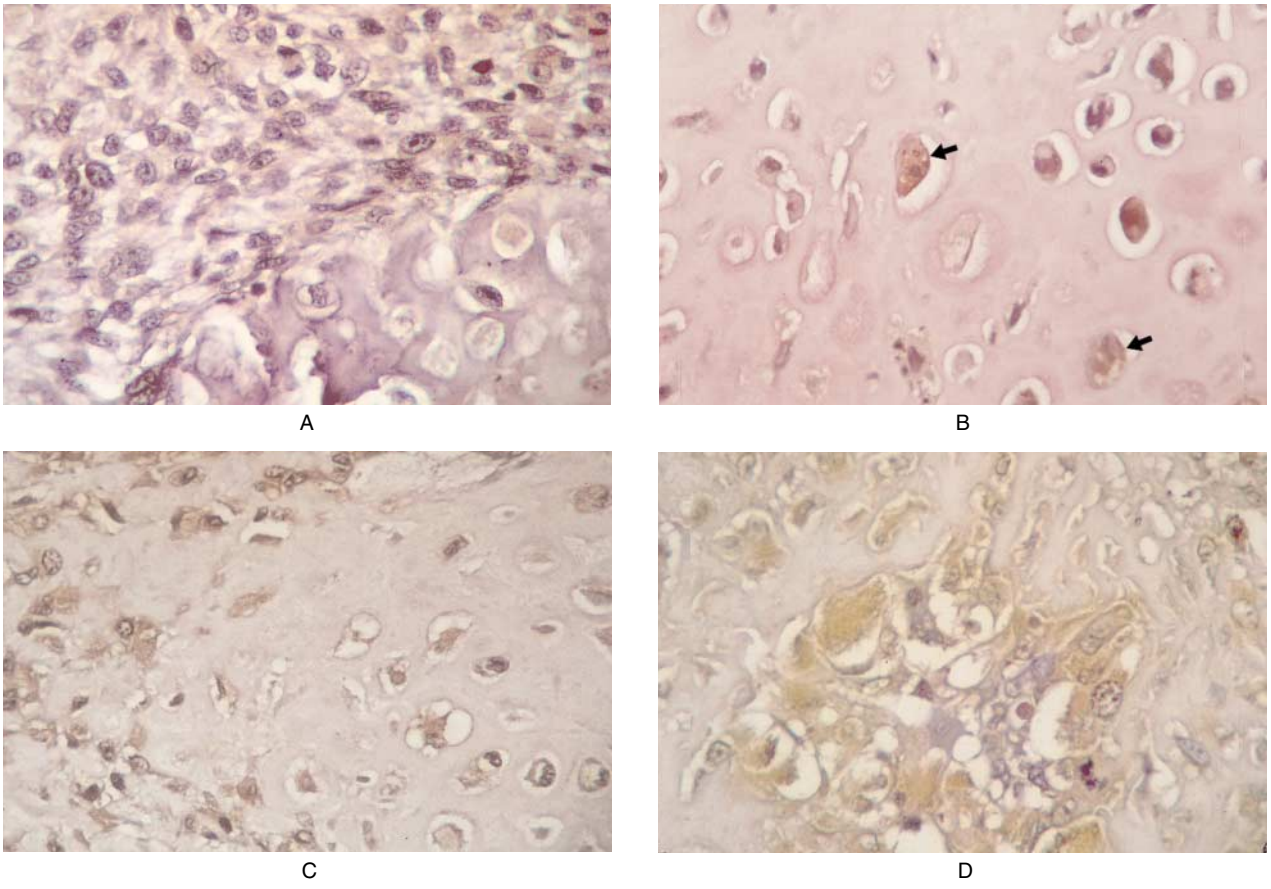

Figure 1. Immunohistochemical Detection of P-Glycoprotein in Osteosarcoma with the Monoclonal Antibody JSB-1.
The brown stain indicates the presence of P-glycoprotein in the tumor cells. There is no detectable staining in Panel A, only a few positive cells (less than 10 percent) in Panel B (arrows), weak reactivity in the majority of cells in Panel C, and intense staining in the majority of cells in Panel D (all panels, ×500). Tumors with immunostaining of more than 10 percent of cells (Panels C and D) were considered to be positive for P-glycoprotein.

toplasmic immunoreactivity was observed on exposure to the monoclonal antibodies JSB-1 and MRK16.

Association of the Expression of P-Glycoprotein with Clinical and Pathological Features

Immunostaining for P-glycoprotein was positive more frequently in female patients than in male patients, but this association was not statistically significant. In contrast, increased levels of P-glycoprotein were more frequent in patients 14 years old or younger than in older patients. Elevated levels of P-glycoprotein were also associated with the anatomical site; proximal tumors, arising in the pelvis (6 patients), proximal femur (5 patients), or proximal humerus (15 patients) were more frequently positive for P-glycoprotein than distal tumors, arising in the distal femur (39 patients), tibia (25 patients), distal humerus (1 patient), or radius (1 patient). No significant difference in the incidence of P-glycoprotein positivity was found among the histologic subtypes. However, P-glycoprotein levels were not elevated in grade 3 tumors, but were elevated in one third of grade 4 osteosarcomas. The level of expression of P-glycoprotein did not differ significantly between patients in whom resection was adequate (radical or wide margins) and those in whom resection was inadequate (marginal or intralesional margins).

No relation was found between the level of expression of P-glycoprotein and the extent of tumor necrosis after preoperative chemotherapy; overexpression of P-glycoprotein was found in tumors from 6 of 25 patients (24 percent) with a poor response ($<$90 percent tumor necrosis) and from 22 of 67 patients (33 percent) with a good response ($\geq$90 percent tumor necrosis) ($P = 0.46$).

The actuarial event-free survival of the subgroup of 92 patients that was the focus of this study was nearly identical to that of the entire group of 164 patients ($P = 0.45$). In the subgroup, increased levels of P-glycoprotein were significantly associated with a decreased probability of remaining event-free after diagnosis ($P = 0.002$) (Fig. 2); the probability of remaining event-free was 42 percent in patients with increased levels of P-glycoprotein and 80 percent in patients without such

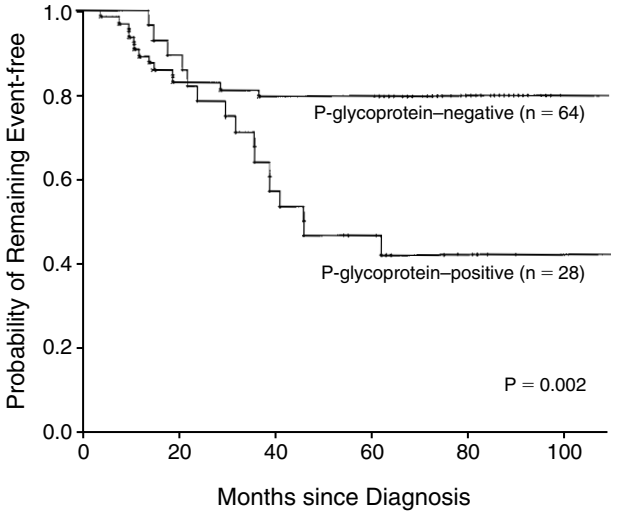

Figure 2. Probability of Event-free Survival in 92 Patients with Osteosarcoma and either P-Glycoprotein–Positive or P-Glycoprotein–Negative Tumors.

Each tick mark represents a patient who had not had any adverse events as of the time of the last follow-up visit.

overexpression. By contrast, the degree of necrosis ($\geq$90 percent or $<$90 percent) after preoperative chemotherapy in the subgroup of 92 patients was not significantly related to the probability of remaining event-free ($P=0.09$), in agreement with the findings in the entire group of 164 patients (Fig. 3).[15] Other clinicopathological features, including age, sex, anatomical site, histologic grade and subtype, and surgical margin, were not associated with the outcome (data not shown).

Table 2 shows the results of Cox proportional-hazards regression analysis in which the response to P-glycoprotein immunostaining and the histologic response after preoperative chemotherapy were adjusted simultaneously to estimate the rate ratios for the occurrence of adverse events in patients with osteosarcoma. After adjustment for possible confounders, positive immunostaining for P-glycoprotein persisted as an independent risk factor for a poor outcome. Patients with increased levels of P-glycoprotein had a significantly higher rate ratio for adverse events (3.37) than patients without such overexpression. Multivariate analysis revealed that a poor response to chemotherapy was also associated with a higher probability of adverse events.

Discussion

We found that high levels of P-glycoprotein in tumor cells were prognostic in patients with osteosarcoma. This highly malignant bone tumor commonly affects adolescents and young adults and, when treated only with surgical removal of the primary lesion, has an extremely aggressive course, with the appearance of metastases in more than 80 percent of cases.[32] The addition of chemotherapy to a regimen of surgery has significantly improved the outcome of osteosarcoma,[6,15,33] but despite an increase in the long-term rate of disease-free survival, metastases develop in a considerable proportion of patients, most of whom die. Since resistance to antineoplastic agents is likely to be a critical factor in the progression of the disease, an understanding of the mechanisms of refractoriness to chemotherapy is important. Multidrug resistance, a well-characterized type of resistance mediated by the *MDR1* gene product P-glycoprotein, involves, among other agents, doxorubicin, the most effective drug in the treatment of osteosarcomas.[34]

We used immunohistochemical methods to examine paraffin-embedded tissue sections of osteosarcoma for the presence of P-glycoprotein. We standardized the approach by using U-2 OS human osteosarcoma cells and their multidrug-resistant derivative, U-2 OS/DX^{30}, as negative and positive controls, respectively. The U-2 OS/DX^{30} cell line is 15 times more resistant to doxorubicin than its parent, U-2 OS.[22] U-2 OS/DX^{30} has a low level of resistance, as is common in most human tumors.[23] The reliability of the monoclonal antibodies against P-glycoprotein that we used (JSB-1 and MRK16) has been extensively investigated in cell lines[20,21,35] and tissue samples.[24,25,35,36] With the use of these antibodies, we were able to evaluate immunohistochemically how representative a specimen was as well as its P-glycoprotein status. Moreover, the availability of archival specimens for this analysis enabled us to evaluate the usefulness of P-glycoprotein status as a prognostic marker after an adequate clinical follow-up period.

Reports of multidrug resistance in sarcomas suggest that levels of P-glycoprotein may be increased at the

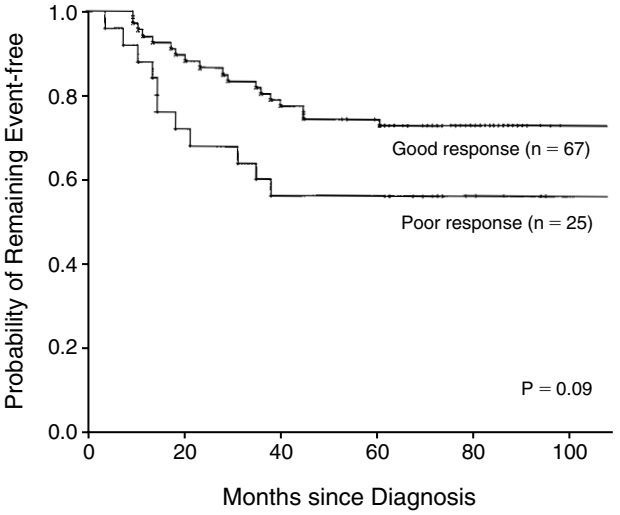

Figure 3. Probability of Event-free Survival in 92 Patients with Osteosarcoma and either Good or Poor Histologic Responses to Preoperative Chemotherapy.

The response to chemotherapy was considered good if the extent of tumor necrosis was 90 percent or greater and poor if it was less than 90 percent. Each tick mark represents a patient who had not had any adverse events as of the time of the last follow-up visit.

Table 2. Results of Cox Proportional-Hazards Regression Analysis, Adjusted for the Response to P-Glycoprotein Immunostaining and the Histologic Response after Preoperative Chemotherapy.*

VARIABLE	ADJUSTED RATE RATIO	95% CI†	P VALUE‡
P-glycoprotein immunoreactivity			
Positive	3.37	1.60–7.10	0.001
Negative	1.00		
Histologic response after chemotherapy			
Poor	2.25	1.05–4.80	0.04
Good	1.00		

*The response to chemotherapy was considered good if the extent of tumor necrosis was 90 percent or greater and poor if it was less than 90 percent.

†CI denotes confidence interval.

‡Chi-square of the model, P = 0.002.

onset of clinical disease,[37-41] and in soft-tissue sarcomas of childhood, a finding of increased levels of P-glycoprotein is strongly associated with a poor outcome.[42] In our study of patients with high-grade osteosarcoma of the extremities, all of whom were treated with the same regimen of chemotherapy in addition to surgical removal of the primary lesion, increased levels of P-glycoprotein were significantly associated with a poorer event-free–survival rate.

Our results indicate that immunoreactivity to P-glycoprotein and, to a lesser extent, chemotherapy-induced necrosis are two variables related to the clinical outcome of high-grade osteosarcoma. The histologic response to chemotherapy is generally considered the most reliable indicator of the clinical course of osteosarcoma,[43] although, at least by univariate analysis, its prognostic value appears to be consistently reduced in the long term.[15]

The absence of a relation between the level of P-glycoprotein and the extent of tumor necrosis might reflect the killing of a majority of tumor cells by a drug that acts independently of P-glycoprotein. It is also possible that P-glycoprotein status and tumor necrosis status identify different phenomena[44]; increased levels of P-glycoprotein may represent not only resistance to chemotherapy, but also tumor progression.

The most important finding of this study is that in patients with high-grade osteosarcomas of the extremities, which had not metastasized at the beginning of the study, the presence of increased levels of P-glycoprotein could be used to identify tumors with a tendency to progress despite chemotherapy. The risk of adverse events was increased more than threefold in patients whose tumors had increased levels of P-glycoprotein. Moreover, in patients whose tumors contained little or no P-glycoprotein, the probability of event-free survival was significantly higher than in patients whose tumors had increased levels of P-glycoprotein, regardless of the histologic response to chemotherapy. Another advantage of the use of P-glycoprotein status over estimation of the extent of necrosis as a prognostic factor is that the former can be determined at the time of initial diagnosis, before any therapy is initiated.

Our results may influence the selection of patients for new treatments for osteosarcoma that combine conventional antineoplastic drugs and agents that can modulate the multidrug-resistance phenotype.[10,11,13,45] Agents that modify the multidrug-resistance phenotype, such as verapamil and cyclosporine, completely reverse the phenotype in vitro by competing with anticancer drugs for binding sites on P-glycoprotein,[46,47] and they have been included in recent protocols for a number of malignant conditions.[12-14,45,48] Patients with osteosarcoma and increased levels of P-glycoprotein may benefit from the addition of such agents to the treatment regimen to enhance the cytotoxic effects of chemotherapy.

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