May P-glycoprotein status be used to stratify high-grade osteosarcoma patients? Results from the Italian/ Scandinavian Sarcoma Group 1 treatment protocol

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Abstract. The aim was to evaluate the clinical impact of P-glycoprotein in primary non-metastatic high-grade osteosarcoma patients, treated with neoadjuvant chemotherapy protocols. P-glycoprotein was assessed by immunohistochemistry on paraffin-embedded tissue samples collected at time of diagnosis from 94 osteosarcoma patients, treated with the Italian Sarcoma Group/Scandinavian Sarcoma Group 1 (ISG/SSG 1) protocol. P-glycoprotein-positivity at diagnosis was found in 53/94 ISG/SSG 1 cases (56%) and emerged as the single factor significantly associated with an unfavourable outcome from survival and multivariate analyses. A comparative analysis of the subgroup of 94 patients considered for P-glycoprotein evaluation and the whole series of ISG/SSG 1 patients showed that this marker retained its prognostic value also in the latter group. In osteosarcoma patients treated with doxorubicin-based chemotherapy protocols, P-glycoprotein overexpression at diagnosis is an important adverse prognostic factor for outcome. P-glycoprotein evaluation can therefore constitute the basis for stratifying, at diagnosis, osteosarcoma patients for whom alternative treatments may be considered.

Introduction

Several studies have focused on the analysis of factors that may be predictive for chemotherapy response and prognosis of high-grade osteosarcoma (OS) patients. The common aim of these studies has been the identification of markers, which may be useful for patient stratification in different subgroups on the basis of tumour biology and individual risk evaluation.

Among prognostic factors at presentation that have been identified so far, the more frequently and constantly reported have been tumour volume, serum alkaline phosphatase levels and P-glycoprotein expression (1-7). P-glycoprotein overexpression is mainly associated with chemoresistance to doxorubicin and, in contrast to tumour volume and alkaline phosphatase level, assessment of P-glycoprotein level in tumour tissue harbours potential predictive as well as prognostic information.

However, there is still a lack of general consensus about the relative prognostic value of P-glycoprotein expression, since uncertain and sometimes conflicting data have been reported. Several factors may be responsible for these discrepancies but, in general, it has to be taken into account that the predictive value of this prognostic factor may be significantly influenced not only by the inherent biology of the tumour but also by the treatment modalities. Therefore, the analysis of the predictive value of P-glycoprotein expression has to be considered in relation to both the clinicopathologic features and characteristics of each treatment protocol.

The adverse prognostic value of P-glycoprotein overexpression, which we have invariably found in our previous studies (8-12), has been confirmed in several analyses performed by different institutions (6,13-15), but also questioned in other reports (16-18). By reviewing the literature on P-glycoprotein's clinical impact in high-grade OS, it appears that the different conclusions obtained in some studies may have several possible reasons, which include not only differences in sample series size or technical approaches (such as tissue processing or methodologies and antibodies used for the assays) but also in treatment protocols. All these parameters vary from study to study and the impact of each of them, although it cannot be precisely estimated and quantified, may significantly influence the biologic and prognostic value of P-glycoprotein.

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In this study, we assessed the expression of P-glycoprotein in 94 high-grade OS patients, who were homogeneously treated with the Italian Sarcoma Group/Scandinavian Sarcoma Group 1 (ISG/SSG 1) treatment protocol. Moreover, we have compared the current data with those obtained in previous doxorubicin-based treatment regimens performed at the Rizzoli Institute (8,9,11).

Patients and methods

Patient eligibility and treatment protocols. From March 1997 to September 2000, 182 patients with newly diagnosed OS entered the ISG/SSG 1 treatment protocol (19). The eligibility criteria for this protocol were: diagnosis of central high-grade OS of the extremity, age younger than 40 years, absence of metastases at the time of diagnosis, and no prior chemotherapy or surgical treatment for bone lesions. Informed consent was collected from all patients in accordance with the standard procedure in each country and with the local ethics committee approval.

Primary treatment consisted of two blocks of high-dose ifosfamide (15 g/m^2), high-dose methotrexate (12 g/m^2), and cisplatin/doxorubicin (cisplatin 120 mg/m^2; doxorubicin 75 mg/m^2). Post-operatively, patients underwent two cycles of doxorubicin (90 mg/m^2) and three cycles each of high-dose ifosfamide (15 g/m^2), methotrexate (12 g/m^2) and cisplatin (120-150 mg/m^2). The total dose of doxorubicin was 330 mg/m^2. Protocol compliance and protocol received dose intensity were calculated as previously described (19).

The type of surgery was chosen depending on the size and location of the tumour, as well as on the patient's age and life quality evaluation, after careful pre-operative staging aimed to achieve adequate surgical margins. Radical and wide surgical margins according to the Enneking score system (20) were considered as adequate, whereas marginal and intralesional margins were considered as inadequate. Three patients did not undergo surgery because of the occurrence of adverse events during the pre-operative phase.

On the basis of the evaluation of tumour necrosis, a good histological response was considered when the extent of necrosis was scored as total or almost total, according to the Picci method (21). A good response defined by using this system roughly corresponds to the Huvos grade 3-4 score.

Tumour volume was evaluated on CT-scans taken at the time of diagnosis. According to volume, tumours were classified in two groups by using the value of 100 ml as cut-off. Alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) serum levels were assessed at time of diagnosis, before starting chemotherapy.

After the end of post-operative chemotherapy, patients were continuously followed and their clinical data was updated. Adverse events were defined as recurrence of the tumour at any site or death during remission. Event-free survival was calculated from the first day of chemotherapy until the date of first relapse, tumour-related death or last follow-up examination. Overall survival was calculated from the first day of chemotherapy until death or the last follow-up examination.

Immunohistochemistry. Tumour samples for immunohistochemical analysis were available from biopsy specimens of 105/182 cases (58%). In the remaining 77 cases, tumour tissue of paraffin-embedded needle biopsies was not sufficient to allow an appropriate immunohistochemical evaluation. Immunohistochemical evaluations were performed in a prospective, blinded fashion during the whole treatment protocol.

Expression of P-glycoprotein was evaluated on paraffin-embedded tumour specimens with the three monoclonal antibodies JSB-1 (Sanbio, Uden, The Netherlands), MRK16 (Kamiya Biomedical, Thousand Oaks, CA, USA) and C494 (Signet Laboratories, Dedham, MA) by using an avidin-biotin peroxidase complex method (Vectastain ABC kit, Vector Laboratories, Burlinghame, CA, USA), as previously described (11). Primary antibodies were incubated overnight at 4°C at the following concentrations: 12.5 μg/ml for JSB-1, 0.003 μg/ml for MRK16, and 0.2 μg/ml for C494. Antibody dilution rates were defined on paraffin-embedded sections of the U-2OS human osteosarcoma cell line and of its doxorubicin-resistant variant U-2OS/DX30, as previously described (8). Under these conditions, only samples with P-glycoprotein levels similar or higher to those of U-2OS/DX30 cells resulted as positive. The final reaction product was revealed by incubation with diaminobenzidine (Sigma, St. Louis, MO) and nuclei were counterstained with Gill's hematoxylin (Sigma).

For each specimen, both negative and positive controls for immunostaining were performed. Negative controls were carried out by replacing the primary antibody with normal horse serum. For each case, one section was also immunostained with the V9 anti-vimentin monoclonal antibody (Roche Molecular Biochemicals, Mannheim, Germany), in order to control the preservation of antigenicity of the tumour specimen: samples which were negative for vimentin were not considered as eligible for the immunohistochemical study. Among the 105 cases considered for this study, 11 were finally excluded for the lack of any positive reaction to vimentin. Sections of normal kidney were used as positive control for P-glycoprotein. Only specimens with a positive immunostaining for at least two out of three monoclonal antibodies in >10% of tumour cells were considered as P-glycoprotein-positive.

Statistics. Differences among means were analysed with the Student's t-test. Two-tailed Fisher's exact test was used to evaluate the statistical association between two variables. Kaplan-Meier and log-rank methods were used to draw and evaluate the significance of survival curves. Cox's proportional-hazards regression analysis was performed to estimate the rate ratios of each possible risk factor for the occurrence of adverse events.

Results

Among the 182 patients, who entered the ISG/SSG 1 chemotherapy protocol, 125 were Italian and 57 were Scandinavian. Tumour tissue samples for immunohistochemistry were available from 105 cases (58%), 94 of which were considered for P-glycoprotein assessment.

Among the 182 ISG/SSG 1 patients, three (two Italians and one Scandinavian) died of chemotherapy-related toxicity and one Italian patient died of pulmonary embolism. These four patients were not considered for the analysis of protocol-

Table I. Comparison of the clinicopathologic characteristics of the 94 cases examined for P-glycoprotein status and of the entire group of 182 ISG/SSG 1 patients.

Variable	94 P-glycoprotein cases (%/total)	182 ISG/SSG 1 cases (%/total)
Group		
ISG	80 (85)	125 (69)
SSG	14 (15)	57 (31)
Gender		
Male	57 (61)	106 (58)
Female	37 (39)	76 (42)
Age (years)		
≤12	18 (19)	42 (23)
>12	76 (81)	140 (77)
Site		
Femur	55 (59)	95 (52)
Tibia	22 (23)	48 (26)
Humerus	14 (15)	30 (16)
Other	3 (3)	9 (5)
Histological subtype		
Osteoblastic	62 (66)	119 (65)
Chondroblastic	6 (6)	13 (7)
Fibroblastic	17 (18)	20 (11)
Telangectatic	2 (2)	6 (3)
Not specified	7 (7)	24 (13)
Surgery[a]		
Resection	87 (93)	164 (92)
Amputation	4 (4)	11 (6)
Rotation plasty	3 (3)	4 (2)
Surgical Margins[b]		
Adequate	83 (97)	147 (94)
Inadequate	3 (3)	10 (6)
Tumor volume[b]		
>100 ml	59 (76)	93 (66)
≤100 ml	19 (24)	47 (34)
ALP[b]		
High	42 (46)	73 (42)
Normal	49 (54)	99 (58)
LDH[b]		
High	19 (22)	46 (28)
Normal	69 (78)	121 (72)
Histologic Response[b]		
Good	14 (15)	30 (17)
Poor	80 (85)	145 (83)

[a]Three patients did not undergo surgery because of the occurrence of adverse events during the pre-operative phase. [b]Total no. does not reach, respectively, 94 or 182 since data were not available for all patients.

Table II. Protocol compliance and protocol received dose intensity in the whole group of ISG/SSG 1 patients and in the subgroup of patients analysed for P-glycoprotein expression.[a]

Group (no. of patients)	PC Mean value ± SE (range)	PRDI Mean value ± SE (range)
ISG/SSG 1 (178)	0.920±0.418 (0.253-1.088)	0.819±0.541 (0.382-1.464)
P-glycoprotein (94)	0.945±0.366 (0.347-1.079)	0.841±0.538 (0.388-1.464)
P-glycoprotein-positive (53)	0.954±0.366 (0.347-1.079)	0.869±0.442 (0.580-1.464)
P-glycoprotein-negative (41)	0.932±0.304 (0.468-1.075)	0.805±0.323 (0.388-1.034)

[a]Patients who died because of chemotherapy-related toxicity (three patients) or pulmonary embolism (one patient) were not considered for these calculations. PC, protocol compliance; PRDI, protocol received dose intensity; SE, standard error.

related factors, relapse rate and survival, and were not among those analysed for P-glycoprotein expression.

Expression of P-glycoprotein and association with clinicopathologic features. The subgroup of 94 patients evaluated for P-glycoprotein resulted in being representative of the whole group of ISG/SSG 1 patients. In fact, no significant differences were found between these two subgroups concerning the distribution of clinicopathologic features (Table I). Protocol compliance and protocol received dose intensity were also similar in these two groups of patients (Table II).

P-glycoprotein-positivity was revealed in 53/94 (56%) cases, all of which presented a positive immunohistochemical reaction in the majority of cells with a moderate to strong intensity of immunostaining.

P-glycoprotein-positivity was not significantly associated with any clinicopathologic feature (Table III). We found a significantly higher frequency of P-glycoprotein-positivity in Scandinavian patients [12/14 cases (86%) compared to 41/80 in Italian patients (51%); P=0.02 by two-tailed Fisher's exact test]. However, this result is most probably biased due to the low number of Scandinavian cases that were available for P-glycoprotein analysis. In fact, the analysis of an additional 45 Scandinavian OS tissue samples from patients treated with the SSG-VIII chemotherapy protocol revealed a positive immunostaining for P-glycoprotein in 17 cases (38%; data not shown), a frequency which was not significantly different to that of Italian cases of the ISG/SSG 1 protocol (P=0.19 by two-tailed Fisher's exact test) and comparable to that of our previous studies (8-12).

Considering the parameters associated with response to chemotherapy, no relationship was found between the expression of P-glycoprotein and the histological response to pre-operative chemotherapy after stratification of patients according to total/almost total (good responders) versus non-total (poor

Table III. Clinicopathologic features of 94 osteosarcomas in relation to positivity for P-glycoprotein at diagnosis.[a]

Variable	Total no. of patients	P-glycoprotein-positive patients		
		No.	%	P value
Group				0.02
ISG	80	41	51	
SSG	14	12	86	
Gender				
Male	57	33	58	
Female	37	20	54	
Age (years)				
≤12	18	11	61	
>12	76	42	55	
Site				
Femur	55	32	58	
Tibia	22	13	59	
Humerus	14	7	50	
Other	3	1	33	
Histological subtype				
Osteoblastic	62	33	53	
Chondroblastic	6	5	83	
Fibroblastic	17	8	47	
Telangectatic	2	2	100	
Not specified	7	5	71	
Surgery				
Resection	87	48	55	
Amputation	4	3	75	
Rotation plasty	3	2	67	
Surgical Margins[b]				
Adequate	83	45	54	
Inadequate	3	2	67	
Tumor volume[b]				
>100 ml	59	33	56	
≤100 ml	19	8	42	
ALP[b]				
High	42	24	57	
Normal	49	27	55	
LDH[b]				
High	19	14	74	
Normal	69	37	54	
Histologic Response				
Good	14	9	64	
Poor	80	44	55	

[a]Only significant P values by two-tailed Fisher's exact test have been reported. [b]Total no. does not reach 94 since data were not available for all patients.

Table IV. Relapse rate in the 94 ISG/SSG 1 patients analysed for P-glycoprotein in relation to P-glycoprotein status and clinicopathologic parameters.[a]

Variable	Total cases	Relapsed cases	Relapse %	P value
P-glycoprotein				0.001
Positive	53	27	51	
Negative	41	7	17	
Gender				
Male	57	23	40	
Female	37	11	30	
Age (years)				
≤12	18	6	33	
>12	76	28	37	
Site				
Femur	55	20	36	
Tibia	22	10	45	
Humerus	14	3	21	
Other	3	1	33	
Histological subtype				
Osteoblastic	62	23	37	
Chondroblastic	6	3	50	
Fibroblastic	17	3	18	
Telangectatic	2	2	100	
Not specified	7	3	43	
Surgery				
Resection	87	32	37	
Amputation	4	2	50	
Rotation plasty	3	0	0	
Surgical Margins[b]				
Adequate	83	29	35	
Inadequate	3	2	67	
Tumor volume[b]				
>100 ml	59	23	39	
≤100 ml	19	5	26	
ALP[b]				
High	42	18	43	
Normal	49	15	31	
LDH[b]				
High	19	8	42	
Normal	69	25	36	
Histologic response				
Good	14	6	43	
Poor	80	28	35	

[a]Only significant P values by two-tailed Fisher's exact test have been reported. [b]Total no. does not reach 94 (Total cases) or 34 (Relapsed cases) since data were not available for all patients.

responders) tumor necrosis (Table III). In order to compare this finding with those of our previous studies, we also evaluated the relationships between P-glycoprotein expression and tumor necrosis by using either the 90% cut-off or the Huvos grade. This analysis further confirmed the lack of correlation between these two parameters (data not shown).

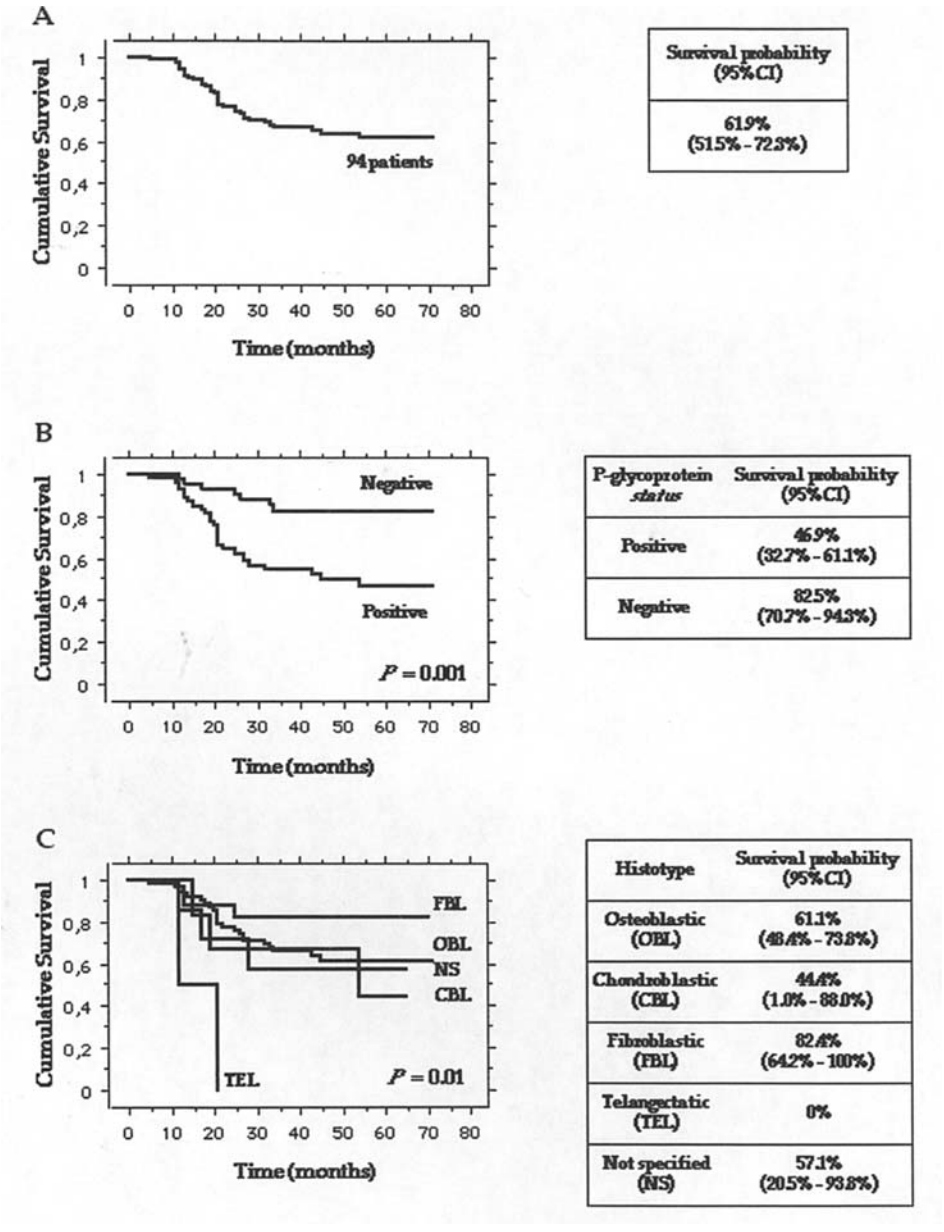

Figure 1. Event-free survival curve in the 94 ISG/SSG 1 osteosarcoma patients analysed for P-glycoprotein expression (A) and according to P-glycoprotein status (B) or histological subtype (C). Survival tables show cumulative survival probabilities with their corresponding 95% confidence intervals (CI). Comparison of survival curves was performed by the log-rank test.

Table V. Time to relapse in the 94 ISG/SSG 1 patients analysed for P-glycoprotein status.

Group (no. of patients)	Time to relapse (months)	
	Median	Range
P-glycoprotein (94)	21	5-54
P-glycoprotein-positive (53)	21	5-54
P-glycoprotein-negative (41)	25	12-34

Regarding adherence to protocol, no difference was found in received dose and dose-intensity between the P-glycoprotein-positive and -negative patients (Table II).

Clinical outcome and survival analyses. Relapse developed in 34/94 patients analysed for P-glycoprotein (36%). Only P-glycoprotein-positivity was significantly associated with a higher relapse rate (P=0.001; Table IV). P-glycoprotein status did not influence the median time to relapse, which was similar in P-glycoprotein-positive and -negative patients (Table V). It is worthwhile noting that all P-glycoprotein-negative patients relapsed within three years from diagnosis whereas P-glycoprotein-positive patients relapsed also at later intervals (Table V). However, no significant difference was found concerning the percentage of patients who relapsed within three years from diagnosis in the two subgroups, since 24 out of 27 P-glycoprotein-positive patients (89%) relapsed in the first 36 months of follow-up.

Survival analyses considered both event-free and overall survival. The global event-free survival curve showed a survival probability of 61.9% (Fig. 1A), with the majority of

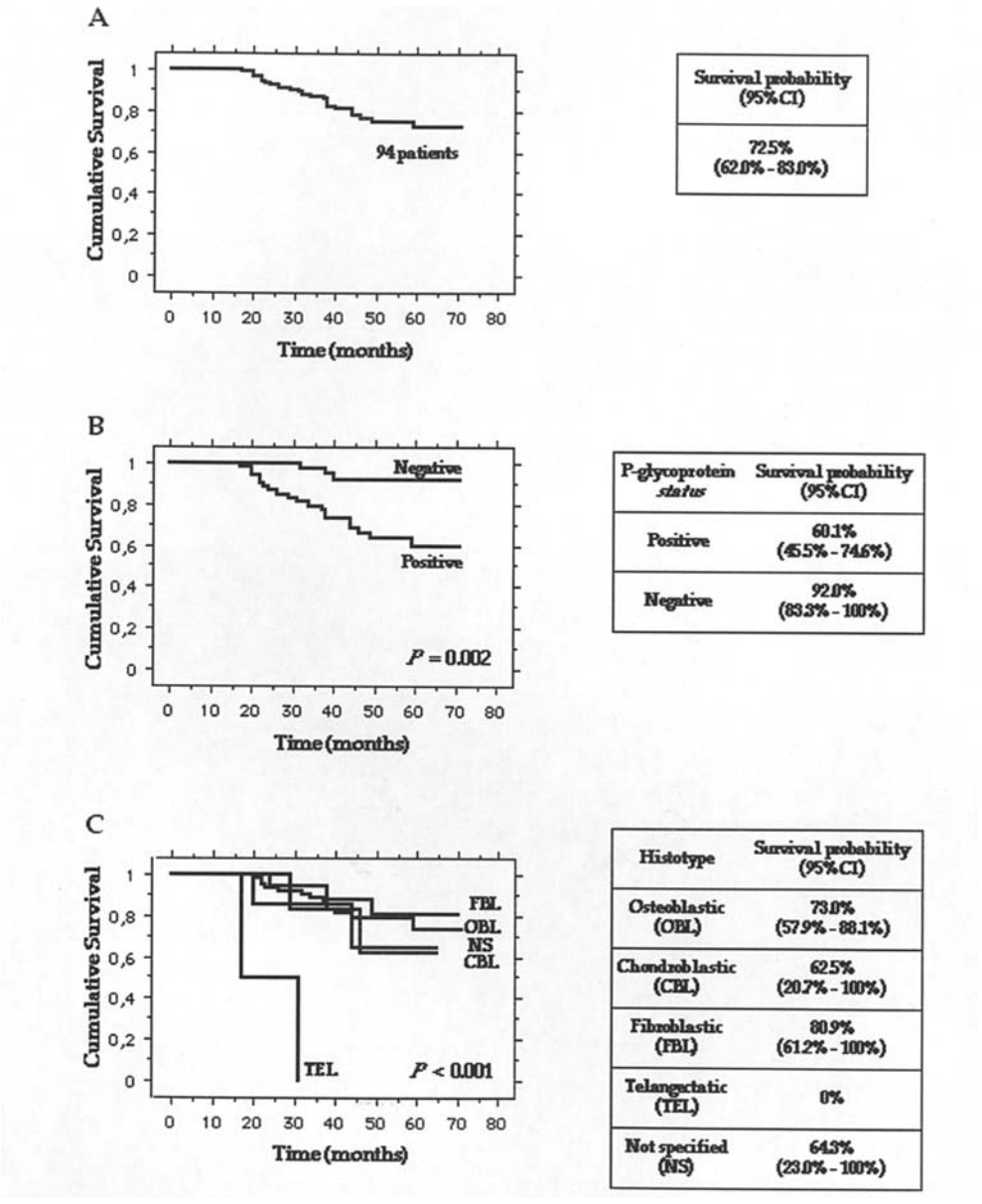

Figure 2. Overall survival in the 94 ISG/SSG 1 osteosarcoma patients analysed for P-glycoprotein expression (A) and according to P-glycoprotein status (B) or histological subtype (C). Survival tables show cumulative survival probabilities with their corresponding 95% confidence intervals (CI). Comparison of survival curves was performed by the log-rank test.

adverse events occurring within the first three years of follow-up. A significantly worse event-free survival was associated only with P-glycoprotein-positivity (P=0.001; Fig. 1B) and telangectatic histotype (P=0.01; Fig. 1C).

Overall survival analyses showed a survival probability of 72.5% (Fig. 2A), and further confirmed the adverse prognostic value of P-glycoprotein positivity (P=0.002; Fig. 2B) and telangectatic histotype (P<0.0001; Fig. 2C), already found by event-free survival analysis. No other clinicopathologic features emerged as significant by survival analyses (data not shown).

Multivariate analysis was performed by considering the parameters that resulted to be significantly associated with clinical outcome by univariate analyses. Cox's proportional-hazards regression analysis applied to event-free survival data revealed that only P-glycoprotein-positivity was significantly associated with a worse outcome and a higher risk ratio for adverse events, whereas telangectatic histotype lost its significance (Table VI). The same evidence was found when the analysis was applied to overall survival data (Table VI).

Multivariate analysis was performed also in the whole group of 178 ISG/SSG 1 patients, by considering P-glycoprotein together with tumour volume at diagnosis and baseline ALP levels, the two parameters which resulted to be significantly associated with clinical outcome in this treatment protocol (19). Only P-glycoprotein-positivity retained its adverse prognostic value for both event-free and overall survival, further confirming its highly significant clinical impact (Table VII).

Evaluation of the prognostic value of P-glycoprotein in relation to different chemotherapy protocols. The adverse prognostic impact of P-glycoprotein-positivity was also

Table VI. Cox's proportional-hazards regression analyses in 94 osteosarcoma patients adjusted for P-glycoprotein status and histologic subtype.

	Variable	Adjusted risk-rate ratio (95% confidence interval)	P value
Event-free survival	P-glycoprotein		0.005
	Positive	3.4 (1.4-7.9)	
	Histotype		ns
	Telangectatic	4.5 (0.7-27.9)	
	Chondroblastic	1.1 (0.2-5.3)	
	Osteoblastic	1.0 (0.3-3.5)	
	Fibroblastic	0.4 (0.1-2.2)	
Overall survival	P-glycoprotein		0.01
	Positive	4.7 (1.4-16.3)	
	Histotype		ns
	Telangectatic	11.2 (1.5-86.1)	
	Chondroblastic	0.9 (0.1-6.7)	
	Osteoblastic	0.9 (0.2-4.0)	
	Fibroblastic	0.7 (0.1-3.9)	

ns, not significant.

Table VII. Cox's proportional-hazards regression analyses in 178 ISG/SSG 1 osteosarcoma patients adjusted for P-glycoprotein status, tumour volume at diagnosis and baseline alkaline phosphatase (ALP) levels.

	Variable	Adjusted risk-rate ratio (95% confidence interval)	P value
Event-free survival	P-glycoprotein		0.01
	Positive	3.0 (1.3-7.1)	
	Tumour volume		ns
	>100 ml	1.3 (0.5-3.6)	
	ALP levels		ns
	High	2.1 (0.9-4.6)	
Overall survival	P-glycoprotein		0.02
	Positive	4.7 (1.3-16.2)	
	Tumour volume		ns
	>100 ml	2.2 (0.5-9.9)	
	ALP levels		ns
	High	1.6 (0.6-4.4)	

ns, not significant.

analysed in relation to different chemotherapy protocols, which had been drawn by considering doxorubicin as the leader drug (Table VIII).

In addition to ISG/SSG 1, the chemotherapy protocols considered for this analysis included the IOR-Adjuvant protocol B (IOR-Adjuvant B) (22) and the neoadjuvant IOR/OS-2, IOR/OS-3a and IOR/OS-3b protocols (3). Among these regimens, there was a progressive decrease of the cumulative doxorubicin dosage, which ranged from 540 mg/m^2 in the IOR-Adjuvant B to 330 mg/m^2 in the ISG/SSG 1 protocol. In parallel, the relative impact of doxorubicin inside each protocol also progressively decreased from the IOR-Adjuvant B, in which doxorubicin was used alone, to the other treatment regimens, in which doxorubicin was administered together with the other three drugs (Table VIII).

As previously demonstrated (8,9,11), the adverse prognostic value of P-glycoprotein-positivity was revealed in all of these protocols. However, in order to compare the results obtained in these different studies with those of ISG/SSG 1, we have calculated the probability of event-free survival at 5 years and of overall survival inside each series in relation to the P-glycoprotein status. This comparative analysis indicated that, in all protocols, the status of P-glycoprotein at diagnosis was able to identify two subgroups of patients with significantly different prognoses. In particular, P-glycoprotein-positive OS patients always presented a worse clinical outcome than P-glycoprotein-negative patients, both in terms of event-free survival probabilities at 5 years and overall survival (Table VIII).

Discussion

Classical OS is the most common malignant tumour of bone and its standard treatment, which includes intensive multiagent chemotherapy and aggressive surgery, results in a cure rate that does not exceed 60-65% (23,24). In the last two decades, several attempts to significantly improve the outcome above this level have failed and, accordingly, inherent or acquired resistance to chemotherapy has emerged as a major obstacle to successful treatment of high-grade OS patients (2,25-27). It is therefore not surprising that, among the different prognostic factors reported so far, overexpression of P-glycoprotein at diagnosis, which is associated with the multidrug-resistant phenotype, is the marker that has been most frequently and constantly reported to be associated with a worse clinical outcome (6). However, a critical analysis of the most relevant studies concerning P-glycoprotein in OS indicates that its actual prognostic value has to be considered not only in relation to clinicopathologic features but also to the characteristics of each treatment regimen.

In this study, we assessed the expression of P-glycoprotein in 94 high-grade OS patients, who were homogeneously treated with the ISG/SSG 1 treatment protocol (19). Evaluation of P-glycoprotein was prospectively performed throughout the whole protocol period and, therefore, investigators were blinded to the clinical outcome data. When the follow-up reached an adequate length, the prognostic value of P-glycoprotein was analysed not only in relation to other clinicopathologic parameters, but also to population origin and treatment protocol characteristics.

The level of P-glycoprotein at diagnosis was assessed on paraffin-embedded OS tissue samples by using the same method, monoclonal antibodies and score system of our

Table VIII. P-glycoprotein prognostic impact in different chemotherapy protocols for high-grade osteosarcoma.

	Protocol (period)			
	IOR-Adjuvant B (1976-1977)	IOR/OS-2 (1986-1989)	IOR/OS-3a IOR/OS-3b (1990-1992)	ISG/SSG 1 (1997-2000)
Drugs (cumulative DX dose)	DX (540 mg/m^2)	DX (480 mg/m^2) MTX, CDDP, IFO	DX (390 mg/m^2) MTX, CDDP, IFO	DX (330 mg/m^2) MTX, CDDP, IFO
Number of cases analysed for P-glycoprotein	37	90	59	94
Event-free survival probability at 5 years ± SE (95% CI)				
PGP-positive	17.6±9.2 (1.0-35.8)	39.3±9.2 (21.2-57.4)	31.6±10.7 (10.7-52.5)	46.9±7.2 (32.7-61.1)
PGP-negative	75.0±9.7 (56.0-94.0)	80.4±5.1 (70.5-90.4)	70.0±7.2 (55.8-84.2)	82.5±6.0 (70.7-94.3)
P value	<0.001	<0.001	0.004	<0.001
Overall survival probability (95% CI)				
PGP-positive	11.8±0.8 (1.0-27.1)	24.1±17.7 (0.1-58.9)	47.4±11.5 (24.9-69.8)	60.1±7.4 (45.5-74.6)
PGP-negative	80.0±0.9 (62.5-97.5)	82.3±17.7 (72.7-91.8)	77.5±6.6 (64.6-90.4)	92.0±4.4 (83.3-100)
P value	0.003	0.04	0.02	<0.001

DX, doxorubicin; MTX, methotrexate; CDDP, cisplatin; IFO, ifosfamide; PGP, P-glycoprotein; SE, standard error; CI, confidence intervals. P values were determined with the Student's t-test for the differences among event-free survival probability average between P-glycoprotein-positive and -negative patients.

previous studies. This has made possible the comparison of data obtained in the present study with those of our previous evaluations, which considered patients treated with an adjuvant protocol with doxorubicin (9) and patients treated with neo-adjuvant multiagent chemotherapy based on doxorubicin, methotrexate and cisplatin (8,11). Our immunohistochemical method was set up in order to reveal levels of P-glycoprotein equal or higher to those exhibited by a doxorubicin-resistant variant of the U-2OS human osteosarcoma cell line (U-2OS/DX30), which has a level of resistance similar to that generally present in clinical settings (8). Moreover, we used three different monoclonal antibodies in order to limit at the minimum level the possibility of false results due to a possible impaired reactivity of any of them.

In addition to the assessment of P-glycoprotein by immunohistochemistry, which has been performed in most OS studies (6), the level of *MDR1* gene expression at diagnosis has been investigated by Wunder *et al* (18). This study is an important contribution to this field and, although it did not reveal any prognostic value for *MDR1* gene overexpression, it showed an incidence of *MDR1* overexpression in the range of those identified by immunohistochemistry. However, it has to be considered that the presence of *MDR1* gene overexpression does not necessarily mean that the level of P-glycoprotein is also increased, and this might partly explain the different results obtained in this study compared to those in which immunohistochemistry was used.

In our study, P-glycoprotein-positivity was found in 56% of ISG/SSG 1 cases, an incidence comparable with those found in our previous evaluations (8-12). We observed a significantly higher incidence of P-glycoprotein-positivity in Scandinavian (86%) compared to Italian patients (51%; P=0.02) but this evidence was not confirmed by the analysis of an additional 45 samples from Scandinavian patients treated with the SSG-VIII protocol, which showed P-glycoprotein-positivity in 38% of cases (data not shown). These data demonstrated that the results concerning the different incidence of P-glycoprotein-positivity in Italian and Scandinavian ISG/SSG 1 patients were most probably biased due to the low number of Scandinavian cases (14) which were available for P-glycoprotein analysis.

Therefore, we can assume that the presence of P-glycoprotein-positivity is not related to the genetic background of Italian and Scandinavian populations.

In order to estimate the prognostic value of biologic and clinicopathologic variables, we evaluated the relapse rate, event-free and overall survival in the subgroup of 94 cases considered for P-glycoprotein analysis.

A higher relapse rate resulted to be significantly associated only with P-glycoprotein-positivity. However, it is worthwhile noting that the presence of increased P-glycoprotein levels did not influence the time to relapse, which was very similar in P-glycoprotein-positive and -negative patients.

Survival analyses confirmed the adverse prognostic value of P-glycoprotein-positivity that, together with the telangectatic histotype, was the only marker invariably and significantly associated with a worse ourcome, either in terms of event-free or overall survival. However, it has to be considered that only two cases were classified as telangectatic and that both these patients were P-glycoprotein-positive, relapsed early and died, probably overestimating the adverse prognostic significance of this histotype. Accordingly, multivariate analyses demonstrated that only P-glycoprotein-positivity was significantly associated with a worse outcome and a higher risk of adverse events, acting as an independent adverse prognostic marker.

The lack of significant influence of ALP levels and tumour volume on outcome, which resulted to be of prognostic value in the whole ISG/SSG 1 group of patients (19), may be explained by the smaller number of patients included in this study. This assumption is supported by the fact that both markers showed a trend toward a higher relapse rate and worse clinical outcome also in the subgroup of 94 patients analysed for P-glycoprotein, although they did not reach statistical significance. Moreover, it has to be taken into consideration that, when considered in a multivariate analysis in the whole group of ISG/SSG 1 patients together with ALP levels and tumour volume, P-glycoprotein status still retained its highly significant prognostic value, further confirming its clinical impact in OS.

The prognostic relevance of P-glycoprotein status in high-grade OS is not surprising, since several studies have clearly demonstrated that doxorubicin is a cornerstone in the chemotherapeutic treatment of this tumour (25,28,29) and that, among the drugs used for OS chemotherapy, increased P-glycoprotein levels are clearly related to a reduced responsiveness to doxorubicin (9). On the other side, it should be taken into consideration that the P-glycoprotein-mediated resistance to doxorubicin can not fully explain the poor outcome of P-glycoprotein-positive patients, since almost one half of them did not relapse and showed a good outcome. One possible explanation is that, despite its high impact on drug response and clinical outcome, P-glycoprotein may act together with other genes involved in resistance to chemotherapy in osteosarcoma patients, as suggested by Wunder *et al* (18).

To better define the clinical impact of P-glycoprotein in high-grade OS, we have analysed its prognostic value in different adjuvant and neoadjuvant chemotherapy protocols, in which doxorubicin was used as the leader drug. This comparison showed that the prognostic value of P-glycoprotein status was retained in all protocols, and that this marker was able to stratify OS patients in two subgroups with significantly different clinical outcome. The subgroup of P-glycoprotein-negative patients always showed a more favourable outcome, independently from the treatment regimen. The subgroup of P-glycoprotein-positive patients presented a significantly worse clinical outcome in all protocols, but the difference in terms of survival with the subgroup of P-glycoprotein-negative patients was much more remarkable in the IOR-Adjuvant B treatment regimen, in which doxorubicin was used alone, compared to the other regimens. This evidence suggests that the adverse prognostic value of increased P-glycoprotein levels at diagnosis appears to be influenced by the relative impact of doxorubicin in the treatment regimen, and that its high clinical impact may be partly reduced by the use of multiagent chemotherapy treatments including drugs which are not substrate for P-glycoprotein.

In this context, treatment strategies aimed to overcome drug unresponsiveness mediated by increased P-glycoprotein expression may improve the clinical outcome and/or reduce the long-term toxicity of high-grade OS. In the absence of new drugs of confirmed clinical efficacy for the treatment of this tumour, several different possibilities may be taken into account.

From the clinical point of view, it has to be noted that the outcome of the P-glycoprotein-positive ISG/SSG 1 patients was comparable to that of previous neoadjuvant treatment regimens with higher cumulative dosages of doxorubicin, in which ifosfamide was used only in the post-operative phase (3). This suggests that ifosfamide in pre-operative treatment can partially replace doxorubicin in a general OS patient cohort with no reduction in activity. Therefore, a frontline strategy to replace doxorubicin with ifosfamide in patients with tumours overexpressing P-glycoprotein could be considered.

Several clinical attempts to block or inhibit P-glycoprotein activity have so far failed, supporting the alternative strategy to normalise P-glycoprotein expression. Exposure of OS cell lines overexpressing P-glycoprotein to interferons has been shown to normalise P-glycoprotein levels and increase chemosensitivity (31). This approach is supported by clinical data demonstrating the activity of interferon-α as a single adjuvant in OS (30).

Another possible approach that has been recently explored is based on the use of a novel antitumour compound, the alkycycline PNU-159548 (ladirubicin), which has been shown to be very active in a large panel of both drug-sensitive and drug-resistant (including P-glycoprotein-overexpressing) OS cell lines (32).

We have also evaluated the impact of HER2/*neu* overexpression in high-grade OS (12), in order to verify whether trastuzumab (Herceptin)-based therapies might be of benefit to OS patients who are unresponsive to conventional treatments (17). Unfortunately, despite the adverse prognostic value of HER2/*neu* overexpression in OS patients, trastuzumab showed a very limited pre-clinical efficacy against OS cells, unless it was associated with additional targeted strategies (12). Therefore, the therapeutic potential of trastuzumab in OS needs to be better examined and considered in combination with other adjuvant approaches.

In conclusion, these findings confirm that the cure probability of high-grade OS patients is highly influenced by the P-glycoprotein status. Moreover, identification of P-glycoprotein-positive cases at diagnosis may be used to stratify

OS patients, in order to define a subgroup of patients who could benefit from alternative treatments based on the use of novel therapeutic regimens, carefully designed on the basis of the available pre-clinical evidence.

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