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Original Paper

Analysis of P-Glycoprotein Expression in Osteosarcoma

M. Serra, K. Scotlandi, M.C. Manara, D. Maurici, S. Benini, M. Sarti, M. Campanacci and N. Baldini

Laboratorio di Ricerca Oncologica, Istituti Ortopedici Rizzoli, Via di Barbiano 1/10, I–40136 Bologna, Italy

Current treatment of high-grade osteosarcoma combines surgical removal of the lesion with chemotherapy. In this study we evaluated whether the expression of P-glycoprotein, a protein closely associated with multidrug resistance, may be helpful in identifying the patients whose tumours will be further resistant to specific agents. By using multidrug-resistant osteosarcoma cell lines as standards, the expression of P-glycoprotein was evaluated in 105 cases of primary and metastatic osteosarcoma by semiquantitative immunofluorescence. Overexpression of the protein was shown in 23% of primary and in 50% of metastatic lesions. In 38 cases, homogeneously treated and followed-up for at least 24 months, overexpression of P-glycoprotein appeared to be associated with a higher relapse rate and with a trend toward a worse outcome. These data support the role of P-glycoprotein in the response to chemotherapy and its involvement in the determination of the outcome of osteosarcoma patients.

Key words: multidrug resistance, P-glycoprotein, osteosarcoma, chemotherapy, immunofluorescence
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INTRODUCTION

DEVELOPMENT OF drug resistance represents a major problem for cancer treatment. Tumours may be intrinsically refractory to antineoplastic agents, and in other instances, patients who are initially sensitive to antineoplastic drugs will subsequently relapse and will not respond to further therapy [1]. At the cellular level, non-responsiveness to drugs may be caused by a variety of mechanisms [2]. The best known, called multidrug resistance (MDR), is characterised by the crossresistance to a wide spectrum of structurally and functionally unrelated lipophilic cytotoxic agents in cells selected for resistance to one of them [3]. MDR is mediated by the *MDR1* gene product, P-glycoprotein, a plasma membrane protein involved in the active efflux of cytotoxic molecules from the cell. P-glycoprotein overexpression has been reported to play an important role in drug resistance in a number of human tumours [4, 5], including sarcomas [6–11], but only a few data are available on the relevance of MDR in osteosarcoma [12,13], the most frequent primary malignant bone tumour. Osteosarcoma is generally considered to be responsive to chemotherapy, although systemic relapses occur in at least 30–40% of the patients, despite the use of aggressive regimens with different antineoplastic drugs [14]. In this type of tumour, preliminary identification of non-responsive cases would be critical to the successful use of more effective regimens of chemotherapy.

Here we report the analysis of P-glycoprotein expression in a series of 105 cases of osteosarcoma, including primary and metastatic lesions, observed at our institution between May 1989 and December 1993. Some of these patients were treated with limb salvage surgery and multiple drug chemotherapy and followed-up for at least 2 years. In those cases, P-glycoprotein expression was compared with the clinical course.

PATIENTS AND METHODS

Clinical series

All the cases were observed at the Istituto Ortopedico Rizzoli between May 1989 and December 1993. Tissue samples were obtained from 79 untreated patients with primary osteosarcoma and from 26 patients with lung metastases of osteosarcoma. In the group of patients with primary tumours, average age was 19.1 ± 1.5 years (Figure 1), male:female sex ratio was 1:4, and the most common sites were, in descending order, femur, tibia and humerus. In all the cases, the specimens were divided into two parts, one for histological evaluation and the other for analysis of P-glycoprotein expression, DNA content and cell proliferation.

The diagnosis of osteosarcoma was established based on conventional criteria [15], and primary tumours were accordingly classified as grade II (2 cases), grade III (10 cases), or grade IV (67 cases) osteosarcoma. With regard to the predominant

Correspondence to M. Serra.
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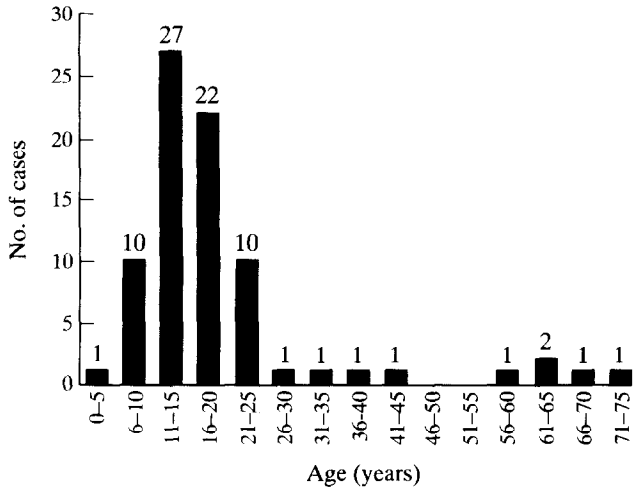

Figure 1. Age distribution of the 79 patients with primary osteosarcoma.

histological appearance and to the clinicopathological features, 48 cases were classified as osteoblastic, 4 cases as chondroblastic, 7 cases as fibroblastic, 6 cases as haemorrhagic, 5 cases as periosteal, 1 case as parosteal, and 2 cases as small cell osteosarcoma. In addition, for 5 cases of high-grade osteosarcoma and 1 case of low-grade osteosarcoma, the pathological subtype was not further determined.

Tumour size was estimated at clinical onset on computed tomography (CT) scan images by determining the three dimesions (a, b, c) of tumour extension and calculating the volume of an ellipsoid according to the formula:

$$\text{Volume} = a*b*c*0.52.$$

38 patients with high-grade, non-metastatic osteosarcoma of the extremities were treated with the same combined protocol, including limb salvage surgery and pre- and postoperative chemotherapy with doxorubicin (60–75 mg/m^2), methotrexate (12 g/m^2), cisplatinum (120–150 mg/m^2) and ifosfamide (2 g/m^2) [14]. These patients were followed-up for 24–44 months (mean 27). The disease-free survival was calculated from the first day of preoperative chemotherapy until the first adverse event or until the date of the most recent follow-up examination. Adverse events included the development of recurrent tumour at any site.

P-Glycoprotein expression

A series of human osteosarcoma MDR cell lines (U-2 OS/DX^{30}, U-2 OS/DX^{100} and U-2 OS/DX^{580}, respectively resistant to 30, 100 and 580 ng/ml doxorubicin) expressing known amount of P-glycoprotein and their parental sensitive cell line U-2 OS [16] were used as standards for the analysis of P-glycoprotein expression in clinical samples.

Human specimens were treated with enzymatic digestion (2 mg/ml collagenase) for 30–90 min at 37°C to obtain a representative cell suspension. Cells were smeared on glass slides and fixed with acetone at room temperature for 10 min. The representativeness of the samples and, in particular, the possible contamination of macrophages, which may overexpress P-glycoprotein [17, 18], were estimated on May–Grünwald–Giemsa-stained cytospins. P-Glycoprotein expression was evaluated by indirect immunofluorescence, using three different monoclonal antibodies: JSB-1 (Sanbio, Uden, The Netherlands) diluted 1:10; C219 (Centocor, Malvern, Pennsylvannia, U.S.A.) diluted 1:5; and MRK16 (Kamiya Biomedical, Thousand Oaks, California, U.S.A.) diluted 1:100. The percentage of P-glycoprotein-positive cells was determined on at least 300 tumour cells, and expressed as P-glycoprotein labeling index (PGP LI). Based on the P-glycoprotein expression of U-2 OS/DX^{30}, the least resistant of the three osteosarcoma MDR cell lines analysed, a PGP LI higher than 15 was considered to identify resistant cases.

DNA cytofluorometry

For ploidy analysis, cytospins were fixed with 70% ethanol, treated with RNase digestion and stained with 50 μg/ml propidium iodide (Sigma, St Louis, Missouri, U.S.A.). The DNA content was analysed by cytofluorometry in 77 cases (61 primaries and 16 metastases), which were classified either as diploid or non-diploid (including euploid-polyploid and aneuploid cases) [19].

Ki-67 immunostaining

Indirect immunofluorescence was used in 52 cases (39 primaries and 13 metastases) to establish the expression of Ki-67 nuclear antigen as an index of proliferative activity of the tumour. Cytospins were fixed with acetone at room temperature for 10 min. For the immunostaining, Ki-67 monoclonal antibody (Dakopats, Glostrup, Denmark) was used at a 1:10 dilution. The percentage of Ki-67-positive cells (Ki-67 labeling index, Ki-67 LI) was estimated on at least 500 tumour cells.

Statistical analysis

The χ^2 test or Fisher's exact test was used to evaluate the statistical significance of differences among groups. Fisher's exact test was used where there were six or fewer items in a group. Student's *t*-test was used to compare pairs of groups of patients. The duration of disease-free survival was estimated with Kaplan–Meier survival tables. Log-rank analysis was used to assess the significance of the disease-free survival curves.

RESULTS

P-Glycoprotein immunostaining

To compare the immunoreactivity of JSB-1, C219 and MRK16 monoclonal antibodies, which bind to different, mutually exclusive epitopes of P-glycoprotein [18], sensitive and MDR osteosarcoma cell lines and tissue samples for 24 clinical cases were stained using the three antibodies. In the MDR cell lines, comparable values of PGP LI, which corresponded to the resistance level, were found for the three different antibodies (Figure 2). Similar results, showing a concordance between the three antibodies, was obtained in tissue samples (Table 1), and further analysis of the clinical series was carried out using the JSB-1 antibody alone.

Primary tumours

Figure 3 shows the range of P-glycoprotein expression in 79 cases of primary osteosarcoma. 18 cases (23%), showing a PGP LI higher than 15, were considered resistant.

The clinical and pathological features observed in sensitive and resistant cases are summarised in Table 2. The percentage of resistant cases was not remarkably different with regard to age and site, but was significantly higher in males than in females ($P = 0.05$). Among high-grade lesions, resistant cases were similarly distributed among grades III and IV. Interestingly, in the two low-grade lesions, P-glycoprotein expression was undetectable. With regard to the pathological subtype, no

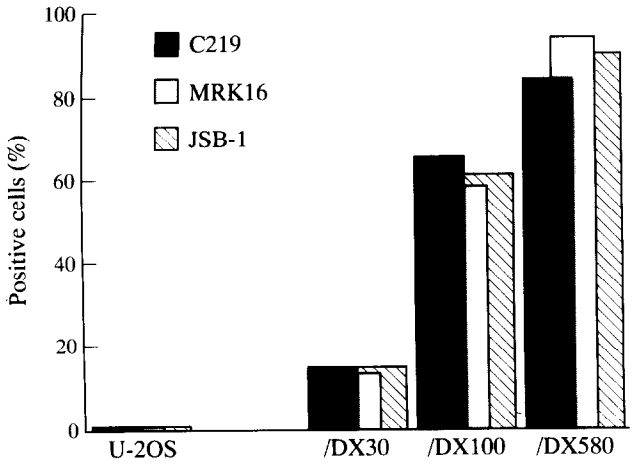

Figure 2. Reactivity of monoclonal antibodies with P-glycoprotein in U-2 OS cells.

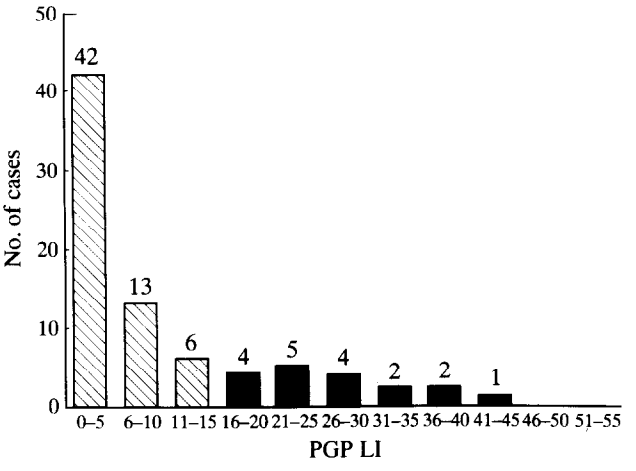

Figure 3. Distribution of PGP LI in 79 cases of primary osteosarcoma. ▨ Sensitive. ■ Resistant.

Table 1. Percentage of cells expressing P-glycoprotein, detected by monoclonal antibodies JSB-1, C219 and MRK16 in 24 patients with osteosarcoma

Patient	JSB-1	C219	MRK16
1	3	0	0
2	1	3	1
3	17	21	23
4	0	0	3
5	28	32	22
6	0	0	2
7	0	2	0
8	21	19	24
9	3	1	2
10	5	2	6
11	9	2	7
12	2	0	1
13	1	0	0
14	33	39	29
15	9	13	11
16	3	1	0
17	1	0	4
18	2	1	3
19	2	4	0
20	2	2	1
21	12	8	15
22	0	1	0
23	25	17	27
24	0	0	0

Table 2. Clinical and histopathological characteristics of 79 cases with primary osteosarcoma

	Total	% Resistant
Gender		
Male	46	30
Female	33	12
Age		
<14 years	29	31
≥14 years	50	18
Site		
Femur	48	19
Tibia	15	40
Humerus	11	27
Pelvis	3	0
Fibula	1	0
Radius	1	0
Histological grade		
I	0	0
II	2	0
III	10	30
IV	67	22
Subtype		
Osteoblastic	48	21
Chondroblastic	4	50
Fibroblastic	7	29
Haemorrhagic	6	0
Small cell	2	100
Periosteal	5	20
Parosteal	1	0
Unclassified	6	17

significant difference in the percentage of resistant cases was found among the osteoblastic, chondroblastic, fibroblastic and periosteal varieties. Interestingly, none of the 6 cases of haemorrhagic osteosarcoma, but both cases of small cell osteosarcoma, were resistant.

Inconclusive results were obtained when comparing parameters of tumour aggressiveness and P-glycoprotein expression. No correlation was found between DNA content and P-glycoprotein expression, the percentage of resistant cases being equally distributed among diploid and non-diploid tumours (2/9, 22% versus 15/52, 29%). However, the analysis of cell proliferation by Ki-67 immunostaining showed a higher Ki-67 LI in the group of resistant cases compared to sensitive cases (15.1 ± 2.4 versus 10.5 ± 1.4, $P = 0.09$). Moreover, mean tumour volume was greater in resistant than in sensitive cases (353 ± 115 versus 233 ± 43, $P = 0.25$).

Metastatic tumours

The 26 metastatic lesions were found to be equally distributed among sensitive and resistant cases (Figure 4). The percentage of metastatic osteosarcoma showing an increased level of P-glycoprotein expression was significantly higher compared to primary lesions (50 and 23%, respectively). The difference is

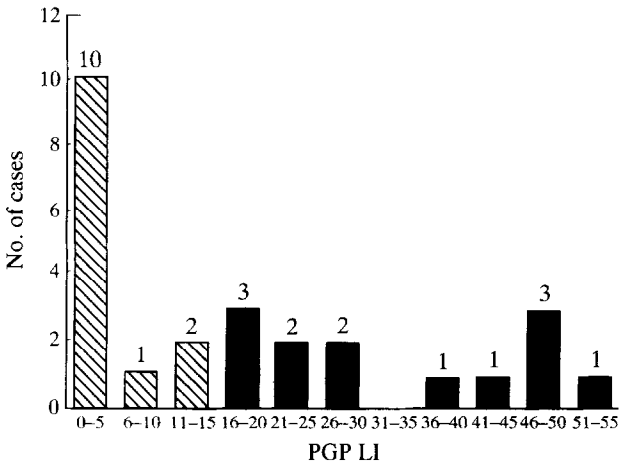

Figure 4. Distribution of PGP LI in 26 cases of metastatic osteosarcoma. ▨ Sensitive. ■ Resistant.

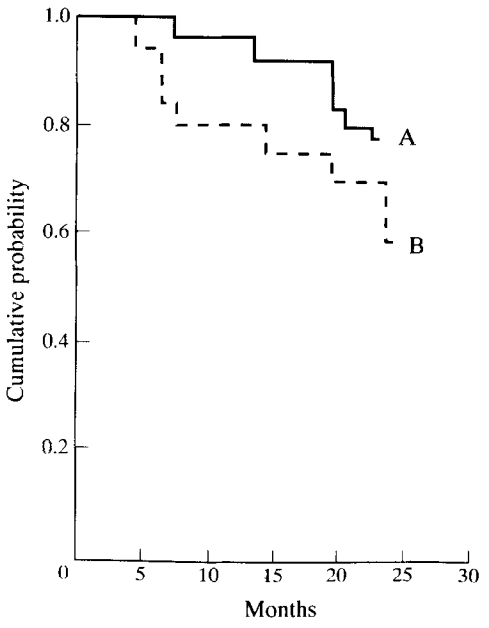

Figure 5. Disease-free survival in 38 patients with high-grade non-metastatic osteosarcoma of the extremities treated with surgery and adjuvant chemotherapy, according to P-glycoprotein expression. (A) PGP LI ≤ 15, (B) PGP LI > 15. Comparison of survival curves by log-rank test ($P = 0.19$).

more relevant when considering only the cases detected during or after chemotherapy (12/21 cases, 57%; $P = 0.003$). In fact, of the 5 cases of metastatic osteosarcoma revealed at the clinical onset, only 1 showed an increased expression of P-glycoprotein.

In 3 cases, samples from the primary lesion and the metastasis were both available for analysis. In 2, the primary tumour was sensitive (PGP LI of 3 and 11, respectively) and the metastasis resistant (PGP LI of 41 and 26, respectively). In the third case, the primary and the metastatic lesion were both resistant, with a higher PGP LI in the metastasis compared to the primary tumour (25 versus 16).

Response to chemotherapy and outcome

The prognostic value of P-glycoprotein in osteosarcoma was analysed in 38 of the 79 primary lesions. These patients, all with a grade III or IV conventional osteosarcoma of the extremities, received the same combined treatment, including limb salvage surgery and multiple drug chemotherapy, and were followed-up for at least 24 months.

15 cases (40%) showed increased levels of P-glycoprotein overepression and were considered resistant. In 36 of the 38 cases, preoperative chemotherapy was given before ablation of the primary tumour, and necrosis after pre-operative chemotherapy was found to be total (good response) in 11 cases (31%), whereas in 25 cases (69%) viable areas were still present (poor response). No relationship was found between P-glycoprotein expression and the amount of necrosis after pre-operative chemotherapy. In fact, sensitive and resistant cases showed similar rates of poor histological response (17/23, 74% and 8/13, 62%, respectively). However, at a 24-month minimum follow-up, the relapse rate was higher in resistant (6/15, 40%) than in sensitive cases (5/23, 22%), and comparison of disease-free survival curves for the two groups showed a trend toward a worse outcome in P-glycoprotein-overexpressing tumours (Figure 5). On the other hand, percentage of necrosis was not predictive of the outcome (data not shown).

DISCUSSION

Although the spontaneous course of high-grade osteosarcoma is characterised by the rapid development of metastases in 80–90% of cases, this tumour may be effectively cured in 60–70% of cases if chemotherapy is added to surgery to prevent the development of secondary lesions [14, 20]. However, despite the use of increasingly aggressive therapeutic regimens, a significant proportion of patients who receive adequate treatment show a relentless progression of the disease, suggesting that, in osteosarcoma, the presence or the development of resistance to chemotherapy play a major role in the clinical course.

MDR, the best known mechanism of cellular resistance to anticancer drugs, is mediated by increased expression of the *MDR1* gene product, the P-glycoprotein. This protein acts at the plasma membrane of resistant cells as an energy-dependent efflux pump for different lipophilic molecules, including some of the agents which are most effective in the treatment of sarcomas. In particular, one of these drugs, the antibiotic doxorubicin, is probably the single most effective drug for osteosarcoma [21], and is always considered, alone or in combination with other agents, in all the regimens for this tumour.

The occurrence of MDR in human sarcomas has already been reported, suggesting that P-glycoprotein overexpression may be present at the clinical onset [6, 7, 9–11]. Moreover, in soft tissue lesions of childhood, a strong correlation between P-glycoprotein expression and the outcome has been reported [8].

Very few data are available on the expression of P-glycoprotein in osteosarcoma, the most frequent primary malignant bone tumour. In a series of adult musculoskeletal sarcomas, Stein and associates [12] found increased levels of *MDR1* gene expression in 10 of 11 cases of osteosarcoma. In 15 cases of osteosarcoma, Wunder and associates [13] showed *MDR1* gene overexpression in 9 patients, and found a trend toward a worse outcome in cases exhibiting high levels of *MDR1* expression.

In this study, we evaluated a series of 105 cases of osteosarcoma, including 79 primary and 26 metastatic lesions, to assess the expression of P-glycoprotein by semiquantitative immunofluorescence. In our opinion, analysis of P-glycoprotein immunostaining in isolated cells, although preventing a tissue distribution evaluation of P-glycoprotein expression, permitted a more accurate quantification of MDR and a reproducible discrimination between sensitive and resistant cases. The

reliability of this method had been previously established *in vitro* in MDR osteosarcoma cell lines [16]. Both in cell lines and in clinical samples, P-glycoprotein immunostaining with JSB-1 monoclonal antibody gave comparable results to those obtained using two other antibodies, C219 and MRK16, which recognise different epitopes of the protein. The U-2 OS/DX^{30} cell line, showing low levels of resistance, was used as a standard to define the cut-off value for the PGP LI, based on the reported evidence that the resistance levels found in clinical samples do not generally exceed a 10–15-fold increase compared to that of corresponding normal tissue [4, 22].

We observed that P-glycoprotein may be overexpressed at the clinical onset, and that the proportion of MDR cases is significantly increased in secondary lesions. These data suggest that MDR may be an inherent feature of osteosarcoma and that, during the course of the disease, possibly under the pressure of anticancer agents, the presence of MDR subpopulations may be enhanced. The administration of cytotoxic drugs may induce the expression of *MDR1*/P-glycoprotein [7, 23]. In our series, this effect was observed in the three cases in which both primary and metachronous secondary lesions were available for the analysis. However, it cannot be excluded that, as described for other tumours [24], in osteosarcoma the observed overexpression of P-glycoprotein may also be associated with the progression of malignancy. In this respect, the positive correlation observed between features representative of tumour progression (size and proliferation rate) and P-glycoprotein overexpression might find a reasonable explanation.

In this study, we evaluated the correlation between the expression of P-glycoprotein, the response to chemotherapy and the clinical outcome in a subgroup of 38 cases with resectable non-metastatic osteosarcoma of the extremities. In this group of patients, P-glycoprotein overepression appeared to be associated with a higher risk of relapse. These preliminary data suggest a possible role for P-glycoprotein as a prognostic indicator of responsiveness to chemotherapy but need to be further evaluated on a larger series. The lack of correlation between P-glycoprotein expression and the histological response to pre-operative chemotherapy might be partly attributed to the possibility that these two parameters identify different phenomena, and, in particular, that P-glycoprotein expression may be representative, not simply of tumour resistance to MDR-related drugs, but also of tumour progression.

The trends observed in this study suggest that evaluation of P-glycoprotein expression in clinical samples at the clinical onset may be useful in identifying those patients who will have a poor response to treatment, and might be important in the planning of innovative chemotherapeutic regimens based on the individual characteristics of the tumour.

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