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The co-expression of p53 protein and P-glycoprotein is correlated to a poor prognosis in osteosarcoma

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Abstract Multidrug resistance (MDR), mediated by P-glycoprotein (P-gp), is an in vitro phenomenon observed within tumour cells, suggesting cross-resistance to unrelated drugs, and expression of P-gp may therefore affect the prognosis and incidence of recurrence after treatment. The mutant p53 protein causes reduced tumour suppression. Co-expression of p53 and P-gp is related to short survival, increased tumour activity and drug resistance.

The purpose of this study was to measure the expression of p53 protein and P-gp in osteosarcoma tissue and assess its prognostic significance. Fifty-two tumour specimens were evaluated. The correlation between p53 and P-gp expression was significant ($P=0.0008$). In univariate analysis of survival, p53 protein was not significant ($P=0.2$) but P-gp was significant ($P=0.0001$). The co-expression of p53 and P-gp was the strongest indicator of a short survival according to multivariate analysis ($P=0.0004$).

Résumé La résistance à une combinaison médicamenteuse, obtenue par le biais du P-glycoprotéin (P-gp), est un phénomène in-vitro des cellules tumorales, montrant une résistance croisée aux médicaments. L'expression du P-gp est liée à la résistance au médicament, au pronostic et à la récurrence. La mutation de la protéine P53 permet de réduire la suppression de la tumeur. D'autres effets de la P53 et du P-gp sont liés à la brève survie, à la malignité et à la résistance au médicament. Cette étude a été pratiquée dans le but d'évaluer le comportement de la protéine P53 et du P-gp dans l'osteosarcome et sa signification pronostique; 52 spécimens ont été testés par histochimie immunologique avec des anti-corps monoclonal. La relation entre le P53 et le P-gp fut positive ($P=0.0008$). Dans l'analyse monovariante de la survie, la

protéine P53 ne fut pas positive ($P=0.2$), mais le P-gp le fut ($P=0.0001$). La co-expression de la P53 et de la P-gp peuvent être un élément de pronostic selon l'analyse de multi-variation ($P=0.0004$).

Introduction

Despite aggressive therapeutic regimes in the treatment of osteosarcoma, there is an incidence of recurrence of between 30% and 50% [2, 15]. The principal cause of failure of treatment is drug resistance. Multidrug resistance (MDR), mediated by P-glycoprotein (P-gp) [21], is an in vitro phenomenon of tumour cells, which become cross-resistant to structurally unrelated drugs. P-gp, encoded by the MDR1 gene, acts as an energy-dependent drug efflux pump [8, 21]. P-gp expression may be associated with drug resistance, recurrence and poor prognosis in patients with osteosarcoma [3, 24].

The p53 tumour-suppressor gene regulates genomic stability [14]. The mutant p53 protein produces a reduction in the ability of cells to suppress tumour activity and the over-expression of this protein has been reported as correlating with rapid, unregulated cell proliferation and poor clinical outcome of malignant tumours [22].

Osteosarcoma cell lines show mutations of the p53 gene, and this may be an important step in the maturation of the tumour [6]. As wild type p53 protein in the SaOS-2 cell line decreases expression of the MDR1 gene, it may be speculated that osteosarcomas containing the mutant p53 protein produce excessive P-gp as a result of the stimulatory effect of the mutant p53 protein on the MDR1 gene.

In this study, we evaluated the relationship between P-gp expression and the nuclear accumulation of p53 protein in osteosarcoma. We also analyzed the effect of co-expression of P-gp and p53 protein on the clinical outcome.

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Materials and methods

Between March 1988 and March 1996, tissue was obtained from 52 of 77 patients with high-grade osteosarcoma (Table 1). Forty-eight patients were classified as Enneking stage II osteosarcoma [12]. They were initially treated with neoadjuvant chemotherapy, and wide resection or amputation. The surgical margins were radical or wide in 40 cases, and marginal in eight. Four patients with stage III osteosarcoma were treated by chemotherapy alone. The mean follow-up was 46 months (minimum 26 months).

Immunohistochemistry. We used mouse monoclonal antibodies, clone DO-7 (Neomarkers, Fremont, Calif., USA) and JSB-1 (Zymed Laboratories, South San Francisco, Calif., USA). Positive control tissue for measurements of p53 protein was obtained from breast carcinoma and for P-gp from hepatocyte and renal tubular cells. Negative controls were obtained by substituting non-immune serum for each antibody. The avidin-biotin-peroxidase procedure was used for immunohistochemical staining [13]. Co-expression of the p53 protein and P-gp was analyzed using double immunohistochemistry combining the ABC (JSB-1; dilution 1:100) and the alkaline phosphatase/anti-alkaline phosphatase (APAAP) methods (DO-7; dilution 1:100) [18]. Two pathologists with no prior knowledge of the clinical outcome assessed the immunohistochemical results. The results were scored as negative when there was absence of staining or only scattered positive cells. Samples were scored as positive when staining was seen in more than 10% of the tumour cells [3, 4].

Table 1 Clinical and pathologic characteristics of the subgroup of 52 patients in whom immunohistochemistry was performed and of the entire group of 77 patients with osteosarcoma

Variable	Subgroup (n=52) No. of patients (%)	Total group (n=77) No. of patients (%)
Age		
Mean	21.4 years	20.6 years
Range	9~53 years	8~67 years
Age group		
≤14 years	9 (17.3%)	24 (31.2%)
>14 years	43 (82.7%)	53 (68.8%)
Sex		
Male	35 (67.3%)	51 (66.2%)
Female	17 (32.7%)	26 (33.8%)
Anatomical site		
Distal femur	21 (40.4%)	32 (41.6%)
Prox. tibia	18 (34.6%)	27 (35.1%)
Prox. humerus	5 (9.6%)	6 (7.8%)
Prox. femur	2 (3.8%)	4 (5.2%)
Others	6 (11.6%)	8 (10.3%)
Histologic subtype		
Osteoblastic	38 (73.1%)	55 (71.4%)
Chondroblastic	4 (7.7%)	10 (13.0%)
Fibroblastic	6 (11.5%)	6 (7.8%)
Telangiectatic	3 (5.8%)	4 (5.2%)
Mixed	1 (1.9%)	2 (2.6%)
Enneking stage		
Ib	0 (0%)	1 (1.3%)
Ia	5 (9.6%)	6 (7.8%)
IIb	43 (82.7%)	65 (84.4%)
III	4 (7.7%)	5 (6.5%)

Statistical methods. Mantel-Haenzel's chi-square test and Fisher's exact test were used to determine the correlation between the p53 protein and the P-gp expression; and correlation between immunostaining and the clinical characteristics was also assessed by Fisher's exact test. Survival analyses were performed according to Kaplan-Meier, and differences were evaluated using the Log-Rank test. Cox's proportional hazard regression analysis was used for multivariate analysis to assess the prognostic value of the clinical characteristics and the p53/P-gp status in osteosarcoma. Tests were performed with the SAS/PC statistical package (SAS Institute, Cary, N.C., USA).

Results

Co-expression of p53 protein and P-gp

Of 52 osteosarcomas, 33 showed nuclear immunoreactivity for the p53 protein with an expression rate of 63%. Eighteen specimens exhibited positive immunostaining for P-gp with an expression rate of 35%. A significant correlation between nuclear p53 expression and P-gp staining was seen ($P=0.0008$; Table 2). Double immunohistochemistry revealed that the p53 protein and P-gp often occurred concomitantly in the same tumour cells.

Correlation of the expression of the p53 protein and P-gp with clinical and pathological states

Expression of the p53 protein was not statistically correlated to age ($P=0.5$), sex ($P=0.4$), histological subtype ($P=0.4$) or Enneking stage ($P=0.6$). No significant difference in the incidence of positive P-gp staining was found in relation to age ($P=0.9$), sex ($P=0.2$), or histological subtype ($P=0.2$). However, P-gp expression was significantly correlated to Enneking stage ($P=0.003$). When comparing the p53/P-gp positivity and the clinical state, p53(+)/P-gp(+) status was not significantly associated with age ($P=0.7$), sex ($P=0.7$) or histological subtype ($P=0.2$). However, a significant association was found between p53(+)/P-gp(+) status and the Enneking stage ($P=0.02$).

Survival analysis

There was no significant difference in survival time between p53 positive and the negative groups ($P=0.2$); however, we did see a significant difference between the

Table 2 Correlation between nuclear p53 accumulation and P-gp expression in the total group of osteosarcoma examined (n=52)

Tumor status	P-gp expression	
	Negative	Positive
p53 negative	18	1
p53 positive	16	17
		$P=0.0008^*$

*Mantel-Haenzel's chi-square test and Fisher's exact test

Fig. 1 Kaplan-Meier survival analysis between the p53(-)/P-gp(-) group and the p53(+)/P-gp(+) group

Table 3 Results of Cox's proportional hazard regression analysis

Variable	Risk ratio	95% CI ^a	P-value
p53/P-gp status			
Positive	10.5	2.86–38.3	0.0004
Negative	1.00		
Age group			
≤14 years	1.00		
>14 years	0.27	0.08–0.90	0.04
Sex			
Male	1.00		
Female	1.42	0.48–4.19	0.52
Anatomical level			
Proximal ^b	1.00		
Distal ^c	0.22	0.05–1.04	0.06
Histologic subtype			
Osteoblastic	1.00		
Others	0.41	0.11–1.46	0.17
Enneking stage			
Ila	1.00		
Iib	4.23	1.02–17.5	0.05

^a Confidence interval

^b Tumors occurring in the proximal femur, proximal humerus or axial skeleton

^c Tumors occurring in the region below the knee and elbow level

P-gp positive and negative groups ($P=0.0001$). Survival of the p53(+)/P-gp(+) patients ($n=17$) was significantly shorter than that of the p53(-)/P-gp(-) patients ($n=18$; 3-year survival 16% vs. 70%; $P=0.0001$; Fig. 1). In a multivariate analysis with regard to age, sex, histologic subtype, and Enneking stage, the p53(+)/P-gp(+) status was the most significant prognostic factor ($P=0.0001$), and other factors such as age and Enneking stage added

prognostic value in osteosarcoma (Table 3). This finding suggests that co-expression of the p53 protein and P-gp is an independent prognostic factor in osteosarcoma.

Discussion

There have been many studies confirming the prognostic significance of biological markers in osteosarcoma [3, 23, 26]. As expression of P-gp has recently been shown to be activated by over-expression of the mutant p53 protein in vitro [9], several attempts have been made to investigate the correlation between these two proteins in malignant tumours [1, 7, 10, 11, 16, 17, 22]. Immunohistochemical studies of the p53 protein show a high correlation between the mutation of the p53 gene and expression of p53 protein. Among monoclonal antibodies for P-gp, JSB-1 reacts with epitope located at the cytoplasm and shows membranous and cytoplasmic staining patterns in osteosarcoma [4]. Immunohistochemical studies for P-gp can reflect the difference of P-gp expression and the characteristics of the tumour [5]. There are differences in immunohistochemical results according to the type of pathological tissue, the types of monoclonal antibodies, and the criteria used to define positivity. Expression of p53 protein was observed in 63% of patients in this study, compared with 72% reported by Bodey et al. [4]. The staining pattern of the p53 protein showed heterogeneity, which resulted from the selection of tumour cells performed by growth potential and aggressiveness. The expression rate of P-gp was not appreciably different in several reports, regardless of the staining methods [3, 19, 20, 24]. There was a significant correlation between p53 protein and P-gp expression ($P=0.0008$). This result was similar to other reports showing a significant correlation between p53 protein and P-gp [7, 11, 16]. Among 18 patients with positive P-gp expression, 17 also showed positive p53 expression. Eighteen of 19 patients who were negative for p53 were also negative for P-gp. This may mean that the probability of P-gp expression is very low when nuclear accumulation of the p53 protein is not seen in osteosarcoma. The reason for the low positivity of P-gp in the p53 positive cases (17 of 33) was based on the fact that the ability to stimulate the MDR1 promotor depends on the sites of the p53 mutation [9].

Survival analysis showed no difference between the p53 negative and positive groups ($P=0.2$). This suggests that it is not a prognostic factor in osteosarcoma [25], although mutation of the p53 gene is common. As over-expression of the p53 protein is related to growth, aggressiveness and poor prognosis in other malignant tumours [1], this result should be interpreted with caution. When considering P-gp expression, survival time was significantly longer in the P-gp negative group than in the positive group ($P=0.0001$), as has also previously been reported by Baldini et al. [3], and thus P-gp expression could be regarded as a poor prognostic factor in osteosarcoma. Evaluation of P-gp expression in clinical speci-

mens may be useful in identifying patients who show a poor response to treatment and important in planning the appropriate chemotherapeutic regime. The p53(+)/P-gp(+) group showed a significantly shorter survival period than the p53(-)/P-gp(-) group. According to multivariate analysis, the p53(+)/P-gp(+) status was the strongest prognostic factor ($P=0.0004$). The prognostic significance of the co-expression of p53 and P-gp has also been reported in cancer of the breast [7, 21] and colorectal cancer [11].

In conclusion, these results suggest that nuclear p53 accumulation may be associated with MDR1 gene expression in osteosarcoma and the co-expression of the p53 protein and P-gp carries a poor prognosis.

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