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Clinical significance of P-glycoprotein immunohistochemistry and doxorubicin binding assay in patients with osteosarcoma

Abstract In 45 osteosarcoma patients, mean age 18 (4–61) years and followed for 14 (5–48) months, we studied the sensitivity to doxorubicin as well as P-glycoprotein expression, and compared these with the extent of tumour necrosis following chemotherapy. Doxorubicin assay was positive in 37 patients in whom necrosis induced by chemotherapy was good in 20 and poor in 17. Metastases developed in nine patients. In eight patients in whom doxorubicin assay indicated tumour resistance, chemonecrosis was poor and all developed pulmonary metastases. P-glycoprotein was studied in pre-treatment biopsies and post-treatment resection specimens. Its expression was positive in 16 patients in whom the necrosis induced by chemotherapy was good in four and poor in 12. In 29 patients with negative P-glycoprotein expression, necrosis was good in 16 and poor in 13. The doxorubicin sensitivity had a high correlation with chemonecrosis (P=0.006) and the incidence of metastases (P<0.001). However, P-glycoprotein expression at the time of diagnosis did not correlate statistically with chemonecrosis (P=0.066). Doxorubicin sensitivity prior to treatment is a better determinant of the response to chemotherapy and clinical outcome than is the P-glycoprotein expression.

Résumé Sur 45 patients atteints d'ostéosarcome, âgés de 18 (4–61) ans et suivis pendant 14 (5–48) mois, nous avons étudié la sensibilité à la doxorubicine et l'expression de la P-glycoprotéine, en comparant avec l'ampleur de la nécrose provoquée par la chimiothérapie. L'essai avec la doxorubicine était positif chez 37 patients, dont 20 avaient une bonne réponse, et 17 en avaient une mauvaise. Des métastases se sont développées chez neuf patients. Pour huit patients résistants à la doxorubicine, la nécrose tissulaire était limité et tous les patients ont développé des métastases pulmonaires. La P-glycoproteine a été étudiée dans les biopsies avant traitement et sur des échantillons de la résection. L'expression de la P-glycoprotéine était positive chez 16 patients. Douze patients ont montrés une faible nécrose et quatre une nécrose importante. Chez 29 patients sans expression de la P-glycoprotéine la nécrose était importante pour 16 et limitée pour 13. La sensibilité à la doxorubicine avait une haute corrélation avec la nécrose (P=0.006) aussi bien qu'avec la survenue de métastases (P<0.001). Cependant l'expression de la P-glycoprotéine à l'étape diagnostique n'a pas de signification statistique pour prédire la nécrose (P=0.066). La sensibilité à la doxorubicine testée avant traitement est un meilleur prédictif de la réponse à la chimiothérapie et du résultat clinique que la P-glycoprotéine.

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Introduction

Osteosarcoma is the most common primary malignant tumour of bone. The survival of patients with this disease has improved in recent years as a result of the development and refining of chemotherapeutic and surgical management. However, a proportion of patients do not respond to chemotherapy and will succumb to the disease. The response of patients to chemotherapy is usually determined following surgical excision of the tumour

and subsequent histological analysis of the resected specimen. This may be too late in those patients in whom there is resistance to drugs, and thus the detection of multi-drug resistance, prior to the initiation of treatment, may help plan alternative strategies for the control of the disease.

Multi-drug resistance is characterised by the loss of sensitivity to a variety of structurally unrelated drugs, including doxorubicin, which is an effective agent in the treatment of high-grade sarcomas. It is an important component of multimodal therapy for osteosarcoma and is a component of most protocols used for the control of this disease. The doxorubicin binding assay tests the sensitivity of osteosarcoma cells to the drug and has been used as a simple clinical test to determine whether patients will respond to multi-agent chemotherapy.

The multi-drug intra-cellular transporter protein (P-glycoprotein) regulates the transport of drugs in and out of the tumour cell. Cells with a high expression of this protein are able to eliminate cytotoxic drugs and remain resistant to chemotherapy. P-glycoprotein (P-gp) has been the focus of a number of studies related to drug resistance [2, 3, 8, 9]. An elevated expression of P-gp in osteosarcoma cells has been linked with a poor prognosis and outcome [2]. We have studied the sensitivity and resistance to doxorubicin as well as P-gp expression in patients with osteosarcoma and compared these with tumour necrosis following neo-adjuvant chemotherapy. The object of this study was to determine whether these tests enable the clinician to identify patients with resistance to chemotherapy prior to the initiation of treatment and whether the tests correlate with the incidence of metastases and the clinical outcome.

Materials and methods

In this prospective study, 45 patients with stage II-B osteosarcoma, with a mean age of 18 (4–61) years, were investigated. Initial data included whole body scintigraphy, plain radiographs and magnetic resonance imaging of the affected extremity. A biopsy was obtained before chemotherapy to confirm the diagnosis and the tissue tested for the doxorubicin binding assay as well as P-gp expression.

The doxorubicin binding assay

The doxorubicin binding assay was performed after releasing osteosarcoma cells from the fresh biopsy specimen using the technique described by Baldini et al. [1] and reported by others [5, 6, 7]. The pattern of intracellular accumulation and the distribution of doxorubicin is different in living sensitive and resistant cells, and may be studied by direct microscopic observation using a fluorescence microscope. This forms the basis of the assay. Fresh tumour tissue samples (obtained at the time of biopsy, as well as representative samples from the resected specimen) were selected and placed in Isocove's modified Dulbecco's medium (Sigmal-7633; Sigma Chemical, St. Louis, Mo., USA). Following a brief period of mechanical agitation, the sample was treated with 0.1% collagenase II (Sigma C-6885) in phosphate buffered saline (PBS), at 37°C, until an adequate cell suspension was obtained. Tissue debris was filtered through a 100-μm Whatman filter paper. After addition of further culture medium to dilute the collagenase, the suspension was centrifuged at 100 rpm for 5 min. The supernatant was removed and an appropriate measured volume of culture medium was added to re-suspend the cells. The cells per unit volume were determined using a counting square and cell viability was calculated using the trypan blue exclusion method. A total of 200,000 from the cell suspension were resuspended in 1 ml of culture medium. Doxorubicin (10 mg) was dissolved in 5 ml of double distilled water to give the desired concentration of 10 μg/ml; 5 ml of this solution was added to 1 ml of the cell suspension obtained and incubated at 37°C under constant motion. Fluorescein diacetate solution (Sigma F-7378; 0.5 mg/ml, pH 7.3) was added with continuous stirring at 60°C and under dark conditions for 10–15 min. Following this 1 ml of PBS was added and the fluid centrifuged at 1000 rpm for 5 min. The pellet obtained was resuspended in 50 ml PBS, of which 1 ml was placed on a slide for fluorescence microscopy. Living cells were identified under blue excitation conditions. Viable cells demonstrated green fluorescence. Sensitive and resistant cells were identified from the viable cells as follows. Osteosarcoma cells with strong nuclear fluorescence and weak cytoplasmic fluorescence were considered sensitive, while cells with diffuse cytoplasmic fluorescence and no nuclear binding were considered resistant. The test was considered positive (sensitive) if more than 80% of the osteosarcoma cells showed nuclear fluorescence and negative (resistant) in less.

P-gp expression

We studied P-gp expression in the pre-chemotherapy biopsy and the post-chemotherapy resection specimens. It was studied in paraffin embedded specimens using an immunohistochemical technique with a monoclonal antibody, JBS-1 (Unbo, The Netherlands). Five-micrometer paraffin sections were deparaffinised in xylene and alcohol and hydrated in PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Sections were then microwave treated twice for 5 min at 700 W in 0.01 M citrate buffer and pH 6.0 and further rinsed in PBS. Sections were incubated with normal goat serum for 30 min to block non-specific antibody binding. Mouse anti-human P-gp antibody was diluted at a ratio of 1:10, in 10% PBS, and sections were incubated overnight. An anti-mouse biotinylated antibody (dilution 1:200) was used for further incubation and an avidin-biotin complex system (LSAB Kit; Dako) with diaminobenzidine (Sigma) as a chromogen used for development. Sections were lightly counterstained with haemotoxylin and examined under light microscopy at a magnification of ×20. P-gp expression was considered positive if more than 25% of the cells in representative paraffin sections showed positive immunostaining.

Chemotherapy

All patients received pre-operative chemotherapy with a standard combination of high dose methotrexate, cis-platin and doxorubicin, irrespective of the doxorubicin sensitivity result or P-gp expression. Following two 4-week courses of chemotherapy, the tumour was resected and examined for chemonecrosis.

Assessment of chemotherapy-induced necrosis

The response to chemotherapy was graded on a scale of I–IV depending on the extent of necrosis [4]: a grade III–IV response was considered good while a grade I–II response was poor. The patients were followed up regularly in a joint orthopaedic oncology clinic for a mean period of 14 (5–48) months.

Statistical analysis was performed using a commercially available programme (SPSS for Windows) and Fischer's exact test was used to calculate the statistical significance between the groups.

Results

Doxorubicin sensitivity tests and tumour necrosis

Doxirubicin sensitivity test before chemotherapy

There were 37 patients in whom the biopsy samples indicated that the tumour was sensitive to doxorubicin. In these chemosensitive patients, good necrosis (grades III–IV) was seen in 20, and poor necrosis (grades I–II) in 17. In eight other patients the test samples studied indicated that the tumour was resistant to doxorubicin and chemonecrosis was poor in these patients. The difference was statistically significant ($P=0.006$, Fischer's exact test; Table 1).

Sensitivity test post-chemotherapy

Following resection of the tumour, doxorubicin sensitivity could only be performed in 22 of 25 patients. This group had poor necrosis, and residual viable tumour was present in resected specimens. Doxorubicin resistance was seen in 19 of these patients. Doxorubicin binding assay performed in the three other patients indicated that the tumour remained sensitive.

P-gp expression and tumour necrosis

In the pre-chemotherapy samples, P-gp expression was positive in 16 patients and negative in 29 (Table 2). Of those with positive expression, chemonecrosis was good in four and poor in 12. In 29 patients with negative expression, chemonecrosis was good in 20 and poor in 13. P-gp expression as a predictor for chemonecrosis was not statistically significant ($P=0.066$, Fischer's exact test).

When considering doxorubicin sensitivity, P-gp expression and chemonecrosis (Table 3), it is seen that of

Table 1 Pre-chemotherapy. Doxorubicin sensitivity and chemonecrosis. Fischer's exact test: significance $P=0.006$

Doxorubicin sensitivity	Chemonecrosis	
	Good	Poor
Sensitive (n=37)	20	17
Resistant (n=8)	0	8

Table 2 Pre-chemotherapy. P-gp expression and chemonecrosis. Fischer's exact test: significance $P=0.066$

P-Glycoprotein expression	Chemonecrosis	
	Good	Poor
P-gp positive (n=16)	4	12
P-gp negative (n=29)	16	13

Table 3 Doxorubicin sensitivity and P-gp expression. Fischer's exact test: significance $P<0.001$

Doxorubicin sensitivity	P-gp expression	
	Positive	Negative
Sensitive (n=37)	8	29
	Necrosis	Necrosis
	Good (0)	Good (20)
	Poor (8)	Poor (9)
Resistant (n=8)	8	0
	Necrosis	–
	Poor (8)	–
Total	16	29

Table 4 Post-chemotherapy. P-gp expression and necrosis. Fischer's exact test: significance $P<0.001$

P-Glycoprotein expression	Chemonecrosis	
	Good	Poor
P-gp positive (n=22)	0	22
P-gp negative (n=23)	20	3

the 37 patients senstive to doxorubicin, P-gp expression was positive in eight and negative in 29. All eight patients with resistance to doxorubicin showed strong P-gp expression. Chemonecrosis in the P-gp positive patients was poor in all patients with or without sensitivity to doxorubicin. Doxorubicin sensitivity had a high correlation with P-gp expression ($P<0.001$). We also studied P-gp expression in post-chemotherapy samples (Table 4). It was not expressed in any patient with good tumour necrosis. In 25 patients with poor chemonecrosis its expression was strongly positive in 22, and in the other three it was weakly positive. These three patients were the same patients with residual "sensitive" tumour after chemotherapy; this correlation was statistically significant ($P<0.001$).

Clinical outcome

We compared the incidence of metastases in patients who were sensitive or resistant to doxorubicin (Table 5). All eight patients who were resistant to doxorubicin developed systemic failure and progression of disease with pulmonary metastases at a mean interval of 5 months after surgery. Six of these died of their disease at a mean interval of 11 months, and two remain alive with the disease.

In the doxorubicin-sensitive group (n=37), two of 20 patients with good chemonecrosis, and seven of 17 with poor necrosis developed pulmonary metastases. Aggressive salvage chemotherapy was instituted in all patients with metastases. The two patients with good chemonecrosis and metastases responded well to salvage chemotherapy and remain alive with no evidence of disease. In

Table 5 Doxorubicin sensitivity and metastases (*NED* no evidence of disease, *AWD* alive with disease, *DOD* died of disease). Fischer's exact test: significance $P<0.001$

Doxorubicin sensitivity	Metastases	
	No metastases	Metastases
Resistant (*n*=8)	0	8 (6 DOD and 2 AWD)
Doxorubicin sensitive (*n*=37)	28 (28 NED)	9 (2+7)* (4 DOD, 3 AWD, 2 NED)

* Two in patients with good chemonecrosis and seven in patients with poor chemonecrosis

the poor chemonecrosis group, four of the seven patients who developed metastases died of their disease and three remain alive with the disease. Doxorubicin sensitivity tests were highly significant in determining the incidence of metastases ($P<0.001$).

Discussion

The reliable assessment of drug resistance before treatment is essential in order to allow a change of management in patients shown to have resistance. Doxorubicin is an anthracycline and is naturally fluorescent under certain conditions of excitement. Its intracellular uptake and nuclear binding can therefore be accurately assessed. As one of the more active agents used in the treatment of osteosarcoma, its sensitivity may be a measure of the response to chemotherapy. However, cautious interpretation of the doxorubicin sensitivity assay is needed as sensitivity to doxorubicin does not guarantee a satisfactory response in terms of chemonecrosis. Despite initial sensitivity, 46% of our patients failed to respond to chemotherapy, and in these patients post-surgery specimens showed high expression of P-gp, indicating that drug resistance had developed during the course of chemotherapy and was thus an acquired rather than an inherent phenomenon. In three patients, failure could be attributed to poor dose intensification and poor patient compliance; the post-chemotherapy sensitivity to doxorubicin was retained (though reduced) and only a weakly positive P-gp expression was seen after treatment.

In the majority of osteosarcoma patients, chemoresistance is usually an acquired phenomenon. The doxorubicin assay indicated that a majority of our patients (82%) were chemosensitive at the time of diagnosis. An earlier study [10] indicated that elevated P-gp expression was seen in 23% of osteosarcomas as compared to 35% in our study. Patients with high initial chemosensitivity often acquire resistance to drugs during the course of their treatment. Thus pre-chemotherapy doxorubicin sensitivity or negative immunohistochemistry for P-gp expression should be interpreted with caution, as some patients may respond unfavourably. In contrast, however, doxorubicin resistance at the time of diagnosis (18% of patients) almost always leads to an unfavourable response in terms of chemonecrosis and metastases formation. The doxorubicin sensitivity test was found to have a high predictive value ($P<0.001$) as compared to the P-gp expression, which was statistically less significant

($P=0.066$). A negative P-gp expression does not necessarily correspond to a good outcome, and apart from P-gp expression a number of other mechanisms may account for loss of sensitivity to anti-chemotherapeutic agents [7]. Doxorubicin resistance was also associated with a high incidence of metastases and a poor outcome ($P<0.001$).

In conclusion, we found that the doxorubicin binding assay provides useful information prior to chemotherapy in patients with osteosarcoma. The predictive diagnostic value of this simple functional assay is better than that of P-gp expression. However, pre-treatment of P-gp expression combined with pre-treatment doxorubicin resistance is a strong indicator that such patients may be prone to failure of treatment. An innovative and aggressive approach needs to be developed for this category of patients with demonstrable pre-treatment resistance to chemotherapy, as conventional treatment modalities do not seem to offer a reasonable chance of cure.

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