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ARTICLE

Human Osteosarcoma Xenografts and Their Sensitivity to Chemotherapy

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Despite the increased survival rates of osteosarcoma patients attributed to adjuvant chemotherapy, at least one third of the patients still die due to their disease. Further improvements in the management of osteosarcoma may rely on a more individualised treatment strategy, as well as on the introduction of new drugs. To aid in the preclinical evaluation of new candidate substances against osteosarcoma, we have established 11 human osteosarcoma xenograft lines and characterised them with regard to response to five different reference drugs. Doxorubicin, cisplatin methotrexate, ifosfamide and lomustine were effective in 3/11, 3/11, 1/10, 5/11 and 4/11 of the xenografts, respectively. Five xenografts were resistant to all compounds tested. We also assessed the mRNA expression levels of the xenografts for the O^6-Methylguanine DNA Methyltransferase (MGMT), DNA topoisomerase II- (Topo II)-α, Gluthathione-S-transferase (GST)-π, Multidrug-resistance related protein (MRP) 1 and Multidrug-resistance (MDR) 1 genes. There was an inverse correlation between the transcript levels of GST-π and doxorubicin growth inhibition ($r= -0.66$; $p<0.05$), and between the transcript levels of MGMT and the effect of lomustine ($r= -0.72$; $p<0.01$), whereas the expression of MRP1 and cisplatin growth inhibition was positively correlated ($r=0.82$; $p<0.005$). This panel of xenografts should constitute a good tool for pharmacological and molecular studies in osteosarcoma. (Pathology Oncology Research Vol 10, No 3, 133–141)

Keywords: osteosarcoma, human tumor xenograft, chemotherapy, drug resistance

Introduction

Chemotherapy has become a cornerstone in the primary treatment of osteosarcoma. Today survival rates of around 60% are achieved, whereas not more than 20% were cured by surgery alone.[1,2] Multimodal treatment regimens usually involve neo-adjuvant chemotherapy with high-dose methotrexate, doxorubicin, cisplatin, and more recently ifosfamide. Reviews of the literature indicate that a survival plateau approaching 60% can be achieved with several different drug combinations, and that inclusion of additional drugs or recent attempts to optimise treatment schedules, have not been convincing in terms of survival benefits.[3] Furthermore, ultraaggressive combination chemotherapy is associated with acute and long-term toxicity. This is of particular concern in patients who either do not have micrometastatic disease, or would have been cured with a simpler and less toxic regimen.[3] Hence, further improvements in the management of osteosarcoma seemingly depend on the inclusion of novel treatments as well as on diagnostic and prognostic tools that may allow for a more individualised and risk-adapted treatment.

Human tumor xenografts in nude mice have been widely used in drug screening and in the preclinical evaluation of new anticancer drugs. Since the biological heterogeneity is pronounced, with considerable differences in chemosensitivity, even within the same histological group of human tumors, it is acknowledged that a potential new drug should be tested in a panel of tumor xenografts. Here we report the establishment and characterisation of 11 human osteosarcoma xenografts, with regards to their sensitivity to drugs used clinically, i.e. doxorubicin, cisplatin, methotrexate and ifosfamide, as well as to lomustine. We

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also assessed the mRNA expression levels of the xenografts for the 0^6-Methylguanine DNA Methyltransferase (MGMT), DNA topoisomerase II-α (Topo II)-α, Gluthathione-S-Transferase (GST)-π, Multidrug-resistance related protein (MRP) 1 and Multidrug-resistance (MDR) 1 genes, in attempts to reveal whether the transcript levels would correlate with the sensitivity to the individual drugs tested.

Materials and methods

Animals and establishment of tumor xenografts

Male and female Balb/c mice, bred at the nude rodent facility at the Norwegian Radium Hospital were used. The animals were maintained under specific pathogen-free conditions, Food and water were supplied *ad libitum*. Housing and all procedures involving animals were performed according to protocols approved by the animal care and use committee, in compliance with the National Committee for Animal Experiments guidelines on animal welfare. The animals were 4-8 weeks of age at the day of tumor implantation. Anaesthesia was obtained with 0.5 mg/g propanidid (SombrevinTM; Gedeon Richter Ltd, Budapest, Hungary). Fragments of tissue from biopsies or surgically removed tumors from patients with osteosarcoma were implanted s.c. into the flanks of nude mice. A xenograft was considered to be established and could be used for therapy experiments when the growth rate had stabilised, usually after 3-5 passages.

Histology

The morphology of archival tumor tissue from patients was compared to different passages of their corresponding xenografts, using conventionally stained paraffin-embedded sections examined by light microscopy.

Drugs, doses and treatment

Drugs were dissolved in saline to obtain solutions of 0.8 mg/ml doxorubicin (Adriamycin™; Pharmacia Upjohn, Stockholm, Sweden), 0.5 mg/ml cisplatin (Bristol-Myers Squibb, NY, USA) and 24 mg/ml ifosfamide (Holoxan™; Asta Medica, Frankfurt, Germany). Infusion concentrate of methotrexate (Methotrexate™, 25 mg/ml; Pharmacia & Upjohn; Stockholm, Sweden) was diluted in saline to obtain a final concentrations of 15 mg/ml. Lomustine (Lomustine™; medac, Hamburg, Germany) was suspended in glycerol to a concentration of 2 mg/ml. Maximal tolerable doses (MTD), inducing a median bodyweight loss of up to 10%, were found to be 8 mg/kg doxorubicin, 5 mg/kg cisplatin, 150 mg/kg methotrexate, 240 mg/kg ifosfamide, 20 mg/kg lomustine. All drugs were administered i.v. on days 0 and 7 i.v., except for lomustine that was given i.p.

Evaluation of antitumor activity

For therapy experiments, tumor fragments of 2x2x2 mm were implanted s.c. in both flanks of nude mice. The animals were randomised for treatment according to tumor size when the average tumor diameters were about 6 mm. Animals bearing tumors with diameters < 4 mm or > 8 mm were excluded. From the first day of treatment tumor diameters were measured one to three times per week. Tumor volume was calculated by the formula 0.5 x length x width^2. Relative tumor volumes (RTV) were defined as 100 for each individual tumor at the start of the treatment, day 0. Construction of growth curves and calculation of the parameters used for assessment of antitumor activity, the specific growth delay (SGD) and the maximal growth inhibition (T/C%), were based on median RTVs. SGD values were calculated according to the formula:

$$SGD = (TD_{treated} - TD_{control}) / TD_{control}$$

where TD is the tumor doubling time from start of treatment. The time for one (TD_{200}) or two (TD_{400}) median RTV doubling times was applied to provide values for SGD_{200} or SGD_{400}.

The maximal growth inhibition was calculated according to the formula:

$$T/C\% = (RTV_{treated} / RTV_{control}) * 100\%$$

The antitumor activity was defined as: (+), SGD >1.0 or T/C% < 50 %; +, SGD >1.0 and T/C% < 50 %; ++, SGD >1.5 and T/C% < 40 %; +++, SGD >2.0 and T/C% < 25 %; ++++, SGD >3.0 and T/C% < 10 %. Deaths occurring within two weeks after the final injection were considered as toxic and the animals were excluded from the study.

Northern blot analysis

Total cellular RNA was prepared by the guanidinium-thiocyanate-caesium chloride method described by Sambrook et al.[4] Samples of five µg total RNA were separated by 1% agarose-formaldehyde gel electrophoresis and transferred onto Hybond-N+ membranes (Amersham Pharmacia Biotec, Uppsala, Sweden). After baking for 2 h and subsequent ultraviolet cross-linking, the membranes were hybridized with DNA probes labeled with ^{32}P according to the random primer technique.[5] The hybridizations were carried out in a buffer containing 0.5 M disodium phosphate (pH 6.6), 7% SDS and 1 mM EDTA at 65°C overnight.[6] The membranes were subsequently washed in 40 mM disodium phosphate (pH 7.2) and 1% SDS. For repeated hybridizations bound probes were stripped off by incubating the filters twice in 0.1% SDS, 0.1x SSC for 5 minutes at 95°C. To

Table 1. **Clinical characteristics of the osteosarcoma patients from whom the xenografted tumor tissue was obtained.**

	Patient characteristics		*Tumor characteristics*					*Chemotherapy* [c]			*Subsequent metastatic disease*
Xenograft	*Age*	*Sex*	*Histology*	*Grade*	*Primary tumor*	*Extent of disease at tissue sampling* [b]	*Origin of xenografted tissue*	*Prior to tissue sampling*	*Other chemotherapy*	*Response* [e]	
TTX	40	M	Fibrobl.	4	Os frontalis	M -	Primary		Mt, Do, Cp	ND	Lungs, and local relapse
OHSX	16	M	Osteobl.	4	Distal femur	Skeleton, multiple	Primary		Do	Progression	Skeleton
AOX	37	M	Osteobl.	2	Femur	M -	Primary		ND	ND	Lungs, bilaterally and arm
SBX	68	F	Osteobl.	4	Femur diafysis	M -	Primary				Lungs, abdomen
ALSKX	42	M	Osteobl.	3-4	Femur	Lungs, bilaterally	Lung metastasis	Mt, Do, Cp	If, Et	Grade II-IV	Lungs, bilaterally
TSX pr.1 [a]	17	M	Osteobl.	4	Distal femur	M -	Primary	Mt, BCD	Grade II		Lungs
TSX pr.2 [a]	17	M	Osteobl.	4	Distal femur	Lungs, bilaterally	Lung metastasis	If	Grade I-II		Lungs and columna
HPBX	19	M	Chondrobl.	4	Tibia	Lungs	Lung metastasis	(Mt, BCD, Do)[d]	If, Ep	Grade II	Lungs and mediastinum
TPX	34	M	Osteobl.	3	Proximal femur	M -	Primary		Mt, Cp, Do, If	Progresion	Lungs, bilaterally and brain
KPDX	8	M	Osteobl.	4	Distal femur	M -	Primary	Mt	Do,Cp	Grade II	Lungs, bilaterally
FTX	17	M	Osteobl.	4	Femur	Lung, solitary	Lung metastasis	Mt, Do, Cp		Grade II	Lungs

[a] TSX pr.1 and TSX pr.2 were established from the primary tumor and lung metastases respectively, of the same patient.
[b] M - : No metastatic disease.
[c] Mt: methotrexate; Do: doxorubicin; Cp: cisplatin; If: ifosfamide; Ep: etoposide; BCD: bleomycin, cyclophosphamide, dactinomycin.
[d] Received Mt and BCD preoperatively to resection of primary tumor 4 years earlier.
[e] Response to chemotherapy according to Huvos grading of chemotherapy effects on primary osteosarcoma.

correct for uneven amounts total RNA loaded in each lane, the filters were rehybridized with a kinase-labeled oligonucleotide probe specific for human 18S rRNA. The levels of specific RNA were calculated relative to the amount of 18S rRNA after scanning of the autoradiograms in a Molecular Dynamics Computing Densitometer. The mRNA expression levels were subsequently classified as follows: -/+, undetectable/low expression, ++ and +++, high or very high expression. The probes used were as follows: Topo II-α cDNA probe was kindly provided by Dr. L. Liu, Johns Hopkins University School of Medicine,[7] GST- was kindly provided by Dr. David Warren, Central Laboratory, Norwegian Radium Hospital,[8] MGMT was provided by Dr. Robert Shoemaker, Laboratory for Drug Discovery Research and Development, NCI, USA[9] and full length MRP 1 cDNA was a gift from Prof. P. Borst, Netherlands Cancer Institute.[10] A probe specific for the MDR1 gene was synthesized by PCR using upper primer 5'-ATATCAGCAGCCCACATCAT-3' and lower primer 5'GAAGCACTGGGATGTCCGGT - 3'.[11]

Statistical analysis

Sample correlation coefficients between treatment efficacy and the mRNA expression of resistanceassociated genes were calculated according to the formula:

$$r = \Sigma(X_i - X_{mean})(Y_i - Y_{mean}) \sqrt{} [\Sigma(X_i - X_{mean})^2] [(Y_i - Y_{mean})^2],$$

where X_i represented the sensitivity (1 -T/C) of xenograft *i* to drug *X* and Y_i represented the expression of gene *Y* in xenograft *i*. X_{mean} represented the mean sensitivity to the drug *X* and Y_{mean} represented the mean expression of gene *Y*. The test statistic $t = (\sqrt{n-2})\ r / \sqrt{1-r^2}$, where d.f. = n-2 for t, was used to test for the level of significance at which the null hypothesis H_o: r = 0 could be rejected in favour of H_1: r # 0.

Results

Approximately 20% of the transplanted osteosarcoma specimens were successfully established as xenografts in nude mice. Eleven xenografts were established from parental tumor tissue received from 10 different patients. Two of the xenografts, TSX pr1 and TSX pr2 were

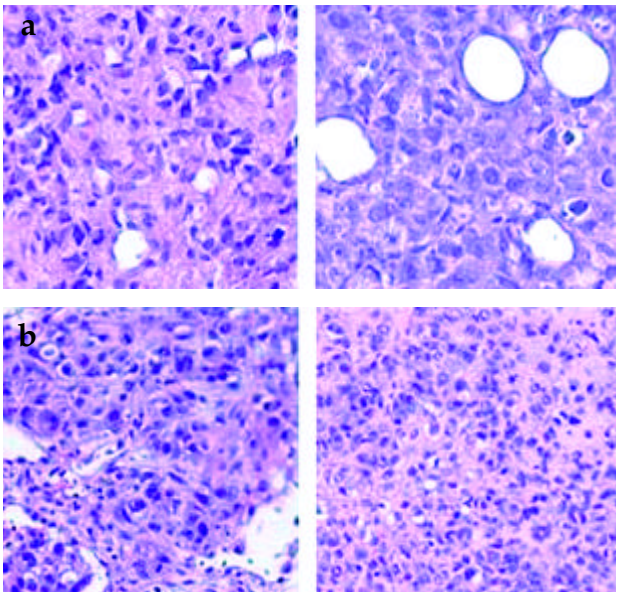

Figure 1. a: *OHSX; Histology from patient primary tumor (left) and histology from the established xenograft.* ***b:*** *HPBX; Histology from patient lung metastasis (left) and histology from the established xenograft.*

established respectively from the primary tumor and a lung metastasis from the same patient. Two patients had previously been treated for retinoblastoma (OHSX and TTX). Seven xenografts originated from primary tumors. Five of these were from primary biopsies and the implanted tissue had therefore not been exposed to any chemotherapy (TTX, OHSX, AOX, SBX, TPX), whereas 2 were from surgically removed primary tumors from patients previously treated with chemotherapy (TSX prl, KPDX). Four xenografts were established from lung metastasis. Three of these patients had received chemotherapy before surgery (ALSKX, TSX pr2, FTX), one of them had received chemotherapy 4 years earlier (HPBX). The origin of the xenografts and clinical characteristics of the corresponding patients are shown in *Table 1*.

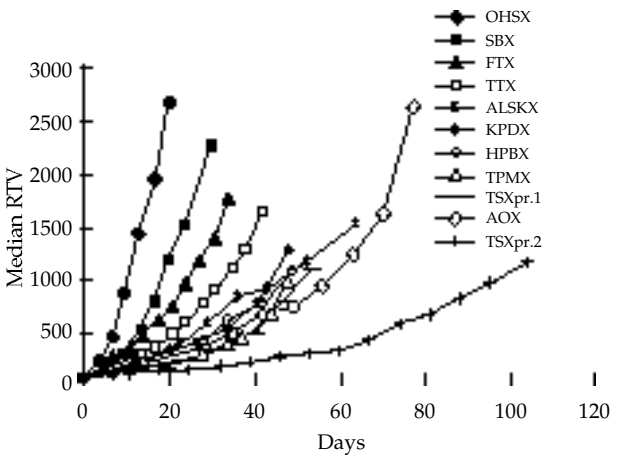

Figure 2. *Median relative tumor volume (median RTV) growth curves of subcutaneous human osteosarcoma xenografts in nude mice.*

Comparison of xenograft tissue and tissue from the original tumor by light microscopy confirmed that the histological features of osteosarcoma were grossly maintained in all cases, as exemplified in *Figure 1*.

Growth characteristics and maximal growth inhibitions (T/C%) and growth delays (SGD) achieved with chemotherapy are listed in *Table 2*. Growth curves for the individual xenografts are shown in *Figure 2*. The median tumor volume doubling times varied between 3 and 20 days.

Defining drug efficacy as SGD>1 and T/C%<50, doxorubicin, cisplatin methotrexate, ifosfamide and lomustine were effective in 3/11, 3/11, 1/10, 5/11 and 4/11 of the xenografts, respectively. Five of the 11 tumors were resistant to all compounds tested. The xenografts established from patients previously treated with chemotherapy were less sensitive than those established from primary biopsies, i.e. 2/6 and 4/5 xenografts respectively were regarded as sensitive to any of the drugs tested. Seemingly there was no difference with regards to growth rates between xenografts established respectively from tumor tissue previously exposed or unexposed to chemotherapy.

Because osteosarcoma patients are treated with multimodal chemotherapy, it was not possible to compare directly responses in the individual patients with those of the respective xenografts. However, in general the patients from whom the xenografted tissue originated responded poorly or moderately to chemotherapy and importantly, all succumbed to their disease. Eight out of ten patients had tumors of the extremities and were without overt metastases at initial diagnosis, hence representing a group of patients with an expected long-term survival of 60-70%. Although we lack data to determine whether the specimens attempted established as xenografts did somehow represent a selected group of patients, our findings suggest that tumors that can be grown in nude mice are clinically aggressive and associated with poor outcome. This is similar to what others previously have shown for soft tissue sarcoma.[12]

Table 3 summarises the antitumor activity of the various drugs and the mRNA expression of MGMT, Topo II-α, GST-π, MRP1 and MDR1. Statistical analysis revealed relatively low, but significant negative correlations between antitumor activity of lomustine and MGMT expression, and between doxorubicin and GST-π levels. The efficacy of cisplatin correlated positively with MRP1 expression (*Figure 3*). No significant correlations between antitumor activities and the expression of MDR1 (r-values from 0.1 to 0.5) or Topo II (r-values from 0.05 to 0.48) were found. Furthermore there were no significant correlations among the expression levels of the different genes (r-values from - 0.21 to 0.5).

Table 2. **Tumor growth characteristics and antitumor effects of ifosfamide, lomustine, cisplatin, doxorubicin and methotrexate in s.c. human osteosarcoma xenografts.**

Growth characteristics[a]				*Antitumor effects*				
Xenograft	*Latency (days)*	TD_{200}[b]	TD_{400}[b]	*Treatment*	*Number of tumors*	SGD_{200}[c]	SGD_{400}[c]	*T/C%*[d]
TTX	27-47	5.0-8.0	9.5-17.5	Ifosfamide	8	0.5	0.4	59.1
				Lomustine	8	>36.8	>18.9	0.4
				Cisplatin	8	15.4	8.2	1.1
				Doxorubicin	9	10.6	6.8	2.9
				Methotrexate	11	0.8	0.4	56.3
OHSX	16	2.5-3.0	6.0-8.5	Ifosfamide	12	>71.4	>29.2	3.6
				Lomustine	12	>71.4	>29.2	2.1
				Cisplatin	12	0.2	2.6	13.4
				Doxorubicin	12	0.0	0.1	54.4
				Methotrexate	12	1.1	0.6	47.1
AOX	65-84	12.5-20.0	30.0-38.5	Ifosfamide	11	>15.9	>6.0	2.5
				Lomustine	11	1.6	1.0	25.4
				Cisplatin	9	1.7	1.1	24.9
				Doxorubicin	11	1.0	1.3	19.1
				Methotrexate	11	0.1	0.1	83.0
SBX	27	7.0	12.0	Ifosfamide	11	0.3	2.4	8.5
				Lomustine	11	0.0	0.3	49.6
				Cisplatin	8	0.2	0.2	58.9
				Doxorubicin	10	0.0	0.0	68.8
				Methotrexate	11	-0.2	0.0	78.0
ALSKX	37-39	10.5-11.0	21.5-22.5	Ifosfamide	9	3.2	1.8	14.2
				Lomustine	14	0.1	0.1	77.4
				Cisplatin	14	0.1	0.2	58.7
				Doxorubicin	11	0.0	0.7	52.7
				Methotrexate	13	-0.2	-0.1	102.6
TSX pr. 1	23-56	6.0-9.0	14.0-34.0	Ifosfamide	9	1.0	0.5	40.6
				Lomustine	8	2.8	1.4	10.7
				Cisplatin	8	0.2	0.1	78.8
				Doxorubicin	11	1.3	1.3	29.4
				Methotrexate	8	0.0	0.0	93.0
TSX pr. 2	63	33.5	64.0	Ifosfamide	11	0.8	0.5	48.9
				Lomustine	12	0.1	-0.1	77.9
				Cisplatin	13	0.8	0.3	51.1
				Doxorubicin	15	0.2	0.1	64.1
				Methotrexate	NA	NA	NA	NA
HPBX	49	15.0	25.0	Ifosfamide	6	0.5	0.7	42.0
				Lomustine	9	-0.1	0	96.9
				Cisplatin	7	0	0.6	45.1
				Doxorubicin	8	0.7	0.9	38.1
				Methotrexate	7	-0.4	-0.1	99.6
TPX	23-27	14.5-21.5	32.5-33.0	Ifosfamide	8	0.0	0.5	43.0
				Lomustine	10	0.3	0.5	57.0
				Cisplatin	8	-0.1	-0.2	86.6
				Doxorubicin	9	0.1	0.1	83.1
				Methotrexate	8	-0.2	0.1	101.2

Table 2. (continue) **Tumor growth characteristics and antitumor effects of ifosfamide, lomustine, cisplatin, doxorubicin and methotrexate in s.c. human osteosarcoma xenografts.**

Growth characteristics[a]				*Antitumor effects*				
Xenograft	*Latency (days)*	TD_{200}[b]	TD_{400}[b]	*Treatment*	*Number of tumors*	SGD_{200}[c]	SGD_{400}[c]	*T/C%*[d]
KPDX	50-57	9.5-13.5	25.5-27.0	Ifosfamide	11	0.3	0.4	74.2
				Lomustine	12	0.2	0.2	82.7
				Cisplatin	10	0.2	0.6	74.2
				Doxorubicin	10	1.0	0.5	56.5
				Methotrexate	10	0.1	0	89.1
FTX	37-39	3.5-5.0	8.5-12.0	Ifosfamide	8	0.5	0.1	62.9
				Lomustine	9	0.2	0.0	76.6
				Cisplatin	10	0.0	0.5	53.9
				Doxorubicin	8	0.1	0.0	55.5
				Methotrexate	12	0.7	0.3	63.2

[a] Median values from single experiments are displayed. Ranges are shown were experience is obtained from more than one experiment.
[b] TD_{200} and TD_{400} were defined as the period (days) required for the median RTV to reach a value of 200 and 400 respectively.
[c] Specific growth delay calculated according to the formula: $SGD = (TD_{treated} - TD_{control}) / TD_{control}$. TD_{200} and TD_{400} were used to obtain values for SGD_{200} and SGD_{400}, respectively. [d] Maximal growth inhibition: The largest difference observed between the median RTVs of the control and the treated group at a particular day during the course of the experiment: $T/C\% = (RTV_{treated} / RTV_{control}) * 100\%$.

Discussion

In a rare tumor type like osteosarcoma a reliable clinical testing of new treatment approaches will take a very long time. Hence, the preclinical evaluation is particularly important. Here we report on the characterisation of a large panel of human osteosarcoma xenograft lines that might be used in the preclinical evaluation of novel therapies. For reference purposes, the chemosensitivity of each line to five drugs was tested. Similar studies have been performed with xenografts from various malignancies, including human soft tissue sarcoma,[13-15] but to our knowledge not with a large panel of osteosarcomas. Although Meyer and colleagues have reported the estab-

Table 3. **Upper panel: Summary of antitumor activities of ifosfamide, cisplatin, lomustine, doxorubicin and methotrexate in s.c. human osteosarcoma xenografts. Lower panel: mRNA expression of MGMT, Topo II-α GST-π, MRP1 and MDR1.**

	Xenograft										
Drug	*TTX*	*OHSX*	*AOX*	*SBX*	*ALSKX*	*TSXpr1*	*TSXpr2*	*HPBX*	*TPMX*	*KPDX*	*FTX*
Ifosfamide	-++++	++++	+++	+++	+	(+)	(+)	(+)	-	-	
Lomustine	++++	++++	++	(+)	-	+++	-	-	-	-	-
Cisplatin	++++	+++	++	-	-	-	-	(+)	-	-	-
Doxorubicin	++++	-	+	-	-	+	-	(+)	-	(+)	-
Methotrexate	-	+	-	-	-	-	-	-	-	-	-
Resistance gene											
MGMT	-	-	-	+++	+	+	ND	++(+)	-	+++	++(+)
GST-	+	++	-	+++	++	+	ND	++	+++	+++	++
MRP1	++(+)	++	++(+)	++(+)	++(+)	(+)	ND	++(+)	-	-	+
MDR1	++	+	+	+	+	-	ND	+	+	++	-
TOPO II-α	+++	++	+	+++	++	++	ND	++(+)	-	++	+

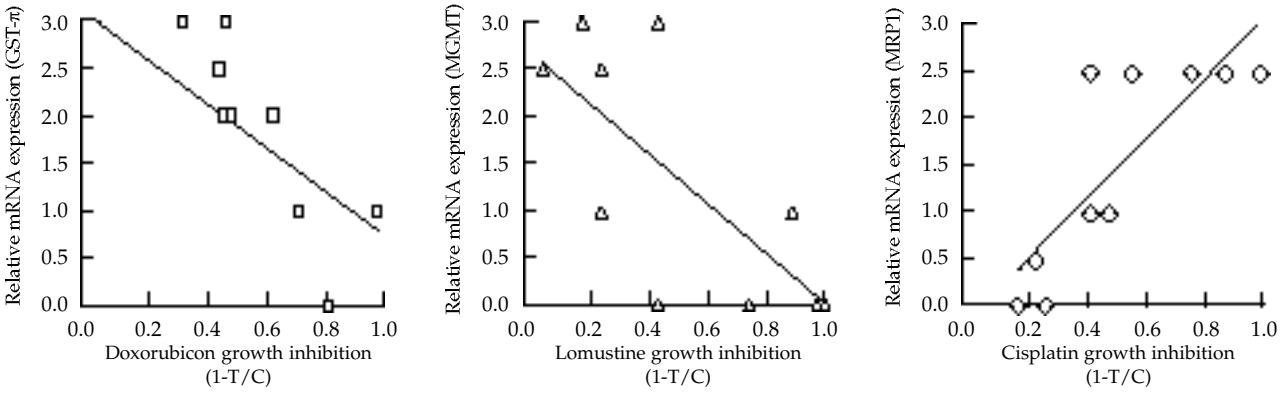

Figure 3. *Left: Correlation between mRNA expression levels of GST-p and doxorubicin growth inhibition (r = -0.66, p < 0.05). Middle: between mRNA expression levels of MGMT and lomustine growth inhibition (r = -0.72, p < 0.01). Right: between mRNA expression levels of MRP1 and cisplatin growth inhibition (r = 0.82, p < 0.005)*

lishment of 7 human osteosarcoma xenografts, these were only characterised histologically and cytogenetically.[16]

For doxorubicin, cisplatin and ifosfamide, the response rates in our xenografts corresponded with response rates around 30 % obtained with these drugs in clinical single agent studies.[1,17] Two of the 3 xenografts responsive to doxorubicin were only weakly sensitive. The efficacy of doxorubicin may therefore be slightly underestimated when compared to its overall clinical activity in osteosarcoma, possibly due to the selection of poor prognosis patients and the pre-treatment of some of the patients. It was not surprising that methotrexate was weakly effective in just one of the xenografts, first, because of the low dose intensity achieved without leucovourin rescue. Secondly, the high circulatory levels of folates found in mice may antagonise the effects of this drug. Lomustine has not been used in treatment of osteosarcoma and comparisons to clinical studies could therefore not be drawn.

Notably, it is likely that tumors successfully established as xenografts represent a selected group with inherent properties associated with aggressive growth and possibly with poor clinical outcome. This might explain, at least in part, the overall modest antitumor activity of the drugs tested. Moreover, some of the xenografts originate from patients having received chemotherapy prior to the sampling of tumor tissue for xenografting. The relative resistance of the xenografts to therapy is, however, not necessarily a disadvantage for further work, as xenograft models often have been claimed to overestimate rather than underestimate drug effects.

The expression of some well-characterised genes linked to drug resistance was assessed to examine whether their transcript levels would correlate with the sensitivity to any of the individual drugs tested.

For osteosarcomas, much of the effort made to find prognostic markers and markers that could allow for a more stratified therapy, has been attributed to the expression of P-glycoprotein. Thus, P-glycoprotein expression in osteosarcoma has been associated with a less malignant phenotype, suggesting a causal relationship to doxorubicin resistance.[18,19] However, the contention of the adverse prognostic value of P-glycoprotein remains to be proven. Thus, whereas some studies have linked expression of P-glycoprotein/MDR1 to a poor clinical outcome,[20-23] others have questioned the relevance of this molecule as a clinical marker.[24-26] In our panel of xenografts, MDR1 mRNA expression level did not correlate to doxorubicin growth inhibition (r = 0.10). This may, however, not contradict the putative value of MDR1/P-glycoprotein as a clinical marker of outcome/chemosensitivity in osteosarcoma because of the possibility that our xenograft panel may represent a selected group of tumors, several of which originates from patients having received prior chemotherapy. Moreover, immunohistochemical studies suggest that a rather small fraction of cells within primary osteosarcomas express high levels of P-glycoprotein, and that these few cells are responsible for the resistant phenotype.[22-24,27,28] This might also help explain why the reported relationship between P-glycoprotein expression and poor clinical outcome was not reflected in our study assessing MDR1 expression in the xenografts at the mRNA level.

The MRP family includes several members that are known from functional studies to mediate cellular efflux of organic anions, for example secondary metabolic reaction conjugates with glutathione, glucuronate or sulphate, including a broad spectrum of anticancer agents.[29] The first member to be linked to drug resistance, MRP1, has been detected in numerous tumor types, including osteosarcoma.[30] However, its impact on clinical resistance is currently not clear. In our xenograft panel, the transcript levels of MRP1 showed significant positive correlation with cisplatin growth inhibition, but did not correlate significantly with the efficacy of any of the

other drugs tested (r = 0.34-0.53). Although an inverse relationship may not be expected because studies with cells suggest that other members of the MRP family, but not MRP1, mediate cisplatin resistance,[29] the biological contention of our finding is elusive. However, it may be speculated if the expression of MRP1 reflects other tumor characteristics that influence the sensitivity to cisplatin.

Elevated expression levels of GST-π- have been found in cell lines resistant to doxorubicin, cisplatin and various alkylating agents[31-35] and increased sensitivity to cisplatin and doxorubicin have been observed in cells transfected to express GST-π antisense.[36] High expression of GST-π have been associated with poor chemosensitivity in patients with breast, non small cell lung and ovary carcinoma, and in head and neck cancer.[37-40] In one study, both prognosis and histological to response chemotherapy of primary osteosarcomas were inversely related to GST-π expression at surgery, but not at primary biopsy.[41] In the present xenograft panel, a significant inverse correlation was found between GST-π expression and doxorubicin growth inhibition. It may therefore be somewhat surprising, at least that there was apparently no relationship to cisplatin growth inhibition (r = -0.22), (ifosfamide, r = 0.01; lomustine, r = -0.34). However, drug resistance can obviously be multifactorial and several mechanisms, functionally dependent or independent of each other may contribute. For example, efficient detoxification by glutathione conjugation may depend on active extrusion of the conjugates by different MRP family members. Thus, whereas glutathion-conjugated doxorubicin efflux seemingly can be mediated both by MRP1 and MRP2, extrusion of glutathione-conjugated cisplatin by MRP1 has not been demonstrated.[29]

There is increasing evidence for a relation between topoisomerase enzyme levels and tumor sensitivity to topoisomerase poisons.[42] The efficacy of doxorubicin did not correlate significantly with the mRNA expression of Topo II-α, although the correlation coefficient (r=0.48, p<0.2) was higher than for the other drugs (r=0.05-0.38). However, a real correlation could also have been overlooked because our sample size provides a relatively weak statistical power.

MGMT is regarded as important for DNA repair after exposure to chloroethylating or methylating agents.[43] The present results are in agreement with this, as only lomustine growth inhibiton was found to correlate significantly with MGMT expression.

In summary, the response rates obtained with doxorubicin, cisplatin and ifosfamide, may give a fair estimate of the general chemosensitivity of human osteosarcomas. Bearing this in mind, the panel should be of value in the preclinical evaluation of novel therapies for this malignancy.

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References

1. *Malawer MM, Link MP, Donaldson SS.* Sarcomas of the Bone. In DeVita VT, Hellmann S, Rosenberg SA, eds. Cancer: Principles and Practice of Oncology 2001; 6th Edition (Volume 2):18911936.
2. *Souhami R, Cannon S.* Osteosarcoma. In Peckham M, Pinedo HM, Veronesi U, eds. Oxford Textbook of Oncology. Oxford, Oxford University Press 1995: 1969-1976.
3. *Bruland OS, Pihl A.* On the current management of osteosarcoma. A critical evaluation and a proposal for a modified treatment strategy. Eur J Cancer 33:1725-1731, 1997
4. *Sambrook J, Fritsh EF, Maniatis T.* Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbour Laboratory Press 1989; 2nd Edition.
5. *Feinberg AP, Vogelstein B.* A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13, 1983
6. *Church GM, Gilbert W.* Genomic sequencing. Proc Natl Acad Sci USA 81:1991-1995, 1984
7. *Tsai-Pflugfelder M, Liu LF, Liu AA, et al:* Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21-22. Proc Natl Acad Sci USA 85:7177-7181, 1988
8. *Moscow JA, Townsend AJ, Goldsmith ME, et al:* Isolation of the human anionic glutathione S-transferase cDNA and the relation of its gene expression to estrogen-receptor content in primary breast cancer. Proc Natl Acad Sci USA 85:6518-6522, 1988
9. *Tano K, Shiota S, Collier J, et al:* Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for 06-alkylguanine. Proc Natl Acad Sci USA 87:686-690, 1990
10. *Zaman GJ, Flens MJ, van Leusden MR, et al:* The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci USA 91:8822-8826, 1994
11. *Fuqua SA, Fitzgerald SD, McGuire WL.* A simple polymerase chain reaction method for detection and cloning of low-abundance transcripts. Biotechniques 9:206-211, 1990
12. *Budach W, Budach V.* Association of tumor growth on nude mice and poor clinical outcome in soft tissue sarcoma patients. J Cancer Res Clin Oncol 127:523-530, 2001
13. *Hoffmann J, Schmidt-Peter P, Hansch W, et al:* Anticancer drug sensitivity and expression of multidrug resistance markers in early passage human sarcomas. Clin Cancer Res 5:2198-204, 1999
14. *Boven E, Pinedo HM, van Hattum AH, et al:* Characterization of human soft-tissue sarcoma xenografts for use in secondary drug screening. Br J Cancer 78:1586-1593, 1998
15. *Budach W, Budach V, Stuschke M, el al:* Efficacy of ifosfamide, dacarbazine, doxorubicin and cisplatin in human sarcoma xenografts. Br J Cancer 70:29-34, 1994
16. *Meyer WH, Houghton JA, Houghton PJ, et al:* Development and characterization of pediatric osteosarcoma xenografts. Cancer Res 50:2781-2785, 1990
17. *Harris MB, Cantor AB, Goorin AM, et al:* Treatment of osteosarcoma with ifosfamide: comparison of response in pedi-

atric patients with recurrent disease versus patients previously untreated: a Pediatric Oncology Group study. Med Pediatr Oncol 24:87-92, 1995
18. *Scotlandi K, Serra M, Nicoletti G, et al:* Multidrug resistance and malignancy in human osteosarcoma. Cancer Res 56:2434-2439, 1996
19. *Scotlandi K, Manara MC, Serra M, et al:* The expression of P-glycoprotein is causally related to a less aggressive phenotype in human osteosarcoma cells. Oncogene 18:739-746, 1999
20. *Serra M, Scotlandi K, Reverter-Branchat G, et al:* Value of P-glycoprotein and clinicopathologic factors as the basis for new treatment strategies in high-grade osteosarcoma of the extremities. J Clin Oncol 21:536-542, 2003
21. *Serra M, Maurici D, Scotlandi K, et al:* Relationship between P-glycoprotein expression and p53 status in high-grade osteosarcoma. Int J Oncol 14:301-307, 1999
22. *Chan HS, Grogan TM, Haddad G, et al:* P-glycoprotein expression: critical determinant in the response to osteosarcoma chemotherapy. J Natl Cancer Inst 89:1706-1715, 1997
23. *Baldini N, Scotlandi K, Barbanti-Brodano G, et al:* Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome. N Engl J Med 333:1380-1385, 1995
24. *Posl M, Amling M, Grahl K, et al:* P-glycoprotein expression in high grade central osteosarcoma and normal bone cells. An immunohistochemical study. Gen Diagn Pathol 142:317-325, 1997
25. *Wunder JS, Bull SB, Aneliunas V, et al:* MDR1 gene expression and outcome in osteosarcoma: a prospective, multicenter study. J Clin Oncol 18:2685-2694, 2000
26. *Gorlick R, Huvos AG, Heller G, et al:* Expression of HER2/erbB-2 correlates with survival in osteosarcoma. J Clin Oncol 17:2781-2788, 1999
27. *Suto R, Abe Y, Nakamura M, et al:* Multidrug resistance mediated by overexpression of P-glycoprotein in human osteosarcoma in vivo. Int J Oncol 12:287-291, 1998
28. *Radig K, Hackel C, Herting J, et al:* Expression of P-glycoprotein in high grade osteosarcomas with special emphasis on chondroblastic subtype. Gen Diagn Pathol 142:139-145, 1997
29. *Borst P, Evers R, Kool M, Wijnholds J.* A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst 92:1295-302, 2000
30. *Ifergan I, Meller I, Issakov J, Assaraf YG.* Reduced folate carrier protein expression in osteosarcoma: implications for the prediction of tumor chemosensitivity. Cancer 98:1958-1966, 2003
31. *Batist G, Tulpule A, Sinha BK, et al:* Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. J Biol Chem 261:15544-15549, 1986
32. *Goto S, Ihara Y, Urata Y, et al.* Doxorubicin-induced DNA intercalation and cavenging by nuclear glutathione S-transferase pi. Faseb J 15:2702-2714, 2001
33. *Saburi Y, Nakagawa M, Ono M, et al.* Increased expression of glutathione S-transferase gene in cis-diamminedichloroplatinum(II)-resistant variants of a Chinese hamster ovary cell line. Cancer Res 1989;49(24 Pt 1):7020-5.
34. *Wang YY, Teicher BA, Shea TC, et al:* Cross-resistance and glutathione-S-transferase-pi levels among four human melanoma cell lines selected for alkylating agent resistance. Cancer Res 49:6185-6192, 1989
35. *Puchalski RB, Fahl WE.* Expression of recombinant glutathione S-transferase pi, Ya, or Ybl confers resistance to alkylating agents. Proc Natl Acad Sci USA 87:2443-2447, 1990
36. *Ban N, Takahashi Y, Takayama T, et al:* Transfection of glutathione S-transferase (GST)-pi antisense complementary DNA increases the sensitivity of a colon cancer cell line to adriamycin, cisplatin, melphalan, and etoposide. Cancer Res 56:3577-3582, 1996
37. *Gilbert L, Elwood LJ, Merino M, et al:* A pilot study of pi-class glutathione S-transferase expression in breast cancer: correlation with estrogen receptor expression and prognosis in node-negative breast cancer. J Clin Oncol 11:49-58, 1993
38. *Shiga H, Heath El, Rasmussen AA, et al:* Prognostic value of p53, glutathione S-transferase pi, and thymidylate synthase for neoadjuvant cisplatin-based chemotherapy in head and neck cancer. Clin Cancer Res 5:4097-4104, 1995
39. *Green JA, Robertson LJ, Clark AH.* Glutathione S-transferase expression in benign and malignant ovarian tumors. Br J Cancer 68:235-239, 1993
40. *Bai F, Nakanishi Y, Kawasaki M, et al:* Immunohistochemical expression of glutathione S-transferase-Pi can predict chemotherapy response in patients with nonsmall cell lung carcinoma. Cancer 78:416-421, 1996
41. *Uozaki H, Horiuchi H, Ishida T, et al:* Overexpression of resistance-related proteins (metallothioneins, glutathione-S-transferase pi, heat shock protein 27, and lung resistance-related protein) in osteosarcoma. Relationship with poor prognosis. Cancer 79:2336-2344, 1997
42. *Kellner U, Sehested M, Jensen PB, et al:* Culprit and victim – DNA topoisomerase II. Lancet Oncol 3:235-243, 2002
43. *Gerson SL.* Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol 20:2388-2399, 2002