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Point Mutations in the mdr1 Promoter of Human Osteosarcomas are Associated With *In vitro* Responsiveness to Multidrug Resistance Relevant Drugs

U. Stein, W. Walther and V. Wunderlich

Among human sarcomas, osteosarcomas usually display high intrinsic mdr1 expression while malignant fibrous histiocytomas (MFH) do not. A comparative polymerase chain reaction (PCR)–based sequence analysis of the mdr1 promoter revealed point mutations in seven out of nine osteosarcomas at nucleotides +103 (2 cases T→C) and +137 (5 cases G→T). No changes were seen in eight MFHs. When COS cells transfected with CAT constructs containing the T→C chloramphenicol acetyltransferase mutant mdr1 promoters were treated with vincristine or doxorubicin, expression of the CAT gene was enhanced to a higher extent than with constructs containing wild-type or G→T–mutant mdr1 promoters. We suggest that there is a correlation between the type of mdr1 promoter mutation and responsiveness to MDR relevant drugs.

Key words: sarcomas, multidrug resistance, mdr1 gene, promoter, mutation
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INTRODUCTION

OVEREXPRESSION OF the human mdr1 gene-encoded P-glycoprotein leads to a multidrug resistant (MDR) phenotype, and allows tumour cells to survive exposure to cytotoxic concentrations of anticancer drugs [1, 2]. Intrinsic or acquired MDR is therefore a major obstacle to successful cancer chemotherapy. Increased mdr1 gene expression has been detected in a variety of human cancers [3]. The mechanism responsible for this overexpression is not yet understood. Several lines of evidence suggest regulation at the transcriptional level [4]. Two transcriptional start sites of the mdr1 gene have been identified [5]: a downstream promoter from which the majority of transcripts are derived, and an upstream promoter region, which has been, until now, poorly characterised. In a collection of human bone and soft tissue sarcomas, we recently discovered high intrinsic mdr1 expression in all osteosarcomas analysed, but low intrinsic expression in malignant fibrous histiocytomas (MFHs), irrespective of bone or soft tissue origin [6]. Thus, sequencing and transient expression analysis of mdr1 promoter regions in osteosarcomas versus MFHs should be helpful to understanding the mechanism of overexpression in more detail. While no sequence alterations were found in MFHs, point mutations were detected in the mdr1 promoter region of osteosarcomas. These mutations may be critical for mdr1 overexpression, as suggested by the inducibility of the reporter gene chloramphenicol acetyltransferase (CAT).

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MATERIALS AND METHODS

Tumours

Tumour samples were obtained from Drs P. Schmidt-Peter and W. Haensch as previously described [6]. Sarcomas with distinctly different intrinsic mdr1 expression levels were chosen for this study: nine osteosarcomas with high mdr1 expression and eight bone or soft tissue derived MFHs with low mdr1 expression [6]. High molecular weight DNA was isolated from frozen tumours (−70°C) according to standard protocols [7].

Northern analysis

Isolation of total cellular RNA from frozen tumours and hybridisation conditions were as previously described [6]. Ten micrograms of each tumour RNA were subjected to northern analysis. Nylon membranes were hybridised to a ^{32}P-labelled mdr1-specific RNA probe pHDR 5.A [8] (ATCC-No. 61360). Hybridisations with a GAPDH (glyceraldehyde-phosphate dehydrogenase) DNA probe were carried out to evaluate uniform RNA loading.

Recombinant plasmids

A region of the human mdr1 downstream promoter sequence (−207 to +158); nomenclature according to [9] was generated by a polymerase chain reaction (PCR) under the following conditions: 94°C 1 min; 45°C 1 min; 72°C 2 min; 30 cycles. The 'Gene Amp PCR Reagent Kit with Ampli Taq DNA Polymerase'

(Perkin-Elmer GmbH, Überlingen, Germany) and two human mdr1 specific oligonucleotides were used: 5'-GAATTC-CTTGCCCTTTCTAG-3' (−207 to −194) including the Eco RI restriction site as the 5'-primer and 5'-GGATCC-AGT-AGCTCCCAGCTT-3' (+144 to +158) including the Bam HI restriction site as the 3'-primer [9]. One hundred nanograms of each high molecular weight DNA from human sarcomas served as template for at least two independent PCR experiments. PCR fragments and the plasmid pBluescript M13 (Stratagene, San Diego, California, U.S.A.) were digested with Eco RI and Bam HI and ligated to generate clones pM13mdr1 to pM13mdr9 (for osteosarcomas) and pM13mdr10 to pM13mdr17 (for MFHs).

To analyse anticancer drug inducibility by CAT-ELISA, PCR fragments of each of two plasmids containing mdr1 promoters with the T→C mutation at +103 (pM13mdr3 and pM13mdr5), or with the G → T mutation at +137 (e.g. pM13mdr7 and pM13mdr8), or with the wild-type promoter (e.g. pM13mdr10 and pM13mdr11), were digested with XbaI. To generate CAT constructs containing the digested mdr1 fragments, the pUCAT vector was created by insertion of a 1.36 kb HindIII/Bam HI fragment of pSV2CAT, with a HindIII site converted to a XbaI site, into the XbaI/Bam HI-digested pUC18. The XbaI-digested PCR fragments were cloned into the XbaI-digested pUCAT, resulting in clones pCATmdr3 and pCATmdr5 (T→C), pCATmdr7 and pCATmdr8 (G→T), and pCATmdr10 and pCATmdr11 (wild-type).

Sequencing

All pM13mdr clones were sequenced according to the 'Sequenase Version 2.0' protocol (United States Biochemicals, Cleveland, Ohio, U.S.A.), by using the universal primer 5'-GTTTTCCCAGTCACGAC-3' supplied with the kit and [^{35}S]dATP (Amersham Buchler GmbH & Co KG, Braunschweig, Germany).

Transient transfection and treatment with MDR-relevant drugs

The African green monkey kidney cell line COS was maintained in DMEM (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum at 37°C and 5% CO_2. COS cells (5 x 10^5 per 60 mm dish) were grown and transfected with 10 μg of each linearised pCATmdr construct (pCATmdr3 and pCATmdr5; pCATmdr7 and pCATmdr8; pCATmdr10 and pCATmdr11) by electroporation (100 V, 250 μF) according to Chu and colleagues [10]. pSV2CAT and pUCAT, respectively, served as positive and negative controls. Forty-eight hours after transfection, 20 ng/ml or 50 ng/ml of each anticancer drug (vincristine from Eli Lilly, Indianapolis, Indiana, U.S.A.; doxorubicin as adriablastine from Farmitalia, Freiburg, Germany), dissolved in 0.15 M NaCl, were added for 24 h at 37 °C before the CAT assay was performed. Untreated controls were harvested at the same times as treated cells.

CAT ELISA

The assay was carried out with a commercially available kit (Boehringer Mannheim, Germany). Promoter activity was determined as CAT expression using a non-radioactive digoxigenin labelled antiCAT antibody, following the conditions recommended by the manufacturer. Cell extracts were prepared by using the supplied lysis buffer. The protein concentration was 150 μg/well of a 96-well microtitre plate. An increase in sensitivity was obtained by using the substrate enhancer, supplied by the manufacturer. The blank value was subtracted automatically in the microplate reader.

Statistical analysis

To evaluate the statistical significance of the drug-induced CAT activity, the control group driven by the wild-type mdr1 promoter sequence was compared with the groups driven by the mutant promoter sequences in each CAT-assay using the Student's *t* - test.

RESULTS

Northern analysis

Tumour samples were selected from a panel of 61 sarcomas of adult patients investigated for frequency and extent of mdr1 expression in our previous study [6]. None of the patients had received chemotherapy. The availability of related tumour types with high versus low mdr1 expression levels was a prerequisite for the experiments reported here. Investigations described concern those types of tumours that had shown high mdr1 expression levels at a high frequency, i.e. the nine osteosarcomas shown in Figure 1a, and tumours for which only low or intermediate mdr1 expression levels at a low frequency had been observed, i.e. the eight MFHs shown in Figure 1b.

mdr1 promoter analysis in human sarcomas

After isolation of DNA from the osteosarcomas and MFHs (Figure 1), the mdr1 promoter region (−207 to +158) was amplified by PCR and sequenced. The reason for analysing this particular region was the presence of a drug-responsive element within this sequence [11–13]. To ensure the valdiity of the results, all experiments (PCR amplification, cloning and sequencing) were carried out at least twice per tumour.

In a series of clones containing the amplified PCR products

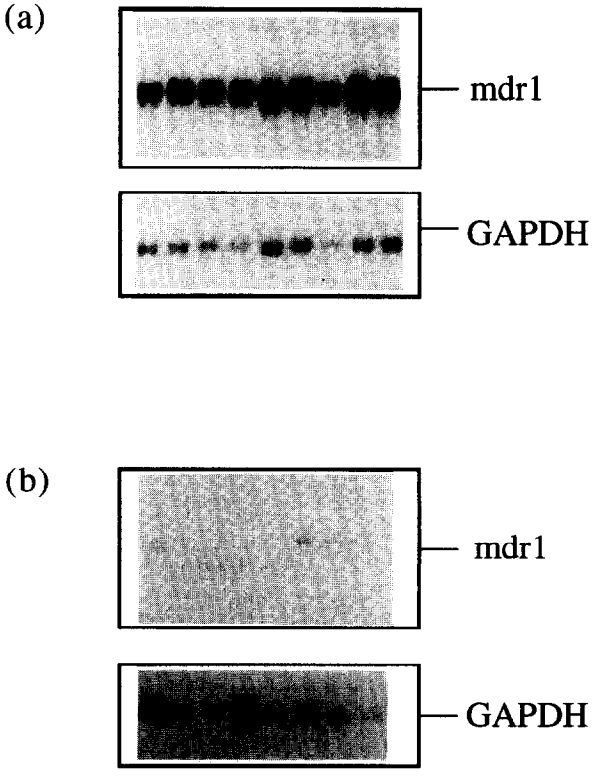

Figure 1. mdr1 expression in human bone and soft tissue sarcomas. (a) Nine osteosarcomas positive for mdr1 expression. (b) Eight malignant fibrous histiocytomas mostly negative for mdr1 expression. Northern hybridisations carried out by using an mdr1-specific riboprobe and compared with GAPDH as an internal standard.

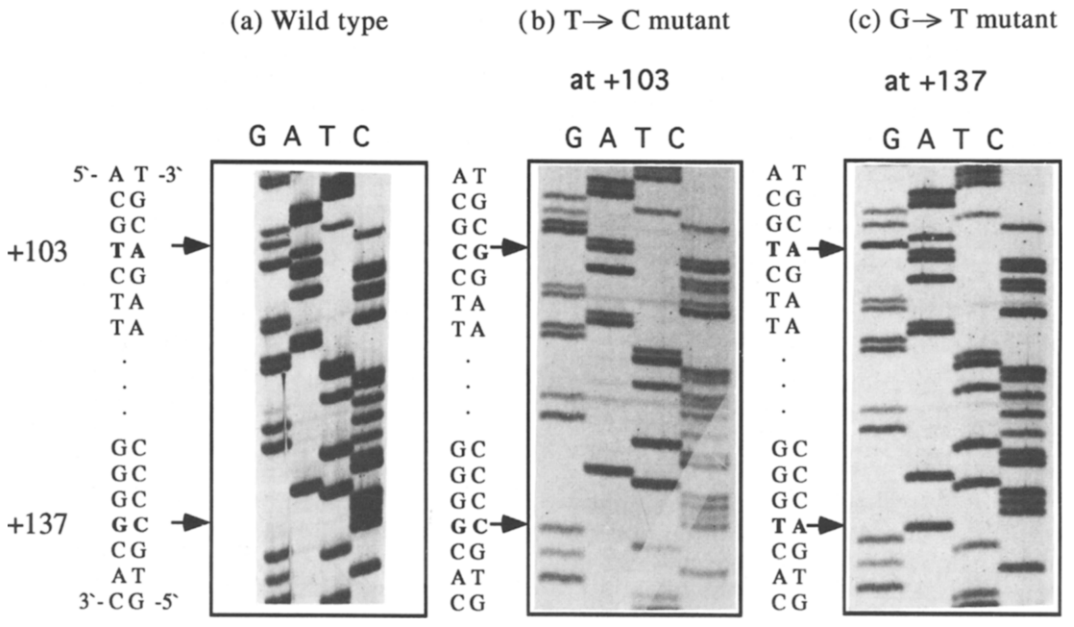

Figure 2. Point mutations within the mdr1 promoter region of human sarcomas. (a) The wild-type mdr1 promoter sequence found in all eight MFHs. (b) The T→C mutant mdr1 promoter sequence (at +103) found in two of nine osteosarcomas. (c) The G→T mutant mdr1 promoter sequence (at +137) found in five of nine osteosarcomas. Bases indicated in bold represent the point mutations and their positions. Sequencing was done with the complementary strand (right strand of indicated sequences).

from nine osteosarcomas, two distinct point mutations were detected (Figure 2): The first was a T→C transition at position +103 present in two of nine osteosarcoma-derived clones (pM13mdr3 and 5). A second type of mutation, a G→T transversion at position +137, was detected in five of nine osteosarcoma-derived clones (pM13mdr2, 4, 7, 8, 9). No other deviations from the wild-type sequence (normal human placenta [9]) were observed within the region analysed. The remaining two osteosarcomas showed no changes in the mdr1 promoter region.

A different picture emerged from the analysis of MFHs, a prototype of human sarcomas with low mdr1 expression. In 8 cases (4 each of bone or soft tissue origin) the sequence was unchanged throughout the entire mdr1 promoter region (Figure 2).

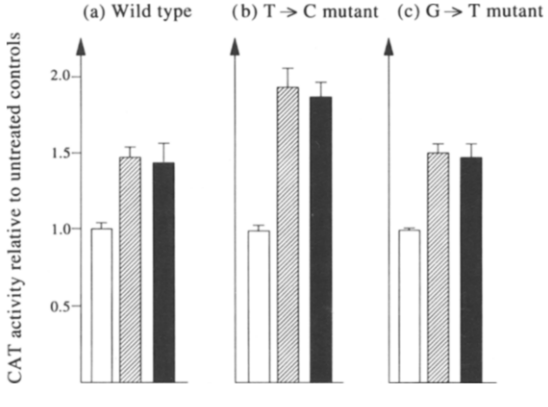

Figure 3. Effects of vincristine and doxorubicin on CAT expression in transfected COS cells driven by (a) the wild-type mdr1 promoter sequence (−207 to +158), untreated (□), +vincristine (▨), +doxorubicin (■); (b) the T→C mutant mdr1 promoter sequence (at +103), untreated (□), +vincristine (▨), +doxorubicin (■); and (c) the G→T mutant mdr1 promoter sequence (at +137), untreated (□), +vincristine (▨), +doxorubicin (■), as measured by CAT ELISA. Average values from five independent assays (±S.E.). Controls: pUCAT (negative) and pSV2CAT (positive).

From these results, it was tentatively concluded that a correlation may exist between mdr1 expression level and the occurrence of certain point mutations in the mdr1 promoter. In order to further substantiate this hypothesis, we performed CAT analyses with mutant and wild-type mdr1 promoter sequences in the presence or absence of drugs that belong to the MDR family.

Effect of anticancer drugs on mutant mdr1 promoter sequences

A set of CAT constructs containing the mutant (T→C or G→T) or wild-type mdr1 promoter sequences was generated in order to analyse the influence of these point mutations on promoter activity and responsiveness to anticancer drugs.

COS cells were transfected with each of these CAT-containing plasmids. Treatments with vincristine and doxorubicin at concentrations of 20 or 50 ng/ml, respectively, were begun 48 h after transfection.

As far as CAT expression driven by the wild-type or mutant mdr1 promoter region is concerned, no differential effects on basal promoter activity were observed (Figure 3). This level of activity was, therefore, taken as 100% to evaluate the potential influence of a treatment with MDR relevant drugs.

CAT expression in pSV2CAT-transfected COS cells (positive control) was more than four to five times higher than in COS cells transfected with the different mdr1 promoter sequences. In pUCAT-transfected cells (negative control), CAT expression was below the level of detection (not shown). Treatment with drugs of the MDR family led to a remarkable induction of CAT expression in all cell clones, irrespective of the mdr1 promoter sequence (Figure 3). Both anticancer drugs caused a comparable degree of induction in a given experiment.

In COS cells transfected with CAT constructs containing the wild-type or the G→T mutant promoters, reporter gene expression was approximately 1.46- and 1.43-fold higher than controls after exposure to vincristine or doxorubicin, respectively. However, in cells transfected with CAT constructs driven by the T→C mutant mdr1 promoter sequence, a significantly

higher induction ($P < 0.05$) was found: in the case of vincristine, CAT expression was 1.91-fold higher and in the case of doxorubicin, CAT expression was 1.84-fold higher (Figure 3) compared with the untreated controls. All results were averaged from five independent experiments. Standard deviations from the means were ≤ 1.5.

These results point to a direct link between the T→C-mutation at position +103 within the mdr1 promoter and increased inducibility by MDR relevant drugs resulting in enhanced expression of the reporter gene, and possibly of the mdr1 gene in cancer cells.

DISCUSSION

There is now general agreement that structural alterations in a number of genes (proto-oncogenes, tumour suppressor genes, etc.) may account for the onset and development of human cancer. Increased mdr1 gene expression is thought to be associated with the progression of many cancers. Whereas amplification of the mdr1 gene is a common event that accompanies increased expression in cell lines but not in clinical tumours [4], the occurence of point mutations has so far appeared to be rather an exception, resulting from mutations in the mdr1 gene coding region in association with drug selection [14]. To our knowledge, point mutations in the non-coding region of the mdr1 gene have as yet not been reported, neither in osteosarcomas nor in any other human tumours. It should be emphasised that both types of point mutation in the promoter region observed in the present study were repeatedly found in *untreated* tumours. With respect to these particular mdr1 promoter mutations it would be interesting to hear, first, whether they occur in both mdr1 alleles; second, whether they occur in other types of tumours and at what frequency; third, whether they are detectable not only in adult (this study) but also in pediatric osteosarcomas, which could lead to the question of somatic versus germline origin; and finally, whether these promoter mutations represent hotspots possibly located at potential binding sites for factors involved in transcriptional regulation. In any event, the appearance of these mutations in independent sarcomas merits further investigation. That these mutations could simply be polymorphisms appears to be unlikely because of the repeated and exclusive occurrence in osteosarcomas.

According to our present knowledge, the expression of mdr1 genes is regulated in a cell-type-specific manner [2, 13]. Differentiation-inducing agents (e.g. retinoic acid) are able to induce mdr1 gene expression [15]. We speculate that the acquisition of +103 or +137 mutations in the mdr1 promoter and the concomitant increase of mdr1 expression may in some way subvert specific differentiation steps in immature precursor cells, subsequently giving rise to osteosarcomas. It remains to be investigated at which development stage, if any, such mutations may occur, by which mechanisms they may be induced, and whether they do indeed predispose to osteosarcomas.

In addition to its role in drug resistance, P-glycoprotein may serve further functions in normal tissues and malignant tumours [1]. For example, in human colon and breast carcinomas, a correlation between mdr1 expression and tumour malignancy has been recently described [16, 17]. This may indicate that cells expressing the mdr1 gene at a high level might possess a selective growth advantage. In the absence of drug exposure, cells may acquire the same property by mutations of the type described in this paper thus explaining the observed high prevalence of such particular mutations.

In view of the occurrence of p53 mutations in human osteosarcomas [18], attention should also be paid to possible specific interactions of mutant p53 with mdr1 promoter sequences [19], leading to transcriptional regulation [20] or to gain-of-function activities [21]. It is conceivable that point mutations beyond the core sequences [20] in the mdr1 promoter could affect this type of interaction.

An interesting result emerged from the functional analysis of the different mdr1 promoter sequences in the CAT assay. Although these constructs could not be distinguished in their effects on basal CAT expression, they responded differently to anticancer drugs. Previous studies have identified responsive elements within mdr1 promoters mediating transcriptional activation by various forms of environmental stress [12, 22]. A specific drug-responsive element, inducible by treatments with anticancer drugs such as vincristine and doxorubicin, has been located at position −136 to −76 [12]. Our finding that, in the presence of a point muation at position +103, mdr1 promoter activity is further stimulated following treatment with the same drugs, suggests a role of additional sequences distant from the known drug-responsive elements. This hypothesis remains to be tested by further experiments. In summary, our results indicate a correlation between a specific point mutation in the mdr1 promoter sequence and responsiveness to drugs of the MDR family.

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The Synergistic and Antagonistic Effects of Cytotoxic and Biological Agents on the *In Vitro* Antitumour Effects of Suramin

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Suramin has shown antitumour activity *in vitro* and *in vivo*. At plasma levels higher than 200 μM there is, however, excessive toxicity. We have, therefore, attempted to improve the antitumour effects of suramin *in vitro* by combining it with several other antitumour agents. The MCF-7 mammary carcinoma and PC3 prostate cancer cell lines were exposed continuously to suramin and the other agents for 6 days. The sulphorhodamine B (SRB) assay was used for the assessment of growth inhibition. The dose–response interactions were evaluated using the median-effect analysis with the Chou and Talalay computer programme. In the MCF-7 cell line, the combination of suramin plus doxorubicin (DXR), cisplatin (CDDP), 5-fluorouracil (5-FU) or tumour necrosis factor (TNF) resulted in synergistic growth inhibition, whilst its combination with miltefosine (HPC) was antagonistic. In the PC-3 cell line, suramin plus CDDP or TNF was synergistic, whilst its combination with DXR, 5-FU and HPC was antagonistic. All tested combinations with interferon-α (IFN-α), interferon-γ (IFN-γ) and with the combination of both IFN-α + IFN-γ were not synergistic. The synergistic effect of suramin with DXR was schedule dependent. Pretreatment (addition of DXR on day 1 and suramin on days 2–5) was additive at the IC_{50} level, in both cell lines. Addition of DXR at day 5 was more effective than simultaneous exposure. We found a synergistic effect for the combination of suramin with CDDP and TNF in both cell lines. In addition the combination with DXR and 5-FU was synergistic in MCF-7. Sequential administration of DXR–suramin or suramin–DXR increased the growth inhibition.

Key words: suramin, prostate cancer, breast cancer, doxorubicin, cisplatin
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INTRODUCTION

SURAMIN, A polysulphonated naphthylurea, was synthesised at the beginning of this century by Bayer (Leverkusen, Germany) and was used for many years in the treatment of trypanosomiasis and onchocerciasis [1]. Recently, it has been shown to possess antitumour properties *in vitro* and *in vivo* [2].

Recently reported phase II studies have demonstrated responses in several human neoplasms including prostate and adrenocortical cancer [3]. These responses have been observed with prolonged administration or higher doses. When the plasma levels of suramin exceeded 200 μM (300 μg/ml), there was, however, an excessive and intolerable toxicity, mainly polyneuropathy and coagulopathy.

The exact mechanism of action of suramin remains unclear.