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Original Paper

MDR1 Gene Expression: Evaluation of its Use as a Molecular Marker for Prognosis and Chemotherapy of Bone and Soft Tissue Sarcomas

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Successful chemotherapeutic treatment of malignant tumours is often limited by the intrinsic or acquired multidrug resistance (MDR). The classical MDR phenotype is characterised by reduced drug accumulation within the cell, caused by overexpression of the *MDR1* gene encoded P-glycoprotein. Some reports have been published evaluating *MDR1* expression as a molecular marker for response to chemotherapy in human bone and soft tissue sarcomas. In this review, an attempt is made to summarise the accuracy of the measurement of *MDR1* expression for use in prognosis, as well as in decisions on chemotherapeutic treatment of sarcomas. In addition, general problems for the performance of such studies is discussed.

Key words: multidrug resistance, *MDR1* gene, P-glycoprotein, bone and soft tissue sarcomas
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INTRODUCTION

BONE AND soft tissue sarcomas represent a major histogenetic class of neoplasms with relatively high malignant potential [1, 2]. Treatment strategies for sarcomas, using polychemotherapy protocols, have been developed in the last decade [3, 4]. However, successful chemotherapy is still often limited by intrinsic drug resistance or resistance acquired following exposure to drugs such as anthracyclins (e.g. doxorubicin), alkaloids (e.g. vincristine), or epipodophyllotoxins (VP-16). This phenomenon, first observed *in vitro* by Biedler and Riehm [5] and termed multidrug resistance (MDR) [for review see [6], is described as a simultaneous resistance against structurally and functionally not related compounds [7]. The classical MDR phenotype is characterised by a reduced drug accumulation within the cells, caused by overexpression of human *MDR1* gene encoded P-glycoprotein, a transmembrane energy-dependent drug-efflux pump with a broad substrate specificity [8–10].

Human *MDR1* expression has been investigated in a variety of normal tissues and tumours of almost all types. In normal tissues, high levels of *MDR1* expression have been found mainly on lumenal surfaces of the colon, jejunum, kidney and liver, in the adrenal cortex, in placental trophoblasts, and in the endothelial cells of brain and testis, representing tissues with secretory or excretory functions [11, 12]. In numerous cancers, *MDR1* overexpression has been detected, either *de novo* or more frequently following chemotherapy; mostly in tumours associated with high *MDR1* expresion levels in their corresponding normal tissue, e.g. carcinomas of colon, kidney and liver [13–15].

In recent years, the expression of the *MDR1* gene and clinical outcome of patients with malignant tumours have been compared. Despite the fact that MDR appears to represent a clinically important resistance mechanism for bone and soft tissue sarcomas and that treatment protocols usually contain MDR relevant cytostatics, comparatively few reports have analysed *MDR1* expression in these sarcomas [12–14, 16–30]. These investigations have generally involved limited numbers of patients and show wide variation in: the tumour material used for the studies (e.g. histological tumour type and grading; tumour material prior or post chemotherapy; childhood versus adult cancer); the methods used for the determination of *MDR1* expression on RNA and protein levels; the definition of "positivity" of *MDR1* expression in tumour samples (e.g. control cell lines); the definition of clinical parameters for response to treatment; and the statistical methods used for correlation analysis and the assessment of prognostic significance for chemotherapy using MDR relevant drugs.

In this review, an attempt has been made to assess the value of *MDR1* expression as a molecular marker for prognosis and chemosensitivity in human sarcomas. The difficulties of such an evaluation will be discussed and the importance of standardised methods for studies of gene expression will be outlined.

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Table 1. Histological tumour types analysed

Histological tumour type	No. of analysed tumours	No. of studies	[References]
Osteosarcoma	153	7	16, 18, 24, 27–30
Rhabdomyosarcoma	94	7	13, 17, 20, 22, 24, 25, 30
Ewing's sarcoma	57	8	13, 18, 20–22, 24–26
Neurogenic sarcoma	43	7	16, 20–24, 30
Malignant fibrous histiocytoma	32	5	16, 22–25
Chondrosarcoma	25	4	18, 23–25
Leiomyosarcoma	18	5	16, 23–25, 30
Liposarcoma	15	5	17, 19, 20, 25
Undifferentiated sarcoma	14	4	17, 19, 20, 25
Synovial sarcoma	11	3	22–24

I. *Bone and soft tissue sarcomas analysed*

Histological tumour type and number of tumour samples. In 16 studies, a total of 462 classified bone and soft tissue sarcomas were investigated. These tumours varied in their histological type and in the number of examined cases per study (Table 1). Thirty rare sarcoma types with only a few representatives (1 or 2 cases per report) were included in this review. Additionally, 11 tumours, only classified as "sarcomas" (one study) as well as 59 tumours described as "soft tissue tumours" (four studies) were analysed. The total number of samples analysed per study is given in Table 2.

In most of the studies, general statements concerning the *MDR1* expression in bone and soft sarcomas as well as a possible correlation with the clinical outcome, were made regardless of the variety and number of the different tumour types analysed. This might be one reason for the contradictory findings of the different research groups, complicating a general comparison and evaluation of these studies.

Grading of tumours. Statements concerning grading were given in only 5 of the 18 analysed studies, using different grading systems even for the same tumour types [31–34]. While in the advanced tumour groups, the number of P-glycoprotein positive tumours was higher and correlated with a poor prognosis [17, 27], no significant correlation was observed between grading and frequency or level of *MDR1* expression and response to chemotherapy, described by Toffoli and associates ([23]; tumours analysed were from G1 to G3), Stein and associates ([24]; grading from 2B to 3B) and Wunder and associates ([28]; high grade osteosarcomas).

Table 2. Number of tumours analysed per study

No. of studies	No. of analysed tumours	[References]
7	21–30	16, 17, 20, 21, 25, 26, 29
4	11–20	12, 14, 28, 30
2	1–10	13, 19
2	61–70	24, 27
1	31–40	23
1	71–80	22
1	91–100	18

Time of determination of MDR1 *gene expression in relation to chemotherapeutic treatment with MDR relevant drugs.* In seven studies, tumour samples were obtained and analysed exclusively from patients prior to any chemotherapy and in four studies, chemotherapeutically treated tumours were described. In another seven studies, sarcomas were investigated prior to and after chemotherapy: both non-sequential (tumours analysed from different patients but at the same time point e.g. at diagnosis only; four reports) as well as longitudinal sequential studies (tumours analysed in the same patient but at different time points, e.g. before and after chemotherapy; three reports), respectively. The percentage of *MDR1* positive sarcomas differed markedly even within uniform untreated or treated tumour groups, as shown in Table 3. Investigations of acquired *MDR1* expression, that is the induction of *MDR1* gene expression caused by chemotherapy using MDR relevant cytostatics, often describe a higher *MDR1* expression level in treated tumour samples compared with tumours analysed at the time of diagnosis. This supports the known effect of inducibility of the *MDR1* expression by certain anticancer drugs [35].

Age of tumour patients. The age of a patient can be crucial for the expression of genes including those associated with drug resistance mechanisms. Despite this fact, there are no data concerning the age of patients whose tumours were examined in 5 of 18 studies. Childhood sarcomas were analysed in six studies, sarcomas of adults in five studies, and two reports covered the results of tumours from both patient age groups.

II. *Methods used for* MDR1/*P-glycoprotein detection*

There are major technical and biological obstacles to the use of *MDR1*/P-glycoprotein expression as a predictor of clinical response to chemotherapy or clinical course: heterogeneity and sampling of the tumour cell population; assessment of tumour cell phenotype versus normal stromal elements (e.g. by immunohistochemistry or *in situ* hybridisation with or without polymerase chain reaction PCR); and other major determinants of response may over-ride the effect of *MDR1*/P-glycoprotein expression (e.g. non-P-glycoprotein mediated MDR phenotypes).

MDR1 *versus P-glycoprotein expression.* To be useful for studies of gene expression, a method should be "relatively easy, sensitive, specific and reproducible" [36]. The most convincing results concerning gene expression are probably obtained by using methods for the detection of the *MDR1*-specific mRNA

Table 3. MDR1 *expression in untreated and chemotherapeutically treated tumours, detected in horizontal studies*

Untreated tumours			Treated tumours		
MDR1 positive	%	[References]	*MDR1* positive	%	[References]
0/11	0	14	0/13	0	25
1/78	1	22	1/4	25	13
1/9	11	25	3/12	25	16
2/14	14	12	6/12	50	23
4/24	17	23	12/21	57	26
3/13	23	16	9/15	60	28
9/26	35	20	24/29	83	29
42/61	69	24			
70/92	76	18			
14/14	100	30			

Table 4. *Methods used for* MDR1 *mRNA and P-glycoprotein detection*

Expression level	Method	[References]
MDR1 mRNA	Slot or dot blot analysis	13, 14, 19, 23, **24**, **25**
	Northern blot analysis	13, 14, 23, **24**, **25**
	RT-PCR	18, 28
	RNase protection assay	14
	In situ hybridisation	**25**
P-glycoprotein	Immunohistochemistry	12, 16, 17, 20–22, **24**, **25**, 26, 27, 29, 30
	Immunoblot	16

References in bold represent those studies performing *MDR1* mRNA and P-glycoprotein detection.

and the appropriate protein, carried out on samples from the same tumour. However, in only 2/18 studies [24, 25] was *MDR1* expression measured at both levels. Six studies examined *MDR1* expression exclusively at the RNA level, whereas 10 studies exclusively focused on the detection of P-glycoprotein (Table 4).

The method most widely used was immunohistochemistry, performed in 11/18 reports. Monoclonal antibodies were used, which differ in their species specificity as well as in their exclusive recognition of the *MDR1* gene product [37–41] (Table 5). Several studies performed immunohistochemistry using two or three antibodies, generating unique results for each antibody. Therefore, comparison and evaluation of immunohistochemical results are complicated, since they are highly dependent on the monoclonal antibody used. However, a small P-glycoprotein positive cell subpopulation within a tumour may be clinically significant, and so sensitive methods of detection, such as immunohistochemistry, are important. In addition, immunohistochemistry permits specific assessment of tumour P-glycoprotein versus those of connective tissue elements.

Techniques for the detection of the *MDR1* mRNA were performed using methods such as slot or dot blot analysis (six studies), respectively, as well as Northern hybridisation techniques (five studies), usually carried out with radioactive labelled probes. Reverse transcriptase-PCR (RT-PCR; two studies) represents the more sensitive quantitative technique to determine gene expression at the mRNA level. Moreover, the advantage of this method is that only a small amount of tumour sample is required. Results on the distribution of transcripts within cells and tissues can also be obtained using *in situ* RT-PCR.

Interestingly, the percentage of *MDR1*-positive tumour samples is higher in those studies using only *MDR1* mRNA detection methods (on average 62%) compare with those reports using immunohistochemistry (on average 46%; calculated using the highest percentage given for several antibodies). Reasons for these apparent discrepancies may not only be due to differing sensitivities of the methods [42], but may also indicate biological differences in the amount of mRNA and protein existing within cells, as illustrated in a panel of cell lines by Wu and coworkers [43]. This study showed that the level of *MDR1* mRNA expression did not result in the same level of P-glycoprotein expression; there is not always a linear correlation between the two parameters.

Verification of MDR1 *specific results.* External and internal standards have to be used to verify gene expression signals

Table 5. *P-glycoprotein detection using monoclonal antibodies*

Monoclonal antibody	Epitope	No. of studies	[References]
C 219	Intracytoplasmic	10	12, 16, 17, 20–22, 25, 27, 29, 30
JSB 1	Intracytoplasmic	6	20–22, 25, 26, 29
C 494	Intracytoplasmic	4	16, 17, 27, 29
MRK 16	Extracellular	3	22, 24, 25
HYB 241	Extracellular	1	12
HYB 612	Extracellular	1	12
MC 57	Extracellular	1	30

on mRNA and on protein level. Therefore, the definition of "positivity" of a tumour sample is a crucial prerequisite. For such a definition, different authors used different criteria, stating that the *MDR1* expression signal was, detectable (requirement for standardised detection methods for both expression levels); higher than the simultaneously analysed sample of appropriate normal tissue (requirement for availability of normal tissue of any analysed histological tumour type); higher than in the sensitive, parental control cell line used as an external standard (requirement for standardised control cell lines for each tumour tissue); higher than in a comparable, simultaneously performed experiment (e.g. PCR, hybridisation or immunohistochemistry, respectively) using e.g. primer or probes for housekeeping enzymes of "negative" monoclonal antibodies as internal standards (requirement for standardised internal controls); or detectable in several areas of the same tumour analysed, with regard to the heterogeneity of a tumour (requirement for more than one analysis per tumour per expression level).

In the majority of the studies (13/18), pairs of control cell lines, the parental and its multidrug resistant derivate(s) were described as external standards, not necessarily derived from the appropriate histological tumour type. In 5/18 studies, there were no data given for control cell lines used for comparative evaluation of the *MDR1* specific signals. The internal controls employed in the reports differed greatly (some reports use only detectability as an *MDR1* positive result). The most convincing approach for the verification of gene expression in a tumour sample is probably by comparison to those of the corresponding normal tissue [12, 44].

III. MDR1/*P-glycoprotein expression in human bone and soft tissue sarcomas—results*

Analyses of intrinsic gene expression in horizontal, non-sequential studies. In 10/18 studies [12, 14, 16, 18, 20, 22–25, 30] intrinsic, non-induced *MDR1* mRNA/P-glycprotein expression was analysed. From a total of 342 tumours examined in these studies, 146 were classified as *MDR1* positive, according to the various criteria of the authors. The apparently conflicting results for *MDR1* positive tumours obtained by the various research groups are given in Table 3 (for immunohistochemical studies which used several monoclonal antibodies, those with the lowest given number of positive evaluated tumours were taken [22, 25]). Summarising these data, an average of 43% of these bone and soft tissue sarcomas showed intrinsic expression of the *MDR1* gene, prior to any chemotherapy with anticancer drugs.

Analyses of MDR1 *gene expression in sarcomas of patients after chemotherapy in horizontal, non-sequential studies.* 7/18 studies [13, 16, 23, 25, 28, 29] examined the *MDR1*/P-glycoprotein expression after chemotherapeutic treatment with MDR relevant drugs. Using the criteria defined by the authors, 55 of the 106 analysed sarcomas were classified as *MDR1* positive (Table 3). In total, 52% of all examined sarcomas from chemotherapeutically treated patients were evaluated as positive for *MDR1* gene expression.

The overall percentage of *MDR1* positive sarcomas was, on average, 43% in tumours obtained from untreated patients compared with 52% in tumours derived from treated patients. In general, the inducibility of *MDR1* gene expression by anticancer drugs belonging to the MDR family has been shown *in vitro* by several groups [35, 46–48]. The percentage of *MDR1* positive tumours from untreated versus treated patients calculated here can only be considered as weak evidence for the inducibility of *MDR1* gene expression possibly occurring in sarcomas, because of all above mentioned limitations. Moreover, an evaluation of *MDR1* expression data from tumours after chemotherapy, without their appropriate untreated counterparts, is complicated.

MDR1 *gene expression analyses in longitudinal, sequential studies.* Possibly the best method of evaluating *MDR1* gene expression as a marker gene for prognosis and therapy of bone and soft tissue sarcomas is the performance of longitudinal, sequental studies, including the examination of several tumour samples from the same patient, prior to and after chemotherapeutic treatment.

There are three impressive longitudinal studies describing *MDR1* expression data of soft tissue sarcomas [17, 19] and of osteogenic sarcomas [27]. All tumours were derived from paediatric patients, treated with MDR relevant anticancer drugs. Tumour samples were available from all patients at the time of diagnosis as well as after chemotherapy: 30 soft tissue sarcoma patients (62 samples) [17], 1 patient with an undifferentiated sarcoma (three samples) [19] and 62 cases of osteogenic sarcomas (313 samples) [27]. Detection of *MDR1* gene expression was carried out using immunohistochemistry with monoclonal antibodies, C219 and C494 [17, 27], and by RNA dot blot analyses [19].

From the 30 cases of soft tissue sarcomas, 9 cases were classified as *MDR1* positive (30%; 4 at diagnosis, 5 at subsequent biopsy) [17]. From the 62 cases of osteogenic sarcomas, 27 were evaluated as *MDR1* positive sarcomas from samples obtained at diagnosis (44%) [27]. One case of undifferentiated sarcoma [19] was an intrinsic *MDR1* positive tumour with increasing expression levels during therapy. Thus, in all three studies, the general inducibility of *MDR1* gene expression by MDR relevant anticancer drugs at different times of treatment was clearly shown, which seems to be independent of the level of *MDR1* expression at diagnosis. These findings confirm the aforementioned data from several *in vitro* analyses [35, 46–48] and from the discussed *MDR1* expression results obtained from "post chemotherapy" studies [13, 16, 23, 25, 26, 28, 29].

IV. *Clinical outcome—definition of parameters and correlation with* MDR1 *gene expression*

Definition of clinical outcome parameters. To correlate *MDR1* gene expression in bone and soft tissue sarcomas with their responsiveness to chemotherapeutic treatment using MDR relevant cytostatics, the definition of clinical response must be clear. The following parameters were primarily used: complete response (CR), partial response (PR), no response (NR) and progressive disease (PD). However, these terms were not defined identically by all authors, creating obvious problems for the comparison of these results. Tumour characteristics, such as tumour size and grade, recurrence-free survival, and overall survival, were also considered for statements concerning correlation of gene expression and clinical data. Moreover, the modalities of treatment (poly- versus monochemotherapy and/or surgery) were also considered.

Correlation of the clinical outcome with MDR1/*Pgp expression.* The *MDR1*/P-glycoprotein expression studies should contribute to the identification of those sarcomas likely to fail to respond to a given drug, to the selection of the drug for therapy of specific histological tumour types, to the decision to continue chemotherapy, as well as for the potential application

of agents to overcome resistance [13]. Of the 18 studies, 11 performed correlation analyses of *MDR1* gene expression levels with clinical outcome parameters.

The final statements concerning correlations were often difficult to interprete. Other parameters potentially influencing the clinical outcome data were not very well documented, e.g. the manner of the chemotherapeutic treatment (mono- versus polychemotherapy), the specific drugs used as well as the doses and the schedules for drug administration.

The results were as varied as the methods used: "The correlation between *MDR1* expression status and disease prognosis for bone and soft tissue sarcomas is significant" is reported in all of the three longitudinal studies and in one study examining different sarcomas prior to and after chemotherapy [16, 17, 19, 27]; "No significant statement is possible" was the summary of another four reports [23, 26, 28, 29], three describing data obtained after chemotherapy and one describing non-sequential data from sarcomas prior to and after chemotherapy. In addition, "No significant correlation is found" was the conclusion of three studies [22, 24, 25], interestingly mostly carried out prior to any chemotherapeutic treatment [22, 24], and including the two reports with data on *MDR1* mRNA and P-glycoprotein expression levels [24, 25].

The evaluation of these results is challenging, since all data were obtained from human tumours and analysed with the final aim of individualising chemotherapy, including or excluding MDR relevant cytostatics. Therefore, in general, it seems to be crucial how studies are conducted (longitudinal, before or after chemotherapy) when evaluating gene expression such as the *MDR1* gene, for use as a molecular marker with consequences for the clinical treatment of patients.

Not withstanding the lack of a standardisation, and the aforementioned limitations of the studies, some conclusions can be made, although these should be viewed with caution. The exclusive detection of intrinsic *MDR1* expression in untreated sarcomas at the time of diagnosis seems to be extremely variable and a significant correlation with response to chemotherapy has not been shown. Data from chemotherapeutically treated patients described here support observations of *MDR1* gene expression inducibility by anticancer drugs and single step selection of drug resistanct cell populations, but do not show significant correlations with clinical outcome; the most useful results were probably obtained from longitudinal studies examining tumour samples from the same patients at several times during the chemotherapeutic treatment. All such studies mentioned here described a significant correlation between induced (high) *MDR1* gene expression (in comparison with the expression level at the time of diagnosis) with an unfavourable prognosis. Therefore, *MDR1* gene expression could be a molecular marker for disease prognosis, response to chemotherapy and for use of MDR overcoming agents, but currently this conclusion can only be considered to be valid for bone and soft tissue sarcomas of childhood.

V. *Requirements for standardised studies to evaluate gene expression as a clinically useful molecular marker*

Well accepted principles of methodology do exist to guide the design, conduct, analysis and reporting of clinical trials, but unfortunately, and perhaps surprisingly, no such guidelines have been developed for prognostic factor studies, which include the analysis of gene expression in human tumours. Some reports discuss the need to establish guidelines [49–51], including the statistical analysis [45]. The fact that only 6/18 reports included statistical analyses in their studies supports the demand for guidelines. Therefore, it is clear from this review that a minimum set of requirements are needed for studies investigating molecular markers, the essential points are described below.

Clearly defined biological working hypothesis. The aim of the study should be clearly defined: is the aim an expression analysis of a certain gene in a certain tumour type (e.g. *MDR1* expression in sarcomas) or a correlation of gene expression in a certain tumour type with clinical outcome?

Definition of the tumours to be included in the study. The histological tumour type and grade should be given. Age of patients should be stated. The number of the tumours examined should be high enough to ensure valid statistical analysis.

Treatment of the patients. Schedules for chemotherapy should be carefully described. In the context of MDR, it is essential that use of MDR or non-MDR relevant drugs is stated as well as how these were given (e.g. mono- or polychemotherapy).

Performance of the study. It is crucial to know whether a study is retrospective or prospective; longitudinal and sequential or horizontal and non-sequential. Moreover, in the context of MDR, the time at which *MDR1* gene expression is measured (before or after chemotherapy) should clearly be stated.

Methods used. Primers, probes, antibodies etc. as well as their sensitivity, specificity and practicability should be well described. The establishment of functional assays for P-glycoprotein in tumour samples (versus gene expression analysis) would represent a valuable addition.

Verification of the results The choice of external and internal controls is important, such as well characterised control cell lines, PCR or hybridisations with primers or probes for "housekeeping enzymes", "negative" antibodies, normal versus tumour tissue, etc.; the criteria for "positivity" should be defined.

These briefly listed criteria emphasises the requirement for guidelines which should represent the framework for any study on molecular prognostic factors.

FURTHER ASPECTS

MDR1 *expression and correlation with clinical outcome in other cancers*

Similar efforts have been made to evaluate and compare *MDR1* gene expression with clinical outcome for a variety of human cancers [6, 12, 14, 15, 36, 52]. Some studies have demonstrated strong correlation between *MDR1* gene expression and poor response to chemotherapy. However, apart from limited data for human carcinomas [e.g. 53], these observations have mainly been described for leukaemias [54, 55], lymphomas [56] and some childhood solid tumours [17, 27, 57, 58]. To date, several clinical trials in multiple myeloma and non-Hodgkin's lymphoma using MDR reversing agents, such as verapamil, cyclosporin A or quinine, have produced evidence that inhibition of P-glycoprotein can increase an individual's response to chemotherapy [59–63]. The ability to modulate clinical MDR by drug resistance modifiers as well as the mechanism of action is well documented for the chemosensitiser cyclosporin A [64, 65].

MDR1 *gene expression and tumour progression*

In addition to its role in drug resistance, *MDR1* gene expression may also be involved in the process of tumour progression [52], reflecting the malignant phenotype and the biological aggressiveness of these cancers. There are such reports in colon carcinomas [66], soft tissue sarcomas [23], and breast tumours [67]. Therefore, *MDR1* overexpression could possibly be a consequence of neoplastic transformation, perhaps suggesting that expression of this gene is a marker of more malignant subpopulations of tumour cells [9].

Other genes possibly associated with MDR or resistance mechanisms in general

MDR is not the only observed resistance mechanism in human cancers [68], so it is possible that there is a panel of resistance-associated genes involved. Other known MDR-associated genes are anionic isozyme-p of the glutathione-S-transferase (GSTp) [69–71] and topoisomerase II [72–74], as well as the recently discovered multidrug resistance related protein (MRP) [75–80], and the 110 kD protein LRP [80,81]; most have been examined in a variety of human cancers. Therefore, another and perhaps more promising approach to an individualised prognosis based on expression of a resistance-associated marker gene could be the so-called "resistance gene monitoring", that is the simultaneous analysis of the expression of several potentially involved genes for the same tumour. It is a challenging issue for the future to prove this procedure is worthwhile and to make it applicable to clinical routine investigations [82].

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