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# Expression of the gene for the multidrug resistance-associated protein in human prostate tissue

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**Abstract** To characterize the clinical relevance of MRP gene in the chemoresistance of prostate carcinomas we determined the multidrug resistance-associated protein (MRP) expression in 30 samples from organ-confined prostate carcinoma, 9 samples from adjacent normal tissue and 4 hormone unresponsive cancers. The measurement of MRP expression was carried out by reverse transcription polymerase chain reaction (RT-PCR) in combination with capillary electrophoresis. Incorporated fluorescence-labeled primers were disclosed by a laser-operated fluorescence detection module. MRP expression was quantified by integration of the peak area and correlated to the ubiquitously expressed β2 microglobulin. As positive control served the adriamycin-resistant HL60-ADR cell line, which overexpresses MRP. MRP expression was found in all samples. All samples showed a lower MRP/β2 ratio than HL60-ADR cells. The expression of the MRP gene was 30% higher in organ-confined tumors than in hormone-unresponsive anaplastic tumors. Normal tissue showed the same MRP mRNA level as the adriamycin-sensitive HL60 cells. A higher tumor stage correlated with an increase of MRP expression (> factor 2), whereas G3 tumors displayed a MRP expression 30% lower than in G2 tumors. The small alterations indicate that MRP expression seems not be involved in the chemoresistance of prostate carcinomas.

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## Introduction

One obstacle in the therapy of advanced metastatic prostate cancer is the poor response to cytotoxic drug treatment. Prostate carcinomas are characterized by a slow proliferation rate which leaves phase-specific cytostatics ineffective. The role of molecular mechanisms in drug resistance, which has been studied in other urologic tumors like renal cell carcinoma [15] and urothelial cancer [10], has not been sufficiently elucidated for the prostate carcinoma. One important resistance mechanism may be the multidrug resistance (MDR) phenotype [8]. MDR is caused in part by the overexpression of the MDR1 gene. This gene encodes a 170 kDa transmembrane protein called P-glycoprotein (P-gp).

P-gp acts as an adenosine triphosphate (ATP)-dependent efflux pump, clearing the cytoplasm from various hydrophobic cytostatics before they can exert their deleterious effects. P-gp is characterized by a widespread set of substrates, comprising anthracyclins, epipodophyllotoxins and vinca alkaloids, as well as the new and promising drug taxol. Compounds that are not transported by P-gp are alkylating agents, platinum compounds, methotrexate and 5-fluoro-2,4(1H, 3H) pyrimidinedione (5-FU).

MDR1 transcript and P-gp are expressed in a multitude of human tissues and tumors [7, 23]. Urologically of relevance is the overexpression of this protein in a high percentage of renal cell carcinomas which may be the cause for the high intrinsic chemoresistance in these tumors [2, 6]. P-gp may also play a role in the chemoresistance of urothelial carcinomas [19]. Clinical relevance was established by the discovery of inhibitors of P-gp. Compounds belonging to the group of calcium antagonists have been studied intensively and used for

the blocking of chemoresistance in renal cell carcinomas. R-verapamil displayed a certain in vivo activity [15].

Recently, another form of chemoresistance was suggested as a possible mechanism for non-P-gp-mediated MDR: the overexpression MRP. The MRP gene encodes a 190-kDa glycoprotein that also belongs to the ATP-binding cassette superfamily of membrane transport proteins. Despite the fact that MRP shares less than 15% amino-acid homology with MDR1 [4] it shows a similar substrate profile. Overexpression of MRP leads to an increase in resistance to adriamycin, vincristine and vinblastine but not to taxol [9, 12]. Unlike P-gp, MRP has the ability to act as a glutathione-S-conjugate carrier [5]. This mechanism may be involved in cisplatin resistance [18].

MRP-mediated MDR, however, differs from P-gp in cell lines expressing MRP, which are weakly sensitive to modulation by the P-gp antagonist verapamil [1].

Carcinomas of the prostate are clinically chemoresistant. In spite of this, the expression and function of specific resistance factors have not been thoroughly examined except for a few cases [17, 21].

In the following we describe a semiquantitative and highly sensitive method which links RT-PCR to subsequent capillary electrophoresis analysis. Using this method, we have examined the expression of MRP in a great number of samples in order to find out if there is a correlation between MRP expression and tumor stage, tumor grade, or hormone-independent growth.

## Material and methods

### Tissue samples

Samples of 30 prostate carcinomas were obtained at radical prostatectomy. Among these carcinomas nine samples from adjacent normal prostate tissues were also dissected. Four samples were obtained from hormone-unresponsive prostate cancers. These four patients had been treated for a longer period with androgen ablation. A section of every sample was characterized by histologic assessment and the tumors were staged and graded. Tissue samples were frozen in liquid nitrogen and stored at −80°C until assessment.

### RNA extraction and cDNA synthesis

Total RNA was extracted by the standard method of Chomczynski and Sacchi [3] by means of acidic phenol. The integrity of the RNA was determined by denaturating agarose gel (1.2%) electrophoresis. First strand cDNA was synthesized from 1 µg of total RNA in 50 mM TRIS-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, 1 mg BSA, 10 U RNasin (Promega), 200 pmol random primers (Gibco) 200 U Mo/MLV reverse transcriptase (Gibco) and DEPC-treated water in a volume of 20 µl for 1 h at 37°C.

### PCR

Ten µl of the RT-reaction were used for each PCR reaction. PCR was carried out with either MRP or β2-microglobulin specific primers. The PCR mixture consisted of 50 mM TRIS-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs. Primers for amplification: human-MRP (MWG-BIOTECH) forward primer 5′-fluorescein-GGA AAC CAT CCA CGA CCC TA-3′; reverse primer 5′-fluorescein-CCT CAT TCG CAT CCA CCT TG-3′ generating a 289-bp PCR product. Human-β2-microglobulin (MWG-BIOTECH) forward primer: 5′-fluorescein-ACC CCC ACT GAA AAA GAT GA-3′; reverse primer 5′-fluorescein-ATC TTC AAA CCT CCA TGA TG-3′ generating a 123-bp PCR product. Amplification of the human-β2-microglobulin transcript was performed to assure the quality of the applied RNA.

To determine the ideal cycling conditions we performed calibration curves for MRP and β2-microglobulin, respectively. These analyses were carried out in triplicate using the same RNA template for three RT-PCR reactions and subsequent capillary electrophoresis/laser-induced fluorescence (CE/LIF) analysis. For each tissue sample RT-PCR and subsequent CE/LIF was performed twice and the mean taken for calculating the MRP/β2 ratio.

### Capillary electrophoresis of PCR amplified products

CE was performed on a P/ACE 2100 (Beckman Instruments, Fullerton, USA) in a reverse-polarity mode. Approximately 2 µl of unpurified PCR product were injected by pressure to a DB-17 capillary (100 µm I.D., 0.2 µm F.D., 50 cm length to the detector) filled with a sieving polymer buffer system containing hydroxypropylmethylcellulose (HPMC) in a TRIS-borate-EDTA (TBE) buffer. This buffer was replaced after each separation.

The generated fluorescent PCR products were detected on-line by an argon-ion laser (excitation 488 nm, emission 520 nm). MRP expression was quantified by integrating the peak area of the PCR product (Gold-Software, Beckman Instruments). To normalize the run-to-run variations in fluorescence signal the relation between the fluorescence signal of the PCR product and total fluorescence signal was determined (= relative fluorescence as percent). MRP expression was normalized by calculating the MRP/β2 microglobulin ratio. Assessment of accuracy of the methodology is described elsewhere [20].

### Statistical analyses

Differences between groups were tested by analysis of variance (ANOVA) followed by Scheffé's test for multiple pairwise comparisons at an error level of at least $P < 0.05$. The program package BiAS was used. Data are expressed as means ± SD unless otherwise indicated.

## Results

In order to determine the gene expression accurately, we had to determine the numbers of cycles for which the PCR amplification curve would be in its linear phase. We created a standard curve for MRP and β2 microglobulin showing ideal conditions at cycle 27 for MRP (Fig. 1) and at cycle 21 for β2 microglobulin (Fig. 2). We performed all PCRs at the determined cycle numbers and calculated the ratio of MRP to β2 microglobulin.

Under these conditions, MRP gene expression could be shown in 34 samples from prostate carcinomas and 9 samples from normal tissue. Four samples in the prostate cancer group were from four hormone-unresponsive cancers. Table 1 shows the characteristics of the samples with regard to the histological classification (staging and grading). MRP gene expression was found in all analyzed samples. No difference in MRP expression was

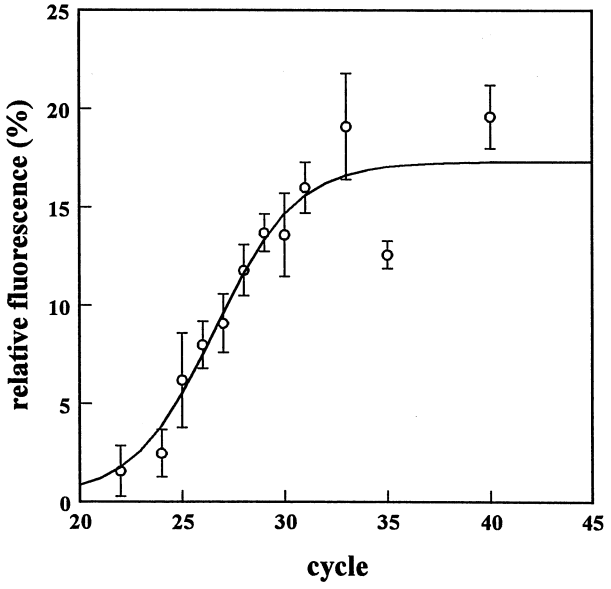


**Fig. 1** Calibration curve for multidrug resistance associated protein (MRP) mRNA. Polymerase chain reaction (PCR) cycles vs. fluorescence signal. Values are means ± SD

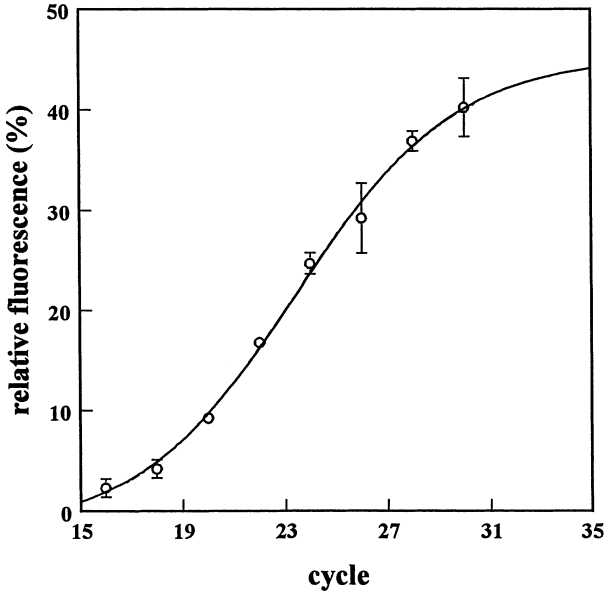


**Fig. 2** Calibration curve for β2-microglobulin mRNA. PCR cycles vs. fluorescence signal. Values are means ± SD

detected between the normal (MRP/β2: 0.35) and the cancerous tissues (Fig. 3).

All prostate cancer samples and the collected normal tissues showed a lower MRP expression than the HL60-ADR cell line. As this cell line expresses MRP in a high degree it was used as a positive control. In this cell line, MRP expression was more than three times higher than the mean values of all other samples. The adriamycin-sensitive cell line HL60 displayed the same MRP mRNA level as normal and prostate cancer tissue (MRP/β2: 0.32).

**Table 1** Characteristics of the samples

| | |
|---|---|
| Adjacent normal prostate tissue | 9 |
| Prostate carcinomas | 34 |
| *Tumor stage* | |
| pT1N0M0 | 0 |
| pT2N0M0 | 4 |
| pT3N0M0 | 23 |
| pTxNxM1 | 4 |
| unclassified | 3 |
| *Tumor grade* | |
| G1 | 1 |
| G2 | 19 |
| G3 | 11 |
| unclassified | 3 |
| Organ confined | 30 |
| Androgen independent | 4 |

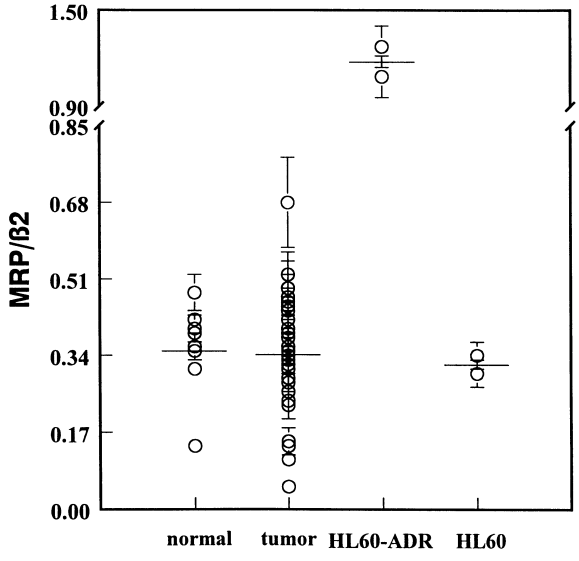


**Fig. 3** MRP/β2 ratio in 9 normal prostate tissues and 34 prostate cancer tissues with their mean values ± SD. Additionally the ratios for the adriamycin-resistant HL60-ADR cells and the adriamycin-sensitive HL60 cells are shown

Tumors histologically graded G2 (MRP/β2: 0.38) revealed a higher MRP expression than G3 (MRP/β2: 0.27) graded tumors (Fig. 4). This decrease in MRP expression with increased histological grading was statistically significant ($P < 0.05$).

Prostate carcinomas showed a correlation between increasing tumor stage and enhanced MRP expression. pT2 classified cancers had a MRP/β2 quotient of 0.18 whereas pT3 tumor reached a quotient of 0.37 (Fig. 5), a significant increase ($P < 0.01$). pT3 cancers (MRP/β2: 0.37), however, displayed nearly the same level of MRP expression as normal tissue (MRP/β2: 0.35).

Thirty of the analyzed cancer samples were obtained from radical prostatectomies without any treatment of the tumor. Four samples were obtained at a transurethral resection. The four hormone-unresponsive tumors showed a slightly reduced (30%) MRP expression compared with the other prostate carcinoma samples (Fig. 6).

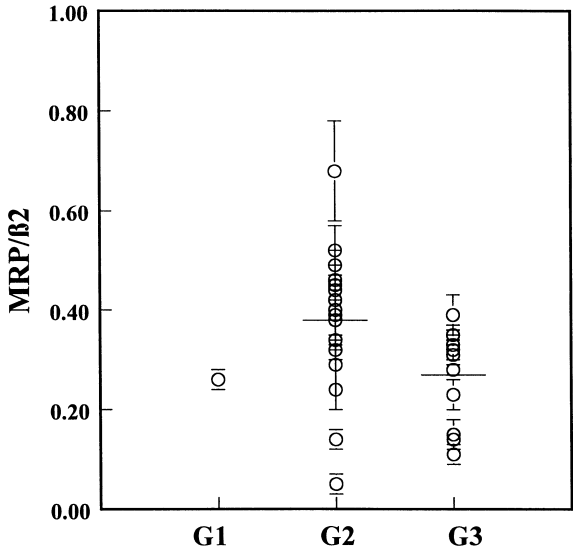


**Fig. 4** MRP expression in relation to the tumor grade of the carcinomas. The figure shows mean values ± SD

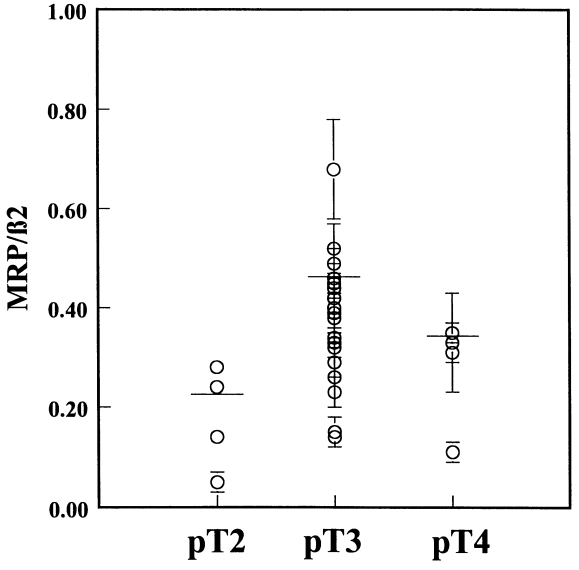


**Fig. 5** MRP expression in relation to the tumor stage. The figure shows mean values ± SD

## Discussion

Prostate carcinomas are intrinsically chemoresistant. They play a unique role in urologic tumors because of their slow proliferation rate. Unlike other types of cancers, the reduced apoptosis but not the enhanced cell growth is responsible for the spreading of the tumor. Hence, tumor growth is caused by a disturbed equilibrium between cell proliferation and apoptosis. As chemotherapeutic substances act on the cell proliferation, this is one reason for the poor response of prostate carcinomas to some phase-specific cytotoxic agents, although others remain to be investigated.

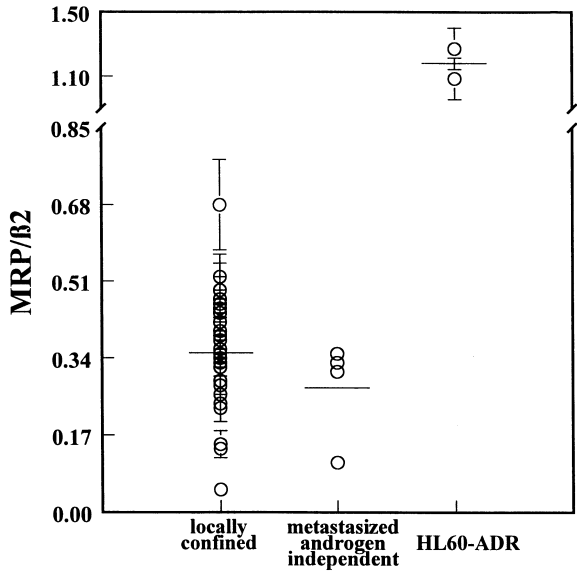


**Fig. 6** MRP/β2 ratios in four androgen-independent prostate cancer tissues and 30 locally confined prostate cancer tissues with their mean values ± SD. Additionally the ratio for the adriamycin-resistant HL60-ADR cells is shown

After the discovery of MDR1 as an anticancer drug pump it was initially thought that the molecule would play a decisive role in tumor responsiveness to chemotherapy in most cancer patients. In the likewise inherently resistant renal cell carcinoma, an involvement of MDR1 was observed [15]. The question whether molecular mechanisms could be involved in the marked cytotoxic drug resistance of prostate cancers has not yet been clarified. Using a combination of RT-PCR and capillary electrophoresis, we studied the mRNA expression of MDR1 in prostate cancers and normal prostate tissue [21]. Using highly sophisticated RT-PCR, MDR1 gene expression could be detected in almost all prostate samples, whereas standard techniques such as immunohistochemistry or Northern blotting were routinely negative. The results also indicate that the MDR1 expression plays no significant role in the MDR phenotype of prostate cancers. The resistance to cytotoxic treatment seems to be rather multifactorial.

Therefore we investigated the expression of another MDR-mediating gene, the recently recognized multidrug resistance-associated (MRP) gene [4] in human prostate tissue.

In our study we determined the expression of the MRP gene in 34 human prostate cancer samples and in 9 normal human prostate tissue samples by semiquantitative RT-PCR. As positive control we used the adriamycin-resistant cell line HL60-ADR which overexpresses MRP [11]. In contrast to their immunoblot results we could demonstrate that adriamycin-sensitive HL60 cells also express MRP mRNA. Further we could show that this level of mRNA expression is approximately four times lower than in adriamycin-resistant HL60-ADR cells.

Our results show that MRP mRNA is expressed regularly in prostate cancer and prostate tissue. These findings indicate that MRP has a physiological function in these tissues. In other cells it could be demonstrated that MRP supports the export of leukotriens and some other glutathione-S-conjugates [13, 16]. Further it should be noted that MRP is able to transport 17 beta-estradiol and possibly some other cholestatic steroid glucuronides [14]. In our study the MRP gene was expressed in G2 tumors at a significantly higher rate than in G3 tumors. This connection between tumor grade and gene expression was also observed for the MDR1 gene [21] and reflects a functional relation between gene expression and the human prostate tissue. Tumors with a low tumor grade tend to maintain the properties of normal tissues whereas an increased tumor grade results in a reduction in the natural gland function. One diagnostically relevant example is prostate-specific antigen (PSA) expression which is reduced in tumors with a high tumor grade. We also investigated the MRP expression in four androgen-independent tumors. They revealed a reduced MRP gene expression and had a high degree of tumor grade.

Studies of the expression of the MRP gene in human prostate tissues are very rare. Nooter et al. [17] examined the MRP mRNA by means of RNase protection assay in 16 histologically unclassified prostate cancer samples. In their study the cancer samples showed a low expression of MRP but normal prostate tissues were not evaluated. The findings of low MRP expression in prostate cancers are in accordance with our results.

In a study by Sugawara et al. [22] MRP expression was examined by RT-PCR but only in normal tissue. They found MRP gene expression but did no quantitation of the expressed mRNA. Our results show that the MRP expression in prostate tissues is generally low. Hence MRP seems not to be involved in the chemoresistance of prostate cancers.

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