Cancer Investigation

ISSN: 0735-7907 (Print) 1532-4192 (Online) Journal homepage: http://www.tandfonline.com/loi/icnv20

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To cite this article: Ogan Demir Abaan, Pelin Kaya Mutlu, Yusuf Baran, Can Atalay & Ufuk Gunduz (2009) Multidrug Resistance Mediated by MRP1 Gene Overexpression in Breast Cancer Patients, Cancer Investigation, 27:2, 201-205, DOI: 10.1080/07357900802173562

To link to this article: http://dx.doi.org/10.1080/07357900802173562

Published online: 11 Jun 2009.

Cancer Investigation, 27:201–205, 2009
ISSN: 0735-7907 print / 1532-4192 online

DOI: 10.1080/07357900802173562

informa
healthcare

ORIGINAL ARTICLE
Clinical Translational Therapeutics

Multidrug Resistance Mediated by MRP1 Gene Overexpression in Breast Cancer Patients

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ABSTRACT

Multidrug resistance (MDR) is a serious handicap towards the effective treatment of breast cancer patients. One of the most prevalent MDR mechanisms is through the overexpression of genes coding the proteins called Multidrug Resistance-associated Proteins (MRPs). The aim of this study was to investigate the expression of MRP1 in tumor tissues from breast cancer patients. In this study, a semi-quantitative RT-PCR approach was utilized. Our results suggest that MRP1 overexpression can mediate MDR in patients. Pre-evaluation of the level of such MDR mediators before chemotherapy can increase the efficacy of the treatment.

INTRODUCTION

Breast cancer is the most common cancer among women. Combination of surgery, chemotherapy and radiation is used for treatment of breast cancer. However, chemotherapy is mostly rendered uneffective by multidrug resistance (MDR) mechanisms towards a variety of chemotherapeutic agents. Increased drug efflux is a common mechanism of resistance in cancer cells, which is frequently seen following exposure of tumor cells to one of the "naturally occurring" anticancer drugs like doxorubicin, vincristine, etoposide, paclitaxel etc. (1). The increased drug efflux is mediated by membrane transporter proteins. These membrane proteins are physiological pumps that remove unwanted chemicals. MDR1 is the most commonly used membrane pump that is involved in conferring resistance to cancer chemotherapy (1). A second family of such pumps has gained attention due to their ability to modulate drug resistance, the multidrug resistance-associated proteins (MRPs). The first MRP protein was reported in 1992 by Cole et al. (2). MRP1 is the first member of the MRP family since many more have been identified in recent years (3). They have been shown to confer resistance when expressed in sensitive cells (4). Studies showed that MRP1 confers resistance to many chemotherapeutic drugs in many different cancers (4). MRP1 can transport uncharged hydrophobic molecules, lipid soluble anions, and hydrophilic anions, including glutathione (GSH) conjugates with multiple and fully dissociating negative charges (5). However, although not essential, GSH provides a modular effect on MRP1. For instance, in the case of doxorubicin, it was shown that conjugation with GSH is not the resistance mechanism in breast cancer cells (6). MRP1 is basally expressed in almost all tissues tested (7). When MRP1 is overexpressed it has the ability to confer resistance to cytotoxic drugs like doxorubicin, vincristine and VP-16 (8). The mechanisms by which MRP mediated MDR can develop could be either intrinsic or acquired after chemotherapy. In this study, MRP1gene expression was determined in various breast cancer tissues from patients. Since overexpression of MRP1 may be responsible for the failure of chemotherapy, the expression levels were quantified and compared to that of controls, which can provide clinicians with a useful tool to assess drug resistance before treatment.

Keywords: Multidrug resistance, MDR, MRP1, Breast cancer, Chemotherapy, MRP Family
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MATERIALS AND METHODS

Cell lines and tissues

Fresh tissue samples (n = 12) from patients (n = 10) were provided by Demetevler Oncology Hospital (Ankara) and stored in RNAlater (Ambion). Formalin fixed paraffin embedded (FFPE) tissues (n = 2) by Gulhane Military Medical Academy, Department of Pathology (Ankara). This study was conducted according to Helsinki Declaration. HL60 cell line was initially provided by Gulhane Military Medical Academy, Department of Hematology (Ankara) and subculturing was performed in RPMI-1640 medium with 10% FCS. Doxorubicin resistant HL60 subline was derived from parental HL60 cells by subculturing in increasing doxorubicin concentrations up to 70 nM. Doxorubicin sensitive and resistant HL60 cell lines were used as negative and positive controls, respectively.

RNA isolation and cDNA preparation

Total RNA was isolated from both the tissues and the cell lines as described previously (9). One to two milligrams of total RNA was reverse transcribed to cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Moloney MLV, Fermentas, Maryland, USA) according to the manufacturer's protocol. After 1 hr incubation at 42°C, the reactions were stopped by 70°C heating for 10 min.

Primers for PCR

MRP1 (10) and β-2-microglobulin (β2M) primers (11) were obtained from IDT Technologies, (Iowa, USA). β2M gene was used as an internal positive control (12). For nested PCR, a second pair of MRP1 primers was designed. The primer sequences were as follows: original MRP1-forward (5′-GACCTGGACTTC GTTCTCA-3′), original MRP1-reverse (5′-ACGTCCAGATTCCTT CATCC-3′, 291 bp); nested MRP1-forward (5′-GACGGGAGCTGGGAAGTCGT-3′), nested MRP1-reverse (5′-GGGAACCCGAAAACAAAACAG-3′, 115 bp) and β2M-forward (5′-ACCC CCACTGAAAAAGATGA-3′), β2M-reverse (5′-ATCTTCAAACCTCCATGATG-3′, 166 bp).

Multiplex PCR

Multiplex PCR was performed for MRP1 and β2M primers. For the nested multiplex PCR, the amplification product from the initial PCR reaction was used. The program used for both of the PCR reactions was as: initial denaturation at 94°C for 30 sec; denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 45 sec with 30 cycles and final extension at 72°C for 5 min.

Densitometric analysis of PCR results

The gel images were processed with the Scion Image Software (Scion Corporation, Maryland, USA). For the patients, the calculated values of MRP1 band intensity of each sample was divided to the β2M band intensity of the same sample. The ratio of these intensities was called Ratio(P). The same ratio was obtained for the sensitive [Ratio(S)] and resistant [Ratio(R)] HL60 cell lines by dividing the band intensity of MRP1 to the band intensity of β2M. Then, the Ratio(P) was divided by the Ratio(S) control [Ratio(P)/Ratio(S)] and Ratio(R) control was divided by the Ratio(S) [Ratio(R)/Ratio(S)]. These standardized ratios were used for determination of MRP1 overexpression relative to a low MRP1 expressing control (sensitive HL60) and a high MRP1 expressing cell line (resistant HL60) (13).

RESULTS

Expression analyses of MRP1 and β2M genes in breast cancer patient samples

The ability of the nested MRP1 primers in amplification was tested since a nested RT-PCR approach is more powerful for the identification of degraded RNA in samples. In the first round of the PCR reaction, cDNA preparations from FFPE tissues were amplified with the original MRP1 primers amplifying a 291 bp fragment (Fig. 1A). In most of the samples, the MRP1 band was too weak to be visualized easily on an agarose gel (Fig. 1A). A second round of PCR amplification was carried out using the nested MRP1 primers, which amplify an internal fragment of 166 bp (Fig. 1B). After the optimization studies RT-PCR was

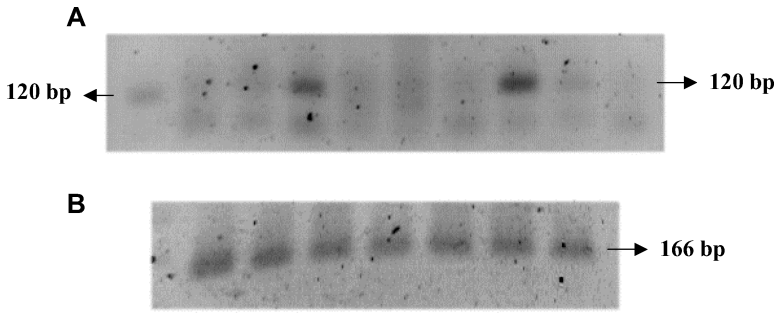

Figure 1. Nested PCR amplification. A) First round of RT-PCR with MRP1 and β2M primers from poor quality RNA. Expected size of MRP1 primer product is 291 bp and β2M is 120 bp. B) The PCR products from panel A was used as a template for a second round of PCR with the nested MRP1 primers. Expected size of nested MRP1 primer product is 166 bp.

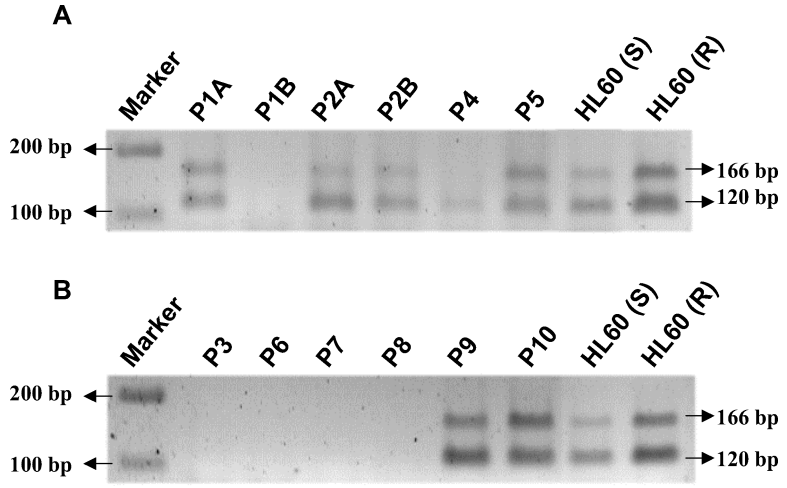

Figure 2. Multiplex PCR results of patient samples. (A) Semi-quantitative multiplex RT-PCR analysis for *MRP1* and *β2M* gene expressions in 6 patient samples (P1A, P2A, P2B, P4 and P5). Sample P1B failed to give detectable PCR product. HL60-S (sensitive) and HL60-R (resistant) cell lines were used for comparison as described in the text. The first lane was the DNA size marker. Expected size of nested MRP1 primer product is 166 bp and β2M is 120 bp. (B) Semi-quantitative multiplex RT-PCR analysis for *MRP1* and *β2M* gene expressions in 6 patient samples (P3, P6, P7, P8, P9 and P10). Samples P3, P6, P7 and P8 failed to give detectable PCR product. Expected size of nested MRP1 primer product is 166 bp and β2M is 120 bp.

performed for all patient samples by using either the nested MRP1 primers or the original MRP1 primers. Sensitive (S) and resistant (R) HL60 cells were used as controls. The agarose gel images of multiplex PCR products (MRP1 and β2M) from samples are shown in Figs. 2 and 3. The multiplex PCR in Fig. 3B was performed using only the MRP1 primers (291 bp) without the use of nested PCR. Samples P1B, P7 and P8 were excluded since the samples failed to give any detectable PCR products (neither with MRP1 nor β2M). It is possible that the samples were not stored adequately therefore no RNA was preserved.

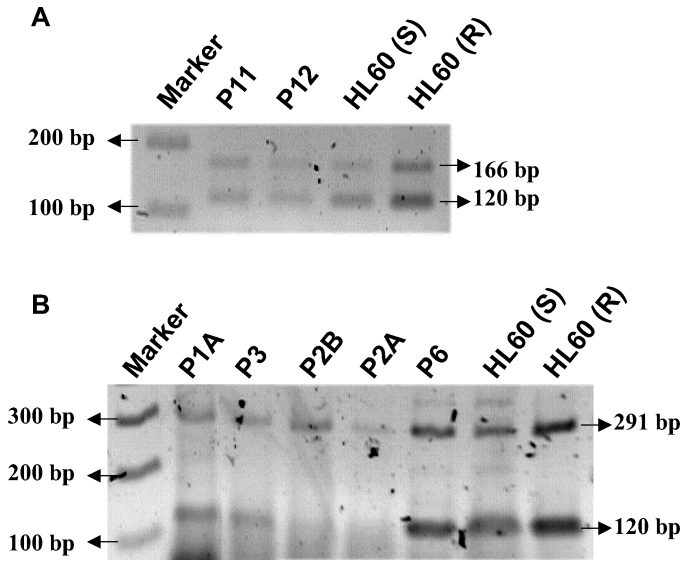

Figure 3. Multiplex PCR results of patient samples. (A) Semi-quantitative multiplex RT-PCR analysis for *MRP1* and *β2M* gene expressions in 2 formalin-fixed paraffin embedded patient tissue samples (P11, P12). HL60-S and HL60-R cell lines were used for comparison as described in the text. The first lane was the DNA size marker. Expected size of nested MRP1 primer product is 166 bp and β2M is 120 bp. (B) Semi-quantitative multiplex RT-PCR analysis for *MRP1* and *β2M* gene expressions in 5 patient samples (P1A, P3, P2B, P2A and P6). Expected size of MRP1 primer product is 291 bp and β2M is 120 bp.

Table 1. Densitometry analysis of the RT-PCR results.

Code	A260	A280	Purity	mg/ml
P1A	0.23	0.141	1.63	0.920
P1B	0.63	0.329	1.91	2.520
P2A	0.3	0.175	1.71	1.200
P2B	0.246	0.172	1.43	0.984
P3	0.285	0.153	1.86	1.140
P4	0.678	0.367	1.85	2.712
P5	1.235	0.783	1.58	4.940
P6	0.096	0.074	1.30	0.384
P7	0.102	0.08	1.28	0.408
P8	0.117	0.078	1.50	0.468
P9	0.268	0.156	1.72	1.072
P10	0.5	0.273	1.83	2.000
P11	FFPE from Gulhane			
P12	FFPE from Gulhane			

Ratio (P) = Ratio of the Patient; Ratio (S) = Ratio of the sensitive HL60 control; Ratio (R) = Ratio of the Resistant HL60 control.

Densitometric analysis

Densitometric analysis of the bands obtained after gel electrophoresis were done as described in the Materials and Methods. The gel images given in Figs. 2 and 3 were quantified using densitometry software in order to determine the band intensities. These values were first standardized against the β2M intensities by simply dividing the values of the MRP1 band intensities of the patient samples to β2M band intensities of the same sample. Then these ratios were re-standardized by dividing to the ratio of the HL60 sensitive control, which has a basal level MRP1 expression (14). The results of these analyses are given in Table 1.

The important values in Table 1 are the Ratio (P)/Ratio (S), which is the sensitive HL60 standardized ratio of the patient, and the Ratio (R)/Ratio (S), which is the sensitive HL60 standardized ratio of the resistant HL60 control. Such standardization was used in order to reduce experiment-to-experiment variability between samples and gels. Densitometry analyses indicated in Table 1 were used for deciding whether patients displayed overexpression or not. If the patient sample displayed a Ratio(P)/Ratio(S) value lower than 1, it was considered as "basal expression." However, if the Ratio(P)/Ratio(S) value was higher than 1, then it was an "overexpression" case. A third category was assigned for the samples that have a higher Ratio(P)/Ratio(S) value than the Ratio(R)/Ratio(S) value, which in this case considered as "high overexpression."

A final decision was made by comparing these values. The value of the patient was compared with that of the controls' value within the same agarose gel. The mean value of the Ratio (R)/Ratio (S) was found to be 1.24 with a standard deviation of 0.12.

DISCUSSION

Before densitometry analysis, the samples must be loaded together with the control onto the gels. There were two controls to limit experiment-to-experiment variations. First control is the β2M, which is definitely necessary to eliminate sample-to-sample variations. However, this is still not enough, since samples are run on different gels or the PCR was performed on different days in most cases. Thus, we need a second control, which is HL60(S), to overcome the gel-to-gel variations. Third and last, HL60(R) was included for comparison of overexpression.

All patients received anthracycline based chemotherapy protocols. The patients P1A, P5, P6, P10, P11 and P12 expressed much higher MRP1 than the doxorubicin resistant HL60 cell line. So, these patients may not respond to chemotherapy as expected. Clinical results after administration of chemotherapeutic agents showed that for P1 the size of the tumor size only reduced 12.5%, which is considered to be not very significant. Interestingly, P2 showed an increase in resistance about 25% after the application of chemotherapy. While P2, P9 and P10 died, and for all the other patients, the chemotherapy is still continuing and a second tissue sample after chemotherapy was not available. Therefore, a definitive conclusion cannot be made. However, here it can be concluded that P1 has an intrinsic resistance, while P2 has gained a higher degree of resistance after chemotherapy. Initially, P2A can be considered as basal level expression since the Ratio(P)/Ratio (S) was 1.02, but the second sample (P2B) from the same patient definitely has an overexpression, which implies the occurrence of an acquired type of resistance. For P4, it is interesting to see the β2M band only. It can be concluded that either the patient has a very low level of expression most probably below the detection limit or there is no expression at all.

In this study we demonstrated that multidrug resistance mediated by MRP1 overexpression could be a determinant factor in multidrug resistance in breast cancer. The therapeutic outcome was correlated with the expression of MRP1, so the precise determination of the levels of MRP1 expression is very important. This work is important in the sense that a protocol for the quantitative determination of one of the multidrug transporters, MRP1, was optimized not only from fresh tissue samples, but also from FFPE tissues. It is difficult to get well-conserved fresh samples for expression analyses since tissues should be preserved fresh after removal. Also doctors are not willing to spare tumor tissue since a thorough pathology report needs to be done. Therefore, it is not easy to get surgeons cooperate in such studies. However, it is very easy to find FFPE tissue for both retrospective and prospective studies. In this manner, this study is valuable especially when there are not many studies in Turkish literature using such approaches described in this work for predicting chemotherapeutic outcome mediated by MDR.

Overexpression of MRP1 results in resistance to different types of anticancer agents used for the treatment of breast cancer patients currently (15–16). It was strongly suggested that expression of MRP1 gene is responsible for a negative response in early breast cancer and thus MRP1 gene expression levels can be used as marker for breast cancer (17). Leonessa et al. have shown that early expression of MRP1 is directly correlated with a worse prognosis (18) while Trock et al. demonstrated that MRP1

gene expression was in low levels before chemotherapy in breast cancer samples as compared to the samples after chemotherapy (19).

Similar studies also aid the determination of the drug transporters involved in MDR in cancer patients and their correlation with and/or prediction of outcome in the clinic. In the future, with the characterization of these MDR transporters, personalized treatment strategies could be developed to increase the chemotherapeutic efficacy.

ABBREVIATIONS

FFPE = Formalin fixed parafin embedded
GSH = glutathione
MDR = Multidrug resistance
MRP1 = Multidrug Resistance-associated Protein-1

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