

# MRP1 but not MDR1 is associated with response to neoadjuvant chemotherapy in breast cancer patients

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**Abstract.** A major problem in the treatment of breast cancer is the development of resistance to chemotherapeutic agents. Although the role of multidrug resistance 1 (MDR1) and multidrug resistance associated protein 1 (MRP1) in inducing drug resistance in many cancers has been widely investigated the clinical significance of expression of these genes in breast cancer remains unclear and the data is still controversial. We investigated the expression of MDR1 and MRP1 in breast cancer patients as well as the possible correlation between MDR1 and MRP1 and clinical response to chemotherapy. In the present study, MDR1 and MRP1 gene expression were investigated by real time reverse transcription polymerase chain reaction (RT-PCR) assay in 54 breast cancer tumors and in corresponding adjacent normal tissues before neoadjuvant chemotherapy. The expression level of MDR1 and MRP1 were significantly higher in breast tumors than normal breast tissues. Although a significant relationship was found between the MRP1 expression and response to treatment no association was observed between MDR1 expression and response to treatment. MDR1 and MRP1 expression levels have been shown to be independent of tumor size, histological grade and the status of progesterone or estrogen receptor.

**Keywords:** Multidrug resistance, real time PCR, breast cancer, MDR1, MRP1

## 1. Introduction

Resistance to chemotherapy limits the effectiveness of anti neoplastic treatments. In multi drug resistance (MDR) tumors become insensitive to many drugs with different structures and functions [1]. Even though breast cancer is one of the sensitive tumors to chemotherapy many of them become multidrug resistance [2].

Neoadjuvant chemotherapy is an approach in the management of locally advanced breast cancer. It gives benefit in the operable breast cancers by increasing the chances of breast conservation. Neoadjuvant chemotherapy followed by surgery allows for assessment of the histopathologic response. Only about 70% of patients demonstrate a clinical response and merely 3%–27% achieve a complete histopathologic response (3). Therefore, roughly 30% of the patients received neoadjuvant chemotherapy are no responsive to this treatment and suffer from the side effects. The patients achieving a complete pathologic response after neoadjuvant chemotherapy have significantly longer disease-free overall and overall survival than nonresponders (3). Thus in any clinical practice it would be very important and beneficial for each patient

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if the patient could be informed in advance about the chance of being responsive or resistance to the neoadjuvant therapy.

One of the mechanisms inducing drug resistance is the efflux of drugs out of cells by increasing the activity of efflux pumps such as ATP Binding Cassette transporters (ABC transporters) [4]. ABC transporters are a family of transmembrane proteins with the ability to transport wide range of substrate molecules across cellular membranes [5].

MDR1 protein or P-gp is a transmembrane glycoprotein with molecular weight of 170 kDa, encodes by MDR gene [6]. It transports many hydrophobic substrates and anti-cancer drugs including etoposide, doxorubicin and Vinblastine [6,7]. The MDR1 gene with 28 exons and 1.2 kb is located on chromosome 7q21.12 [5].

Like p-gp, MRP is a member of ABC transporter that transport a wide variety of substrates, such as organic anion, leukotriene C4 as well as a number of chemotherapeutic agents, including doxorubicin, daunorubicin, vincristine and colchicines. Also it transports glutathione conjugates and cyclic nucleotides [8].

Although the role of MDR1 and MRP1 in inducing drug resistance in many cancers has been widely investigated the data is still controversial (2, 9, 10, 11). Therefore, the aim of the present study was to analyze the expression of MDR1 and MRP1 in breast cancer patients to see whether or not the expression of either of these genes or in combination or could be used as a marker to predict of drug sensitivity.

## 2. Material and methods

### 2.1. Patients

The project was approved by the local ethics committee of National Institute for Genetic Engineering and Biotechnology (NIGEB) and written informed consent was obtained from each case. Fifty four patients with locally advanced breast cancer admitted to Imam Khomeini Hospital in Tehran were enrolled in this study. The breast cancer patients had not yet received any chemotherapy treatment.

The tumor size and the axillary lymph node status were measured clinically and by using ultrasonography. At this stage, fresh tissue specimens (tumor and normal tissue adjacent to tumor) were collected by the clinicians in separated sterile tubes. Tissue samples were frozen and stored at  $-70^{\circ}\text{C}$ .

The patients were then received three cycles of anthracycline based chemotherapy (FAC/FEC) regime at three weekly intervals.

Routine and metastatic work up was done including complete blood examination (total blood count, platelet count), chest radiograph, ECG (Echocardiography when ECG had a positive finding), liver function tests, Bone Scan.

The patients were assessed both clinically and by ultrasonography for response in the form of reduction in breast tumor size and axillary lymph node status according to UICC criteria [12].

Complete response and partial response were considered when the tumor disappeared completely and when more than half of the tumor diameters showed regression with no local progression respectively. No response was considered for the patients whose tumor diameters had less than 50% regression.

### 2.2. RNA extraction and cDNA synthesis

After histologic diagnosis was confirmed for all samples (and thus cancer tissues could be distinguished from normal tissues) RNA was isolated from 100 mg tissue using Tripure Isolation Reagent (Roche Applied Sciences, Indianapolis, USA). For cDNA synthesis, 1  $\mu\text{g}$  of total RNA from each sample was used to synthesize first-strand cDNA according to the manufacturer's protocol (Fremontas, Mannheim, Germany).

### 2.3. Real-time RT-PCR

mRNA expression of the MDR1 and MRP1 gene was quantified as described and validated previously [13] by real-time RT-PCR using a lightcycler<sup>TM</sup> system (Roche Applied Sciences, Indianapolis, USA.) with Fast-Start DNA Master SYBR-Green I kit (Roche Applied Sciences, Indianapolis, USA.) using  $\beta$ -actin as housekeeping gene.

The PCR was performed in 20  $\mu\text{L}$  of solution, consisting of 2  $\mu\text{L}$  of Fast Start Master solution and 0.6  $\mu\text{M}$  of each primer. A total of 18  $\mu\text{L}$  of this reaction mix was placed into glass capillaries, and 2  $\mu\text{L}$  of cDNA was added as template.

A standard Lightcycler PCR program was established for each gene. Thermal Cycling were as follows:  $95^{\circ}\text{C}$  for 10 sec followed by an amplification program 55 cycles with temperature ramp rate of  $20^{\circ}\text{C}/\text{sec}$ . The amplification program was  $95^{\circ}\text{C}$  for 10 sec, primer  $T_m$  (Table 1) for 15 sec and  $72^{\circ}\text{C}$  for 15 sec with a single fluorescence acquisition at the end of the elon-

Table 1  
Sequences of primers

Primer	Amplicon size	Sequence	$T_m$
MRP1-Forward	294 bp	5'-CGG AAA CCA TCC ACG ACC CTA ATC-3'	64°
MRP1-Reversed		5'-ACC TCC TCA TTC GCA TCC ACC TGG-3'	
MDR1-Forward	308 bp	5' TGA CAT TTA TTC AAA GTT AAA AGC 3'	62°
MDR1-Reversed		5' TAG ACA CTT TAT GCA AAC ATT TCA A3'	
$\beta$ -actin-Forward	161 bp	5'-GAG ACC TTC AAC ACC CCA GCC-3'	60°
$\beta$ -actin-Reversed		5'-AGA CGC AGG ATG GCA TGG G-3'	

Table 2  
Comparison between tumor size and response to chemotherapy

	Tumor size							
	< 5 cm		5–8 cm		8–10 cm		> 10 cm	
	N	%	N	%	N	%	N	%
Responder	21	38.9	8	14.8	5	9.3	6	11.1
Non responder	6	11.1	5	9.3	1	1.8	2	3.7

gation step. The third segment consisted of a melting curve program at 95°C for 0 sec, 70°C for 10 sec and 95°C for 0 sec with a liner temperature transition rate of 0.1°C/sec with continuous fluorescence acquisition. Finally, a cooling program cooled the reaction mixture to 40°C. To ascertain that fluorescence signals were associated with specific products, melting curves were constructed for each reaction, and the PCR products were electrophoresed on 1.5% agarose gel.

#### 2.4. Data analysis

The raw data were analyzed using version 3.03 of the Lightcycler software as described previously [13]. The software calculates the relative amount of the target gene and the reference gene (housekeeping gene) based on the crossing point which was defined as the cycle number at which the fitted line in the log-linear portion of the plot intersected the threshold level. An external standard curve for MDR1 and  $\beta$ -actin was generated from a serial dilution of mRNA of each gene. For each sample, the amounts of MDR1 and the housekeeping gene were measured. Finally, the relative expression was calculated as the ratio of MDR1 to  $\beta$ -actin in each sample. Statistical analysis was performed using SPSS for software V16.0 (SPSS, Inc., Chicago, IL). Differences between groups were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. Spearman correla-

Table 3  
Comparison between histological grade and response to chemotherapy

	Histological grade					
	Stage I		Stage II		Stage III	
	N	%	N	%	N	%
Responder	6	11.1	22	40.7	12	22.2
Non responder	3	5.6	8	14.8	3	5.6

tion coefficient was used to determine the relationship between expression levels of MRP1 and LRP. Association between clinical characteristics and an expression level was determined using Chi-squared test. A *P* value less than 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Patients' clinical and pathological data

In total 54 breast cancer patients were studied (all female). The median age of the patients was 47.26 year (SD 13.89). Invasive ductal carcinoma and invasive lobular carcinoma were diagnosed in 85% and 11% of the patients respectively. Thirty two patients (59%) were diagnosed at postmenopause and 22 (41%) at premenopause status. Grade I, II, and III were diagnosed by pathology examination in 17%, 55% and 28% of the patients respectively. Fifty percentage of the patients had tumor size smaller than 5 cm ( $n = 27$ ), about 35% ( $n = 19$ ) of the patients had tumor size between 5–8 cm, while 15% of the patients had tumors size larger than 10 cm ( $n = 8$ ). Thirty four (63%) of the patients had lymph node metastasis. Estrogen and progesterone receptors positive were seen in 69% and 62% of the patients respectively.

The patients were divided into two groups according to the results of the neoadjuvant therapy; patients classified as having partial response formed the positive response group, while patients classified as showing no response made up the negative chemotherapy response group.

Forty patients had partial response (75%) and fourteen had no response (25%). There was no patient with complete response.

#### 3.2. Association between clinicopathology markers and response to chemotherapy

We investigated whether or not traditional clinical prognostic factors as stage of tumor, tumor size and

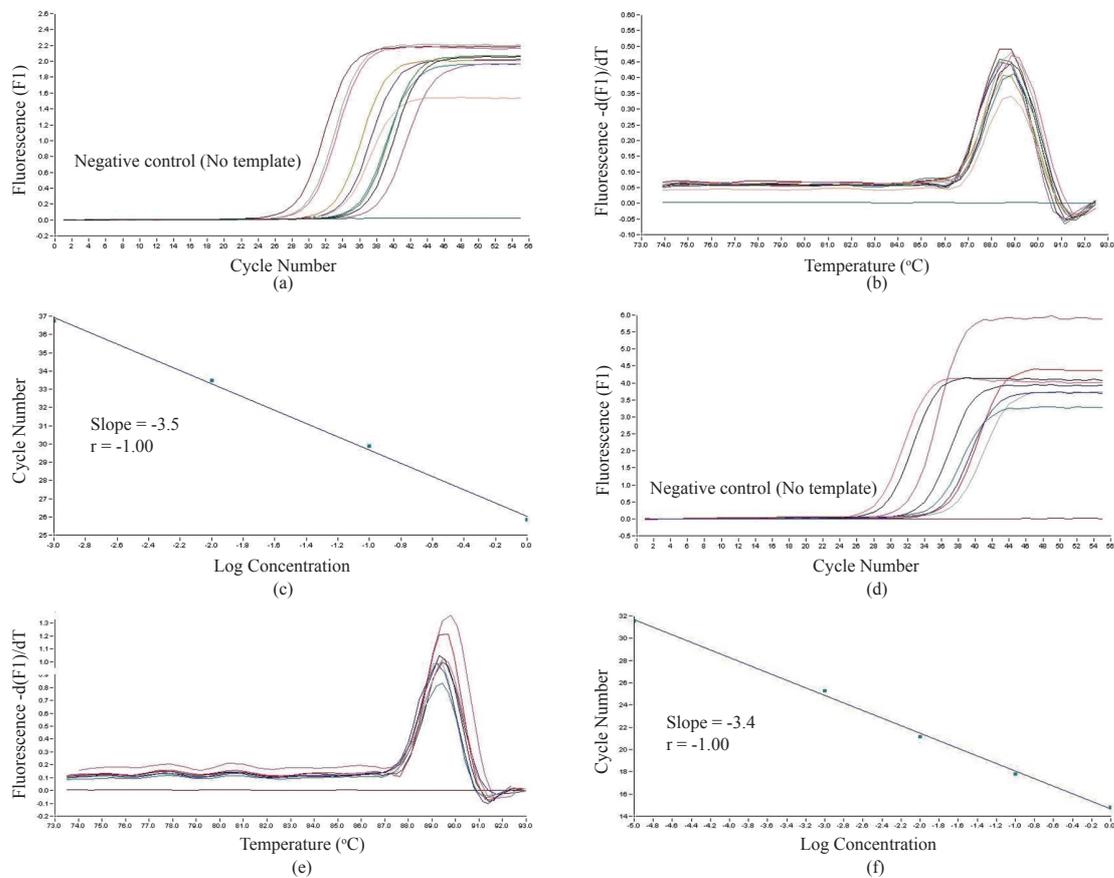


Fig. 1. Real-time RT-PCR for quantification of MRP1 (1a-1c) and  $\beta$ -actin (1d-1e) mRNA with amplification curves (1a,1d) and melting point analysis (1b,1e). Standard curves (1c, 1f) were generated by plotting the Ct value vs. the log of the dilution of the calibrator cDNA. (Colours are visible in the online version of the article; <http://dx.doi.org/10.3233/DMA-130985>)

lymph node involvement could be predictor of response to chemotherapy. There was no relationship between tumor size (Table 1), histological grade (Table 2) and lymph node involvement with response to drug ( $P = 0.823$ ,  $P = 0.069$ , and  $P = 0.06$  respectively).

### 3.3. Real-time PCR validation

For the mRNA expression of the target genes and the housekeeping gene a reliable and reproducible relative quantification method based on LightCycler<sup>TM</sup> technology was established and validated. Melting curve analysis showed only one peak for each reaction and this also confirmed by electrophoresis of PCR products that showed only one band of the expected size.

For high PCR efficiency the slope of the calibration curve approach was 3.402 (optimum curve slope) and the y-intercepts close to the Ct value of the negative control. The amplification curves, melting curve analy-

sis, and standard curves are exemplified for MRP1 and  $\beta$ -actin in Fig. 1.

### 3.4. Expression of MDR1 and MRP1 in normal and tumoral breast tissue

The expression levels of MDR1 and MRP1 in normal and tumoral tissues of the patients are shown in Fig. 2. Mean expression level of MDR1 in normal breast tissues were  $0.7221 \pm 0.1098$  and in tumoral breast tissues were  $0.7859 \pm 0.1108$ . A statistically significant increase in MDR1 expression was observed when samples from normal breast tissue were compared with tumoral breast tissue (Fig. 2). The expression level of MRP1 in normal and tumor tissues were  $0.7560 \pm 0.13348$  and  $0.8709 \pm 0.11724$ , respectively. As shown in Fig. 2, the expression level of MRP1 was significantly higher in breast tumor than normal breast tissue ( $P = 0.0001$ ). Subsequent analysis with regard

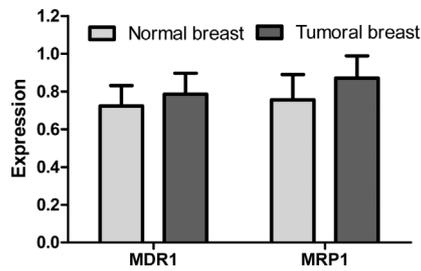


Fig. 2. Mean expression level of MDR1 and MRP1 in normal and tumoral breast tissue.

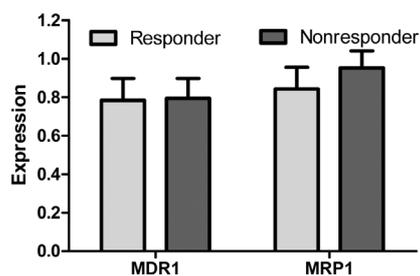


Fig. 3. Mean expression level of MDR1 and MRP1 in responder and non-responder.

to potential interrelationships of these drug resistance genes revealed that the mRNA levels of MDR1 were not related to MRP1 ( $P > 0.05$ ).

### 3.5. Correlation between mRNA expression of MDR1 and MRP1 and response to chemotherapy

A comparison between the tumors of responder versus nonresponder patients with respect to the relative expression levels of MRP1 showed that the expression level of MRP1 was significantly higher in nonresponder than responder patients ( $P < 0.05$ ). Mean expression level of MRP1 in responder and nonresponder were  $0.8422 \pm 0.11331$  and  $0.9529 \pm 0.08809$ . While the mean expression level of MDR1 in responder and nonresponder were  $0.7832 \pm 0.1144$  and  $0.7936 \pm 0.1035$ , respectively (Fig. 3). Although this value was higher in nonresponder this difference was not statistically significant.

## 4. Discussion

Breast cancer with 1 million new cases in every year is the most common malignancy in women [14]. Despite the advances in therapeutic methods about 40–50% of patients will eventually die from the disease [14]. Therefore researchers are looking for appro-

prate therapeutic methods in order to increase the survival rate. This is facilitated by finding suitable markers which might predict clinical outcome (prognostic markers) and response to a particular type of therapy (predictive markers).

The development of multidrug resistance is a major obstacle to successful chemotherapy in cancers. Various mechanisms, such as reduced drug uptake, the efflux of intracellular drugs, the activation of DNA repair pathways, and the induction of anti-apoptotic machineries can confer multidrug resistance in cancer cells [6]. Despite the intensive studies regarding the role of these genes in inducing drug resistance in cancer patients the data is still controversial and debated. A possible role of the MDR1 and MRP1 in clinical breast cancer has previously been studied but the results are not conclusive [2,9,11,15].

In the present study we examined the expression of these two genes in breast cancer patients by real time RT PCR. We examined whether the expression of either or combination of these two genes in breast cancer tumor cells correlates with response to neoadjuvant chemotherapy.

The expression levels of MDR1 and MRP1 was found to be significantly different between tumoral tissues and normal breast tissues. MDR1 and MRP1 are apparently expressed at higher levels in carcinoma cells than non-cancer cells. However, we found no significant correlation between MDR1 expression and response to treatment. Arnal and co workers (2000) demonstrated comparatively to normal tissues, a significant induction of MDR1 expression in untreated tumors but after treatment no differences were detected between tumor and normal samples. Also no significant relationship was found between MDR1 expression and clinical response to chemotherapy neither before nor after treatment [15].

Faneyte et al. [16] compared the post-chemotherapy expression of MDR1 in tumors that had microscopic evidence of response with that did not respond. The difference was not statistically significant. In pre-chemotherapy samples, responders did not differ significantly from non-responders. They found no correlation between clinical response and MDR1 expression [16]. This is similar to finding of Rudas et al. [9] that in a study on 80 patients with locally advanced breast cancer demonstrated that the expression of MRP1, and MDR1 increase after preoperative chemotherapy but there was no association between their expression and response to chemotherapy [9]. In contrast to these studies Burger et al. [2] reported a

significant correlation between MDR1 gene expression and response to chemotherapy. MDR1 gene expression occurred in 68% of resistant patients, but in only 17% of clinically responsive patients [2]. Similar to this Atalay et al. [17] showed a strong correlation between MDR1 expression and response to treatment. While the expression rate of MDR1 was 100% in clinically resistant patients only 5% of the clinically responsive patients had MDR1 gene expression [17].

We found a significant positive correlation between MRP1 expression and response to treatment. Nooter et al. [18] evaluated the role of MRP1 expression in the prognosis and response to chemotherapy of breast cancer patients. They showed the presence of MRP1 protein was associated with lower response rate in previously untreated patients who received first-line chemotherapy [18]. Similarly Filipits and co workers [11] suggested that MRP1 expression plays an important role in the clinical resistance to adjuvant CMF chemotherapy [11].

In contrast to the above findings Burger et al. [2] showed an inverse relation between MRP1 gene expression and clinical response to chemotherapy but expression of this gene was not significantly different in clinically responsive and unresponsive patients [2]. Atalay et al. [17] showed that although 80% of the clinically unresponsive patients had higher MRP1 gene expression, but the relation between MRP1 expression and clinical drug response was not significant [17].

In regards to relationship between the expression level and clinicopathology markers such as stage of tumor, tumor size, hormone status and lymph node involvement there was no association between expression of these genes and clinicopathology markers. Further studies showed similar results [2,10,19].

In conclusion, conflicting results have been obtained from several studies regarding the role of these genes in inducing MDR. This may be partly due to the methods applied for investigation of gene expression. For this reason it seems that a gold standard method should be presented in order to study the expression of these genes and their role in drug resistance. Hence we recommend Real time PCR as a good method for evaluation of these gene expressions at mRNA level because Real-time PCR is simple to use, and requires only minimal experience and skills. It is the method that allows quantification and provides maximum sensitivity (e.g. 10,000 times more sensitive than northern blot).

Furthermore, future prospective studies on large patient populations are required to further explore the impact of drug resistance genes on the clinical outcome of breast cancer patients treated with chemotherapy.

## Acknowledgements

This project was supported by a NIGEB grant (No:412). The authors would like to thanks all patients who willingly participated in the study.

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