RESEARCH ARTICLE

Expression of *LRP* Gene in Breast Cancer Patients Correlated with *MRP1* as Two Independent Predictive Biomarkers in Breast Cancer

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Abstract

   Background: Breast cancer is the most common malignancy in women. Multidrug resistance (MDR) is still a great obstacle of breast cancer chemotherapy. We have previously shown that multidrug resistance-associated protein 1 (*MRP1*) is associated with response to neoadjuvant chemotherapy. The lung resistance-related protein (*LRP*) is identified as a prognostic marker and response to treatment factor which has been studied mainly in hematological malignancy and leukemia. In this study, we aimed to analyze *LRP* expression and possible correlation between the expression level of this gene with *MRP1* as a candidate marker for chemotherapy resistance. **Materials and Methods:** We collected 54 breast tumors and adjacent normal tissues from Iranian breast cancer patients and Real time RT-PCR was employed to measure the gene expression level in our samples. **Results:** *MRP1* and *LRP* expression level were significantly lower in tumor tissues of the patients responding to chemotherapy compared to non-responding patients. No relation between the expression level of either of these genes and clinicopathology markers was found. **Conclusion:** Our results suggest that *LRP* gene expression is correlated to *MRP1* in human breast cancer cells and may affect the clinical response to treatment.

Keywords: Multidrug resistance- Breast cancer- LRP- MVP- MRP1

Introduction

   Breast cancer with an estimated 1.7 million cases and 521,900 deaths in 2012 is known as the most common malignancy in women. Breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer deaths among females. In developed countries, breast cancer consists about 38% of deaths (Torre et al., 2015) and in Iran with 23% frequency in women is the most prevalent cancer in women (Mousavi et al., 2008).

   Among solid tumors, breast cancer is considered as one of the most responsive to chemotherapy, but most of the tumors that initially response to drugs become resistance (Burger et al., 2003) and the incidence of resistance increases with breast cancer progression (Martin et al., 2014). This phenomenon is named as Multi Drug Resistance (MDR) in which cancer cells become resistant to the wide spectrum of drugs with different structure and function (Gottesman, 2002). Several mechanisms responsible for drug resistance have been identified including mutation in or overexpression of the drug's target, drug inactivation and efflux of the drug from the cell by ATP Binding Cassette (ABC) transporters

(Motalebzadeh et al., 2017). These transporters are capable of decreasing the intracellular drug concentration in vitro (Dean, 2009).

   The Multi Drug Resistance Related Protein1 (*MRP1*) is a member of the ABC-transporter that located on chromosome 16p13 and transports a wide range of compounds including glutathione conjugates and cyclic nucleotides out of cells. It has been shown *MRP1* molecule has a role in the efficiency of the drugs used for the treatment of nonmalignant diseases such as antibiotics and antiviral drugs (Cole, 2014).

   Another mechanism of drug resistance is the intracellular redistribution of drugs without changing their intracellular accumulation (Lee et al., 2017). Lung Resistance Protein (*LRP*) is not a member of ABC transporter family but it is a major vault protein (MVP) and is found in cytoplasm and nuclear membrane and can transport drugs out of nucleus. In cells, *LRP* is usually associated with vesicles and lysosomes and is thought to be responsible for the uptake of drugs in cytoplasmic vesicles that are then probably extruded from the cell by exocytose (Triller et al., 2006). *LRP* gene is located on chromosome 16p11.2 close to the *MRP1* gene. Because of

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its position on nuclear membrane it is thought to have an effect on the transport of drugs that their main target is DNA therefore it plays an important role in drug resistance (Lu and Shervington, 2008).

The role of *MRP1* in inducing drug resistance in many cancers has been widely investigated. In our previous study we found that *MRP1* is correlated with response to the chemotherapy in Iranian breast cancer patients (Taheri and Mahjoubi, 2013). The effect of *LRP* in prognosis and response to treatment has been studied mainly in hematological malignancy and leukemia and rarely on solid tumors (Bhatia et al., 2015).

The aim of the present study was to investigate the role of *LRP* and *MRP1* genes in response to chemotherapy and the prognostic value of these genes in Iranian breast cancer patients.

Materials and Methods

Patients sample collection

Fifty four patients with breast cancer who had undergone breast cancer surgery were enrolled in this study. The project was approved by the local ethical committee of National Institute of Genetic Engineering and Biotechnology (NIGEB). Written informed consent was obtained from all cases. Tissue specimens (tumor and normal tissue adjacent to tumor) were collected by the surgeons. Histologic diagnosis was confirmed for all samples. The patients' features are shown in Table 1. The patients then were received anthracycline based chemotherapy (FAC/FEC). Response to treatment was evaluated by UICC criteria. Routine clinical examination comprises complete blood examination, chest radiography, ECG (Echocardiography), bone scan and liver function tests were done.

RNA extraction and cDNA synthesis

RNA extraction was carried out by Tripure Isolation Reagent (Roch applied sciences). For cDNA synthesis, 1 µg of total RNA from each sample was used to synthesize first-strand cDNA according to the manufacturer's protocol (Frementas).

Real-time qRT-PCR

The mRNA levels of *LRP* gene was measured by real-time qRT-PCR using a lightcycler™ system (Roche Applied Sciences) with Fast-Start DNA Master SYBR-Green I kit (Roche Applied Sciences). In addition, the mRNA level of β-actin as internal control was measured and used to normalize the mRNA levels of the drug resistance gene.

The primers were designed using Primer Premier 5.0 software (Table 2).

All reactions were carried out in a total volume of 20 µL in capillary tubes. Each reaction mix contained 0.6 µM of each primer, 2.5 mM $MgCl_2$ and 2 µL of Fast Start Master solution. A total of 18 µL of this reaction mix was placed into glass capillaries, and 2 µL of cDNA was added as template. The capillary tubes were capped and placed in the carousel under reduced light conditions. Thermal cycling consisted of an initial denaturation step

95 °C for 10 min followed by an amplification program (denaturation, amplification and extension) repeated for 55 cycles with temperature ramp rate of 20 °C/sec. The amplification program was 95 °C for 10 sec, primer Tm (Table 2) for 15 sec and 72 °C for 15 sec with a single fluorescence acquisition at the end of the elongation 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 linear temperature transition rate of 0.1 °C/sec with continuous fluorescence acquisition. Finally, a cooling program cooled the reaction mixture to 40 °C.

For confidence accuracy and specificity of Real Time PCR, PCR products were checked on 1.5% agarose gel.

A standard Lightcycler PCR program was established using logarithmic regression for each gene.

Data Analysis

The raw data were analyzed using version 3.03 of the Lightcycler software as described previously (Golalipour et al., 2007). 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 the Tuckey multiple comparison tests. Association between clinical characteristics and an expression level was determined using Chi-squared test. A p-value less than 0.05 were considered statistically significant. Also to determine the relationship between the expression level of *LRP* and *MRP1* gene the spearman correlation coefficient was used.

Results

This study was done on 54 breast cancer patients to assess the expression levels of *MRP1* and *LRP* on clinicopathology criteria and response to treatment.

LRP gene expression level in tumor and normal breast tissues was assessed by Real Time RT PCR. The final results were expressed as the ratio of each gene to β-Actin, an internal control gene, in each sample as described previously (Motalebzadeh et al., 2018).

Expression of LRP in normal and tumoral breast tissues and its correlation with MRP1

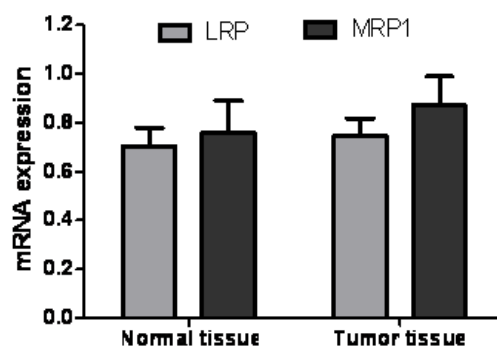According to our previous study, we found the expression level of *MRP1* in normal and tumor tissues

Figure 1. Relative Mean Expression of *MRP1* and *LRP* in Normal and Tumor Breast Tissue

Table 1. Patients and Tumor Characteristics

Characteristic	No (%)
All of the Patients	54 (100%)
Menopausal Status	
Premenopausal	22 (41%)
Postmenopausal	32 (59%)
Pathological Type	
Ductal carcinoma	48 (89%)
Lobular carcinoma	6 (11%)
Histological Grade	
Grade-I	9 (17%)
Grade-II	30 (55%)
Grade-III	15 (28%)
Tumor Size	
>5cm	27 (50%)
5-8cm	13 (24%)
8-10cm	6 (11%)
>10cm	8 (15%)
Lymph Node Metastasis	
Positive	34 (63%)
Negative	20 (37%)
Response to Chemotherapy	
Responder	40 (74%)
Non-responder	14 (26%)

Figure 2. Correlation between the Expression Levels of *MRP1* and *LPR* in Breast Tumor Tissue (2A) and Normal Breast Tissue (2B).

were 0.7560 ± 0.13348 and 0.8709 ± 0.11724, respectively (Taheri and Mahjoubi, 2013).

As shown in Figure 1, the *LRP* mRNA level of breast tumoral tissues (0.7458 ± 0.07198) was significantly (p=0.003) higher than normal tissue (0.7029 ± 0.07544).

The results showed that there was a positive significant correlation between expression levels of *MRP1* and LPR in breast tumor tissues (r=0.6420, P<0.0001) and normal breast tissues (r=0.4937, P=0.0002) (Figure 2).

Response to chemotherapy and correlation with LRP gene expression level

The clinical response showed that 40 (74%) patients were classified as responsive (R) and 14 (26%) as nonresponsive (NR). Relative expression of *LRP* gene was 0.7264 ± 0.07035 and 0.8012 ± 0.04244, respectively (Figure 3). The results showed that the expression level of *LRP* was significantly higher in nonresponder than responder patients (P<0.05).

Clinicopathology characteristics and LRP gene expression level
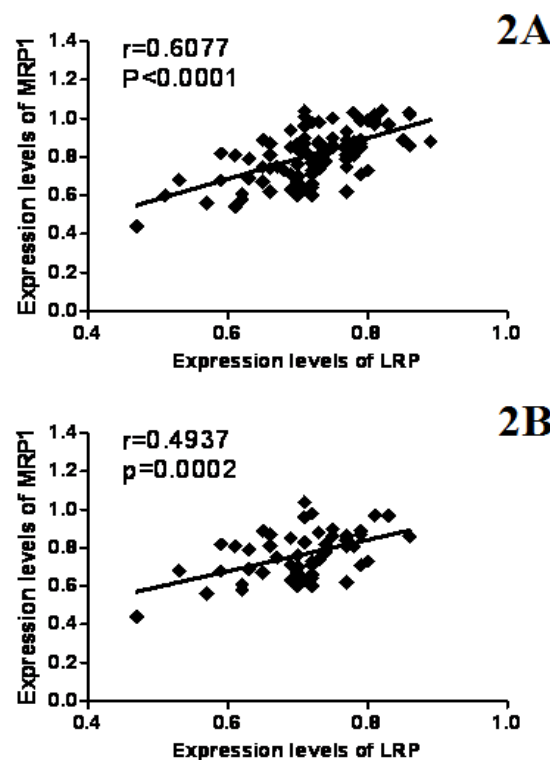
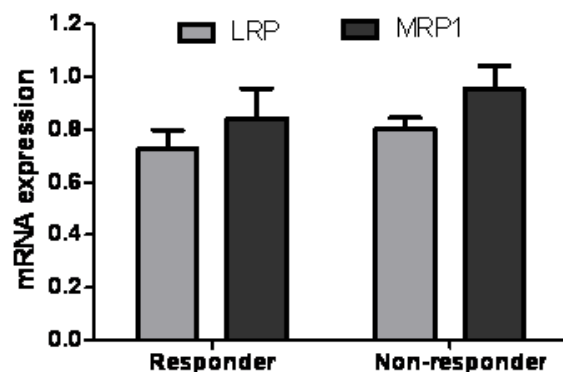We investigated the mRNA expression level of *LRP*

Figure 3. Relative Mean Expression of *MRP1* and *LRP* in Responder and Non-responder

gene in relation to traditional clinical prognostic factors as listed in Table 2. The average age of the patients was calculated 47.26 (SD=13.89) years. Of 54 patients, 9 (17%) were in grade-I, 30 (55%) were in grade-II and 15 (28%) were in grade-III. There was no association between the expression of *LRP* and grade of breast tumor. Concerning to tumor size, 27 (50%) patients had tumor

Table 2. Sequences of the Primers

Primer	Amplicon size	Sequence	T_m
LRP-Forward	137bp	5' GGC AGG ACA ATG AGA GGG TAC T 3'	60°
LRP-Reversed		5' CCG AAC TTG CCC TGT GAC AT 3'	
β-actin-Forward	161bp	5'-GAG ACC TTC AAC ACC CCA GCC-3'	60°
β-actin-Reversed		5'-AGA CGC AGG ATG GCA TGG G-3'	

size <5 cm, 13 patients (24%) had tumor size between 5-8 cm, 6 (11%) between 8-10 cm and 8 (15%) over 10 cm. Our results indicated there was not any association between tumor size and the expression level of *LRP*. Sixty-nine percent of patients were estrogen receptor positive and 62% were progesterone receptor positive. In 63% of patient's lymph node was involved. There was no association between hormone status, lymph node involvement and expression level.

Discussion

Although breast cancer survival rates have increased in recent years many women still die from the disease. Resistance to chemotherapy with metastasis is the most important cause of death (Fonseca, 2012). Resistance to chemotherapy limits the effectiveness of anti neoplastic treatment. Several proteins have been identified that are able to prevent the intracellular accumulation of anticancer agents by efflux mechanism such as MDR1 and *MRP1* (Fojo and Coley, 2007). Several studies showed that *LRP* promotes cancer progression and the expression of this gene is an adverse prognostic factor for response to chemotherapy (Huh et al., 2006; Lee et al., 2017). The majority of the studies have been performed in hematological malignancies. Only a few studies have addressed the role of *LRP* expression in solid tumors and no clear relationship between *LRP* and outcome of the patients was observed (Gillet and Gottesman, 2010; Martin et al., 2014).

In the present study, we examined the expression level of *LRP* gene and the correlation of this gene with *MRP1* in breast cancer patients. The *LRP* and *MRP1* were studied by real time RT PCR and their clinical significance in chemoresistance were also investigated.

With respect to *LRP*, we found that a statistically significant correlation between the expression level and response to treatment in this group of patients. Herman Burger et al in a study done on breast cancer patients found a statistically significant correlation between the expression level and length of PFS (progression-free survival) (Burger et al., 2003). In another study by Schneider and coworkers, they found *LRP* expression in locally advanced breast cancer was associated with the presence of axillary nodal metastasis after induction chemotherapy, and they suggested that *LRP* may play a role in the resistance phenotype of intrinsically resistant clones that persist after induction chemotherapy (Schneider et al., 2001). The fact that ABC transporter expression levels and resistance to chemotherapy are positively correlated in some but not all breast cancer may reflect differences in analytic methods, patients' population, or the chemotherapeutic drugs used.

Overexpression of *MRP1* has been observed in several human cancers, including breast cancer, gastric hepatoid adenocarcinoma, and several types of leukemia (Faneyte et al., 2004; Mahjoubi et al., 2008; Iseri et al., 2011). We previously found and reported a significant positive correlation between *MRP1* expression and response to treatment and the importance of *MRP1* rather than MDR1 in breast cancer patients (Taheri and Mahjoubi, 2013).

Atalay and co-workers observed increased expression of *MRP1* after chemotherapy in almost all locally advanced breast cancer patients but this increase was not statistically significant (Atalay et al., 2006). Rudas et al demonstrated that the expression of *MRP1*, *LRP* and p-gp increase after preoperative chemotherapy but there was no association between their expression and response to chemotherapy (Rudas et al., 2003). A correlation between *MRP1* expression and patient survival rates after chemotherapy has been noted in some studies (Burger et al., 2003; Rudas et al., 2003), whereas other reports showed no correlation between *MRP1* expression and prognosis (Faneyte et al., 2004; Huh et al., 2006).

We also found that the expression level of *LRP* was positively related with *MRP1* gene and this may indicate that the expression of these genes is likely to be coordinately regulated. Similar correlations between the expression of these genes were observed previously (Burger et al., 2003)

Regarding to relationship between the expression and clinicopathology markers, no association was found between expression of these genes and stage of tumor. There was also no association between tumor size, hormone status, lymph node involvement and expression level of *LRP* and *MRP1* genes.

In conclusion the results showed that *MRP1*/*LRP* expression has been associated with unfavorable outcome in our breast cancer patients. Both *LRP* and *MRP1* seem to contribute to drug resistance. Determination of *MRP1* and *LRP* (either alone or in combination) may be important for the prediction of the chemotherapy outcome in breast cancer patients. It may also be valuable for developing anticancer drugs with more effectiveness on the patients.

Further studies should focus on the molecular basis of how the expression of these transporters is regulated in normal breast cells and in their malignant counterparts.

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