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Effect of MDR1 polymorphism on multidrug resistance expression in breast cancer patients

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ABSTRACT. One of the limitations in the treatment of cancer patients with chemotherapy is the development of multidrug resistance (MDR). A well-known mechanism responsible for drug resistance is over-expression of ABC-transporter genes such as MDR1. This gene encodes p-glycoprotein (P-gp), a transmembrane glycoprotein that transports many hydrophobic substrates and anti-cancer drugs out of the cell. MDR1 gene polymorphisms could alter the expression level of P-gp and consequently result in drug resistance. We investigated a possible association between MDR1 gene C3435T polymorphism and its expression in Iranian breast cancer patients. PCR-RFLP was used for the detection of C3435T single nucleotide polymorphism in 54 breast cancer patients and 50 healthy individuals. The expression level of MDR1 was determined by real-time quantitative PCR. We observed no difference in the frequency of C3435T polymorphism between breast cancer patients and healthy controls. However,

there was a significant association between MDR1 expression levels and C3435T polymorphism in the patients. C3435T polymorphism may play a role in inducing drug resistance by altering the expression level of the MDR1 gene.

Key words: Multidrug resistance; MDR1 gene; C3435T polymorphism; Real-time polymerase chain reaction; Breast cancer

INTRODUCTION

One of the problems in the treatment of cancer patients with chemotherapy is multidrug resistance (MDR). This means that tumor cells become insensitive to a wide range of cytotoxic drugs that are structurally and functionally different.

Breast cancer is known to be the most prevalent type of cancer among women and the second leading cause of death after lung cancer. Although chemotherapy has been one of the most widely used treatments for breast cancer patients, the development of MDR in these patients also limits the success rate (Atalay et al., 2006).

One of the best known mechanisms responsible for the MDR phenotype in cancer patients is the increased ability of tumor cells to transport drugs out of the cell, employing one or more ATP-dependent transporters (Gottesman et al., 2002).

P-gp is a transmembrane glycoprotein with a molecular weight of 170 kDa. It is one of the ATP-dependent transporters encoded by MDR1 gene (van der Deen et al., 2005). The role of P-gp in MDR in cell lines and in many cancers has been confirmed by many investigators (Linn et al., 1995, Decleves et al., 2002).

The MDR1 gene with 28 exon and 1.2 kb is located on chromosome 7q21.12, and the coding region accounts for less than 5% of the total (Sakaeda, 2005). Over 50 single nucleotide polymorphisms (SNPs) have been identified in the human MDR1 gene (Kimchi-Sarfaty et al., 2007). Among them, C3435T SNP on exon 26 as a silent mutation, seems to affect the expression level of MDR1 and consequently drug resistance of cells (Hoffmeyer et al., 2000).

The aim of the present study was to determine the frequency of C3435T polymorphism among breast cancer patients and healthy individuals as well as the possible association between C3435T polymorphism and MDR1 expression level.

MATERIAL AND METHODS

Fifty-four patients with primary breast cancer and 50 women as the control group were enrolled in this study. There was no significant difference regarding age between case (46.72 ± 13.89) and control groups (43.28 ± 12.12) ($P > 0.05$). Tumor, normal tissue adjacent to tumor and blood were obtained from each patient and blood from each healthy individual.

The breast cancer patients had not yet received any treatment. The project was approved by the local Ethics Committee of the National Institute for Genetic Engineering and Biotechnology (NIGEB), and written informed consent was obtained in all cases. Tissue specimens were collected from the Cancer Institute of Imam Khomeini Hospital between April 2007 and January 2009. Histologic diagnosis was confirmed for all samples.

RNA extraction and cDNA synthesis

RNA extraction was carried out with the Tripure Isolation Reagent (Roche Applied Sciences). For cDNA synthesis, 1 µg total RNA from each sample was used to synthesize first-strand cDNA according to the manufacturer protocol (Fermentas).

Real-time RT-PCR

Evaluation of the expression level of MDR1 was performed by real-time quantitative polymerase chain reaction (PCR) using the Lightcycler™ system (Roche Applied Sciences) and Fast-Start DNA Master SYBR-Green I kit (Roche Applied Sciences). The following primers were used for evaluating MDR1 expression: MDR1 forward 5'-TGACATTTATTCAAAGTTA AAAGCA-3' MDR1-reversed 5'-TAGACACTTTATGCAAACATTTCAA-3'. β-actin was selected as the housekeeping gene for assessment of expression. The primer sequences for β-actin were as follows: forward 5'-GAGACCTTCAACACCCCAGCC-3' and reverse 5'-AGACGCAG GATGGCATGGG-3'.

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 in glass capillaries, and 2 µL 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 at 95°C for 10 min followed by an amplification program (primer annealing, amplification and quantification) repeated for 55 cycles with temperature ramp rate of 20°C/s. The amplification program was 95°C for 10 s, 62°C for 15 s and 72°C for 15 s, 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 s, 70°C for 10 s and 95°C for 0 s with a liner temperature transition rate of 0.1°C/s with continuous fluorescence acquisition. Finally, a cooling program cooled the reaction mixture to 40°C.

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

DNA extraction and genotyping

Genomic DNA extraction from blood samples (54 patients and 50 control individuals) was carried out by Diatom DNA Prep 200 (Isogen Lab Ltd. Russ). MDR1 C3435T polymorphism was detected using a PCR-RFLP assay. The following primers were used: 5'-GCTGG TCCTGAAGTTGATCTGTGAAC-3' as forward and 5'-ACATTAGGCAGTGACTCGATG AAGGCA-3' as reverse primer (Turgut et al., 2007).

The PCR mixture included 1 µM primer, 200 µM of each dNTP (Sigma), Taq DNA polymerase 1X buffer with 1.5 mM $MgCl_2$, and 2.5 units Taq polymerase (5 U/µL, Sigma).

The PCR protocol was as follows: initial denaturation at 94°C for 2 min followed by 35 cycles, consisting of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s. Final extension was performed at 72°C for 4 min. Amplified segments were analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and observed under ultraviolet light.

The PCR product (248 bp in size) was digested for 3 h at 37°C with 2 U *Mbo*I restriction enzyme (Fermentas, Germany). The expected fragment sizes were: a 238-bp fragment for TT genotype, 172- and 60-bp fragments for the CC genotype, and 238, 170 and 60 bp for the CT genotype. DNA fragments generated after restriction enzyme digestion were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed with an ultraviolet transilluminator.

Data analysis

The raw data were analyzed using the Lightcycler software, version 3.03. 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 β-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 β-actin in each sample. Statistical analysis was performed using the SPSS for software V16.0 (SPSS, Inc., Chicago, IL). Differences between groups were analyzed by one-way analysis of variance (ANOVA) and the Tukey multiple comparison test. The difference in genotype frequencies between controls and breast cancer patients was determined using the chi-square test. A P value less than 0.05 was considered to be statistically significant.

RESULTS

For assessments of C3435T polymorphism of MDR1 gene and its association with MDR1 expression level, we analyzed 54 patients with breast cancer. The patient and tumor characteristics, gathered from the pathology reports, are listed in Table 1.

Table 1. Patients and tumor characteristics.

Characteristics	
Total patients	54 (100%)
Menopausal status	
Premenopausal	22 (41%)
Postmenopausal	32 (59%)
Histological grade	
Grade I	9 (17%)
Grade II	30 (55%)
Grade III	15 (28%)
Tumor size	
<5 cm	27 (50%)
5-8 cm	13 (24%)
8-10 cm	6 (11%)
>10 cm	8 (15%)
Lymph node metastasis	
Positive	34 (63%)
Negative	20 (37%)

Data are reported as number with percent in parentheses.

The allele and genotype frequencies in patient and control groups were determined. In the patient group, the C allele frequency was 46.3% and the T allele frequency was 53.7%, and in the control group the frequencies were 47% for C allele and 53% for T allele. The allele frequency was not significantly different between controls and patients ($P > 0.05$). The MDR1 wild-type genotype (CC) was observed in 10 patients (18%), whereas 30 patients (56%) were heterozygous (CT) and 14 patients (26%) were homozygous (TT). In the control group, genotype frequencies were 10 (20%) for CC, 27 (54%) for CT and 13 (26%) for TT (Table 2). This difference was not found to be statistically significant ($P = 0.980$).

Table 2. Genotype frequency of C3435T MDR1 polymorphism.

	Genotype		
	CC	CT	TT
Breast cancer (N = 54)	10 (18%)	30 (56%)	14 (26%)
Control (N = 50)	10 (20%)	27 (54%)	13 (26%)

Data are reported as number with percent in parentheses.

MDR1 expression level was assessed in 54 patients and 50 healthy controls by real-time RT-PCR. The final results were expressed as the ratio of MDR1 to β-actin in each sample, as described previously (Golalipour et al., 2007). We evaluated MDR expression in tumor, normal tissue adjacent to tumor, and blood for each patient, and blood for each healthy control. Since the expression level of MDR1 in blood and tumor tissues was similar for patients, the final comparison between patients and controls was based on the data from tumor of patients and blood of controls. There was a significant association between MDR1 expression and C3435T polymorphism in patients ($P = 0.001$). Mean expression levels of CC, CT and TT genotypes in patients were 0.8350 ± 0.1249, 0.8127 ± 0.0850 and 0.6936 ± 0.1024, respectively (Figure 1). While the expression level of MDR1 in the TT genotype was significantly lower than in CC and CT genotypes ($P = 0.001$), this difference was not significant between CC and CT genotypes ($P = 0.806$).

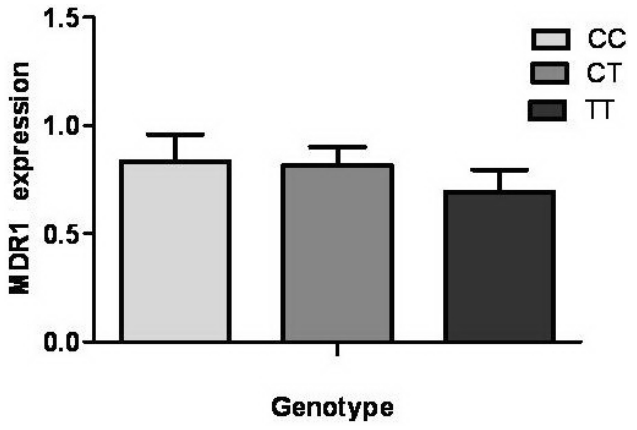

Figure 1. MDR1 expression in patients with different genotypes.

In the control group, mean expression level of MDR1 in individuals with the TT genotype was lower than in others, but this difference was not significant (P = 0.584). Mean expression levels of CC, CT and TT genotypes were 0.7539 ± 0.07802, 0.7177 ± 0.12212 and 0.7068 ± 0.1105, respectively.

Of 54 patients, 9 (17%) were in stage I, 30 (55%) in stage II and 15 (28%) in stage III. The genotype frequency was not significantly different regarding stage of disease (P > 0.05). Concerning tumor size, 27 (50%) patients had tumor 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. In 34 patients (63%), lymph nodes were involved. The diversity of the genotype did not correlate statistically with tumor size or lymph node involvement; although we must admit that the study sample was small.

Concerning hormone receptor status, 69% of patients were estrogen receptor positive and 62% progesterone receptor positive, and there was no statistical difference between the three genotypes (P > 0.05).

DISCUSSION

Resistance to drugs with different structure and function is one of the basic problems in the treatment of cancers. The overexpression of MDR1 gene is known to result in drug resistance in cancer cells. There are many proposed mechanisms, including gene amplification, which may change the expression level of a particular gene. Previously, we have shown that gene amplification was not responsible for the up-regulation of MRP1 gene, and we suggested that the presence of a particular polymorphism may alter the level of expression (Golalipour et al., 2007). Therefore, in this study we attempted to see whether or not the expression of MDR1 gene is changed by a particular genotype.

We first compared genotype frequency in breast cancer patients with healthy controls. We observed no difference in the frequency of C3435T polymorphism between breast cancer patients and healthy controls. Furthermore, we investigated the possible association between C3435T polymorphism and expression level. In contrast with healthy control, MDR1 expression level in breast cancer patients was significantly lower among patients with the TT genotype (P = 0.001) than CT and CC genotypes.

Chang and co-workers (2009) in a study on metastatic breast cancer patients found that polymorphisms of MDR1 C3435T did not correlate with drug resistance. However, they found that patients with the CT genotype had a significantly lower disease control rate compared to the CC genotype. They indicated that the CT genotype correlated with shorter overall survival.

A study conducted by Rodrigues et al. (2008) on 41 Brazilian women with stage II and III breast cancers showed that there was no statistically significant correlation between the diverse genotypes and the clinical and pathological data. However, patients with complete pathological response had only the polymorphic genotype (CT, TT) and not the wild-type genotype (Rodrigues et al., 2008). Other studies on different cancers have found no statistical difference in the frequency of this genotype between patients and controls (Urayama et al., 2002; Kurzawski et al., 2005). In contrast, in a study performed in Turkey, a difference in the frequency of C3435T polymorphism between breast cancer patients and healthy controls was observed. However, they did not find any significant difference between clinicopathologic parameters and this polymorphism in breast cancer patients (Turgut et al., 2007). The result of the present study also revealed that the TT allele correlated with lower expression of MDR1 gene, which could explain why the patients with this genotype responded better than CC and CT genotypes to drug.

It has been shown that the T allele is linked to weaker expression/activity of P-gp in cancer cells. In this situation, the CC genotype would be protected from cytotoxicity by more efficient drug efflux than in other genotypes (Jamroziak et al., 2004).

In conclusion, the results show that there was no association between MDR1 C3435T polymorphism and clinicopathologic characteristics such as tumor histologic grade, size and hormone receptor status, and lymph node involvement. However, there was an association between the MDR1 genotype and its expression. Further studies aimed at determining the possible relation between MDR1 expression and treatment outcome in this group of patients would be of value.

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