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# MRP1 polymorphisms (T2684C, C2007T, C2012T, and C2665T) are not associated with multidrug resistance in leukemic patients

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**ABSTRACT.** One of the main problems in treating cancer patients is that cancer cells can develop drug resistance. Resistance to multiple anticancer drugs, so called multidrug resistance (MDR), most likely involves a nonspecific mode of resistance, through drug-efflux transporters. One of the most extensively studied genes involved in MDR is multidrug resistance protein 1 (*MRP1*). We investigated a possible association between the expression level of *MRP1* and the occurrence of MDR in leukemic patients, and we tested the hypothesis that *MRP1* polymorphisms are predictive of MDR in patients with acute leukemia. The mRNA level of *MRP1* was determined in 111 patients with acute leukemia (including 52 patients with acute myeloid leukemia and 59 patients with acute lymphoblastic leukemia), by quantitative real-time PCR, to determine how it af-

 

fected the response to chemotherapy. We typed T2684C, C2007T, C2012T, and C2665T *MRP1* polymorphisms in 111 patients classified as either drug-resistant or drug-responsive. We found that high expression of *MRP1* was associated with the MDR phenotype in both acute myeloid leukemia and acute lymphoblastic leukemia patients. There was no effect of a particular genotype on the expression level of the *MRP1* gene. We found no significant differences in chemosensitivity among any of these genotypes.

**Key words:** Multidrug resistance protein; Multidrug resistance; Single nucleotide polymorphism

## INTRODUCTION

Drug resistance that develops in cancer cells often results from elevated expression of particular proteins, such as cell membrane transporters. This can result in an increased efflux of cytotoxic drugs from the cancer cells, thus lowering their intracellular concentration (Childs and Ling, 1994).

The human multidrug resistance protein 1 *(MRP1*) was discovered to induce drug resistance in a drug-selected lung cancer cell. It encodes a 190-kDa polytopic transmembrane protein comprising 1531 amino acids and belongs to subfamily C of the ATP-binding cassette (ABC)-transporters (Cole et al., 1992; Hipfner et al., 1997; Kuwano et al., 1999). In tumor cells, *MRP1* is able to confer resistance against a variety of natural product drugs, such as anthracyclines, vinca alkaloids and VP-16 (Zaman et al., 1993; Cole et al., 1994), and this resistance is associated with a reduced intra-cellular drug accumulation. Furthermore, the overexpression of *MRP1* in cancer patients has been reported by many researchers (Kuss et al., 1994; Nooter et al., 1995; Kruh et al., 1995; Norris et al., 1996; Chan et al., 1997; Golalipour et al., 2007, and Mahjoubi F and Akbari S, unpublished data). However, it is not clear why this phenomenon occurs in some patients with the same type of leukemia and not in others.

We have previously shown that neither chromosomal changes nor gene amplification in cancer cells is responsible for increased *MRP1* expression (Golalipour et al., 2007).

There have been reports of the influence of some single nucleotide polymorphisms on the structure and expression of the ABC transporter gene, consequently inducing drug resistance (Siddiqui et al., 2003). Therefore, we aimed to investigate the possible effect of some known *MRP1* polymorphisms on *MRP1* overexpression in patients with leukemia.

## MATERIAL AND METHODS

All patients were recruited through the same hematology clinic at the two major government hospitals in Tehran.

After obtaining informed consent, we collected peripheral blood from 111 patients

with acute myelogenous leukemia (AML) or acute lymphoblastic leukemia (ALL). Patients were divided into three groups: in complete remission (CR), in relapse and sensitive to drug (RS), and in relapse and resistant to drug (RR). Patients were considered to be in the complete remission group if established criteria were met, which included cellular marrow with <5% blast cells, neurophil count >1.5 × $10^9$/L, platelet count ≥100 × $10^9$/L, and no evidence of leukemia in other sites, observed over six months. Finally, the relapsed group consisted of patients with relapse within 6 months after remission. The resistant HL-60 cell line known to overexpress *MRP1* and peripheral blood of 30 healthy individuals were used as overexpressed and normal controls, respectively.

## Total RNA isolation and cDNA synthesis

RNA extraction and cDNA synthesis were performed as previously described (Golalipour et al., 2007).

## Real-time RT-PCR

The sequences of primers for assessment of *MRP1* expression were: forward 5'-CGGAAACCATCCACGACCCTAATC-3' and reverse 5'-ACCTCCTCATTCGCATCC ACCTGG-3'. The sequences of primers used for assessment of β-2-microglobulin expression were forward 5'-CTATCCAGCGTACTCCAAAG-3' and reverse 5'-GACAAGTCTGAATG CTCCAC-3'. For quantification of gene expression, we used the Lightcycler™ system (Roche Applied Sciences) and the Fast-Start DNA Master SYBR-Green I kit (Roche Applied Sciences). A standard Lightcycler polymerase chain reaction (PCR) program was established for each gene. Standard curves were generated using logarithmic regression. Thermal cycling consisted of an initial denaturation step at 95ºC for 10 min followed by a 3-step (primer annealing, amplification and quantification) program repeated for 50 cycles with temperature ramp rate of 20°C/s. The program was 95°C for 0 s, 64°C for 10 s and 72°C for 40 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, 72°C for 10 s and 95°C for 0 s with a linear temperature transition rate of 0.1°C/s with continuous fluorescence acquisition. Finally, a cooling program cooled the reaction mixture to 40ºC. The β-2-microglobulin PCR program was the same except that the annealing temperature in the second segment was 50°C for 10 s. To ascertain that fluorescence signals were associated with specific products, 1.5% agarose gel electrophoresis was performed to check for the absence of nonspecific bands.

An external standard curve for *MRP1* and β-2-microglobulin was generated from serial dilution of mRNA of each gene. The standard curve was constructed from the plot of crossing points against the copy number of serially diluted standard samples. For each sample, the amounts of *MRP1* and the housekeeping gene were measured. Finally, the relative copy number was calculated by the ratio of *MRP1* to β-2-microglobulin copy number in each sample.

Statistical calculations and tests were performed using the SPSS 13.0 software (SPSS, Inc., Chicago, USA). Normality of data was tested using the Shapiro-Wilk normality test. Differences between groups were analyzed by one-way ANOVA and the limit of statistical significance was defined as $P < 0.05$.

## DNA extraction and PCR

Genomic DNA was isolated from enriched peripheral blood blasts of patients using the Nucleose DNA Prep kit (Stratagene, La Jolla, USA) and TRI-ZOL reagent (Gibco BRL, Gaithersburg, USA), respectively. Pelleted DNA was resuspended in extragene E solution.

The primer pairs used to amplify genomic DNA were designed based on the genomic sequence of *MRP1* reported in GenBank. PCR was performed using the thermal profile described below. The standard PCR was carried out in a 25-µL volume containing 200 ng genomic DNA. PCR was performed using the primers below:

For exon 16, forward primer was 5'-CAGGTGTGTTGTGTGTCGTTTC-3' and reverse primer was 5'-TCTGGCACAGGGACAGTC-3'.

For exon 20, forward primer was 5'-GCATCTGCCTCATATAACCC-3' and reverse primer was 5'-GGCAGGGTCCTTAGGTTG-3'.

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 program was as follows for exons 16 and 20: The initial denaturation step at 94°C for 5 min, followed by the amplification program 30 cycles of: 94°C for 30 s; 52°C for 35 s, and 72°C for 35 s, followed by one cycle of 72°C for 10 min.

The PCR products were used for single-strand conformation polymorphism (SSCP) and/or sequencing, using an automated sequencer (moel377, ABI).

## RESULTS

### mRNA expression of *MRP1* in CR compared to RS and RR groups

A statistically significant ($P < 0.05$) difference between *MRP1* expression level between CR and RS/RR groups was observed in both AML and ALL patients. Mean expression in the CR group (0.032 ± 0.031 in AML, and 0.3104 ± 0.4791 in ALL patients) was significantly lower than mean expression in the RS (0.422 ± 0.297 in AML and 0.55 ± 0.1638 in ALL patients) and RR (0.619 ± 0.284 in AML and 0.7598 ± 0.21 in ALL patients) groups (Table 1 and Figure 1).

**Table 1.** Number of patients in complete remission (CR), relapse and sensitive to drug (RS), and relapse and resistant to drug (RR) groups, with the range of *MRP1* expression.

| Groups | Patients (N = 111) | *MRP1* expression level |
|---|---|---|
| CR | 53 | $1.00\ E^{-11}$-$1.00\ E^{-02}$ |
| RS | 34 | $1.00\ E^{-10}$-$1.00\ E^{+05}$ |
| RR | 24 | $1.00\ E^{-01}$-$1.00\ E^{+16}$ |

### Genotyping at *MRP1* 2684, 2007, 2012, and 2665

To evaluate *MRP1* involvement in drug resistance in leukemia patients and to explore its pharmacogenomics, we typed the 4 polymorphisms in 111 patients classified as either drug-resistant or drug-responsive.

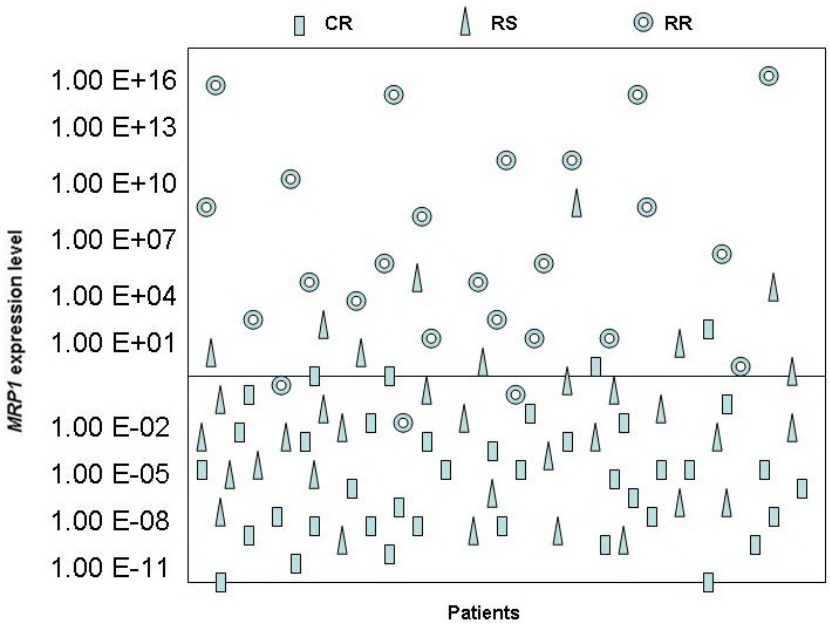


**Figure 1.** Expression of *MRP1* mRNA in peripheral blood of patients. Each symbol represents one patient. CR = complete remission; RS = relapse and sensitive to drug; RR = relapse and resistant to drug.

In order to identify genetic variations, PCR-SSCP analysis of all fragments was carried out with DNA isolated from blood of 111 patients. Since there was no fragment variations by SSCP among any of these patients, PCR products were sequenced, and comparison was made with the published *MRP1* sequences. GenBank accession numbers AC003026 and U91318, which included the complete genomic sequence of exon 12 to exon 31, were used as wild-type sequences. The sequences were analyzed with the use of the Sequencer Navigator program. The sequencing procedure resulted in the identification of no mutation.

## DISCUSSION

*MRP1* gene is an important candidate gene potentially influencing the response to anticancer drugs (Chan et al., 1997; Golalipour et al., 2007). We have previously shown that in a group of AML patients, overexpression of *MRP1* seemed to contribute to the multidrug resistance phenotype (Golalipour et al., 2007, and Mahjoubi F and Akbari S, unpublished data). Comparing *MRP1* gene copy number in all those patients with a high level of *MRP1* mRNA with normal healthy individuals, no sign of gene amplification could be seen in any of those cases (Golalipour et al., 2007). Since mutations affecting *MRP1* expression and/or *MRP1* transport activity can be expected to influence the pharmacokinetic properties of chemotherapeutic drugs and consequently their desired and adverse effects, we attempted to examine whether the genotype of *MRP1* correlates with the expression status.

In this study enrolling a larger number of patients, we were able to confirm that the *MRP1* gene could be responsible for inducing drug resistance in both AML and ALL patients. However, our data suggest that none of the common *MRP1* polymorphisms (T2684C, C2007T, C2012T, and C2665T) are associated with multidrug resistance in these groups of patients.

In conclusion, we presented evidence based on quantitative real-time PCR that up-regulation of *MRP1* gene may induce multidrug resistance in leukemic patients, but none of the known polymorphisms seemed to have effect on the expression level of this gene in our patient group. It should be noted that patients with drug resistance and drug sensitive leukemia selected for this study were seen at only two referral centers and thus may not represent the whole Iranian population.

In the future, an investigation with a larger number of cases should be addressed.

## ACKNOWLEDGMENTS

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