RESEARCH COMMUNICATION

Multidrug Resistance-Associated Protein 1 Predicts Relapse in Iranian Childhood Acute Lymphoblastic Leukemia

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Abstract

Multidrug resistance (MDR) is a main cause of failure in the chemotherapeutic treatment of malignant disorders. One of the well-known genes responsible for drug resistance encodes the multidrug resistance-associated protein (MRP1). The association of MRP1 with clinical drug resistance has not systematically been investigated in Iranian pediatric leukemia patients. We therefore applied real-time RT-PCR technology to study the association between the *MRP1* gene and MDR phenotype in Iranian pediatric leukemia patients. We found that overexpression of MRP1 occurred in most Iranian pediatric leukemia patients at relapse. However, no relation between *MRP1* mRNA levels and other clinical characteristics, including cytogenetic subgroups and FAB subtypes, was found.

Keywords: MDR - multidrug resistance-associated protein (MRP1) gene - leukemia - Real-time RT-PCR

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Introduction

Multidrug resistance is a phenomenon whereby tumor cells in vitro that have been exposed to one cytotoxic agent develop cross-resistance to a range of structurally and functionally unrelated compounds (Simon and Schindler, 1994). The drug resistance that develops in cancer cells often results from elevated expression of particular proteins, such as cell-membrane transporters, which can result in an increased efflux of the cytotoxic drugs from the cancer cells, thus lowering their intracellular concentration (Ambudkar et al., 1999; Gottesman et al., 2002). In addition, MDR occurs intrinsically in some cancers without previous exposure to chemotherapy agents (Fardel, 1996). The cytotoxic drugs that are most frequently associated with MDR are hydrophobic, amphipathic natural products, such as the taxanes (paclitaxel, docetaxel) vinca alkaloids (vinorelbine, vincristine, vinblastine), anthracyclines (doxorubicin, daunorubicin, epirubicin), epipodophyllotoxins (etoposide, teniposide), topotecan, dactinomycin and mitomycin C (Ambudkar et al., 1999; Krishna, 2000).

A number of different mechanisms can mediate the development of MDR, including increased drug efflux from the cell by adenosine triphosphate (ATP)-dependent transporters, decreased drug uptake into the cell, activation of detoxifying enzymes and defective apoptotic pathways (Gottesman, 2002). One of these is the overexpression of adenosine triphosphate (ATP)-dependent membrane proteins that function as drug-efflux pumps (Cole et al., 1992).

ABC (ATP-binding cassette) proteins form one of the largest protein families and members of this family are found in all living organisms from microbes to humans. The wild-spread presence of these proteins with a relatively conserved structure and function suggests a fundamental role (Endicott and Ling, 1989; Higgins, 1992; Gottesman and Pastan, 1993; Ueda, 1997; Klein, 1999; Gottesman, 2002).

Drug resistance markers are often predictive of treatment response and outcome in patients with acute myeloid leukemia. The immunologic detection of drug efflux pumps such as P-glycoprotein (Pgp) and multidrug resistance associated protein 1 (MRP1) correlate with functional assays of drug resistance and these drug accumulation defects also appear operable in ALL (Thomas, 2000).

The multidrug resistance protein MRP1 is a member of the superfamily of ATP-binding cassette (ABC) transporters to which P-glycoprotein (P-gp), encoded by the MDR1 gene, also belongs. MRP1, a 190-kd protein, is encoded by the MRP1 gene located on chromosome 16p13. Overexpression of MRP1 results in resistance to different classes of chemotherapeutic agents. During the past few years, additional MRP1 homologues were identified (MRP2-MRP9). However, the role of these MRP1 isoforms in MDR is not well defined yet (Taniguchi et al., 1996; Kool et al., 1997; Kool et al., 1999). The human multidrug resistance-associated protein ABCC1/MRP1 was discovered as the basis of a non-p-glycoprotein associated multidrug resistance (Cole et al., 1992.). This protein is responsible for the transport of a remarkable

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range of drugs as it confers a cancer cell resistance to hydrophobic drugs. However, further functional characterization revealed that the preferred substrates for MRP1 are organic anions, including drugs conjugated to glutathione (GSH), glucuronate, or sulfate (Strautnieks et al., 1998; Borst et al., 2000). MRP1 also mediates the cotransport of unconjugated amphiphilic anions, together with free GSH. The major physiological function of ABCC1 is presumably leukoteriene C4 Transport (Borst et al., 2000). In addition to protecting cells within the body against drugs, environmental toxins and heavy metals, MRP1 has also been implicated to contribute to cellular antioxidative defense system and inflammation (Leslie, 2001; Hipfner, 1999). Haematological neoplasms are usually primarily sensitive to chemotherapy, but the relapse rate is still high. Drug resistance is therefore a major cause of chemotherapeutic and patient death in haemato-oncology (Marie and Legrand, 2003). Cellular mechanisms of drug resistance have been increasingly better defined for patients with acute leukemia and other hematologic malignancies (Sonneveid, 2000).

In this study, our goal was to develop an accurate and reproducible method to enable the quantification of MRP1 expression. We have applied quantitative real-time RT PCR to the analysis of gene expression in peripheral blood obtained from pediatric patients. We aimed to investigate the expression of MRP1 in ALL pediatric patients at transcription level and examined whether the mRNA level of MRP1 correlated with clinical outcome and could be used as a prognosis factor.

Materials and Methods

Patients and samples

After obtaining informed consent we collected peripheral blood (PB) from 42 pediatric patients with acute lymphoblastic leukemia (ALL). For each patient several clinical and pathological characteristics including age, sex, leukemia FAB subtype and karyotype were considered. Patients were divided into two groups, at complete remission (CR) and at relapse (Relapsed). Patients were considered in complete remission group if established criteria wich include cellular marrow with < 5% blast cell, neurophill count > 1.5×10^9/L, platelet count ≥ 100×10^9/L and no evidence of leukemia in other sites observed in six months. Finally, Relapsed group consist of patients with relapse within 6 months after remission. The resistant HL-60 cell line known to overexpress MRP1 and peripheral blood of 10 healthy individuals were used, respectively, as overexpression and normal controls.

Total RNA isolation and cDNA synthesis

White blood cells from PB samples were separated by red blood cell lysis buffer (RBCL) and then suspended in PBS. Total RNA was isolated from white blood cells using a High Pure RNA Isolation kit (Roche kit). Its concentration was determined by spectrophotometery and its purity was assessed by an OD260/OD280 absorption ratio greater than 1.7. RNA was stored at -70 °C until use. cDNA was then made using a standard amount of RNA with a commercially available kit (Fermantas Revert AID First Strand cDNA Synthesis kit .(The resulting cDNA was diluted in DEPC-treated pure water and used in real-time RT-PCR reaction.

Real-time RT- PCR

The sequences of primers for assessment of MRP1 expression were as followes: forward 5-CGGAAACCATCCACGACCCTAATC-3 and reverse 5-ACCTCCTCATTCGCATCCACCTGG-3. The sequences of used primers for assessment of β_2M (β-2-microglobulin) expression were forward 5-CTATCCAGCGTACTCCAAAG-3 and reverse 5-GACAAGTCTGAATGCTCCAC-3. The primers were designed using primer premier 5.0 software and synthesized by MWG. All primer sequences were checked by ASePCR program (http:// genome. Ewha. Ac. Kr/ Ecgene/) for absence of any false priming sites. The length of the amplicon was 294 bp for MRP1 and 147 bp for β_2M.

For quantification of gene expression, we used the lightcyclerTMsystem (Roche Applied Sciences) and the Fast-Start DNA Master SYBR-Green I kit (Roche Applied Sciences). A standard Light cycler PCR program was established for each gene. Standard curves were generated using logarithmic regression. Thermal cycling consisted of an initial denaturation step at 95 °C for 10 minutes followed by a three step (primer annealing, amplification and quantification) program repeated for 50 cycles with temperature ramp rate of 20 °C/sec. The program was 95 °C for 0 °C, 64 °C for 10 sec and 72 °C for 40 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, 72 °C for 10sec 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. The β-2-microglobulin PCR program was the same except that the annealing temperature in the second segment was 50 °C for 10 sec. To ascertain that fluorescence signals were associated with specific products, samples were checked by 1.5% agarose gel electrophoresis for the absence of nonspecific bands.

Data analysis

Raw data were analyzed using version 3.03 of the light cycler software. Crossing point defined as cycle number at which the fit line in the log-linear portion of plot intersected the threshold level. An external standard curve for MRP1 and β_2M 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_2M copy number in each sample.

Statistical calculation and tests were performed using SPSS 13.0 software. Normality of data was tested using the Shapiro-Wilk normality test. Differences between groups were analyzed by One-way ANOVA test. The limit for conclusion of statistical significance was defined as $p \leq 0.05$.

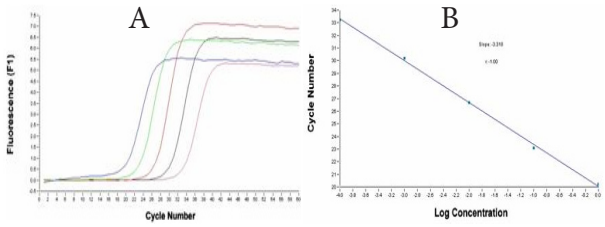

Figure 1. Amplification (A) and Calibration Curve (B) of Serial Dilution of MRP1. The calibration curve showed a good correlation between transcript copy number and threshold cycle (r = -1.00). The calibration curve slope (-3.318) is near to optimum curve slope and represent high PCR efficiency (0.98)

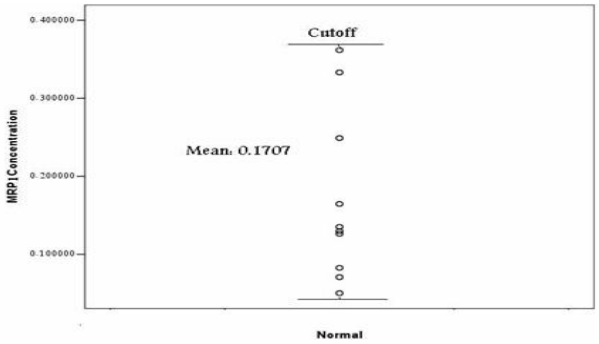

Figure 2. Expression of MRP1 mRNA in Peripheral Blood of Healthy Individuals. The concentration of MRP1 was expressed as the ratio of MRP1 to β_2M concentration (each circle represents a sample). According to MRP1 expression in healthy individual cutoff defined as mean±2SD

Results

Real time RT-PCR validation

RT-PCR products showed only one band of expected size upon electrophoresis and only a single melting temperature peak was observed for each reaction, suggesting non specific amplification did not occur. To establish optimal conditions for quantitative analysis, a calibration curve was prepared using serial dilution of cDNA (Figure 1A). The calibration curve showed a good correlation between transcript copy number and threshold cycle (r = -1.00). To ensure high PCR efficiency, we tried to reach a calibration curve slope near to -3.322 (optimum curve slope) and y-intercepts close to Ct value of negative control (Figure 1B).

Determination of cutoff values

To obtain a cutoff value to discriminate between normal and upregulated states of samples, we examined the expression of MRP1 mRNA in cell line and healthy blood by real time RT-PCR. The final results were expressed as the ratio of MRP1 to β_2M copy number in each sample. MRP1 expression mean was 0.1707 in healthy samples (Figure 2). On the basis of MRP1 expression value in healthy samples, we defined the cutoff for MRP1 as 0.219 (Mean±2SD). Therefore all values above this cutoff were assumed to represent overexpression.

Expression of MRP1 and clinical outcomes

The expression of MRP1 was analyzed according to clinical response to drugs. A total of 42 patients with de novo ALL were studied with ages ranging between 2 and 16 years. A statistically significant ($p<0.01$) increased in MRP1 expression was observed when samples from relapsed groups of ALL patients were compared with samples from patients with CR. MRP1 overxpression was observed in many ALL cases at relapse stage (Figure 3).

Table 1. MRP1 Expression Level in ALL Samples

Phase (i)	Phase (j)	Mean difference (i-j)	Standard error	P-value
Normal	CR	0.3104	0.2395	0.203
	Relapse	0.7598	0.5819	0.005

Expression of MRP1 in Normal group (phase i) compared to CR and Relapse groups (phase j) mean difference between normal and CR is not significant (P-value = 0.203) but normal and Relapse groups MRP1 level is significantly different (P-value = 0.005)

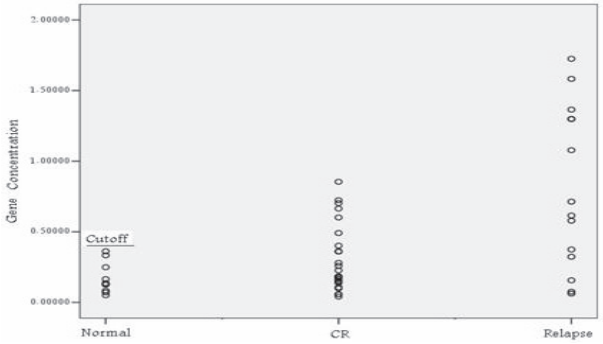

Figure 3. The Expression of MRP1 mRNA at CR and Relapse in ALL is Shown (each circle represents a sample). Distribution of copy numbers in CR and healthy individuals is different from relapsed patients.

Mean expression in the CR group (0.3104±0.4791) was significantly lower than mean expression in the relapse (0.7598±1.1638) groups. Table 1 shows mean difference among normal and ALL samples and P-value of each. Significant difference observed between normal and relapse groups (P-value = 0.005) but mean difference between normal and CR groups is not significant (P-value = 0.203). lack of MRP1 expression in patients in the CR group was not the cause of mean difference between and relapse patients because the expression upper-limit in CR patients was near to the lower limit of relapse patients (P-value = 0.059).

Correlation of MRP1 expression with other clinical characteristics

The correlation of other well-known variables such as gender, age, WBC count at diagnosis. CD34 expression, FAB subtype and cytogenetic finding with MRP1 mRNA concentration was also analyzed. The patients were assigned to different genetic subgroups according to the chromosomal abnormalities identified in the leukemic cells and the finding were described according to the international nomenclature. There was no clear relationship between MRP1 expression and gender, age, WBC count and CD34 expression, cytogenetic risk group and FAB subtype.

Discussion

Drug resistance is an important factor that limits

the successful treatment of acute leukemia. One of the most important causes of treatment failure in childhood ALL is the emergence of multidrug resistance. Various mechanisms can give rise to clinical drug resistance, but best studied is the overexpression of transmembrane transport proteins. It is assumed that pharmacogenomic analysis of other ABC transporters, such as the MRP family, will reveal similarly important roles in handling many different drugs. In the present article, we focused on the clinical relevance and prognostic significance of MRP1 efflux pumps in childhood ALL.

The knowledge that expression of a drug-resistance gene, such as MRP1, can result in clinically significant drug resistance, has led to the idea that drug-resistance genes can be used as selected markers in gene therapy (Gottesman, 1995). One of the barriers to successful gene therapy is the inefficiency of transfer of therapeutic genes into target cells. Use of cancer drug-resistance genes as linked markers to allow selection of cells to which therapeutic genes have been transferred has been suggested as a mean to improve efficiency of gene therapy (Gottesman et al., 2000).

This is the first study describing the association between MRP1 expression and clinical outcome in Iranian childhood ALL. We found that relapsed patients had a higher expression of MRP1 gene, than patients who remained in complete remission. Therefore, in Iranian children, high expression of MRP1 is possibly associated with unfavorable outcome.

Childhood ALL originates from the more committed lymphoid progenitor cell. The level of the transforming event is not strictly connected to a certain age group because there is an overlapping spectrum of the course of the disease between children and adults with ALL (Sabine, 2005). The association between maturation stage and ABC transporters has already been observed in normal hematopoiesis. The expression and functional activity of P-glycoprotein and breast cancer resistance protein were found to be higher in normal hematopoietic stem cells with an immature phenotype. In human normal bone marrow cells, a higher MRP expression was observed compared with ALL cells. In contrast, healthy normal B and T lymphocytes were found to have a lower MRP expression than ALL cells (Wijnholds et al., 2000; Yoshida, 2001; Bunting, 2002; Ros, 2003)

Many studies have addressed the role of a number of MRPs in ALL. No association between expression levels and clinical outcome was observed for mrp1 in childhood ALL in some of these studies (Sauerbrey et al., 2002; Plasschaert et al., 2003).

Other studies, like ours, contradicted these findings (Sabine et al., 2005). This discrepancy with our study for MRP1 might be caused by the limited number of cases with childhood ALL patients and an unfavorable outcome studied in their study. By including more relapsed patients in our study population, we were able to describe and detect a possible influence of MRP1 expression on prognosis. A remarkable finding in our study is that MRP1 have a higher expression in relapsed pediatric ALL.

However, since we selected more children with an unfavorable outcome, our observations about the prognostic significance of MRP1 expression may possibly not be generalized to the usual ALL population. In addition, we found no differences in important prognostic factors, such as WBC counts, age and unfavorable chromosomal rearrangements among our cases.

The profile with high expression of multiple MRPs indicates that it will be of limited value to counteract the effects of a single MRP with regard to drug resistance. A more general approach is required, especially because the different MRPs have strongly overlapping substrate specificities and are capable of transporting many different chemotherapeutic drugs. Thus far, no clinical trials in leukemia are known that make use of specific MRP inhibitors. This study underscores the importance of the design of a more general MRP inhibitor that can modulate all MRP transporters simultaneously (Sabine et al., 2005).

Recently, the results of microarray analysis were reported in patients with childhood ALL in relation to drug sensitivity and clinical outcome Holleman et al.. The gene expression patterns were investigated in relation to in vitro cellular drug resistance to prednisone, vincristine, asparaginase and daunorubicin. Early recognition of a profile with high MRP expression could identify patients with an increased risk for relapse that could benefit from treatment adaptations based on this knowledge (Gottesman, 1995). In summary, the present study shows that a subset of ALL patients with high MRP1 expression has an unfavorable prognosis independently of all other prognostic factors.

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