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Multi-Drug Resistance (*MDR1*) Gene Expression in de novo Acute Leukemia Cells: Correlations with CD Surface Markers and Treatment Outcome

One important mechanism of drug resistance in acute leukemia is the over-expression of the multi-drug resistance (*MDR1*) gene that encodes a 170-kDa membrane protein called P-glycoprotein. To estimate the incidence and role of *MDR1* gene expression in patients with acute leukemia, we investigated the expression of *MDR1* by using the RT-PCR method in blast cells from 40 cases of de novo acute leukemia. We found a high frequency of *MDR1* gene expression: 10 out of 20 with de novo acute myeloid leukemia (AML), 8 out of 17 with de novo acute lymphoblastic leukemia (ALL), and none of the 3 with de novo acute mixed leukemia, were *MDR1* mRNA-positive. No correlation between cluster designation (CD) surface markers (CD19, CD7, CD13, CD33, CD34, CD14, HLA-DR) and *MDR1* gene expression in AML was found. The complete remission rate was correlated with *MDR1* gene expression. Among 40 evaluable patients examined, 17% (3 of 18) with *MDR1* mRNA-positive reached complete remission versus 77% (17 of 22) with *MDR1* mRNA-negative (p=0.044). These results suggest that *MDR1* gene expression can be used as a prognostic factor and may be helpful in determining chemotherapeutic protocol for patients with acute leukemia.

Key Words : *Genes, MDR1; P-glycoprotein; Leukemia, acute; Polymerase chain reaction; CD marker*

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INTRODUCTION

Drug resistance remains a problem in cancer chemotherapy. A particularly important type of tumor drug resistance, multi-drug resistance (*MDR1*), is manifested by cross resistance to a number of structurally and functionally unrelated lipophilic drugs. Several mechanisms of drug resistance exist, but one important mechanism is the over expression of the *MDR1* gene that encodes a 170-kDa integral membrane protein named P-glycoprotein (P-gp) (1-3). P-gp acts as a drug efflux pump that actively extrudes a wide variety of drugs out of the cell, thereby preventing them from accomplishing their intracellular action (4, 5). *MDR1* gene expression is frequently observed in different human tumors, both untreated and treated with chemotherapeutic drugs, as well as in some normal tissues with excretory function, such as kidney, adrenal gland, liver and colon (6-8), where it most likely functions as a transport protein.

High expression of the *MDR1* gene has been reported in several human cancers (7, 8) as well as acute leukemias (9, 10), though initial studies showed a low incidence of *MDR1* phenotype (11, 12). The various incidences of *MDR1* gene expression might be partially due to the different methods used in these studies. Expression of the *MDR1* gene was found in approximately 50% of patients with de novo untreated acute myeloid leukemia (AML) (10, 13). Phenotypically, cluster designation (CD) 7 and CD34 were the only surface markers that were significantly associated with *MDR1* gene expression, *MDR1* mRNA was frequently expressed in $CD7^+$ AML and $CD7^+CD4^-CD8^-$ ALL (14). High *MDR1* levels usually indicate unfavourable outcome to conventional chemotherapy (15-17). In this study, we examined the *MDR1* gene expression in 43 samples from 40 patients with de novo acute leukemia together with the immunophenotype by using qualitative reversed transcriptase-polymerase chain reaction (RT-PCR) and direct-immunofluorescence staining measured with flow cytometry. The aims of the present study were to determine the incidence of

detectable *MDR1* mRNA and its relations to the CD surface marker and clinical outcome in acute leukemias.

MATERIALS AND METHODS

Patients

Forty-three samples from 40 patients with de novo acute leukemia were obtained between July 1996 and July 1997 in Kyungpook National University Hospital, Taegu, Korea. The patients involved in this study were classified as follows: 20 with de novo AML [according to the French-American-British (FAB) classification, 1 M0, 9 M2, 4 M3, 1 M4, 4 M5, 1 M6], 17 with de novo acute lymphoblastic leukemia (ALL) (6 L1, 10 L2, 1 L3), 3 with mixed leukemia (1 pre-B and monocytic, 2 myeloid and T-lymphoid). The mean age of the patients was 25.6 ± 20.6 (mean±standard deviation, SD) with a range of 2-65 years. The mean white blood cell count was $47.9 \pm 66.2 \times 10^9$/L (mean±SD). The specimens were obtained from bone marrow or peripheral blood with $69.7 \pm 21.9\%$ (mean±SD) of blast cells. Mononuclear cells were isolated from bone marrow or peripheral blood by centrifugation on Histopaque-1077 (Sigma Co., USA), frozen at -70℃ in RPMI 1640 medium (Gibco BRL, USA), was supplemented with 10% fetal bovine serum and 10% dimethylsulfoxide. Immunophenotyping of each sample was done with the Acute Leukemia Phenotyping Kit (Becton Dickinson Co., USA), which contained a series of monoclonal antibodies which react directly to myeloid and lymphoid markers: CD10 (CALLA), CD19 (Leu-12), CD20 (Leu-16), CD5 (Leu-1), CD3 (Leu-4), CD22 (Leu-14), CD7 (Leu-9), CD33 (Leu-M9), Anti-HLA-DR, CD13 (Leu-M7), control IgG2a and control IgG1. CD34 (Becton Dickinson Co., USA), and CD14 (mouse anti-human CD14) were purchased from Becton Dickinson Immunocytometry Systems (USA) and Serotec, U.K., respectively. The cells were evaluated using FACScan and the Lysis II software program (Becton Dickinson Co., USA). The patients were treated with Adriamycin+Ara-C or Idarubicin+Arc-C for AML and Adriamycin+ Vincristine for ALL as induction regimens. Clinical outcome was studied at least 6 months after induction therapy in all patients. Complete remission (CR) was defined as normal hematopoiesis in the bone marrow with less than 5% blast cells and a parallel return of the peripheral blood counts to normal after one course of remission induction therapy.

Cell culture

Cell line KG-1 (as a *MDR1* positive control) and CCRF-CEM (as a *MDR1* negative control) derived from human leukemia were purchased from Korean Cell Line Bank (KCLB). The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and kept at 37℃ in a humidified atmosphere containing 5% CO_2.

RNA extraction and reverse transcription

The cell line and patients' mononuclear cells were washed twice in ice-cooled PBS and RNA was extracted using the TRI reagent-phenol-chloroform method. RNA yield and purity were determined spectrophotometrically at 260/280 nm (Ultrospec 3000, Pharmacia Biotech, U.K.). The cDNA was synthesized from 0.5-1 μg of total celluar RNA in 20 μL of a solution containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 1 mM of each dNTP, 2.5 μM oligo d(T)16 or random hexamers, 20 U RNAse inhibitor and 50 U murine leukemia virus (MuLV) reverse transcriptase. The reverse transcription mixture was incubated at 42℃ for 15 min, followed by 99℃ for 5 min. The cDNA was stored at -20℃ until use.

Oligonucleotide primers

The following oligonucleotides were used as primers (18) for PCR amplification of specific *MDR1* and beta-actin fragment, giving rise to 242 bp and 838 bp PCR products, respectively.

MDR1 sense 5' AAG CTT AGT ACC AAA GAG GCT CTG 3' (2041-2064)
antisense 5' GGC TAG AAA CAA TAG TGA AAA CAA 3' (2260-2282)
β-actin sense 5' ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG 3' (294-325)
antisense 5' CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC 3' (1100-1131)

PCR amplification

PCR amplification of cDNA was performed in 40 μL of a solution containing 1 mM $MgCl_2$, 40 mM KCl, 8 mM Tris (pH 8.3), 8 mol for each specific primers, 8 μL of RT product and 2 U AmpliTaq DNA polymerase with a DNA thermal cycler (Perkin-Elmer, 9600, Roche Diagnostic System, USA) under the following conditions: the samples were first denatured at 95℃ for 2 min and followed by 95℃, 1 min denaturation, annealing of the primers at 58℃, 1 min and elongation at 72℃, 1 min

for 35 cycles. The final step was then kept at 72℃ for 7 min. The products were electrophoresed in 1 x Tris-borate electrophoresis buffer with 2 mM EDTA on 2% agarose gels containing 0.5 μg/mL ethidium bromide.

Statistical analysis

The significant differences between groups were analyzed using chi-square test, except for the differences in the percentages of blast cells in bone marrow and peripheral blood, which were tested with Student's t test. The Fischer exact test was used when the expected frequency in at least one cell is less than 5. Differences were considered significant if $p<0.05$.

RESULTS

Incidence of *MDR1* gene expression in leukemic cells

In 40 patients with acute leukemia at diagnosis, *MDR1* mRNA levels in blast cells obtained from bone marrow aspirates or peripheral blood were determined by the RT-PCR method. The 242 bp size of *MDR1* gene RT-PCR products was in agreement with that predicted by the nucleotide sequences of the PCR primers (Fig. 1). Ten out of 20 (50%) de novo AML cases, 8 out of 17 (47%) de novo ALL and none of the 3 de novo mixed leukemia cases had detectable *MDR1* mRNA level (Table 1).

Table 1. Multi-drug resistance (*MDR1*) gene expression in de novo acute leukemia patients

Type	No. of cases	No. of *MDR1* (+) (%)
AML*	20	10 (50%)
ALL†	17	8 (47%)
Mixed AL‡	3	0 (0%)

*AML, acute myeloid leukemia
†ALL, acute lymphoblastic leukemia
‡AL, acute leukemia

Table 2. Multi-drug resistance (*MDR1*) gene expression in acute myeloid leukemia patients, classified according to FAB* group

FAB type	No. of case	No. of *MDR1* (+) (%)
M0	1	0 (0%)
M2	9	5 (56%)
M3	4	1 (25%)
M4	1	0 (0%)
M5	4	4 (100%)
M6	1	0 (0%)

*FAB, French-American-British

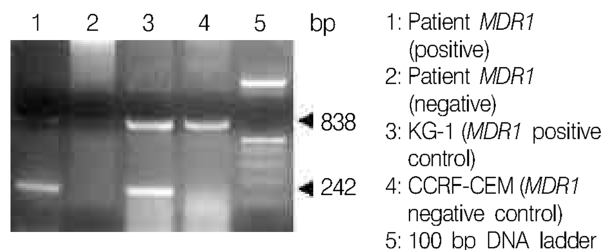

Fig. 1. RT-PCR for *MDR1* mRNA in leukemia cells were determined. β-actin (arrowed at 838 bp) of each specimen was used as a control to compare with its corresponding *MDR1* gene (arrowed at 242 bp).

Relationship between clinical data and *MDR1* gene expression

The *MDR1* expression of the de novo AML cells, classified according to FAB group, are shown in Table 2. The *MDR1* gene expression was higher in AML-M5 (100 %) and in AML-M2 (56%). But no statistical relationship between *MDR1* expression and the FAB classification was found. Patients older than 40 years, sex, white blood cell counts and percentage of blasts at diagnosis were not significantly different between *MDR1* mRNA-positive and *MDR1* mRNA-negative patients (Table 3). Serial determinations of *MDR1* levels in the three patients showed that *MDR1* mRNA levels became positive in two negative cases after treatment. One positive patient remained positive for *MDR1* expression after treatment (Table 4). In terms of immunophenotype of AML, no obvious correlation of *MDR1* gene expression to the CD surface markers (CD19, CD7, CD13, CD33, CD34, CD14, HLA-DR) was found (Table 5).

Correlation of *MDR1* gene expression and the outcome of chemotherapy

The analysis of the relationship between *MDR1* gene expression and the outcome of chemotherapy over 6

Table 3. Clinical data and multi-drug resistance (*MDR1*) gene expression in patients with acue leukemia

Characteristics	*MDR1* gene (+)	*MDR1* gene (−)	p value
Age ≥ 40 yr	4/13*	10/13	0.331
< 40 yr	14/27	12/27	0.933
Sex male	10/20	9/20	0.928
female	8/20	13/20	0.538
WBC count ($\times10^9$/L)	41.9±56.9†	52.8±92.3	0.665
Blast %	68.2±21.6	71.2±22.9	0.675

*Number of positive samples/number of corresponding characteristics examined
†Mean±standard deviation

Table 4. Multi-drug resistance (*MDR1*) gene expression before and after chemotherapy in three acute leukemia patients

Case	Age/sex	FAB* type	*MDR1* gene	
			before	after
1	4/F	ALL†-L1	−	+
2	7/F	ALL-L2	+	+
3	32/M	AML‡-M0	−	+

*FAB, French-American-British
†ALL, acute lymphoblastic leukemia
‡AML, acute myeloid leukemia

Table 5. Multi-drug resistance (*MDR1*) gene expression in relation to immunophenotype of acute myeloid leukemia patients

CD* marker		*MDR1* gene (+)	p value
CD19	+	3/5†	1.000
	−	7/15	
CD7	+	1/3	1.000
	−	9/17	
CD13	+	9/18	1.000
	−	1/2	
CD33	+	10/19	1.000
	−	0/1	
CD34	+	7/9	0.260
	−	3/11	
CD14	+	4/5	0.431
	−	6/15	
HLA-DR	+	9/15	0.633
	−	1/5	

*CD, cluster designation
†Number of positive samples/number of samples examined

Table 6. Multi-drug resistance (*MDR1*) gene expression and clinical outcome in patients with acute leukemia

MDR1 gene	CR* rate (%)	Persistence (%)	Expired (%)
+	3/18† (17)	9/18 (50)	6/18 (33)
−	17/22 (77)	3/22 (14)	2/22 (9)

*CR, complete remission
†Number of samples with corresponding outcome/number of samples examined
p values: CR group, p=0.044; Persistent group, p=0.135; Expired group, p=0.245

months after induction was restricted to those who were treated with at least one MDR-related drug. The association of *MDR1* gene expression with CR rates is shown in Table 6. The CR rate was significantly higher for patients with no detectable *MDR1* expression than those with detectable expression (77% versus 17%; p=0.044). For the rates of patients with persistent leukemia and death in aplasia, they were slightly higher in *MDR1* RNA-positive group than in *MDR1* RNA-negative group, but there was no significant difference (p=0.135 and p=0.245, respectively).

DISCUSSION

The findings described here demonstrate that expression of the *MDR1* gene is common in acute leukemia cells at diagnosis. The high incidence of *MDR1* gene expression in our cases with AML (50%) is consistent with some others (13-15, 19-21) ranged from 39-71%. It differs from those of Ito et al. (12) and Goldstein et al. (8) which reported a very low frequency of *MDR1* gene expression. For de novo ALL cells, the expression of *MDR1* here (47%) was higher than that in published reports (20, 21) but similar to the result of Gruber et al. (10). None of the 3 acute mixed leukemia at diagnosis were *MDR1* RNA-positive in this study. No other references could be found which may contribute to the new concept of mixed leukemia. The variations among studies may reflect differences in patient population, differences between various techniques in sensitivity and specificity, or differences in using positive control.

We did not find correlations between the levels of *MDR1* expression and clinical data, such as patient age, sex, WBC count, blast percentage of mononuclear cells and FAB type, although Pirker et al. found that *MDR1* gene expression was present more often in women than in men (15). Miwa et al. reported that CD7 and CD34 were the only surface markers which were significantly associated with the *MDR1* gene expression (14, 22). Zhou et al. revealed a significant inverse association between CD33 and *MDR1* gene expression (13). In our studies, we did not confirm the correlation between CD surface markers and the *MDR1* gene expression in AML. Zochbauer et al. (23) reported similar results that *MDR1* RNA or P-glycoprotein expression were not associated with the expression of CD surface markers (CD7, CD13, CD19, CD34) except HLA-DR. They found that P-gp expression was more frequent in HLA-DR positive than in HLA-DR negative patients (47% versus 10%, p=0.04).

In agreement with reports from others (11, 15, 24-26), the overexpression of the *MDR1* gene in acute leukemia cells is associated with the treatment outcome. The CR rate among patients with *MDR1* mRNA-negative was more than four times higher than in those with *MDR1* mRNA-positive, 77% versus 17% (p=0.044). There was a significant inverse correlation of *MDR1* expression and achievement of a CR in patients with acute leukemia. However, with regard to persistence and death in aplasia rates, levels of statistical significance were not reached. Three of 22 patients who were clinically resistant to MDR drugs did not exhibit *MDR1* expression, suggest-

ing that other mechanisms of drug resistance were present. Seventeen percent of patients with detectable *MDR1* expression attained a CR which can partly be explained by the fact that these patients were also treated with non-MDR-related drugs.

We found overexpression of *MDR1* gene in two patients with acute leukemia after chemotherapy, implying that *MDR1* gene expression could be induced by some therapeutic drugs.

In this study, we determined *MDR1* gene expression by RT-PCR which has a potential advantage over other RNA methods due to increased sensitivity and minimal amount of samples necessary. Noonan et al. reported that 27 out of 51 de novo acute nonlymphoblastic leukemia (ANLL) had detectable *MDR1* mRNA with PCR, but no *MDR1* gene expression was measured by filter hybridization (27). Brophy et al. (28) compared four methods for determining *MDR1* expression: slot blotting, RT-PCR, in situ *MDR1* mRNA, and immunohistochemistry with the MRK-16 monoclonal antibody. The results of the four techniques were not completely in agreement. They recommended RT-PCR as a standard technique for assessing clinical cases because of its relative simplicity and specificity for the *MDR1* gene. We also obtained similar results between *MDR1* mRNA RT-PCR and P-glycoprotein assay with the C219 monoclonal antibody using flow cytometry, however we could obtain some correlations between RT-PCR and daunorubicin efflux as P-glycoprotein functional assay in a few patients (data not shown).

Our study provides further evidence that *MDR1* gene expression may be an important mechanism of MDR in acute leukemia, and may serve as a prognostic factor. Moreover, *MDR1* gene expression could alter chemotherapeutic strategies to improve the treatment outcome. Nooter et al. (29) added cyclosporin A, a drug known to inhibit the P-gp pump, to the incubation medium which resulted in an increase in daunorubicin uptake by the leukemic cells from patients with *MDR1* mRNA-positive. MDR could be overcome by cyclosporin A (29-32) or verapamil in patients with ANLL and ALL (31). Further studies for MDR reversal with cyclosporin A, verapamil or less toxic verapamil analogue will offer the best chemotherapeutic regimens in acute leukemia in order to enhance the CR rates and prolong the patient's survival.

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