Quantitative Analysis of Human Multidrug Resistance 1 (*MDR1*) Gene Expression by Nonisotopic Competitive Reverse Transcriptase Polymerase Chain Reaction Assay

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We have established competitive reverse transcriptase polymerase chain reaction (RT-PCR) assay for the quantification of *MDR1* mRNA encoding P-glycoprotein (P-gp) by analyzing leukemia sublines of MOLT-3 with various expression of *MDR1*. The expression was quantified by simultaneous RT-PCR of cellular RNA with decreasing amounts of heterologous competitor RNA, which shares the *MDR1* primer sequences with the cellular *MDR1* mRNA, but yields a different-sized PCR product. This allows resolution of the amplified cDNA fragments. The amounts of *MDR1* mRNA measured by the assay were accurate and reproducible over wide range, and were determined as 31.6, 100, and 316 amol/ μ g total RNA in MOLT-3/TMQ₇₀, MOLT-3/

TMQ₈₀₀, and MOLT-3/VCR_{1,000}, respectively. The relative ratio of *MDR1* mRNA measured by the competitive RT-PCR among three sublines was similar to that of *MDR1* transcript determined by Northern analysis (1:4:12) and to that of P-gp measured by flow cytometry (FCM) analysis. In mononuclear cells from patients with leukemia, *MDR1* mRNA could be sufficiently quantified by the competitive RT-PCR established, while FCM assay could scarcely detect P-gp. This study demonstrated that the competitive RT-PCR assay using heterologous competitor RNA is a rapid, reliable, and non-radioactive procedure and is acceptable for the evaluation of *MDR1* expression in clinical samples. J. Clin. Lab. Anal. 11:258–266, 1997. © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Prolonged exposure of tumor cells to natural hydrophobic cytotoxic drugs may result in the acquisition of resistance by cells not only to the drug used but also to a series of structurally and functionally unrelated drugs. This phenomenon, called multidrug resistance (MDR), is one of the major obstacles in cancer chemotherapy. MDR is frequently mediated by the overproduction of P-glycoprotein (P-gp) (1), a 170 kDa cellular transmembrane protein that can act as a drug efflux pump (2). P-gp is encoded by *MDR1* (*PGY1*) (3). Although there is a homologous gene called *MDR2*, only *MDR1* is known to confer drug resistance (4). *MDR1* is frequently expressed in intrinsically drug-resistant human tumors and certain cancers at relapse after chemotherapy (5). In the treatment of acute non-lymphoblastic leukemia, complete remission rates were significantly lower and survival terms shorter

in P-gp-positive than in P-gp-negative patients (6). These findings indicate that the *MDR1* expression must contribute to MDR phenotype in many human cancers and leukemias.

In efforts to predict outcome of cancer chemotherapy in clinics, a rapid, simple assay for quantification of *MDR1* in clinical samples should be established. MDR phenotype can be detected by using gene-specific probes for *MDR1* or monoclonal antibodies against P-gp (7). Conventional Northern or dot blot analysis requires large amount of material, but this may not always be obtained in clinical samples. The flow cytometry (FCM) analysis using anti P-gp monoclonal anti-

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body cannot detect minimal expression of P-gp in clinical samples because of its low sensitivity, while conventional reverse transcriptase polymerase chain reaction (RT-PCR) shows higher sensitivity, but has problems for quantitation. These backgrounds urged us to develop competitive RT-PCR method for the measurement of *MDR1* mRNA amount. At least three other groups have quantified *MDR1* expression by the assay based on the competitive RT-PCR (8–10). Two of them used mutated or deleted competitor of *MDR1*. Such homologous competitor fragments may form heteroduplexes between competitor and target sequences during PCR cycles, subsequently interfering with quantitation.

In this study, we designed a multiple, heterologous competitor RNA template that would be available not only for quantifying *MDR1* gene expression but also for other drug-resistance-related gene expressions such as thymidylate synthase (*TS*). To evaluate the accuracy and reproducibility of the competitive RT-PCR assay developed, the results of the assay were thoroughly compared with those of Northern blot or FCM assay by analyzing human leukemia sublines of MOLT-3 with various magnitude of MDR phenotype. The competitive RT-PCR assay established was then applied to clinical samples.

MATERIALS AND METHODS

Cell lines

The parent MOLT-3 human acute lymphoblastic leukemia cell line (11) and multidrug-resistant sublines, MOLT-3/TMQ₇₀ (70-fold trimetrexate [TMQ] resistant), MOLT-3/TMQ₈₀₀ (800-fold TMQ resistant), and MOLT-3/VCR_{1,000} (1,000-fold vincristine [VCR] resistant) (12, 13) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and fed twice a week with fresh medium. The latter two sublines were generously provided by Dr. Takao Ohnuma at Mount Sinai School of Medicine, New York, NY.

Clinical Samples

Peripheral blood or bone marrow aspirates were obtained from ten patients with leukemia at Saitama Medical Center, Keio University Hospital, and Shinshu University Hospital, Japan. Two of them were obtained at the time of initial diagnosis before chemotherapy, while the others had previously received anticancer chemotherapy. Mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients and used for total RNA extraction and FCM analysis. The samples with > 70% leukemic cells were used for competitive RT-PCR assay except one case with chronic myelogenous leukemia in blastic crisis.

RNA Extraction

Total cellular RNA was isolated using TRIZOL Reagent™ (Life Technologies, Gaithersburg, MD) as described by

Chomczynski (14). RNA concentration and purity were determined spectrophotometrically.

Drug-sensitivity studies

The *in vitro* sensitivity of parent and resistant MOLT-3 cells to anticancer drugs was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with minor modifications (15). Briefly, 4×10^3 viable cells were inoculated in 0.2 ml 96-well culture dishes (Falcon; Becton Dickinson, Cockeysville, MD) in the presence of graded concentrations of anticancer drugs and incubated for 72 hours at 37°C in a humidified 5% CO₂/95% air atmosphere. At the end of the incubation, 10 µl MTT (Sigma, St. Louis, MO) solution (4 mg/ml in phosphate-buffered saline [PBS]) and 10 µl of 0.1 M sodium succinate (Sigma) were added to each well, and the plate was further incubated for 4 hours at 37°C. Following incubation, the plate was centrifuged at 650 g for 5 minutes and supernatants were removed. The formazan crystals produced were solubilized by the addition of 150 µl dimethylsulfoxide (Sigma), then the contents of the wells were thoroughly mixed on a plate shaker for 5 minutes and the absorbance of each well was immediately measured at 540 nm wavelength using a plate reader (model MTP-120; Corona, Katsuta, Japan). Dose-response curves were drawn by plotting the absorbance, expressed as a percentage of the control value without drug treatment, against drug concentrations.

FCM Analysis of P-gp Expression

Cell surface expression of P-gp was analyzed with anti-human P-gp mouse monoclonal antibody MRK16 (16) (Kyowa Medicus, Tokyo, Japan), using a standard indirect immunofluorescence assay. Briefly, logarithmically growing 10^6 cells in 0.1–0.5 ml of PBS containing 1 mM EDTA were incubated with heat-inactivated human γ-globulin (Sigma) at the final concentration of 50 µg/ml at 4°C for 30 minutes in order to block non-specific binding of the antibody to Fc receptors. Then the cell suspension was incubated with 10 µl of MRK16 at 4°C for 1 hour. After washing twice, the cells were incubated with 10 µl of FITC-conjugated goat anti-mouse IgG (Ortho Diagnostic System, Raritan, NJ) or PE-conjugated rat anti-mouse IgG_{2a+b} (Becton Dickinson) at 4°C for another 1 hour, washed again, and analyzed on FACScan (Becton Dickinson). For negative control, FBS or isotype antibody (mouse IgG_{2a}) was added instead of MRK 16.

Northern Blot Analysis of *MDR1* Expression

The expression of *MDR1* was analyzed as previously described (17). The cDNA for the human *MDR1* (pMDR 2000XS (18) was provided from Dr. Michael M. Gottesman at the National Cancer Institute, Bethesda, MD). The *Bst*UI-*Pme*I fragment was used for the probe. The cDNA for human

glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) (pHcGAP) was obtained from the ATCC (Rockville, MD). The *Pst*I-*Xba*I fragment was used for the probe as an internal control. Ten µg of total cellular RNA per lane was size-fractionated by electrophoresis in 0.41 M formaldehyde/1% agarose gels. The size-fractionated RNA was transferred onto Maximum Strength Nytran nylon membrane using Turboblotter Rapid Downward Transfer Systems (Schleicher and Schuell, Keene, NH) as described by Chomczynski (19). The membranes were exposed to ultraviolet light (254 nm) for fixation of RNA and hybridized with ³²P-labeled cDNA probes. Specific hybridization signals were visualized by autoradiography using Konica SR-G film (Konica, Tokyo, Japan) between 2 intensifying screens at -80°C for 4–24 hours.

Construct of Competitor RNA (Fig. 1)

For the preparation of competitor template, PCR MIMIC Construction Kit (Clontech, Palo Alto, CA) was utilized. First, the following oligonucleotides (sense, +; antisense, -) were used as composite primers for the primary PCR to generate the first MIMIC product; *MDR1-TSC*(+), 5' TTTCATTTTGGTGCCT

Fig. 1. Scheme for the construction of the vector pcDNA3-*MDR1TSC*. RNA transcribed *in vitro* (indicated as bold line) from vector linearized by *Xho*I was used as competitor in the competitive RT-PCR assay. T7, T7 RNA polymerase promoter sequence.

GGCAGCATCCAACACATCCTCCGCTGCCGCAAGTG-AAATCTCCTCCG3' (single underline, 5' *MDR1* specific primer sequence [398–419, exon 6]; double underline, 5' *TS* specific primer sequence [109–129]; the other, 20-base sequence that anneals to neutral DNA from MIMIC Kit; the numbers used in the primer sequences, the nucleotide numbers, taking +1 for the A of the initiation codon of human *MDR1* (20) or *TS* cDNA (21)); *TS-MDR1C*(-), 5' TCTCCCGATCTCTGG-TACAGCAGAAGGCCAGAGCATAAGATGCTTGAG-TCCATGGGGAGCTTT3' (single underline, 3' *MDR1* specific primer sequence [943–922, exon 9]; double underline, 3' *TS* specific primer sequence [653–633]; the other, 20-base sequence that anneals to neutral DNA from MIMIC Kit). Next, gene specific primers, *MDR1*(+) (5' TTTTCATTTTGGTGCCTGGCAGC3') and *TS*(-) (5' TCTCCCGATCTCTGGTACAGC3'), were used for the second round PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA) to generate pure PCR MIMIC product. The resultant PCR product was gel-purified using GENECLEAN III (BIO101, La Jolla, CA), 5'-phosphorylated by T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and then ligated to *Eco*R V-digested pcDNA3 (Invitrogen, San Diego, CA) (22). TOP10F⁺ competent cells (Invitrogen) were transformed, and the colonies were screened by PCR with T7 primer and SP6 primer (Promega, Madison, WI) to check the existence of the insert. The right clones were expanded, and the plasmid DNA was purified by JETSTAR Plasmid Midiprep Kit (GENOMED Inc., Research Triangle Park, NC) without RNase. The orientation and fidelity of the insert in the vector were confirmed by DNA sequencing using Dye Terminator Cycle Sequencing FS Kit and a DNA sequencer, Model 373A (Perkin-Elmer ABI, Foster City, CA).

Mimic competitor RNA was synthesized from *Xho*I-linearized pcDNA3-*MDR1TSC* recombinant by T7 RNA polymerase according to the manufacturer's recommended reaction conditions (New England Biolabs). Template DNA was degraded with RQ1 RNase free DNase (Promega) and the synthetic competitor RNA was extracted by TRIZOL ReagentTM followed by ethanol precipitation and spectrophotometric quantitation. Competitor RNA was tested to be free of DNA contamination by DNA-PCR using AmpliTaq[®] (Perkin-Elmer) (without reverse transcription step).

Competitive RT-PCR

Total cellular RNA (100 ng) in combination with semi-logarithmic serial dilutions of mimic competitor RNA from 100 to 1 amol was amplified by RT-PCR using GeneAmp[®] EZ *rTth* RNA PCR Kit (Perkin-Elmer) according to the following method; RNAs, 0.45 µM *MDR1*(+) primer and *MDR1*(-) primer (5' AGAAGGCCAGAGCATAAGATGC3'), 1 × EZ buffer (50 mM bicine, pH 8.2; 115 mM potassium acetate; 8% (w/v) glycerol), 300 µM of each dNTP, 2.5 mM Mn(OAc)₂ and 1 unit of *rTth* DNA polymerase in 10 µl were incubated at 65°C

for 1 hour (reverse transcription), followed by the following PCR cycles, an initial step of 95°C for 1 minute, 30~40 cycles of a 2-temperature PCR (95°C for 15 seconds, 65°C for 40 seconds) and end with 65°C for 7 minutes. Each PCR product was then size-fractionated through a 1.5% agarose gel. The amplified cellular fragment (target) was 544 bp and the mimic competitor was 621 bp. The amount of competitor RNA yielding equal molar amounts of products gave that of target *MDR1* mRNA.

To verify the quality of total cellular RNA extracted, RT-PCR of *GAPDH* was performed separately. Total cellular RNA was amplified by RT-PCR using GeneAmp® EZ *rTth* RNA PCR Kit with human *GAPDH* control amplicon set (Clontech) (60°C for 30 minutes to reverse-transcribe and PCR cycles; an initial step of 95°C for 1 minute, 30 cycles of a 3-temperature PCR [95°C for 15 seconds, 60°C for 45 seconds, and 72°C for 2 minutes] and end with 72°C for 5 minutes). The band intensities derived from each 100 ng total RNA were virtually identical.

Direct Sequencing of PCR Products

The amplified cellular fragments (544 bp) were gel-purified using GENECLEAN III and directly sequenced using Dye Terminator Cycle Sequencing FS Kit.

RESULTS

Drug Sensitivities of the Cells Determined by MTT Assay

IC₅₀ (drug concentration producing 50% inhibition of cell growth) values of and relative resistances to TMQ, VCR and doxorubicin (DXR) in the sensitive and resistant cells are given in Table 1. MDR phenotype was upgraded in order; MOLT-3 (sensitive) < MOLT-3/TMQ₇₀ < MOLT-3/TMQ₈₀₀ < MOLT-3/VCR_{1,000}, as determined by sensitivity to VCR.

P-gp Expression of Cell Surface Measured by Indirect Immunofluorescence FCM Using MRK16

Figure 2 shows fluorescence histograms of the sensitive and resistant MOLT-3 cells stained with MRK16. The parent

Fig. 2. Expression of P-glycoprotein on MOLT-3 and its resistant sublines with various magnitude of multidrug resistance phenotype, being stained with MRK16 mouse monoclonal antibody against human P-glycoprotein and FITC labeled goat anti-mouse IgG. (A) control; (B) MOLT-3 cells; (C) MOLT-3/TMQ₇₀ cells; (D) MOLT-3/TMQ₈₀₀ cells; (E) MOLT-3/VCR_{1,000} cells.

MOLT-3 did not express P-gp on cell surface. In the resistant sublines, various degree of P-gp expression was observed, displaying the mean fluorescence intensity of 10, 62, 390, and 780 for MOLT-3, MOLT-3/TMQ₇₀, MOLT-3/TMQ₈₀₀, and MOLT-3/VCR_{1,000} cells, respectively.

MDR1 Expression Determined by Northern Blot Analysis

Figure 3 shows the result of Northern blot analysis for *MDR1* mRNA. No *MDR1* signal was observed in the parent MOLT-3 cells even after prolonged exposure. The intensities of *MDR1* mRNA signal (after normalization to *GAPDH* expression) measured by a densitometer were MOLT-3/TMQ₇₀, 1; MOLT-3/TMQ₈₀₀, 4; MOLT-3/VCR_{1,000}, 12 in a relative ratio.

Exponential PCR Amplification of *MDR1* RNA

The *MDR1*-specific cDNA sequence was amplified with a pair of primers, *MDR1*(+) and *MDR1*(-) derived from the different exons, 6 and 9 of the *MDR1* gene (20), respectively, to identify the PCR products as results originated from mRNA but not from genomic DNA. The *MDR1*-specific primers were selected so that they would not amplify the closely related *MDR2* cDNA sequence.

To determine the exponential range of PCR amplification for *MDR1* mRNA and *in vitro* transcribed, mimic competitor RNA (Fig. 1), we co-amplified 100 ng total cellular RNA isolated from MOLT-3/TMQ₈₀₀ cells and 10 amol of mimic competitor RNA. The samples were taken every 2 PCR cycles from 30–42 cycles. PCR products were size-fractionated

TABLE 1. IC₅₀ Values of the Drugs Used Against the Sensitive and Resistant Cell Lines and the Degree of Resistance to Antineoplastic Drugs

Cell line	IC ₅₀ (μM) ^a /(Relative resistance ^b)		
	TMQ	VCR	DXR
MOLT-3	0.012	0.009	0.022
MOLT-3/TMQ ₇₀	0.84 (70)	0.0015 (1.7)	0.055 (2.5)
MOLT-3/TMQ ₈₀₀	9.6 (800)	0.25 (280)	0.3 (14)
MOLT-3/VCR _{1,000}	0.042 (3.5)	0.9 (1,000)	0.18 (8)

^aIC₅₀ values were obtained from dose-response curves of each cell line after exposure of the cells for 72 hours to a drug. Each data value represents the mean of at least 2 independent experiments performed in quadruplicate. All experimental values were within 15% of the mean.

^bIC₅₀ values of the resistant cells/IC₅₀ value of the parent cells.

Fig. 3. Northern blot analysis of *MDR1* mRNA in the sensitive and resistant cells. *GAPDH* expression was used as an internal standard. Lane 1,

MOLT-3; lane 2, MOLT-3/TMQ₇₀; lane 3, MOLT-3/TMQ₈₀₀; lane 4, MOLT-3/VCR_{1,000}.

through a 2% agarose gel, stained with ethidium bromide, and the band intensities were compared with the 603-bp band of serially diluted ØX174DNA-*Hae*III digest DNA marker (New England Biolabs). As shown in Fig. 4, both the *in vitro* transcribed mimic and the cellular *MDR1* RNA were amplified in the same exponential fashion from 34–40 PCR cycles.

Quantitation of *MDR1* mRNA in the Parent and Resistant MOLT-3 Sublines by the Competitive RT-PCR

The amounts of *MDR1* mRNA in MOLT-3 sublines were measured using 38 RT-PCR cycles, which produce an exponential amplification. A constant amount of cellular RNA (100 ng) was mixed with decreasing amounts of synthetic *MDR1* mimic RNA (from 100 to 1 amol). Four representative gels are shown in Fig. 5. No detectable *MDR1* mRNA was found in the parent MOLT-3 cells (A). The amount of *MDR1* mRNA was 31.6, 100, and 316 amol/μg total RNA in MOLT-3/TMQ₇₀, MOLT-3/TMQ₈₀₀, and MOLT-3/VCR_{1,000}, respectively (B, C, D). The relative ratio of *MDR1* mRNA measured by competitive RT-PCR among three resistant sublines was similar to those of *MDR1* transcript determined by Northern blot analysis (1:4:12; Fig. 3). The results of competitive RT-PCR assay were in parallel not only with those of Northern blot analysis, but also with the expression of P-gp measured by indirect FCM analysis. The competitive RT-PCR assay in each MOLT-3 subline was repeated at least three times, and the standard deviation was within 10%.

The direct sequencing of PCR products amplified confirmed that the sequence of each PCR product was completely identical to the *MDR1* sequence reported (20) (data not shown).

Fig. 4. Exponential range of amplification for 100 ng of total cellular RNA obtained from MOLT-3/TMQ₈₀₀ and 10 amol of synthetic mimic competitor RNA. Cellular and competitor RNAs were co-amplified by RT-PCR for various numbers of cycles. The intensity of each PCR product stained with ethidium bromide was compared with the 603-bp band of serially diluted ØX174DNA-*Hae*III digest DNA marker, and the relative band intensity was estimated.

Fig. 5. Competitive RT-PCR assay of *MDR1* in parent and resistant MOLT-3 cells. One hundred ng of total cellular RNA extracted from each MOLT-3 subline in combination with serial dilutions of the competitor RNA was subjected to the assay. After 38 cycles of amplification, the products were resolved on a 1.5% agarose gel and stained with ethidium bromide. The amount of competitor RNA yielding equal molar amounts of products gave that of

target *MDR1* mRNA. (A) MOLT-3 cell line; (B) MOLT-3/TMQ₇₀ cell line; (C) MOLT-3/TMQ₈₀₀ cell line; (D) MOLT-3/VCR_{1,000} cell line. M: DNA size standard, ØX174DNA-*Hae*III digest; lanes 1–5: 100, 31.6, 10, 3.16, and 1 amol of *MDR1* mimic competitor RNA, respectively. The positions of the 621- (competitor origin) and 544-bp (cellular *MDR1* mRNA origin) fragments are indicated.

Quantitation of *MDR1* mRNA in Clinical Samples

Ten clinical samples obtained from patients with leukemia were measured for *MDR1* mRNA expression by the competitive RT-PCR established. Mononuclear cells isolated by centrifugation on Ficoll-Hypaque gradients were used for the competitive RT-PCR assay for *MDR1* mRNA. *MDR1* expression in the tested samples is shown in Table 2. In four out of the ten samples, *MDR1* mRNA was detectable. Three out of the four cases with *MDR1* expression did not respond to chemotherapy, and case 1 responded to chemotherapy was dead of pneumonia during the recovery phase from myelosuppression.

On the other hand, four out of the six cases without *MDR1* expression responded to chemotherapy except AML M0 and CML mixed crisis.

Since P-gp antigen expression measured by FCM analysis using an anti P-gp monoclonal antibody appeared parallel to *MDR1* mRNA expression quantitated by competitive RT-PCR assay (Fig. 2 and 5), the comparison of competitive RT-PCR assay and FCM analysis was also evaluated in clinical samples. Figure 6 represented a typical case. Fluorescence intensity of cells stained with MRK16 plus PE-conjugated secondary antibody scarcely shifted as

TABLE 2. Analysis of *MDR1* mRNA Expression in Human Leukemias

Case	Age/Sex	Diagnosis	Chemotherapy	Sample	(% of Blasts)	<i>MDR1</i> ^a	Response to subsequent therapy
1	68/M	AML(M4)	–	PB	(78)	100	CR
2	32/F	AML (M4Eo) (3rd relapse)	+	PB	(84)	40	NR
3	71/M	AML (transformed from MDS)	+	PB	(82)	8	NR
4	60/M	CLL	+	PB	(93)	<1	PR
5	63/M	CML-BC	+	BP	(72)	<1	PR
6	22/F	ALL	–	BM	(98.4)	<1	CR
7	52/M	CML-BC (mixed)	+	BM	(90)	<1	NR
8	55/M	AML (M0) (1st relapse)	+	BM	(90)	<1	NR
9	72/M	CML-BC (mixed)	+	PB	(60)	20	NR
10	54/M	NK cell leukemia	+	PB	(81)	<1	CR

^aamol/μg total RNA

AML, acute myelogenous leukemia (French-American-British classification); MDS, myelodysplastic syndrome; CLL, chronic lymphocytic leukemia; CML-BC, chronic myelogenous leukemia in blastic crisis; ALL, acute lymphoblastic leukemia; NK, natural killer; PB, peripheral blood; BM, bone marrow; CR, complete response; RP, partial response; NR, no response.

Fig. 6. A typical clinical case in which P-gp antigen expression was scarcely detected by flow cytometry analysis using anti P-gp monoclonal antibody (left; A, mouse IgG_{2a} control antibody plus PE-conjugated secondary anti-

body; B, MRK16 plus PE-conjugated secondary antibody). The expression of *MDR1* in the same case was quantitated as 20 amol/ μ g RNA by the competitive RT-PCR assay (right).

compared with control (left), while *MDR1* mRNA expression was clearly quantitated as 20 amol/ μ g RNA by the competitive RT-PCR assay (right).

DISCUSSION

A rapid, simple, non-radioactive assay based on the competitive PCR have been developed for the quantification of *MDR1* mRNA expression (8–10, 23). The synthetic competitor RNA is also employed as an internal standard for this assay to verify the efficiency of both the reverse transcription reaction and the PCR. The synthetic RNA has the same primer sequences as the target mRNA so that there must be no differences in efficiencies of primer annealing. Indeed, both the synthetic competitor RNA and cellular *MDR1* mRNA were amplified in the same exponential fashion (Fig. 4). Further, the difference in base-pair size between competitor and target (621 vs. 544 bp, respectively) allows sufficient separation of their corresponding amplification products in an agarose gel electrophoresis. All the steps in the assay established can be easily carried out in clinical laboratories without use of radioisotope. The results of the competitive RT-PCR assay showed a good correlation with those of both FCM and Northern blot analyses. Although Northern blot analysis is the standard method for the quantification of gene expressions, it requires large amounts of material. On the contrary, competitive RT-PCR assay developed needs only less than 1/20 of total RNA as compared with Northern blot analysis and no radioisotope with retaining accuracy and sensitivity, thus it is particularly suitable for the detection of the *MDR1* gene expression in clinical samples at low levels of resistance. As expected, this assay system was shown to work properly in clinical samples obtained

from patients with leukemias, giving four positive samples among ten. On the other hand, by the present FCM analysis utilizing an anti-P-gp monoclonal antibody, minimally expressed P-gp in the same clinical samples could not clearly be detected (Fig. 6). In spite of the same amount of *MDR1* mRNA per μ g RNA, the smaller the cell size of most clinical samples than that of cell lines, the smaller amount of P-gp single cell has on the cell surface. Accordingly, the fluorescence intensity obtained from clinical samples tends to become weak as compared with cell lines, suggesting that FCM analysis utilizing an anti-P-gp monoclonal antibody might be disadvantageous to most clinical samples at low levels of P-gp expression. Therefore, in a clinical laboratory setting RT-PCR assay is strongly recommended as the standard technique to measure *MDR1* levels because of its high sensitivity, relative simplicity, and specificity. In addition to RT-PCR assay, a complementary technique able to detect heterogeneity of P-gp function would be useful (24). Because the co-existence of normal cells expressing *MDR1* in clinical samples, for example hematopoietic stem cells, some lymphoid subpopulations or natural killer cells (25–27), may potentially interfere in evaluating the *MDR1* expression in tumor cells particularly when measured by conventional RT-PCR assay. As a suitable, additional method, rhodamine 123 (Rh 123) dye exclusion assay (24, 28) seems better than FCM analysis utilizing an anti-P-gp monoclonal antibody because of its sensitivity (unpublished data). In contrast to conventional RT-PCR assay, very small fraction of hematopoietic stem cells in normal bone marrow had negligible *MDR1* expression and mononuclear cells obtained normal peripheral blood had minimal *MDR1* expression (4–8 amol/ μ g RNA) when measured by this quantitative, competitive RT-PCR assay (unpublished data). As a consequence of the foregoing, it is pos-

sible to set up a cutoff point and evaluate *MDR1* expression in leukemic cells accurately even when samples are contaminated with normal cells expressing *MDR1*. This is a great advantage of quantitative assay utilizing competitive RT-PCR.

Conventional RT-PCR assay has been employed to quantify the level of gene expression with the use of "housekeeping" genes as internal controls (29–31) on the assumption that expression of the control gene does not change during malignant progression. However, mRNA concentrations of these genes do not always remain constant during malignant progression (32, 33), and their pseudogenes or family genes were reported to result in false-positive PCR products (34). Furthermore, the RT-PCR assay with an internal control digresses from linearity at high PCR cycles, especially with β -actin, as reported previously (35), indicating another potential pitfall of conventional RT-PCR.

To establish a reliable competitive RT-PCR assay, the design of competitor is one of the important factors. Other studies used homologous competitor fragments which were synthesized by introduction of deletion or insertion for changing sizes of PCR product, or introduction of mutation for creating new restriction sites, however, they require time-consuming site-directed mutagenesis and multiple cloning steps (8, 10). In this study, we designed competitor RNAs to have the same primer sequences as the target genes and an unrelated insert in the middle (heterologous competitor). This strategy allows easy resolution of competitor and target PCR products by agarose gel electrophoresis, followed by quantification of band intensities by comparison. Heterologous competitor would be preferable for competitive PCR assay as compared with homologous competitor, because they do not form heteroduplexes between the competitor and target sequences. Formation of heteroduplexes makes quantitation of the PCR products intricate, since PCR products resulted from the competitor or target cannot be clearly separated, particularly when restriction enzyme digestion is required to distinguish them (36). In the present study, we also devised the multiple competitor template, cloned in a plasmid vector, and this allows the supply of unlimited amounts of competitor by *in vitro* transcription for the analysis of various drug resistance genes. Our multiple competitor could be used for the quantification of not only *MDR1* but also *TS* mRNA by competitive RT-PCR (unpublished data). Since drug resistance is multifactorial (37), this kind of multiple competitor is of great practical value in clinical samples.

The various conditions for the quantitative analysis of *MDR1* mRNA using competitive RT-PCR were made up by measuring *MDR1* mRNA in multidrug resistant MOLT-3 sublines with known magnitude of MDR phenotype. In future, the assay method should be standardized for the comparison of the results in different laboratories since diversification in competitors, primers, and other conditions used might provide a large fluctuation in quantification. For such standard-

ization, our three multidrug resistant MOLT-3 sublines with known *MDR1* expression can be utilized as standard MDR cell lines. In fact, the use of standard MDR cell lines has been recommended for calibrating the results obtained from any detection method and for subsequent analyses of clinical samples on "the St. Jude MDR Workshop on Methods to Detect P-Glycoprotein-associated Multidrug Resistance" (38).

In this study, visual inspection of staining gels was performed for quantitating RT-PCR product because of simplicity and availability in any laboratories. The intensity of the ethidium-bromide luminescence can be also measured by CCD imaging system (39), however, the quantitation values were almost equal to the data produced by visual inspection in our preliminary experience and the steps for gel electrophoresis and staining are still required. In the future the quantitation of product might be automated by coupling with HPLC, to put it concretely, by a single-step separation from nucleotides and remaining primers through an HPLC column and a computerized device (40, 41). In fact, Warren et al. demonstrated that HPLC utilizing a high efficiency anion-exchange column provided a rapid and easily automated technique for the quantitative analysis of subnanogram to microgram amounts of PCR products and that the accuracy, precision, and linearity of the method exceeded those attainable with electrophoretic techniques (42).

In conclusion, competitive RT-PCR assay using heterologous competitor RNA is a rapid, simple, reliable, and non-radioactive procedure with high sensitivity for the quantification of *MDR1* mRNA and is acceptable for the evaluation of *MDR1* expression in clinical samples in any clinical laboratory with molecular technology. Further investigation with more clinical samples is now ongoing to elucidate the importance of *MDR1* overexpression on the clinicopathological features of cancer patients.

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