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Inhibitory Effect of Curcumin on *MDR1* Gene Expression in Patient Leukemic Cells

Songyot Anuchapreeda, Pattra Thanarattanakorn[1], Somjai Sittipreechacharn[1], Singkome Tima, Prasit Chanarat, and Pornngarm Limtrakul[2]

Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, 50200, Thailand, [1]Department of Pediatrics, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand, and [2]Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand

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When patients with cancers are treated with chemotherapeutic agents a long time, some of the cancer cells develop the multidrug resistance (MDR) phenotype. MDR cancer cells are characterized by the overexpression of multidrug resistance1 (*MDR1*) gene which encodes P-glycoprotein (Pgp), a surface protein of tumor cells that functions to produce an excessive efflux and thereby an insufficient intracellular concentration of chemotherapeutic agents. A variety of studies have sought potent MDR modulators to decrease *MDR1* gene expression in cancer cells. Our previous study has shown that curcumin exhibits characteristics of a MDR modulator in KB-V1 multidrug-resistant cells. The aim of this study was to further investigate the effect of curcumin on *MDR1* gene expression in patient leukemic cells. The leukemic cells were collected from 78 childhood leukemia patients admitted at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand, in the period from July 2003 to February 2005. There were 61 cases of acute lymphoblastic leukemia (ALL), 14 cases of acute myeloblastic leukemia (AML), and 3 cases of chronic myelocytic leukemia (CML). There were 47 males and 31 females ranging from 1 to 15 years old. Bone marrows were collected. The leukemic cells were separated and cultured in the presence or absence of 10 μM curcumin for 48 hours. MDR1 mRNA levels were determined by RT-PCR. It was found that curcumin reduced *MDR1* gene expression in the cells from 33 patients (42%). Curcumin affected the *MDR1* gene expression in 5 of 11 relapsed cases (45%), 10 of 26 cases of drug maintenance (38%), 7 of 18 cases of completed treatment (39%), and 11 of 23 cases of new patients (48%). The expression levels of *MDR1* gene in leukemic patient cells as compared to that of KB-V1 cells were classified as low level (1-20%) in 5 of 20 cases (25%), medium level (21-60%) in 14 of 32 cases (44%), and high level (61-100%) in 14 of 20 cases (70%). In summary, curcumin decreased MDR1 mRNA level in patient leukemic cells, especially in high level of *MDR1* gene groups. Thus, curcumin treatment may provide a lead for clinical treatment of leukemia patients in the future.

Key words: Curcumin, Multidrug resistance, P-Glycoprotein, Patient leukemic cells

INTRODUCTION

Leukemia is a group of blood diseases characterized by malignancies (cancer) of blood- forming tissues. Leukemia is the most common childhood cancer. The highest incidence of leukemia in Thailand is in the North region, especially in Lampang province (Age-standardized world incidence rate (ASR) = 6.4 in males and ASR = 3.5 in females) in the years 1995 to1997. In Chiang Mai province, the ASRs were 4.0 in males and 3.8 in females in the same years (Sriplung *et al.*, 2003). From data on leukemia patients in Maharaj Nakorn Chiang Mai Hospital in the period 2000-2002, there were 603 leukemia patients (144 childhood cases, 79 males and 65 females; and 459 adult cases, 255 males and 204 females) (Lorvidhaya *et al.*, 2002). When patients with cancer are treated with a cytotoxic agent, the pharmacological goal is to deliver as much active drug as possible to the molecular target in

Correspondence to: Songyot Anuchapreeda, Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
Tel: 66-53-949237; Fax: 66-53-945066
E-mail: sanuchapreeda@yahoo.com

cancer cells in order to cause sufficient molecular damage to cause the death of the cell. On the other hand, the emergence of the MDR phenotype renders cells resistant not only to the drug used in the chemotherapy, but also to a broad spectrum of unrelated cytotoxic drugs as well. When human tumor cells express this phenotype, they often overexpress the drug export protein called plasma membrane P-glycoprotein (Pgp). It has a molecular weight of approximately 150-170 kDa. This phosphoglycoprotein, which belongs to the superfamily of ATP-binding cassette (ABC) transporters, consists of two halves that share a high degree of sequence homology (Ambudkar *et al.*, 1999). One of the major obstacles to successful chemotherapeutic treatment is the drug resistance of leukemic cells to chemotherapeutic agents. Assessing the drug resistance in leukemic cells is important for treatment of leukemia. Quantitative real time RT-PCR is one method developed to evaluate drug resistance and used in clinical samples. The study of drug resistance in acute leukemia revealed high concentrations of MDR1 mRNA in all relapsed patients by RT-PCR (Fujimaki *et al.*, 2002). NASBA (Nucleic acid sequence-based amplification) was also developed to determine *MDR1* gene expression in clinical samples and is widely used in clinical MDR detection (Hayashi *et al.*, 2004). Moreover, multiplex PCR in combination with nested PCR was used for quantitative analysis of mRNA expression of MDR1, MRP, and topoisomerase IIa in small amounts of tumor tissue (Schwarzenbach, 2002).

Curcumin (diferuloyl methane), a phenolic compound responsible for the bright yellow color of turmeric, is believed to be the main pharmacological agent in this spice. It possesses antioxidant activity *in vitro* (Kunchandy and Rao, 1990; Soudamini and Kuttan, 1989; Kuo *et al.*, 1996; Selvam *et al.*, 1995) and is used in lipid peroxidation tests (Sreejayan and Roa, 1994; Salirnath *et al.*, 1989; Asai *et al.*, 1999). Curcuminoids are effective in preventing and ameliorating gastric lesions and have anti-inflamatory (Huang *et al.*, 1992; Ammon *et al.*, 1993), antibacterial (Banerjee and Nigam, 1978; Fitzpatrick, 1954; Shankar and Murthy, 1978), anti-fungal, and anti-yeast (Sawada *et al.*, 1971) antihypocholesterolemic (Rao *et al.*, 1970; Patii and Srinivasan, 1971), anticancer (Ruby *et al.*, 1995; Rao *et al.*, 1995; Limtrakul *et al.*, 1997; Singh *et al.*, 1998; mohan *et al.*, 1998; Limtrakul *et al.*, 2001; Anuchapreeda *et al.*, 2006), antimutagen (Nagabhushan *et al.*, 1987; Polasa *et al.*, 1992), antiparasitic (Roy *et al.*, 1976), antitumor-promoting (Azuine and Bhide, 1992), and antiproliferative activities (Dorai *et al.*, 2001). Recently, we have described dual modulation of *MDR1* expression and Pgp function by crude curcuminoids (Anuchapreeda *et al.*, 2002).

The rhizomes of *Curcuma longa* Linn contain three major pigments of curcuminoids: curcumin (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III). All commercial curcuminoids sold as "curcumin" (Sigma-Aldrich, ICN, GNC and etc.) are mixtures of the three curcuminoids. The pharmacological activities of the three curcuminoids have been compared in many studies. The effect of curcumin on the level and function of Pgp in dexamethasone-induced primary hepatocytes, showed that 25 mM curcumin inhibited Pgp function by the rhodamine123 test and decreased the Pgp level (Romiti *et al.*, 1998). Curcumin at 50 μM concentration induced cell death in multidrug resistant CEM and LoVo cells (Piwocha *et al.*, 2002). Commercial grade curcumin has been reported by us to inhibit *MDR1* gene expression and Pgp function. Moreover, it also increased drug sensitivity in human cervical carcinoma KB-V1 cells (Anuchapreeda *et al.*, 2002; Waiwut *et al.*, 2002; Limtrakul *et al.*, 2004; Cheaware *et al.*, 2004). This study aims to determine the inhibitory effect of curcumin on *MDR1* gene expression in patient leukemic cells.

MATERIALS AND METHODS

Materials

Commercial grade curcumin (77% curcumin I, 17% curcumin II and 3% curcuminIII), SuperScript' One-step RT-PCR System with Platinum® *Taq* DNA polymerase reagent, TRIZOL® reagent, RNaseOUT™, and primers were purchased from Invitrogen™ Life Technology (Carlsbad, CA, U.S.A.). RPMI1640, penicillin-streptomycin, and L-glutamine were purchased from GIBCO-BRL (Grand Island, NY, U.S.A.).

Cell line and patient leukemic cells culture conditions

The multidrug-resistant cell line KB-V1 (multidrug resistance cervical carcinoma cell line) was a generous gift from Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, U.S.A.). This cell line was cultured in DMEM with 4.5 g of glucose/liter plus 10% fetal calf serum, L-glutamine, penicillin (50 units/mL), and streptomycin (50 mg/mL); 1 mg/mL of vinblastine was added to the KB-V1 culture medium. This cell line was maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C. When the cells reached confluency, they were harvested and plated for consequent passages or for drug treatments.

The bone marrow (BM) leukemic cells were collected from BM of 78 leukemia patients; 61 cases of acute lymphoblastic leukemia (ALL), 14 cases of acute myelogenous leukemia (AML), and 3 cases of chronic myelocytic leukemia (CML).There were 47 males and 31 females ranging from 1 to 15 years old. Each cell type of leukemia was identified by French-American-British (FAB) Coo-

peration Group classification. Treatment was classified as follows: completed treatment; relapse case; drug maintenance, and new case. These samples were collected at Maharaj Nakornchiangmai Hospital, Chiang Mai, Thailand. Leukemic cells were washed twice with ice-cold phosphate-buffered saline (PBS). After that, red blood cells were lysed by hypotonic buffer solution. Cells were cultured in completed RPMI1640 in the presence and absence of 10 μM curcumin for 48 h in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C (Anuchapreeda *et al.*, 2006). The KB-V1 cell line was used as a positive control cell in this experiment because of its high expression level of MDR1 mRNA.

The effects of curcumin on cell growth were observed by examining morphology of the cultures with an inverted phase contrast microscope. The MTT test was used throughout the experimental observations to check cell viability.

RNA extraction and quantitative RT-PCR

RNA of leukemic patient cells and KB-V1 cells was isolated by TRIZOL® Reagent. RNaseOUT™ was added to the RNA extraction products for RNA protection (40 units/20 μL of reaction mixture). The amount of RNA was determined by OD measurement at λ = 260; one OD unit = 40 μg/mL. RT-PCR was performed using SuperScript' III One-step RT-PCR System with Platinum® *Taq* DNA polymerase reagent. For *MDR1*, the forward primer sequence used was GCCTGGCAGCTGGAAGACAAA-TACACAAAATT and the reverse primer sequence used was CAGACAGCAGCTGACAGTCCAAGAACAGGACT, corresponding to residues 406-437 and residues 657-688, respectively, of the published cDNA sequence (Murphy *et al.*, 1990). Using these primers, PCR yielded a 283-bp product. Evaluation of β-*actin* gene expression, used as control of the RNA amount, was carried out by using the forward primer sequence CAGAGCAAGAGAGGCATCCT and the reverse primer sequence TTGAAGGTCTCAAAC ATGAT, corresponding to residues 216-235 and residues 405-424, respectively, yielding a 201-bp product. cDNA was synthesized from mRNA at 55°C for 30 min and denatured at 94°C for 2 min. Amplification was performed in 30 cycles of sequential denaturation (94°C, 1 min); annealing (55°C, 1 min); and extension (72°C, 1 min). For negative control, water was amplified a total of 30 cycles to detect possible contamination. A total 15 μL of each PCR product was electrophoresed in 1X Tris/acetate/EDTA (TAE) electrophoresis buffer on a 1% agarose gel. Gels were stained with 2 μg/mL ethidium bromide and analyzed by scanning densitometry (BIO-RAD). The KB-V1 cDNAs were used as a positive control in every experiment.

Data and Statistical analysis

After curcumin treatment, the values of *MDR1* gene expression were compared with vehicle control of the same sample. An inhibitory effect was defined as one in which the difference between treatment and control was ≥ 3%. Data were the mean ± standard deviation of mean from triplicate samples of three independent experiments. Differences between the means were analyzed by one-way ANOVA analysis of variance. Probability values $p < 0.05$ were considered to be a statistical significance.

RESULTS

This study of *MDR1* gene expression in patient leukemic cells in four classes of leukemia patients (relapsed cases, drug maintenance, completed treatment, and new cases) found that three of four groups showed similar MDR1 mRNA levels (drug maintenance (45.9 ± 33.8), completed treatment (42.1 ± 30.7), and new cases (44.2 ± 30.8)). Relapsed cases showed a lower MDR1 mRNA level than those of the other three groups (27.4 ± 22.4) (Fig. 1). However, the difference was not statistically significant. When *MDR1* gene expression was analyzed by leukemic cell type (ALL, AML, and CML) it was found that the ALL group (45.4 ± 31.6) and CML group (54 ± 36.3) were not significantly different. The AML group (25.1 ± 19.4) was

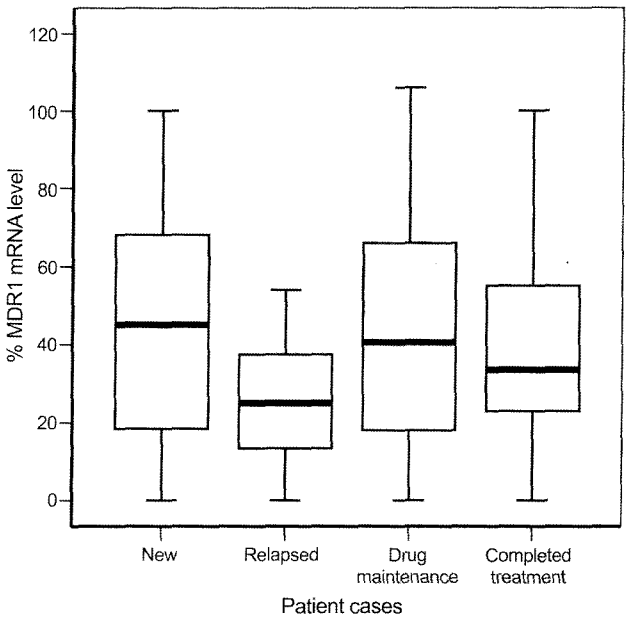

Fig. 1. *MDR1* gene expression in patient leukemic cells (78 samples) in groups of leukemic patients; relapse cases, drug maintenance, completed treatment, and new cases. The MDR1 mRNAs were determined by RT-PCR and quantitated by scan densitometer. The relative levels of *MDR1* gene expression in each patient cell (defined in KB-V1 cell line as 100% expression). PCRs for MDR1 and β-actin were performed 30 cycles.

significantly different from the ALL group, but was not significantly different from the CML group (Fig. 2). Patient leukemic cells were treated with a non-toxic dose of curcumin (10 μM) for 48 h. Cell viability after curcumin treatment was 86 ± 13% by MTT assay. The effect of curcumin on *MDR1* gene expression in patient leukemic cells was determined by RT-PCR. The amounts of MDR1 mRNA after ethidium bromide staining were measured by densitometer scan. This revealed that in 33 cases (42%) MDR1 mRNA levels were decreased by curcumin treatment, including 5 of 11 relapsed cases (45%), 10 of 26 drug maintenance cases (38%), 7 of 18 completed treatment (39%), and 11 of 23 new patients (48%), as shown in Table I. Curcumin-responsive leukemic cell samples were ALL (28 of 61; 47%), AML (4 of 14; 28%), and CML (1 of 3; 33%), as shown in Table I. Curcumin affected both males and females with the values of 49% and 32% of total samples, respectively (Table I). The levels of *MDR1* gene expression in these 78 patients showed low level (1-20%), medium level (21-60%) and high level (61-100%). Six patient leukemic cells did not express *MDR1* gene. MDR1 mRNA levels were decreased by curcumin treatment in 5 of 20 in the low level group (25%), 14 of 32 in the medium level group (44%), and 14 of 20 in the high level group (70%), as shown in Figs. 3, 4a, 4b and 4c. In the low level group, drug maintenance showed the highest number of samples in which *MDR1* gene expression was

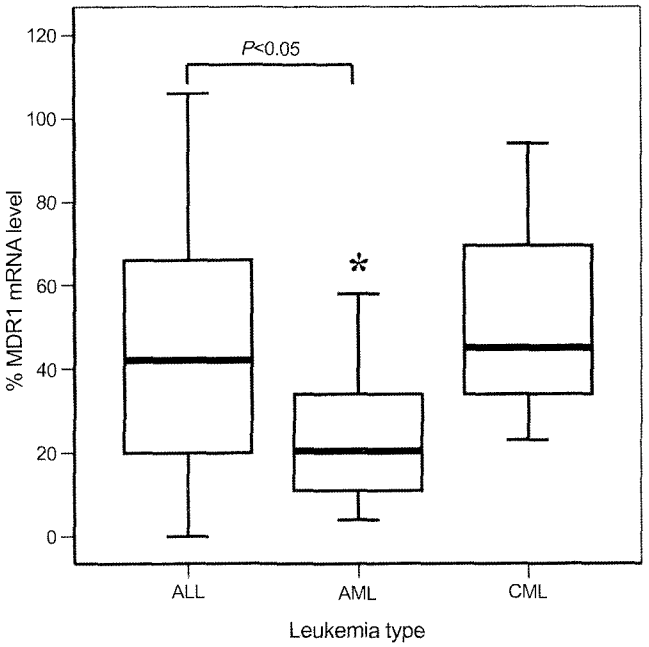

Fig. 2. *MDR1* gene expression in patient leukemic cells (78 samples) in group of patient leukemic types; ALL, AML, and CML. The MDR1 mRNAs were determined by RT-PCR and quantitated by scan densitometer. The relative levels of *MDR1* gene expression in each patient cell (defined in KB-V1 cell line as 100% expression). PCRs for MDR1 and β-actin were performed 30 cycles. Key: (*) significantly different from the control ($p< 0.05$).

Table I. Samples with MDR1 mRNA levels decreased by curcumin in different clinical patient groups

MDR1 mRNA levels	Patient groups	Fraction of total samples with MDR1 mRNA level decreased by curcumin (%)			Fraction of total samples*	Percent of total samples*
		ALL	AML	CML		
0	New case	0/2	-	-	-	-
	Completed treatment	0/1	-	-	-	-
	Drug maintenance	0/2	-	-	-	-
	Relapsed case	0/1	-	-	-	-
Low (1-20%)	New case	1/4 (25)	0/2 (0)	-	1/5	20
	Completed treatment	0/2 (0)	1/1 (100)	-	1/5	20
	Drug maintenance	3/6 (50)	0/1 (0)	-	3/5	60
	Relapsed case	0/1 (0)	0/3 (0)	-	0/5	0
Medium (21-60%)	New case	3/5 (60)	0/1 (0)	1/1 (100)	4/14	29
	Completed treatment	4/8 (50)	0/1 (0)	-	4/14	29
	Drug maintenance	2/8 (25)	0/2 (0)	0/1 (0)	2/14	14
	Relapsed case	2/3 (67)	2/2 (100)	-	4/14	29
High (61-100%)	New case	5/7 (71)	1/1 (100)	-	6/14	43
	Completed treatment	2/4 (50)	-	-	2/14	14
	Drug maintenance	5/6 (83)	-	0/1 (0)	5/14	36
	Relapsed case	1/1 (100)	-	-	1/14	7

* Samples in this case are total samples with decreased MDR1 mRNA after curcumin treatment.

inhibited by curcumin (60%), followed by new cases (20%), and completed treatment (20%). Within the medium level group new cases (22.6%), drug maintenances (22.6%) and completed treatment (22.6%) showed higher numbers of leukemia samples than relapsed cases (14.2%). Within the high level group, new cases showed highest number of leukemia samples (43%), followed by drug maintenance (36%), completed treatment (14%), and relapsed cases (7%) (Table I).

DISCUSSION

Overexpression of the *MDR1* gene is a major problem of cancer chemotherapy. One of the major obstacles to successful treatment is the drug resistance of leukemic cells to chemotherapeutic agents. MDR has been shown to be partly mediated by enhanced expression of the *MDR1* gene, which is expressed widely in healthy tissues and physiologically functions as an ATP-dependent efflux pump. Overexpression of the *MDR1* gene has been well established as the cause of the MDR phenotype in many selected drug-resistant cell lines *in vitro*. In many human cancers, the presence of Pgp/*MDR*1 has been demonstrated using monoclonal antibodies or gene probes (Bourhis *et al.*, 1989; Chan *et al.*, 1990; Noonan *et al.*, 1990). Drug-resistant KB-V1 cells have been shown to express Pgp at high level on their plasma membrane (Schoenlein *et al.*, 1992). Therefore the MDR1mRNA level from KB-V1 cells was used in the same batch as a positive control (100%) and all samples were analyzed by semiquantitative RT-PCR as measured by densitometer scan. Chemotherapuetic agents in the treatment of leukemia have been identified as substrates for Pgp, so it is reasonable that *MDR1* gene is overexpressed in their cells. In this study, we found that drug maintenance and completed treatment showed high levels of MDR1 mRNA. Surprisingly, new cases also showed high MDR1 mRNA levels, perhaps because they were treated with other chemicals before admission. Relapsed cases showed the lowest MDR1 mRNA levels. When different leukemia types (ALL, AML and CML) were observed, the result revealed that the MDR1 mRNA levels in ALL were higher than those of CML and AML. However, this result was inconclusive because more than 70% of samples derived from ALL leukemia patients. Thus, this result contrasts to the previous reports describing higher *MDR1* gene expression detected in AML cases than ALL cases (Fujimaki *et al.*, 2002). The relationship between *MDR1* gene expression and clinical status was more evident in AML than in ALL.

To improve the clinical outcome of leukemia it is essential to develop an effective strategy to reverse this resistance. Scientists are currently trying to identify new compounds as MDR modulators. Curcumin, a major active component of the food flavor turmeric (*Curcuma longa* Linn.), consists of three major active ingredients; curcumin I, curcumin II, and curcumin III. The commercial product which is usually called "curcumin" is a mixture of the three curcuminoids. Curcumin in particular displays numerous biological properties, including antioxidant and anti-inflammatory effects, as well as anti-mutagen and anticancer properties. Moreover, curcumin is also an MDR modulator. The MDR modulator properties of curcumin are found in many reports, including our group (Anuchapreeda *et al.*, 2002; Waiwut *et al.*, 2002; Limtrakul *et al.*, 2004; Cheaware *et al.*, 2004). We found that nontoxic doses of curcumin I increased sensitivity to vinblastine in the KB-V1 cell line, and curcumin I retained the drug in KB-V1 cells more effectively than curcumin II and curcumin III (Limtrakul *et al.*, 2004; Cheaware *et al.*, 2004). Bielak-Zmijewska *et al.* have shown that curcumin is an agent which at a concentration of 50 μM, induces caspase-3-independent apoptosis in many normal and cancer lymphoid cells (Bielak-Zmijewska *et al.*, 2000). According to our preliminary study of the three curcuminoids on patient leukemic cells, curcumin I was also more effective than curcumin II and curcumin III (data not shown). In the present report we used patient leukemic cells from bone marrow to assess the effect of 10 μM curcumin (noncytotoxic dose) on *MDR1* gene expression. The effect of curcumin on patient leukemic cells showed an inhibitory effect in 42% of total samples. One important question is whether hereditary variants of the *MDR1* gene account for the inter-individual variability in the pharmacokinetics and pharmacodynamics of drugs. Mickley has reported the first evidence of polymorphisms in the human *MDR1* gene (Mickley *et al.*, 1998). Single nucleotide polymorphisms (SNPs) in exons 21 and 24 were observed in a population of tumor patients, in drug-resistant cell lines, in cell refractory malignant melanomas, and in healthy volunteers. The transporter polymorphisms can be used to understand the individual variability in drug response in individual patients. Curcumin had effects in all leukemic cells; ALL, AML, and CML (47%, 28% and 33%, respectively). It could not be concluded that curcumin affected ALL more than AML and CML, because the majority of the samples were ALL. Curcumin affected males 35% more than females. Curcumin affected the *MDR1* gene expression in all four clinical cases. New cases and relapsed cases seemed to be more responsive than completed treatment and drug maintenance cases. Thus curcumin may be applied to new cases and relapsed cases, especially in relapsed cases, because it is the major problem in chemotherapy of childhood leukemic patients. *MDR1* gene expression was divided into three groups according to levels (low, medium and high levels). Curcumin decreased MDR1 mRNA in all three of these groups of patient leukemic

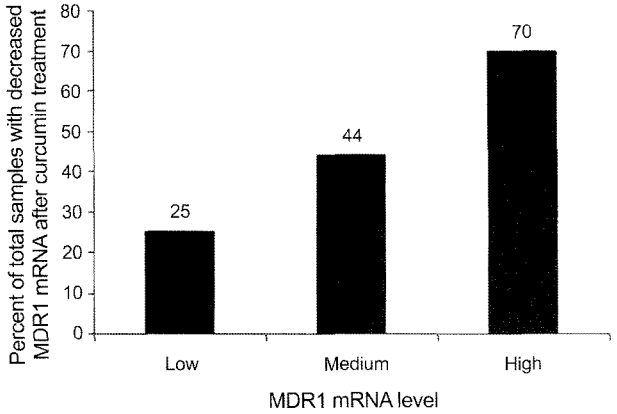

Fig. 3 *MDR1* gene expression in each level (low, medium and high) in leukemic patient cells which were decreased by curcumin (78 samples). The MDR1 mRNAs were determined by RT-PCR and quantitated by scan densitometer. The relative levels of *MDR1* gene expression in each patient cell (defined in KB-V1 cells as 100% expression). PCRs for MDR1 and β-actin were performed 30 cycles.

cells. It is an important new result that curcumin produced a greater decrease of MDR1 mRNA levels in the high and medium level groups than it had in the low level group. In the case of high *MDR1* gene expression level (61-100%), curcumin significantly inhibited *MDR1* gene expression ($p<0.05$). Curcumin slightly inhibited *MDR1* gene expression level in the low MDR1 expression group (1-20%), and medium MDR1 expression group (21-60%), however the data are not significantly different.

In conclusion, the present study suggests that curcumin could be considered as a promising lead compound for the design of more efficacious MDR modulators. Although the present experiments demonstrate that curcumin is an effective inhibitor of *MDR1* gene expression *in vitro*, animal experimentation is required to determine if curcumin has potential as an effective and safe chemosensitizer for treating cancers expressing Pgp-mediated MDR.

Abbreviation

Age-Standardized Rate (ASR) is a summary measure of the cancer incidence rate that a population will have if it has a standard age structure. It is calculated first by estimating the age-specific incidence rates and then applying these rates to the standard population. The world standard population is used in this study. It is expressed in units of incidence per 100,000 population. Standardization is necessary when comparing several populations that differ with respect to age, because age has a powerful influence on the risk of cancer.

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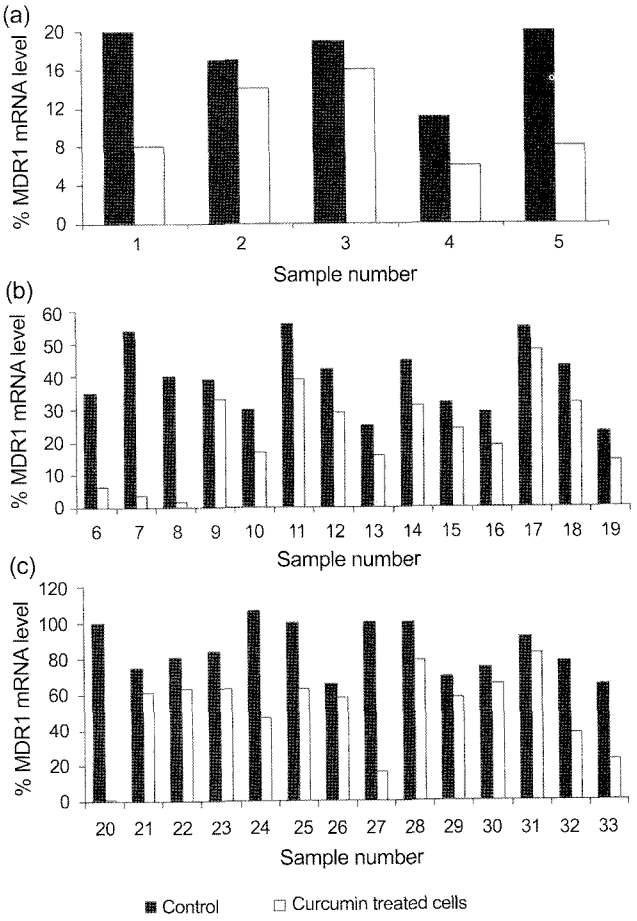

Fig. 4 Sample analysis of the effects of curcumin treatment on MDR1 mRNA in (a) low, (b) medium, and (c) high level group samples of leukemic patient cells. The MDR1 and β-actin mRNA levels following treatment with 10 μM curcumin for 48 h were determined by RT-PCR. The PCR products (283 bp MDR1 and 201 bp β-actin) were run in 1% agarose gel. The bands were quantitated by scan densitometer. *MDR1* gene expression was measured and normalized to β-actin expression. The relative levels of *MDR1* gene expression in each patient cell (defined in KB-V1 cell line as 100% expression).

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