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Establishment of Real-Time Polymerase Chain Reaction Method for Quantitative Analysis of Asparagine Synthetase Expression

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We established a real-time quantitative PCR (RQ-PCR) with which to measure abundance of the asparagine synthetase (AS) mRNA. The level of *AS* mRNA paralleled AS enzyme activity, as well as the AS protein level detected by Western blotting and by *in situ* immunostaining. Cytotoxicity tests *in vitro* showed that the *AS* mRNA level also synchronized with cellular resistance to L-asparaginase in cell lines. Cellular levels of AS enzyme activity correlated with resistance to L-asparaginase. These results indicate that the *AS* mRNA level is an index of resistance to L-asparaginase. RQ-PCR is superior to enzyme assays, Western blotting, and immunostaining in the following ways: less labor and time, accurate and reproducible quantitativity, and broad dynamic range. In addition, RQ-PCR could evaluate differences in L-asparaginase sensitivity although immunostaining could not. And in clinical samples, we analyzed eight pediatric leukemia cases by this RQ-PCR to evaluate whether this method was applicable to clinical laboratories and the expression level of *AS* mRNA in each case were predictable for the effectiveness of L-asparaginase treatment. Consequently, this method was useful enough in defining candidates for selective therapy that targets an AS deficiency. *(J Mol Diagn 2004, 6:217–224)*

Asparagine is not an essential amino acid obtained from outside the body because it is synthesized by using the hydrolysis energy of ATP from aspartic acid and glutamine via asparagine synthetase (AS). Even when the asparagine supply is reduced, normal cells can compensate by synthesizing L-asparagine. However, lymphoblastic cells require external asparagine for growth as they lack sufficient AS activity.[1–3] Thus, L-asparaginase is effective against childhood acute lymphoblastic leukemia (ALL) during the induction of remission or the intensification phases of treatment.[4,5] Asparagine in the blood, cerebrospinal fluid and bone marrow is depleted by L-asparaginase. A reduction of asparagine leads to cell death, since exposure to L-asparaginase *in vitro* induces the fragmentation of DNA and morphological changes typical of apoptosis in a mouse lymphoma cell line[6] and in NIH3T3 cells.[7] An asparagine deficiency can be evoked by the intracellular depletion of glutamate and glutamine.[7] The apoptosis of leukemia cells induced by L-asparaginase may be associated with cell cycle arrest in the G1 phase because DNA strand breaks can be seen in G1 phase cells as early as 8 hours after L-asparaginase exposure.[8] L-asparaginase activates AS expression and the overexpression of human AS protein can induce the L-asparaginase-resistance phenotype.[9] The expression of AS and sensitivity to L-asparaginase were correlated not only in leukemic, but also in ovarian cancer cells.[10] These observations indicate the importance of monitoring AS activity as a marker for clinical decisions regarding L-asparaginase therapy. The present study established real-time quantitative PCR (RQ-PCR) as a method of measuring *AS* expression. We discuss the clinical application of this method compared with immunohistochemistry, Western blotting, and enzyme activity.

Materials and Methods

Cell Lines and Culture Conditions

K562, HL60, U937, and MOLT4 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cells were suspension-cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA) containing 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and penicillin and streptomycin under a 5% CO_2 atmosphere. MOLT4/R[11] and U937/R[1] were established by sequential

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incubation of parental MOLT4 and U937 cells with increasing concentrations (from 10^{-5} to 1.0 U/ml) of L-asparaginase. After 2 months, they were kept in the medium containing 1.0 U/ml of L-asparaginase. Cells were subjected to following analyses under logarithmically confluent growing.

Patients and Isolation of Leukemic Cells

The study included four patients with childhood ALL aged from 4 to 15 years and four with childhood acute myeloblastic leukemia (AML), aged from 2 to 15 years. All patients were diagnosed based on French-American-British (FAB) classification[12] and World Health Organization (WHO) classification[13] at the Shiga Medical Center for Children between 1998 and 2002. All clinical samples were obtained under informed consents of the patients or their guardians. The local ethics committee at Shiga Medical Center Hospital approved the study. Of the four patients with ALL, one had FAB-L1, two had FAB-L2, and one had mixed lineage leukemia with a biphenotype (AMLL).[14,15] All patient samples subjected to this study contained more than 70% blasts in bone marrow.

Heparinized bone marrow samples were obtained by aspiration, placed on ice, and immediately transported to the laboratory for RNA extraction. Mononuclear cells were isolated by density gradient centrifugation with Ficoll-Paque (Amersham, Piscataway, NJ) in phosphate-buffered saline (PBS).

Western Blotting

We used here a monoclonal antibody against AS, which was produced by immunizing female BALB/c mice with the purified human asparagine synthetase (rHAS) extracted from *Saccharomyces cerevisiae*. Hybridoma cells were produced by fusion of the spleen cells in the ratio of 7:1.[16] The selected hybridoma cells were cloned by the limiting dilution technique and screened by antibody-capturing enzyme-linked immunosorbent assay (ELISA) with the purified rHAS. Subsequently, the 3G6 selected hybridoma cells were grown in Dulbecco's Modified Eagle Medium with high glucose (4.5 g/L) and L-glutamine (Invitrogen) containing 10% FBS (Sigma). Supernatants of these cells were used in the following experiments.[11,17]

We extracted total protein from 1×10^5 cells that were washed three times in cold PBS and lysed in RIPA buffer (150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.9), 0.5% sodium deoxycholate). Aliquots of protein (10 μg) were resolved by electrophoresis on 10% polyacrylamide-SDS gels and blotted onto Hybond-P polyvinylidene difluoride membrane (Amersham) using a mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad, Hercules, CA). Non-specific binding was blocked using Block Ace (Dainippon Pharmaceutical, Osaka, Japan), then the blots were probed using an anti-AS monoclonal antibody. A mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Chemicon, Temecula, CA) was used as internal control in 10% Block Ace for 1 hour at room temperature (RT). The blots were washed and probed with secondary horseradish peroxidase (HRP)-conjugated antibody (Amersham) and visualized using ECL reagents (Amersham). The intensity of the bands was quantified using a Luminous Imager (Aisin Cosmos, Tokyo, Japan).

Enzyme Activity Assay

The activity of AS was measured by the ability of whole cell lysate to convert L-[^{14}C] aspartic acid to L-[^{14}C] asparagine as described.[1,18,19] Whole lysates of cell lines were prepared by two freeze-thaw cycles in homogenizing buffer (10 mmol/L Tris, pH7.5, 5 mmol/L EDTA, 1 mmol/L dithiothreitol, 20% glycerol). The protein concentrations of the samples were determined using the BCA assay (Pierce, Rockford, IL). Homogenates were centrifuged at 30,000 $\times$ *g* for 20 minutes at 4°C. Portions of the supernatants were assayed in a final volume of 0.2 ml containing 140 mmol/L Tris/HCl (pH7.5), 3 mmol/L L-[^{14}C] aspartic acid (7.66 GBq/mmol), 8 mmol/L $MgCl_2$, 8 mmol/L ATP, and 30 mmol/L L-glutamine. After incubation at 37°C for 30 minutes, the reactions were terminated by heating at 60°C for 3 minutes. Under these conditions, the assay was linear for at least 2 hours when up to 1.5 mg of lysate protein was included. Unlabeled asparagine (5 μmol/L) was added, then the mixture was loaded onto an ion exchange column containing AG 1X8 acetate form (Bio-Rad) and eluted with 3 ml of H_2O. The elute (1 ml) was added to 8 ml of scintillation fluid (Nacalai Tesque, Kyoto, Japan) and amounts of ^{14}C were measured in a liquid scintillation counter (Aloka, Tokyo, Japan).

Immunostaining

Specimens of cell lines prepared using Cytospin 3 (Shandon, Pittsburgh, PA) and leukemic bone marrow samples smeared on glass slides were dried and fixed with a methanol and acetone mixture (1:1) for 90 seconds at 4°C. Thereafter, the specimens were incubated at 37°C, washed, inhibited with H_2O_2 and exposed to the primary monoclonal antibody against AS for 16 minutes at 37°C. Slides were stained using an automated immunohistochemistry system (EX-IHC, Ventana Medical Systems, Tucson, AZ) and the DAB Detection Kit (Ventana Medical Systems). The instrument blocked non-specific binding with normal horse serum, applied secondary antibody conjugated to the avidin-biotin peroxidase complex, and visualized cells using 3,3-diaminobenzidine (DAB) as a substrate over a standard period of development.[20] Primary antibody was replaced with non-immune mouse immunoglobulins (Vector Laboratories, Burlingame, CA) diluted to the concentration of the primary antibody as the negative control. Slides were counterstained with hematoxylin, dehydrated, cleared, and cover slipped.

For fluorescence staining, secondary antibody was replaced with FITC-labeled anti-mouse immunoglobulin (Dako, Glostrup, Denmark). Cell nuclei were also stained with propidium iodide (PI, Dojindo, Kumamoto, Japan) in PBS for 20 minutes at RT. The cells were washed twice in TBS and mounted using Vectashield mounting medium

(Vector Laboratories). The fluorescence of FITC and PI was visualized and photographed using an Olympus FV300 laser confocal microscope (Olympus, Tokyo, Japan).

MTT Assay of Cell Lines and Blast Cells from Patients

In vitro sensitivity was tested using slightly modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction.[21] Round-bottomed 96-well microculture plates (Sumilon, Tokyo, Japan) containing 20 μl of L-asparaginase at various concentrations were stored at −20°C. After two washes in RPMI 1640 medium, aliquots of 180 μl of cell suspension (2×10^6 cells/ml) were cultured under the same conditions as the cell lines.[11] Four wells contained medium without drugs or cells as a blank for the plate reader and eight wells contained cells and no drug to measure control cell viability. We tested six concentrations of L-asparaginase ranging from 0.01 to 5 U/ml in duplicate. Plates were incubated under 5% CO_2 for 4 days at 37°C. On day 4, pelleted cells were resuspended in 100 μl RPMI 1640 medium (without phenol red) containing 0.5 mg/ml of the tetrazolium dye MTT (Sigma). After 4 hours incubation, the supernatant was removed and 100 μl of isopropyl alcohol was added to dissolve crystals. The optical density of the wells, which linearly reflects the cell number, was determined using a plate reader (Bio-Rad) at 570 nm with a reference wavelength of 650 nm. The drug concentration lethal to 50% of the cells (ID_{50}) was calculated from the dose-response curves.

Leukemic cells from patients were examined using the MTT assay in a similar manner. After a 4-day incubation in a 5% CO_2 atmosphere at 37°C, the results were not evaluated if the OD in control wells was below 0.050 to avoid inconsistencies.[22]

Reverse Transcription (RT)-RQ-PCR Assay of AS *and* GAPDH *mRNAs*

Total RNA was isolated from the cells described above using QIAamp RNA Blood Mini (Qiagen, Hilden, Germany) as described by the manufacturer. First strand cDNA (15 μmol) was synthesized from 1 μg of total RNA with random hexamer primers using the First Strand cDNA Kit (Amersham).

We performed RQ-PCR analysis using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Sequences of primers and a TaqMan probe labeled with FAM (6-carboxyfluorescein) for *AS* were designed by the Primer Express v1.5 software (Applied Biosystems) based on the GenBank database. These sequences were as follows: forward, 5′- CCT CTC CAG ACA TTT GCA ATT G -3′; reverse, 5′-ACT TCA TCC AGA GCC TGA ATGC -3′; probe, FAM- TGC CAC CTT TCT AGC AGC CAG TAA ATC G -TAMRA. To analyze expression values of *GAPDH*, we applied the Pre-Developed TaqMan Assay Reagents Endogenous Control (Applied Biosystems). Reactions proceeded at least twice in duplicate in 25 μl with about 1.0 μl of cDNA, 12.5 μl of 2X TaqMan Universal PCR Master Mix, 200 nmol/L of TaqMan probe, and 200 nmol/L of forward and reverse primers for each gene. The amplification conditions were an initial 2 minutes at 50°C (to allow UNG to destroy any contaminating templates), 10 minutes at 95°C (to activate the enzyme), followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. Amplification proceeded on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Data were collected and analyzed by Sequence Detector v1.6 software (Applied Biosystems).

To construct standard curves for detection of expression value of each gene, RQ-PCR proceeded using plasmids that included the PCR products of *AS* and *GAPDH*. The purified PCR amplified with the respective primers described above products were directly ligated into the pGEM T-vector (Promega, Madison, WI). The sequences of the cloned plasmids were confirmed. Each plasmid was diluted 10-fold from 10^1 to 10^7 copies/μl as templates. The means of duplicate experimental expression values of *AS* and *GAPDH* were determined based on the respective standard curves. The expression value of *AS* was then divided by that of *GAPDH*, yielding the normalized *AS* expression value (AS/GAPDH).

Statistical Analysis

Correlations among AS enzyme activities, protein amounts, and *AS* expression values in the cell lines were determined using by Pearson's correlation coefficient. If the values were not determined in any categories, statistical analysis was not carried out.

Results

Western Blotting

We tested the sensitivity of Western blotting using a series of 10-fold dilutions of K562 cells. We decided that the K562 cell line was suitable as a standard for calibration curves of Western blotting because of the highest level expression among the parent cell lines. Total protein (10 μg) extracted from K562 cells was designated as 1. The upper column of Figure 1A shows that the intensities of 64-kd bands gradually decreased. This is consistent with the molecular weight estimated from the amino acid sequence of AS. The intensity of bands obtained from above Western blotting was plotted to construct a calibration curve (Figure 1A, lower column).

Total protein extracted from 1×10^5 K562 cells was Western blotted using a monoclonal antibody for AS and GAPDH. The expression level of each cell line was obtained relative to the series of dilutions described above (Figure 1B, upper column). GAPDH expression of all cell lines as internal control showed almost equivalent level, indicating that the same amounts were loaded (Figure 1B, lower column). The HL60 expression level was 48.7 ± 8.8 when K562 was established at 100. The expression

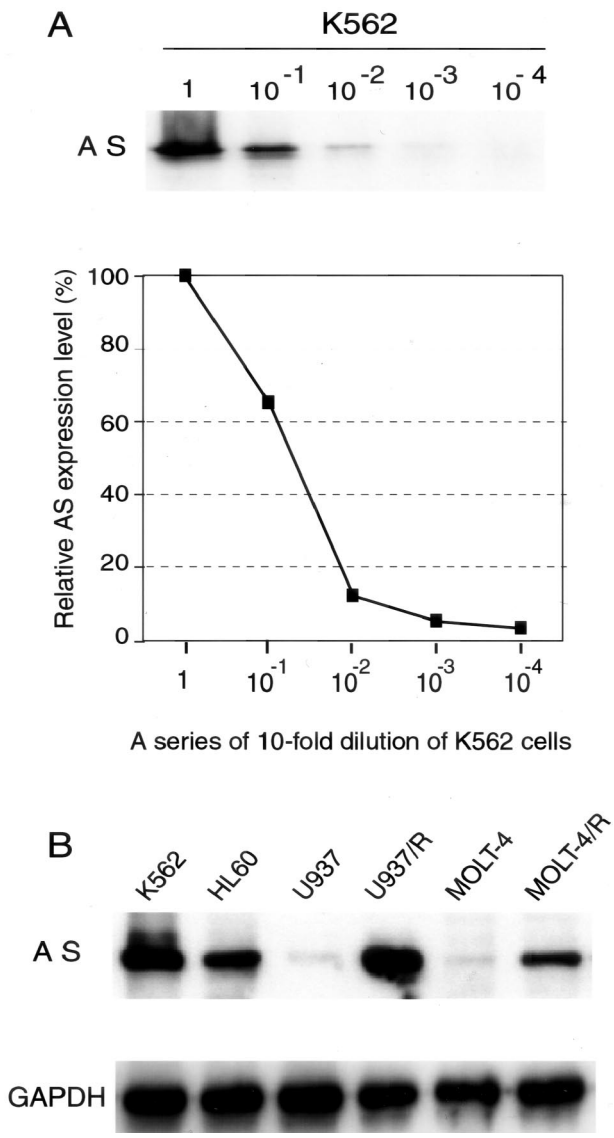

Figure 1. AS protein amounts by Western blotting. **A:** A series of 10-fold dilutions of K562 cells subjected to construct calibration curve. Total protein (10 μg) extracted from K562 cells was designated as 1. Western blotting showed that intensity of 64 kd bands gradually decreased (**upper column**). The intensity of the bands was quantified, and a calibration curve was obtained (**lower column**). **B:** AS and GAPDH expression of each cell line. GAPDH expression of all cell lines as internal controls showed almost equivalent level, indicating that the same amounts were loaded. High-level expression was observed in K562, and intensities of HL60, U937, and MOLT4 gradually reduced in order. Expression level of AS was higher in U937/R and MOLT4/R than that in respective parent cells.

levels of U937 and of MOLT4 were 6.4 ± 2.0 and 0.7 ± 0.5, respectively, whereas that of AS was higher in U937/R (>100) and in MOLT4/R (24.9 ± 3.2) than in the parent cells. The relative value of U937/R could not be calculated since it was expressed at levels higher than those of K562 and which were beyond the possible range of calculation.

Assay of AS Enzyme Activity

The proteins with which to assay the enzyme activity of AS were extracted from the cell aliquots collected for Western blotting (Table 1). When the value of K562 was established at 100, the activity values of HL60, U937, and MOLT4 were 42.6 ± 23.3, 9.1 ± 7.4 and 5.8 ± 4.1, respectively. The activities of AS were higher in U937/R (165.4 ± 49.0) and MOLT4/R (24.3 ± 6.9) than in the parent cells. The correlations were consistent between the expression level analyzed by Western blotting and enzyme activity and the AS enzyme activity significantly correlated with the amount of protein (γ>0.99).

Immunostaining

The intensity of cell lines that were immunostained with DAB was in the order of K562 (+ + +), HL60 (+ +) and U937 (+) (Figure 2, Table 1). Although DAB staining did not detect any signals in MOLT4 (Figure 2), a slight signal was detected by fluorescent immunostaining (Figure 3). The staining of AS was more intense in U937/R (+ + +) and MOLT4/R (+) than in the parent cells. These findings were parallel to those of the AS enzyme activities and the Western blots described above (Table 1).

MTT Assays of Blast Cells from Patients and Cell Lines

By *in vitro* MTT assays, ID_{50} values indicating cellular resistance to L-asparaginase correlated with the expression level of *AS* mRNA in all cell lines (Table 1). The ID_{50} values for 8 pediatric patients with leukemia could be analyzed (Table 2). Clinical and laboratory features of patients are shown with the blast ratios (%) of the samples assayed. We evaluated the correlation between ID_{50} values and following *AS* mRNA expression in these eight cases.

Table 1. *AS* Expression Level and *in Vitro* L-Asparaginase Sensitivity in Leukemia Cell Lines

Cell lines	Relative protein amount (%) (Western blot)	Relative enzyme activity (%)	*AS* mRNA (AS/GAPDH)	Immuno-staining intensity*	ID50 for L-asp (U/ml)
K562	100	100	1.10E-1	+++	>5
HL60	48.7 ± 8.8	42.6l ± 23.3	3.60E-2	++	0.35
U937	6.4 ± 2.0	9.1 ± 7.4	7.90E-3	+	0.125
U937/R	>100	165.4 ± 49.0	3.68E-1	+++	>5
MOLT4	0.7 ± 0.5	5.8 ± 4.1	8.07E-4	−	<0.01
MOLT4/R	24.9 ± 3.2	24.3 ± 6.9	2.00E-2	+	0.2

*+++, Strong; ++, moderate; +, mild; −, negative.

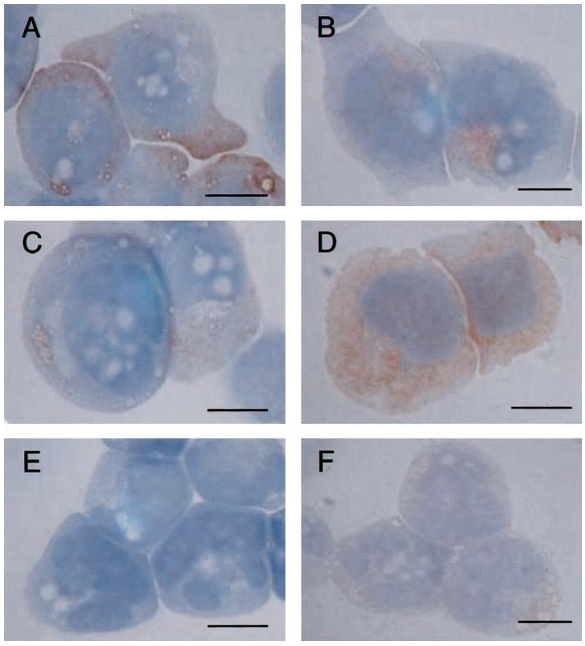

Figure 2. Immunostaining cell lines. **A**, K562; **B**, HL60; **C**, U937; **D**, U937/R; **E**, MOLT4; **F**, MOLT4/R. Signals of each cell line reflect amount of AS protein quantified by Western blotting shown in Figure 1. Signals were undetectable in MOLT4. **Bars**, 10 μm.

RT-RQ-PCR

To prepare standard curves, plasmids including the RT-PCR product of *AS* and *GAPDH* were constructed. The dilution test determined the sensitivity of the assay. Each plasmid was 10-fold diluted. Based on the amplification plots (Figure 4, A and C), linearity remained until a 10^6 dilution according to the calibration curves of both *AS* and *GAPDH* (Figure 4, B and D). Thus, the method could quantify only 10 copies of both genes. The correlation coefficients of both standard curves were above 0.99, indicating that these assays were practical.

We examined the inter-assay reproducibility of both *AS* and *GAPDH*. Each plasmid was serially diluted in a range from 10^7 to 10^1 copies/well to create standard curves that were run at least in duplicate 10 times on different days. An evaluation of the inter-assay variances of the mean Ct showed that those for both *AS* and *GAPDH* were below 4%. We also determined that the intra-assay variances of 10 replicates of the same serial dilutions were below 4% (Table 3).

The normalized *AS* expression value (AS/GAPDH) of each cell line was determined by dividing the expression value of *AS* by that of *GAPDH*. The AS/GAPDH values were relatively higher in K562 (1.10E-1) and in HL60 (3.60E-2), but lower in the monocytic lymphoma cell line U937 (7.90E-3) and in the T-ALL cell line MOLT4 (8.07E-4). The L-asparaginase-resistant cell lines U937/R and MOLT4/R had higher AS/GAPDH values than parent cells (U937/R, 3.68E-1; MOLT4/R, 2.00E-2; Figure 5, Cell Lines). These results were parallel to those of immunostaining and Western blotting. The AS/GAPDH value was correlated with the protein amount ($\gamma = 0.98$) and with enzyme activity ($\gamma = 0.96$).

Table 2 shows the relationship between the level of *AS* mRNA expression and *in vitro* L-asparaginase sensitivity

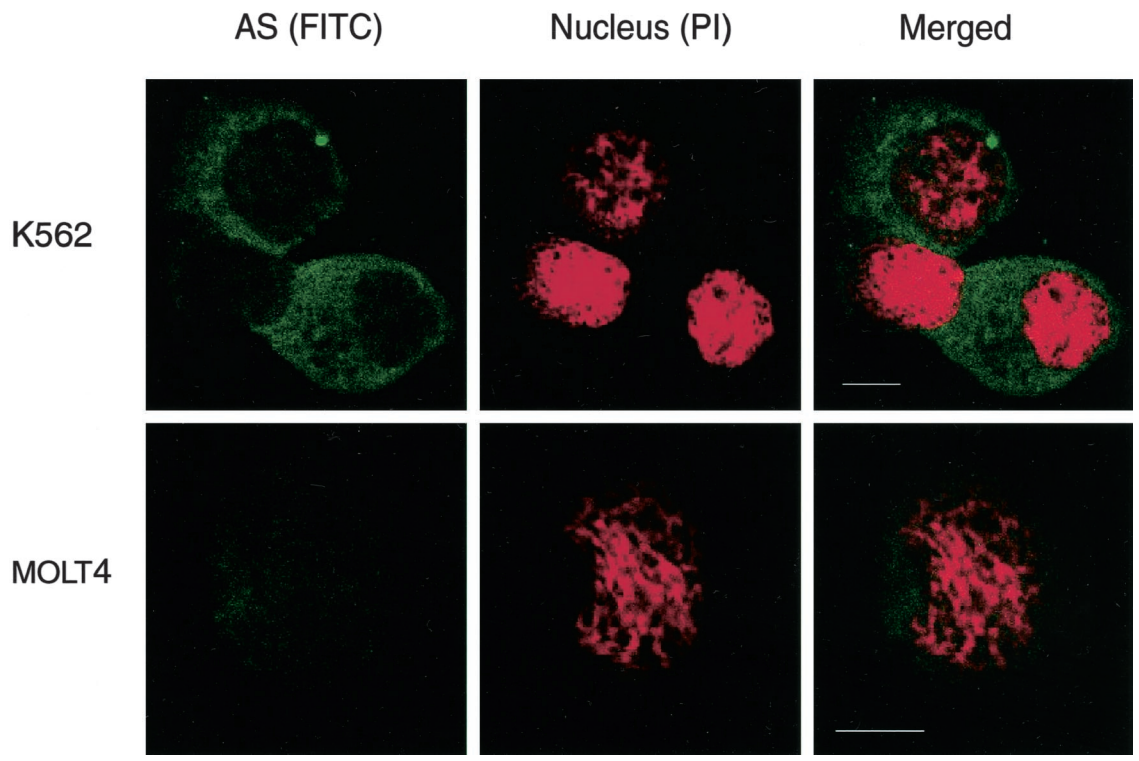

Figure 3. Confocal microscope images of fluorescent immunostaining. Green represents FITC fluorescence indicating AS expression, and red represents PI-stained cell nuclei. K562 cells emit green signals throughout cytoplasm and did not overlap with nucleus region (**top**). In contrast to Figure 2, slight green signals were emitted in MOLT4 cytoplasm (**bottom**). **Bars**, 10 μm.

Table 2. Expression Value of *AS* mRNA and *in Vitro* L-Asparaginase Sensitivity in Pediatric Patients with Leukemia

Patient	Age	Sex	Diagnosis	WBC (/fl)	Blast in BM (%)	ID50 for L-asp (U/ml)	AS mRNA (AS/GAPDH)	Clinical response to L-asparaginase*
1	13	F	ALL-L1	7400	90	<0.01	7.58E-04	CR
2	15	F	ALL-L1	6700	95	<0.01	8.33E-04	CR
3	4	F	ALL-L2	5100	86	0.01	9.46E-04	CR
4	8	M	AMLL†	9700	75	5.0	3.01E-02	NR
5	14	F	AML-M0	5820	70	<0.01	9.46E-04	CR
6	6	M	AML-M2	6460	88	>5.0	2.52E-02	NA
7	15	M	AML-M5	377000	99	0.01	7.52E-04	UK
8	2	F	AML-M7	18900	80	<0.01	2.28E-03	NA

*Clinical response to L-asparaginase is shown whether or not remission was induced by VPL (Vincristine, Predonisolone, L-asparaginase combination therapy for 4 weeks). CR, complete remission means residual leukemic blasts less than 5%. PR, partial remission means residual leukemic blasts more than 5% and less than 25%. NR, no response; residual leukemic blasts more than 25%. NA, not available; L-asparaginase was not employed for first line treatment. UK, unknown; the patient died before remission induction treatment was initiated.

†AMLL, mixed lineage leukemia with biphenotype.

in 8 pediatric patients with leukemia. Table 2 and Figure 5 (Leukemia column) show that the AS/GAPDH values of eight pediatric patients were widely distributed between those of HL60 and MOLT4. When the value of ID_{50} for L-asparaginase was low, that of AS/GAPDH also tended to be low. The ID_{50} values for each patient synchronized with the *AS* mRNA expression levels. Blast cells derived from two patients with AMLL (Table 2, Patient 4) and AML-M2 (Patient 6) with obviously high *AS* mRNA expression were resistant to L-asparaginase *in vitro* even at concentrations of at least 5 U/ml. Findings of 0.01 or less than 0.01 U/ml ID_{50} for L-asparaginase are plotted near the level of MOLT4 (Figure 5, black triangles in Leukemia). Clinical response to L-asparaginase was shown in Table 2. Complete remission was induced in not only ALL but also AML cases with low *AS* mRNA expression.

Discussion

The absence of sufficient AS cellular activity in blast cells compared with normal cells is thought to be the basis of the anti-leukemic effect of L-asparaginase in ALL. Although L-asparaginase is routinely applied in ALL, tools are required to study its role and value in other types of leukemia in more detail. Here, we established an RQ-PCR

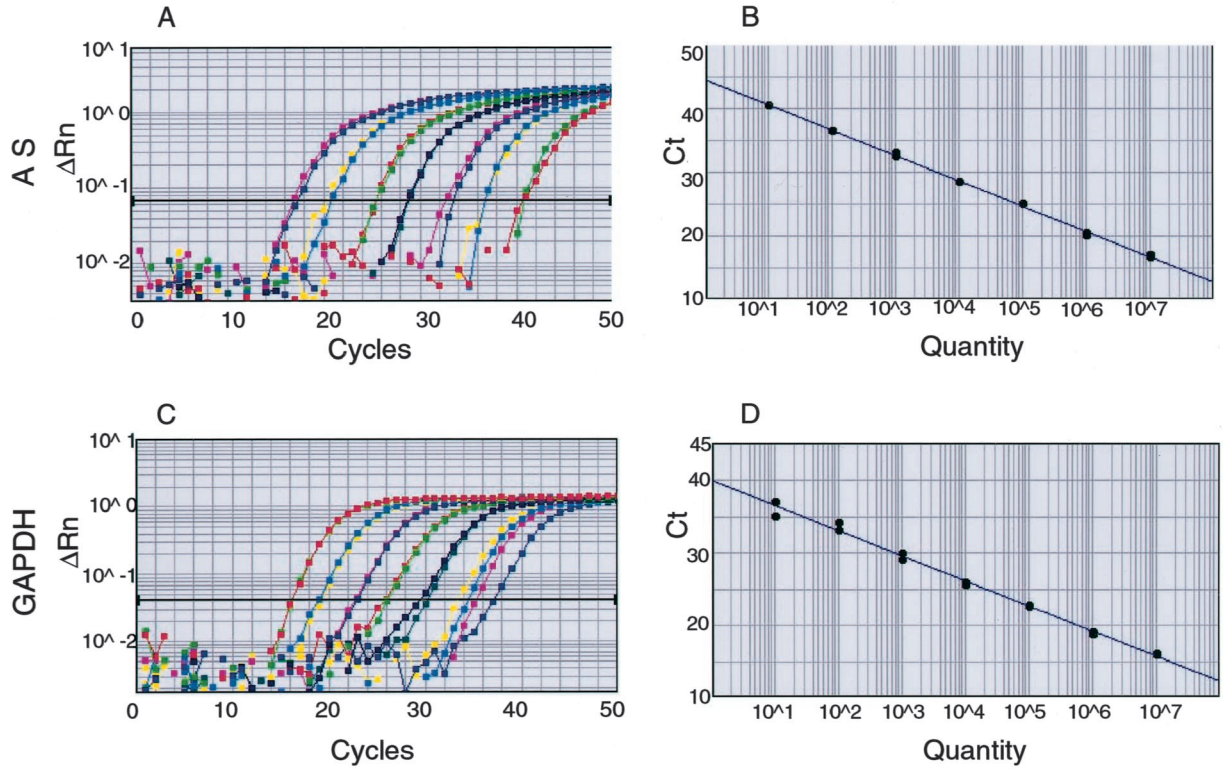

Figure 4. Amplification plots and standard curves of cDNAs for *AS* (**A** and **B**) and *GAPDH* (**C** and **D**) analyzed by real-time PCR. Relative fluorescence (ΔRn) is plotted at numbers of PCR cycles for *AS* (**A**) and *GAPDH* (**C**). Ct (cycle threshold) values were established in logarithmic growth phases, and plotted at each dilution to construct standard curves for *AS* (**B**) and *GAPDH* (**D**). Correlations of *AS* and *GAPDH* were linear with correlation coefficients (γ) of 0.996 and 0.993 respectively, and slope values were −3.96 and −3.45.

Table 3. Inter-/Intra-Assay Variation (%CV) of *AS* and *GAPDH* by RQ-PCR

	Copies/well	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Inter-assay	AS	2.05	1.99	1.85	1.08	1.19	1.86	3.24
	GAPDH	0.84	0.93	0.56	1.05	0.51	1.03	2.50
Intra-assay	AS	1.38	1.51	1.09	1.26	1.78	1.46	3.01
	GAPDH	0.59	0.49	1.23	0.76	0.88	0.63	2.11

CV, coefficient of variation.

method to analyze *AS* expression and we compared this method with semi-quantitative immunostaining, Western blotting, and enzyme activity measurements.

The RQ-PCR method measures PCR product accumulation using a dual-labeled fluorogenic probe. The PCR product is monitored in real time and not only at the end point, thus avoiding the danger of estimating the product at the plateau stage. Moreover, compared with enzyme activity measurements, RQ-PCR is much more specific. The RQ-PCR quantified the template DNA or RNA at the cycle when specific PCR products are first detectable.[23] This allows accurate and reproducible quantitation of gene copies. Post-PCR sample handling is not required, thus preventing potential PCR product carry-over contamination, resulting in faster and higher throughput assays. Because it is highly accurate and less labor intensive, the assay can be applied to many quantitative analyses in the clinical laboratory.

Although post-transcriptional regulation cannot be addressed by this method, this is not true of *AS* mRNA expression and activity. Expression levels of *AS* determined by RQ-PCR correlated with those obtained by immunostaining, Western blotting, and enzyme activity. We conventionally examined AS protein expression in leukemic cells using a monoclonal antibody directed to human AS protein. Immunostaining has been mainly used to determine AS expression levels in individual leukemia blast cells.[24] Generally, this type of method is not practical for quantitative analysis or high-throughput clinical applications. The present study identified a close correlation between the results obtained by RQ-PCR and by immunostaining at a relatively higher expression level. We also detected *AS* expression by RQ-PCR even in a patient with a very low (10^{-4}) AS/GAPDH value. Therefore, measuring *AS* by RQ-PCR is quantitative and linear, with a dynamic range that is wide enough for use in clinical laboratories.

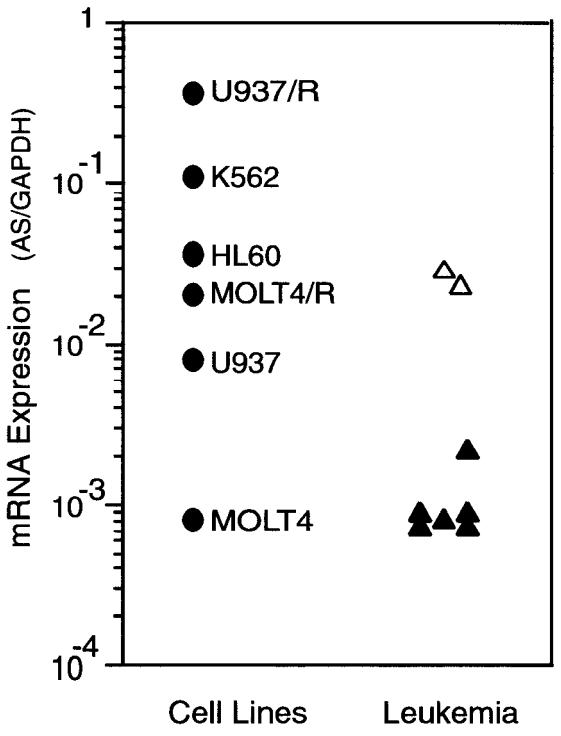

Figure 5. Values of *AS* mRNA expression. Plots of AS/GAPDH ratio. **Black triangles** represent patients with values the ID50 of 0.01 or less than 0.01 U/ml for L-asparaginase. **White triangles** indicate those with values above the ID50 of 0.01 U/ml for L-asparaginase.

In the present study, cell lines that were intensely stained such as K562, HL60, and U937/R also had high AS/GAPDH and L-asparaginase ID_{50} values. In U937 and MOLT4/R, the immunostaining intensity was almost equivalent. However, the AS/GAPDH value of U937 determined by RQ-PCR was about 2.5-fold higher than that of MOLT4/R. This result was parallel to those of the Western blots and the AS enzyme activities. The MTT assay cannot evaluate material from all patients because leukemic cells sometimes die before assays. This assay, as well as Western blotting and enzyme activity measurement is also complex, rendering it unsuitable for routine analyses. Immunostaining alone could not evaluate differences in L-asparaginase sensitivity, whereas RQ-PCR could recognize differences between these two cell lines in detail. Thus, the AS/GAPDH determined by RQ-PCR provides more useful information.

Although we analyzed only a few patients, some of those with AML had significantly low *AS* mRNA levels (Patient 5, 7, and 8 in Table 2). Samples from these patients were also sensitive to L-asparaginase *in vitro*. Complete remission was induced by combination therapy with L-asparaginase in one case of AML as well as in ALL. Dübbers et al[3] have found significantly lower AS activity in B-lineage ALL subgroups as well as in AML-M5. Results of MTT assays indicated that AML-M5 is as sensitive to L-asparaginase as B-precursor ALL.[22,25] We intend to apply RQ-PCR to more clinical patients to evaluate AS expression levels as a useful marker on which to base clinical decisions regarding L-asparaginase therapy.

References

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