Quantification of ALL-Type BCR/ABL Fusion Transcripts in Patients with Chronic Myelogenous Leukemia in Chronic Phase Using Real-Time Quantitative Polymerase Chain Reaction

Akihiko Yokohama,¹ Atsushi Isoda,¹ Hideki Uchiumi,¹

Takafumi Matsushima,¹ Norifumi Tsukamoto,¹ Shunichi Shimano,²

Hirokazu Murakami,³ Masamitsu Karasawa⁴ and Yoshihisa Nojima¹

Background and Aims : A diagnostic feature of chronic myelogenous leukemia (CML) is the presence of BCR/ABL fusion genes. There are two types BCR/ABL transcripts: CML-and acute lymphoblastic leukemia (ALL)-types. ALL-type BCR/ABL transcript is detectable in approximately 50% of CML patients, and its abundance is generally less than that of CML-type BCR/ABL. However, the exact stoichiometry has not been established. In this report, we sought to quantify these two types of BCR/ABL transcripts in patients with CML in chronic phase (CP) by highly sensitive quantitative PCR methods. **Methods :** We examined two types of the BCR/ABL transcripts in seven patients with CML in chronic phase using reverse transcription and real-time quantitative polymerase chain reaction (RQ-PCR). The BCR/ABL expression was normalized to the expression of GAPDH. **Results :** In 5 out of 7 patients, ALL-type BCR/ABL could be detected. The ratio of CML-type BCR/ABL to GAPDH ranged from 4.94×10^{-3} to 81.8×10^{-3} , and that of ALL-type to GAPDH ranged from 0.15×10^{-3} to 0.85×10^{-3} . Thus, ALL-type transcript levels ranged from 1.3 to 54.3% of the CML-type levels. **Conclusions :** In CML patients, expression of ALL-type BCR/ABL was 1 to 2 orders of magnitude lower than expression of CML-type BCR/ABL. (Kitakanto Med J 2003 ; 53 : 115~119)

Key words : CML, real-time PCR, ALL-type BCR/ABL, CML-type BCR/ABL

Introduction

Philadelphia Chromosome (Ph1), t (9 ; 22) (q34 ; q11), is a hallmark for diagnosis of chronic myelogenous leukemia (CML). As a result of reciprocal translocation, t (9 ; 22) (q34 ; q11) generates an abnormal fusion gene, designated BCR/ABL. Compared to the normal ABL protein, the BCR/ABL product has enhanced tyrosine kinase activity, which plays a critical role in leukemogenesis of CML.^{1~3} Since conventional chromosomal analysis occasionally fails to detect Ph1 in CML patients (masked Ph1), more sensitive tests have been recommended to detect

the fusion gene. These include reverse transcriptase polymerase chain reaction (RT-PCR), Southern blot, and fluorescence in situ hybridization (FISH). In the majority of CML patients, the breakpoint in BCR is located in a 5.8kb region known as the major breakpoint cluster (M-BCR).⁴ The resulting BCR/ABL fusions express an mRNA called the CML-type or p210-type transcript. On the other hand, 20% of adult patients with acute lymphoblastic leukemia (ALL) have Ph1 chromosomes, and in half of them, the breakpoints in BCR are in the minor breakpoint cluster region (m-BCR). These fusion genes express the ALL-type or p190-type BCR/ABL mRNA.^{5,6}

¹ Third Department of Internal Medicine, Gunma University School of Medicine, Maebashi, Gunma 371-8511, Japan

² Gunma Cancer Center, Ota, Gunma 373-8550, Japan

³ School of Health Sciences, Faculty of Medicine, Gunma University School of Medicine, Maebashi, Gunma 371-8511, Japan

⁴ Blood Transfusion Service, Gunma University School of Medicine, Maebashi, Gunma 371-8511, Japan

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Address : AKIHIKO YOKOHAMA Third Department of Internal Medicine, Gunma University School of Medicine, Showa-machi, Maebashi, Gunma 371-8511, Japan

Recent reports demonstrated that a few CML patients exclusively express ALL-type BCR/ABL mRNA. The clinical features of these patients were intermediate between CML and chronic myelomonocytic leukemia (CMML).^{7,8} Meanwhile, some patients having clinical features typical for CML were reported to have ALL- and CML-type BCR/ABL mRNAs simultaneously.^{9,10} Thus, the specificity and clinical significance of ALL- and CML-type BCL/ABL remain to be clarified.

Recently, we reported that ALL-type mRNA was detectable in 13 out of 25 CML patients by qualitative RT-PCR,¹¹ which confirmed previous observations by other investigators.¹² However, the abundance of ALL-type BCR/ABL mRNA in CML has been controversial. van Rhee et al reported that expression levels of ALL-type BCL/ABL in CML patients were lower than CML-type by 2.5×10^{-5} – $7.4 \times 10^{-3.9}$. In contrast, CML patients analyzed by Saglio et al expressed ALL-type BCR/ABL at levels 8–32% of those of CML-types.¹⁰ Moreover, CML-and ALL-type BCR/ABL mRNA are reported to be expressed even in some normal individuals.^{13,14} It is plausible, therefore, that non-pathogenic circulating BCR/ABL-positive cells cause a false-positive result in assays of extremely high sensitivity. This presents a problem in evaluating minimal residual disease after chemotherapy or stem cell transplantation for CML. Therefore, an accurate and sensitive quantification of BCR/ABL transcripts is indispensable to diagnose and monitor patients with CML. In this regard, our method in the previous study was only qualitative. To accurately measure the abundance of ALL-type BCL/ABL in CML patients, we developed here a highly sensitive and quantitative method based on real-time quantitative polymerase chain reaction (RQ-PCR).

Material and Methods

RNA preparation and cDNA synthesis

We studied peripheral blood of seven patients with CML in chronic phase (CP) after obtaining informed consent from them. All patients received no treatment prior to the time of sample collection. White blood cells were isolated with 6 % dextran in saline, and total RNA was extracted using RNA-Bee (TEL-TEST, Friendswoods, TX) according to the manufacturer's recommendation. Five μ g of total RNA was reverse-transcribed in 20 μ l reactions containing 100 ng random hexamer, 10mM DTT, 40U RNase inhibitor, 1x RT buffer and 200U Superscripts II Reverse transcriptase (Invitrogen, Carlsbad, CA). Reaction was performed for 60 minutes at 42°C.

Real-time Quantitative PCR and threshold cycle (Ct)

RQ-PCR was performed with an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). PCR was carried out for ALL-or CML-type BCR/ABL, together with GAPDH as an endogenous control to normalize each sample (multiplex PCR). Primers and probes for detecting sequences were designed using Primer Express software version 1.0 (PE Applied Biosystems). Taqman Fluorogenic Probes were labeled at the 3' end with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) and at the 5' end with reporter dye FAM (6-carboxy-fluorescein) by PE Applied Biosystems. Pre-Developed Taqman Assay Reagent for GAPDH (PE Applied Biosystems) contained Taqman Probe, which is labeled with VIC as the reporter dye. All the primers were made by commercial laboratory (SIGMA Genosys Japan, Hokkaido, Japan). The sequences of the primers and the probes were as follows,

b2a2F1 :

5'-TGTGAAACTCCAGACTGTCCACA-3'

b2a2R :

5'-TCAGACCCTGAGGCTCAAAGTC-3'

b2a2Probe :

5'-AGAGCCCTTCAGCGGCCAGTAGCAT-3'

ela2F :

5'-CAGATCTGGCCCAACGATG-3'

ela2R2 :

5'-TGAGGCTCAAAGTCAGATGCTACT-3'

ela2Probe :

5'-TCCATGGAGACGCAGAGCCCTTCA-3'

Reactions of 25 μ l contained 1x Taqman Universal PCR Master Mix, no uracil-N-glycosylase (UNG) (PE Applied Biosystems), 800nM of each primer, 100nM of probe and 2 μ l of sample cDNA, 1.25 μ l of Taqman GAPDH Assay Reagent. After a 2 minute incubation at 4°C and denaturation for 10 minutes at 95°C, the reaction mixtures were subjected to 45 cycles of 15 seconds of denaturation at 95°C and 45 seconds of annealing and extension at 58°C for ALL-type BCR/ABL assay. For CML-type, the annealing and extension step was changed to 1 min at 60°C. We used ela2F primer, ela2R2 primer and ela2 probe for detection of ALL-type, and b2a2F1 primer, b2a2R primer and b2a2 probe for CML-type BCR/ABL. The length of amplicons was 85bp for ALL-type and 149bp (b3a2 type) or 74bp (b2a2 type) for CML-type BCR/ABL. Rigorous precautions were taken to prevent cross-contamination of samples, and all experiments included a negative control of water in place of sample cDNA taken through all stages of the reactions. After the amplification, the threshold cycle (Ct) was set at the middle of the exponential phase of amplification

Table 1 The characteristics of patients

Cane No.	sex/age	Hepatomegaly	Splenomegaly	Hb (g/L)	WBC ($\times 10^9/L$)	Blast+ProM	(%)					Plt ($\times 10^9/L$)	LDH (U/L)	Ph1 (%)
							Sg	Mono	Ly	Ba	Eo			
1	F/19	+	+	95	235	5.5	28.0	0.0	4.0	5.0	3.5	766	3000	100
2	M/44	+	+	129	31.2	0	65.0	6.0	6.0	2.0	1.0	278	646	100
3	M/19	—	+	129	137.4	1.0	44.0	2.0	2.0	2.0	3.0	304	1219	95
4	M/67	+	—	146	31.1	0	75.5	3.0	12.0	5.0	1.0	396	809	100
5	M/19	—	+	125	175	1.0	37.5	4.0	1.0	4.5	4.0	1756	1577	100
6	M/73	—	+	97	206	10.0	40.5	0.0	5.0	9.0	3.5	1027	1942	100
7	F/65	—	+	110	81.8	0	72.0	0.0	7.0	11.0	3.0	643	662	100

on a logarithmic scale. The accumulated fluorescence data were available for calculation of gene expression.

Construction of Standard Curves

The standard samples were prepared by cloning PCR products of GAPDH and CML-type b3a2 BCR/ABL from K562 cells and ALL-type BCR/ABL from a patient with ALL, respectively, that were amplified with the primers described using a GeneAmp PCR System 9700 thermal cycler (PE Biosystems). Each PCR product was inserted into pGEM T easy vector system I (Promega, Madison, Wis.) according to manufacturer's recommendation. Recombinant plasmid was isolated and purified by Plasmid Miniprep kit (Bio-Rad Laboratories, Hercules, CA). The sequence of each insert was confirmed using an ABI 310 autosequencer (PE Applied Biosystems). The copy number of each vector was quantified by ethidium bromide staining after agarose gel electrophoresis. Vectors were serially diluted in distilled water. For the construction of standard curves, 2 μ l of vector at known concentrations was added to 23 μ l PCR reaction mixture containing 1x Taqman Universal PCR Master Mix, no UNG, 800nM of each primer, 100nM of probe, 2 μ l of cDNA from normal peripheral blood or yeast RNA and 1.25 μ l of Pre-Developed Taqman Assay Reagent for GAPDH. Each standard sample was amplified in triplicate.

Calculation of the expression values

The number of BCR/ABL and GAPDH transcripts in samples were determined from the standard curves. Values below the level of the lowest standard were defined as a negative result. All samples were tested in quadruplicate. The numbers of BCR/ABL transcripts were expressed as the ratio to the number of GAPDH transcript.

Results

Patient characteristics

Seven patients were diagnosed with CML in CP from 1999 to 2002, based on myeloproliferative features and the presence of Ph1 chromosomes in their bone marrow. None of the patients received chemotherapy

prior to this study. Clinical characteristics of these patients are shown in Table 1. All patients had CML-type BCR/ABL fusion transcript (b3a2), which was detected with the qualitative PCR used in our previous study.¹¹

Standard Curve of RQ-PCR for ALL- and CML-type BCR/ABL mRNA

A standard curve was established for ALL-type BCR/ABL, CML-type BCR/ABL and GAPDH, based on known concentrations of plasmids bearing each gene. Each curve displayed a linear relation between the Ct and the logarithm of initial template (Fig. 1a, b). The dynamic range of the method spanned five to six orders of magnitude for both the ALL-type and the CML-type assay. The amplification plots of serially diluted BCR/ABL and GAPDH standards represented log increments. Each standard was analyzed in triplicate. Ct was set at the middle of the exponential phase of amplification on a logarithmic scale (Fig. 1c, d). Highly reproducible results were obtained with a minimum of 10 copies of CML-type template and 25 of ALL-type template.

Sensitivity

To determine the sensitivity of our method, we used RNA from K562 cells, which express CML-type BCR/ABL. K562 RNA was serially diluted with RNA of healthy normal peripheral blood before cDNA synthesis. Our method could detect BCR/ABL mRNA from a single K562 cell in a background of 10^5 normal cells. This method was more sensitive than the conventional single-step RT-PCR methods reported previously.¹⁵⁻¹⁷

Quantification of ALL-type and CML-type BCR/ABL mRNA in clinical samples

In seven patients with CML in CP, the ALL-type and CML-type BCR/ABL transcripts were quantified. The results are summarized in Table 2. In 2 out of the 7 patients (patient numbers 3 and 7), the ALL-type transcript was undetectable. The ratio of CML-type BCR/ABL transcript to GAPDH transcript ranged from 1.2×10^{-3} to 81.8×10^{-3} (median 6.29×10^{-3}). Among the 5 patients whose ALL-type transcripts were

Fig. 1 a, b: ALL-type (a) and CML-type (b) BCR/ABL amplification plots of plasmid vector at known copy number. For ALL-type analysis, the concentration of vectors ranged from 25 to 106 copies and for CML-type analysis the concentration ranged from 10 to 10⁶ copies. Each vector at known concentration was amplified in triplicate
c, d: Standard curves for ALL-type (c) and CML-type (d) BCR/ABL by plotting the known concentration versus Ct using 6 standards.

Table 2 Copy number of ALL- and CML-type BCR/ABL

Case No.	ALL-type/ GAPDH($\times 10^3$)	CML-type/ GAPDH($\times 10^3$)	ALL/ CML(%)
1	0.17	4.94	3.4
2	0.15	11.6	1.3
3	—	1.2	—
4	0.17	5.05	3.4
5	7.40	13.7	54.3
6	0.85	6.29	13.4
7	—	81.8	—

detectable, the BCR/ABL to GAPDH ratio ranged from 0.15×10^{-3} to 7.4×10^{-3} (median, 0.17×10^{-3}). The median ratio of ALL-type to CML-type transcripts was 13.4% (range 1.3–54.3%). Thus, the expression of ALL-type BCR/ABL transcripts is 10^1 to 10^2 times lower than that of CML-type.

Discussion

We developed here an accurate and sensitive quantitative method for ALL-type and CML-type BCR/ABL transcripts. In the literature, single-step RT-PCR has been reported to be sufficiently sensitive to detect CML-type BCR/ABL transcripts contained from one K562 cell in a background of up to 10^4 control cells lacking BCR/ABL.^{15–17} In the assay presented here, we achieved 10-fold higher sensitivity by the same measure. Although the reason for the increased sensitivity is unknown, possibilities include the usage of higher amounts (5 μ g) of total RNA, short PCR amplicons (149 and 85bp), and the addition of Taqman probe. Saglio et al also used 5 μ g of total RNA as starting material for reverse transcription and detected ALL-type BCR/ABL by nested PCR in 25 out

of 25 CML patients.¹⁰ We used single-step PCR, since nested PCR could increase the sensitivity but reduce the accuracy of quantification.

Three previous reports described simultaneous expression of CML- and ALL-type BCR/ABL in CML patients. Two of them quantified ALL-type BCR/ABL mRNA, but the values reported differed by more than ten fold. As both results were based on conventional quantitative nested PCR, it is questionable whether they accurately reflect the BCR/ABL expression. In the present study, RQ-PCR was used to overcome this uncertainty. We measured the median ratio of ALL-type BCR/ABL to GAPDH as 0.17×10^{-3} (range 0.15×10^{-3} to 7.4×10^{-3}), and the median ratio of ALL-type to CML-type BCR/ABL as 13.4% (range 1.3–54.3%). This result was close to the 8–32% reported by Saglio et al. Moreover, in their study the ALL-type BCR-ABL fusion protein was also detected by Western blot, which is less sensitive than PCR, suggesting that ALL-type BCR-ABL transcript cannot be very rare. This observation favors our and Saglio's estimation and argues against van Rhee's report that the ratio of ALL-type to CML-type ranged from 3.0×10^{-5} to 7.4×10^{-3} .

The presence of ALL-type BCR/ABL may be associated with some clinical features of CML. Lichty et al reported that, compared with CML patients who had no ALL-type transcripts, co-expressors had significantly higher leukocyte counts and blast cell counts. However, there was no significant difference in disease progression or Sokal scores.¹² In our previous report, ALL-type transcripts tended to be more

prevalent in patients with blastic crisis or in accelerated phase than in chronic phase. Other laboratory data including blood cell counts and NAP scores were not significantly different.¹¹ Since we analyzed here only small numbers of patients, it is hard to determine whether the expression levels of ALL-type or CML-type BCR/ABL are correlated with clinical features.

In summary, we performed quantitative analysis of ALL- and CML-type BCR/ABL simultaneous expression in chronic CML, and demonstrated that the expression of ALL-type transcripts was 10^1 to 10^2 -fold lower than that of CML-type. Further study is needed to test whether such low levels of ALL-type transcripts have any clinicopathological associations.

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