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Original Article

Monitoring Twenty-Six Chronic Myeloid Leukemia Patients by *BCR-ABL* mRNA Level in Bone Marrow: A Single Hospital Experience

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Chronic myeloid leukemia (CML) is caused by the *BCR-ABL* oncogene. The Philadelphia chromosome (Ph) from a reciprocal translocation, t(9;22) (q34;q11) causes a fusion gene, *BCR-ABL*, that encodes a constitutively active tyrosine kinase. Treatment of CML by imatinib is effective to control the tyrosyl phosphorylation of the protein related to the cell signaling. *BCR-ABL* mRNA is overexpressed in the minimal residual disease (MRD), known as an early sign of relapse. Between December 2005 and June 2008, we measured *BCR-ABL* mRNA levels in the bone marrow (BM) from patients by quantitative real-time polymerase chain reaction (RQ-PCR) in Aomori Prefectural Central Hospital. Eighty-six samples from 26 patients were collected. Among the 26 CML patients, 11 patients (42%) were in the pretreatment group. Seven (64%) of the 11 patients achieved complete molecular response (CMR). In the post-treatment group consisting of the remaining 15 patients, 9 (60%) patients achieved CMR. The patients receiving imatinib at a dose over 300 mg per day required 13 (6–77) months [median (range)] to achieve CMR. On the other hand, the patients receiving a dose below 300 mg per day required 29.5 (11–84) months [median (range)]. When *BCR-ABL* mRNA was detected during the treatment course of patients with CMR, careful observation of *BCR-ABL* mRNA was useful for tracking the clinical course of patients. In conclusion, the *BCR-ABL* mRNA level was useful for monitoring the clinical course in 26 patients with CML.

Key words: chronic myeloid leukemia (CML), *BCR-ABL*, minimal residual disease (MRD), imatinib mesylate, real-time quantitative PCR (RQ-PCR)

Chronic myeloid leukemia (CML) is a malignant hematological disorder caused by the *BCR-ABL* oncogene [1]. The Philadelphia chromosome (Ph) results from a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22) (q34;q11) [2]. This translocation causes a fusion gene, *BCR-ABL*, which encodes a constitutively active tyrosine kinase [3]. Historically, interferon α plus cytarabine was a mainstay as a standard therapy for patients with CML who couldn't undergo hematopoietic stem cell transplantation [4, 5]. However, imatinib mesylate (Gleevec®, Novartis Pharmaceuticals, East Hanover, NJ, USA), a selective *BCR-ABL* tyrosine kinase inhibitor (TKI), has

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become the first-line treatment for patients with CML [6–10]. Recently, in addition to imatinib, effective second-line *BCR-ABL* inhibitors have begun to be used for patients who don't respond to imatinib because they are resistant or relapsed. These drugs have increased specificity and potency as *BCR-ABL* inhibitors compared with imatinib [11, 12].

As long-term follow-up for CML therapy with *BCR-ABL* TKI, minimal residual disease (MRD) monitoring is required. MRD is known as an early sign of recurrence and represents the condition with low levels of leukemia cells in the bone marrow. The level of *BCR-ABL* mRNA was reported to reflect the presence of MRD in patients with CML and to be a marker by which physicians can monitor disease status and treatment response, even when the morphological findings indicate complete remission (CR) [13].

We began measuring *BCR-ABL* mRNA in bone marrow (BM) samples using real-time quantitative polymerase chain reaction (PCR) methods in our hospital in December 2005. The purpose of the present study was to investigate retrospectively whether the *BCR-ABL* mRNA value could allow us to monitor patients with CML, using a real-time quantitative PCR technique.

Materials and Methods

Patients and materials. BM samples were collected from outpatients and inpatients diagnosed and treated in the Department of Hematology at Aomori Prefectural Central Hospital from December 2005 to August 2008. The BM samples were collected at diagnosis, during therapy, and during post-therapy follow-up. Most samples were obtained as a part of diagnostic procedures. Eighty-six samples from 26 CML patients were collected.

The 26 CML patients were divided into a pretreatment group of 11 patients and a post-treatment group of 15 patients (Table 1). The pretreatment group received no remission induction therapy at diagnosis. The post-treatment group had already received various types of treatment. The 26 CML patients included 15 male and 10 female patients. Their median age was 56.5 years old (range, 27–77 years). Written informed consent for study participation was obtained from all of the patients. The protocol was approved by the ethical committee of Aomori Prefectural Central Hospital.

Clinical criteria and treatment strategy. For this study, the chronic phase (CP) of CML was defined by the presence of less than 15 percent blasts, less than 20 percent basophils, and less than 30 percent blasts plus promyelocytes in the peripheral blood and marrow.

A complete hematologic response (CHR) was defined as a white blood cell (WBC) count of $< 10 \times 10^9$/L, a platelet count of $< 450 \times 10^9$/L, no immature cells (blasts, promyelocytes, myelocytes) in the peripheral blood, and disappearance of all signs and symptoms related to leukemia. A cytogenetic response was categorized as complete (0% Ph-positive), partial (1–34% Ph-positive), or minor (35–90% Ph-positive). A major cytogenetic response included complete plus partial cytogenetic responses (Ph-positive $< 35\%$). Cytogenetic response was judged by standard cytogenetic analysis. We propose that a reduction in *BCR-ABL* mRNA levels of at least 3 log be used to define a major molecular response. A complete molecular response was defined as undetectable levels of *BCR-ABL* mRNA [13, 14].

Patients orally received imatinib 100–400 mg/day. The imatinib dose was adjusted according to response and tolerance. The change of treatment strategy and the result based on the level of *BCR-ABL* mRNA were observed. Drug toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria (version 2.0).

RNA extraction and cDNA synthesis. The BM from the patients was processed with RBC Lysis Buffer (Roche Diagnostics, Basel, Switzerland). Total RNA was extracted using the High Pure RNA Isolation Kit (Roche), following the manufacturer's instructions. BM was stirred at 25℃ for 10 min, and total RNA was extracted. Quality of the extracted RNA was checked by a spectrophotometer/ optical density instrument, the Gene Quant pro (GE Healthcare Bio-Sciences, Tokyo, Japan). We performed 1% agarose gel electrophoresis of the RNA, For complementary DNA (cDNA) synthesis, reverse transcription (RT) was performed with the Transcripter First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instruction. The synthesized cDNA was stored at −80℃ until use.

Real-time quantitative PCR of BCR-ABL

Table 1 All CML patients and samples

No.	Pre or post treatment	Age	Sex	Phase	First *BCR-ABL* mRNA (copies/μg RNA)	Therapy	Response	Adverse events of imatinib	CTC grade
1	Pre	65	F	CP	21,000	Dasatinib + PSL	CHR		
2	Pre	50	F	CP	21,000	Imatinib 400 mg	CMR (6)	T	3
3	Pre	27	M	CP	10,000	Imatinib 400 mg	CMR (9)	M	1
4	Pre	42	M	CP	9,600	Imatinib 300 mg	CMR (8)	S	2
5	Pre	60	M	CP	11,000	Imatinib 100 mg	CHR		
6	Pre	27	F	CP	15,000	Imatinib 200 mg	CMR (15)	M, S	2, 2
7	Pre	51	M	CP » AP	8,800	Imatinib » BMT	CMR (7)	N	3
8	Pre	73	F	CP	8,800	Imatinib 200 mg	CCR	N, T	2, 2
9	Pre	74	M	CP	28,000	Imatinib 400 mg	–		
10	Pre	51	M	CP	17,000	Imatinib 400 mg	CMR (9)	T	2
11	Pre	35	F	CP	9,200	Ara-C » imatinib 300 mg	MMR	N, T	2, 2
12	Post	66	F	CP	0	Imatinib 400 mg	CMR (13)		
13	Post	54	F	CP	0	Imatinib 400 » 300 mg	CMR (77)	E	2
14	Post	66	M	CP	0	Imatinib 400 » 200 mg	CMR (44)	S	2
15	Post	73	F	CP	0	Imatinib 100 mg	CMR (84)	S	2
16	Post	75	F	CP	0	Imatinib 400 mg	CMR (48)		
17	Post	46	M	CP	0	Imatinib 400 mg	CMR (42)		
18	Post	82	F	CP » BC	19,000	HU + IFN » Imatinib 400 mg	CHR		
19	Post	59	M	CP	0	Imatinib 300 mg	CCR	E	2
20	Post	72	M	CP	4,000	Imatinib 300 mg	CCR	C	1
21	Post	44	M	CP	0	Imatinib 400 mg	CCR		
22	Post	77	M	CP	0	Imatinib 300 mg	CMR (31)		
23	Post	41	M	AP	0	Imatinib 300 mg	MMR	N	3
24	Post	44	M	CP	0	Imatinib 400 » 200 mg	CMR (11)	S	2
25	Post	42	M	CP	0	Imatinib 400 » 300 mg	CMR (25)	M	2
26	Post	61	M	CP	6,500	Imatinib 400 » 300 mg	MCR	S	2

Samples were collected in the Department of Hematology at Aomori Prefectural Central Hospital between October 2005 and July 2008. Twenty-six CML patients were divided into 15 post-treatment patients and 11 pretreatment patients. ( ) indicated months to achieve CMR. AP, accelerated phase; Ara-C, cytarabine; BC, blastic crisis; BMT, bone marrow transplantation; C, elevated creatinine; CCR, complete cytogenetic response; CHR, complete hematological response; CMR, complete molecular response; CP, chronic phase; E, edema; HU, hydroxyurea; IFN, interferon; M, myalgia; MCR, major cytogenic response; MMR, major molecular response; N, neutropenia; T, thrombocytopenia; S, skin lesion; –, no follow up.

mRNA. Real-time quantitative polymerase chain reaction (RQ-PCR) reactions and fluorescence measurements were performed on the LightCycler 2.0 instrument (Roche). A LightCycler primer and probe set (Roche) was used for quantitative assessment. Primers for Human Major bcr/abl (Roche, GenBank Accession: AJ131466) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH: Roche, GenBank Accession: M33197), and LightCyler-FastStart DNA Master Hybridization Probe (Roche) were used. PCR was performed on the LightCycler 2.0 instrument (Roche) according to the manufacturer's instruction. The resulting data were treated with LightCycler Software (Roche).

The normal range of *BCR-ABL* mRNA expression levels was determined in peripheral blood from 34 healthy volunteers with their informed consent and normalized to the GAPDH levels as an internal control. When *BCR-ABL* mRNA expression levels were detected, the samples were judged as positive. All 34 samples from healthy volunteers were negative.

Statistical analysis. All of these analyses were carried out using Microsoft Excel software (Microsoft Corp., Redmond, WA, USA) and JMP®8 software (SAS Institute, Cary, NC, USA). A two-tailed unpaired *t*-test analysis was performed to compare the duration of treatment.

Results

At diagnosis, all patients in the pretreatment group showed an overexpression of *BCR-ABL* mRNA (11/11, 100%). The median value was 11,000 copies/μg RNA, with a range of 8,800 to 21,000 (Table 1). Two (18%), 1 (9%), 1 (9%), and 6 (55%) of them achieved CHR, complete cytogenic response (CCR), major molecular response (MMR), and CMR, respectively, after the start of imatinib therapy (Fig. 1). One patient (No. 9) could not continue imatinib treatment. In the post-treatment group, 1 (6.66%), 1 (6.66%), 3 (20%), 1 (6.66%), and 9 (60%) of the 15 patients achieved CHR, MCR, CCR, MMR, and CMR, respectively.

In the post-treatment group, there were 3 patients (nos. 18, 20, and 26) in whom values of the *BCR-ABL* mRNA were above the cutoff. Patient no. 18 was treated with hydroxyurea and interferon (HU + IFN). *BCR-ABL* mRNA was high after blastic crisis in this patient, in spite of the treatment with imatinib for 5 years. In patient no. 20, the first measurement of *BCR-ABL* mRNA levels was high. CCR but not CMR was achieved due to resistance against imatinib treatment. Additional measurement of this patient will occur in the future. Patient no. 26 achieved MCR at 9 months after the completion of treatment because of bad compliance with imatinib treatment. Patient no. 23 achieved MMR and continued imatinib therapy at a dose of 300 mg. *BCR-ABL* mRNA was elevated to 91 copies/ml in this patient. Also, in patient no. 13, *BCR-ABL* mRNA rose to 100 copies/ml in the clinical course. With careful observation during imatinib treatment, *BCR-ABL* mRNA became negative again in these patients. Maintaining the previous treatment resulted in continued CMR in most patients of the post-treatment group.

The patients receiving imatinib at a dose over 300 mg per day (nos. 2, 3, 4, 7, 9, 10, 11, 12, 13, 16, 17, 18, 19, 20, 21, 22, 23, 25, and 26) were

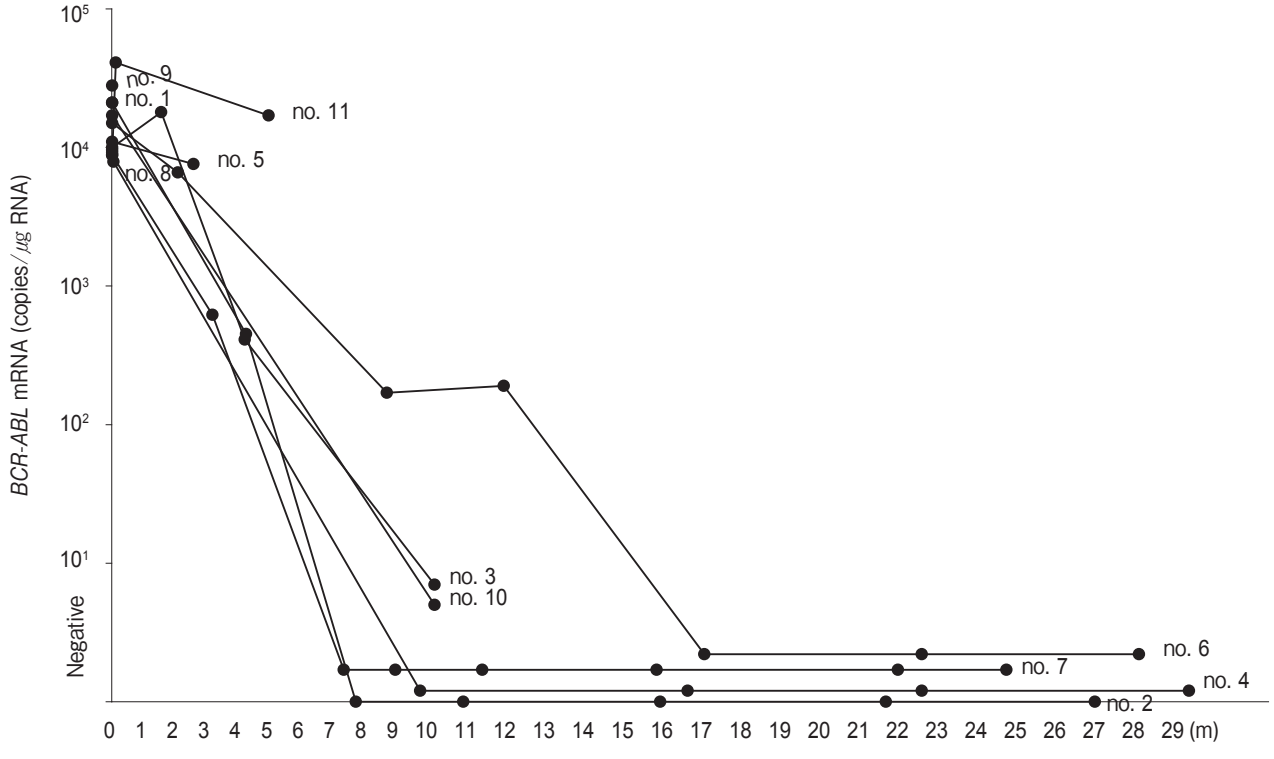

Fig. 1 *BCR-ABL* mRNA levels of 11 pretreatment patients.
Patient no. 2 was treated with 400 mg/day of imatinib, and CMR was continuously achieved. Patient no. 7 received BMT after imatinib therapy. After CMR, the patient was treated with BMT and followed with immunosuppressive agents. *BCR-ABL* mRNA levels remained negative. m: months.

compared with the patients receiving imatinib at a dose below 300 mg per day (nos. 5, 6, 8, 14, 15, and 24). The ratio of CHR, MCR, CCR, MMR, and CMR among the patients receiving over 300 mg of imatinib per day was 5.3% (1/19), 5.3% (1/19), 15.8% (3/19), 10.5% (2/19), and 57.9% (11/19), respectively. One patient (No. 9) could not continue imatinib treatment. In the patients below that treatment level, the ratio of CHR, CCR, and CMR was 16.7% (1/6), 16.7% (1/6), and 66.6% (4/6), respectively. The patients receiving imatinib at a dose over 300 mg per day required 13 (6–77) months [median (range)] to achieve CMR. On the other hand, the patients receiving imatinib at a dose below 300 mg per day required 29.5 (11–84) months [median (range)] to achieve CMR. The effectiveness of imatinib therapy was dose-volume dependent. Unfortunately, there was no significant difference between the 2 groups ($p = 0.384$).

Adverse effects of imatinib therapy in the 26 CML patients are shown in Table 2. For over half of the patients (17/26), the dose of 400 mg/day of imatinib was discontinued because of adverse events. Hematological toxicity was seen in 6 patients (26.1%; nos. 2, 7, 8, 10, 11, and 23). Non-hematological toxicity was seen in 11 patients (42.3%; nos. 3, 4, 6, 13, 14, 15, 19, 20, 24, 25, and 26). In the post-treatment group, some patients required imatinib dose reduction. When *BCR-ABL* mRNA was detected in the treatment course of the patients with CMR, careful observation of *BCR-ABL* mRNA was useful to survey the clinical course of CML patients (Fig. 2).

Table 2 Adverse events of imatinib in 26 CML patients

Events	Cases	CTC grade
Hematological toxicity		
Thrombocytopenia	4	2, 2, 2, 3
Neutropenia	4	2, 2, 3, 3
Non-hematological toxicity		
Elevated serum creatinine level	1	1
Myalgia	3	1, 2, 2
Rash	6	2, 2, 2, 2, 2, 2
Superficial edema	2	2, 2
All	20 (17 patients)	

The dose of 400 mg/day of imatinib was not continued due to adverse events in 14 cases. Hematological toxicity was seen in 8 cases (6 patients, 23.1%). Non-hematological toxicity was seen in 12 cases (11 patients, 42.3%).

Patient no. 1 with CML was treated with dasatinib. This patient was resistant to imatinib as a first regimen with the dosage of 400 mg/day. This change to dasatinib allowed the patient to achieve CHR but not CCR. *BCR-ABL* mRNA was not analyzed again because of the presence of blast cells in the BM analysis for this patient.

Discussion

The efficacy of imatinib has been reported in newly diagnosed CML patients [16, 17]. Frequent monitoring of *BCR-ABL* mRNA is required among the imatinib treatment patients, except in patients remaining susceptible to imatinib for 2–3 years. However, annual rates of treatment failure during the International Randomized Study of Interferon and STI571 (IRIS) were 3.3–7.5% during the first 3 years [18]. Thirty to fifty percent of CML patients require substitute or additional treatment, and the disease statuses of these patients need to be frequently monitored. Frequent monitoring by molecular methods every 2 to 3 months in patients with CCR were reported [19]. In our study, when bone marrow was analyzed, the level of *BCR-ABL* mRNA in BM was simultaneously monitored at an interval of 1–3 months.

BCR-ABL-dependent factors associated with imatinib resistance included a lot of amplification of *BCR-ABL* and the mutation of *BCR-ABL* kinase domain [20–22]. The point mutations can change the structure of *BCR-ABL* kinase protein, which makes imatinib unable to bind to *BCR-ABL* kinase. The T315I mutation gives rise to a highly resistant kinase present in 50–90% of relapsed CML patients [23–26]. The screening for *BCR-ABL* mutations is not necessarily required in patients that respond to imatinib. However, when the effectiveness of imatinib is reduced, or the *BCR-ABL* mRNA is overexpressed at least 2-fold of the MMR, the mutation should be checked [27–29]. In our study, imatinib was not effective in patient no. 1, who was later treated with dasatinib. The mutation should be checked in this patient, but could not yet be done within the timeframe of this study.

BCR-ABL non-dependent factors of imatinib resis-

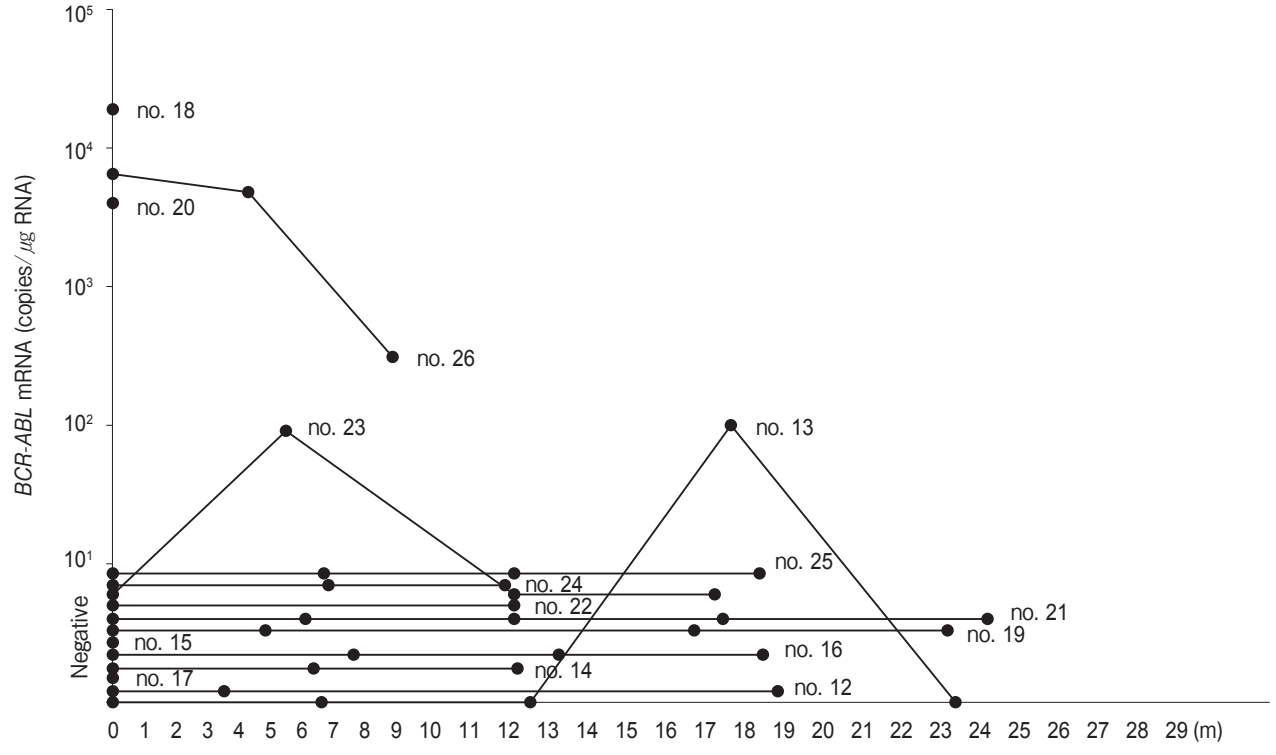

Fig. 2 *BCR-ABL* mRNA levels of 15 post-treatment patients.
In patients no. 23 and no. 13 with CMR, *BCR-ABL* mRNA was detected intermittently. Patient no. 23 achieved CMR and continued imatinib at a dose of 300 mg/day. *BCR-ABL* mRNA was elevated to 91 copies/ml. Also, in patient no. 13, *BCR-ABL* mRNA was elevated to 100 copies/ml during the clinical course. With careful observation during imatinib treatment, *BCR-ABL* mRNA became negative again in these patients. In patient no. 18, *BCR-ABL* mRNA was high after blastic crisis. Patient no. 20 could achieve CCR but not CMR due to resistance against imatinib. Patient no. 26 achieved MCR at 9 months after the imatinib treatment because of bad compliance with treatment. m: months.

tance have been reported, for example, when *p*-glycoprotein encoded by the *MDR-1* gene excretes imatinib and plasma binding of α-1 acid glycoprotein with imatinib prevents the drug from reaching its target protein [30, 31]. Dasatinib (Sprycel®, Bristol-Myers Squibb, Princeton, NJ, USA) has both *BCR-ABL* and *SRC* inhibitory activity. In patient no. 1, who was resistant to imatinib therapy, dasatinib therapy was started and the patient achieved CHR.

Overexpression of *BCR-ABL* mRNA is predictive of a subsequent loss of response, and increased doses of imatinib may be required [20, 27, 32, 33]. In patients with CCR, it is important to achieve CMR by quantifying *BCR-ABL* mRNA levels continuously. RQ-PCR analysis to quantify the amount of CML residual disease, particularly in patients with CCR, showed a 3-log reduction of disease in approximately 40% to 60% of patients [8, 14, 16, 21]. Some studies have associated a two-fold, a 0.5-log, or a 1-log increase of molecular disease with a higher rate of detection of mutations and/or a higher relapse rate. In the present study, 7 patients (nos. 2, 3, 4, 6, 7, 10, and 11) achieved marked reduction of *BCR-ABL* mRNA levels with imatinib treatment. When *BCR-ABL* mRNA exceeds the cutoff level during the clinical course, disease status should be observed more carefully and the treatment strategy might need to be changed.

Frequent monitoring using a sensitive RQ-PCR method is important. However, different sources of samples, different handling procedures of samples, and a mixture of data from different laboratories can confuse the medical staff about the results. In our study, the bone marrow was used as a sample to evaluate the patient at the same time as we evaluated *BCR-ABL* mRNA. In preliminary experiments, there

was no difference in *BCR-ABL* mRNA levels between the marrow and the peripheral blood (data not shown). Our hospital established a technique of *BCR-ABL* mRNA quantification, and this method is more useful than past methods for assessing the absolute quantity of *BCR-ABL*. Standardization is needed to improve the commercial kits in the future, including the selection of the housekeeping gene used as an internal control.

Severe adverse events make some patients reduce the dose of imatinib or stop treatment temporarily, despite the relatively good tolerability of imatinib. Imatinib therapy at a dose over 300 mg/day has better effectiveness than therapy at a dose below 300 mg/day [10]. Reducing the dose to 200 mg/day after achieving CMR has been found to be effective in imatinib-intolerant patients. However, it remains unclear whether low-dose imatinib or treatment interruption after CMR is effective compared to the standard dose in maintaining CMR [15, 21, 31, 34, 35]. Three patients in our study (nos. 6, 14, and 24) were treated with 200 mg/day imatinib. They achieved and maintained CMR. In this context, continuous monitoring of *BCR-ABL* mRNA levels is required.

In the present study, we investigated the clinical utility of *BCR-ABL* mRNA in 26 CML patients. Using our RQ-PCR methods, careful observation of *BCR-ABL* mRNA was useful to survey the clinical course of CML patients.

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