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Retention but significant reduction of BCR-ABL transcript in hematopoietic stem cells in chronic myelogenous leukemia after imatinib therapy

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Abstract

Chronic myelogenous leukemia (CML) is effectively treated with imatinib mesylate (IM), a small molecule inhibitor of the BCR-ABL tyrosine kinase that is expressed in the entire hematopoietic compartment including stem cells (HSC) and progenitors in CML patients. While IM induces disease remission, it does not appear to eradicate BCR-ABL-positive stem cells. We investigated the residual CML cells in HSC and myeloid progenitors isolated using fluorescence-activated cell sorting after IM-therapy. Quantitative real-time polymerase chain reaction detecting *BCR-ABL* transcripts showed that CML progenitors were eradicated within 12 months while the BCR-ABL-positive HSC remained. However, IM-therapy continuation could significantly decrease the ratio of *BCR-ABL* to *BCR* also in the HSC population. Our results implicate that the sorted and purified stem cells are useful for more sensitive quantification of BCR-ABL-positive minimal residual disease.

Keywords

CML; Imatinib; Leukemic stem cells; MRD

1 Introduction

The clinical success of the ABL kinase inhibitor imatinib mesylate (IM) in chronic myelogenous leukemia (CML) serves as a model for molecular targeted therapy of cancer [1–5]. However, despite unprecedented rates of complete cytogenetic response, residual disease remains detectable in the majority of patients [6–8], with disease recurrence upon discontinuation of IM-therapy [9–11]. It has been reported that primitive quiescent, malignant hematopoietic progenitor cells from patients with CML are insensitive to IM [12]. Recently, granulocyte–macrophage progenitors (GMP) with an aberrant potential for self-renewal were detected in CML blast crisis (BC) [7,13], indicating GMP might also function as leukemia stem cells. Mathematical models of clinical response to IM-therapy have also suggested that CML stem cells may be resistant to this drug, thus accounting for the persistence of minimal residual disease and the development of drug resistance [14]. In this study, we investigated the residual disease in hematopoietic stem cells (HSC) and myeloid progenitors from patients with CML chronic phase (CP) after IM-therapy, and show retention but significant reduction of *BCR-ABL* transcript in HSC.

2 Study design

2.1 Patients and evaluation

Patients with a confirmed diagnosis of CML were investigated at indicated points before and after the start of IM-therapy. Bone marrow samples were harvested after written informed consent. Hematologic, cytogenetic and molecular responses were determined according to the European LeukemiaNet recommendations [15]. Briefly, complete hematological response (CHR) was defined as disappearance of signs and symptoms of disease, no splenomegaly, and complete blood counts within institutional normal limits. Complete cytogenetic response (CCR) was defined as 0% Ph metaphases among at least 20 metaphases in the bone marrow. Major molecular response (MMR) was defined either by *BCR-ABL* transcript levels below 100 copy per microgram of RNA quantified with reverse-transcriptase-polymerase-chain-reaction (RT-PCR) or transcription-mediated amplification (TMA) [16], or by 3 log reduction from initial levels at diagnosis [17,18]. Quantification of the *BCR-ABL* transcripts by TMA method was performed using Amp-CML kit (Fujirebio, Tokyo, Japan).

2.2 Separation of HSC and progenitors

For the detection of MRD of HSC or progenitors from CML CP after IM-treatment, the mononuclear cells were freshly prepared within 24 hr after bone marrow harvest. For the detection of *BCR-ABL* and *BCR* transcripts of HSC or progenitors from CML CP before IM-treatment, if the fresh bone marrow samples were not available, frozen cells were thawed and subjected to FACS analysis. Mononuclear cells were stained with lineage-associated PE-Cy5.5-conjugated antibodies including CD2, CD3, CD4, CD8, CD14, CD19, CD20 and CD56 from Caltag (South San Francisco, CA). Flow-cytometric analysis and cell sorting were performed as previously published [12,19]. The cells with the lineage cocktail antibodies were further incubated either with HSC-associated antibodies consisting of APC-conjugated anti-CD34 (HPCA-2; BD Pharmingen, San Diego, CA), biotinylated anti-CD38 (Caltag), FITC-labeled CD47 and phycoerythrin-conjugated anti-CD90 (Thy-1) followed by staining with streptavidin-Cy7PE (Invitrogen, Carlsbad, CA) to visualize CD38-biotin-stained cells or with progenitor-associated antibodies consisting of APC-conjugated anti-CD34, biotinylated anti-CD38, streptavidin-Cy7PE, phycoerythrin-conjugated anti-IL-3 receptor (9F5; BD Pharmingen) and FITC-conjugated anti-CD45RA (MEM56; Caltag).

Unstained samples and isotype controls were included to assess background fluorescence. After staining, cells were analyzed and sorted by using FACSaria (BD Immunocytometry Systems, San Jose, CA). HSC identified as CD34⁺CD38⁻Lin⁻, were separated to Thy-1⁺ (HSC/Thy-1⁺) and Thy-1⁻ (HSC/Thy-1⁻) cells. Common myeloid progenitors (CMP) were identified based on CD34⁺CD38⁺IL-3Rα⁺CD45RA⁻Lin⁻ staining, and their progeny including GMP were CD34⁺CD38⁺IL-3Rα⁺CD45RA⁺Lin⁻, whereas megakaryocyte/erythroid progenitors (MEP) were identified based on CD34⁺CD38⁺IL-3Rα⁻CD45RA⁻Lin⁻ staining [20].

2.3 Quantification of *BCR-ABL* transcripts

RNA was isolated from HSC/Thy-1⁺, HSC/Thy-1⁻, CMP, GMP, or MEP using the RNA STAT-60™ (TEL-TEST, INC. Friendswood, TX), and reversely transcribed into cDNA using TaqMan Gold RT-PCR Kit™ with random hexamers (Applied Biosystems, Foster City, CA). Primers and probes used in this study were described previously as *BCR-ABL* [21], and *BCR* [12]. Quantitative RT-PCR analysis of the expression of *BCR-ABL* and *BCR* was performed with 50 cycles of two-step PCR (15 s at 95°C and 60 s at 60°C) after initial denaturation (95°C for 10 min) using an ABI Prism 7700 Sequence Detector System (Applied Biosystems). *BCR* was used as the control gene and the *BCR-ABL* levels for each sample were expressed as a ratio of *BCR-ABL* to *BCR*.

Quantification standards were prepared by cloning PCR products of *BCR-ABL* and *BCR* from CML samples. Each PCR product was cloned into pBluescript sk(-) vector by the TA cloning method, sequenced and ligated into the same vector. The resulting plasmids were digested with the appropriate restriction enzymes and used for stable standards to keep the same copy number of *BCR-ABL* and *BCR*.

3 Results and discussion

3.1 Analysis of HSC and progenitor profiles

FACS analysis revealed higher levels of the HSC/Thy-1⁻ cells and progenitors (CD34⁺CD38⁺Lin⁻ cells) in bone marrow from patients with CML CP than in normal bone marrow although the level of long term HSC (HSC/Thy-1⁺) in CML CP is similar with normal bone marrow (Fig. 1a). The proportion of HSC/Thy-1⁻ within stem cells (CD34⁺CD38⁻Lin⁻) was expanded in CML CP (Fig. 1b). After the IM-therapy the proportion of progenitor pools (CD34⁺Lin⁻ cells) within Lin⁻ were remarkably reduced, especially that of HSC/Thy-1⁻ cells and progenitor cells. They are even significantly lower compared with

their counterparts in normal bone marrow. This would seem to indicate that imatinib suppresses progenitor pools more than matured pools of hematopoietic cells. With based on the nucleated cell number of bone marrow samples, it was estimated that the absolute number of HSC/Thy-1⁺ was increased in CML CP, but decreased during IM-therapy (data not shown).

The proportion of MEP was increased and that of GMP was decreased in bone marrow from patients with CML CP as compared with their normal counterparts, which were consistent with previously described results (Fig. 1c) [12,22]. The reason for the reduced proportion of GMP and the increased proportion of MEP in CML CP is not clear. One hypothesis is a differentiation block of late erythropoietic progenitors and a promoted differentiation of GMP with expansion failure as suggested previously [23,24]. Another unique characteristic of CML is an increased proliferation of the granulocytic cell lineage with marked basophilia and eosinophilia. The detection of their progenitors has been reported [25,26] and progenitors of basophil and eosinophil have been identified within GMP, whereas GMP are failed to expand in CML. Analysis of individual progenitors may clear the abnormal proliferation and differentiation destined for basophil and eosinophil in future study.

Expanded HSC/Thy-1⁺ and progenitors were inhibited and the ratio of MEP and GMP was normalized after IM-therapy. Increased population of HSC/Thy-1⁺ within HSC in CML CP was also inhibited after IM-therapy. These data suggested that the progenitor pools of CML are expanded in HSC/Thy-1⁺ population.

3.2 Analysis of *BCR-ABL* transcripts in each population after IM-therapy

We compared the *ABL*, *BCR* or *GAPDH* genes as internal control for the quantification and evaluation of *BCR-ABL* mRNA expression in HSC from CML CP before IM-therapy. When we got less number of sorted cells from frozen samples, there were some cases, in which *BCR-ABL* transcript was positive but *ABL* transcript was negative. The transcript level of *GAPDH* was about 20–200 times higher than that of *BCR-ABL*, so that the *BCR-ABL/GAPDH* ratio was significantly affected by the number of sorted cells especially in cases that the sorted cell counts were small. On the other hand, the transcript level of *BCR-ABL* was within 0.2–2 times of *BCR* transcript and the ratio of *BCR-ABL* to *BCR* was not affected by the sorted cell counts. For these reasons, we believed that the *BCR* transcript as an internal control is the best for our study. An another advantage to use the *BCR* is that the level of minimal residual CML cells are able to be estimated as two times of the *BCR-ABL/BCR* ratio, if we assume that the promoter activities of *BCR* and *BCR-ABL* are the same and the mRNA stability of the transcripts are the same.

Quantitative real-time polymerase chain reaction detecting *BCR-ABL* transcripts showed that *BCR-ABL*-positive progenitors in bone marrow were eradicated within 12-month in 5 patients. *BCR-ABL*-positive cells, however, remained in the stem cell population (Fig. 2). They were positive even after achieving undetectable levels of *BCR-ABL* transcript in total RNA isolated from the bone marrow. Differential retention of CML cells in HSC/Thy-1⁺ or HSC/Thy-1⁺ were observed in case 1, 8 or 3, 4, 7, respectively. MRD in HSC/Thy-1⁺ was under the detectable level in case 3, 4, 7 although the MRD in HSC/Thy-1⁺ was positive. In those cases, HSC/Thy-1⁺ population was much smaller than HSC/Thy-1⁺ population as the mean proportion of sorted cell number of HSC/Thy-1⁺ to that of HSC/Thy-1⁺ was 0.24 (SD = 0.13) (Supplementary Table 1). When MRD in stem cell was small, the detected MRD in stem cell population in patient with MMR was sometimes on border line of our technical sensitivity, so that MRD in HSC/Thy-1⁺ might become under the detectable level due to the paucity of the sorted cells in these cases.

3.3 Ratio of *BCR-ABL/BCR* transcripts in each population after IM-therapy (Fig. 2)

The ratio of *BCR-ABL* and *BCR* was significantly decreased with the continuation of imatinib, however the retention of *BCR-ABL*-positive cells was observed in HSC/Thy-1[−] or HSC/Thy-1⁺ populations except case 9. In case 6, residual CML was still detected in all populations after 50 months of IM-therapy although the *BCR-ABL/BCR* ratio was significantly lower. The *BCR-ABL/BCR* values are known to correlate with the percentage of the Philadelphia chromosome in matched bone marrow samples [9–11]. We also examined *ABL* transcript as an internal control. However, in the case that we got less number of sorted cells after IM-therapy, we could not detect *ABL* transcript even if we could detect *BCR-ABL* transcript (data not shown).

It has been reported that primitive quiescent leukemic progenitor cells of CML patients are insensitive to IM [27], and Ph positive cells are persistent in the bone marrow of CML patients even in complete cytogenetic remission following IM-therapy [7,13]. Mathematical models of clinical response to IM-therapy have also suggested that CML stem cells may be resistant to this drug [28]. However, there have been no reports investigating the direct quantification of Ph positive stem cells in bone marrow during IM-therapy. We purified individual HSC and progenitors populations using fluorescence-activated cell sorting and quantified the ratio of *BCR-ABL/BCR*, which correlates closely with the CML cell ratio in the bone marrow. The *BCR-ABL*-positive cells were always detected in progenitor pools even after the CCR was achieved. When the MMR was achieved, the retention of CML remained in the HSC. The retention in HSC was observed both in Thy-1⁺ and Thy-1[−], or each individual population. Recently, this Thy-1[−] population in human cord blood was detected as candidate human multipotent progenitors (MPP). However, we could not determine the role of Thy-1 expression in residual CML cells. Importantly, IM-therapy continuation could significantly decrease the ratio of *BCR-ABL* to *BCR* also in the HSC population. In this report, we demonstrated the sorted and purified stem cells are useful for more sensitive quantification of *BCR-ABL*-positive minimal residual disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Analysis of the proportion of HSC and progenitors. (a) Mean proportion of HSC/Thy-1⁻ (CD34⁺CD38⁻Thy-1⁻Lin⁻), HSC/Thy-1⁺ (CD34⁺CD38⁻Thy-1⁺Lin⁻), and progenitors (CD34⁺CD38⁺Lin⁻). Percentage of individual population is expressed as the percentage within Lin⁻ cells in bone marrow from 3 control subjects, 14 patients with CML CP, 6 patients with CML BC and 14 samples from 10 patients after treatment with imatinib (CML STI) for 6 months and over. (b) Mean proportion of HSC/Thy-1⁻ within CD34⁺CD38⁻Lin⁻ cells. (c) Mean proportion of myeloid progenitor populations, including common myeloid progenitors (CMP), granulocyte–macrophage progenitors (GMP), and megakaryocyte–erythroid progenitors (MEP). Percentage of individual progenitors within CD34⁺CD38⁺Lin⁻ population

is depicted. *Error bars* represent standard deviation. Statistical analyses were performed with the use of Excel software and Student's two-tailed unpaired *t* test

Case	Age at diagnosis	Sex	Karyotype at diagnosis	Periods of IM-therapy (months)	Clinical response	Frozen or fresh cells	viability (%)	BCR-ABL/BCR					
								Total BM	HSC/Thy-1+	HSC/Thy-1-	CMP	GMP	MEP
1	51	M	46,XY,t(9;22)	0		frozen	24.1	0.23	0.44	0.24	0.69	0.47	0.51
				6	MMR	fresh	85.7	0	0.10	NA	0	0	0
				8	MMR	fresh	85.0	0	0.016	0	0	0	0.0018
				12	MMR	fresh	81.9	0	0.012	0	0	0	0
2	57	F	46,XX,t(9;22)	0		fresh	43.2	0.49	0.27	0.73	0.23	0.52	0.25
				1	CHR	fresh	76.3	0.016	0.10	0.42	0.30	0.032	1.00
				6	MMR	fresh	82.4	0.00040	0.0016	0.0017	0	0	0.0012
3	38	M	46,XY,t(9;22)	0		frozen	64.3	0.58	0.39	0.19	0.83	1.62	0.39
				10	MMR	fresh	79.3	0	0	0.00018	0	0	0
4	55	M	46,XY,t(9;22)	0		frozen	19.9	0.15	0.55	0.66	0.63	0.44	0.72
				54	MMR	fresh	78.3	0.00023	0	0.0026	0	0	0
5	63	F	46,XX,t(9;22)	3	CCR	fresh	56.7	0.013	0.30	0.068	0.15	0	0.17
				6	MMR	fresh	67.3	0	0.0069	0.0012	0	0	0
6	32	M	46,XY,t(9;22)	0		frozen	50.6	0.15	0.23	0.37	0.41	0.32	0.44
				50	MMR	fresh	72.9	0.00063	0.0049	0.0010	0.0012	0.0038	0.00032
7	63	F	46,XX,t(9;22)	0		frozen	24.2	0.68	1.63	1.31	0.70	1.09	0.71
				6	MMR	fresh	60.5	0	0.0028	0.0044	0.0052	0.0081	0.0014
				12	MMR	fresh	49.6	0	0	0.00052	0	0	0
8	43	M	46,XY,t(9;22)	7	MMR	fresh	79.7	0.00004	0.0049	0.017	0.015	0.0036	0
				12	MMR	fresh	59.9	0	0.00016	0	0	0	0
9	25	M	46,XY,t(9;22)	0		frozen	50.6	0.24	0.29	1.31	0.27	0.30	0.36
				18	MMR	fresh	76.6	0	0	0	0	0	0

 >0.1
 >0.01
 >0.001
 >0.0001
 >0.00001
 0 (under sensitivity)

Fig. 2.

Characteristics of the patients and results of quantitative PCR. Patient profiles, the time points of examination, the conditions of samples and the cell viability are shown. Residual CML cells are evaluated by the ratio of *BCR-ABL* to *BCR*. The percentage of cell viability was determined by dividing the number of PI negative cells by the total cell counts. Sorted cell numbers are shown in Supplementary Table 1. *CHR* complete hematological response, *CCR* complete cytogenetic response, *MMR* major molecular response, *NA* not available