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ORIGINAL ARTICLE

Analysis of major BCR-ABL1 mRNA by digital polymerase chain reaction is useful for prediction of international scale

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Abstract

Background Major BCR-ABL1 mRNA in patients with chronic myeloid leukemia (CML) has generally been analysed by real-time polymerase chain reaction (PCR). Application of the international scale (IS) for the quantification of major BCR-ABL1 mRNA has been recommended in several sets of guidelines, including those of the European LeukemiaNet. The aim of this study was to clarify the efficacy of digital PCR technology for the IS of BCR-ABL1 mRNA in the patients with CML by comparing with real-time PCR.

Methods The analysis of BCR-ABL1 mRNA was carried out by the Ipsogen® BCR-ABL1 Mbcr IS-MMR DX Kit (Qiagen), and the QuantStudio 3D Digital PCR System (Thermo Fisher Scientific) using 20 peripheral blood samples obtained from the 9 patients with CML at Sapporo Medical University Hospital.

Results The correlation between the data obtained by digital PCR and by real-time PCR was really high at $R = 0.96$. The detection limit of digital PCR was up to 0.003% and was equal to IS with 0.01% or less in comparison with real-time PCR.

Conclusions Digital PCR technology is promising for predicting the IS value with similar efficacy to real-time PCR and should be useful for simple monitoring of the effects of tyrosine kinase inhibitor (TKI) treatments.

Keywords Chronic myeloid leukemia (CML) · Major BCR-ABL1 · International scale (IS) · Digital PCR · Real-time PCR

Introduction

Major BCR-ABL1 fusion mRNA of Ph1 chromosome in patients with chronic myeloid leukemia (CML) has been measured by quantitative real-time polymerase chain reaction (PCR) and used for monitoring the drug response of tyrosine kinase inhibitor (TKI) [1]. The expression of major BCR-ABL1 mRNA and ABL1 mRNA has generally been analysed with a standard curve using the World Health Organisation (WHO) international standard substances and the international scale (IS) was calculated as follows: (major BCR-ABL1 mRNA expression/ABL1 mRNA expression) × 100 [2]. In the guidelines of the European LeukemiaNet (ELN), the National Comprehensive Cancer Network (NCCN), and the Japanese Society of Hematology (JSH), the application of IS has been recommended [3–5]. However, the distribution of the first WHO International Genetic Reference Panel for quantitation of major BCR-ABL1 mRNA is restricted to laboratories calibrating secondary standards or those supplied with commercial kits. It is not intended for the calibration of in-house assays for local use only. Although it is relatively easy to use WHO secondary standards, it is necessary to acquire laboratory-specific conversion factors to correct for differences between facilities [6], which is time consuming. Therefore, we have to use expensive commercial kits.

In recent years, digital PCR has been used for absolute quantification of genes accurately without requiring a standard curve [7, 8]. In the chip-based digital PCR technology, PCR mixture is added to a chip with 20,000 wells, so that the copy number of target molecule in each well becomes 1 or less, and endpoint PCR is performed using Taqman chemistry. The number of wells with/without positive signals is

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determined from the fluorescence intensity of dye for detecting the target molecule. The final copy number of the chip is calculated by a correction factor using a Poisson model to correct wells containing more than one target molecule. Several reports have been published about the use of digital PCR for detecting BCR-ABL1 mRNA using the QX100 (QX200) Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) or the BioMark Real-Time PCR System with the 12.765 Digital Array (Fluidigm Corporation, South San Francisco, CA, USA) [9–12].

Based on this background, we evaluated the analysis of major BCR-ABL1 using the QuantStudio 3D Digital PCR System (Thermo Fisher Scientific, Waltham, MA, USA) in this study.

Materials and methods

Study population

The retrospective study, "the development of minimal residual disease detection methods by digital PCR", was approved by the institutional review board of Sapporo Medical University Hospital on 2015 [8]. From this study, 20 peripheral blood samples from 9 patients with CML and 20 peripheral blood samples from 20 healthy volunteers as negative control were included. These clinical samples were collected after informed consents. Total RNA was extracted by PureLink® RNA Mini Kit (Thermo Fisher Scientific) and the concentration was determined by a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). The synthesis of complementary DNA (cDNA) was carried out by SuperScript® IV VILO™ Master Mix (Thermo Fisher Scientific). The cDNA reaction mixture (20 µL) included 4 µL SuperScript® IV VILO™ Master Mix, RNA (2.5 µg), and distilled water. The reaction was carried out using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific) under the following conditions: anneal primers at 25 °C for 10 min, reverse transcribe RNA at 50 °C for 20 min, inactivate enzyme at 85 °C for 5 min, followed by cooling step at 4 °C. In advance, an in-house real-time PCR assay was used to confirm positivity for the expression of major BCR-ABL1 in all clinical samples.

Plasmid control

A plasmid control of major BCR-ABL1 was made from the cDNA derived from the K562 cell line purchased from American Type Culture Collection (Manassas, VA, USA), and that of ABL1 was also made from the cDNA derived from peripheral blood of a healthy volunteer. Transformation was carried out by the TOPO® TA Cloning Kit, Dual Promoter, with One Shot TOP10 chemically competent *E. coli* cells (Thermo Fisher Scientific). Using colony selected by blue-white screening using S-Gal®/LB Agar/Kanamycin Blend (Sigma-Aldrich, St. Louis, MO, USA), confirmation of insertion by PCR, culture, and plasmid extraction by QIAfilter Plasmid Kits (Qiagen GmbH, Hilden, Germany) were performed. The plasmid concentration was measured by a Qubit® 3.0 Fluorometer, and the plasmid copy number per litters was calculated, according to the following formula: 6.02×10^{23} (copy/mol) × plasmid DNA concentration (g/L)/plasmid DNA length (bp) × 660 (g/mol/bp) [13]. The range of digital PCR was measured using plasmid control with a plasmid copy number from 10^0 to 10^6. Distilled water was used instead of plasmid in negative control.

Real-time PCR

IS from the patient samples was analysed using the Ipsogen® BCR-ABL1 Mbcr IS-MMR DX Kit (Qiagen), in accordance with the manufacturer's procedures. The amplification and analysis were performed by LightCycler® 480 (Roche Diagnostics GmbH, Mannheim, Germany).

Digital PCR

For primer and probe, the major BCR-ABL1 fusion (assay ID; Hs03024646_ft) and ABL1 (assay ID; Hs01104728_m1) were purchased from TaqMan® Gene Expression Assays (Thermo Fisher Scientific). Digital PCR mixture (15 µL) included 7.5 µL 2 × QuantStudio® 3D Digital PCR Master Mix v2 (Thermo Fisher Scientific), 0.75 µL TaqMan® Gene Expression Assay, 6.75 µL cDNA (from 84.375 to 843.75 ng) and distilled water. By QuantStudio 3D Digital PCR Chip Loader (Thermo Fisher Scientific), the mixture was loaded on a chip of QuantStudio 3D Digital PCR 20K Chip Kit v2 (Thermo Fisher Scientific). For the detection of BCR-ABL1, the chip was amplified using the ProFlex PCR System (Thermo Fisher Scientific) under the following conditions: 96 °C for 10 min, followed by 39 amplification cycles (2 min at 56 °C, 30 s at 98 °C), 2 min at 60 °C, followed by cooling step at 10 °C. Similarly, ABL1 was amplified under the following conditions: 96 °C for 10 min, followed by 39 amplification cycles (2 min at 60 °C, 30 s at 98 °C), 2 min at 60 °C, followed by cooling step at 10 °C. After the amplification, the chip was read using QuantStudio 3D and analysed with QuantStudio 3D Analysis Suite Cloud Software. Using the expression levels of BCR-ABL1 and ABL1 mRNA determined by digital PCR, the result calculated from the following formula (major BCR-ABL1 mRNA expression/ABL1 mRNA expression × 100) was defined as equivalent IS (eIS) in this study.

Results

Measurement of the range in digital PCR

The measurement range of major BCR-ABL1 and ABL1 by digital PCR was analysed using plasmid control with 10^0 to 10^6 plasmid copy number. Distilled water was used instead of plasmid in negative control. Triple measurements were performed twice independently and the results were expressed as mean ± SE. The ranges of major BCR-ABL1 in digital PCR were linear with 10^0 to 10^5 copies, and that of ABL1 were with 10^1 to 10^5 copies (Fig. 1). The experimental value of BCR-ABL1 and ABL1 was underestimated in 10^6 copies. Slight amplified signals of less than 10 copies were confirmed in the negative control (distilled water) of BCR-ABL1 and ABL1.

The correlations between digital PCR and real-time PCR in major BCR-ABL1 and ABL1 expression level

The major BCR-ABL1 expression levels in 20 clinical samples ranged from 4 to 62,488 copies in digital PCR and from 4 to 53,700 copies in real-time PCR (Fig. 2). The median levels of BCR-ABL1 were 1025 copies in digital PCR and 554 copies in real-time PCR. The major BCR-ABL1 expression by digital PCR in the negative control detected noise at 7 ± 4 copies for 20 healthy volunteers, and at 1 ± 2 copies for no template control (cDNA reaction mixture to which distilled water was added instead of RNA). This noise was not detected by the real-time PCR. Similarly, the ABL1 ranged from 17,771 to 123,228 copies in digital PCR and from 16,800 to 59,000 copies in real-time PCR. The median levels of ABL1 were 63,001 copies in digital PCR and 41,400 copies in real-time PCR. The correlation between real-time PCR and digital PCR calculated by Pearson's correlation coefficients (*R*) was 0.93 for the major BCR-ABL1 and 0.89 for ABL1, showing a very strong correlation.

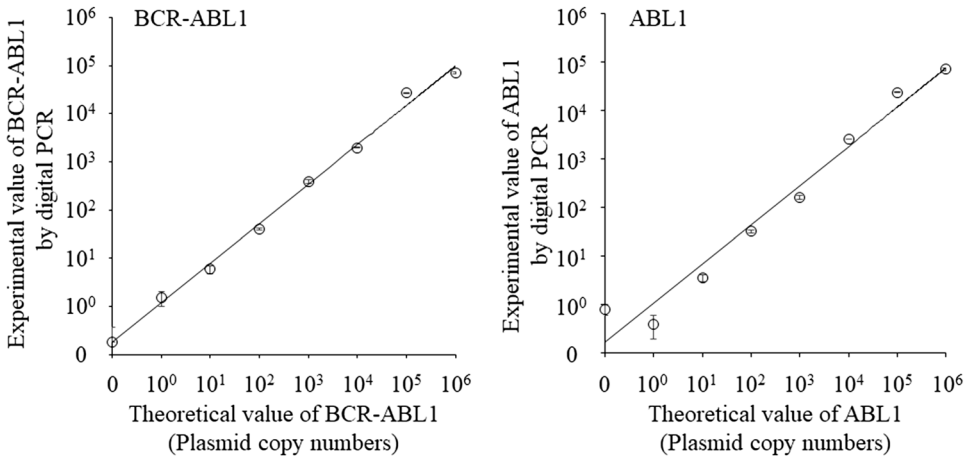

Fig. 1 The measurement range of major BCR-ABL1 and ABL1 by digital PCR using plasmid control with 10^0 to 10^6 plasmid copy number. Distilled water was used instead of plasmid in negative control. Triple measurements were performed twice independently and the results were expressed as mean ± SE

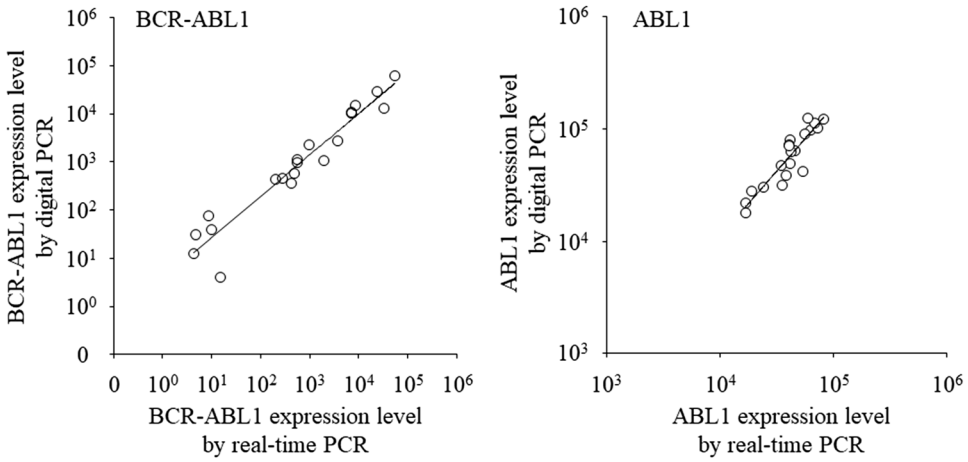

Fig. 2 The correlation between digital PCR and real-time PCR in major BCR-ABL1 and ABL1 expression level ($N = 20$). Pearson's correlation coefficient (R) was 0.93 for major BCR-ABL1 and 0.89 for ABL1

Correlation between eIS by digital PCR and IS by real-time PCR

Using the expression levels of BCR-ABL1 and ABL1 mRNA determined by digital PCR, the result calculated from the following formula (major BCR-ABL1 mRNA expression/ABL1 mRNA expression × 100) was defined as equivalent IS (eIS) in this study. The eIS obtained by digital PCR ranged from 0.003 to 51%, and the IS by real-time PCR ranged from 0.008 to 41% (Fig. 3). The correlation between the eIS and the IS was very high at $R = 0.96$. Two out of 9 patients could be followed up (Fig. 4). The correlations between eIS and IS showed strong at $R = 0.98$ in case 1 and at $R = 0.99$ in case 2.

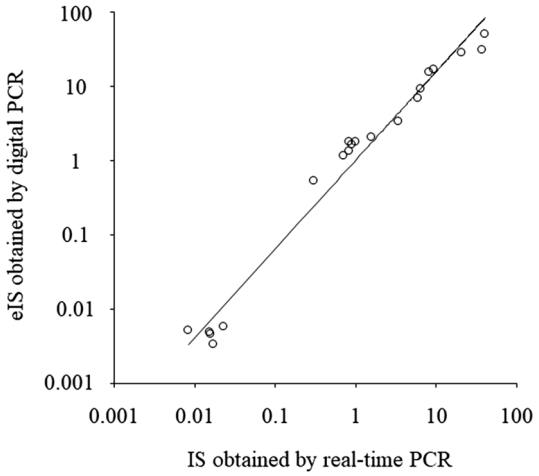

Fig. 3 The correlation between eIS by digital PCR and IS by real-time PCR ($R = 0.96$). Using the expression levels of BCR-ABL1 and ABL1 mRNA determined by digital PCR, the result calculated from the following formula (major BCR-ABL1 mRNA expression/ABL1 mRNA expression × 100) was defined as equivalent IS (eIS)

Discussion

The analysis of major BCR-ABL1 mRNA determined using the QuantStudio 3D Digital PCR System was evaluated in this study. In real-time PCR, there is always a risk that results differ among individuals and facilities due to differences in the efficiency of the reverse transcription, the selection of primer and probe, PCR conditions, or the created standard curve [6]. Since digital PCR can quantify gene expression without the need of a standard curve, it may be useful for reducing these troubles [8]. Reverse transcription reagents, primers and probes used in this study were available for commercial use, and PCR conditions were in accordance with the manufacture's manual. We confirmed that the correlation coefficient between eIS obtained by digital PCR and IS by real-time PCR was high at 0.96. Therefore, digital PCR may be able to predict IS value without difference among individuals and facilities. In the guidelines of the ELN, the NCCN, and the JSH [3–5], it has been recommended that IS of major BCR-ABL1 was used, but this required either the submission of samples to an external clinical laboratory company or the purchasing of an expensive kit. Like this, digital PCR allowed to predict the IS value easily, quickly, cheaply, and accurately.

Accuracy in measuring very low copy number of BCR-ABL1 mRNA is important, since hematologists are now trying to keep the BCR-ABL1 level less than 0.01% in CML

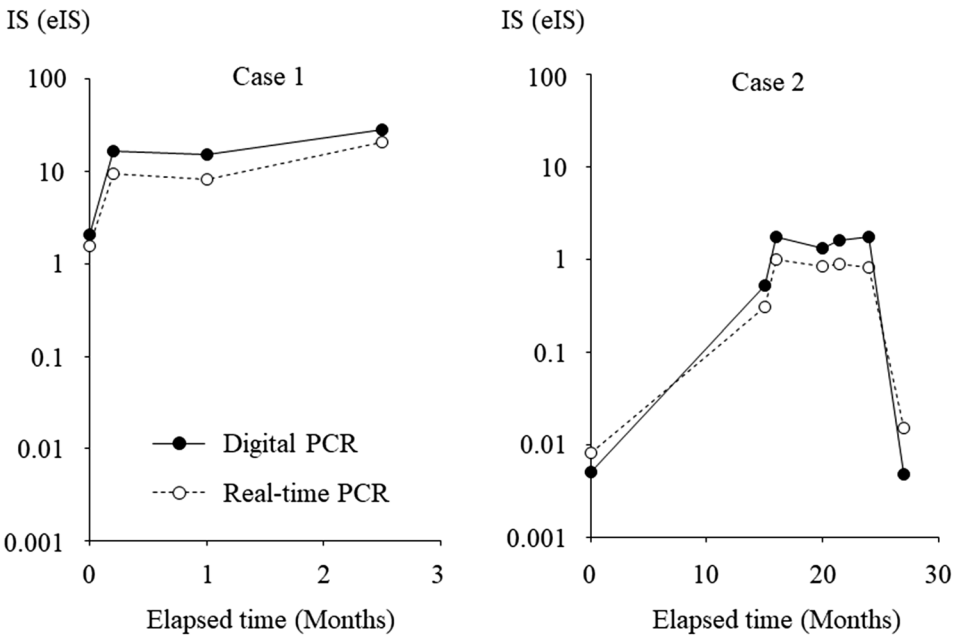

Fig. 4 Follow-up of 2 cases. The correlation between eIS values obtained by digital PCR (closed circle and solid line) and IS by real-time PCR (open circle and broken line) had well at $R = 0.98$ in case 1, and at $R = 0.99$ in case 2

patients by TKI treatments. Using QuantStudio 3D Digital PCR System, BCR-ABL1 mRNA could be detected with high sensitivity up to eIS 0.003%. However, there was divergence in the results obtained by digital and real-time PCR methods at a low level of eIS (IS) in preliminary studies. In the analysis of 84.375 ng of cDNA added to the digital PCR mixture on detection of BCR-ABL1, digital PCR failed to detect the low level of eIS with high sensitivity. Jennings et al. have reported that the limit of detection and limit of quantification in digital PCR are largely determined by the amount of total RNA tested and the number reactions, and IS was detected at up to 0.001% using the QX100 Droplet Digital PCR System [10]. Alikian et al. also have reported that adaptions to the protocol to increase the amount of RNA measured are likely to be necessary to improve the analytical sensitivity of BCR-ABL testing on a digital PCR platform [12]. To overcome this problem, when eIS is less than 0.5, the amount of cDNA added to the digital PCR mixture on the detection of BCR-ABL1 was increased 10-fold from 84.375 to 843.75 ng. As a result of this improvement, the sensitivity of digital PCR increased to the same level as real-time PCR. Sometimes, the amount of cDNA (i.e. the copy number of the target molecules) used for the digital PCR may affect the accurate eIS measurements. That is, the quantification of both BCR-ABL1 and ABL1 was lacking at 10^5 copies or more, and the number of wells with positive signals was approximately 100% (saturated) in the measurement at 10^6 copies. Therefore, when the copy number in the digital PCR mixture is approximately 6667 copies/μL (10^5 copies/15 μL) or more, accuracy is insufficient and re-examination of the diluted sample may be necessary.

Important information regarding drug response is obtained from the major BCR-ABL1 mRNA quantitation between 0.1 and 0.001% on the IS [14, 15]. In this study, only 5 samples could be confirmed. However, eIS and IS were correlated well even if IS was less than 0.1 as shown in case 2 of Fig. 4. Background noise was also detected in negative controls such as healthy volunteers and no template control. In the future, this normal background noise may make it difficult to diagnose samples with BCR-ABL1 expression levels at borderline [12]. Therefore, further research is desired.

In conclusion, digital PCR technology is promising for predicting the IS value with similar efficacy to real-time PCR and should be useful for simple monitoring of the effects of TKI treatments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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