Advantages of digital PCR in the detection of low abundance BCR-ABL1 gene in patients with chronic myeloid leukemia

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Abstract. Quantitative monitoring of BCR-ABL1IS gene using reverse transcription quantitative-PCR (RT-qPCR) is an important method for evaluating the treatment effects in patients with chronic myeloid leukemia (CML). Digital-PCR (dPCR) can be applied to detect the BCR-ABL1 gene with high sensitivity. In the present study, the results of the Clarity™ dPCR system were compared with those of the RT-qPCR in order to determine whether dPCR can be applied in the clinical setting. A total of 83 patients were included in the present study, and they were divided into two groups according to the results of BCR-ABL1IS during ongoing monitoring. A total of 43 patients with undetectable BCR-ABL1IS where enrolled in group A. BCR-ABL1 testing was performed using the dPCR system on the same peripheral blood samples of patients from group A, and the association between dPCR results and relapse was analyzed. The RT-qPCR platform and dPCR system were used simultaneously to detect the BCR-ABL1 gene of another 40 patients who achieved either partial cytogenetic response (PCyR) or further response. Among patients with undetectable BCR-ABL1IS, patients with dPCR-positive disease (BCR-ABL1 >0.1%) were more likely to undergo molecular relapse (P=0.018). The results of dPCR detection of BCR-ABL1% were consistent with the RT-qPCR results (R²=0.9510) in patients who achieved PCyR or further response. For samples with BCR-ABL1IS <1.0%, the consistency of the dPCR and RT-qPCR results was better than that of BCR-ABL1IS >1.0% (R²=0.9488 vs. R²=0.9264 for BCR-ABL1IS). The detection results of the BCR-ABL1 gene in patients with CML using dPCR matched well with those from the RT-qPCR. To conclude, the results of the dPCR system can be applied as a supplement to the RT-qPCR platform, particularly for those with BCR-ABL1IS <1.0%.

Introduction

The BCR-ABL1 gene is a molecular marker of chronic myeloid leukemia (CML), and its transcript level can accurately reflect tumor burden (1). Molecular monitoring refers to the detection of BCR-ABL1 transcripts in the peripheral blood through the use of reverse transcription quantitative-PCR (RT-qPCR) (2,3). In October 2005, an International Standard was proposed for molecular testing, which included changing BCR-ABL1 gene detection in every laboratory via a conversion factor (4). Therefore, BCR-ABL1 gene detection was also converted to the international standard value BCR-ABL1IS in the present study (5,6).

Quantitative monitoring of BCR-ABL1IS via RT-qPCR is currently the gold standard method of evaluating patient response to tyrosine kinase inhibitors (TKIs) and subsequent classification into prognostic subgroups (7). However, RT-qPCR has a number of shortcomings: The difference in amplification efficiency between the reference gene and the target gene, and the difference between platforms and personnel skill level among different laboratories leads to errors in amplification efficiency (8). Therefore, a consistent conversion factor must be established and verified on a regular basis (9).

Digital PCR (dPCR) is used in the detection and quantification of nucleic acids (10). First, dPCR evenly distributes the reaction system into a large number of reaction units, and the number of nucleic acid sequences of interest conforms to the Poisson distribution (7). PCR amplification is then performed independently in each reaction unit. Following the end of the amplification, the fluorescence signal of each reaction unit is detected, and finally the copy number of the nucleic acid sequence of interest is calculated based on the ratio of the Poisson distribution and the reaction unit that is positive for the fluorescent signal in all the reaction units (7). The primary advantage of dPCR over RT-qPCR is that it can be performed without the requirement for a calibration curve,
therefore offering more straightforward means of ensuring interlaboratory reproducibility (9,11).

Materials and methods

Patients and samples. A total of 83 patients were included in the present study, and these were divided into two groups: Groups A and B. All patients were diagnosed in the Department of Hematology of The Affiliated Hospital of Xuzhou Medical University between September 10, 2016 and March 4, 2017. A total of 43 patients with undetectable BCR-ABL1 result from peripheral blood were selected as the group A. The median age of patients in group A was 45 years (age range, 13-72 years), including 22 men and 21 women. Only 25 patients from group A had sokal scores (1). The same blood samples of Group A patients were tested using the Clarity™ dPCR system (JN Medsys). Group B comprised of 40 patients who achieved either cytogenetic response or further response between January 3, 2017 and May 3, 2017. The median age of patients in group B was 49 years (age range, 10-68 years), including 24 men and 16 women. Only 24 patients from groups B had sokal scores. A RT-qPCR platform (Roche Diagnostics) and Clarity™ dPCR system was used to detect the BCR-ABL1 fusion gene within the same peripheral blood sample, simultaneously. There was no BCR-ABL1 kinase domain mutation detected in patients enrolled in the present study. The study protocol was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University, and all patients included in the present study provided written informed consent.

Quantification of the human BCR-ABL1 fusion gene using the Clarity™ dPCR system. DNA was diluted 10- or 100-fold prior to quantifying the human BCR-ABL1 fusion gene using the Clarity™ dPCR system (JN Medsys). A RT-qPCR platform (Roche Diagnostics) and Clarity™ dPCR system was used to detect the BCR-ABL1 fusion gene within the same peripheral blood sample, simultaneously. There was no BCR-ABL1 kinase domain mutation detected in patients enrolled in the present study. The data were analyzed using Clarity™ software (version 1.0; JN Medsys), and a proprietary algorithm was used for setting each threshold based on fluorescent intensities to determine the proportion of positive partitions out of the total. Based on this information, the software determines the DNA copies/µl of the dPCR mix using the Poisson statistics. The mean partition volume of 1.336 nl was used to calculate the copy number. Each dPCR test was performed twice, and the average value was taken as the final result.

Statistical analysis. The results of BCR-ABL1 transcripts were statistically analyzed, with descriptions of the data including the calculation of mediums, ranges, standard deviations (SDs), coefficients of variation (CVs). The association between age and relapse was evaluated using the χ² test. Age and dPCR outcome were assessed via Kaplan-Meier analysis and log-rank test. Linear regression analysis was used to analyze the results of dPCR and RT-qPCR, and R² represents the coefficient of determination. P<0.05 was considered to indicate a statistically significant difference.

Results

Baseline characteristics of patients. In the present study, 39 patients were MR4.5 (BCR-ABL1<0.003 2% or undetectable disease in cDNA over 32,000 ABL1 transcripts) when enrolled in Group A, and 4 patients were MR5. Table II presents the clinical features of Group A patients. The median age of the patients at diagnosis was 45 years (range, 13-72 years). The disease stage of patients in group A was chronic phase, no patients were in accelerated phase or blast phase, and 68.0% of these patients had a low Sokal Score (1). The median white blood cell (WBC) count at the time of initial diagnosis was 62.7x10⁹/l (17.8x10⁹/l-263x10⁹/l), median hemoglobin (HB) level was 94 g/l (53 g/l-145 g/l), median platelet (PLT) count was 414x10⁹/l (153x10⁹/l-2886x10⁹/l). Of the total patients, 37 received hydroxyurea; 41 received TKIs; 3 patients had been treated with interferon; and four had received a hematopoietic stem cell transplantation (HSCT). The median duration of TKI treatment of the patients in Group A was 46.5 months (range, 14.1-149.0 months) prior to enrollment in the present study. When BCR-ABL1 gene was undetectable by RT-qPCR, the maximum of the ABL1 control transcripts was 228,110 copies. dPCR was used to detect peripheral blood samples from patients at the time that their BCR-ABL1 results were undetectable. The % of BCR-ABL1 was between 0.0030 and 9.2390% and the median was 0.0517%. The BCR-ABL1% was 0.1% in 24 patients, and was >0.1% in other 19 patients.

There were two patients already at PCyR when they were enrolled in Group B; 32 patients were CCyR; and 6 patients were MMR. Table II presents the clinical features of Group B patients. The median age at diagnosis was 47 years (range, 10-68 years). The median WBC count at the time of initial diagnosis was 126.7x10⁹/l (10.8x10⁹/l-700.7x10⁹/l); HB level was 97 g/l (70 g/l-140 g/l); PLT count was 445x10⁹/l (184x10⁹/l-1,536x10⁹/l). Of the total patients, 39 received hydroxyurea; 34 received TKIs; three received both TKIs and interferon; 2 patients received a HSCT; and 1 patient was diagnosed in the accelerated phase of disease at the initial diagnosis. The median duration of TKI treatment of patients in Group B was 24.7 months (3.4-127.9 months) prior to enrollment in the present study. When the BCR-ABL1% results detected via dPCR achieved PCyR, the PCyR or MR5 outcome were assessed via Kaplan-Meier analysis. R² represents the coefficient of determination. P<0.05 was considered to indicate a statistically significant difference.

Molecular relapse of Group A patients. RT-qPCR was used to detect BCR-ABL1 level in the peripheral blood of patients...
in Group A every 1-3 months starting from their initial enrollment date in the present study. Molecular relapse was defined as a $BCR-ABL1$ level >0.1%. Relapsed patients ended their follow-up at the time of recurrence. Patients who did not relapse were followed up until October 31, 2018. During the follow-up period, 37 patients received TKIs according to the original protocol; two received both TKIs and interferon; and 4 patients who received HSCT did not take TKIs after the transplantation. At the end of the follow-up, depth of remission in two patients maintained MR5, 21 patients maintained MR4.5, and 20 patients had a molecular relapse. None of the patients included in the present study succumbed. No mutations in the $BCR-ABL1$ kinase domain were detected. The median time to molecular relapse was 11.0 months (range, 3.6-18.4 months). The median follow-up time for all patients was 20.1 months (range, 3.6-25.3 months). Of the 24 patients with $BCR-ABL1$ level <0.1% detected by dPCR, seven relapsed. Of the 19 patients with $BCR-ABL1$ >0.1%, 13 experienced recurrence. (Fig. 1A; $\chi^2=5.560$; $P=0.018$).

The median $BCR-ABL1$ level detected by dPCR system in the relapsed patients was 0.1860% (range, 0.0062-9.2390%), and the median $BCR-ABL1$% in patients without recurrence was 0.0537% (range, 0.0003-1.5323%) ($P=0.080$). Fig. 1B and Table III present relapse by age (≤45 years or >45 years) ($\chi^2=1.773$; $P=0.183$). There was no statistically significant difference in the Kaplan-Meier survival curve (Fig. 1B; $\chi^2=6.731$; $P=0.081$).

### Discussion
At present, RT-qPCR is the preferred method for detecting $BCR-ABL1$ for the initial diagnosis and ongoing monitoring of CML (3,9). However, RT-qPCR can lead to errors in $BCR-ABL1$ detection. As such, appropriate correction factors

#### Table I. Oligonucleotides used for $BCR-ABL1$ amplification.

| Oligonucleotide | Sequence (5’-3’) |
|-----------------|------------------|
| $BCR-ABL1$ forward | TCCGCTGACCACCATCAAAGGA |
| $BCR-ABL1$ reverse | CACTCAGACCCTGAGGCTCAA |
| $ABL$ forward | TGGAGATAACACTTAAGCATAACTAAAGGT |
| $ABL$ reverse | GATGTAGTTGCTTGGGACCCA |
| $BCR-ABL$ probe | CCCCCTAGGCCGAGTGAGCATCTGA |
| $ABL$ probe | CCATTTTGGTTTGGGCTTCACACATT |

Figure 1. Risk of relapse associated with the dPCR results in Group A patients ($P=0.018$). (A) The association between the risk of relapse and age and the dPCR results of Group A patients ($P=0.081$). (B) Relapse was defined as $BCR-ABL1$I5 >0.1%. dPCR+ indicates $BCR-ABL1$ >0.1%; dPCR- indicates $BCR-ABL1$ <0.1%; ▼ indicates censored observations. dPCR, digital PCR.

Comparison of $BCR-ABL1$ detected by RT-qPCR and dPCR in Group B patients. Table IV presents the SD and CV of the $BCR-ABL1$ gene in Group B patients detected by two platforms. The SD and CV of dPCR detection results were lower than RT-qPCR in the two groups with $BCR-ABL1$I5 <1.0% or >1.0%. Scatter plots demonstrate the linear relationship between the quantification of $BCR-ABL1$ transcript copies (green plots), $ABL$ (blue plots) and $BCR-ABL1$ (red plots) (Fig. 2). Quantification of the cDNA derived from clinical samples by RT-qPCR was compared with the dPCR platform. $BCR-ABL1$ transcript copy numbers, $ABL$ transcript copy numbers and $BCR-ABL1$% measured by dPCR platform revealed a good association with RT-qPCR across all sample groups (Fig. 2). Fig. 3 presents $BCR-ABL1$% of the two disease levels ($BCR-ABL1$I5 <1.0% or $BCR-ABL1$I5 >1.0%) and the matched non-CML controls measured by dPCR and RT-qPCR. The samples for blank controls were generated with water instead of cDNA. The non-CML control groups also revealed a positive measurement for $BCR-ABL1$ and can be distinguished from the test for patients with CML.
need to be established and periodically verified (8,9). In the present study, only dPCR could be applied to the detection of *BCR-ABL1* gene, as dPCR is able detect a lower level of *BCR-ABL1* gene than RT-qPCR (8). dPCR has a higher sensitivity, which is an advantage when detecting very low levels of the *BCR-ABL1* gene (12). For molecular response monitoring of rare fusion transcripts associated with CML, dPCR is a very useful tool (13). Patients who intend to discontinue TKIs must achieve deep molecular remission, as when the RT-qPCR result is undetectable, a positive dPCR result may indicate a higher risk of relapse (14). The data from the present study demonstrated that there was no statistically significant difference between the *BCR-ABL1* level detected by dPCR in relapsed and non-relapsed groups (P=0.080), but patients with *BCR-ABL1* >0.1% were more likely to experience molecular relapse (P=0.018), which is consistent with a previous study (14). The results from the present study also demonstrated that patients aged <45 years were more likely to relapse, but this was not statistically significant and therefore further larger scale studies are required (14). In the present study, the molecular relapse rate of patients in Group A was 46.5%, even if they achieved MR4.5 or MR5, suggesting it is necessary to closely monitor the *BCR-ABL1* gene for patients who achieved further MR (3,14). An ideal strategy would be to determine the *BCR-ABL1* level every 1-3 months, in order to expose early molecular relapse (3).

The main advantage of dPCR is that it can be performed without the need for a calibration curve, therefore offering a simpler method of ensuring reproducibility between different laboratories (11). In addition, dPCR can provide greater confidence in detecting low *BCR-ABL1* copy number concentrations at the limits of current RT-qPCR technology (12). Goh et al (15) compared dPCR and RT-qPCR to detect *BCR-ABL1* fusion gene in patients with CML and revealed that its sensitivity was 3 times higher than RT-qPCR. The data from the present study revealed that the results of dPCR for *BCR-ABL1* transcription, ABL1 transcription and *BCR-ABL1*% were in accordance with those of RT-qPCR, and the coherence at *BCR-ABL1* IS <1.0% was better than that at >1.0%. Normalization using the ABL1 gene appeared to lead to error in the results, as there was significant difference between the *BCR-ABL1* level detected by dPCR in relapsed and non-relapsed groups (P=0.080), but patients with *BCR-ABL1* >0.1% were more likely to experience molecular relapse (P=0.018), which is consistent with a previous study (14). The results from the present study also demonstrated that patients aged <45 years were more likely to relapse, but this was not statistically significant and therefore further larger scale studies are required (14). In the present study, the molecular relapse rate of patients in Group A was 46.5%, even if they achieved MR4.5 or MR5, suggesting it is necessary to closely monitor the *BCR-ABL1* gene for patients who achieved further MR (3,14). An ideal strategy would be to determine the *BCR-ABL1* level every 1-3 months, in order to expose early molecular relapse (3).

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generally a better agreement between dPCR and RT-qPCR when measuring BCR-ABL1 absolute values than when measuring ABL1 (7). These results suggest that ABL1 may be a good choice for a reference gene for RT-qPCR, rather than the ideal internal reference gene for dPCR (14).

In conclusion, the detection results of the BCR-ABL1 gene in patients with CML using dPCR apply well with the results obtained by RT-qPCR, particularly in the detection of low abundance BCR-ABL1 gene (BCR-ABL1 IS <1.0%). dPCR has advantages for patients with CML, who have a deep molecular response, as the results of dPCR can be applied as a supplement to RT-qPCR before planning TKIs discontinuation (16).

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZY and QS conceived and designed the study. HZ, YH and QS collected the samples and carried out the research. ZY, HZ, QS, SZ, KZ, QW, JC, MN, JQ and HC analyzed the data and wrote the manuscript. JC and MN revised the manuscript. ZY, QS, JQ and HC participated in the translation of the manuscript. The study was conducted under the guidance of KX, LZ and ZL. All the authors accepted the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Clinical Trials of the Affiliated Hospital of Xuzhou Medical College (approval number, XYFY2015-KL005-01). Certain patients received hematopoietic stem cell transplantation. No organs/tissues were obtained from prisoners. Patients donated hematopoietic stem cells in the Affiliated Hospital of Xuzhou Medical College. The informed consent was signed by all participants.

Patient consent for publication

Written informed consent was provided by all patients.

Competing interests

The authors declare that they have no competing interests.

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