Clinical application of catalytically cleavable fluorescence probe technology for multiplexing quantification of BCR–ABL1 fusion transcripts

Kyoung-Jin Park a,b,1, Young Min Woo a,b,1, Kwangwoo Kim c, Seung-Tae Lee d, Chang-Seok Ki d, Hee-Jin Kim d, Sun-Hee Kim d, Jong-Won Kim d,*

a Department of Health Sciences and Technology, Samsung Advanced Institute for Health Sciences and Technology, Sungkyunkwan University, Seoul, Republic of Korea
b Samsung Biomedical Research Institute, Samsung Medical Center, Seoul, Republic of Korea
c Bio Technology Group, Advanced Technology Center, Samsung Technoin Co., Ltd., Seongnam, Republic of Korea
d Department of Laboratory Medicine & Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea

A R T I C L E   I N F O

Article history:
Received 23 September 2013
Received in revised form 16 October 2013
Accepted 17 October 2013
Available online 25 October 2013

Keywords:
BCR–ABL1
CataCleave probe (catalytically cleavable fluorescence probe)
Chronic myelogenous leukemia
Performance
RT-PCR (reverse transcription—polymerase chain reaction)

A B S T R A C T

Background: Accurate measurement of BCR–ABL1 fusion transcripts is critical for therapeutic stratification in patients with chronic myelogenous leukemia (CML). Previous studies have reported the variable performance of the existing quantitative reverse transcription polymerase chain reaction (RQ-PCR). Here, we developed a one-step multiplex RQ-PCR method based on the catalytically cleavable fluorescence probe technology for quantification of BCR–ABL1 transcripts.

Methods: Performance was evaluated with respect to the limit of detection (LoD), linearity, precision, and comparison on the VBA7 Real-Time PCR system. Multiplex RQ-PCR was performed by the one-step and one-well reaction without the hands-on time.

Results: Our assay showed a LoD of 1.5 pg with linearity in the range of more than 4 logs of dilution. Intraassay, interassay, and total percent CVs at the concentration of 150 ng were 12.8%, 22.6%, and 28.0%, respectively. The assay correlated well with Asuragen’s BCR/ABL1 Quant™ kit over a 6 log concentration range (r = 0.9967).

Conclusion: Our assay demonstrated comparable performance characteristics in comparison with previous RQ-PCR based on the TaqMan probe technology. We conclude that our method could be a reliable tool in the clinical setting.

© 2013 Published by Elsevier B.V.

1. Introduction

Chronic myelogenous leukemia (CML) is characterized by the presence of Philadelphia (Ph) chromosome and the BCR–ABL1 fusion gene. This fusion gene shows constitutive tyrosine kinase activity, which leads to leukemia development [1]. It is also identified in other Ph-positive leukemias such as acute lymphoblastic leukemia (ALL) [2,3]. Previous studies have reported three main subtypes of the BCR–ABL1 fusion gene according to the location of the breakpoints of the BCR gene: major breakpoint cluster region (M-bcr; e14a2, e13a2), minor bcr (e1a2), and micro bcr (e19a2) [3–6]. Furthermore, clinical variability and different prognostic implications among the subtypes according to the breakpoints have been reported [3–7]. Therefore, identification and discrimination of BCR–ABL1 subtypes in a multiplex format could be critical for the clinical management as well as molecular diagnosis of Ph-positive leukemia.

In addition to qualitative detection of BCR–ABL1 subtypes, serial measurement by quantitative reverse transcription polymerase chain reaction (RQ-PCR) is essential for the assessment of treatment response [8–10]. The current practice guidelines of the National Comprehensive Cancer Network recommend regular measurement of BCR–ABL1 mRNA level every 3 months for patients with CML undergoing treatment [10]. The International Randomized Study of Interferon versus STI571 (IRIS) trial has demonstrated that major molecular response (MMR), which is a ≥3 log reduction of BCR–ABL1 transcript level from the baseline level, at 12 months of imatinib has prognostic implications with regard to progression free survival [11]. Furthermore, for monitoring of minimal residual disease (MRD), at least a 4-log reduction below the standardized baseline in 2 consecutive blood samples has been recommended [12,13]. Also, detection of the rising level of BCR–ABL1...
would be an important direct clue of treatment failure such as imatinib resistance. This indicates that measurement of BCR-ABL1 should be performed in a linear range of at least 4 logs and with high precision to guide therapeutic decision. Therefore, more sensitive and precise quantification of BCR-ABL1 fusion transcripts would be a prerequisite for the management of patients with BCR-ABL1 positive leukemia.

Definitive clinical utility of quantitative detection of BCR-ABL1 fusion transcripts has given rise to a number of analytical systems, which have shown significant differences in performance [14–17]. Recently, there has been a study with regard to the performance of Asuragen BCR/ABL1 Quant™ kit (Asuragen Inc.) based on TaqMan probe technology [15]. The kit was developed to simultaneously quantitate three BCR-ABL1 subtypes and ABL1 in a single reaction. In a multiplex quantification format, the assay has analytical sensitivity with at least $10^{-5}$ dilution over a linear range of 6 logs and a total precision of 78% at a concentration of $10^{-5}$ dilution [15]. These performance characteristics are superior to that of other commercially available kits including Cepheid Xpert® BCR-ABL monitor assay, although there are some differences in the analytical system including the evaluation protocol and operator [16,17].

Here, we developed a multiplex RQ-PCR assay using catalytically cleavable fluorescence probe (CataCleave probe) technology [18]. CataCleave probe technology is based on a chimeric DNA–RNA–DNA probe and RNase H, which cleaves specifically only the RNA portion of the DNA/RNA hybrid [18]. The advantage of CataCleave probe over the Taqman probe lies in detection sensitivity in the case of low copies of the target in a multiplex format [18]. The aim of the current study was to evaluate the performance of multiplex RQ-PCR based on CataCleave probe technology with comparison to Asuragen BCR/ABL1 Quant™ kit and to determine whether CataCleave technology could be used in the clinical field of human RNA quantification.

2. Materials and methods

2.1. Samples

A total of 73 samples from patients with BCR-ABL1 positive leukemia were collected for molecular monitoring from March 2012 to September 2012. This study was approved by the Institutional Review Board of Samsung Medical Center. The patient population included 10 samples with minor BCR-ABL1 subtypes and 63 samples with major subtypes. The peripheral blood samples were collected in EDTA tubes and RNA was extracted within 24 h after venipuncture. A BCR-ABL1 positive K562 cell line (Ambion) and a BCR-ABL1 negative HL60 cell line (Ambion) were prepared to evaluate the limit of detection (LoD), linearity, and precision. RNA was extracted using TRIzol Reagent (Invitrogen) and was stored at $-70^\circ$C until analysis. The concentration and purity of the RNA were evaluated using the standard spectrophotometric method and the OD260/280 ratio.

2.2. Primer and CataCleave probe design

For quantification of three BCR–ABL1 transcripts, 5 primers and 2 probes were designed using CataCleave probe technology. For b3a2, b2a2, and e1a2, forward primers were designed to be specific for BCR exon b2 and e1, and the probe consisted of a FAM fluorophore at the 5' end with Black Hole Quencher (BHQ1) at the 3' end (Fig. 1). For ABL1, which was used as the endogenous control, forward primers were designed to be specific for ABL1 exon a2, and the probe was labeled with the TEX 615 fluorophore at the 5' end and BHQ2 at the 3' end (Fig. 1). The sequences of primers and probes include the following: forward primer (FP) for b3a2 and b2a2: 5'-CTGCAGATGCTGACCAACTCGTGT-3', e1a2-FP: 5'-CACACCTACCGATTTCCG-3', ABL1-FP: 5'-GTGACGTTCTGGGCGTCG-3'; reverse primer (RP) for b3a2, b2a2, and e1a2: 5'-GGGTCCAGGAGAGTTTCTTCT-3'; ABL1-RP: 5'-GGTGGAAGCCCTTCCG-3'; Probe for BCR-ABL1: FAM-AAGCCCCTrCrArGCGCCAGTAG-BHQ1, Probe for ABL1: TEX615-ACCTGAGGrGrArGrTGCAACCGG-BHQ2. Specificity of the primers and probes was checked using a BLAST sequence query. There was no significant sequence homology with other sites in the human genome. In a multiplex format, the amplicon sizes of b2a2, b3a2, e1a2, and ABL1 were 168 bp, 243 bp, 304 bp, and 348 bp, respectively (PCR results available upon request). The 3 types of transcripts were produced by in vitro transcription using T7 RNA polymerase and DNA templates containing T7 promoter. The DNA templates were manufactured by overlap extension PCR.

2.3. One-step multiplex RQ-PCR assay

The reagents consist of an enzyme mix, a probe/primer mix, and standard materials with respect to both BCR-ABL1 and ABL1. Three standard materials for BCR–ABL1 contained $10^5$, $10^4$, and $10^3$ copies and 3 standard materials for ABL1 contained $10^7$, $10^6$, and $10^4$ copies. A volume of 15 μl of RNA input adjusted to 1500 ng was added into 13 μl of the enzyme mix and 2 μl of the primer/probe mix. The reverse transcription (RT) reaction, which was performed at 55°C for 60 min, was followed by PCR without the hands-on assay step. Amplification of both BCR–ABL1 and ABL1 was performed in one reaction using the VIIA 7 Real-Time PCR System (Applied Biosystems). After the initial denaturation at 95°C for 5 min, a 3-step cycle (at 95°C for 10 s, at

![Fig 1. Primers and probes design. Locations of primers specific for BCR exon b2, exon e1, and ABL1 exon a3 are indicated by gray arrows. The probe is labeled with 5' fluorophore (FAM) and 3' quencher (BHQ1) for b2a2, b3a2, and e1a2. ABL1 was used as an endogenous control.](image-url)
60°C for 10 s and at 65°C for 30 s) was repeated 45 times. Standard materials and negative control were tested into each run. The BCR–ABL1 transcripts and ABL1 transcript were detected on the FAM channel and TEX615 channel of a real-time instrument, respectively.

2.4. Determination of linearity, LoD, precision, and comparison

Performance was evaluated with respect to linearity, LoD, precision, and comparison according to the CLSI guidelines [12,19–21]. Linearity, LoD, and precision were assessed by serially diluting RNA from the K562 cell line into that from the HL-60 cell line. To determine linearity, 6 samples of K562 RNA (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 2 \times 10^{-5} dilutions) were produced and measured 5 times for each concentration. The LoD was assessed by 12 replicates from 7 samples (10, 8, 6, 4, 2, 1 and 0.8 pg). Precision was obtained from samples with three concentrations, 150 ng (10^{-1} dilution of K562 RNA), 150 pg (10^{-4} dilution of K562 RNA) and 10 pg (concentration close to LoD), for 5 days. Within-run precision was determined by 5 repeated measurements using the same samples. Between-run precision was assessed by 2 independent measurements in a day with an interval of more than 2 h in a day. A comparison study with the Asuragen kit was performed using 73 patient samples. Both tests were performed on the VIIA7 Real-Time PCR system (Applied Biosystems) for comparison by the same operator.

2.5. Determination of conversion factor

To convert BCR–ABL1 values to an international scale (IS), the first World Health Organization (WHO) international standard (NIBSC code: 09/138) was used [22]. The reference panel consisted of 4 concentrations (ampule code 08/192, 08/194, 08/196, 08/198), which were used as primary reference materials. These 4 materials were measured in triplicate. The value of the percentage of ratio of BCR–ABL1 to ABL1 transcript (% ratio) and the log transformed % ratio was calculated. To determine conversion factor, we calculated the difference of log transformed % ratio between the assigned value and local value. After regression analysis of difference against mean value, conversion factor was defined as the anti-log of mean difference.

2.6. Data analysis

Standard curves, threshold cycle value and the copy number of each sample were automatically generated through ViiA™ 7 software (Applied Biosystems). The % ratio and the log transformed % ratio were calculated. To achieve an IS, the log transformed % ratio values were multiplied by the method-specific conversion factors. The data were analyzed using Analyse-it (ver 2.30, Analyse-it Software) and R ver 2.15.2 (R Foundation for Statistical Computing).

3. Results

3.1. One-step multiplex RQ-PCR assay

Multiplex RQ-PCR assay was performed by the one-step and one-well reaction without the hands-on assay step. The total required time for reverse transcription, multiplexed amplification, and fluorescent signal detection was 2 h and 25 min on the VIIA7 Real-Time PCR system. Representative amplification plots of b2a2, b3a2, and e1a2 were shown in Fig. 2. The 3 subtypes were all detected at the concentrations of 10^{7}, 10^{5}, 10^{3}, and 10 copies of BCR–ABL1 transcripts (Fig. 2).

3.2. Linearity, LoD, precision, and comparison

The assay was linear in a range of concentrations from 150 ng to 7.5 pg of K562 RNA (from 1:10^{-1} to 2 \times 10^{-5} dilutions), which is over a 4-log range (r^2 = 0.9981) (Fig. 3). Probit analyses showed that LoD was the concentration of 1.5 pg (3 copies of BCR–ABL1 transcripts) with 95% confidence. All 12 replicates showed positive results at a concentration of 2 pg, while 6 out of 12 replicates were positive at a concentration of 0.8 pg of K562 RNA. With respect to % ratio, the total coefficients of variation (CV) at a concentration of 150 ng, 150 pg, and 10 pg were 28.0%, 56.6%, and 64.8%, respectively (Table 1). Within-run precision and between-run precision were 12.8–35.4% and 22.6–55.9%, respectively (Table 1) (the total CV based on the BCR–ABL1 Ct value was 2.20–2.46% for all three concentrations; the total CV based on ABL1 Ct value was 1.34–3.17%). The assay was well correlated with Asuragen’s BCR/ ABL1 Quant™ kit (Asuragen Inc., Austin, TX, USA) over a 6 log concentration range (r = 0.9967, P < 0.0001) (Fig. 4).

3.3. Conversion factor

The log transformed % ratio was linear between the known values and measured values (r^2 = 0.9775). The mean difference between the known values and measured value was 0.244% ratio on a logarithmic scale, corresponding to a conversion factor of 0.57 (95% confidence interval of 0.446 to 0.727). The constant bias was disappeared after the application of conversion factor. There was not observed proportional bias between known values and measured value.

4. Discussion

Previous studies have proven that quantification of BCR–ABL1 fusion transcripts using RQ-PCR is critical for therapeutic stratification of patients with Ph-positive leukemia [10,11]. This would necessitate the introduction of highly sensitive and accurate methods. In practice, a number of RQ-PCR analytical systems have been used for quantification of BCR–ABL1 fusion transcripts. However, considerable variations have been reported in the analytical system, including the control gene, primer, probe chemistry, instrumentation, reverse transcription procedure,
quantitative PCR procedure, and operator [14]. Subsequently, these variations caused variations in the reported BCR–ABL1 values. Therefore, the development of an accurate analytical method as well as a standardized reporting scale would be highly recommended for clinical application. Here, we developed a multiplex RQ-PCR assay based on CataCleave probe technology and demonstrated that the performance of our assay was comparable to that of the Asuragen kit based on TaqMan probe technology. In addition, we calculated method-specific conversion factors to test the feasibility of reporting BCR–ABL1 values on the IS.

Our multiplex RQ-PCR assay was performed in a one-step process from the RT reaction to the quantitative PCR. Current RQ-PCR methods are highly complex and have many steps including sample preparation, RNA extraction, RT reaction, multiplexed amplification, and quantification. Due to the introduction of potential errors in each step, it is highly desirable to reduce the hands-on time. One of the advantages of our assay is that it was performed in a one-step process without any hands-on time. As a result, this may shorten the required time for RQ-PCR. The total time for our RQ-PCR assay was 2h and 25min. In contrast, our assay was superior to the previous assay [15]. When tested at 10−5 dilution at 1500 ng input using the Asuragen kit, our method may show comparable performance characteristics to the Asuragen kit [15].

Because of the variability associated with reverse transcription, it is widely accepted that the current RQ-PCR assay is performed in duplicate or triplicate [25]. With respect to repeatability and reproducibility, our method was superior to the previous assay [15]. When tested at 10−5 dilution at 1500 ng input using the Asuragen kit in quadruplicate over 5 days, the within-run precision, between-run precision, and total precision based on % ratio were reported to be 74.1%, 24.9%, and 78.0%, respectively [15]. Brown et al. described that high variability at very low concentrations was due to the current limitations of RQ-PCR [15]. When performed in quintuplicate over 5 days, our assay precision was comparable to that of the Asuragen kit even at the lower concentration, although there are some differences in the analytical system (Table 1). Furthermore, our assay was well correlated with Asuragen kit over a 6 log concentration range, when performed by the same operator using the same amount of input RNA on the same day (Fig. 4). This indicates that our method could be a reliable alternative for the diagnosis and follow-up in patients with CML.

Excellent performance of our assay could be attributed to the application of CataCleave probe technology. Until now, CataCleave technology has been reported to be used to detect of target DNA, such as SNP, deletion, and partially methylated DNA as well as microorganisms [18,26]. For the first time, we showed the clinical application of CataCleave probe technology for quantification of human RNA such as BCR–ABL1 fusion transcripts in a multiplex assay format. With modification of CataCleave probe length and position of fluorescence, it would be possible to develop a high-throughput assay according to clinical needs. Excellent performance characteristics of CataCleave technology would lead to extensive applications including development of a number of commercial kits in medical oncology.

Table 1

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Within-run precision</th>
<th>Between-run precision</th>
<th>Total precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% ratio SD</td>
<td>CV (%)</td>
<td>% ratio SD</td>
</tr>
<tr>
<td>150 ng</td>
<td>0.016</td>
<td>12.8</td>
<td>0.023</td>
</tr>
<tr>
<td>150 pg</td>
<td>0.00007</td>
<td>31.3</td>
<td>0.00009</td>
</tr>
<tr>
<td>10 pg</td>
<td>0.0000009</td>
<td>35.4</td>
<td>0.000014</td>
</tr>
</tbody>
</table>

Abbreviations: CV, coefficients of variation; % ratio, the percentage of ratio of BCR–ABL1 to ABL1 transcript; SD, standard deviation.
In the current study, a conversion factor was not validated in collaboration with the reference laboratory. However, when applied the first WHO international standard (NIBSC code: 09/138) consisting of 4 different levels, there was no proportional bias between the measured value and known value. This indicated that it is feasible to express BCR–ABL1 values on an IS by application of a method-specific conversion factor. Further studies to verify and validate the method-specific conversion factor are required for IS standardization.

5. Conclusions

Our multiplex RQ-PCR assay based on CataCleave technology demonstrated comparable performance in comparison with the previous assay based on TaqMan technology. This method could be a reliable and accurate tool for the diagnosis and follow-up of BCR–ABL1 positive leukemia. To our knowledge, this is the first report regarding the clinical application of CataCleave probe technology in the field of human RNA quantification. Our study conclusively establishes that CataCleave technology can be reliably used for quantification of RNA in the clinical setting.

Acknowledgments

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry for Health & Welfare Affairs, Republic of Korea (A092255).

References