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Original Article

Detecting T315I BCR-ABL mutants in leukemia between RT-qPCR and conventional Sanger sequencing: a comparative study

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Abstract: Objective: To explore the clinical significance of real-time quantitative reverse transcription PCR (RT-qPCR) in detecting T315I BCR-ABL mutants in leukemia patients. Methods: A total of 62 leukemia patients were enrolled in this study. Peripheral blood from the patients was analyzed to determine which BCR-ABL mutants had occurred in the leukemia patients using Sanger sequencing. RT-qPCR was then introduced to detect the BCR-ABL mutants in the leukemia patients, and the results were compared with the Sanger sequencing results in terms of accuracy. Results: The T315I (C944T) BCR-ABL mutant was the most common mutation in the chronic myelogenous leukemia (CML) patients. RT-qPCR demonstrated a complete concordance with Sanger sequencing in terms of detecting the BCR-ABL mutations. An ROC curve analysis indicated that the RT-qPCR method was of value in detecting the T315I BCR-ABL mutants, and the differences were statistically significant ($P<0.05$). Conclusion: The RT-qPCR approach is equally accurate in detecting the T315I mutation compared with conventional Sanger sequencing. In addition, RT-qPCR is easy to carry out, is less expensive, and is more non-specific, suggesting it will have broad prospects for application in the detection and monitoring of the T315I mutation in leukemia patients in the future.

Keywords: Chronic myeloid leukemia, BCR-ABL mutations, T315I, RT-qPCR

Introduction

Chronic myelogenous leukemia (CML) is a type of leukemia that accounts for about 15% of adult leukemia cases, as characterized by the presence of Philadelphia chromosome (Ph) [1]. The Ph results from the t (9; 22) (q34; q11) balanced reciprocal translocation, which produces the BCR-ABL oncogene, encoding a chimeric BCR-ABL protein that constitutively activates the tyrosine kinase [2]. The activation of tyrosine kinase induces increased proliferation, the activation of transcription factors and the evasion of apoptosis, which ultimately cause uncontrolled cell growth and differentiation. Mutations in BCR-ABL have been discovered along the length of the BCR-ABL protein [3, 4], which change the shape of the tyrosine kinase inhibitors (TKI) binding site or the overall protein conformation, preventing TKI from binding directly or indirectly or affecting the activity at the catalytic site or ATP-binding loop [5, 6]. Some have studies reported that patients with mutations in BCR-ABL may be resistant to the first- and second-line therapies [7-11]. Thus it can be seen that point mutations in BCR-ABL may cause patients to relapse or to be resistant to treatment.

Acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) are a heterogeneous group of hematologic malignancies that occur as a result of monoclonal proliferation and the expansion of lymphoid precursors in the bone marrow, blood, and other organs [12]. The Ph chromosome has been detected in about a third of adult AML and ALL patients [13]. As with CML treatment, the first-line therapy for AML

Table 1. The primer sequences of the T315I BCR-ABL mutant, the Y253H BCR-ABL mutant, the E255K BCR-ABL mutant, and the E255V BCR-ABL mutant

Target gene	Primer sequences (5'-3')
T315I BCR-ABL mutant	F: GCCCCCGTTCTATATCATAAT
	R: GGATGAAGTTTTTCTTCTCCAG
Y253H BCR-ABL mutant	F: CGTACACCTCCCCGTG
	R: ACTCCAGACTGTCCACAGCAT
E255K BCR-ABL mutant	F: GCGGGGGCCAGTACGGGA
	R: GCCAATGAAGCCCTCGGAC
E255V BCR-ABL mutant	F: ACACGCCCTCGTACATCA
	R: CGCTGACCATCAATAAGG

and ALL patients treated with dasatinib is ineffective in patients carrying the T315I mutation of BCR-ABL [14].

Mutations in the BCR-ABL, especially at position T315I, may significantly affect the efficacy of present treatment strategies in both AML and ALL patients. As a result, the analysis for BCR-ABL mutation status plays an important role in the choices of leukemia treatments. The Sanger sequencing method is widely used, and it has become the gold standard for detecting the BCR-ABL mutation [15]. However, the method has some limitations, such as its high cost and the fact that it is complicated to carry out. RT-qPCR has come to be regarded as a tool for the rapid and sensitive detection of the fusion gene, for example, to monitor minimal residual disease (MRD) in leukemic patients [16]. This technique is easy to carry out and less costly. However, there are few studies on the use of RT-qPCR for detecting BCR-ABL mutants in leukemia patients. Therefore, this study was designed to explore the efficacy of the RT-qPCR method for detecting BCR-ABL mutants in leukemia patients, hoping to provide an experimental basis for its use in the clinical setting.

Methods

Patients and methodology

A total of 62 patients diagnosed with leukemia at the First Affiliated Hospital of Bengbu Medical College from April 2013 to July 2013 were enrolled in this study. Peripheral blood was collected from the patients and further applied for RNA extraction. This study complied with the Helsinki Declaration and was approved by the Research Ethics Committee of Bengbu Medical College. An informed consent was collected from every patient enrolled in this study.

Patients enrolled in this study were further classified by the types of leukemia. Patients were eligible if the diagnosis met the leukemia criterion as follows [17]: patients had the symptoms of anemia, bleeding, infection, and tissue or organ infiltration and so on; abnormal blood smear and circumference blood cells; they were confirmed by spinal marrow chromosome karyotyping analysis; the type of leukemia included CML, AML or ALL. Patients were ineligible if they did not actively cooperate in this study. Nine healthy people at the hospital during the same time period were also included in this study. They were used as a negative control and to determine the false positive rate of RT-qPCR. Sanger sequencing was employed to determine the mutations in the patients with different types of leukemia. Samples from 40 CML patients were first analyzed to determine the main mutations occurring in the CML patients. Then samples from the rest of patients in this study were further analyzed by both Sanger sequencing and RT-qPCR.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from peripheral blood by utilizing the TRIzol reagent (Life Technology, CA, USA) following the manufacturer's instructions. cDNA was obtained from 5 μg of total RNA with a high-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, PA, USA) according to the manufacturer's instructions. The resulting cDNA was used as the template for the RT-qPCR analyses to examine the expressions of certain mutants. According to the BCR-ABL mutants sequences in the GeneBank database, the primers were designed by Primer Premier software version 5. The primer sequences are shown in **Table 1**.

The reaction system 25 uL included PCR Mix-Buffer (1:200) 12 μL, forward and reverse primers of 1 μL each, cDNA 5 μL, dH_2O 6 μL. The Applied Biosystems 7500 quantitative PCR instrument was centrifugally inserted for amplification. The reaction conditions included initial

Table 2. Basic clinical information of the patients and healthy people

Parameter	Leukemia patients	Healthy people
Number of cases	62	9
CML	40	
AML	4	
ALL	18	
Sex		
Male	37	5
Female	25	4
Average age (years)	41.2±2.5	40.8±2.0
Course of disease		
Chronic phase	47	
Accelerated phase	12	
Blast crisis	3	
Treatment		
TKI	30	
Chemotherapy	32	

Note: TKI: Tyrosine kinase lymphoid; CML: Chronic myelogenous leukemia; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia.

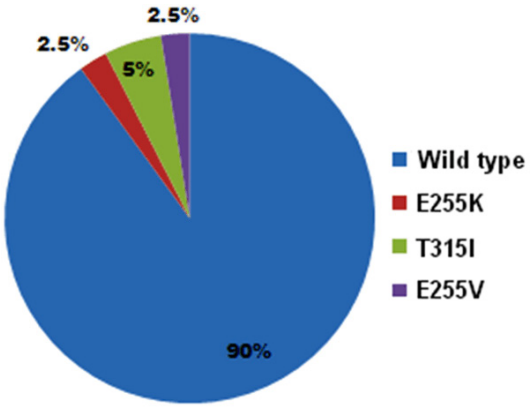

Figure 1. Mutations found in the CML patients. Wild type: 36 cases. T315I BCR-ABL mutant: 2 cases. E255K BCR-ABL mutant: 1 case. E255V BCR-ABL mutant: 1 case. CML: chronic myelogenous leukemia.

denaturation at 95°C for 1 min, denaturation at 95°C for 10 sec, renaturation at 60°C for 80 sec, extension at 72°C for 90 sec, with a total of 40 cycles.

Sanger sequencing

The BCR-ABL cDNA transcripts from the PCR amplification were sequenced utilizing a PRIME-Perfect Pure RNA Purification kit (Thermo Fisher Scientific, Pittsburgh, PA, USA) and a cDNA Synthesis kit (R&D, Shanghai, China). The mutant sequences were compared with the wild-type sequences utilizing Sequencher sequence analysis software. All the analyses were performed by BGI (Beijing, China).

Table 3. Mutations in the patients with different types of leukemia by Sanger sequencing

Phenotypes	AML	ALL	Total
Wild type	2 (50.0%)	12 (66.7%)	14 (63.6%)
T315I	2 (50.0%)	5 (27.8%)	7 (31.8%)
Y235H		1 (5.6%)	1 (4.5%)
E255K			
E255V			
Total	4 (100%)	18 (100%)	22 (100%)

Note: AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia.

Sensitive test for RT-qPCR detecting T315I BCR-ABL mutants

Total RNA was extracted from the peripheral blood of the 9 healthy people, and RT-qPCR was performed to detect any T315I BCR-ABL mutants. The false positive rate of RT-qPCR for detecting T315I BCR-ABL mutants was observed. The Sanger sequencing method for detecting T315I BCR-ABL mutants in leukemia patients was selected as a control, and the sensitivity of RT-qPCR was observed.

Statistical analysis

SPSS 21.0 software was used for the statistical analysis. All measurement data had a normal distribution and homogeneity of variance, and they were expressed as the mean ± standard deviation and compared using an independent sample *t*-test. Dichotomous data were expressed as a percentage, and compared using an χ^2 test. The receiver operating characteristics (ROC) curve was established to assess RT-qPCR in detecting the T315I BCR-ABL mutants based on the area under the curves. $P<0.05$ (bilateral) was considered statistically significant.

Results

Clinical information of the leukemia patients and the healthy people

The clinical information of the patients and the healthy people is summarized in **Table 2**. There were 40 CML patients, 4 AML patients, 18 ALL

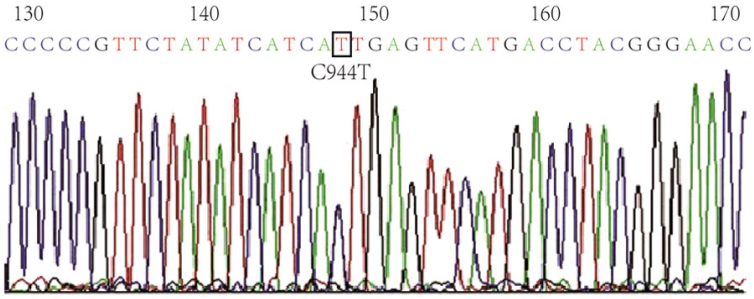

Figure 2. T315I (C944T) BCR-ABL mutant sites.

patients, and 9 healthy people. The average age of the leukemia patients was 41.2±2.5 years, and the leukemia patients included 37 males 25 females. The average age of the healthy people was 40.8±2.0 years, and the healthy people included 5 males and 4 females.

T315I (C944T) and E255K/V BCR-ABL mutants in CML patients by Sanger sequencing

To develop a sensitive method to monitor the mutations in patients with leukemia, we first examined the possible mutations that can occur in CML patients. We primarily detected the BCR-ABL mutants in the 40 CML patients using Sanger sequencing. The Sanger sequencing results indicated that among the 40 CML patients with drug resistance or poor therapeutic responses, there were 4 patients who presented with BCR-ABL mutations that were mainly T315I (C944T) and E255K/V mutations, as shown in **Figure 1**.

T315I BCR-ABL mutants in AML patients and ALL patients by Sanger sequencing

Four acute myeloid leukemia (AML) patients and 18 acute lymphoblastic leukemia (ALL) patients were enrolled in this part of the study. The Sanger sequencing showed that there were different types of mutations, and they included T315I and Y235H, as shown in **Table 3**. Among the AML and ALL patients, the T315I (C944T) BCR-ABL mutant was the main type, as seen in **Figure 2**.

Identical accuracy between RT-qPCR and Sanger sequencing for the BCR-ABL mutant

We then tried to develop a RT-qPCR method to monitor the T315I BCR-ABL mutant and utilized these 12 patients to examine the accuracy of the new method. We designed the specific primers to detect the possible T315I BCR-ABL mutants in the process of leukemia treatments. As shown in **Table 4**, all the T315I BCR-ABL mutants revealed by Sanger sequencing had also been detected by RT-qPCR. Meanwhile, RT-qPCR also detected other mutations found by Sanger sequencing. Taking the conveniences into account, RT-qPCR was an easier and faster way to monitor the harmful mutations resulting from the treatments, especially T315I.

The false positive rate and the ROC curve of RT-qPCR for the T315I BCR-ABL mutant

The T315I BCR-ABL mutant was not detected in the mononuclear cells of the peripheral blood from the 9 healthy people using the RT-qPCR method, nor by Sanger sequencing. The false positive rate of RT-qPCR for detecting the T315I BCR-ABL mutants was 0%. All the leukemia patients with the T315I BCR-ABL mutants were observed using the RT-qPCR method. And the sensitivity of RT-qPCR was 100%. According to the ROC curve analysis showing the value of RT-qPCR for detecting T315I BCR-ABL mutants, the AUC for RT-qPCR was 0.938, suggesting that the RT-qPCR had dramatically different values in detecting T315I BCR-ABL mutants ($P<0.05$), as illustrated in **Figure 3**.

Discussion

The choice of therapy for leukemia should be determined based on the BCR-ABL mutation status, as the BCR-ABL kinase domain mutations are the most frequent mechanism of resistance to TKI therapy. The TKI imatinib, as the first-line clinical drug for CML patients, has excellent efficacy and safety, but it's often invalidated in patients with ABL kinase mutations, especially at the position T315I in the gatekeeper region [18]. The second generation TKIs, such as nilotinib, dasatinib, and later bafetinib were developed and have been demonstrated to overcome most of the imatinib resistance. Unfortunately, they still fail to react to the BCR-ABL mutation at the T315I position [19, 20]. In this case, the remaining treatment

Table 4. A comparison of the accuracy of detecting leukemia resistance mutations using RT-qPCR and Sanger sequencing

Sample	T315I		Y253H		E255K/V	
	Sequencing	RT-qPCR	Sequencing	RT-qPCR	Sequencing	RT-qPCR
1	Positive	Positive				
2	Positive	Positive				
3	Positive	Positive				
4	Positive	Positive				
5	Positive	Positive				
6	Positive	Positive				
7	Positive	Positive				
8	Positive	Positive				
9	Positive	Positive				
10			Positive	Positive		
11					Positive	Positive
12					Positive	Positive

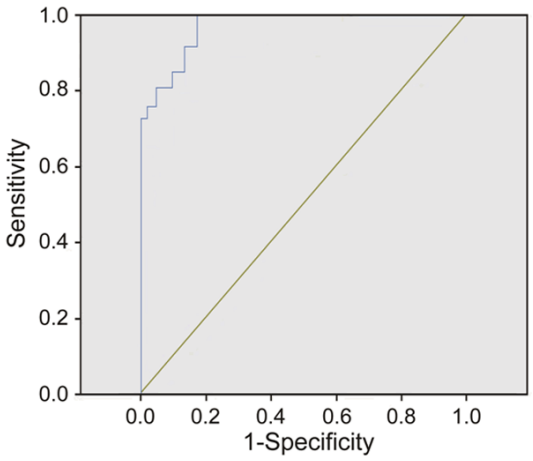

Figure 3. The ROC curves for detecting the T315I BCR-ABL mutants using RT-qPCR. The AUC for RT-qPCR was 0.938. ROC: The receiver operating characteristics.

therapies are limited mainly to the third-generation BCR-ABL inhibitors targeting the T315I mutations, such as potatinib and omacetaxine [21, 22]. Similarly, for the Ph + ALL patients, the most frequently used drugs are imatinib and dasatinib, and the patients who relapse during treatment with dasatinib usually carry the T315I mutation [14]. Several agents are in early-stage development to act against T315I, such as homoharringtonine [23]. As a result, the detection of possible mutations, especially the T315I mutation, in leukemia patients would be significant in the determination of therapy strategies. In this study, the Sanger sequencing results indicated that among leukemia patients with resistance to treatment or poor therapeutic responses, about 19.4% of the patients presented gene mutations, which were mainly mutations in the BCR-ABL at the position T315I. This finding is consistent with the findings of previous studies [24]. We also observed main mutations in the positions T315I, Y253H, E255K, and E255V in different types of leukemia patients, in line with some previous studies [25]. Thus it can be seen that gene mutations of BCR-ABL are common in leukemia patients, especially in patients who are insensitive to most current treatments.

In the present study, we utilized two strategies, RT-qPCR and Sanger sequencing, to detect the CML resistance mutations and compared their accuracy. The results indicated a parallel accuracy in the detection of the common CML mutations T315I, Y253H, and E255K/V between the different methods. Sanger sequencing is a conventional method and has been used clinically to identify the BCR-ABL mutations associated with TKI resistance. However, the Sanger sequencing has low sensitivity and could not detect the mutations until the mutation rate was over 20%, which greatly limits its application in monitoring the effectiveness of treatments [26].

Compared with Sanger sequencing, RT-qPCR presents many advantages in terms of monitoring the effects of the treatments. With the aid of spectroscopy and computer technology, RT-qPCR is easy to carry out, is less expensive, and is an accurate and sensitive approach which could detect even a single copy of a gene from the samples [27]. It is recognized as the standard tool for MRD monitoring in acute lymphoblastic leukemia (ALL) [28]. In addition, it was also used to quantify the BCR-ABL transcripts in the bone marrow and peripheral blood of the CML patients, which will greatly aid in the initial diagnosis of CML [29]. Our study utilized specific primers against the T315I mutation in the BCR-ABL kinase domain and

develops an easy and sensitive approach to monitoring the effects of treatments for CML. Our RT-qPCR results were in complete agreement with the Sanger sequencing, indicating its important practical application in the clinic.

However, there are still some limitations in this study, such as its small sample size, the fact that it was single-center study, and that it showed no results in terms of the clinical application of RT-qPCR detecting the T315I mutation, etc. Prospective randomized controlled trials with larger sample sizes and multi-center studies are needed in future research to further validate the advantages of RT-qPCR in detecting the T315I mutation in leukemia patients.

The mutation at position T315I is the main reason for treatment resistance in leukemia patients. Our strategy of employing the RT-qPCR method showed identical accuracy in detecting the T315I mutation compared with conventional Sanger sequencing, while it has many other advantages such as being easy to carry out, it is less expensive, and it is more sensitive. It has a broad potential for application t in detecting and monitoring the T315I mutation in leukemia patients in the future.

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Disclosure of conflict of interest

None.

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