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Analysis of Two Mutations in the BCR-ABL Fusion Gene Relevant for Monitoring Chronic Myeloid Leukemia Patients

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Abstract: Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm characterized by a structural chromosomal aberration. The BCR-ABL tyrosine kinase produced by the t(9;22)(q34;q11) translocation (also known as the Philadelphia chromosome - Ph^+) is the initiating event in CML. In the present paper, we proposed a new and efficient method based on reverse transcription and nested PCR amplification for highlighting E255K/V and T315I mutations in the BCR-ABL fusion gene. We included 20 patients (13 males and 7 females) with CML (mean age of 55 ±17 years) based on the presence of Ph+ and data regarding the molecular response to Imatinib therapy. The obtained results indicate the presence of E255K/V and T315I mutations in two patients from this research. Furthermore, it was observed that patients with CML who had acquired mutations did not respond to Imatinib therapy and required personalized therapy. We concluded that this protocol is easy to implement and relatively rapid and inexpensive compared with other techniques to identify the mutations.

Keywords: chronic myeloid leukemia; BCR-ABL; nested PCR; T315I; E255K/V; mutations.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative syndrome caused by mutations in a hematopoietic compartment that lead to the proliferation and accumulation of bone marrow cells and progenitor cells [1,2]. This change subsequently results in excessive production of granulocytes in the bone marrow, causing both splenomegaly and hyperleukocytosis [3,4]. Currently available data show that the distribution of CML incidence varies by age, gender, and region. The annual incidence of CML varies from 0.4 / 100.000 to 1.75 / 100.000 depending on the country. The incidence of CML increases with age and is rare in children under 14 (0.7 / million / year). In addition, CML is more common in men than women, with a male-to-female ratio of 1.2-1.7. However, as far as we know, no studies have characterized CML's global prevalence and burden [5].

In patients with CML, mutations are one of the most common determinants of tyrosine kinase inhibitor (TKI) resistance in the breakpoint cluster region protein-tyrosine-protein kinase ABL1 (BCR-ABL1) kinase domain, which occurs in 40-50% of all TKI-resistant cases [6–8]. In addition, CML that recurs and progresses to advanced stages of the disease is often associated with recurrent oncogenesis mutations frequently mutated in other hematological malignancies. Despite the great clinical relevance of mutation-associated TKI resistance in

CML, the underlying mechanism of oncogenic acquisition of resistant CML cells remains poorly understood [9,10].

The majority of chronic myeloid leukemia (CML) patients are carriers of t(9; 22) (Philadelphia chromosome, Ph) [11,12], while the others (5-8%) have complex chromosomal rearrangements [13–15]. The BCR-ABL fusion gene encodes for the p210 protein, which has tyrosine kinase activity [16,17].

Targeting BCR-ABL with tyrosine kinase inhibitors (TKIs) has resulted in rapid clinical responses for most patients with CML. However, long-term use of TKIs can predispose to a selection of clones with mutations in the BCR-ABL kinase domain and therapy resistance[18]. The substitutions of glutamic acid to lysine or valine at codon 255 [E255K/V] or threonine to isoleucine at codon 315 [T315I] were associated with an impaired response to second-generation of TKIs [12,19]. The so-called "gatekeeper residue" T315I alters the association of imatinib and all second-generation TKIs [20,21]. This mutation occurs in 20% of patients with resistant CML [22,23]. Identifying these translocations and mutations in the ABL kinase domain is useful for CML diagnostic, prognosis, and personalized treatment [14,16,24].

The main methods that can be used to detect the BCR-ABL transcript are RT-PCR, multiplex PCR, nested PCR, quantitative PCR, FISH method, etc. Each of these methods has a number of advantages and disadvantages. It should be noted that in many cases, several methods can be used to increase the sensitivity and specificity with which mutations of interest are detected.

The analysis of fusion genes by qualitative RT-PCR is based on the design of oligonucleotide primers placed in opposite directions to the breakpoints of the fusion genes so that the RT-PCR product contains tumor-specific sequences. In most types of chromosomal aberrations related to fusion gene breakpoints, there will be values above 10kb, which makes it difficult to perform a classical technique by DNA PCR from patients [22]. This would mean that site-specific recombination at the DNA breakpoint would be determined in each individual patient to perform the PCR analysis correctly. Because of these criteria, it has been observed that in patients with CML, testing is performed on the BCR-ABL fusion gene transcript. This transcript is reverse transcribed into cDNA (complementary DNA), then used in PCR. Qualitative PCR shows the presence or absence of the BCR-ABL fusion gene [23].

Multiplex RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) is an accessible, fast and sensitive method for detecting BCR-ABL variants. The appearance of these transcripts associated with CML may help in the prognosis and treatment of the disease. This technique allows patients undergoing treatment to be monitored when conventional cytogenetic methods and quantitative RT-PCR can no longer detect the minimum residual disease. The advantage of the Multiplex RT-PCR technique is that the BCR allele is amplified as an internal control (this way, false-negative results are avoided). A great benefit for those who work with the method is that it can work several tests simultaneously, a relatively simple and convenient technique [25].

Multiplex RT-PCR can simultaneously detect multiple target sequences in a single reaction tube, using multiple pairs of primers. The benefits of this technique are simplicity and shorter analysis time compared to the RT-PCR method. This co-amplification of several target sequences depends on the compatibility of the primers used in the reaction. Therefore, detecting p210 and p190 proteins in a single patient requires multiple procedures, which is costly and

requires a longer analysis time. Clinically, this technique is beneficial and effective for the detection of genetic changes present in patients with CML [26].

Nested PCR is a variant of classical PCR, but it involves 2 successive amplification steps: 2 pairs of primers (“external” and “internal”) are used to amplify the same sequence of interest. The first pair of primers ("external") amplifies a DNA sequence in the template like any standard PCR. The second pair of primers ("internal") are called nested primers and align with the sequence of amplicons obtained in the first PCR reaction. Thus, the latter will generate an amplicon smaller in size but identical to a sector of the template used in the first PCR reaction. 2 partially separated and successive PCR reactions perform this type of PCR. In the case of this technique, to control the quality of the samples and to validate the negative results correctly, ABL is amplified as an internal standard in a separate reaction. Thus, when the amplification result is negative and the expression of the ABL gene is sufficient, the method must be repeated to confirm the result. Nested PCR is an affordable technique for a small number of samples, especially when quantification is not essential. Therefore, sensitivity and specificity are significantly improved. In addition, this method benefits less developed countries because it requires less material and financial resources [27].

Real-Time qRT-PCR is the only method to detect minimal residual disease after the patient reaches optimal values determined by karyotyping or FISH. FISH is a cytological technique used to determine the temporal expression of RNA molecules or various types of proteins [28].

FISH is a targeted assay that can visualize the BCR-ABL fusion gene. Samples are used for the ABL and BCR target genes and control sequences. The FISH method can be applied to nucleated cells or metaphase chromosomes. An epifluorescent microscope is required for analysis [29]. If the analysis is performed on interphase nuclei, then the analysis of several at least 200 cells is recommended. The FISH method has a higher sensitivity to conventional karyotyping (less than one Ph + cell per 100 nucleated cells can be detected) [30] but requires greater material and financial resources than these.

Due to the much lower costs than quantitative PCR, the Nested PCR method proves to be much more sensitive than quantitative PCR and can detect less than 106 copies of BCR-ABL transcript. Given these qualities, the Nested PCR can be used to monitor the effect qualitatively during treatment administration.

This paper aims to test a new identification of mutations in the BCR-ABL fusion gene to establish existing clinical methods and customize therapy according to the needs of patients. The idea of this study arose primarily due to the limitations of current detection methods (mainly Real-Time PCR and FISH) that require special logistical, material, and human resources or require significant effort to analyze the data obtained.

2. Materials and Methods

Our study was performed in the Molecular Biology Department of Fundeni Clinical Institute, Bucharest, with the hospital's Ethics Committee's agreement. Twenty patients were selected based on the Philadelphia chromosome (Ph+) presence and data regarding the molecular response to imatinib therapy (Table 1). Every subject signed the informed consent before inclusion in the study.

Table 1. The clinical and paraclinical characteristics of subjects selected for this study.

Variable	No. of patients
Female / Male	7 / 13
Age (years)	55 ±17
T315I mutation	1
E255K/V mutation	1
Major molecular response (MMR)	13
Transcript type B3a2	15
Transcript type B2a2	5
Response to Imatinib therapy: Yes/ No/ other situation	9 / 3/ 8
Treatment duration (months): 0-12 / 13-24 / 25-36 / 37-48/ >49 / Deceased/other situation)	3/ 2/ 2/ 7/ 1/ 5
Associated diseases (number of patients)	16

2.1.RNA extraction and cDNA synthesis.

Total RNA was extracted from peripheral blood leukocytes (12 mL) using an *in-house"* protocol. Its concentration was tested on a NanoDrop spectrophotometer (Spectrophotometer ND-1000). Total RNA was used for cDNA synthesis using M-MLV reverse transcriptase (Invitrogen).

2.2.PCR amplification and mutation analysis.

Nested PCR-RFLP was performed to identify mutations of the ABL domain. pBCR-F 5'GAGCAGCAGAAGAAGTGTTTCAGA3' and pABL-R 5'CTGAGATGCCTCACTCCAAG3' primers were used for the first PCR amplification, whereas pFWD1A 5'CATCATTCAACGGTGGCCGACGG3' and pREV4A 5'GTTGCACTCCCTCAGGTAGTC3' were used for the second PCR amplification. Both PCR reactions were carried out in 20 µL reaction volume (18 µL mix reaction – Qiagen; Tip Molbiol Berlin and 2 µL of DNA). Two µL of cDNA were used for the first PCR, whereas 1 µL of the amplicon was used for the second PCR. The first PCR amplification program consists in: initial step at 95ºC/ 15 seconds, 40x cycles (95ºC/ 30s, 60ºC/ 30s and 72 ºC/ 150s) and a final extension step at 72ºC / 7 minutes. The program used for the second PCR was: initial denaturation step at 95ºC / 15 seconds, 35x cycles (95ºC/ 30s, 61ºC/ 30s, 72 ºC/ 40s), followed by a final extension step at 72ºC / 7 minutes.

The PCR products were analyzed on 3% agarose gel stained with ethidium bromide.

Table 2. The estimated size of restriction fragments**.**

Mutation	Restriction enzyme	Size (bp) of restriction fragments	
		***"Wild-type"* allele**	**Mutant allele**
T315I	Dde I	344+35+14	379+14
E255K/V	Mnl I	166+9+51+12+131+15+9	175+51+12+131+15+ 9

3. Results and Discussion

3.1. Nested PCR.

The Nested PCR step was performed to amplify the BCR-ABL fusion gene and the ABL fragment. Following the first stage of PCR, the samples were analyzed by 3% agarose gel electrophoresis (Figure 1). The molecular weight marker can be seen in the first well. In the following wells (2 - 5), the amplified BCR-ABL fusion genes from the DNA template were migrated from 4 patients confirmed with CML (Figure 1A). The transcripts obtained after amplification have approximately 5600 bp. Following the completion of the second stage of PCR, the samples were analyzed similarly to the first stage of PCR, in 3% agarose gel. The

molecular weight marker can be seen in the first well. In the following wells (2 - 5), the amplicons of the ABL fragment from the BCR-ABL fusion gene were migrated. The transcripts obtained after amplification have 393 bp (Figure 1B).

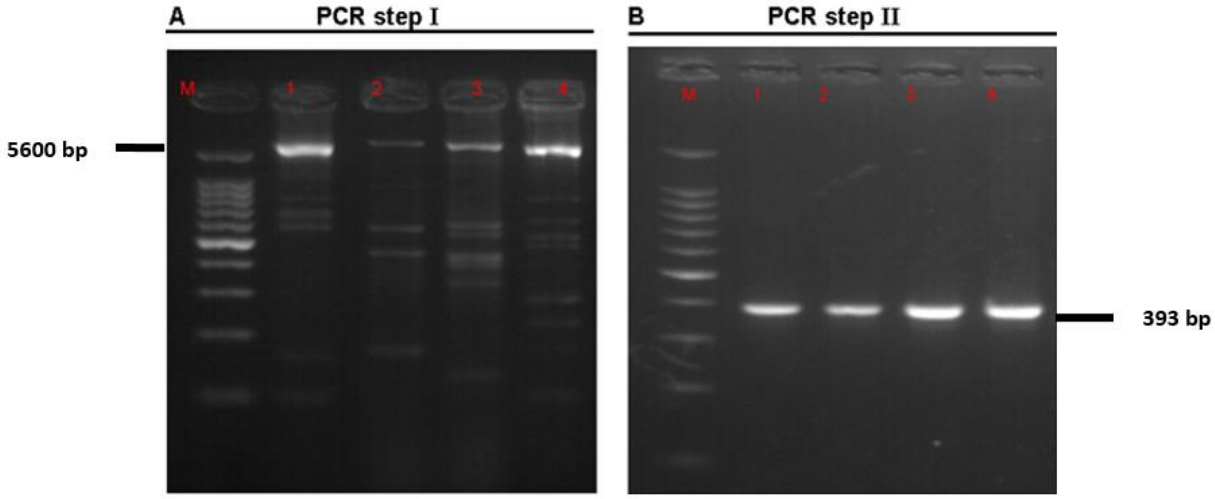

Figure 1. The results of nested PCR (ethidium bromide-stained agarose gel). M: molecular weight marker BenchTop 100 bp DNA Ladder (Promega); (A) 1-4: BCR-ABL fusion gene amplification products (5600bp); (B) 1-4: ABL fragment amplification products (393pb).

3.2. Restriction endonuclease digestion.

Identification of T315I and E255K/V mutations was performed by restriction endonuclease digestion [12,31] and separation by polyacrylamide gel electrophoresis (PAGE 8%) (Figure 2). This step was implemented to highlight the presence or absence of mutations in the BCR-ABL fusion gene.

In the case of the T315I mutation, the restriction of amplicons with the Dde I enzyme was performed. Following the restriction, 3 restriction fragments were obtained for "wild-type" patients (344 bp, 35 bp, 14 bp - not visible on the gel), and for patients who had the T315I mutation, 2 fragments (379bp and 14bp) were obtained (Table 2).

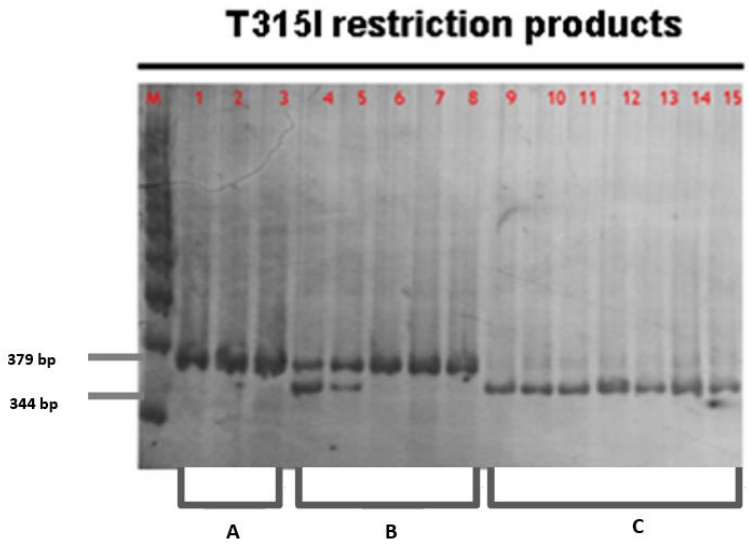

Figure 2. Electrophoresis of the restriction products was obtained after digestion of the amplicons with the restriction enzymes (PAGE 8%, silver). M: Molecular weight marker BenchTop 100 bp DNA Ladder (Promega); (**A**) Amplicons obtained from nested PCR; (**B**) 5 samples from the patient confirmed with T315I mutation from the years 2012, 2014, and 2015; (**C**) Samples without T315I mutation.

The results were exemplified using images in and in polyacrylamide gel. As the 344 bp and 379 bp bands in agarose gel could not be differentiated, an 8% polyacrylamide gel was made. In this situation, several samples of the patient confirmed with the T315I mutation were used. The samples were collected in different years: 2012 (the patient was confirmed with the T315I mutation and the presence of a large number of leukemic cells) and from 2014 to 2015 (the patient was given specific TKI to inhibit the T315I mutation). The 2012 test shows in the polyacrylamide gel that the patient has both a "wild-type" allele and a mutated allele. In the 2014-2015 trials, there is no longer a “wild-type” allele; the patient presented only with a mutated allele.

The method used for highlighting E255K/V and T315I mutations in the BCR-ABL fusion gene was based on reverse transcription, nested PCR, and RFLP. This method identified these mutations in two patients involved in the study. The estimated frequency of each mutation in our lot was 2.5%.

Twelve CML patients were in the first line of TKI therapy. Among them, three developed Imatinib resistance, and two had E255K/V or T315I mutation.

Three of the patients involved in the study were excluded from other statistical analyses because they decided to be treated in another hospital.

3.3. Discussion.

The protocol developed in this study tries to be complementary to current detection methods (e.g., Real-Time PCR and FISH). This method is relatively fast and does not require special equipment to detect T315I and E255K/V. It can be useful for laboratories which few financial resources.

Molecular analysis of mutations in the BCR-ABL fusion gene is helpful for personalized therapy and genetic counseling. In our study, the frequency of both T315I and E255K/V mutations was estimated to be 2.5%. This value is by those reported for other populations. Globally, T315I mutation is present in (6.78%) of all CML patients [32,33]. The E255K mutation was reported to be present in 11% of Asian and caucasian CML patients [34].

The resistance to Imatinib therapy depends on the acquired mutation [35,36]. Both patients with T315I or E255K/V mutation were resistant to Imatinib therapy in this study. The male with T315I mutation was 78 years old and had been under therapy for 11 years. T315I mutation was detected after 7 years of trying different TKIs without significant therapeutic. The patient achieved MMR after two years of treatment with ponatinib. The E255K/V mutation was detected in a male who died shortly after inclusion in this study.

4. Conclusions

The method proposed in this study revealed T315I or E255K/V mutation in two patients with CML. The performed protocol is faster and more affordable than other techniques for identifying tested mutations. The proposed method can complement the results provided by the Real-Time PCR and FISH methods, and the results can be useful for personalized therapy and genetic counseling. The protocol must be tested for sensitivity and specificity with the inclusion in the study group of a larger number of patients with CML.

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Conflicts of Interest

The authors declare no conflict of interest.

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