
Molecular diagnosis of bcr-abl fusion gene in CML patients using Multiplex-Reverse Transcription-Polymerase Chain Reaction

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Abstract

Background: Chronic myeloid leukemia (CML) is a stem cell disorder results from chromosomal abnormality, Philadelphia chromosome (Ph), which arises from the reciprocal translocation of part of long arm of chromosome 9, in which proto-oncogene ABL gene (ablson) is located, to long arm of chromosome 22, in which BCR gene (break point cluster region) is located forming BCR-ABL fusion gene, a molecular marker of CML. The BCR-ABL gene can be detected using several molecular methods, including southern blotting, fluorescence *in situ* hybridization (FISH) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). For its simplicity, rapidity, and sensitivity, RT-PCR is one of the most common techniques used for analyzing whether a target gene is being expressed or not.

Objective: This study was designed as a try to apply molecular techniques as conformational diagnosis of BCR-ABL gene and its variants in CML patients.

Patients & Methods: Venous blood sample from 34 CML patients, 12 ALL patients, 1 AML patients, 1 CMML patient and 2 healthy individuals were collected. RNA was extracted from these samples using specific kit for this purpose. Molecular screening for the presence of *bcr-abl* in those samples was done using Multiplex-Single Step-Reverse Transcription-Polymerase Chain Reaction (M-SS-RT-PCR). Amplified products were electrophoresed in 1.5% agarose gel.

Results: The results showed that all CML patients were positive for *bcr-abl* while all the others were negative for this gene.

Conclusion: qualitative molecular diagnosis of *bcr-abl* using M-SS-RT-PCR considered as Conformational diagnosis for CML patients before starting treatment.

Key Word: CML - BCR/ABL -Multiplex Reverse Transcription Polymerase Chain Reaction.

Introduction

The term "breakpoint cluster region (bcr)" was first applied to a DNA on the long arm of chromosome 22 (22q11), which is disrupted in patients with chronic myeloid leukemia (CML) bearing the Philadelphia (Ph) translocation t (9; 22) (q34; q11) ^[1, 2]. *bcr* is situated in a 5' to 3' orientation with the 5' end closer to the centromere of chromosome 22^[3]. The entire gene spans 130 kb and contains 23 exons. The first exon of *bcr* gene is of critical significance, because it is the one exon of *bcr* included in all known variants of *bcr-abl* fusion gene^[4].

The ABL gene is about 225 kb in size and is expressed as both a 6 or 7kb mRNA transcript, with alternatively spliced first exons, exons 1b and 1a, respectively, spliced to the common exons 2-11. Exon 1b is approximately 200 kb 5' of exon 1a^[5]. The very long Intron is a target for translocations in CML patients, in which ABL gene is translocated from chromosome 9 to the center of the BCR gene on chromosome 22 to produce a chimeric BCR-ABL RNA translated into an abnormal ABL protein with tyrosin kinase activity, a key role in the malignant transformation, with a molecular weight of 210 KD in Ph-positive CML patients and 190 KD in Ph-positive acute lymphoid leukemia (ALL) patients^[6].

The fusion of BCR and ABL on the Ph

chromosome occurs in a head-to-tail manner, with the 3' end of ABL joined to the 5' end of BCR. This configuration places the fusion gene under the control of the BCR promoter^[3].

Regardless, the fusion transcript almost always includes exon 2 of ABL (a2). In contrast, in CML, the break on chromosome 22 is restricted in most patients to an area of 5.8kb termed the major-bcr (M-bcr)^[7]. M-bcr consists of five exons termed M-bcr exons e12-e16 (or e1-e5). These exons are actually located within the central region of the BCR gene. Most breaks occur immediately downstream of exon 2 or 3 of the M-bcr region and result in b2a2 or b3a2 fusion transcripts^[3]. In acute leukemia, however, the breakage can also occur outside M-bcr in about half the cases^[8], usually within the 3' end of Intron 1 of the BCR gene termed the minor bcr (m-bcr)^[9], resulting in an e1a2 fusion transcript. Other unique breakpoint sites have been found. These include a micro 3' site termed μ -bcr (BCR exons 19 and 20), which can yield a protein of 230 KD^[10]. Patients with the fusion between BCR exon 19(e19) and ABL exon 2(a2) were classified as having neutrophilic CML (19). Other BCR-ABL variants include a b3a3, e6a2, e2/a1a, e8/a2 and e13/a2 fusion transcripts were indicated^[11].

Several techniques exist simultaneous analysis of multiple target genes.

Multiplex-Reverse Transcriptase-polymerase chain reaction (M-RT-PCR) provides an alternative and accurate method to detect multiple gene expression by amplifying the entire gene under the same conditions. In compare with basic PCR in which one pair of primer is used to amplified a specific sequence, M-RT-PCR undergo using multiple pairs of primers to amplified many sequences simultaneously.

This procedure can run either using two steps-M-RT-PCR (TS-M-RT-PCR) or single step-M-RT-PCR (SS-M-RT-PCR). For diagnostic samples, the use of M-RT-PCR has been suggested to detect simultaneously several kinds of BCR/ABL and BCR transcripts as internal controls in one reaction by using three BCR and one ABL primers^[12, 13]. This method allows the reliable detection of typical BCR/ABL transcripts, such as b2a2 or b3a2, and atypical types such as transcripts lacking ABL exon a2 (b2a3 and b3a3), transcripts resulting from BCR breakpoints outside the M-bcr, such as e1a2 or e6a2 or transcripts with inserts between BCR and ABL exons^[14, 15].

Patients, Materials & Methods

Patients

This is a prospective study enrolled (59) patients at The National Center of Hematology (NCH)/Al-Mustansirya University from February 2006 to August 2008. They were diagnosed clinically and hematologically as CML (42 patients), ALL (15 patients), AML (1 patients), CMML (1 patient). Patients were randomly selected concerning age, gender, disease duration, disease phase and pre-treatment.

Positive controls

Commercially available extracted RNA from BCR-ABL cell line K562 (Ambion, USA) was used as positive controls in amplification reactions. Also, blood samples from two patients diagnosed cytogenetically and molecularly as CML Ph-positive, *bcr-abl* positive in another lab (Molecular Biology Lab/College of Medicine/Jordanian University) were used as a positive control.

Negative controls

Two healthy individuals were included as healthy negative control. They were evaluated hematologically and molecularly in the same manner as the patients.

Sample collection

One ml of venous blood (VB) sample was obtained from each subject, placed in tube containing EDTA (as anti-coagulant).

Then, 100µl of VB was mixed with 400µl of lysis reagent contain Guanidinium Thiocyanate (GTC) and the suspension was passed several times through a pipette for about 5min at 25°C. The

lysate was used in RNA extraction or kept at -80°C until used.

Methods

RNA extraction

Total RNA was extracted from 100 µl VB using BD tract RNA /DNA isolation kit (Maxim Biotech, USA) following manufacturer information. To the cell lysate, 150µl of cold RNA precipitate reagent was added and mixed well by inversion several times for 5min at 25°C. Then, 350µl of chloroform was added to the mixture and inverted for 1min. The mixture was placed on ice for 15min then, centrifuged at 12000 rpm for 15 min at 4°C using bench top cool microcentrifuge (Jouan MR 23i, France). Aqueous phase was transferred to a sterile eppendorf tube; 650µl ice cold Isopropanol was added to the aqueous phase and the mixture was kept in -20°C for 30min. The mixture was centrifuged at the same speed, period and temperature as above.

RNA precipitate forms a white pellet at the bottom of the tube. The supernatant was removed and RNA pellet was washed with 75% ethanol.

RNA pellet was briefly dried from ethanol (5-10min at 25°C), then dissolved in about (75µl) DEPC-H₂O and stored at -80°C until used.

RNA concentration and purity were estimated using UV-spectrophotometer as in^[16].

Formaldehyde agarose gel electrophoresis

The integrity of the RNA was checked by size fractionation on a formaldehyde agarose gel electrophoresis as in^[16].

Multiplex-Single Step-Reverse Transcriptase-Polymerase Chain Reaction (M-SS-RT-PCR)

M-SS-RT-PCR reaction was preformed using SS-RT-PCR kit supplied from (Maxim, U.S.A.). Master Mix was prepared according to the number of patient's samples and controls plus one additional reaction to ensure adequate volume. It was planned to perform one positive control reaction tube (using RNA extracted from CML p210 positive cell line K562), and negative controls, one no-template control (NTC) reaction tube and one no amplified control reaction tube (NAC) using DEPC-H₂O and RNA extracted from healthy individual, respectively, in addition to the patients reaction tubes. M-SS- RT-PCR master mix was prepared contains the following components per one reaction: 25 µl of 2X SSRT buffer, 5 µl of 10X Oligo-dT, 0.5µl of 100X RNase inhibitor, 1µl of 50X SSRT-Polymerase, 0.5µM of each of the following primers :

BCR-e1:5'-ACCGCATGTTCCGGGACAAAAG-3',

BCR-b2:5'-

ACAGAATTCCGCTGACCATCAATAAG-3',

BCR-rev: 5'-
ATAGGATCCTTTGCAACCGGGYCYGAA-3'
ABL-a2:5'-

TGTTGACTGGCGTGATGTAGTTGCTTGG-3'

DEPC-H₂O was added to the final volume of 50 µl. The components were mixed well by aspirating several times into a pipette. Then, 5µl of RNA extracted from patients VB (equivalent to 0.25µg total RNA) was added to patient's reaction tubes, 5µl of DEPC-H₂O was added to NTC reaction tube, 5µl of healthy RNA was added to NAC reaction tube, 5µl of K 562 RNA was added to CML p210 positive control reaction tube. Reaction tubes were placed into the thermal cycler (XP-thermal cycler, Bioer) and M-SS-RT-PCR reaction was running using the following program: 50°C for 30 min (X1), 95°C for 5 min (X1), (95°C for 30sec, 67°C for 30sec and 72°C for 10 sec)(X35) and 72 °C for 10 min(X1). PCR amplified products were electrophoresed on 1.5% agarose gels as in [16].

Results

A total of 59 VB samples related to 42 CML

patients, 15 ALL patients, 1 AML patient, 1 CMML patients and 2 healthy individuals were monitored for the presence of *bcr-abl* using M-SS-RT-PCR assay. Samples that gave a sufficient RNA purity (≥ 1.7) and quality (as estimated by denatured agarose gel electrophoresis) were selected for M-SS-RT-PCR. Samples that showed degraded RNA, even after repeated the extraction from the same sample or from new sample obtained from the patient were neglected.

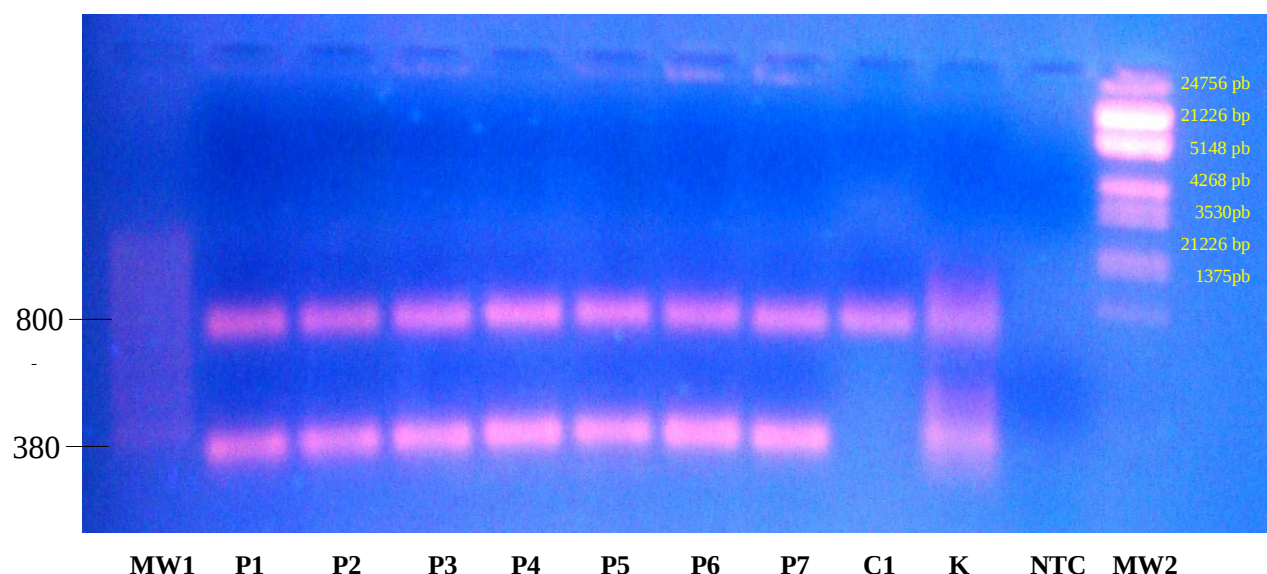
So, only (50) samples related to (34) CML patients, (12) ALL patients, (1) AML patient, (1) CMML patient and (2) healthy individuals were monitored.

CML patient's characteristics involved in M-SS-RT-PCR assay, including gender, age, CML phase, prior treatment, hematological response and cytogenetic response were shown in **table (1)**. All these factors studied at time point of molecular analysis.

Table 1: CML patients characteristics involved in M-SS-RT-PCR assay.

Factors	Patient No.	(%)
Gender :	11	32.35
- Male	23	67.64
- Female		
Age :		
- ≤ 40 ys.	25	73.52
- > 40 ys.	9	26.47
Prior treatment :		
- Previously treated	26	76.47
- First line IM	8	23.52
CML phase :		
- CP	30(88.23)	
- AP	4(11.77)	
-CML= Chronic myeloid leukemia, CP=Chronic phase, AP=Accelerated phase, IM=imatinib		

Figure .1: M-SS-RT-PCR analysis of *bcr-abl*. Lane (P1-P7) indicated amplified products of CML patients.



The results of M-SS-PCR for all CML patient's samples included in this study indicated that they were *bcr-abl* positive when detected in different intervals from starting imatinib (IM) treatment.

The result of agarose gel electrophoresis assay for CML amplified products using M-SSRT-PCR was shown in **figure (1)**.

The assay considered satisfactory for interpretation when the internal control (normal *bcr* allele) was present, PCR product of the fusion gene positive control (K652) was positive and the NTC shown no amplified product. Amplified products of RNA extracted from CML patients Lane (C1): Amplified product of RNA extracted from healthy individual. Lane (K): Amplified product of standard RNA extracted from cell line K652. Lane (NTC): No template control. (MW1): DNA ladder. (MW2): Molecular weight marker of lambda DNA restricted with *Eco RI*+*Hind III*. Electrophoresis was carried out in 1.5% agarose gel supplied with ethidium bromide at (5V/cm) for 60 minutes.

As shown, there were two bands detected in lane (P1-P7) referred to amplification products from (7) CML patients samples. The first band with a molecular size approximately of 800 bp (referred to *bcr* allele, as internal control) and the second one with approximately of 350 bp (referred to *bcr-abl* fusion transcript) in comparison with standard curve using molecular weight marker and in comparison with amplified product of K 652.

The type of transcript was identified in (29/34) CML patients using M-SSRT-PCR as b3a2 (according to molecular size). Interestingly, two of those patients were identified as *bcr-abl*, b3a2 in other lab (as mentioned in *Positive controls*).

In this study, none of the *bcr-abl* fusion variants were detected in amplified products of ALL, AML, CMML or healthy individuals. Also, fusion variant e1a2 was not identified in CML patient's amplified products.

Discussion:

Diagnoses of leukemia have been improved by classical karyotyping, FISH and PCR analysis^[17]. As numerical and structural chromosome aberrations and unknown balanced alterations are detected solely by cytogenetic analysis. RT-PCR as a conventional method, form an important platform for molecular characterization of genetic aberrations. There are two main advantages of PCR methods. First, this technique might be applied in less than two days, and one can perform PCR reactions of different aberrations and different patients at the same time. Second, in detection of known submicroscopic aberrations, usage of this method provides more certain results and ensures detection of one malignant cell among one million normal cells. Moreover, molecular techniques can be used when the material is of little quantity or insufficient for classical cytogenetic^[18].

The aim of this study was to validate the application of M-SS-RT-PCR assay for the detection of *bcr-abl* fusion gene in leukemic patients at the RNA level, the reason for using such technique in alternative of using genomic DNA (gDNA), due to the long Introns in DNA in which, breaks occur so, a classical PCR using gDNA is difficult to carry out for characterization of the transcript such as *bcr-abl*.

Quality of extracted RNA is a critical point that limited the success of further steps in RT-PCR analysis. It is affected since sample collection. The best way to store the sample is as lysates in GTC and at -20°C, in which the RNA is stable for months or stored at -80°C in which the RNA is stable for years^[12].

Any system as complex as RT-PCR is effected by a number of factors^[19]. Annealing temperature is considered as a critical factor in the PCR reaction. Common causes of PCR reaction failure are lack of primer binding because the annealing temperature is too high, or inefficient to strands disassociation owing to the denaturing temperature being too low^[12]. The effectiveness of melting temperature (*T_m*) was optimized for the four primers used in M-SS-RT-PCR and 67°C was chosen rather than 62°C (that mentioned by the manufacturer), for sufficient amplification results. This temperature acts as a median for the *T_m* of those four primers used in this reaction.

In M-SS-RT-PCR, oligo-dT primers were used for generation of cDNA template. It was recommended that this primer is preferred to be used for successful generation of cDNA rather than a hexamer primers because the last one can interfere with the subsequent steps in M-SS-RT-PCR.

All CML patients were *bcr-abl* positive after different IM treatment periods. The observation of the vast majority of patients on IM treatment who achieved CHR and /or MCyR but still have measurable disease after at least 12 to ≥18 ms of IM therapy is consistent with experimental observations of Hughes *et al*^[20]. Reason behind this status is that IM acts mainly by inhibiting proliferation rather than by inducing apoptosis in *bcr-abl* positive leukemic cells. Long follow up with dose escalation is required to determine if IM can eventually eradicate the leukemic clone.

It is crucial to confirm that primers used in this method do not generate an amplification product from gDNA that may be confused with that from cDNA since RNA preparations always contain variable amounts of contaminated gDNA. For this purpose, control reaction was done using gDNA (extracted from the same blood sample of RNA) as a template. No PCR product was detected in any reaction, indicating that these primers had high target specificity.

Adult ALL patients were included in this study in order to screen for the presence of ALL Ph-positive. No e1a2 fusion gene product was detected in any of samples screened. In one study, they detected e1a2 variants in two of nineteen (11%) ALL pediatric patients^[18]. Also, other studies referred to that 20-50% of adult ALL cases had such breakpoint^[21]. May be more number of ALL patients are required to be screened.

AML and CMML patients included in this study were at first, diagnosed hematologically as had myeloproliferative disease and they showed *bcr-abl* negative using M-SS-RT-PCR. In one study, they found that the p190 was detected in two of six AML patients^[18]. Other study referred to that 2-10% of pediatric AML cases were *bcr-abl* positive^[21].

In this study, p190 transcripts was included in monitoring assay for CML patients because a previous report referred to that p190 transcripts has been rarely detected in p210 positive CML patients^[22]. One study reported that one from seven p210 positive CML patient has additional p190 transcript^[18]. P190 expression was not detected in p210 positive CML patients included in this study. That is consistent with what reported by Jones *et al.*^[23]. Saffroy and his Cowarkares^[24] examined 186 CML specimens using RT-PCR and Elmaagacli and his colleges^[25] examined 343 CML specimens using RT-PCR and compared their results with the results of cytogenetic and FISH analysis. However, they were neither measured nor validated the p190 transcript. It has been reported that, all CML patients at the time of diagnosis have some p190 protein by alternative splicing mechanism in addition to p210 transcripts^[22].

As a consequence, it might be stated that both p210 and p190 transcripts status of all pediatric and adult CML, ALL and AML cases should be checked routinely for diagnostic, prognostic and therapeutic purposes. An amplified product from the BCR gene is the only band detected in those healthy individuals who were considered as negative control. The sole presence of this band indicates that the quality of RNA was good, thus the individual confidently can be considered to be negative for *bcr-abl*.

Although, it was initially felt that *bcr-abl* transcripts are specific for leukemia, surprisingly, other experiments suggest that small amounts of such transcripts can be found in normal individuals if very sensitive PCR techniques are used^[26]. It is possible that these transcripts represent "errors" that occur because of the close proximity of chromosome nine and chromosome twenty two during S to G2 transition of the cell cycle, and that immune surveillance prevents the emergence of CML in individuals who remain healthy. Like this error occurs in approximately $1/10^8$ WBCs^[27].

Characterization of the junction region of transcript coding for p210 *bcr-abl* (if b2a2 or b3a2) is based on the size of the PCR products that detecting using agarose gel electrophoresis.

The type of *bcr-abl* transcript was identified in patients included in this study mostly as b3a2 but some patient's samples show a figure in-between b2a2 and b3a2.

Standard positive controls must be included to check for efficiency and sensitivity. Convenient positive control used in this study was commercially extracted RNA from cell line K652 (b3a2) which is recommended in other studies^[28].

For the purposes of validation, unselected series of specimens were tested, rather than evaluation samples from only a selected group of patients. So, the study included specimens across the spectrum of specimen's types, collection variables and patient's variables.

In conclusion, the concomitant evolution of multiple leukemia translocations in the same specimen and correlation with standard cytogenetic analysis serves as a powerful internal quality control for the assays, analytic specificities and sensitivity. The genetic analysis of leukemia has prognostic and therapeutic implication. This, together with the advent of targeted therapy, underscores the need for an accurate genetic diagnosis that can be rapidly be obtained by molecular approaches and which is key to rational risk stratification and institution of appropriate therapy.

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