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Frequency of BCR-ABL Fusion Transcript Types with Chronic Myeloid Leukemia by Multiplex PCR in Srinagarind Hospital, Khon Kaen Thailand

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ABSTRACT

Context: *Chronic myeloid leukemia (CML) is characterized by the consistent involvement of the Philadelphia chromosome (Ph), which is derived from a reciprocal translocation between chromosome 9 and 22, the main product of the t(9;22)(q34;q11) translocation, is found in the leukemic clone of at least 95% of CML patients. There are two major forms of the BCR/ABL fusion gene, involving ABL exon 2, but including different exons of BCR gene. The transcripts b2a2 or b3a2 code for a p210 protein. Another fusion gene leads to the expression of an e1a2 transcript, which codes for a p190 protein. Other less common fusion genes are b3a3 or b2a3, which codes for a p203 protein and e19a2 (c3a2) transcript, which codes for a p230 protein. Its frequency varies in different populations. In this study we aimed to report the frequency of BCR-ABL fusion transcript types with CML by multiplex PCR in Srinagarind hospital, Khon Kaen Thailand. Multiplex PCR for BCR-ABL was performed on 58 patients, to detect different types of BCR- ABL transcripts of the t(9; 22). All patients examined were positive for some type of BCR/ABL rearrangement. The majority of the patients (93.10%) expressed one of the p210 BCR-ABL transcripts, b3a2 and b2a2 transcripts were detected in 53.45% and 39.65% respectively. The expression of an e1a2 transcript showed 3.75%. Co-expression of p210/p230 were detected in 3.45%. Co-expression of p210/p190 were not detected. Multiplex PCR is useful, saves time and reliable in the detection of BCR- ABL transcript types. The frequencies of one or other rearrangement in CML varies in different population.*

Keywords

BCR-ABL Fusion Transcript Type, Chronic Myeloid Leukemia, Multiple PCR.

Introduction

The Philadelphia (Ph) chromosome (derivative chromosome 22). This chromosome is the product of a reciprocal translocation between the long arms of chromosomes 9 and 22 t(9;22), was the first disease-specific chromosomal abnormality to be associated with a malignancy namely, chronic myelogenous leukemia (CML) [1]. Breaks in the c-abl gene typically occur in the first intron. Breaks in bcr generally occur in one of three regions: the major breakpoint cluster region (M-bcr), the minor breakpoint cluster region (m-bcr) and the micro breakpoint cluster region (m-bcr) [2]. Breakpoints occurring in M-bcr involve introns 13 or 14 and join exon13 (also known as b2) or 14 (also known as b3) with exon 2 of abl (a2) resulting in the fusion transcripts b2a2 (e13a2) and b3a2 (e14a2), respectively. These transcripts lead to the production of an 8.5 kb transcript coding for a 210 KDa (p210) chimeric protein [3,4]. Another breakpoints occurring in m-bcr leads to the expression of an e1a2 transcript, which codes for a p190 KDa (p190) protein. Breakpoints in m-bcr involve intron 19 and result in the joining of exon 19 (e19) of bcr with a2, e19a2 (c3a2), coding for a 230 KDa (p230) protein.5Fusion transcriptsb3a2 and b2a2 account for the majority of CML cases. The e1a2 fusion transcript is seen primarily in t(9;22) positive acute lymphoblastic leukemia and lymphoid blast phase of chronic myelogenous leukemia, but rarely in chronic myelogenous leukemia in chronic phase. There are e19a2 fusion transcript is found in cases of chronic myelogenous leukemia with prominent neutrophilic maturation [6]. Populations also showed different percentages of the two most common transcripts b2a2 and b3a2, and of the rarer transcripts in

their CML patients [7,8]. This study was designed to determine the frequency of BCR-ABL fusion transcript types with CML by multiplex PCR in Srinagarind Hospital, Khon Kaen Thailand.

Material and Methods

Multiplex PCR for BCR-ABL was performed on 58 patients with CML attending the Hematology clinic Srinagarind Hospital, Khon Kaen between January 2016- October 2016, to detect different types of BCR-ABL transcripts. All patients have been diagnosed as Ph-positive CML.

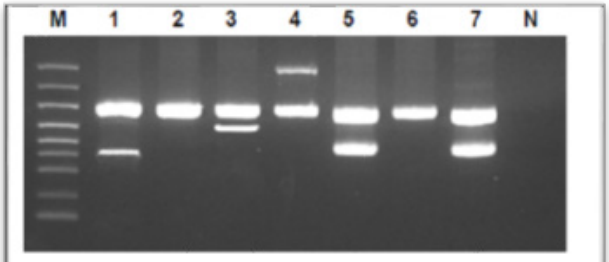
Ribonucleic acid (RNA) extraction and cDNA synthesis: 6-12 ml of Peripheral Blood (PB) or 1-3 ml of Bone marrow aspiration (BMA) samples were diluted in an NH4Cl: Tris solution to lyse the red cells and the white cell fraction was pelleted and washed once in PBS. Total RNA was extracted from peripheral the white cell pellets using Trizol (Invitrogen, Grand Island, USA) according to the manufacturer instructions. For cDNA synthesis, the RNA quantity and quality was assessed using a Nano Dropspectrophotometer (Nano Drop Technologies, Wilmington, Delaware, USA) and if 260/280 ratio in the Nano Drop monitor less than 1.8, repeated RNA extraction can be carried out [9]. Then cDNA synthesized using first strand cDNA synthesis kit (Fermentas UAB, Lithuania). One micro gram RNA was reversely transcribed with 10U/µl MMLV, in 1x RT buffer, 25ng/µl random hexamer primer, 25µM dNTP, 0.01M DTT and 2U/µl RNasin. At 75ºC for 2 min, 42 ºC for 1 hr, and 75ºC for 10 min.

Multiplex RT-PCR conditions: The cDNA samples were also tested of BCR-ABL transcript types using the Seeplex® Leukemia BCR/ABL research kit (Seegene, Korea). Prepare the master mix which includes; 4 µl of 5X Leukemia BCR/ABL PM (primer pair for BCR/ABL detection and primer pair for internal control), 3 µl of 8-MOP solution (DNA polymerase, Buffer containing dNTPs and MgCl2+stabilizer), and 10µl of 2X multiplex master mix (System to prevent carry-over contamination). Aliquot 17µl of master mix in 0.2 ml PCR tube then add 3 µl of cDNA samples product. For positive control use 3µl of the Leukemia BCR/ABL PC. And use 3µl the distilled water for negative control. Immediately run the PCR reaction using the following program; 1 cycler of denaturation at 94 ºC for 15 min, 37 cycler of annealing at 94ºC for 0.5 min, 60ºC for 1.5 min, 72ºC for 1.0 min and 1 Cycler of extension at 72ºC for 10 min. Electrophoresis 5µl of the PCR products and 5µl of BCR/ABL marker (Use to determine the approximate size target product) on a 2% agarose gel stained with ethidium bromide to analyze the size of the amplicons. Procedure overview shown in Figure 3.

This project was conducted according to the principles of the Helsinki Declaration, and was approved by the Khon Kaen University Ethical Review Board. Each patient has an assigned written informed consent. Reference No. HE591203.

Results

The expected bands were as follows: 1012 bp (c3a2), 764 bp (b1a1), 600 bp (Internal control), 476 bp (b3a2), 401 bp (b2a2), 348 bp (e1a2), 299 bp (b3a3), 224 bp (b2a3), 174 bp (e2a3). The quality of RNA and efficiency of cDNA synthesis was analyzed by amplification of BCR gene as an internal control. The amplified product (600bp) from the BCR gene was the only band detected in BCR/ABL negative patients. The absence of this bands indicated procedural failure. The results of multiplex RT-PCR for some different patients are shown in Figure 1, 2 and Table 1.



Samples No	Result
1	*e1a2*
2	*No BCR/ABL translocation*
3	*b3a2*
4	*c3a2*
5	*b2a2*
6	*No BCR/ABL translocation*
7	*b2a2*

Figure 1: Determination of BCR-ABL fusion transcript product obtained by real-time reverse transcription-polymerase chain reaction (qRT-PCR) in agarose gel. M: BCR/ABL Marker, Line1: e1a2, Line2: No BCR/ABL translocation, Line3: b3a2, Line4: c3a2, Line5: b2a2, Line 6: No BCR/ABL translocation, Line7: b2a2 and Lane N: Distilled water as negative control.

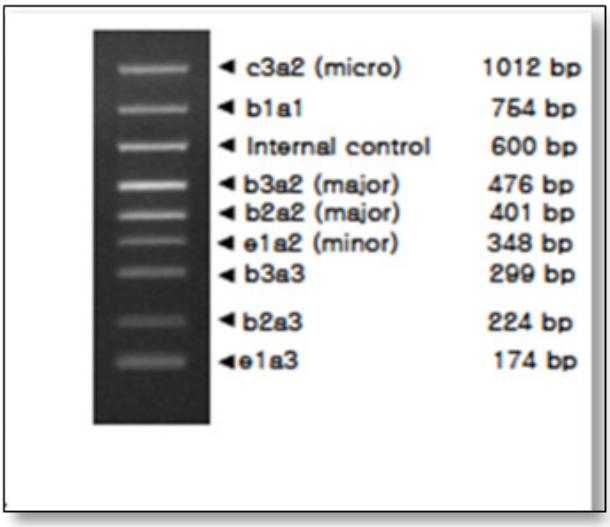


Figure 2: Gel of BCR/ABL Marker results. Band1: c3a2 or e19a2 (1012bp); Band2: b1a2 (764bp), Band3: Internal control (600bp), Band4: b3a2major (476 bp), Band5: b2a2major (401bp), Band6: e1a2minor (348 bp), Band 7: b3a3 (299bp), Band8 b2a2 (224bp), Band9 e1a3 (174 bp).

Leukemia BCR/ABL	Size in agarose gel (bp)
Internal control	600
c3a2 (micro) or e19a2	1012
b1a1	754
b3a2 (major)	476
2a2 (major)	401
e1a2 (minor)	348
3a3	299
b2a3	224
e1a3	174

Table 1: Amplicon Information.

The primer combinations in multiplex RT-PCR allowed simultaneous detection of all known types of BCR-ABL and BCR transcripts in one reaction simultaneously.

We were able for the reliable detection of typical p210 transcripts, such b2a2 or b3a2 and atypical types, such as transcripts lacking ABL exon a2 (b2a3 and b3a3), or p190BCR/ABL transcripts, such as e1a2 in 58 patients at the time of presentation (Figure 2).

Using the Seeplex® Leukemia BCR/ABL research kit, all patients examined were positive for some type of BCR/ABL rearrangement. The majority of the patients (93.10%) expressed one of the p210 BCR-ABL transcripts, b3a2 and b2a2 transcripts were detected in 53.45% and 39.65% respectively. Co-expression of p210/p230 was detected in 3.45%. The expression of an e1a2 transcript, which codes for a p190 protein showed 3.75 %. Co-expression of p210/p190 was not detected. Shows in Table 2.

Determine the sensitivity of the Seeplex® Leukemia BCR/ABL, a standard serial dilution has been set up from 105 to 10-1 plasmid DNA copy/reaction and was analyzed with the Seeplex® Leukemia BCR/ABL. The detection limit of the Seeplex® Leukemia BCR/ABL is 10 copies/reaction (10 copies/3 µL nucleic acid). Reproducibility tests were carried out at 5 different points of time in the course of 2 weeks by 3 different experimenters. The same results were obtained in every test, confirming the reproducibility of the product.

BCR-ABL fusion transcripts	Protein	Percent (%)
b3a2	210	61.25
b2a2	210	35
e1a2	190	3.75
b3a2/e19a2	210/230	3.45

Table 2: Frequencies of BCR/ABL Transcriptsin KhonKaen CML patients.

Discussion

RT-PCR is useful for analyzing the transcriptional activity of genes and gene isoforms. Multiplex RT-PCR is similar to conventional PCR but includes more than one pair of primers, so that all the known bcr-abl transcripts can be detected. For diagnostic samples, the use of multiplex 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.

Seeplex® Leukemia BCR/ABL which can detect the major leukemia translocations by RT-PCR has numerous advantages over conventional cytogenetics, including no requirement for dividing cells, grouping for related genes, rapid and efficient for test, detection of translocations that may be missed by conventional cytogenetics. This product detects type and variants type of leukemia with only one PCR reaction by using DPO™ after cDNA synthesis and maximize specificity, sensitivity and accuracy.

The Internal Control (IC) has been added to check for substances in the processed specimens that may interfere with PCR amplification. The Internal Control is introduced into each amplification reaction and is co-amplified with target nucleic acid from the clinical specimens. In addition, 8-methoxypsoralen (8-MOP) is used to terminate the template activity of contaminated DNAs. 8-MOP is known to intercalate into double-stranded nucleic acids and forma covalent inter strand crosslink after photo-activation with incident light of wavelength of 320-400 nm. Please discard the PCR tube after UV irradiation (365 nm) for 20 minutes to prevent carry-over contamination.

BCR-ABL gene rearrangement studies in 58 Khon Kaen Ph+ CML patients showed the frequency of b3a2 and b2a2 transcripts to be 53.45% and 39.65 % respectively. In a study by Marjan Yaghmaie et al., 102007 the incidence of b3a2 and b2a2 transcripts in Ph+ CML patients was 62% and 20% respectively. Sulaf Farhat-Maghribi et al., 2016 found that the frequency of b3a2 and b2a2 transcripts to be 57.1% and 14.3% respectively. Its frequency varies in different populations. In Khon Kaen, Thailand CML patients, the frequency of b3a2 transcripts was found higher than that of b2a2.

In this study we found a low incidence of CML patients (3.45%) expressing more than one type of mRNA. For example, one patient showed co-expression of b3a2 and e19a2 fusion genes. Co-expression of more than one type of fusion transcript in a patient could be due to alternative splicing or for rare type due to existence of several leukemia cell lines with different BCR-ABL transcript expression.

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