นิพนธ์ต้นฉบับ ▪ Original Article

เทคนิค Multiplex PCR เพื่อระบุชนิดการกลายพันธ์ของจีน BCR/ABL ในผู้ป่วยโรคมะเร็งเม็ดเลือดขาว ชนิด Chronic Myeloid Leukemia พื้นที่ภาคตะวันออกเฉียงเหนือ ประเทศไทย

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Multiplex PCR for Identifying BCR-ABL Fusion Transcript Types in Northeastern Thailand Chronic Myeloid Leukemia Patients

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หลักการและวัตถุประสงค์: ฟิลาเดลเฟียโครโมโซมเป็นโครโมโซมที่มีต้นกำเนิดมาจากการแลกเปลี่ยนซึ่งกันและกันระหว่างแขนยาวของโครโมโซม 9 และโครโมโซม 22 t(9;22) (q34;q11) พบได้ร้อยละ 90 ถึงร้อยละ 95 ในผู้ป่วยมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอิลอยด์ (CML) การแลกเปลี่ยนโครโมโซมดังกล่าวทำให้เกิดการสร้างจีนกลายพันธุ์ BCR-ABL โดยพบได้บ่อย ๆ มี 2 ชนิดประกอบด้วยชนิด b3a2 และ b2a2 ซึ่งอยู่บนโปรตีนขนาด 210 กิโลดาตัน (210 KDa) ชนิดอื่น เช่น e1a2 อยู่บนโปรตีนขนาด 190 กิโลดาตัน (190 KDa) และที่พบได้น้อยคือชนิด e19a2 อยู่บนโปรตีนขนาด 230 กิโลดาตัน (230 KDa) ชนิดของจีนกลายพันธุ์ของจีน BCR-ABL มีรายงานการค้นพบที่แตกต่างกันไปในแต่ละพื้นที่ แต่ยังไม่มีการรายงานความความถี่ของชนิดการกลายพันธ์ของจีน BCR-ABL ในผู้ป่วยมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอิลอยด์ในพื้นที่ภาคตะวันออกเฉียงเหนือประเทศไทย การศึกษาครั้งนี้มีจุดประสงค์เพื่อรายงานชนิดการกลายพันธุ์ของจีน BCR-ABL ในภูมิภาคตะวันออกเฉียงเหนือโดยใช้เทคนิคมัลติเพลกพีซีอาร์

วิธีการศึกษา: เป็นการศึกษาย้อนหลังศึกษาในในผู้ป่วยมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอิลอยด์ผู้ใหญ่ในโรงพยาบาลศรีนครินทร์และโรงพยาบาลศูนย์ขอนแก่น ซึ่งเป็นโรงพยาบาลระดับตติยภูมิในพื้นที่ภาคตะวันออกเฉียงเหนือ ประเทศไทย โดยเก็บข้อมูลจากทะเบียนผู้ป่วยเป็นระยะเวลา 3 ปี (มกราคม 2560 ถึงมกราคม 2563) เลือดหรือไขกระดูกของผู้ป่วยมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอิลอยด์ผู้ใหญ่ถูกเก็บมาตรวจวิเคราะห์โดยใช้เทคนิคมัลติเพลกพีซีอาร์จากผู้ป่วยจำนวน 177 ราย มีจุดประสงค์เพื่อจะระบุชนิดการกลายพันธุ์ของจีน BCR-ABL ในกลุ่มผู้ป่วยมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอิลอยด์ ในภูมิภาคตะวันออกเฉียงเหนือประเทศไทย โดยใช้เทคนิคมัลติเพล

Background and Objective: Philadelphia chromosome is the chromosome originating from the reciprocal translocation between long arms of chromosome 9 and chromosome 22 t (9;22) (q34; q11), it presents in 90 - 95% of patients with chronic myeloid leukemia. The aberration results from a reciprocal translocation are creating a BCR-ABL fusion gene. There are two major forms of the BCR-ABL fusion gene, involving ABL exon 2, but including different exons of BCR gene. The transcript b3a2 and b2a2 codes for a p210 protein. Other fusion gene leads to the expression of an e1a2 transcript, which codes for a p190 protein. Its frequency varies in different populations but there are no data from northeastern Thailand. In this study we aimed to report BCR-ABL fusion transcript types in northeastern Thailand CML patients by Multiple Reverse Transcriptase-Polymerase Chain Reaction (Multiplex PCR).

Materials and Methods Retrospective descriptive and analytical study of all adult CML patients in Srinagarind and Khon Kaen hospitals, which is the main tertiary medical center in the northeastern region Thailand. Data has been collected from medical records for 3 years (January 2017 to January 2020). Peripheral blood or bone marrow samples were analyzed by multiplex PCR from 177 adult northeastern Thailand CML patients. Aim to identify BCR-ABL fusion transcript types in northeastern Thailand CML patients. Multiplex

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กพีซีอาร์
ผลการศึกษา: ผู้ป่วยทั้งหมดให้ผลบวกชนิดใดชนิดหนึ่งต่อจีนกลายพันธุ์ BCR-ABL ผู้ป่วยส่วนใหญ่ร้อยละ 93.79 ตรวจพบว่าเป็นกลุ่ม Major โดยระบุว่าเป็นชนิด b3a2 และ b2a2 ร้อยละ 58.19 และ 35.59 ตามลำดับ ตรวจพบว่ามีจีนซ้อนกันระหว่างชนิด b3a2(p210) และ e1a2(p230) ร้อยละ 0.56 สำหรับชนิด e1a2 ซึ่งอยู่บนโปรตีนขนาด 190 นั้นถูกค้นพบร้อยละ 4.52 และชนิด e19a2 ซึ่งอยู่บนโปรตีนขนาด 230 นั้นพบจำนวนร้อยละ 1.14 และไม่พบการการซ้อนกันระหว่างโปรตีน 210 กับโปรตีน 190
สรุป: เทคนิคมัลติเพลกพีซีอาร์มีประโยชน์และประหยัดเวลาในการตรวจเพื่อระบุชนิดจีนกลายพันธุ์ BCR-ABL ซึ่งจะสามารถช่วยในการพยากรณ์โรคและการรักษาโรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอิลอยด์

คำสำคัญ: ชนิดการกลายพันธุ์ของจีน BCR-ABL; มัลติเพลกพีซีอาร์; โรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยลิลอยด์

PCR.
Results: All patients examined were positive for some type of BCR/ABL rearrangement. The majority of the patients (93.79%) expressed one of the p210 BCR-ABL transcripts, b3a2 and b2a2 transcript types were detected in 58.19% and 35.59% respectively. Co-expression of b3a2 (p210)/e1a2 (p230) was detected in 0.56%. The expression of an e1a2 transcript type, which codes for a p190 protein showed 4.52%. And 1.14% was detected in e19a2 transcript type. Co-expression of p210/p190 was not detected.
Conclusions: Multiplex RT-PCR is useful and saves time in the detection of BCR-ABL fusion transcript types; the occurrence of these transcripts associated with CML can assist in prognosis and treatment of disease.

Keyword: Multiplex PCR, BCR-ABL Fusion Transcript Types, Chronic Myeloid Leukemia (CML).

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Introduction

The Philadelphia (Ph) chromosome that results from a reciprocal translocation involving the long arms of chromosomes 9 and 22, t (9,22)(q34, q11), is one of the most frequent cytogenetic abnormalities in hematological disorders.[1] It is found in 98% of chronic myeloid leukemia (CML), 20-40% of adult acute lymphoblastic leukemia (ALL), 5% of childhood ALL, and 2% of acute myeloblastic leukemia (AML).[2] At the molecular level, t (9,22) is characterized by the fusion of the proto-oncogene ABL (9q34.1) and the breakpoint cluster region (BCR) at band 11.2q of chromosome 22, giving rise to a chimeric gene, BCR-ABL, which is translated into a fusion protein with transforming ability.[3] Diagnosis of CML is based on the detection of BCR-ABL gene. This reciprocal translocation gives rise to BCR-ABL fusion oncogene, which translates a chimeric protein, p210 BCR-ABL that is characterized by constitutive activation of its tyrosine kinase activity. The BCR-ABL gene encodes different fusion proteins that vary in size depending on the breakpoint in the BCR gene. In general, three breakpoint cluster regions in the BCR gene have been described: M-BCR, m-BCR, and μ-BCR.[4] 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.[5,6] As a result of alternate splicing these transcripts lead to the production of a 8.5 kb transcript coding for a 210 kDa (p210) chimeric protein.[7,8] Approximately 70% of Ph-positive ALL cases have breakpoints that result in the fusion of BCR exon e1 to ABL exon a2 (e1a2 transcript), which encodes a smaller (190 kDa) BCR-ABL protein (p190) The remaining 30% have rearrangements that are identical to those in CML.[9] In some cases, the breakpoint between BCR exon 19 and 20 in the u-BCR region induces a larger p230 BCR-ABL protein.[10-13] Rarely, patients present with a BCR-ABL oncogene containing a breakpoint within the μ-bcr region at exone19 that produces a230 kDa tyrosine kinase (p230).[14] In addition, some of the previous reports performed showed that the frequencies of BCR-ABL mRNA transcripts in CML patients differ in different ethnic groups.[15,16] Not only does the frequency of different subtypes among CML patients vary between different studies, but also the impact of these subtypes on the phenotypic characteristics of CML patients is a conflicting issue.[17] Polymerase Chain Reaction (PCR) is a rapid and sensitive method used for in vitro amplification of size-limited DNA segments. A multiplex-PCR assay for the identification of BCR-ABL transcript types that allows the simultaneous detection of two or more genes in the same reaction.[18] This study was designed to identify BCR-ABL fusion transcript types in

northeastern Thailand CML patients by Multiplex RT-PCR.

Materials and Methods

Study Design

We designed a retrospective descriptive and analytical study of all adult CML patients in Srinagarind and Khon Kaen hospitals, which is the main tertiary medical center in the northeastern region Thailand. Data has been collected from medical records for 3 years (January 2017 to January 2020).

Methods

Multiplex PCR for BCR-ABL was performed on 177 patients with CML, who attending the Hematology clinic Srinagarind hospital and Khon Kaen hospital. Peripheral blood and bone marrow samples were analyzed by multiplex PCR, to identify BCR/ABL fusion transcript types in northeastern Thailand CML patients. All patients have been diagnosed as Ph-positive CML.

RNA Isolation

Ribonucleic acid (RNA) extraction and cDNA synthesis: 6 ml of Peripheral Blood (PB) or 1-3 ml of Bone Marrow Aspiration (BMA) samples were diluted in a 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 NanoDrop spectro-photometer (NanoDrop-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.[19]

cDNA synthesized

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 75°C for 2 min, 42 °C for 1 hr, and 75°C 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 $MgCl^{2+}$ 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 °C for 15 min, 37 cycler of annealing at 94 °C for 0.5 min, 60°C for 1.5 min, 72 °C for 1.0 min and 1 Cycler of extension at 72 °C 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.

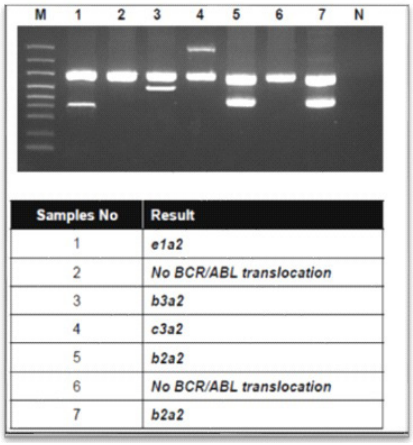
Ethical consideration

This study protocol has been approved and reviewed by the Khon Kaen University Ethic Committee for Human Research based on the declaration of Helsinki and the ICH Good Clinical Practice Guideline, reference No. HE 621519.

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.

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 p190 BCR-ABL transcripts, such as e1a2 in 177 patients at the time of presentation in the Figure2. Using the Seeplex® Leukemia BCR-ABL research kit, all patients examined



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 (RT-PCR) in agarose gel. M: BCR/ABL Marker, Line1: e1a2, Line2: No BCR/ABL translocation, Line3: b3a2, Line4: c3a2, Line5: b2a2, Line6: No BCR/ABL translocation, Line7: b2a2 and Lane N: Distilled water as negative control.

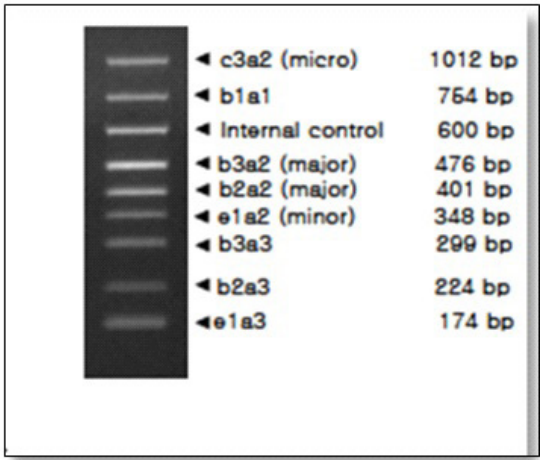


Figure 2 Shown gel of BCR/ABL Marker results. Band1: c3a2 or e19a2 (1012bp); Band2: b1a2 (764bp), Band3: Internal control (600bp), Band4: b3a2major (476 bp), Band5: b2a2major (401bp), Band6: e1a2minor (348 bp), Band7: b3a3 (299bp), Band8 b2a2 (224bp), Band9 e1a3 (174 bp)

were positive for some type of BCR/ABL rearrangement. The majority of the patients (93.79%) expressed one of the p210 BCR-ABL transcripts, b3a2 and b2a2 transcript types were detected in 58.19% and 35.59 % respectively. Co-expression of b3a2 (p210) / e1a2 (p230) was detected in 0.56%. The expression of an e1a2 transcript type, which codes for a p190 protein showed 4.52 %. And 1.14% was detected in e19a2 or c3a2 transcript type. Co-expression of p210/p190 was not detected as shown in Figure 3.

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

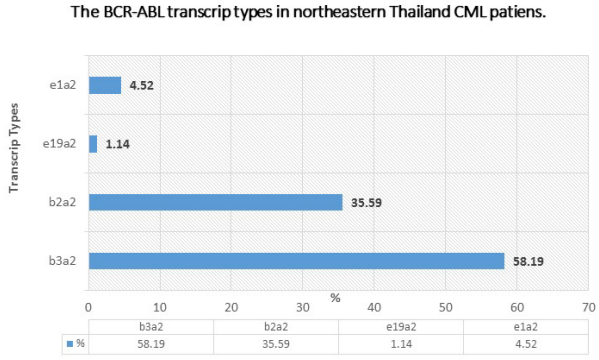


Figure 3 Definite the BCR-ABL transcript types in northeastern Thailand CML patients.

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.

Discussion

RT-PCR is useful for analyzing the transcriptional activity of genes and gene isoforms. Multiplex 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 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 preventcarry-over contamination.

BCR-ABL gene rearrangement studies in 177 adult northeastern Thailand, The Philadelphia chromosome Positive (Ph+) CML patients showed the frequency of b3a2 and b2a2 transcripts to be 58.19% and 35.59 % respectively. In a study by Marjan Yaghmaie et al[20], the incidence of b3a2 and b2a2 transcripts in Ph+ in Iranian CML patients was 62% and 20% respectively. Goh et al[21], found that Korean CML patients (538/548, 98.18%) were found to have b3a2 or b2a2, and total frequency of occurrence of c3a2, e1a2, b2a3, b1a1, and e1a3 or coexpression of b2a2 and b3a2 was less than 2.00%. In Eastern India found out of 122 cases, 33 b2a2, 69 b3a2, 2 e1a2, and 2 e19a2 cases have been detected. Six coexpressed both b2a2 and b3a2 transcripts in study by Mondal et al.[22] 250 Mexican patients with chronic myeloid leukaemia found 226 (90.4%) patients it was p210, while the remaining 9.6% showed coexpression or one of the transcripts of p190/p210/p230. In 7% of patients with p210 expression there are both isoforms (b3a2/b2a2) in a study by R.M. Arana-Trejo et al.[23] Its frequency varies in different populations. In adult northeastern Thailand Ph+ 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 (0.56%) 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.

Conclusions

Multiplex RT-PCR is a simple, useful and time-saving technique that allows specific and simultaneous detection of BCR-ABL transcripts; the occurrence of these transcripts associated with CML can assist in prognosis and treatment of disease.

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