การตรวจหาการกลายของยีนลูกผสมชนิด BCR-ABL
ในผู้ป่วยโรคมะเร็งเม็ดโลหิตขาวชนิดยี่ยมออลอยด์
แบบเร็ววิ่ง ขาวไทย

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บทกลมย่อ
การกลายของยีน BCR-ABL ในบริเวณ kinase domain (KD) มักเป็นสาเหตุสำคัญของการดื้อยาตัวแทนในผู้ป่วยโรคมะเร็งเม็ดโลหิตขาวชนิดยี่ยมออลอยด์แบบเร็ววิ่งขาวไทย ซึ่งการกลายนี้จะลดความสามารถของการต้านภัยที่จะไปยัง kinase activity การตรวจสอบกลายในระยะแรกจะช่วยพวกเรื่องใน การวางแผนการรักษาผู้ป่วยให้เหมาะสม เพื่อป้องกันการไม่ให้เกิดการกลายที่ วัตถุประสงค์ของการค้นหา เทคโนโลยีที่มีผลต่อการกลายของยีน BCR-ABL ในผู้ป่วยมะเร็งเม็ดโลหิตขาวชนิดยี่มออลอยด์แบบเร็ววิ่งที่มีผลต่อสมัคติในโพรเจกต์การทางคลินิกแบบทางตรง จากด้านย่อยแสดงหรือใช้บูรณาการจำนวน 59 ตัวอย่าง

ผลการทดสอบพบว่าผู้ป่วยที่มีการกลายของยีนลูกผสมชนิด BCR-ABL จำนวน 29 ราย (ร้อยละ 49.15)
ของผู้ป่วยทั้งหมด โดยพบ multiple mutation ในผู้ป่วยจำนวน 3 ราย (ร้อยละ 10.34 ของผู้ป่วยทั้งหมด)
และการกลายที่มีมากที่สุดคือ ซีดี T315I และ F359V โดยพบทั้งหมด 8 ราย (ร้อยละ 13.56) นอกจาก
นั้นพบการกลายชนิด G250E, Y253H, Q252H, F359C, Y253F, E275K และ F317L โดยพบจำนวน
7 ราย (ร้อยละ 11.86), 6 ราย (ร้อยละ 10.17), 2 ราย (ร้อยละ 3.39), 2 ราย (ร้อยละ 3.39), 1 ราย
(ร้อยละ 1.69), 1 ราย (ร้อยละ 1.69) และ 1 ราย (ร้อยละ 1.69) ตามลำดับ
โดยสรุปพบที่มีการกลายที่พบสูงที่สุดคือ T315I และ F359V

คำวิพากษ์: โรคมะเร็งเม็ดโลหิตขาวชนิดยี่มออลอยด์แบบเร็ว BCR-ABL การกลาย ยาตัวแทนการที่
พบพบว่า สามารถตรวจสอบการกลายที่ปรากฏในไม้ได้
และสามารถตรวจสอบการกลายดังกล่าวที่ exon 4 ถึง 9

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BCR-ABL Mutation Detection in Thai Chronic Myeloid Leukemia Patients

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Abstract

Mutations in the kinase domain (KD) of BCR-ABL lead to drug resistance by reducing the capacity of imatinib to inhibit kinase activity. The early detection of such mutation may allow timely treatment intervention to prevent or overcome the resistance. The objective of this report is to develop a direct sequencing method for detection of BCR-ABL mutations in imatinib mesylate resistance CML patients. The detection method involved RT-PCR amplification of the entire kinase domain of the BCR-ABL and then detected all BCR-ABL point mutation by direct sequencing.

Twenty-nine cases of 59 CML patients (49.15\%) were reported for the incidence of BCR-ABL mutations. Multiple mutations were detected in 10.34 \% of the patients and the most frequently detected mutations were T315I and F359V for 13.56 \% each. Moreover, the detected mutations were G250E, Y253H, Q252H, F359C, Y283F, E275K, and F317L for 7 cases (11.86\%), 6 cases (10.17\%), 2 cases (3.39\%), 2 cases (3.39\%), 1 case (1.69\%), 1 case (1.69\%), 1 case (1.69\%), respectively.

For summary, direct sequencing of the BCR-ABL kinase domain allowed detection of emerging mutations and was able to detect mutations for a broad range of BCR-ABL kinase domain exon 4 to 9 in CML patients.

Keywords: Chronic Myeloid Leukemia, BCR-ABL, Mutation Imatinib Mesylate, Direct Sequencing

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Introduction

Chronic myeloid leukemia (CML) results from the balanced translocation of c-ABL from chromosome 9 and BCR on chromosome 22, [(9;22) (q34;q11)]. This fusion gene plays an important role in signal transduction pathways resulting in enhanced cellular proliferation and blocked apoptosis. (1)

Imatinib mesylate (Gleevec, Novartis, Basel, Switzerland) is the current therapy of first choice for CML. (2) It is a potent and selective inhibitor of the BCR-ABL tyrosine kinase. (3) Although, most of the patients have a good response to Imatinib mesylate but some still showed relapse after an initial response (acquired resistance) or primary resistance. (4-8) To date, more than 90 different mutations leading to 50 amino acid substitutions in the BCR-ABL kinase domain have been identified (Fig. 1). It is believed that the mutations emerge under the selective pressure of Imatinib mesylate therapy. (6-8) The different mutations lead to varying levels of the drug resistance depending on locations of the mutations. (9) Some studies have suggested that mutations in the adenosine triphosphate binding site (P-loop) are associated with poor prognosis. (10) Thus, identification of specific mutations can assist physicians to develop an appropriate therapeutic strategy such as dose escalation, selection of new tyrosine kinase inhibitor, combination therapy, and bone marrow transplantation. For this reason, the early detection of the BCR-ABL kinase domain to screen for mutation may play important role in the management to prevent or overcome resistance. (11-12)

Here we described the cDNA sequencing method for detecting BCR-ABL mutations in Thai CML patients. Our method involved reverse transcription polymerase chain reaction (RT-PCR) amplification of BCR-ABL kinase domain from exon 4 to 9 which covered all the reported kinase domain mutations. (9) The present data showed that direct sequencing method was an efficient technique for single or multiple detection of BCR-ABL mutations.

Materials and Methods

Biological samples

A total of 59 bone marrow (BM) or peripheral blood (PB) samples was obtained from routine service of Human Genetics Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok from January 2008 to February 2010. All were previously diagnosed as CML by clinical and laboratory characteristics that were obtained through medical record chart review with confidentiality. This study (No.2009/1514) was approved by the Ethical Committee for Human Research, Faculty of Medicine Ramathibodi Hospital, Mahidol University.
Methods

RNA extraction and RT-PCR

Total cellular RNA was extracted from WBC of each sample using QIAGEN RNA Blood Mini Kits (QIAGEN, USA). The quantity of RNA was checked by absorbance at 260 and 280 nm using Nanodrop spectrophotometry (Delaware, USA). Four microgram of total cellular RNA was reverse transcribed to cDNA using random primers according to manufacturer’s instruction (10X hexanucleotide mixture, Boehringer Mannheim) and 100U of Superscript III reverse transcriptase.

We initially established singleplex RT-PCR reactions individually for each specific primer set to screen the optimal conditions that would diminish primer dimer formation (Table 1). The PCR reactions were carried in a final volume of 25 µl containing 5 µmol of each primer, 1U Taq polymerase, 200 µM each dNTP, 1X PCR buffer, and 1.5 mM MgCl₂. After PCR completion the size of each amplification product was separated by electrophoresis on 2 % agarose gel.

Direct sequencing

Direct sequencing was performed using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3130 DNA Analyzer (Applied Biosystems). Sequences were compared with the wild-type sequence using BLAST (ABL accession number M14752). Sequence analysis was performed on both strands of each fragment.

Table 1 Oligonucleotide primers used for the amplification of the ABL kinase domain

<table>
<thead>
<tr>
<th>ABL kinase domain</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABL exon 4-7</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>5'CGCAACAAGCCCACCTGTC3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'GCATGGGCTGTGTAGGTGTC3'</td>
</tr>
<tr>
<td>Amplicon size</td>
<td>534 bp</td>
</tr>
<tr>
<td>ABL exon 7-9</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>5'AGAGATCTTTGCTGCCCCGAAA3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'CCACTTTGCTGTAGATCTGGATT3'</td>
</tr>
<tr>
<td>Amplicon size</td>
<td>436 bp</td>
</tr>
</tbody>
</table>
**Results**

**Sequence Analysis for ABL Kinase Mutations**

Direct sequencing was done to confirm the presence of a point mutation in all cases. Results of sequence analyses are listed in Table 2. A total of 9 different point mutations was detected. These mutations involved an amino acid substitution. In twenty-nine of 59 patients (49.15%) were reported mutations. Multiple mutations were detected in 10.34% of the patients with mutations, and the most frequently detected mutations in our patients were T315I and F359V. These patients represented all phases of the disease, and virtually all mutations led to loss of imatinib response. Seventeen patients showed mutations (G250E, Y253F, Y253H, and E255K) falling within the nucleotide-binding loop (P-loop) in Fig. 2. Nineteen patients showed mutations outside the P-loop which located in other regions of the kinase domain (T315I, F317L, F359C, 259V).
Table 2  ABL kinase domain mutations detected in the present study

<table>
<thead>
<tr>
<th>BCR-ABL mutation</th>
<th>Nucleotide exchange (M14752)</th>
<th>Amino acid substitution</th>
<th>No. of cases with the mutation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G250E</td>
<td>1113G &gt; A</td>
<td>Gly 250 Glu</td>
<td>7 (11.86%)</td>
</tr>
<tr>
<td>Q252H</td>
<td>1120G &gt; T</td>
<td>Gln252His</td>
<td>2 (3.39%)</td>
</tr>
<tr>
<td>Y253H</td>
<td>1121T &gt; C</td>
<td>Tyr253His</td>
<td>6 (10.17%)</td>
</tr>
<tr>
<td>Y253F</td>
<td>1122A &gt; T</td>
<td>Tyr253Phe</td>
<td>1 (1.69%)</td>
</tr>
<tr>
<td>E275K</td>
<td>1127G &gt; A</td>
<td>Glu275Lys</td>
<td>1 (1.69%)</td>
</tr>
<tr>
<td>T315I</td>
<td>1308C &gt; T</td>
<td>Thr315 Ile</td>
<td>8 (13.56%)</td>
</tr>
<tr>
<td>F317L</td>
<td>1315C &gt; G</td>
<td>Phe317Leu</td>
<td>1 (1.69%)</td>
</tr>
<tr>
<td>F359C</td>
<td>1439T &gt; G</td>
<td>Phe359Val</td>
<td>2 (3.39%)</td>
</tr>
<tr>
<td>F359V</td>
<td>1440T &gt; G</td>
<td>Phe359Ala</td>
<td>8 (13.56%)</td>
</tr>
</tbody>
</table>

NOTE. Base numbers are detailed according to GenBank accession No.M14752

Fig. 2  Kinase domain mutations reported in Imatinib resistance Thai CML patients. The most frequent mutations fell into 2 categories that emphasized their structural role in Imatinib binding: nucleotide binding P-loop mutations, and direct contact points of Imatinib. For space considerations, single-letter amino acid codes were used here in place of 3-letter codes.
Discussion

Recently, there were several reports involving the identification of activating KD mutations in the BCR-ABL fusion gene which was the most common mutations in CML. Numerous studies have confirmed and extended these finding to the extent that BCR-ABL mutations are currently frequent single mutation described in CML with reported incidence approximately 10% in early CP, 30% in late CP, and 90% in blast crisis.\(^{14}\) The first BCR-ABL mutation identified in 2001 was T315I mutation at the base of ATP- binging pocket.\(^{11}\) Since no data previously existed in Thailand with respect to BCR-ABL mutations in CML, therefore we attempted to characterize the incidence and type of mutations in the KD of adult Thai CML patients.

The studies of BCR-ABL mutations in Asian countries were previously known only from Singapore and South Korea patients.\(^{1}\) This study represented the first series of BCR-ABL mutations in CML ever reported from Thailand. The incidence of BCR-ABL mutation in the present study compared to Singapore, and South Korea was 49.15%, 48.33%, and 65%, respectively. The incidence of BCR-ABL mutation was similar in Thai and Singaporian CML patients but lower than South Korian patients. This may be due to the different techniques used or the total number of studies samples in each study. All BCR-ABL mutations may reflect the geographic heterogeneity of the studied CML populations. The majority of mutations (29/59) in this study involved entered KD on ABL gene of the BCR-ABL fusion gene. Most BCR-ABL mutations reported were T315, F359, G250, Y253H and less frequently in Q252, E275, and F317. We found mutiple mutations in 10.34% of the patients with mutations. In other study, Bandford et al. reported that the frequency of BCR-ABL mutations in Australain was 66/279 cases (24%), most frequently represented M351T, E255K, F359V, T315I, and multiple mutations have been detected in 23% of the patients\(^{6}\) whereas from our study more mutation frequency was found 49.15%, but our data revealed less frequency of multiple mutations in the patients. The most frequent mutations in our patients were T315I and F359V that were similar to the report by Bandford et al. Therefore, the inconsistent distribution of BCR-ABL mutations may be attributed to the variation of ethnicity, genetic susceptibility and environmental exposure. The incidence of BCR-ABL hot spot mutations in Thai CML were P-loop and Imatinib contact site but was slightly less frequent than those reported from other parts of the world as shown in Table 3. This may be due to the different techniques.
used or the total number of sample size. It could be speculated that multiple mechanisms of the relation to Imatinib mesylate resistance across all countries included BCR-ABL mutations, BCR-ABL gene amplification, and clonal cytogenetic evolution.\(^{(3)}\) The BCR-ABL mutation is the common cause of secondary resistance occurring in 50 to 90% of resistance cases.\(^{(7)}\) For example, the C to T transition of residue T315 leads to the substitution of wild type threonine by isoleucine. T315I mutations ranged from 4-19% is one of the most frequent mutations.\(^{(11)}\) Moreover, it is reported that the different BCR-ABL mutations lead to different degree of drug resistance. F317L was detected in our study leading to partial drug resistance, whereas Y253F, Y253H, E255K and T315I were detected and would lead to complete drug resistance. Only T315I was detected by conferred resistance to all targeted tyrosine kinase inhibitors. Therefore, it can be managed clinically by bone marrow transplant. Thus identification of specific mutations can assist physicians to develop an appropriate therapeutic strategy for their patients.

### Table 3  Frequency of BCR-ABL mutations in CML by various reports

<table>
<thead>
<tr>
<th>Country</th>
<th>No. of sample (n)</th>
<th>Method</th>
<th>% Mutation positive</th>
<th>Mutation location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singapore</td>
<td>60</td>
<td>Direct sequencing</td>
<td>48.33%</td>
<td>P-loop, IM binding site, C-loop, A-loop, Other sites</td>
</tr>
<tr>
<td>South Korea</td>
<td>68</td>
<td>ASO-PCR</td>
<td>65%</td>
<td>P-loop, IM binding site, C-loop, A-loop</td>
</tr>
<tr>
<td>Australia</td>
<td>279</td>
<td>Direct sequencing</td>
<td>24%</td>
<td>P-loop, IM binding site, C-loop, A-loop</td>
</tr>
<tr>
<td>Germany</td>
<td>66</td>
<td>ASO-PCR</td>
<td>22.7%</td>
<td>P-loop, IM binding site, C-loop, A-loop</td>
</tr>
<tr>
<td>Thailand</td>
<td>59</td>
<td>Direct sequencing</td>
<td>49.15%</td>
<td>P-loop, IM binding</td>
</tr>
</tbody>
</table>
Conclusions

Direct sequencing of the BCR-ABL kinase domain is relatively rapid and allows detection of emerging mutations in CML patients. This technique permits detection of single or multiple mutations covering amino acids 236–486 and is used in clinical labs worldwide. Identification of specific mutations can assist physicians to develop an appropriate therapeutic strategy for their patients by indicating whether a higher dose of imatinib, use of dasatinib or nilotinib or other therapies which might be more beneficial. It is therefore important that emerging mutations could be detected early to allow timely treatment intervention before overt relapse.

Acknowledgements

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References


