RAPID IDENTIFICATION OF β-THALASSEmia MUTATIONS BY MUTAGENICALLY SEPARATED POLYMERASE CHAIN REACTION

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Abstract  β-thalassemia is a syndrome characterized by a reduction or complete absence of the β-globin chain. More than 25 mutations within and flanking the β-globin gene have been identified as the cause of this syndrome in Thailand. To date, several PCR-based techniques have been employed to rapidly identify the types of β-thalassemia mutations. The recently introduced MS-PCR is among those techniques used for this particular purpose. However, the identification of the β-thalassemia mutations by this technique has never been carried out in Thailand. The main objective of this study was to demonstrate the potential of MS-PCR in the swift and accurate characterization of the β-thalassemia mutations commonly seen in northern Thailand. The study was conducted in 60 β-thalassemic patients attending the Thalassemia Clinic, Department of Pediatrics, Maharaj Nakorn Chiang Mai Hospital. The direct DNA preparation from the buffy coat was accomplished using the Chelex method. The MS-PCR was performed in all samples with 3 primers including common, mutant and wild type. The cycle sequencing reaction using the Big Dye™ Nucleotide Sequencing kit was carried out to confirm the results obtained from the MS-PCR technique. The MS-PCR technique was proved to be accurately capable of detecting 6 common β-thalassemia mutations found in Thailand including codons (Cds) 41/42 (-TTCT), codon (Cd) 26 (G-A), codon (Cd) 17 (A-T), condons(Cds) 71/72 (+A), nt-28 (A-G) and IVS2 – nt 654. By employing the MS-PCR technique, 111 alleles including 54 Cds 41/42 (-TTCT), 30 Cd 26 (G-A), 20 Cd 17 (A-T) and 7 nt-28 (A-G) of the β-globin gene mutations were identified in 56 thalassemic patients. This included 93.3% of the β-thalassemic patients studied in this project. Only 9 alleles in 5 patients, or 6.7% of the studied subjects, were left unidentified and nucleotide sequencing was accomplished to complete the task. This study demonstrated the potential application of the MS-PCR technique in the detection of β-thalassemia mutations. It also indicated that the MS-PCR technique could be an alternative choice for the identification of β-thalassemia mutations which are commonly encountered in northern Thailand. Chiang Mai Med Bull 2004;43(4):133-141.

Keywords: β-thalassemia mutation, mutagenically separated polymerase chain reaction

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β-thalassemia is a syndrome characterized by a reduction or complete absence of the β-globin chain. A resulting imbalance in the globin chain synthesis occurs, in which the α-globin chain is relatively excessive, and the forms homotetramer formed is composed entirely of the α-globin chain (α₄). The α₄-homotetramer tends to precipitate and proteolyse inside young red blood cells that die prematurely inside bone marrow, thus resulting in ineffective erythropoiesis. About 3-9% of Thai people carry a β-thalassemia gene and 8-70% possess an HbE gene. These people could generate new cases of β-thalassemia, which would be overwhelmingly problematic for Thailand as a whole. Affected individuals suffer from chronic anemia, which requires frequent blood transfusions; jaundice; hepatosplenomegaly; growth retardation; bone changes which generates the “Mongoloid face” appearance; and other fatal complications. Finally, these patients will die prematurely at a young age. (1-4)

β-thalassemia is mainly caused by point mutations within and flanking the β-globin gene. Locations at which these mutations occur reflect the clinical phenotype of the patients. Mutations within and adjacent to the coding regions mostly result in a clinical outcome more severe than that occurring elsewhere. (5,6) To date, more than 300 mutations that cause β-thalassemia have been identified across the world (http://globin.cse.psu.edu/). In Thailand, 26 such mutations have been identified, which in a homozygous or compound heterozygous state could generate life-threatening β-thalassemia. (7)

Several PCR-based techniques have been employed to identify the β-globin mutations that cause β-thalassemia. These include the restriction enzyme digestion of PCR products, allele-specific oligonucleotide hybridization (ASO dot blot), reverse dot blot (RBD), allele specific amplification such as the amplification refractory mutation system (ARMS) and nucleotide sequencing. (8) These techniques have their own strong and weak points. The mutagenically separated polymerase chain reaction (MS-PCR) was originally described by Rust et al. (9) as a general technique for the analysis of any point mutations. This technique was successfully used in the detection of β-globin gene mutations among Chinese β-thalassemic patients. (10)

**Objectives**

The main objective of this study was to demonstrate the potential application of MS-PCR for a rapid and accurate detection of common β-globin gene mutations in Thailand.

**Materials and methods**

The study was conducted with 60 β-thalassemic patients who attended the Thalassemia Clinics, Department of Pediatrics, Maharaj Nakorn Chiang Mai Hospital. Genomic DNA was directly prepared using the Chelex™ method. The detection of the common β-globin mutations was undertaken using the MS-PCR method using 3 oligonucleotide primers (common, normal and mutant),
all of which were specifically designed (Table 1). Nucleotide sequencing by the BigDye™ Nucleotide Sequencing Kit (ABI Prism) was carried out to confirm the results generated from the MS-PCR using the strategy introduced by Sirichotiyakul et al. (11).

**The MS-PCR**

The principle of the technique involves the use of two allele-specific primers of different lengths that are separately complementary to a given DNA sequence except for a mismatch near the 3’-end of the primers. These additional and deliberately designed differences can markedly reduce cross-reaction in subsequent PCR cycles. A typical MS-PCR test contains three primers in the PCR mixture; different products can be identified by their varied sizes that are determined by various primer sizes. Both normal and mutant alleles are amplified in the same reaction tube. Subsequent gel electrophoresis shows at least one of the two allelic products at the same locus (Fig. 1).

The PCR was performed in a total volume of 25 µl containing 250 ng genomic DNA, 200 µM of each dNTP; 0.5 units Taq DNA polymerase (QIAGEN); 3 primers, each of which was added at the optimal amount as shown in Table 2; 10 mM Tris pH 8.8; 50 mM KCl and an
optimal amount of MgCl₂ as also shown in Table 2. A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 94°C for 1 min, primer annealing at the optimal annealing temperature for each mutation (Table 2) and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min. The amplified products were separated in 2.5% agarose gel electrophoresis at 120 volts for 15-20 minutes before visualizing with a UV-transilluminator. The fragment size of each mutation is shown in Table 3.

Results

1. Detection of β-globin gene mutations using the MS-PCR

To determine the validity and reliability of the MS-PCR in detecting 6 common mutations in the β-globin gene, DNA samples with known mutations, after characterization using the standard nucleotide sequencing technique, were amplified with a set of oligonucleotide primers designed for the MS-PCR method (Table 1). All six β-globin gene mutations, which were characterized by both techniques, were completely consistent as shown in Fig. 2, 3, 4, 5, 6 and 7.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Annealing temp (C)</th>
<th>MgCl₂ (mM)</th>
<th>Primer (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd41/42(-TTCT)</td>
<td>56</td>
<td>1.50</td>
<td>N : 150, M : 250</td>
</tr>
<tr>
<td>Cd17(A-T)</td>
<td>59</td>
<td>1.50</td>
<td>N : 150, M : 100</td>
</tr>
<tr>
<td>Cd 71/72(+A)</td>
<td>55</td>
<td>2.50</td>
<td>N : 100, M : 150</td>
</tr>
<tr>
<td>Nt-28(A-G)</td>
<td>59</td>
<td>1.50</td>
<td>N : 100, M : 100</td>
</tr>
<tr>
<td>HbE(CD26;G-A)</td>
<td>56</td>
<td>1.25</td>
<td>N : 100, M : 150</td>
</tr>
<tr>
<td>IVS2-nt 654</td>
<td>59</td>
<td>1.50</td>
<td>N : 100, M : 100</td>
</tr>
</tbody>
</table>

Table 3. Sizes of the amplified products for each type of β-thalassemia mutation analysed in the study (Neg = Negative, Het = Heterozygote, Homo = Homozygote)

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Neg (bp)</th>
<th>Het (bp)</th>
<th>Homo (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd 41/42(-TTCT)</td>
<td>351, 324</td>
<td>351, 324</td>
<td>324</td>
</tr>
<tr>
<td>Cd17(A-T)</td>
<td>190, 170</td>
<td>190, 170</td>
<td>170</td>
</tr>
<tr>
<td>Cd 71/72(+A)</td>
<td>115, 135</td>
<td>115, 135</td>
<td>135</td>
</tr>
<tr>
<td>Nt-28(A-G)</td>
<td>305, 287</td>
<td>305, 287</td>
<td>287</td>
</tr>
<tr>
<td>HbE (CD26; G-A)</td>
<td>138, 160</td>
<td>138, 160</td>
<td>160</td>
</tr>
<tr>
<td>IVS 2-nt 654 (C-T)</td>
<td>134, 115</td>
<td>134, 115</td>
<td>115</td>
</tr>
</tbody>
</table>

2. Incidence of β-globin gene mutations in the analysed subjects after the MS-PCR analysis

The MS-PCR was subsequently used to identify 6 common β-thalassemia mutations in 120 chromosomes of 60 β-thalassemic patients. Interestingly, this technique could identify 111 alleles including 54 (45%) codons 41/42 (-TTCT), 30 (25%) codon 26 (G-A) or HbE, 20 (16.6%) codon 17 (A-T) and 7 (5.83%) nt-28 (A-G) of the β-globin gene mutations, which were in 56 (93.3%) thalassemic patients. Only 9 alleles in 5 patients (6.7%) could not be identified and the nucleotide sequencing was employed to complete the task. The results in this category are shown in Table 4.
Discussion
Since β-globin gene mutations are responsible for the clinical outcome of most β-thalassemic patients, detection of these particular genetic defects is important, particularly for genetic counselling and prenatal diagnosis. Several PCR-based detections of these mutations have been collectively introduced. The MS-PCR employed in this report was firstly used to characterize the β-globin mutation in other groups, with promising success. In Thailand, β-thalassemic patients also carry point mutations, which could make the MS-PCR applicable. We have clearly shown that the simple MS-PCR technique can accurately identify common β-thalassemia mutations in Thai β-thalassemic patients. This could mean that the characterization of these mutations can be easily performed by only a single PCR step followed by agarose gel electrophoresis without any further steps being taken. However, the primers used in this study could detect only common β-thalassemia mutations, leaving some cases unidentified. In those problematic individuals, the mutation detection was accomplished by direct nucleotide sequencing.

Figure 2. (A) Result of the MS-PCR for detection of the 4bp (-TTCT) deletion at codons 41/42 [codons 41/42 (-TTCT)]. Lane M indicates the φX 174 Hae III digested DNA size marker. Lanes 1 and 3 represent those negative individuals for codons 41/42 (-TTCT). Lane 2, 4 and 5 are heterozygote codons 41/42 (-TTCT). The corresponding nucleotide sequences that cover this region of individual # 5 are shown in “B”.

Figure 3. (A) Result of the MS-PCR for detection at Codon 17 [Codon 17 (A-T)]. Lane M indicates the φX 174 Hae III digested DNA size marker. Lane 4, 5, 6 and 8 represent negative individuals, producing only 190-bp fragments. Lane 1, 2 and 3 are heterozygote with 190-bp and 170-bp amplified fragments. Lane 7 is a homozygous individual with only a 170-bp amplified fragment generation, of which the corresponding nucleotide sequencing is shown in “B”.
Summary

The MS-PCR technique has a strong potential application for the identification of Thai β-thalassemia mutations. It can be applied for β-thalassemia genotype characterization in the Thai population and as an alternative choice for carrier screening and prenatal diagnosis of β-thalassemia; a commonly encountered disease in northern Thailand.

Table 4. Incidence of β-globin mutations observed in the study

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Allele observed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codons 41/42 (-TTCT)</td>
<td>54 (45)</td>
</tr>
<tr>
<td>Codon 26 (G-A)</td>
<td>30 (25)</td>
</tr>
<tr>
<td>Codon 17 (A-T)</td>
<td>20 (16.6)</td>
</tr>
<tr>
<td>Nt-28 (A-G)</td>
<td>7 (5.83)</td>
</tr>
<tr>
<td>IVS1-NT1 (G-T)</td>
<td>6 (5)</td>
</tr>
<tr>
<td>Codon 43 (G-T)</td>
<td>1 (0.83)</td>
</tr>
<tr>
<td>Codons 27/28 (+C)</td>
<td>1 (0.83)</td>
</tr>
<tr>
<td>NT-87 (C-A)</td>
<td>1 (0.83)</td>
</tr>
</tbody>
</table>

Figure 4. (A) Result of the MS-PCR for the determination of the alanine addition at codons 71/72 [codons 71/72 (+A)]. Lane M indicates the φX 174 Hae III digested DNA size marker. Lane 1, 2 and 3 represent the heterozygous state with 135-bp and 115-bp amplified fragments. The 115-bp amplified fragments seen in lane 4 and 5 are homozygous for this mutation. The corresponding nucleotide sequencing of the mutation in lane 3 is shown in “B”.

Figure 5. (A) Result of the MS-PCR for determination of the A-G substitution at nucleotide-28 of the β-globin gene promoter [NT-28 (A-G)]. Lane M indicates the φX 174 Hae III digested DNA size marker. Lane 1 and 2 represent negative individuals with the 305-bp fragment. Lane 3 and 4 represent the heterozygous state with the 305-bp and 287-bp fragments. The corresponding nucleotide sequencing of the mutation in lane 3 is shown in “B”.

Remark

This work is part of a study on Molecular Characterization of β-Thalassemia Major and β-Thalassemia Intermedia at Maharaj Nakorn Chiang Mai Hospital under the granted ethics clearance.
number 125/2002 issued by The Research Ethics Committee, Faculty of Medicine, Chiang Mai University.

References

Figure 6. (A) Result of the MS-PCR for determination of the G-A substitution at codon 26 (HbE). Lane M indicates the φX 174 Hae III digested DNA size marker. Lane 1 are those negative individual for the HbE allele. Lane 2 represents the heterozygote state for this mutation. The corresponding nucleotide sequencing of this individual mutation #2 is shown in “B”.

Figure 7. Result of the MS-PCR for determination of the C-T substitution at nucleotide 654 within IVS2 (IVS2 nt 654). Lane M indicates the φX 174 Hae III digested DNA size marker. Lanes 1 and 2 represent heterozygotes with 134-bp and 115-bp amplified fragments. Lanes 3 and 4 are negative individuals for this particular mutation. The corresponding nucleotide sequencing of this mutation in individual #1 is shown in “B”.
MS-PCR for β-thalassemia mutation detection

การตรวจหา β-thalassemia mutations อย่างรวดเร็วด้วย mutagenically separated polymerase chain reaction

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บทคัดย่อ β-thalassemia เป็นกลุ่มอาการที่เกิดจากการลดลงหรือหายไปของการสร้าง β-globin chain ปัจจุบันในประเทศไทยพบ mutations มากกว่า 25 ชนิดทั้งด้านภายในและด้านภายนอก β-globin gene ซึ่งเป็นสาเหตุของ β-thalassemia และทั้งนี้มีการพัฒนาเทคนิค polymerase chain reaction หลายแบบเพื่อการตรวจวินิจฉัย mutations เหล่านี้ เทคนิค Mutagenically Separated Polymerase Chain Reaction (MS-PCR) เป็นเทคนิคหนึ่งในบรรดาเทคนิค PCR เหล่านี้ แต่อย่างไรก็ตาม MS-PCR ยังไม่เคยถูกนำมาใช้ในการตรวจ β-thalassemia mutation ในประเทศไทยเลย ดังนั้นวัตถุประสงค์ของการศึกษาครั้งนี้เพื่อพบและตรวจสอบข้อผิดพลาดของเทคนิค MS-PCR ในการตรวจ β-thalassemia mutations ในผู้ป่วยชายไทยภาคเหนือ ทำการศึกษาในผู้ป่วย β-thalassemia จำนวน 60 คนที่เข้ารับการตรวจรักษาที่ศูนย์การแพทย์ภาคเหนือ ภาควิชากุมารเวชศาสตร์, โรงพยาบาลเชียงใหม่จุฬาลงกรณ์ พบว่าสามารถตรวจวินิจฉัย β-thalassemia mutations ได้อย่างถูกต้อง ซึ่งประกอบด้วย MS-PCR โดยใช้ primers 3 เส้นคือ common, mutant และ normal primers ทำ DNA sequencing โดย Big Dye™ Nucleotide Sequencing kit เพื่อคัดเลือกผลที่ได้จาก MS-PCR จากการศึกษาพบว่า เทคนิค MS-PCR สามารถตรวจหา β-thalassemia mutations ที่พบบ่อยในประเทศไทย ได้ 6 ชนิดคือ codons (Cds) 41/42 (-TTCT), codon (Cd) 26 (G-A), codon (Cd) 17 (A-T), condons(Cds) 71/72 (+A), nt-28 (A-G) และ IVS2 – nt 654 ได้อย่างถูกต้อง และเทคนิค MS-PCR สามารถตรวจหา β-thalassemia mutations จำนวน 111 alleles ซึ่งประกอบด้วย Cds 41/42 (-TTCT) จำนวน 54 alleles, Cd 26 (G-A) จำนวน 30 alleles, Cd 17 (A-T) จำนวน 20 alleles และ nt-28 (A-G) จำนวน 7 alleles ในผู้ป่วย β-thalassemia ประมาณ 56 ราย หรือร้อยละ 93.3 ของผู้ป่วยทั้งหมด ส่วนผู้ป่วยที่เหลือ 5 ราย (ร้อยละ 6.7 ของผู้ป่วยทั้งหมด) ใช้เทคนิค nucleotide sequencing ในการตรวจ β-thalassemia mutation หรือศึกษาครั้งนี้แสดงให้เห็นคุณค่าของ MS-PCR ในการตรวจ β-thalassemia mutations และ MS-PCR สามารถเป็นอีกทางเลือกหนึ่งที่จะใช้ในการตรวจหา β-thalassemia mutations ซึ่งพบบ่อยทางภาคเหนือของประเทศไทย เชิงนาวารสาร 2547:43 (4):133-141.

คำสำคัญ: β-thalassemia mutation, mutagenically separated polymerase chain reaction