Diagnosis of thalassemia carriers commonly found in Northern Thailand via a combination of MCV or MCH and PCR-based methods

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Abstract

Background: Conventional diagnostic strategy for thalassemia carriers is time-consuming and requires many types of laboratory tests.

Objective : To demonstrate the possibility of a combination of MCV or MCH and a PCR-based technique in identifying α- and β-thalassemia carriers in the population of the Northern Thailand in which molecular defects causing thalassemia are well documented.

Materials and methods: Seventy northern Thai healthy adults with Hb12 g/dL, MCV 80 fL and MCH 27 pg were tested for the α- and β-globin gene mutations commonly found in Northern Thailand by a modified multiplex allele-specific PCR. The nucleotide sequencing was employed to confirm the results obtained from the PCR-based method.

Results: A combination of MCV 80 fL or MCH 27 pg and a modified multiplex allele-specific PCR was able to definitely diagnose α- and β-thalassemia carriers in 81.4% of the samples tested. These included single carriers of SEA-α thalassemia 1, of α-thalassemia 2 (3.7-kb deletion), of Hb Constant Spring, of HbE, of $\beta^{41/42(-TTCT)}$-thalassemia and of $\beta^{17(A-T)}$-thalassemia. These also included double carriers of α-thalassemia 2 (3.7-kb deletion) and $\beta^{17(A-T)}$-thalassemia, double carrier of Hb Constant Spring and HbE, double carrier of SEA-α thalassemia 1 and $\beta^{17A-T)}$-thalassemia and double carrier of α-thalassemia 2 (3.7-kb deletion) and HbE. The results obtained from the modified multiplex allele-specific PCR completely agreed with those generated from the standard nucleotide sequencing.

Conclusion: Combination of MCV or MCH and a modified multiplex allele-specific PCR might be an alternative means in detecting common α- and β-thalassemia carriers in northern Thailand. This strategy may be applied in other regions where thalassemia is endemic and globin gene mutations are well documented. ***Bull Chiang Mai Assoc Med Sci 2013; 46(1): 22-32***

Keywords: *Thalassemia carrier, thalassemia screen, red cell indices, multiplex allele-specific PCR, globin gene mutations*

การใช้ MCV หรือ MCH ร่วมกับเทคนิค PCR เพื่อวินิจฉัยพาหะธาลัสซีเมียชนิดที่พบบ่อยในภาคเหนือของประเทศไทย

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บทคัดย่อ

บทนำ: การวินิจฉัยพาหะธาลัสซีมียในปัจจุบันใช้เวลานานและอาศัยการตรวจทางห้องปฏิบัติการหลายชนิดเพื่อให้ได้ผลการวินิจฉัยที่ถูกต้อง

วัตถุประสงค์: เพื่อแสดงความเป็นไปได้ของการใช้ MCV หรือ MCH ร่วมกับเทคนิค PCR ในการตรวจวินิจฉัยพาหะธาลัสซีเมียได้อย่างรวดเร็วในกลุ่มประชากรทางภาคเหนือของไทยซึ่งทราบความผิดปกติระดับโมเลกุลของโรคธาลัสซีเมียเป็นอย่างดี

วัสดุและวิธีการศึกษา: ทำการศึกษาในตัวอย่างเลือดจากผู้มีสุขภาพดีและมีระดับฮีโมโกลบินมากกว่า 12 g/dL จำนวน 70 ราย ที่มีค่า MCV <80 fL และ MCH <27 pg โดยตรวจหาความผิดปกติของ α- และ β-globin genes ด้วยเทคนิค multiplex allele-specific PCR ชนิดปรับปรุง และใช้เทคนิคการหาลำดับเบสในการยืนยันความถูกต้อง

ผลการศึกษา : การใช้ MCV หรือ MCH ร่วมกับเทคนิค multiplex allele-specific PCR ที่ปรับปรุงสามารถวินิจฉัยชนิดของพาหะ α- และ β-thalassemia ได้อย่างถูกต้องใน 81.4% ของจำนวนตัวอย่างซึ่งประกอบด้วยพาหะเดี่ยวของ SEA-α thalassemia 1, α-thalassemia 2 (3.7-kb deletion), Hb Constant Spring, of HbE, $\beta^{41/42(-TTCT)}$-thalassemia และพาหะ $\beta^{17(A-T)}$-thalassemia ได้ และพาหะซ้อนของ α-thalassemia 2 (3.7-kb deletion) ร่วมกับ $\beta^{17(A-T)}$-thalassemia, Hb Constant Spring ร่วมกับ HbE, SEA-α thalassemia 1 ร่วมกับ $\beta^{17(A-T)}$-thalassemia และ α-thalassemia 2 (3.7-kb deletion) ร่วมกับ HbE ผลการตรวจที่ได้จากวิธี multiplex allele-specific PCR ตรงกับผลที่ได้จากวิธีการหาลำดับเบส

สรุปผลการศึกษา: การใช้ MCV หรือ MCH ร่วมกับ multiplex allele-specific PCR อาจจะเป็นทางเลือกในการตรวจวินิจฉัยพาหะ α- และ β-thalassemia ที่พบบ่อยในภาคเหนือของประเทศไทย การตรวจทางห้องปฏิบัติการรูปแบบนี้อาจจะนำไปใช้ในพื้นที่ที่มีอุบัติการณ์ของธาลัสซีเมียสูงและทราบความผิดปกติของ globin gene เป็นอย่างดี ***วารสารเทคนิคการแพทย์เชียงใหม่ 2556; 46(1): 22-32***

คำหัส: *พาหะธาลัสซีเมีย การคัดกรองธาลัสซีเมีย ดัชนีเม็ดเลือดแดง การขยายยีนแบบจำเพาะ ความผิดปกติของยีนโกลบิน*

Introduction

Thalassemia is an inherited hemolytic anemia caused by abnormality of globin chain production. It is caused by defects of globin genes which are inherited in an autosomal recessive manner. Those homozygous and compound heterozygous for abnormal globin genes are affected by the disease characterized by chronic anemia requiring frequent blood transfusions followed by several fatal complications. In contrast, those heterozygous and doubly heterozygous for these abnormalities are clinically asymptomatic with obvious alteration of erythrocytic

parameters. Alpha (α)- and beta (β)-thalassemias are the most common types of thalassemia worldwide.[1]

Thalassemia is a globally distributed hemolytic anemia spreading from the Mediteranean through Far Eastern regions including Thailand. In Thailand, approximately 40% of its population are heterozygotes or carriers of thalassemia comprising 20-30% α-thalassemia carriers, 3-9% β-thalassemia carriers, 10-60% HbE carriers and 1-6% Hb Constant Spring carriers.[2] High incidence of thalassemia carriers in Thailand leads to a high probability of producing new cases of thalassemia in this country. Thus, correct diagnosis of a thalassemia carrier should be essential in managing this disorder.

Presently, diagnostic strategy for thalassemia carriers consists of 3 consecutive steps including initial screening by the one-tube osmotic fragility test (OFT) with or without mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), followed by hemoglobin identification by high performance liquid chromatography (HPLC), low pressure liquid chromatography (LPLC), capillary zone electrophoresis (CZE) and finally by analysis for globin gene defects by allele-specific polymerase chain reaction (allele-specific PCR).[3-9] This conventional strategy is time-consuming and requires many steps to obtain definite diagnosis. Employing this strategy, a rapid and definite diagnosis of the thalassemia carriers is unlikely. To overcome this problem, a rapid and accurate protocol needs to be established.

In Northern Thailand, successful diagnosis of carriers of SEA-α thalassemia 1 has been demonstrated by a combination of OFT and Gap-PCR for α-globin gene deletion.10 However, this concept has never been tested for other types of thalassemia in this region. Thus, this study is aimed to test the possibility of a combination of MCV or MCH and the PCR-based method in making a definite diagnosis of both α- and β-thalassemia carriers common in the northern Thailand where specific globin gene mutations are well characterized. It is anticipated that the proposed strategy would be a prototype that can be applied in other regions where α- and β-thalassemia are common and globin gene mutations are also well documented.

Materials and Methods

This study was ethically approved by The Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University. EDTA blood samples were collected from 70 healthy subjects of the Northern Thailand. Hb, MCV and MCH levels were analyzed by an automated blood cell analyzer (Sysmex KX-21; Sysmex Corporation, Kobe, Japan). DNA was prepared from EDTA blood using the Chelex extraction method with some modifications.[11] A 100-microliter EDTA blood sample was firstly washed in 1 mL 0.5% (v/v) Triton X-100 and then in 1 mL deionised water (DI) before adding 110 µL DI and 1-2 drops of Chelex suspension (5% (w/v) in DI). The mixture was then incubated in a 56°C-waterbath for 2 hours and then in a boiling water-bath for 10 minutes. Finally, the tube was centrifuged at 15,000 g for 1 minute, supernatant harvested and kept at -20°C until use.

Characterization of α-globin gene defects commonly found in Northern Thailand was performed using the protocols described elsewhere with some modifications.[12-14] A 50 µL-PCR reaction was performed containing 0.4 µM each of the following primers; SEA1 (5'-TGA CTC CAA TAA ATG GAT GAG GA-3'), SEA3 (5'-GCC TGC GCC GGG GAA CGT AAC CA-3'), CS2 (5'- CCA TTG TTG GCA CAT TCC GG -3') and C3 (5'- CCA TTG TTG GCA CAT TCC GG -3') as well as 0.025 µM of αG-17 (5'- AGA TGG CAC CTT CCT CTC AGG-3'), 1.2 µM each of Alpha 3.7A (5'- CCC AGA GCC AGG TTT GTT TAT CTG-3') and Alpha3.7B (5'- GAG GCC CAA GGG GCA AGA AGC AT-3') in 10 mM Tris pH 8.5, 50 mM KCl, 1 mM MgCl2, 140 µM dNTPs, 2% DMSO, 2M .betaine, 0.1 unit DNA polymerase (iTaq; iNtRON Biotechnology, Inc.) and 5 µL DNA. The amplification reaction was carried out in 29 thermal cycles comprising denaturation at 94°C for 6 minutes 30 seconds in the first cycle and for 1 minute 30 seconds in subsequent 28 cycles, primer annealing at 60°C for 1 minute 30 seconds and extension at 72°C for 2 minute 20 seconds in the first 28 cycles and for 7 minutes during the last cycle. 762-bp amplified products generated from SEA1 and SEA3 primers were produced in the presence of SEA deletion of α-thalassemia 1. 180-bp fragments were produced from C3 and CS2

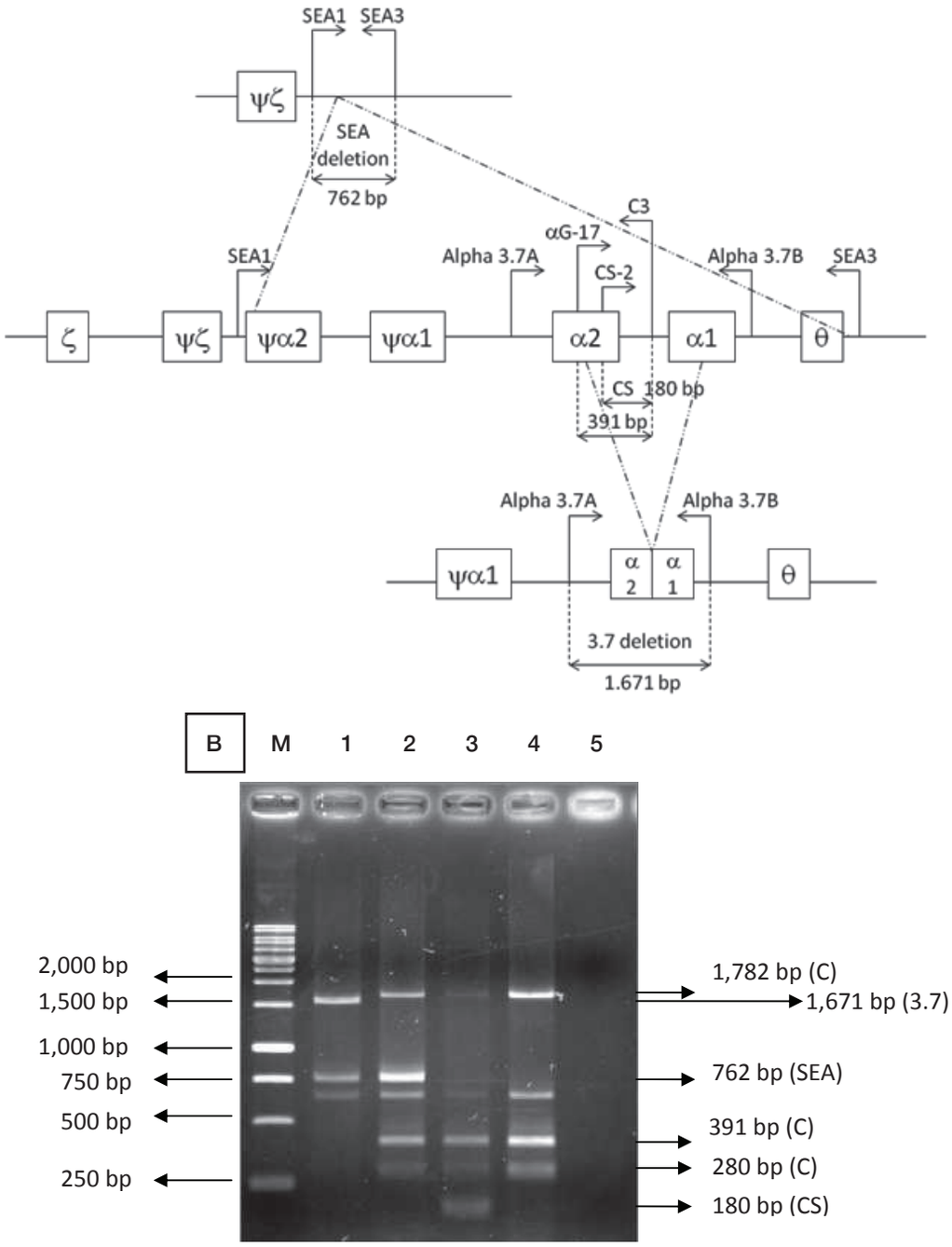

Figure 1 (A) Locations of binding sites of primers used in multiplex allele-specific PCR to identify α-globin gene defects. The corresponding amplified products of each primer pair are indicated. (B) Agarose gel pattern of amplified products in which the specific amplified products were produced. Lane 1 is the DNA from HbH disease (- α3.7/- -SEA with 1,671-bp and 762-bp products), Lane 2 is the DNA from carrier of SEA-α thalassemia (- -SEA/αα with the762-.bp products), Lane 3 is the DNA from carrier of Hb Constant Spring (αCSα/αα with the 180-bp products), Lane 4 is the DNA from normal individual in which only the 280-bp, 391-bp and 1,782-bp internal controls products are seen, Lane 5 is a blank control. M is a 1-kb DNA size marker. Note: "C" is internal control. "3.7" is 3.7-kb α-thalassemia 2. "SEA" is SEA-α thalassemia 1. "CS" is Hb Constant Spring.

primers in the presence of HbCS allele whereas 1,671-bp PCR products from Alpha3.7A and Alpha3.7B primers were generated in the presence of 3.7-kb deletion of α-thalassemia 2. 391-bp fragments generated from αG17 and C3 primers served as internal control (Figure 1).

A modification of the amplification protocol described by Tatu et *al* was performed to characterize the β-globin gene defects commonly found in Thailand. [14] PCR was carried out in a 25 µL PCR reaction containing 0.032 µM of Beta-common multiplex primer (5'-AAG AGC CAA GGA CAG GTA CGG CTG T-3'), 0.056 µM of Beta41/42 multiplex primer (5'-AGA TCC CCA AAG GAC TCA ACC T-3'), 0.054 µM of Beta17 multiplex primer (5'-CCA ACT TCA TCC ACG TTC ACG TA-3'), 0.018 µM of Beta E multiplex primer (5'-CGT ACC AAC CTG CCC AGG GCC AT-3') and 0.14 µM of M-28M1 primer (5'-AGA AGC AAA TGT AAG CAA TAC ATG GCT CTG CCC TGC CAT C -3') in 10 mM Tris pH 8.5, 50 mM KCl, 4 mM MgCl2, 140 µM dNTPs, 2% DMSO, 0.1 unit DNA polymerase (iTaq; iNtRON Biotechnology, Inc.) and 5 µl DNA. The amplification reaction was carried out in 27 thermal cycles comprising denaturation at 95°C for 5 minutes in the first cycle and for 1 minute in subsequent 26 cycles, primer annealing at 62°C for 30 seconds and extension at 72°C for 30 seconds in the first 26 cycles and for 7 minutes during the last cycle. 469-bp amplified products generated from Beta-common multiplex and Beta41/42 multiplex primers were produced in the presence of TTCT deletion at codons 41/42. 267-bp fragments were produced from Beta-common multiplex and Beta17 multiplex primers in the presence of "T" instead of "A" at codon 17. 290-bp products were produced from Beta-common multiplex and Beta E multiplex primers in the presence of HbE allele. Finally, 136-bp fragments were produced from Beta-common multiplex and M-28M1primers in the presence "G" instead of "A" at the nucleotide -28 in β-globin gene promoter. 391-bp fragments generated from αG-17 and C3 primers were used as an internal control (Figure 2). Blood samples positive for the multiplex allele-specific PCR were randomly selected for nucleotide sequencing to validate the mutation identification.

Results

The mean Hb level in 70 blood samples

Table 1 Frequencies of globin gene mutations in thalassemia carriers with MCV < 80 fL and MCH < 27 pg detected by the modified multiplex allele-specific PCR

	Number of samples (%)
α-thalassemia-1 (SEA type)	18 (25.7)
α-thalassemia-2 (3.7 type)	12 (17.1)
HbCS	1 (1.4)
β^{17}-thalassemia	1 (1.4)
$\beta^{41/42}$-thalassemia	2 (2.8)
HbE	15 (21.4)
HbCS/HbE	2 (2.8)
α-thalassemia-1 (SEA type)/β^{17}-thalassemia	1 (1.4)
α-thalassemia-2 (3.7 type)/β^{17}-thalassemia	3 (4.2)
α-thalassemia-2 (3.7 type)/HbE	2 (2.8)
Negative for PCR analysis	13 (18.5)
Total	70

analysed was 13.1-1.0 g/dl (Mean-SD). The MCV and MCH levels were 73.1-5.6 fL (Mean-SD) and 23.-2.2 pg (Mean-D), respectively. In the cases with MCV<80 fL and MCH<27 pg, the modified multiplex allele-specific PCR analysis revealed that 57 samples (81.4%) were carriers of α- and β- thalassemia in both sin-

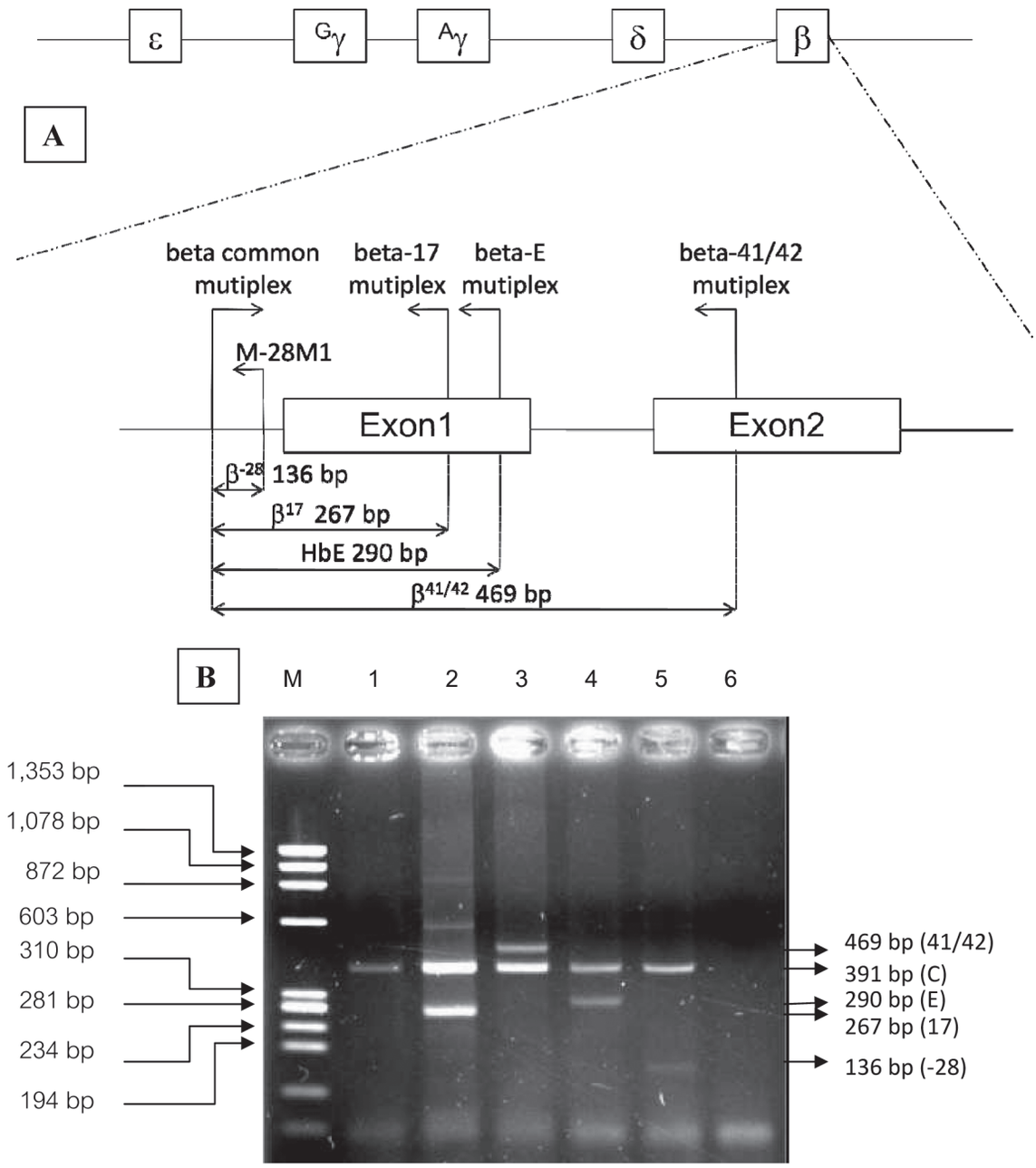

Figure 2 (A) Locations of binding sites of primers used in multiplex allele-specific PCR to identify β-globin gene defects. The corresponding amplified products of each primer pair are indicated. (B) Agarose gel pattern of amplified products in which the specific amplified products were produced. Lane 1 is the DNA from normal individual in which only the 391-bp internal control products is seen. Lane 2 is the DNA from carrier of $\beta^{17(A-T)}$ (β/β^{17} with the 267-.bp products), Lane 3 is the DNA from carrier of $\beta^{41/42(-TTCT)}$ ($\beta/\beta^{41/42}$ with the 469-bp products), Lane 4 is the DNA from carrier of HbE (β/β^{E} with the 290-bp products), Lane 5 is the DNA from carrier of $\beta\text{-}^{28(A-G)}$ ($\beta/\beta\text{-}^{28}$ with the 136-bp products), Lane 6 is the blank control. M is a 1-kb DNA size marker. Note : "C" is internal control. "41/42" is $\beta^{41/42(-TTCT)}$. "E" is HbE. "17" is $\beta^{17(A-T)}$and "-28" is $\beta\text{-}^{28(A-G)}$.

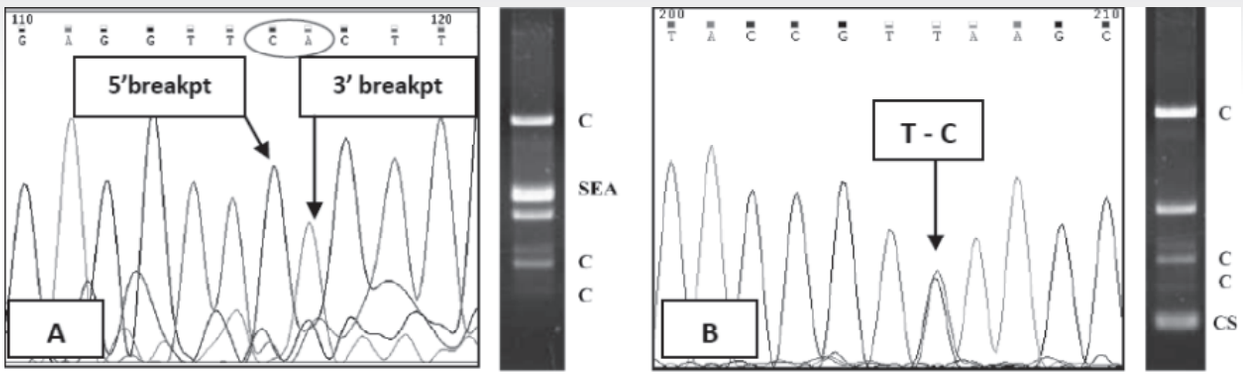

Figure 3 Comparison of α-globin gene mutations identified by the nucleotide sequencing (left box) and by the multiplex allele-specific PCR (right box) for SEA-.α thalassemia 1 (A) and Hb Constant Spring (B). Note: "C" is internal control. "SEA" is SEA-α thalassemia 1. "CS" is Hb Constant Spring.

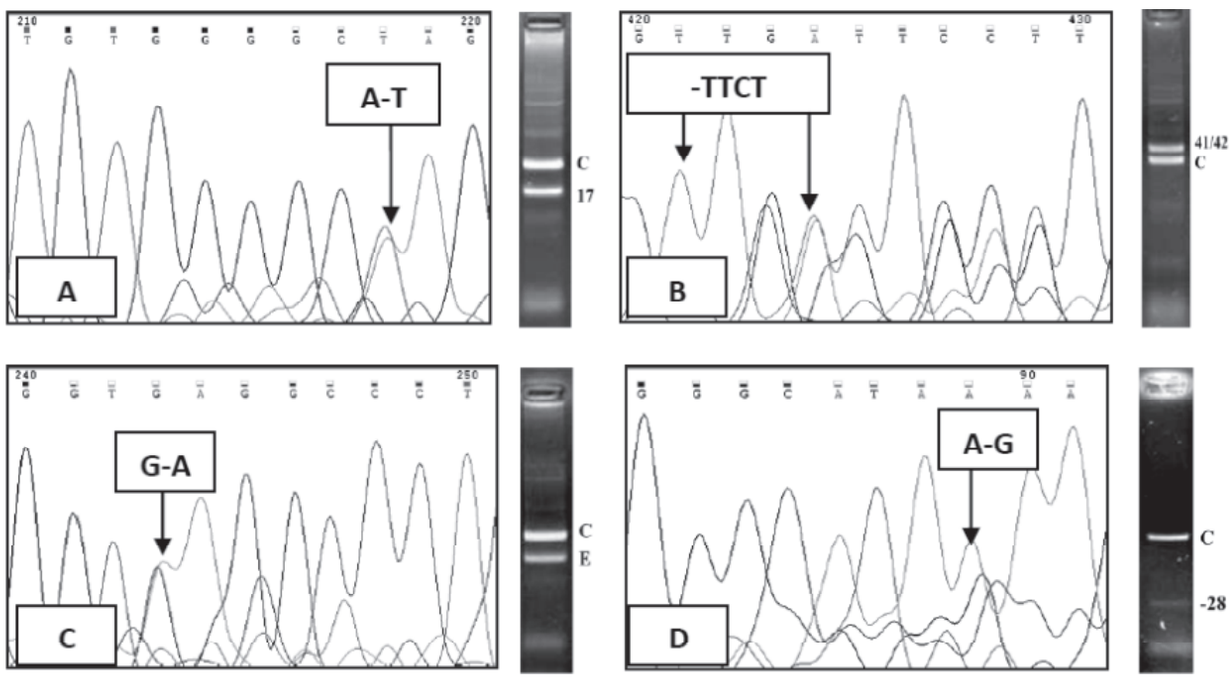

Figure 4 Comparison of β-globin gene mutations identified by the nucleotide sequencing (left box) and by the multiplex allele-specific PCR (right box) for $b^{17(A-T)}$ (A), $b^{41/42(-TTCT)}$(B), HbE (C) and $b^{-28(A-G)}$ (D). Note : "C" is internal control. "41/42" is $\beta^{41/42(-TTCT)}$. "E" is HbE. "17" is $\beta^{17(A-T)}$and "-28" is $\beta^{-28(A-G)}$.

gle and double states. These included single carriers of SEA-α thalassemia 1 (- $-^{SEA}$/αα), HbE (β/β^{E}), Hb Constant Spring (α^{CS}α/αα), α-thalassemia 2 (3.7-kb deletion) (- $\alpha^{3.7}$/αα), $\beta^{41/42(-TTCT)}$-thalassemia (β/$\beta^{41/42}$) and $\beta^{17(A-T)}$-thalassemia (β/β^{17}). These also included double carriers of α-thalassemia 2 (3.7-kb deletion) (- $\alpha^{3.7}$/αα) and $\beta^{17(A-T)}$-thalassemia (β^{17}), double carrier of Hb Constant Spring (α^{CS}α/αα) and HbE (β/β^{E}), double carrier of SEA-α thalassemia 1 (- $-^{SEA}$/αα) and $\beta^{17(A-T)}$-thalassemia (β^{17}) and double carrier of α-thalassemia 2 (3.7-kb deletion) (- $\alpha^{3.7}$/αα) and HbE (β/β^{E}) as shown in Table 1. The globin gene mutations identified by the modified multiplex allele-specific PCR were completely identical to those generated by the standard nucleotide sequencing (Figures 3, 4).

Discussion

Conventional detection of thalassemia carriers in Thailand starts with initial screening tests including OFT or MCV and DCIP tests. [8,15,16] Those positive for the initial screening tests are subjected to Hb identification

to determine HbA2/E levels to identify carrier states of β-thalassemia and HbE.[3] Employing these two laboratory stages, one cannot determine types of β-globin mutations, α-thalassemia and double carriers of α- and β-thalassemia which require DNA analysis.

Rund et al and Sanguansermsri demonstrated that it was possible to carry out mutation analysis just after the initial screening of α- and β-thalassemia carriers by MCV and OFT in the area where mutations are well documented.[10,17] Based on these notions, we attempted to characterize the α- and β-globin gene mutations common in northern Thailand by the modified multiplex allele-specific PCR in the 70 samples previously tested for MCV and MCH. The results clearly showed that common thalassemia in either single or double states was detected in the majority of samples having MCV and MCH below the cut-off points. This showed that the MCV and MCH were effective in screening thalassemia carriers and that a combination of these red cell indices and the modified multiplex allele-specific PCR was possible in detecting α- and β-thalassemia carriers of all statuses. MCV and MCH are the parameters accurately obtained by an automated blood cell analyzer. They have been intensively evaluated and proven to be effective parameters in screening of thalassemia carriers and the cut-off points of 80 fL for MCV and 27 pg for MCH are mostly widely accepted.[6,8,9,17-22] However, MCV and MCH values of HbE carriers may overlapped with those of normal population and approximately 5% HbE carriers will be missed when this indices are relied on.[23,24,25] Thus, HbE screening tests may be included in order to increase efficiency of this proposed protocol.

In Northern Thailand, the $\beta^{41/42}$, β^{17}, β^{-28}, β^{E} and $--^{SEA}$, $-\alpha^{3.7}$, $\alpha^{CS}\alpha$ are the common β- and α-globin gene defects.[26,27] Molecular analysis of these mutations have been well established as stated earlier and we have modified these PCR methodologies to establish an in-house protocol. Employing this modified PCR protocol to test the 70 blood samples whose screening results were positive, the majority of the samples were diagnosed to be carriers of the common types of globin gene defects. This indicated that the PCR analysis of common globin gene mutations directly after the MCV or MCH analysis was capable in identifying common α- and β-thalassemia in Northern Thailand. The modified multiplex allele-specific PCR employed in this study had previously been validated against the known samples. The standard nucleotide sequencing was employed to confirm the results generated by this multiplex allele-specific PCR. As shown, the mutations identified by both techniques were completely identical. This should indicate that the specificity and sensitivity of the proposed strategy were significant. In the minor portion of the samples that had positive screening results, the proposed multiplex allele-specific PCR failed to identify any globin gene mutations. These samples certainly harbored rare types of globin gene mutations and other tests, e.g. Hb identification is still required to make the definite diagnosis.

Another most significant advantage of molecular analysis of globin gene defects is an ability to detect the carriers of α-globin thalassemia either in a single carrier state or in double carrier state with β-thalassemia and HbE. In the Northern Thailand, double carriers of α- and β-thalassemia or HbE are fairly common.[14] These double carriers cannot be detected by the conventional Hb identification techniques such as HPLC.

Skipping the Hb identification step as proposed in this report raised 2 major questions. These included ability of this new approach in nationwide carrier screen and in detecting complex thalassemia encountering in Thailand. As a national strategy for thalassemia screen, all samples having positive screening results are subjected for Hb identification (e.g. by HPLC, LPLC or CZE). Despite Hb identification is capable of identifying only carriers of β-thalassemia, δβ-thalassemia, but α-thalassemia and α/β-thalassemia cannot be detected by this method. Detection of α-thalassemia still needs DNA analysis. More importantly, mutation analysis of β-globin gene in those diagnosed as being the β-thalassemia carrier is always required in case prenatal diagnosis is planned. Thus, implementing this proposed strategy may be helpful in this situation. As stated, the carriers of δβ-thalassemia or abnormal hemoglobins can only be indicated by the

Hb identification and could be missed in situation where the proposed strategy is applied.[3,28-31] Thus in situation where the δβ -thalassemia and abnormal hemoglobins are expected, the Hb identification is still necessary.

Although the presented strategy seemed to violate the national thalassemia diagnosis strategy, we herein only proposed an alternative means in detecting thalassemia carriers. Hb identification by HPLC or LPLC or CZE is still necessary in this context. Further intensive investigations are required to clarify this concept.

In conclusion, we have attempted to demonstrate possibility of combination of MCV or MCH and a multiplex allele-specific PCR in detecting common α- and β-thalassemia carriers. However, this strategy was only a preliminary model that could not identify all types of globin gene mutations. Hb identification is still required in detecting thalassemia carriers. Additional studies are needed to extent its efficacy.

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References

1. Weatherall DJ, Clegg JB. The Thalassaemia Syndromes (Fourth edition). Oxford: Blackwell Scientific; 2001.
2. Fucharoen S, Winichagoon P. Thalassemia in SouthEast Asia: problems and strategy for prevention and control. Southeast Asian J Trop Med Public Health. 1992; 23: 647-55.
3. Fucharoen S, Winichagoon P, Piankijagum A. Standardization on laboratory diagnosis of thalassemia and abnormal hemoglobin. Southeast Asian J Trop Med Public Health. 1999; 30 Suppl 3: 90-8.
4. Fucharoen G, Sanchaisuriya K, Sae-ung N, Dangwibul S, Fucharoen S. A simplified screening strategy for thalassaemia and haemoglobin E in rural communities in south-east Asia. Bull World Health Organ. 2004; 82: 364-72.
5. Kattamis C, Efremov G, Pootrakul S. Effectiveness of one tube osmotic fragility screening in detecting beta- thalassaemia trait. J Med Genet. 1981; 18: 266-70.
6. Pranpanus S, Sirichotiyakul S, Srisupundit K, Tongsong T. Sensitivity and specificity of mean corpuscular hemoglobin (MCH): for screening α-thalassemia-1 trait and β-thalassemia trait. J Med Assoc Thai. 2009; 92: 739-43.
7. Sirichotiyakul S, Tantipalakorn C, Sanguansermsri T, Wanapirak C, Tongsong T. Erythrocyte osmotic fragility test for screening of α-thalassemia-1 and β-thalassemia trait in pregnancy. Int J Gynaecol Obstet. 2004; 86: 347-50.
8. Sirichotiyakul S, Maneerat J, Sa-nguansermsri T, Dhananjayanonda P, Tongsong T. Sensitivity and specificity of mean corpuscular volume testing for screening for α-thalassemia-1 and β-thalassemia traits. J Obstet Gynaecol Res. 2005; 31: 198-201.

9. The Working Party of the General Haematology Task Force of the British Committee for Standards in Haematology. The laboratory diagnosis of haemoglobinopathies. Br J Haematol. 1998; 101: 783-92.
10. Sanguansermsri T, Phumyu N, Chomchven S, Steger HF. Screening for α-thalassemia-1 heterozygotes in expecting couples by combination of a simple erytrocyte osmotic fragility test and a PCR-based method. Community Genetics. 1999; 2: 26-39.
11. Polski JM, Kimzey S, Percival RW, Grosso LE. Rapid and effective processing of blood specimens for diagnostic PCR using filter paper and Chelex-100. Mol Pathol. 1998; 51: 215-7.
12. Fucharoen S, Sanchaisuriya K, Fucharoen G, Panyasai S, Devenish R, Luy L. Interaction of hemoglobin E and several forms of α-thalassemia in Cambodian families. Haematologica. 2003; 88: 1092-8.
13. Sutcharitchan P, Wang W, Settapiboon R, Amornsiriwat S, Tan AS, Chong SS. Hemoglobin H disease classification by isoelectric focusing: molecular verification of 110 cases from Thailand. Clin Chem. 2005; 51: 641-4.
14. Tatu T, Kiewkarnkha T, Khuntarak S, Khamrin S, Suwannasin S, Kasinrerk W. Screening for co-existence of α-thalassemia in β-thalassemia and in HbE heterozygotes via an enzyme-linked immunosorbent assay for Hb Bart's and embryonic ζ-globin chain. Int J Hematol. 2012; 95: 386-93.
15. Winichagoon P, Thitivichianlert A, Lebnak T, Piankijagum A, Fucharoen S. Screening for the carriers of thalassemias and abnormal hemoglobins at the community level. Southeast Asian J Trop Med Public Health. 2002; 33 Suppl 2: 145-50.
16. Wiwanitkit V, Suwansaksri J, Paritpokee N. Combined one-tube osmotic fragility (OF) test and dichlorophenol- indolphenol (DCIP) test screening for hemoglobin disorders, an experience in 213 Thai pregnant women. Clin Lab. 2002; 48: 525-8.
17. Rund D, Filon D, Strauss N, Rachmilewitz EA, Oppenheim A. Mean corpuscular volume of heterozygotes for β-thalassemia correlates with the severity of mutations. Blood. 1992; 79: 238-43.
18. Jindadamrongwech S, Wisedpanichkij R, Bunyaratvej A, Hathirat P. Red cell parameters in α-thalassemia with and without β-thalassemia trait or hemoglobin E trait. Southeast Asian J Trop Med Public Health. 1997; 28 Suppl 3: 97-9.
19. Lafferty JD, Crowther MA, Ali MA, Levine M. The evaluation of various mathematical RBC indices and their efficacy in discriminating between thalassemic and non-thalassemic microcytosis. Am J Clin Pathol. 1996; 106: 201-5.
20. Liao C, Xie XM, Zhong HZ, Zhou JY, Li DZ. Proposed screening criteria for β-thalassemia trait during early pregnancy in southern China. Hemoglobin. 2009; 33: 528-33.
21. Karimi M, Rasekhi AR. Efficiency of premarital screening of β-thalassemia trait using MCH rather than MCV in the population of Fars Province, Iran. Haematologia (Budap). 2002; 32: 129-33.

22. Schubert S, Christofi Y, Papadatou A. [Routine hematologic parameters for thalassemia screening]. Folia Haematol Int Mag Klin Morphol Blutforsch. 1990; 117: 867-73.
23. Yeo GS, Tan KH, Liu TC. Screening for β thalassaemia and HbE traits with the mean red cell volume in pregnant women. Ann Acad Med Singapore. 1994; 23: 363-6.
24. Ittarat W, Ongcharoenjai S, Rayatong O, Pirat N. Correlation between some discrimination functions and hemoglobin E. J Med Assoc Thai. 2000; 83: 259-65.
25. Tatu T, Prakunwisit D, Chayajak S, Chiampanichayakul S, Kasinrerk W. RBC count and its differentiation potential between α-thalassemia (SEA type), β-thalassemia and HbE heterozygotes. CMUJ for natural sciences 2007; 6: 57-64.
26. Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia: molecular biology and clinical medicine. Hemoglobin. 1997; 21: 299-319.
27. Lemmens-Zygulska M, Eigel A, Helbig B, Sanguansermsri T, Horst J, Flatz G. Prevalence of α-thalassemias in northern Thailand. Hum Genet. 1996; 98: 345-7.
28. Bunn HF, Forget BG, Ranney HM. Human Hemoglobins. Toronto: W.B. Saunders Company; 1977.
29. Wood WG, Clegg JB, Weatherall DJ. Hereditary persistence of fetal haemoglobin (HPFH) and δβ thalassaemia. British Journal of Haematology. 1979; 43: 509-20.
30. Bollekens JA, Forget BG. δβ Thalassemia and hereditary persistence of fetal hemoglobin. Hematology/Oncology Clinics of North America. Vol. 5; 1991: 399-422.
31. Wood WG. Hereditary Persistence of Fetal Hemoglobin and dβ Thalassemia. In: Steinberg MH, Forget BG, Higgs DR, Nagel RL, eds. Disorder of Hemoglobin: Genetics, Patho physiology and Clinical Management. Cambridge: Cambridge University Press; 2001: 356-88.