GENOTYPING OF BETA THALASSEMIA TRAIT BY HIGH-RESOLUTION DNA MELTING ANALYSIS

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Abstract. Beta thalassemia is a common hereditary hemalogogical disease in Thailand, with a prevalence of 5-8%. In this study, we evaluated the high resolution DNA melting (HRM) assay to identify beta thalassemia mutation in samples from 143 carriers of the beta thalassemia traits in at risk couples. The DNA was isolated from venous blood samples and tested for mutation under a series of 5 PCR-HRM (A, B, C, D and E primers) protocols. The A primers were for detection of beta thalassemia mutations in the HBB promoter region, the B primers for mutations in exon I, the C primers for exon II, the D primers for exon III and the E primers for the 3.4 kb deletion mutation. The mutations were diagnosed by comparing the complete melting curve profiles of a wild type control with those for each mutant sample. With the PCR-HRM technique, fourteen types of beta thalassemia mutations were detected. Each mutation had a unique and specific melting profile. The mutations included 36.4% (52 cases) codon 41/42-CTTT, 26.6% (38 cases) codon 17 A-T, 11.2% (16 cases) IVS1-1 G-T, 8.4 % (12 cases) codon 71/72 +A, 8.4% (12 cases) of the 3.4 kb deletion and 3.5% (5 cases) -28 A-G. The remainder included one instance each of -87 C-A, -31 A-C, codon 27/28 +C, codon 30 G-A, IVS1-5 G-C, codon 35 C-A, codon 41-C and IVSII -654 C-T. Of the total cases, 85.8% of the mutations could be detected by primers B and C. The PCR-HRM method provides a rapid, simple and highly feasible strategy for mutation screening of beta thalassemia traits.

Keywords: bata thalassemia trait, multation, PCR-HRM, risk couples

INTRODUCTION

Beta thalassemia is a common hereditary hematological disease in Thailand (Fucharoen and Winichagoon,1987; Winichagoon *et al*, 1990; Wong *et al*, 2004). There is a 5-8% prevalence of the beta thalassemia trait in northern Thailand (Tienthavorn *et al*, 2006). One infant with beta thalassemia major is born out of every thousand life births. Prevention programs have tried to identify at-risk couples during pregnancy to obtain genetic counseling and prenatal testing for thalassemia and of affected decide on the pregnancy outcome (Sirichotiyakul *et al*, 2009). However, some beta thalassemia mutations have a mild clinical presentation making genetic counselling problematic. Therefore, beta thalassemia mutations in at-risk couples need to be

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specifically identified. In Thailand about 20 different types of beta thalassemia mutations have been reported, most of which are point mutations: -87 C-A, -31 A-G, -28 A-G, codon (CD) 15 G-A, CD 17 A-T, CD 26 G-T, CD 27/28 + C, CD 30 G- A, IVSI-1 G- T, IVSI-5 G- C, CD 35 C-A, CD 41 - C, CD 41/42--CTTT, CD 43 G-T, CD 71/72 + A, CD 95+A and IVSII- 654 C-T. Others include 3.4 kb, 619 and 105 base pair deletions (Laig *et al*, 1989; Sanguansermsri *et al*, 1990; Thein *et al*, 1990; Lynch *et al*, 1991; Fukumaki *et al*, 1992; Winichagoon *et al*, 1992). Currently, molecular analysis of beta thalassemia mutations includes the multiplex amplification refractory mutation system (MARMS) and direct DNA sequencing. MARM-PCR, which is more widely used, is inexpensive and rapid. However, the method is tedious and limited in its ability to detect some beta thalassemia mutations (El-Gawhary *et al*, 2007; Mirasena *et al*, 2008). Nearly all beta thalassemia mutations can be detected by direct DNA sequencing but this is not the favored method due to difficulties with the techique, amount of time required and cost (Sirichotiyakul *et al*, 2003). Recently, there have been reports of the possible application of the high resolution DNA melting (HRM) assay as a tool for thalassemia mutation identification (Pornprasert *et al*, 2008; Prathomtanapong *et al*, 2008; Shih *et al*, 2009). In this study we evaluated the use of HRM as a method for beta thalassemia mutation detection.

MATERIALS AND METHODS

Blood samples from 143 carriers of beta thalassemia trait in couples at risk for offspring with beta thalassemia major were recruited from the antenatal care clinic and referred to us as part of the National Thalassemia Prevention Program. All had tested positive for beta thalassemia trait with a positive MCV test and a hemoglobin A_2 concentration between 4.0 and 8.0%.

DNA-isolation

Genomic DNA was isolated from 200 µl of whole blood according to the manufacturer's protocol with the QIA-amp DNA Mini Kit (QIAGEN, Hilden, Germany). The extracted DNA was quantified with a spectrophotometer (SPECORD Plus, Germany) at 260 and 280 nm, with A260/A280 ratios between 1.6 and 1.8. The isolated DNA was diluted with 1 X PCR buffer to 5 ng/µl and stored at -20ºC until analysis.

HBB gene

The DNA sequence of the HBB gene was derived from NCBI's GenBank: U01317.1 (HBB gene; nucleotide numbers 62137-63472). The HBB gene's promoter region is defined by nucleotide numbers 61921-62137, exon I is 62190-62279, exon II is 62409-62631 and exon III is 63482-63607. The previously characterized beta thalassemia mutations and their locations in the HBB gene were taken into consideration when designing primers (Laig *et al*, 1989; Sirichotiyakul *et al*, 2003; Mirasena *et al*, 2008).

Primer design

All primer pairs were designed according to the Primer-BLAST program. Four primer pairs (A, B, C and D) were designed to cover the four regions of the HBB gene where the common beta thalassemia mutations are located (Fig 1). The A-primers were for the amplification of the promoter DNA segment, the B-primers for exon I, the C-primers for exon II, and the D-primers for the IVSII and exon III DNA segments. The A-forward primer 5′ ACTTAGACCTCACCCTGTGGA 3′ (nucleotide numbers 62022-42) and the A-

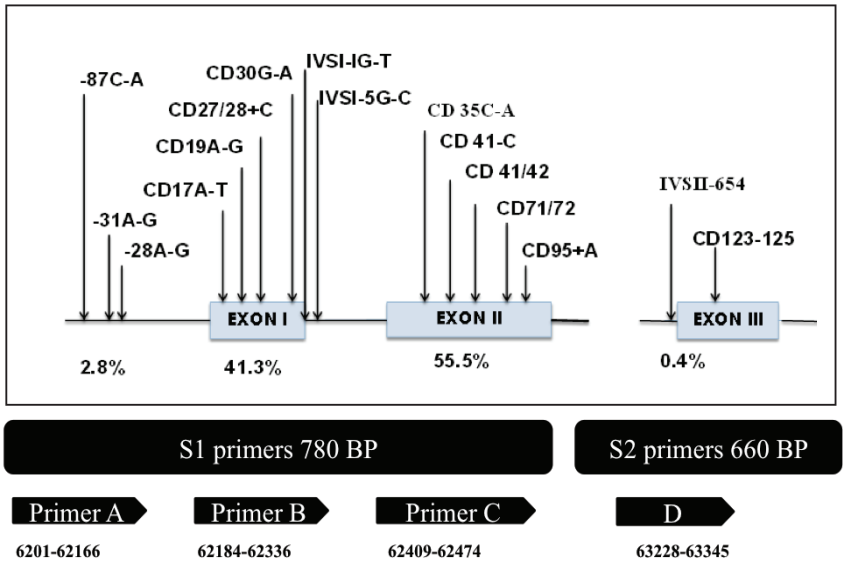

Fig 1–Schematic representation showing the positions of four PCR-HRM primer pairs (A, B, C and D) and two DNA sequencing primer pairs (S1 and S2). The A, B, C and D primer pairs were responsible for detecting beta-thalassemia mutations in the promoter region, exon I, II and III regions, respectively. S1 was used to analyze the DNA sequence of the HBB gene from the promoter region to the 3′ end of IVSII, while S2 was used to analyze the whole exon III gene sequence.

reverse primer 5′ TGGTGTCTGTTTGAGGTTGC 3′ (62168-87) were used to amplify the promoter region and generate a PCR product 166 base pairs (bp) in length and used to detect -87 C-A, -31 A- G and -28 A-G mutations. HBB gene exon I was amplified with the B-forward primer 5′ CCTGAGGAGAAGTCGCCGTT 3′ (62202-21) and B-reverse primer 5′ GTCTCCACATGCCCAGTTTCT 3′ (62319-39). The PCR product was 137 BP in length and used to detect CD 17 A-T, CD 27/28 + C, CD 30 G-A, IVSI-1 G- T and IVSI-5 G-C mutations. HBB gene exon II was amplified with the C-forward primer 5′ TTCCCACCCTTAGGCTGCTGGT 3′ (62396-417) and C-reverse primer 5′TGGCAAAGGTGCCCTTGAGGT 3′ (62557-78). The PCR product was 183 bp and was used to detect CD 35 C- A, CD 41 - C, CD 41/42 - CTTT and CD 71/72 + A. The IVSII and exon 3 DNA segment was amplified using the D-forward primer 5′ CTTTCTTTCAGGGCAATAATGA 3′ (63199-321) and D-reverse primer 5′ AGAAATATTTATAGTCAGAAATATTG 3′ (63291-316). The PCR product length was 117 BP and was used to detect the IVSII-654 C- T mutation. To detect the 3.4 kb deletion, three different primer pairs were designed. These were E1-forward, E1-reverse and E2-reverse. The E1-forward primer 5′ GTCACACTTTGGGTTGTAAGTGAC 3′ (61360-83) and E1-reverse primer 5′ TCAATGTGCTCTGTGCATTAGT 3′ (61462-83) were used to amplify the wild type gene at the 5′ DNA break point of the 3.4 kb deletion (U01317; 61417). The product size was 124 bp in length. To verify the 3.4 kb mutant, the DNA sequence of the deleted junction (U01317, HBB gene; 61417/64902) was amplified with the E1-forward primer and the E2-reverse primer 5′ TTCCTTTTGTTGCCTTTGCT 3′ (64960-79). The product obtained was 135

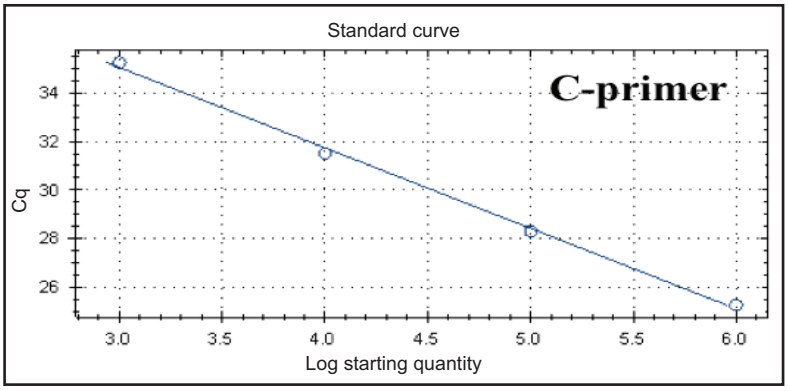

Fig 2–Image of the standard curve for the C primer. It was tested against normal control DNA by 5-fold dilution and revealing 100% efficiency, a correlation coefficient (R^2) of 0.998 and a slope of -3.319.

bp. All primers were purchased through Invitrogen (Carlsbad, CA).

PCR performance evaluation

The performance of all 5 PCR protocols was evaluated by real-time PCR against serial DNA dilutions of 1:10, 1:100, 1:1,000 and 1:10,000 starting with 62.4 ng/µl DNA on the Bio-Rad CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA). The PCR efficiency, the slope of the standard curve and correlation coefficient (R^2) were automatically calculated with the provided software.

Real-time PCR and high resolution DNA melting analysis

To identify beta thalassemia mutations, two series of five PCR protocols were carried out simultaneously. One was conducted with a normal DNA sample and the other with an unknown beta thalassemia trait sample. Each test sample contained one of 5 specific primer pairs and PCR mixtures and was performed in two replicates. To detect the 3.4 kb deletion mutant, E1 forward / E1 reverse primers were used to amplify normal control DNA and E1 forward/ E2 reverse primers were used for unknown samples. The 25 µl PCR mixture included 5 µl of DNA, 1.5 µM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate, 2 µM of SYTO9, 1 unit of Platinum® *Taq* DNA polymerase, 0.2 µM of each primer (Invitrogen, Carlsbad, CA) and the rest was a 1X PCR buffer. Thermal cycling was performed using the Bio-Rad CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA), starting with an initial step of activating the *Taq* DNA polymerase at 94ºC for 2 minutes, followed by 40 cycles of denaturation at 94ºC for 15 seconds, annealing at 64ºC for 15 seconds and extension at 72ºC for 20 seconds. Fluorescence activity was measured on a SYBR Green I channel (533 nm) at the end of each cycle. The HRM melting program started at 95ºC for 10 seconds, followed by a melting cycle from 75ºC to 90ºC with a transitional rate of 0.2ºC per 10 seconds. Fluorescence was monitored continuously during the melting process. HRM analysis was performed using Bio-Rad Precision Melt Analysis Software (Bio-Rad, Hercules, CA). The different fluorescence temperature-shifted curves were compared between the wild type control and the unknown sample with each primer protocol. The positive primer set revealed curves that were not in alignment (Fig 3).

Direct DNA sequencing

To identify the molecular mutations characteristic of beta thalassemia trait,

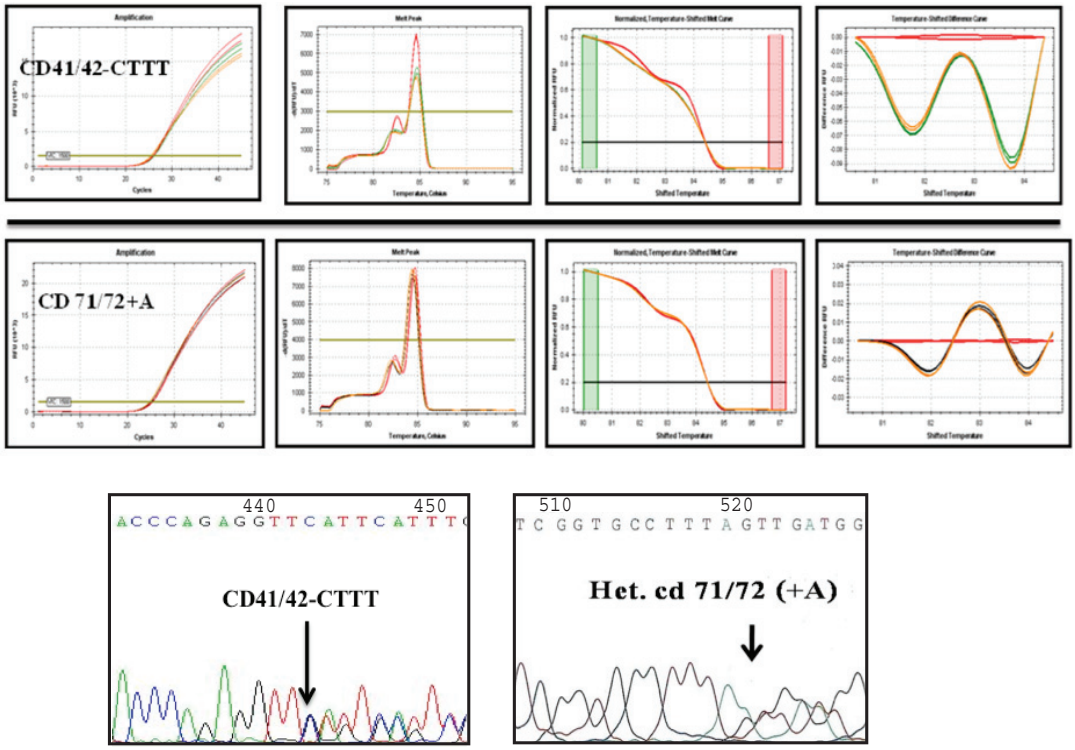

Fig 3–Example of complete melting profiles (HRM images) generated by the C primer set for beta thalassemia traits CD 41/42 -CTTT and CD 71/72 + A. Each mutation was detected by comparing the complete melting profile of a normal DNA sample with the mutant gene. The results were in accordance with the direct DNA sequencing technique.

two primer pairs were designed (S1 and S2). The S1 forward primer 5 ' ACTCCTA-AGCCAGTGCCAGA 3' (61974-93) and S1 reverse primer 5' TGTACCCTGT-TACTTCTCCCC 3' (62700-20) were used to amplify the HBB gene nucleotide sequence numbers 61974-62720. The PCR product was 780 bp in length, including the promoter region and the exon I and II regions where most beta thalassemia mutations occurs.

The S2 forward primer 5' ACAATG-TATCAGCCTCTTTGCAC 3' (63223-46) and S2 reverse primer 5' TGCATTAGCT-GTTTGCAGCC 3' (63884-903) were used to amplify the 3' part of- the-IVSII and exon III on the HBB gene (660 bp; 63223-63939) where some rare mutations might be discovered.

The S1 protocol PCR mixture consisted of 50 µl in a 0.2 ml thin wall tube containing 5 µl genomic DNA, 200 µM of each dNTP, 1.0 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 4 µM of mixed S1 forward and S1 reverse primers in 10 mM Tris pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$ and the rest being 1X buffer. A total of 40 thermal cycles was carried out, with each cycle involed in DNA denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute and primer extension at 72°C for 2

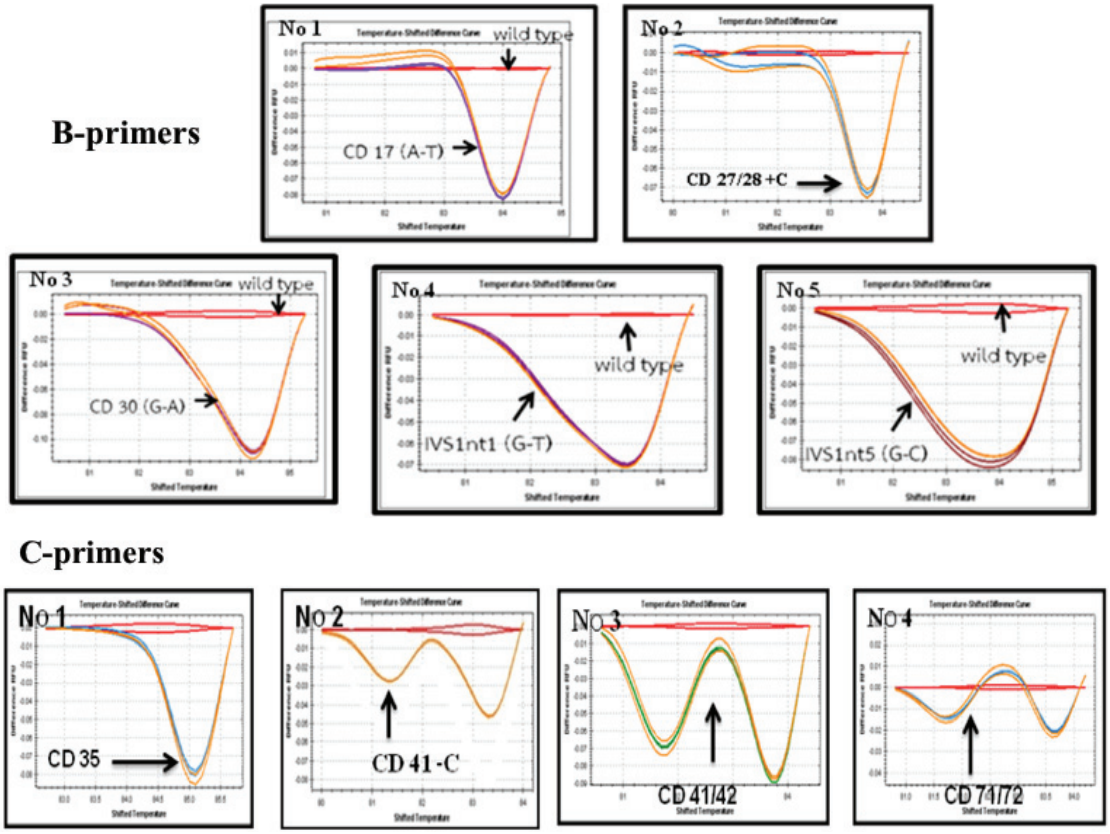

Fig 4–HRM images for beta thalassemia mutations detected with the PCR-HRM method using B- and C-primer protocols. Each mutation gave a unique temperature-shift curve pattern.

minutes. The initiation denaturation was extended to 95ºC for 15 minutes while the final extension was prolonged for 7 minutes.

The S2 protocol PCR mixture of 50 µl contained 5 µl genomic DNA, 200 µM of each dNTP, 1.0 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.5 µM of mixed S2 forward and S2 reverse primers in 10 mM Tris pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$ and the rest being 1X buffer. A total of 35 thermal cycles was carried out, with each cycle involved in DNA denaturation at 94ºC for 45 seconds, primer annealing at 55ºC for 1 minute and primer extension at 72ºC for 90 seconds. The initiation denaturation was extended to 95ºC for 15 minutes while the final extension was prolonged for 7 minutes.

The PCR products were then checked by electrophoresis on 1.5% agarose gel. The DNA bands were made visible by staining with ethidium bromide. The S1 and S2 DNA bands were 780 and 660 base pairs in length, respectively. The PCR products were further purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). DNA purification was performed according to the manufacturer's instructions. Purified DNA obtained in 20 µl buffer was further processed for cycle sequencing reactions carried out in a 0.2 ml PCR tube. The reaction mixture contained 8 µl of ABI Prism BigDye Terminator Ready Reaction Mix (Applied

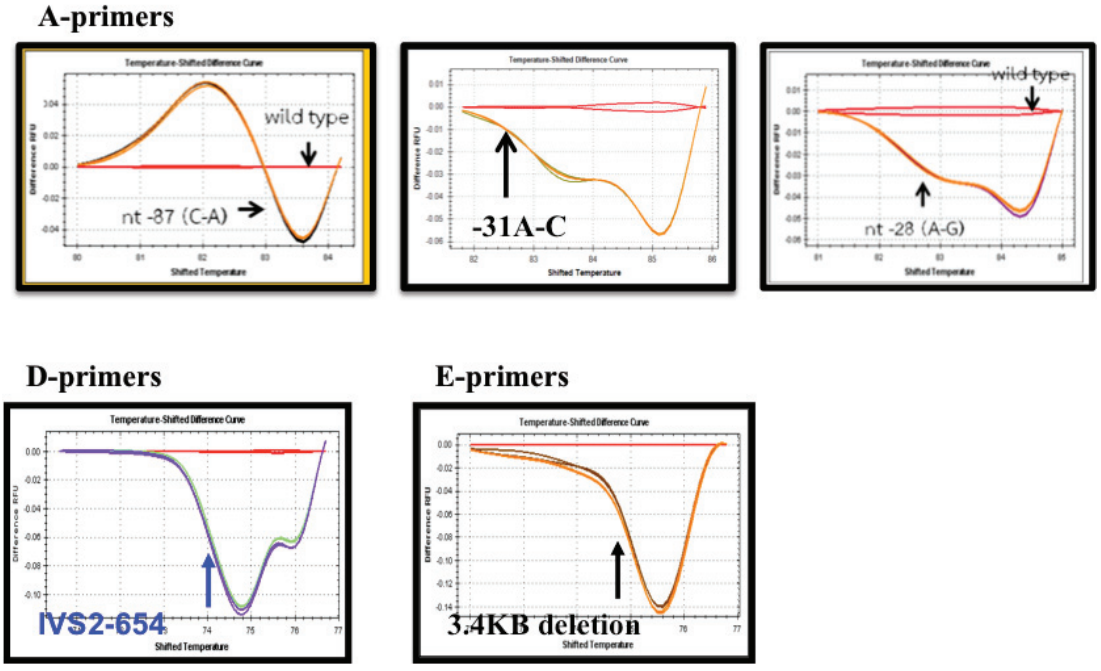

Fig 5–HRM images of beta thalassemia mutations detected with the PCR-HRM method using A-, D- and E-primer protocols. Each mutation gave a unique temperature-shift curve pattern.

Biosystems, Carlsbad, CA), 3.2 µM of the S1 forward primers (or S2 forward primers), 5 µl of purified DNA and the rest was distilled water to give a volume of 20 µl. The thermal cycle program was used for DNA denaturation at 96ºC for 10 seconds, primer annealing at 50ºC for 5 seconds and primer extension at 60ºC for 4 minutes for a total of 25 cycles. The entire content of extension product was then transferred into a tube containing 2 µl of 3 M sodium acetate at a pH of 4.6 and 50 µl of 95% ethanol. The tube was mixed thoroughly and left at room temperature for 15 minutes and then spun at 14,000 rpm in a micro-centrifuge for 30 minutes and the supernatant was discarded. The pellet was washed with 250 µl of 70% ethanol, spun at 14,000 rpm for 25 minutes and dried for 1 minute on a heat block at 90ºC. Direct DNA sequencing was carried out by suspending the precipitated extension products in 20 µl of template suppression reagent. The suspension was mixed, spun down and then heated at 95ºC for 2 minutes; it was spun down immediately after chilling on ice for 10 minutes. The suspension was further analyzed in an automated DNA Sequencer (four-capillary 3100-Avant Genetic analyzer).

Conventional Gap PCR detection of the 3.4 kb deletion

To detect the 3.4 kb deletion three primer pairs were developed. Two of them were the E1- forward primer 5′ GTCACACTTTGGGTTGTAAGTGAC 3′ (61360-83) and the E1-reverse primer 5′ TCAATGTGCTCTGTGCATTAGT 3′ (61462-83) used for amplifying the wild type control DNA segment and yielding a PCR product of 124 base pairs. The third primer was the E3-reverse primer 5′-TGCCATTTCATGGTTCACCTTTCA

3′ (65045-68). The E1-forward primer and E3-reverse primer were used to detect the 3.4 kb deletion, producing a product of 224 base pairs. The PCR mixture in a total volume of 25 µl contained 5 µl of DNA template, 200 µM of each dNTP, 2.0 Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), 0.38 µM of each primer, 50 mM KCl, 1.5 mM $MgCl_2$ and the rest was 1XPCR buffer. The mixture was placed in a conventional PCR apparatus and preheated at 95ºC for 3 minutes. The PCR was cycled 40 times at 95ºC for 30 seconds, 62ºC for 30 seconds, and 72ºC for 45 seconds, with a final extension at 72ºC for 7 minutes. The PCR products were analyzed with 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

RESULTS

For the PCR-HRM protocol the optimal concentration in the master mixed were 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.3 µM primers, 0.3 µM SYTO9 and 1.5 units of (platinum) Taq DNA polymerase. The average annealing temperature was 60ºC and for HRM analysis the temperature was between 70 and 90ºC. The performance of primer sets A to E in the PCR-HRM protocols tested revealed an average efficiency of 99.8%, a R^2 of 0.99 and a slope of -3.32 (Fig 2). PCR-HRM analysis of the 143 DNA samples showed 14 specific and recognizable normalized temperature-shifted melt curves and temperature-shifted difference curves (Fig 3). Mutations -87C-A, -31 A-C and -28 (A-G) were detected by primer pair A (Fig 5). The CD17 A-T, CD27/28 +C, CD30 G-A, IVS1nt1 G-T and IVS1nt5 G-C mutations were detected by primer pair B (Fig 4). The CD 35 C-A, CD 41-C, CD 41/42 -CTTT and CD71/72 +A were detected by primer pair C (Fig 4). IVS2nt654 (C-T) was detected by primer pair D (Fig 5) and the 3.4 KB deletion by primer pair E (Fig 5). There were 52 instances (37.6%) of CD41/42 -CTTT, 38 instances (27 %) of CD17 A-T, 16 instances (11.3%) of IVS1nt1G-T, 12 instances (8.5 %) of CD71/72 +A, 12 instances (8.5%) of the 3.4 kb deletion and 5 instances (3.5 %) of -28 A-G. There was also one example each of -87 C-A, -31 A-C, CD27/28 +C , CD 30 G-A, IVS1nt5 G-C, CD 41-C and IVSII 654 C-T. Primers B and C were responsible for 85.8% of all mutations detected. The three most common mutations were CD41/42 -CTTT, CD17 A-T and IVS1nt1 G-T. The temperature-shift difference curves for similar mutations were reproducible and aligned almost exactly. The 3.4 kb deletion results were in accordance with the conventional Gap-PCR technique. Beta thalassemia mutations detected by PCR-HRM with the A, B, C and D primer sets could clearly distinguish between normal DNA and any genotype of beta thalassemia trait and also presented the same results as direct DNA sequencing.

DISCUSSION

HRM technology is a recently developed method for fast high-throughput post-PCR analysis of genetic mutations (Wittwer *et al*, 2003; Montgomery *et al*, 2007; Erali *et al*, 2008; Wittwer, 2009). This method could be suitable to screen for beta thalassemia mutations. This is because the types of mutations and their locations in the HBB gene, including the SNP location, have been well characterized. This information made setting up a high performance PCR-HRM to screen for beta thalassemia mutations easier. Therefore, appropriate primers could potentially be designed using the Primer-Blast program to cover all mutations, including

deletion mutations. Moreover, automated PCR-HRM apparatuses provided with precision melting software are readily available, which makes analysis of beta thalassemia mutations simpler and more convenient. A series of five PCR protocols has been developed. The primer sets were designed based on beta thalassemia mutation prevalences. Mutation detection should be nearly absolute. A few non-Thai ethnic beta thalassemia traits might escape detection due to the uncommon mutations and they might need additional evaluation with conventional Gap-PCR or direct DNA sequencing.

The PCR master mix was modified by the addition of intercalating dye SYTO9 instead of SYBR Green I. SYTO9 is less toxic in the amplification reaction and produced a less dynamic dye redistribution to non-denatured regions of the nucleic strand during melting than SYBR Green I. This creates melting profiles with better resolution and reproducibility (Pornprasert *et al*, 2010; Monis *et al*, 2005). The appropriated PCR master mix contained 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM primers, 3.0 μM SYTO9 and 1.5 unit platinum Taq DNA polymerase. The annealing temperature was 60ºC while the high-resolution DNA melting temperature was between 70 and 90ºC. The PCR protocols were assessed after optimization and revealed acceptably high efficiency (99-100%).

The PCR-HRM method with precision melting was able to differentiate fourteen types of common beta thalassemia mutations. This was because each genotype had a specific temperature-shift difference curve (HRM image). This method allows diagnosis of 143 beta thalassemia mutations with 100% accuracy. Furthermore, in the process of beta thalassemia screening, mutations could be detected with the B and C primer protocols with a success rate of 85.8%. These results can help reduce workload if initial screening with just the B and C primer protocols is carried out, instead of with 5 PCR-HRM protocols.

In summary, the PCR-HRM technique may be used to identify all types of beta thalassemia mutations. It is a convenient and accurate tool for beta thalassemia mutation scanning. These methods may be beneficial and cost effective for beta thalassemia screening in the national thalassemia prevention program.

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