RESEARCH NOTE

DETECTION OF DELETION α^+-THALASSEMIA MUTATION [-α (3.7), -α (4.2)] BY QUANTITATIVE PCR ASSAY

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Abstract. In Thailand, Hb H (α^0-thal/α^+-thal) disease is highly prevalent. We designed 3 primer sets (A, B and C) to detect -α (3.7) and -α (4.2) deletion types of α^+-thal by quantitative (q)PCR. The A and C primer sets were used to amplify DNA sequences at the 3′ terminal regions of HBA2 and HBA1 gene, respectively, and the B primer set was used to amplify an upstream DNA sequence at the 5′ flanking region of HBA1 gene. The relative quantities of the PCR products (based on threshold cycle (CT) values) of the 3 primer sets were calculated according to the equation $R = 2^{-\Delta\Delta CT}$, and these values were used to distinguish between -α (3.7) and -α (4.2) deletion mutations. The type of α^+-thal mutations was determined by calculating the difference between $R_{(C-A)}$ and $R_{(C-B)}$, yielding a value either of 0.5 or 1.0, which indicates the copy number of the target DNA compared with normal diploid control. Measured values that are close to 0.5 indicate there is a single allele of the target DNA. This method was applied to 250 DNA samples recruited for this study, and the $R_{(C-A)}$ and $R_{(C-B)}$ value determined for 185 cases of non α-thal was 1.03 ± 0.04 and 0.95 ± 0.08, respectively, for 41 cases of -α (3.7) α^+-thal trait 0.49 ± 0.04 and 0.45 ± 0.04, respectively, and for 2 cases of -α (4.2) α^+-thal trait 0.5 ± 0.1 and 1.01± 0.06, respectively. The allele frequency of -α (3.7) and -α (4.2) mutation was 0.092 and 0.004, respectively. These results were in concordance with those obtained by conventional gap-PCR. The method described here is simple, accurate and feasible for screening of α^+-thal carriers and should provide valuable information for genetic counselling of patients at risk of having a child with Hb H disease.

Keywords: α^+-thal deletion mutation**,** quantitative PCR, relative gene quantification assay

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INTRODUCTION

There are two α-globins genes, HBA2 and HBA1, located on chromosome 16 (http//www.ncbi.nlm.nih.gov/nuccore/NG_000006). α^+-Thalassemia (thal) is

caused by a deletion of one of the two α-globin genes leaving only one gene intact (Higgs *et al*, 2001). The most common α^+-thal mutation in ethnic Thai people is -α (3.7) deletion, followed by -α (4.2) deletions (Lemmens-Zygulska *et al*, 1996). The most common type of non deletion α^+-thal mutation is $\alpha^{\text{Constant Spring (CS)}}$, followed by $\alpha^{\text{Pakse (PS)}}$. α^{CS}-Globin mRNA has a point mutation at stop codon 142 (UAA>CAA), and α^{PS}-globin mRNA has a stop codon mutation of UAU (both encoded by HBA2 gene) (Pichanun *et al*, 2010; Sornkayasit *et al*, 2012). Deletion and non-deletion (α^{CS} and α^{PS}) α^+-thal are present in Thailand with an average prevalence of 18%, 2.5% and 0.5%, respectively (Pichanun *et al*, 2010; Sornkayasit *et al*, 2012).

α^0-Thal mutations (deletion of 2 α-globin genes in *cis*) also are quite common in Thailand, and the percent of population who are carriers can be as high as 8%-15% in some parts of the country (Suwannakhon *et al*, 2014). The combination of α^0-thal and α^+-thal genes results in Hb H disease, which has an estimated prevalence of 1%-2% of the population. Clinically, in the steady state, patients with Hb H disease have mild to moderate anemia but may develop acute hemolytic crisis after severe infection and may occasionally require blood transfusion (Charoenkwan *et al*, 2005; Fucharoen *et al*, 2009; Laosombat *et al*, 2009). Fetuses with Hb H disease may on occasion progress to a fatal hydrops fetalis syndrome (Lorey *et al*, 2001; Chui *et al*, 2003). When one partner of a couple carries an α^0-thal mutation, it is necessary to screen the other for α^+-thal mutation to provide appropriate genetic counselling.

Hematological parameters, such as Hb concentration, MCV and MCH are normal or near normal in α^+-thal carriers, whose deletion genotypes can only be detected using DNA-based methods. The current method for detecting the common α^+-thal deletions is conventional gap-PCR, which is tedious and technically difficult (Liu *et al*, 2000). We describe an assay using quantitative (q)PCR to detect the presence and type of α^+-thal deletion in such carriers.

MATERIALS AND METHODS

Blood samples and DNA isolation

Two hundred fifty blood samples were recruited from the antenatal care clinic as part of the National Thalassemia Prevention Program. Genomic DNA from 200 μl of whole blood was isolated using the QIA-amp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Extracted DNA concentration was determined by measuring absorbance at 260 nm and 280 nm using a spectrophotometer (SPECORD Plus, Analytik, Jena AG, Jena, Germany), with A260/A280 ratios of between 1.6 and 1.8 accepted as of suitable quality. DNA was diluted with 1X PCR buffer to 5ng/μl and stored at -20°C until analysis.

Primers design

DNA sequences of HBA2 and HBA1 genes (Z84721: nucleotide numbers 33710-34573 and 37543-38384, respectively) were derived from NCBI's GenBank (http: //www.ncbi.nlm.nih.gov/nuccore/NG_000006). Primer pairs (Invitrogen, Carlsbad, CA) were designed using the Primer-BLAST program (http://www.ncbi.nlm.NIH.Gov/tools/primer-blast). Three primer pairs (A, B and C) were designed to detect the α^+-thal [-α (3.7) and -α (4.2)] genotypes (Fig 1). Two primer pairs were designed to target the 3' flanking regions of HBA2 [A primer pair: forward A-primer 5'TCCTGGCTTCTGTGAGCACCGT3' (34420-42), and reverse A-primer 5'ACCAGGAAGGGCCGGTGCAAG 3' (34526-

46)] and HBA1 [C primer pair: forward C-primer 5′ACGCCTCCCTGGACAAGTTC 3′ (38213-32), and reverse C-primer 5′AGAAGCATGGCCACCGAGGCTC3′ (38279-300)] genes. The PCR product of A prime pair is 127 base pairs (bp) covering the terminal region of Exon III of HBA2 gene, and the amplicon of C primer pair is 80 bp, covering the terminal region of Exon III of HBA1 gene. The third (B) primer set [forward B-primer 5′CACCTCCATTCTCCAACCACAG 3′ (36372-93), and reverse B-primer 5′AGTCTGGAGGTGTGGACGAGGC 3′ (36526-47)] amplified a region of the 5′ flanking region of HBA1 generating a 176 bp amplicon.

qPCR assay

PCR mixture (25 μl) included 5 μl of DNA, 1X PCR buffer, 1.5 μM $MgCl_2$, 200 μM each dNTP, 2 μM SYTO9 (Invitrogen, Carlsbad, CA), 1 U Platinum® (Invitrogen) *Taq* DNA polymerase and 0.2 μM each primer. Thermocycling was performed using CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA) as follows: 94°C for 2 minutes; followed by 40 cycles of 94°C for 15 seconds, 64°C for 15 seconds ND and 72°C for 20 seconds. Fluorescence was measured on the SYBR channel (533 nm) at the end of each cycle (Seeratanachot *et al*, 2013). The ratio (R) of target DNA sequence copy numbers was calculated using the equation $R = 2^{-\Delta\Delta CT}$, where CT is the threshold cycle. $R_{(C-A)}$ represents the ratio of CT values of the C and A primer sets of the sample compared with normal DNA. Thus, $\Delta\Delta CT_{(C-A)} = [CT_C - CT_A]_{normal} - [CT_C - CT_A]_{sample}$, and similarly in $R_{(C-B)}$ $\Delta\Delta CT_{(C-B)} = [CT_C - CT_B]_{normal} - [CT_C - CT_B]_{sample}$. Calculation of each R value was carried out using Microsoft Excel.

α^0-Thal carriers and Hb H subjects were not included in this study because they have a single or no copy of the target DNA. These samples were analysed by assessing the presence versus the absence of individual PCR products. The failure to amplify PCR product from the A primer set indicates that the sample was Hb H and had -α (4.2) deletion, whereas failure to amplify PCR product from either A or B primer set indicates that the sample is Hb H with -α (3.7) deletion.

In SEA α^0-thal type, HBA1 and HBA2 genes are missing due to a 19,304 bases deletion (Winichagoon *et al*, 1995). The 5′ DNA deletion break point is located at NCBI reference Z84721 nucleotide number 26259, and the 3′ break point at NCBI reference Z69706 nucleotide number 2608. The primers, designed according to the principles of conventional gap-PCR and to meet the requirements for qPCR conditions (http://sg.idtdna.com/primerquest) were forward primer P1 [5′GTCGTCCCCACTGTCGTC3′ (26202-19)] and reverse primer P3 [5′GGCTTACTGCAGCCTTGAAC3′ (Z69706 2647-67)] to detect the SEA-DNA deletion (117mbp amplicon), and P1 and reverse primer P2 [5′ACGCCGTCCGACTCAAGGA3′ (26334-53)] to detect the normal DNA sequence Z84721 26161-26341 (152 bp amplicon). For qPCR, 0.1 μM each primer (P1, P2 and P3) was added to the mixture and the PCR reaction was performed as described above. PCR reactions using control DNA generated a DNA melting profile with a single Tm of 92.4 °C, whereas PCR reactions with DNA from SEA samples produced a DNA melting profile with two Tms of 88.4 and 92.4 °C. This method was also tested using DNA from Hb Bart's hydrops fetalis (homozygous SEA-type) cases, which generated a DNA melting profile with a single Tm of 88.4°C (Pornprasert *et al*, 2008). All DNA samples were assayed in duplicate.

Table 1
Primes used in the study.

Primer	Sequence (5′→3′)	Position[b]	Product (bp)	Tm[a] (°C)
A-Forward	TCCTGGCTTCTGTGAGCACCGT	34420-42	127	88
A-Reverse	ACCAGGAAGGGCCGGTGCAAG	34526-46		
B-Forward	CACCTCCATTCTCCAACCACAG	36372-93	176	84.8
B-Reverse	AGTCTGGAGGTGTGGACGAGGC	36526-47		
C-Forward	ACGCCTCCCTGGACAAGTTC	38213-32	88	84
C-Reverse	AGAAGCATGGCCACCGAGGCTC	38279-300		

[a]Melting temperature. [b]Based on GenBank accession no. Z84721.

Conventional gap-PCR assay

Detection of -α (3.7) and -α (4.2) mutations were carried out using two sets of multiplex PCR protocols (Liu *et al*, 2000). Three primer pairs were used to detect the -α (3.7) deletion mutant: forward primer (common) 3′AAGTCCACCCCTTCCTTCCTCACC5′ (Z84721 positions 32755-32778), reverse primer (mutant) 3′TCCATCCCCTCCTCCCGCCCCTGCCTTTTC5′ (Z84721 positions 38492-38521), and reverse primer (normal) 3′ATGAGAGAAATGTTCTGGCACCTGCACTTG5′ (Z84721 positions 34942-34971), generating products of 1963 and 2217 bp for -α (3.7) and the normal control, respectively. Three additional primer pairs were used to detect the -α (4.2) deletion mutant: forward primer (common) 3′TCCTGATCTTTGAATGAAGTCCGAGTAGGC5′ (Z84721 positions 30283-30312), reverse primer (mutant) 3′ATCACTGATAAGTCATTTCCTGGGGGTCTG5′ (Z84721 positions 36176-362050), and reverse primer (normal) 3′TGGGGGTGGGTGTGAGGAGACAGGAAAGAGAGA5′ (Z84721 positions 31760-31789), generating products of 1725 and 1510 bp for -α (4.2) and normal control, respectively. PCRs were performed in 25 μl containing 0.75 M betaine, 5% DMSO, 200 μmol of dNTPs, 1.25 U AmpliTaq Gold® DNA polymerase, 1X GeneAmp® buffer (Applied Biosystems, Foster City, CA), 0.2 μmol of each primer, and 5 μl of genomic DNA. Thermocycling (performed in a T100™ Thermo Cycler; Bio-Rad) conditions were as follows: 15 minutes at 95°C; 35 cycles of 95°C for 1 minute, 65°C for 1 minute, and 72°C for 2.5 minutes; with a final step of 72°C for 10 minutes. Amplicons were analysed by 1.5% agarose gel-electrophoresis and visualised using ethidium bromide staining under UV illumination (Liu *et al*, 2000).

RESULTS

Single tube qPCR assays of normal DNA samples with each of the A, B and C primer pairs revealed specific DNA melting profiles with Tm of 88.0ºC, 84.8ºC and 84.0°C, respectively (Fig 2). Application of relative qPCR using DNA samples of known α^+-thal deletion mutants (Seeratanachot *et al*, 2013) revealed that the average $R_{(C-A)}$ and $R_{(C-B)}$ values corresponded to the predicted values (data not shown). From 250 DNA samples tested, there were 41 cases of -α (3.7) and 2 of -α (4.2) α^+-thal traits. Two cases showed no amplification with both A and B primer

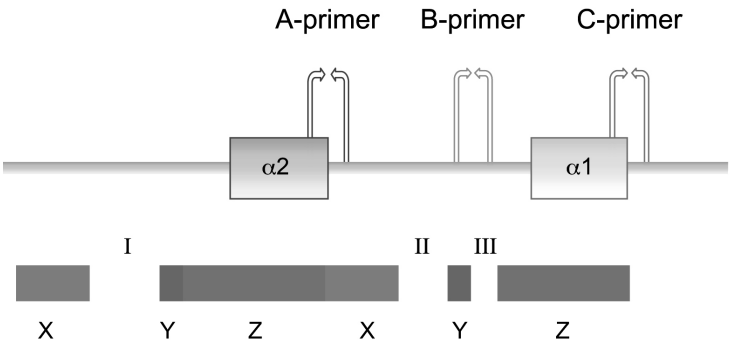

Fig 1–Schematic representation of the positions of A, B and C primer sets. Duplicated α-globin genes are divided into three homologous sub-segments (X, Y, and Z boxes) with regions of non homologous elements (I, II, III) (Higgs *et al*, 2001).

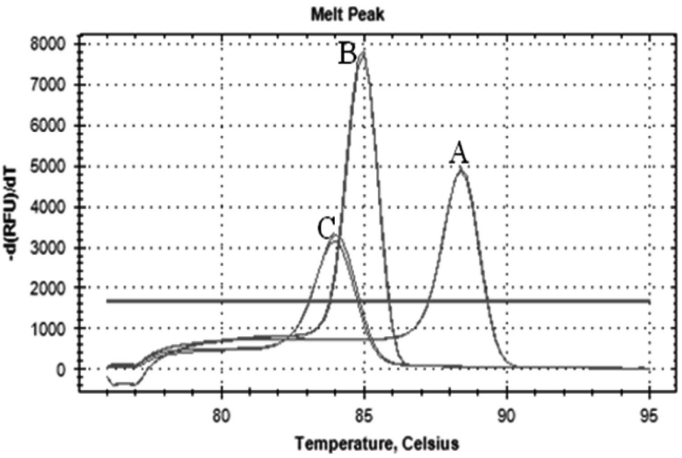

Fig 2–DNA melting profiles of amplicons using A, B and C primer pairs.

pairs and were considered to be homozygous -α (3.7) α^+-thal. There were 18 cases [--(SEA)] α^0-thal trait, and 1 case of Hb H disease [--(SEA) / -α (3.7)]. The values of $R_{(C-A)}$ and $R_{(C-B)}$ determined for the 185 non α^+-thal carriers was 1.03 ± 0.04 (mean ± SD; range 0.84-1.18; %CV 7.31) and 0.95 ± 0.08 (mean ± SD; range 0.81-1.15; %CV 8.16), respectively; for the 41 -α (3.7) α^+-thal traits 0.49 ± 0.04 (mean ± SD; range 0.41-0.61; %CV 8.28) and 0.45 ± 0.04 (mean ± SD; range 0.40-0.54; %CV 7.90), respectively; and for the 2 cases of -α (4.2) α^+-thal traits 0.5 ± 0.1 (mean ± SD) and 1.01± 0.06 (mean ± SD), respectively. The allele frequency of the -α (3.7), -α (4.2) and --α (SEA) deletion mutation was 0.092, 0.004 and 0.036, respectively. These findings of α^+-thal deletion types were in concordance with those obtained using conventional gap-PCR technique.

DISCUSSION

This study showed that it was possible to determine the occurrence as well as the deletion type (-α (3.7) or-α (4.2) of α^+-thal trait by using qPCR to measure the copy number of the deletion region in HBA1 and HBA2 genes on chromosome 16. As homologous elements are contained in the α-globin gene clusters, non-specific products and primer dimers can be generated in the PCR process and might influence the outcome of the quantification analysis. Therefore, it is very important to perform the assay using appropriately designed primers and PCR conditions.

The α^+-thal and α^0-thal allele deletion frequency was calculated to be 0.096 and 0.036, respectively, giving rise to an expected birth of 7 cases of Hb H disease per 1,000 pregnancies in northern Thailand. These data demonstrate the importance of detecting of α^+- and α^0-thal carriers

in order to provide appropriate genetic counselling to couples at risk, as fetuses with Hb H disease may succumb to hydrops fetalis syndrome *in utero*.

In summary, the relative gene quantification assessment by qPCR technique described here offers an alternative approach than conventional gap-PCR to identify the presence of deletion α^+-thal carriers. The method is simple and highly accurate, and should provide beneficial information for public health policies regarding management of Hb H disease in Thailand and other similarly affected countries.

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