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ORIGINAL ARTICLE

Detection of alpha-thalassemia-1 Southeast Asian type using real-time gap-PCR with SYBR Green1 and high resolution melting analysis

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Abstract

α-Thalassemia-1 Southeast Asian (SEA) type is the most common genetic disorder in the Asian population. Couples who are both carriers have a 25% chance of conceiving Bart's hydrops fetalis. Therefore, results from carrier screening and prenatal diagnosis frequently need to be available rapidly. A rapid technique for diagnosis of α-thalassemia-1 SEA type was implemented. The technique used is based on real-time gap-PCR and high resolution melting (HRM) analysis of the amplified fragment using the Rotor-Gene 6000™. The DNA samples used for amplification were obtained from whole blood, cord blood, and chorionic villus sampling (CVS). With this method, the α-thalassemia-1 SEA allele can be easily distinguished from wild type α-globin gene allele. The real-time gap-PCR and HRM analysis offers additional benefits including minimal labor, rapid turnaround time, and a decreased risk of PCR carryover contamination. It is cost-effective and safe, does not require fluorescently labeled probe and hazardous chemicals. Moreover, it is accurate showing 100% concordance with conventional gap-PCR analysis.

Key words Real-time; gap-PCR; SYBR Green1; alpha-thalassemia-1; high resolution melting analysis

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α-Thalassemia is one of the most serious genetically transmitted diseases creating health problems in many countries, with gene frequencies varying between 1% and 98% throughout the tropics and subtropics. More than 95% of recognized α-thalassemia involved deletion of 1 or both α-globin genes from chromosome 16p13.3 (1, 2). These gene deletions caused mild α-thalassemia-2 and severe thalassemia-1, respectively. The most common type of thalassemia-1 in the Asian population is the Southeast Asian type (SEA) (3). Even thought carriers of the α-thalassemia-1 with SEA type do not manifest any clinical symptoms, couples who are both carriers have a 25% chance of conceiving a homozygous fetus, which manifests as Bart's hydrops fetalis, the most severe thalassemic syndrome. All of these fetuses die either *in utero* or soon after birth (4–6). In addition, approximately 75% of mothers carrying fetuses with homozygous for the α-thalassemia-1 SEA type will develop toxemia of pregnancy (7). An investigation of α-thalassemia-1 SEA type is therefore essential for carrier couples and for prenatal diagnosis of fetus conceived by couples who are both carriers of this type of gene deletion.

The gap-PCR analysis currently used to diagnose α-thalassemia-1 SEA type is based on multiplex amplification at the breakpoint area of thalassemia-1 with SEA type and the wide type α-globin gene allele. The technique requires labor intensive, time-consuming, and post-PCR processing steps (8). In an effort to develop a more straightforward diagnostic test, quantitative real-time PCR with specific probes (9, 10) has been used for detection of α-thalassemia-1 SEA type. Probe-based assays are generally used for multiplex real-time PCR analysis. However, they are relatively expensive. Real-time PCR with SYBR Green1 followed by melting curve analysis has also been use to enhance the speed

of α-thalassemia-1 SEA detection (11, 12). However, this technique is not suitable for gap-PCR. Recently, a high resolution melting (HRM) analysis incorporating the fluorescent dye has been used for detecting heterozygous and homozygous sequence variants for genotyping and variation scanning (13–15). This approach is a closed system that does not require a fluorescently labeled probe or a separation step. In contrast to traditional melting curve analysis, HRM reliably detects single-base differences in homozygous and heterozygous sequence (16). This technique is cost-effective, has high sensitivity and specificity, rapid turnaround time, and decreases a risk of PCR carryover contamination.

In this study, we report a novel and powerful scheme combining real-time gap-PCR with SYBR Green1 and high resolution melting analysis for rapid detection of α-thalassemia-1 SEA type.

Materials and methods

DNA samples

DNA was extracted from a hundred samples including 80 whole blood, 10 cord blood, and 10 chorionic villus sampling (CVS) using the Chelex method (Chelex 100 Resin, Sigma, CA, USA) (17). According to the thalassemia screening protocol, the genotype from each sample was firstly determined by the conventional gap-PCR analysis. This study was approved by the Ethics Committee of the Faculty of Associated Medical Sciences, Chiang-Mai University.

Real-time gap-PCR with SYBR Green1 and HRM analysis

DNA amplification was carried out in a 20 μL reaction volume containing : 10 μL of 2× SYBR Green1 PCR master mix (Quantimix easy SYG kit, Biotools, Madrid, Spain) and 0.38 μM of each primer with nucleotide sequences as shown in Table 1 and 7 μL of DNA sample. The gap-PCR with SYBR Green1 was performed on Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia). The mixture was preheated at 95°C for 3 min and then the PCR was cycled 40 times at 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s. The amplification cycles were followed by high resolution melting cycle from 85°C to 95°C at a rate of 0.1°C per 2 s. When the melting temperature (Tm) is reached, double stranded DNA is denatured and the SYBR Green1 is released which cause a dramatic decrease in fluorescence intensity. The rate of this change was determined by plotting the derivative of the fluorescence relative to the temperature (dF/dT) vs. temperature by data analysis software of the real-time PCR instrument. The temperature at which a peak occurs on the plot corresponds to the Tm of the DNA duplex.

Results

In α-thalassemia-1 SEA trait, two amplified fragments from the α-thalassemia-1 SEA allele and from wild type α-globin gene allele were generated. The amplified fragments from α-thalassemia-1 SEA allele had the specific peak at Tm of 88 ± 1°C whereas the amplified fragments from wild type α-globin gene allele had the specific peak at Tm of 91 ± 1°C (Fig. 1A–C). Only an amplified fragment from the α-thalassemia-1 SEA allele with the specific peak at Tm of 88 ± 1°C was found in Bart's hydrops fetalis (Fig. 1B and C), while only an amplified fragment from the wild type α-globin gene allele with the specific peak at Tm of 91 ± 1°C was found in normal individual (Fig. 1A–C).

DNA samples extracted from cord blood of normal individual, thalassemia-1 SEA trait and Bart's hydrops fetalis showed similar specific peak heights as observed in those extracted from CVS (Fig. 1B and C, respectively). These specific peak heights were also found similarly in DNA samples extracted from whole blood of normal individual and thalassemia-1 SEA trait (Fig. 1A).

Moreover, the results of real-time gap-PCR with SYBR Green1 and HRM analysis of a hundred assessed samples including 80 whole blood, 10 cord blood and 10 CVS were completely consistent with those of conventional gap-PCR analysis (Table 2).

Discussion

This study shows that the real-time gap-PCR with SYBR Green1 and HRM analysis could facilitate rapid screening of α-thalassemia-1 SEA type. It could assay the DNA samples processed from CVS that is of benefit for prenatal diagnosis of Bart's hydrops fetalis in the fetus and is offered to all couples who are both carriers of the α-thalassemia-1 with SEA type allele. This technique

Table 1 Primer sequences used in real-time gap-PCR with SYBR Green1 for detection of α-thalassemia-1 SEA type

Primer	Sequence (5′ → 3′)	Product length (bp)	GenBank accession no.
NaI-Forward primer	AGA AGC TGA GTG ATG GGT CCG	–	Z84721
NaII-Reverse primer	ACA AAC GCC CGT CCG ACT CAA	196	Z84721
NaIII-Reverse primer	TGG ACT TAA GTG ATC CTC CTG CCC	134	Z69706

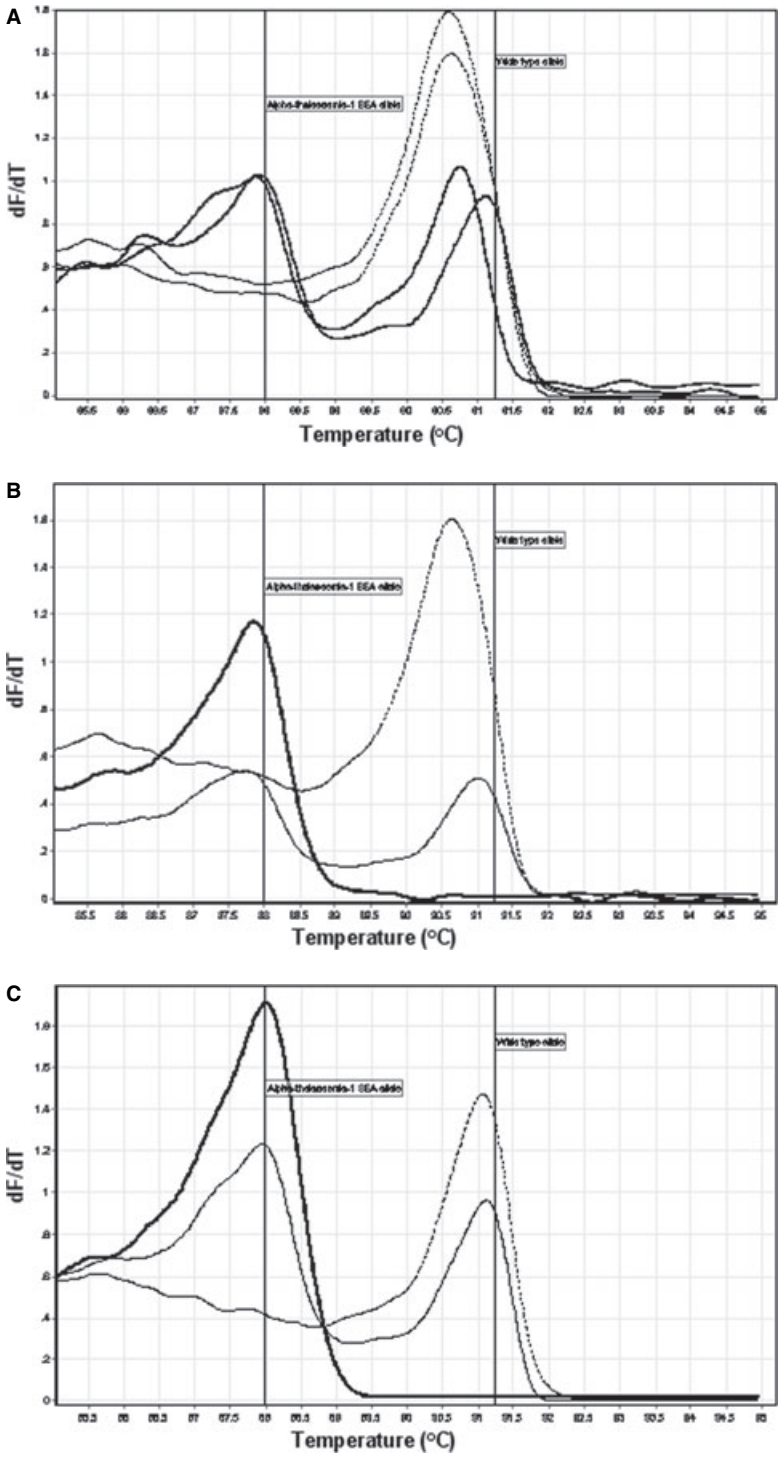

Figure 1 Dissociation curve analysis of thalassemia-1 SEA type. DNA samples were extracted from whole blood (A), cord blood (B), and chorionic villus sampling (C) of normal individual (……), thalassemia-1 SEA trait (——) and Bart's hydrops fetalis (▬).

does not require fluorescently labeled probes or separation steps. It therefore offers additional benefits including minimal labor, rapid turnaround time, and decreased risk of PCR carryover contamination. The gap-SYBR Green1-PCR with HRM analysis for detection of α-thalassemia-1 SEA can supply timely information. Such results from carrier screening frequently need to be available rapidly, especially when couples present after establishment of a pregnancy (18).

Without HRM methodology, the real-time gap-PCR with SYBR Green1 and melting analysis had been performed for detection of α-thalassemia-1 SEA type. By using primers with nucleotide sequence similar to those used in conventional gap-PCR, the melting curve analy-

Table 2 The results of α-thalassemia SEA type of a hundred DNA samples

Specimen	gap-PCR and HRM analysis		Genotype tested by conventional gap-PCR	Case number
	134 bp Tm 88 ± 1°C	196 bp Tm 91 ± 1°C		
Whole	–	+	αα/αα	73
blood	+	+	_SEA/αα	7
(*n* = 80)				
Cord	–	+	αα/αα	3
blood	+	+	_SEA/αα	4
(*n* = 10)	+	–	_SEA/_SEA	3
CVS	–	+	αα/αα	3
(*n* = 10)	+	+	_SEA/αα	3
	+	–	_SEA/_SEA	4

sis was clearly differentiated between Bart's hydrops fetalis and normal individuals. However, the melting curves of the heterozygous were not clearly distinguishable from those of normal subjects (19). Based on rapid detection and appropriation for HRM analysis, three oligonucleotide primers were designed for amplifying small DNA fragments, 134 and 196 bp, from the α-thalassemia-1 SEA allele and wild type α-globin gene allele, respectively. After a high resolution melting temperature analysis, peak heights of Tm were observed specifically in each fragment. These specific peak heights could facilitate rapid screening of heterozygous and homozygous α-thalassemia-1 of SEA type.

A previous study suggested that SYBR Green1 was not suitable for HRM analysis since it can only be used at non-saturating concentration. Therefore, SYBR Green1 that dissociated from DNA during melting can re-intercalate into regions of unmelted double stranded DNA (20). This phenomenon of fluorescence dye redistribution can mask the small differences in melting behaviour (21, 22). However, the re-intercalation of SYBR Green1 did not affect our study. Because the gap-PCR with SYBR Green1 and HRM analysis was set up for detecting of α-globin gene deletion and is unlike the analysis of single gene mutation, the discrimination of amplicons with sufficiently different Tm values can be provided in the present study.

In conclusion, our results indicated the real-time gap-PCR with SYBR Green1 and HRM analysis for diagnosis of α-thalassemia-1 SEA type is much safer than conventional gap-PCR method because it does not use hazardous chemicals such as acrylamide and ethidium bromide. This technique is cost-effective, uses minimal labor, has rapid turnaround time, and decreases risk of PCR carryover contamination. The real-time gap-PCR with SYBR Green1 may be one alternative technology available for routine clinical diagnosis of α-thalassemia-1 with the SEA type.

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