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One single-tube PCR assay to detect both CD17 (A>T) and IVS-II nt-654 (C>T) homozygous mutations of β-thalassemia

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Aim: Several methods can be used to detect gene mutations associated with β-thalassemia, but it is difficult to achieve reliable and reproducible results. In this study, we introduce a new method, a single-tube multiplex polymerase chain reaction (PCR) assay, to detect both CD17 (A>T) and IVS-II nt-654 (C>T) homozygous mutations.

Materials and methods: This new method designs specific primers to diagnose homozygous mutations and normal controls.

Results: After PCR amplification, homozygous mutations produce different fragments from normal controls.

Conclusion: This study represents an important step towards the development of a novel protocol to diagnose β-thalassemia and other diseases that target numerous mutations.

Key words: β-Thalassemia, single-tube, CD17, IVS-II nt-654

1. Introduction

β-Thalassemia (β-thal) is a severe disorder of β-globin gene expression characterized by hypochromic, hemolytic anemia and inherited as an autosomal disorder (1,2). In general, each ethnic group appears to carry its own profile of mutant alleles. Currently, 265 different point mutations (http://globiin.bx.psu.edu) have been characterized to be associated with β-thal (3–6), 4 of which can account for about 87% of the occurrence of β-thal. In descending frequencies, these 4 mutations are codon CD41/42(-CTTT), IVS-II nt-654 (C>T), CD17 (A>T), and TATA box nt-28 (A>G) (7). In China, β-thal, with carrier frequencies ranging from 0.02% to 4.8%, affects an estimated 20 million people that are predominantly distributed in Guangxi, Guangdong, Hainan, and Yunnan provinces (8). The universality of this disease produces great mental and economic pressure upon society and the patients' families, which no doubt prevents the development of the public health in southern China (9).

The main clinical diagnostic methods of β-thal are polymerase chain reaction-reverse dot blot (PCR-RDB), real-Time PCR, the polymerase chain reaction-amplification refractory mutation system (PCR-ARMS), restriction fragment length polymorphism (RFLP), antisense oligodeoxynucleotide (ASO), single-strand conformation polymorphism (SSCP), and Gene Chip (10–13). It took us about 8 h to complete the procedure including PCR and RDB. Previously, the PCR method has been proven to be simple, very sensitive, and specific for the routine diagnosis of β-thal mutation. However, it is difficult to achieve reliable and reproducible results in comparison with a single-tube multiplex PCR assay. In this study, a single-tube multiplex PCR assay has been successfully used to diagnose the 2 most widely occurring β-globin gene mutations in China.

2. Materials and methods

2.1. Patients and samples

In total, 40 unrelated patients (19 females and 21 males, mean age of 40.5 ± 12.9 years) and 20 control patients (12 females and 8 males, mean age of 41.3 ± 6.8 years) were investigated in this study. All patients had no other diseases. All individuals enrolled gave informed written consent as approved by the local institutional review board. The investigated mutations included 20 CD17 (A>T) homozygous mutations and 20 IVS-II-654 (C>T)

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homozygous mutations. Genomic DNA was isolated from whole blood of participants using the Puregene DNA Isolation Kit (QIAGEN, Hong Kong).

2.2. Primer designs

Giving the detection of the CD17 (A>T) homozygous mutation for an example, we carried out a parallel multiplex PCR reaction by using a pair of primers, 5'-ATTGCTTACATTTGCTTCTG-3' and 5'-ACTTCATCCACGTTCAGCTT-3', for the specific amplification of the CD17A and CD17T alleles, respectively (128-bp fragments; Table 1). The reverse primer differs only in the mutated nucleotide at its 3' end. We inlet a C > G change (the modified nucleotide is underlined), which will cause an additional mismatch to increase the allele specificity of the reaction, shorten the annealing temperature, and rule out the nonspecific PCR amplification. The specific mismatch site could provide a relatively large difference of melting temperature. We also introduced another pair of primers, 5'-TGATAATTTCTGGGTTATGGT-3' and 5'-AATATCCCCCAGTTTAGTAG-3', for the detection of the IVS-II-654 (C>T) homozygous mutation (Table 1). GeneTool (GeneBio, Switzerland) software was used to design the primers.

2.3. PCR reaction

PCR reaction was carried out in a Bio-Rad S1000 PCR System (Bio-Rad Laboratories, USA) with 1 cycle of denaturation at 95 °C for 5 min; 5 touch-down cycles of denaturation at 94 °C for 40 s, annealing for 40 s at 53 °C for the first cycle, and a 1 °C reduction per cycle; 25 cycles of denaturation at 94 °C for 40 s and annealing at 48 °C for 40 s; and a final extension step at 72 °C for 7 min. The reaction was carried out in a final volume of 50 μL at the same concentration of 4 primers for CD17 (A>T) and IVS-II-654 (C>T) homozygous mutations, with a final concentration of 1 or 2.5 mM magnesium chloride and 100 ng of DNA template, using Taq DNA polymerase (TaKaRa, China). PCR products were separated by agarose gel electrophoresis (2.0% agarose gel; Figure 1) and visualized using Bio-Rad Chemi Doc XRS (Bio-Rad Laboratories). Fragment size was determined by comparison to a 2-kb DNA marker (Invitrogen, USA). DNA sequencing of the β-globin gene was performed using 2 pairs of primers flanking the 3 exons of β-globin genes, and DNA was amplified using Taq DNA polymerase (TaKaRa) in the Bio-Rad S1000 PCR System (Bio-Rad Laboratories). The primers and product lengths are shown in Table 2. The PCR product was sequenced using the ABI3700 (ABI, USA) and visualized using the Chromas program.

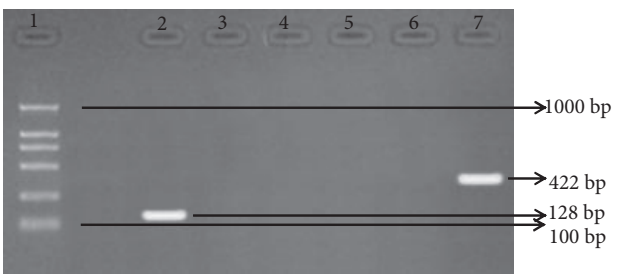

Figure 1. One specific single-tube polymerase chain reaction for both CD17 (A>T) and IVS-II-654 (C>T) homozygous mutations. Lane 1 was 2-kb DNA marker; 2 was CD17T-specific PCR; 7 was IVS-II-654T–specific PCR; 3, 4, 5, and 6 were normal controls.

Table 1. PCR primer sequences, fragment sizes, and gene location.

ID	Primer sequence (5'-3')	Fragment size (bp)	GenBank No. : nucleotides
No.1	ATTGCTTACATTTGCTTCTG	128	NG_000007 : 70538-70557
No.2	ACTTCATCCACGTTCAGCTT		NG_000007 : 70646-70665
No.3	TGATAATTTCTGGGTTATGGT	422	NG_000007 : 71673-71693
No.4	AATATCCCCCAGTTTAGTAG		NG_000007 : 72075-72094

Table 2. β-Globin primers for PCR and sequencing.

Gene	Primer sequence (5'-3')	Fragment size (bp)	GenBank No. : nucleotides
β1 (exon1-2)	Forward: GTACGGCTGTCATCACTTAGACCTCA	602	NG_000007 : 70416-70441
	Reverse: TGCAGCTTGTCACAGTGCAGCTCACT		NG_000007 : 70992-71017
β2 (exon3)	Forward: GTGTACACATATTGACCAAA	423	NG_000007 : 71496-71515
	Reverse: AGCACACAGACCAGCACGTT		NG_000007 : 71899-71918

3. Results

The electrophoresis results showed that the single-tube multiplex PCR assay worked well (Figure 2). CD17 (A>T) and IVS-II-654 (C>T) homozygous mutations could be detected in a single tube. After amplification, the CD17 (A>T) samples could produce a 128-bp fragment with the combination of 4 primers. Meanwhile, the IVS-II-654 (C>T) samples could produce a 422-bp fragment with the combination of 4 primers. However, the normal control samples produced no fragment using the combination of 4 primers. In a verification test, all samples that had been detected by DNA sequencing were analyzed by single-tube PCR assay, and the results showed complete agreement between the new method and sequencing analysis. To evaluate the reproducibility of the single-tube PCR assay, all samples were tested 5 times, and all the mutant and wild-type blots showed the same depth of color every time. Heterozygous samples were excluded since this method was a qualitative analysis and could not discriminate between heterozygous samples and homozygous samples. For detecting more homozygous mutations in future studies, reducing the requirements of multiple reaction conditions in a single tube, we did not use internal control in this PCR assay, but negative nontemplate controls were included in each PCR. PCR products were subjected to electrophoresis on agarose gels and sequenced.

4. Discussion

Although β-thal is a genetic disease that has been well characterized at the DNA level, mutation detection in the β-globin gene is still based on time-consuming methodologies, requiring cumbersome sample manipulation. Commonly, the occurring mutations of the β-globin gene are detected by a number of PCR-based procedures, such as RDB analysis or primer-specific amplification with a set of probes (10,13). Other methods based on real-time PCR or microarray technology (11,12) are incompatible for the routine clinical laboratory because of their cost and complexity.

In view of the above drawbacks, in this study, we have explored a different approach based on specific primers. It is indicated that single-tube multiplex PCR assay could quickly and simply detect both CD17 (A>T) and IVS-II-654 (C>T) homozygous mutations. The frequency of CD17 (A>T) and IVS-II-654 (C>T) mutations in China is 0.10%–1.59% and 0.57%–0.63%, respectively (14,15).We found that this method is less laborious, less expensive, and faster, which makes it more suitable for routine diagnosis. Therefore, this method can be well extended to detect other gene mutations.

It should also be noted that the single-tube multiplex PCR assay has some limitations. For example, we can only detect 2 homozygous mutations in a single tube; a heterozygous mutation (a mutation of only 1 allele) and

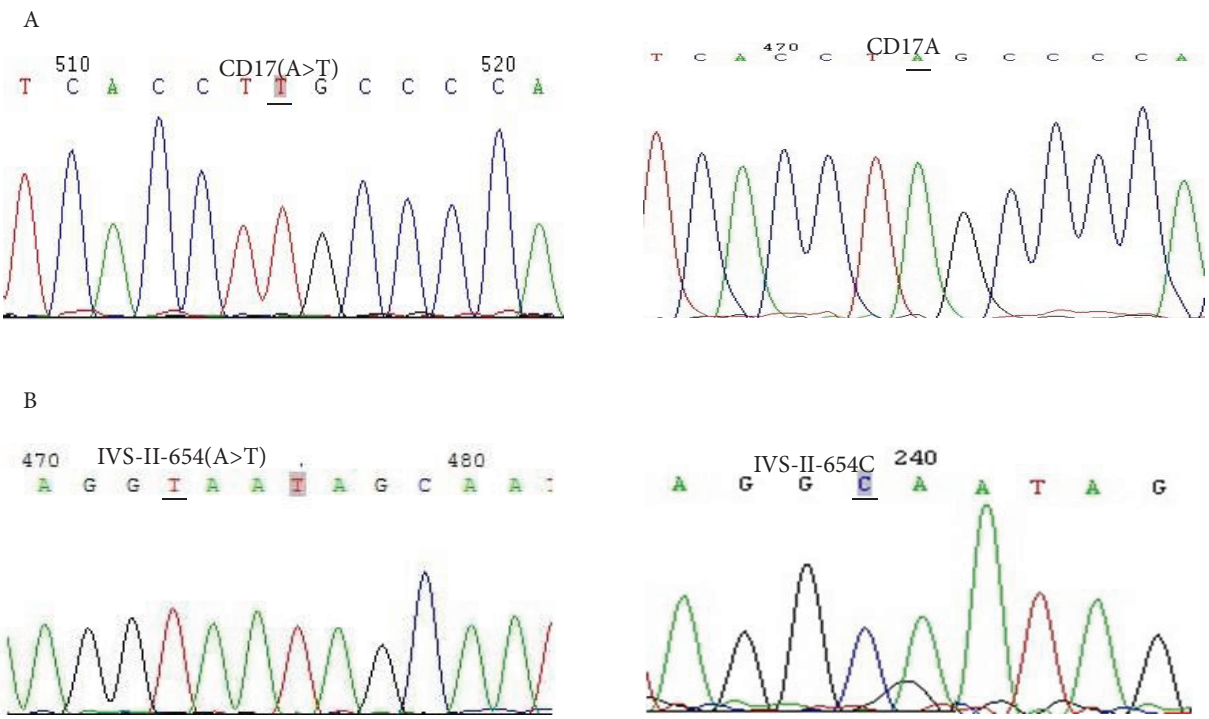

Figure 2. Sequencing results of (A) homozygous CD17 (A>T) mutation and wild type, (B) homozygous IVS-II-654 (C>T) mutation and wild type.

compound heterozygous mutations (2 different mutations in the paternal and maternal alleles) could not be detected at present. However, we expect that this method will be perfected with continuous improvement of various analytical techniques.

In summary, this study represents an important step toward the development of a novel protocol to diagnose β-thal and other diseases that target numerous mutations.

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