



## ORIGINAL ARTICLE

## Primer-Introduced Restriction Analysis Polymerase Chain Reaction Method for Non-Invasive Prenatal Testing of $\beta$ -Thalassemia

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### Abstract

We have developed a new method for non-invasive prenatal testing (NIPT) of paternally inherited fetal mutants for  $\beta$ -thalassemia ( $\beta$ -thal). Specially designed primer-introduced restriction analysis-polymerase chain reaction (PIRA-PCR) were used to detect four major mutations [IVS-II-654, *HBB*: c.316-197C>T; codon 17 (A>T), *HBB*: c.52A>T; –28 (A>G), *HBB*: c.-78A>G and codons 41/42 (–TTCT), *HBB*: c.126\_129delCTTT] causing  $\beta$ -thal in China. The PIRA-PCR assay was first tested in a series of mixed DNA with different concentrations and mixed proportions. Subsequently, this assay was further tested in 10 plasma DNA samples collected from pregnant women. In the DNA mixture simulation test, the PIRA-PCR assay was able to detect 3.0% target genomic DNA (gDNA) mixed in 97.0% wild-type gDNA isolated from whole blood. For plasma DNA testing, the results detected by PIRA-PCR assay achieved 100.0% consistency with those obtained from the amniocentesis analysis. This new method could potentially be used for NIPT of paternally inherited fetal mutants for  $\beta$ -thal.

### Keywords

$\beta$ -Thalassemia ( $\beta$ -thal), non-invasive prenatal testing (NIPT), primer-introduced restriction analysis-polymerase chain reaction (PIRA-PCR)

### History

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### Introduction

$\beta$ -Thalassemia ( $\beta$ -thal), is one of the most common monogenic diseases in China, caused by mutations of the human  $\beta$ -globin gene. To date, more than 50 different  $\beta$ -thal mutations have been reported in the Chinese population, among which the four most common mutations [IVS-II-654, *HBB*: c.316-197C>T; codon 17 (A>T), *HBB*: c.52A>T; –28 (A>G), *HBB*: c.-78A>G and codons 41/42 (–TTCT), *HBB*: c.126\_129delCTTT] (Table 1) account for around 90.0% of these cases (1–3).  $\beta$ -Thalassemia patients gradually develop severe anemia and organ injury, and regular blood transfusions are necessary to maintain their normal growth and development. Considering the high-cost and limited treatment solutions, a better choice is to prevent the birth of new  $\beta$ -thal case. And one of the most effective ways for prevention is performing a prenatal genetic test (4).

Nowadays, most of prenatal genetic tests for monogenic disease use fetal genetic material collected through invasive obstetric procedures that carry a small but significant risk of miscarriage (1.0%) (5,6). In 1997, Lo *et al.* (7) discovered the presence of fetal DNA in the maternal blood, opening the door for non-invasive prenatal diagnosis (PND) using cell-free fetal DNA (cffDNA) in maternal plasma. non-invasive prenatal testing (NIPT) for fetal sex determination, Rhesus-D (RhD) genotyping and trisomy 21 detection had been used in clinical practices (8–10). However, maternal plasma DNA exists in extremely low concentrations. Moreover, it is a mixture of maternal DNA and fetal DNA, with cffDNA only comprising around 3.0–10.0% of the total cell-free DNA (cfDNA). Moreover, half of the cffDNA are fetal of maternal origin which is difficult to discern from the background DNA of the mother due to the high level of molecular similarity between them (11). Therefore, it is difficult to test the fetal DNA in low concentration of maternal plasma DNA with interference from large background of maternal DNA. Although many methods of NIPT for  $\beta$ -thal have been reported (12–15), how to make the technique more effective has still been a challenge for NIPT of  $\beta$ -thal.

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Table 1 Different nomenclature of the four  $\beta$ -globin gene mutations found in the People's Republic of China.

$\beta$ Mutations	HGVS Nomenclature	dbSNP ID
IVS-II-654 (C>T)	<i>HBB</i> : c.316-197C>T	rs34451549
Codon 17 (A>T)	<i>HBB</i> : c.52A>T	rs33986703
–28 (A>G)	<i>HBB</i> : c.–78A>G	rs33931746
Codons 41/42 (–TTCT)	<i>HBB</i> : c.126_129delCTTT	rs80356821

Primer-introduced restriction analysis-polymerase chain reaction (PIRA-PCR) was first reported in tumor studies for detection of low frequency mutations. The PIRA-PCR combined with the restriction fragment length polymorphism (RFLP) analysis or sequencing was able to detect <3.0% mutant alleles in a background of >97.0% wild-type alleles (16,17). In this study, we developed a PIRA-PCR assay that was able to enrich and detect fetal DNA of paternal origin with one of four common  $\beta$ -thal mutations in maternal plasma. Our results showed that the mutations the proportion of which in the DNA mixture was 3.0%, can be detected after enrichment of the PIRA-PCR assay. The assay was further validated using maternal plasma DNA. The PIRA-PCR assay is a rapid, cost effective and highly accurate method that could potentially be used for non-invasive prenatal detection of paternally inherited fetal mutants and would greatly reduce the pressure of miscarriage from traditional sampling.

## Material and methods

### Sample collection

We collected peripheral blood from one normal person and four  $\beta$ -thal carriers. The carriers had different heterozygous *HBB* mutations, that were the IVS-II-654, codon 17, –28 and codons 41/42, respectively. We also recruited 10 pregnant women who carried a different *HBB* mutation from that of their husbands who carried one of the mentioned common  $\beta$ -thal mutations in the People's Republic of China (PRC). They were all at high-risk of having a fetus affected with  $\beta$ -thal and were going to receive invasive prenatal genetic testing for *HBB* mutations. Maternal peripheral blood samples were drawn from the recruited pregnant women at 12–23 weeks of gestation (Table 2); all maternal peripheral blood samples were drawn before the invasive procedure was performed. This study was approved by the Institutional Review Board of BGI-Shenzhen and the participating hospitals. Informed consent was obtained from all the participants.

### DNA isolation

Plasma was separated from maternal blood by two-step centrifugation (18) (4 °C, 1600 g centrifuge for 10 min., and then 4 °C, 16,000 g centrifuge for another 10 min. to remove residual blood cells) within 4 hours after drawing blood. Plasma samples were stored at –80 °C before they were used for DNA extraction. DNA was isolated from 1 mL plasma sample or 200  $\mu$ L) peripheral blood sample by the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Plasma DNA was eluted with 10  $\mu$ L of nuclease-free water (Promega, Madison, WI, USA) and genomic DNA (gDNA) was eluted with 30  $\mu$ L of nuclease-free water.

Table 2. Overview of the information of the mutations in each family and the results of non-invasive prenatal diagnosis that were detected by primer-introduced restriction analysis-polymerase chain reaction in maternal plasma.

Family	Members	Gestation age (weeks)	$\beta$ Genotype	Result of PIRA-PCR assay
1	Father	20	$\beta^{\text{codons 41/42}}/\beta^{\text{A}}$	$\beta^{\text{codons 41/42}}$
	Mother		$\beta^{\text{codon 43}}/\beta^{\text{A}}$	
	Fetus		$\beta^{\text{codons 41/42}}/\beta^{\text{codon 43}}$	
2	Father	17	$\beta^{\text{codon 17}}/\beta^{\text{A}}$	$\beta^{\text{codon 17}}$
	Mother		$\beta^{\text{codons 41/43}}/\beta^{\text{A}}$	
	Fetus		$\beta^{\text{codon 17}}/\beta^{\text{codons 4/42}}$	
3	Father	23	$\beta^{\text{codon 17}}/\beta^{\text{A}}$	$\beta^{\text{codon 17}}$
	Mother		$\beta^{\text{codons 41/43}}/\beta^{\text{A}}$	
	Fetus		$\beta^{\text{codon 17}}/\beta^{\text{A}}$	
4	Father	22	$\beta^{\text{codon 17}}/\beta^{\text{A}}$	$\beta^{\text{codon 17}}$
	Mother		$\beta^{\text{codons 41/43}}/\beta^{\text{codon 17}}$	
	Fetus		$\beta^{\text{codon 17}}/\beta^{\text{codons 41/42}}$	
5	Father	12	$\beta^{\text{IVS-II-654}}/\beta^{\text{A}}$	$\beta^{\text{A}}$
	Mother		$\beta^{\text{codons 41/43}}/\beta^{\text{A}}$	
	Fetus		$\beta^{\text{A}}/\beta^{\text{A}}$	
6	Father	16	$\beta^{\text{IVS-II-654}}/\beta^{\text{A}}$	$\beta^{\text{IVS-II-654}}$
	Mother		$\beta^{\text{codon 17}}/\beta^{\text{A}}$	
	Fetus		$\beta^{\text{IVS-II-654}}/\beta^{\text{codon 17}}$	
7	Father	18	$\beta^{\text{IVS-II-654}}/\beta^{\text{A}}$	$\beta^{\text{A}}$
	Mother		$\beta^{\text{codons 41/43}}/\beta^{\text{A}}$	
	Fetus		$\beta^{\text{A}}/\beta^{\text{codons 41/43}}$	
8	Father	21	$\beta^{\text{IVS-II-654}}/\beta^{\text{A}}$	$\beta^{\text{A}}$
	Mother		$\beta^{\text{codons 41/42}}/\beta^{\text{A}}$	
	Fetus		$\beta^{\text{A}}/\beta^{\text{A}}$	
9	Father	18	$\beta^{-28}/\beta^{\text{A}}$	$\beta^{\text{A}}$
	Mother		$\beta^{\text{codons 41/42}}/\beta^{\text{A}}$	
	Fetus		$\beta^{\text{A}}/\beta^{\text{codons 41/42}}$	
10	Father	18	$\beta^{-28}/\beta^{\text{A}}$	$\beta^{-28}$
	Mother		$\beta^{\text{codons 41/42}}/\beta^{\text{A}}$	
	Fetus		$\beta^{-28}/\beta^{\text{A}}$	

### DNA mixture preparation

In order to simulate the cfDNA of maternal plasma (14), gDNA was first fragmented by sonication with a Covaris S2 system (Covaris Inc., Woburn, MA, USA) using the following settings: duty cycle 10.0%, intensity 5 cycles/burst 200, time 60 seconds, 12 cycles). We then tested the sensitivity of the PCR assay with a series concentration (20, 2, 0.2 and 0.02 ng/ $\mu$ L) of fragmented DNA as template. After confirming the template concentration, we mixed fragmented wild-type gDNA from a normal person with fragmented mutant-type gDNA from  $\beta$ -thal carriers in a series of proportion (1.0, 3.0, 6.0 and 10.0%). The DNA mixtures were prepared for a simulation test.

### Primer-introduced restriction analysis-polymerase chain reaction assay

This PIRA-PCR assay is a two-step PCR procedure with an intermittent step of restriction digestion to selectively eliminate wild-type alleles. In first-step PCR, both wild-type and mutant alleles in maternal plasma DNA were amplified with the same primer pairs by which an artificial restriction site was introduced into the PCR products of wild-type alleles. After that, the PCR products were digested with corresponding restriction enzymes that was used to eliminate the wild-type alleles. Subsequently, the products digested by

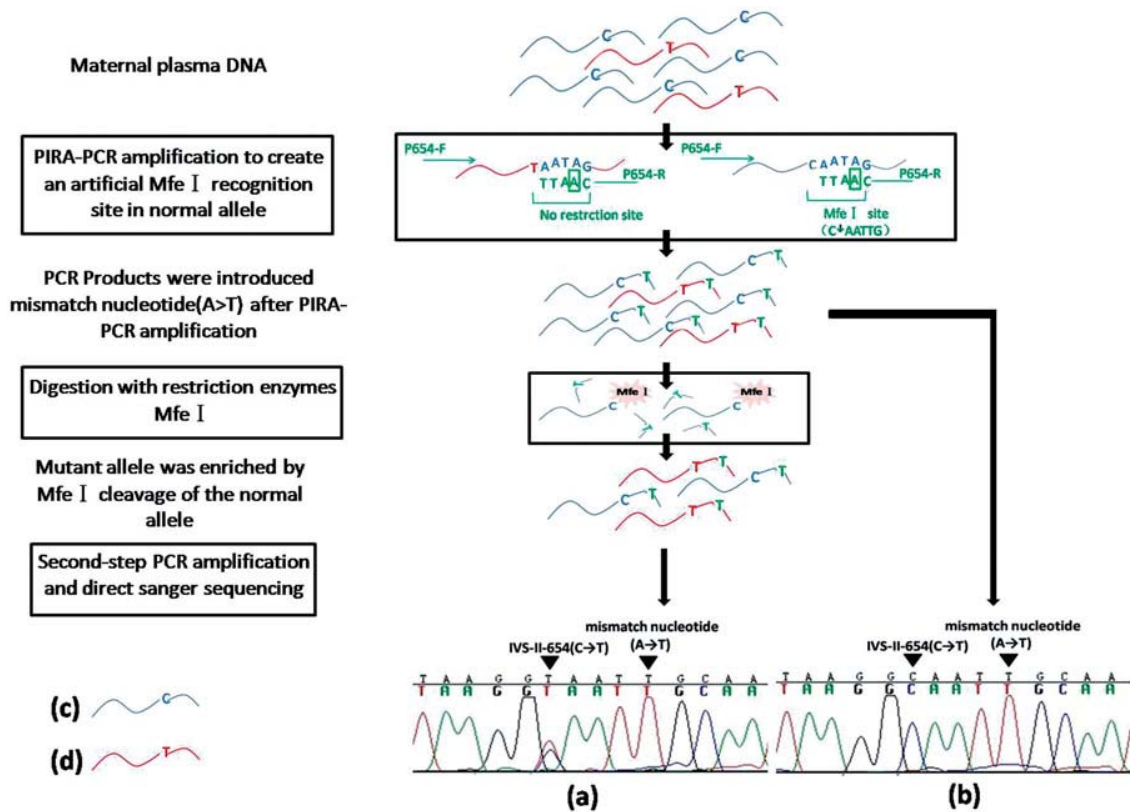


Figure 1. Schematic illustration of the PIRA-PCR method for fetal  $\beta$ -thal mutation detection in maternal DNA. The detection of the IVS-II-654 mutation was described as an example. The percentage of fetal mutation allele increased to over 50.0% in maternal DNA *via* the PIRA-PCR assay. Primer characters in frames are mismatched nucleotides T>A by artificial introduction. (a) The enrichment of the mutated fetal allele after PIRA-PCR and sequencing. (b) The result of direct PCR-sequencing before enrichment by digestion of the restriction enzyme. (c) The paternally inherited fetal IVS-II-654 mutation allele.

restriction enzymes were used as template for the second-step PCR, during which the mutation alleles were amplified. Lastly, the second-step PCR products were analyzed by direct sequencing (Figure 1).

The principle of primer design for the PIRA-PCR assay is as follows: (i) the mismatch nucleotide should not be introduced at the last two bases of the 3' terminal of the primer; (ii), the number of the mismatch nucleotide must be no more than 2; (iii), the restriction site that will be introduced by PIRA-PCR should not present in the amplified region of DNA; (iv), it is suggested that the length range of PCR product should be 100–130 bp. Based on the above principle, PIRA-PCR primers were designed to amplify the four different regions covering the IVS-II-654, codon 17, –28 and codons 41/42 mutations in the *HBB* gene separately with the use of primer premier 5 software (<http://www.premierbiosoft.com/primerdesign/index.html>). Respectively, an artificial *Mfe*I site was created to detect the IVS-II-654 mutation, *Hind*III restriction site was created to detect the codon 17 mutation, and the *Dra*I restriction site was generated to detect the –28 and codons 41/42 mutations (Table 3).

The first-step PCR reaction was performed in a 25  $\mu$ L, volume containing 9.3  $\mu$ L of plasma DNA or DNA mixtures, 2  $\times$  GC buffer (TaKaRa, Dalian, PRC), 0.2 mM dNTPs (TaKaRa), 20 pmol of each primer and 1 unit Taq polymerase (TaKaRa). After an initial denaturation at 94  $^{\circ}$ C for 2 min., the reaction was cycled for 30 seconds at 94  $^{\circ}$ C, 30 seconds at

50  $^{\circ}$ C and 15 seconds at 72  $^{\circ}$ C for 40 cycles, followed by a final extension for 5 min. at 72  $^{\circ}$ C. Subsequently, the PCR products were digested with the corresponding restriction enzyme. The restriction enzyme digestion reaction was performed in a 25  $\mu$ L volume containing 10  $\times$  NE buffer (New England Biolabs Inc., Ipswich, MA, USA), 2  $\mu$ L PCR product and 0.5  $\mu$ L restriction enzyme (NEB) at 37  $^{\circ}$ C for 2 hours. Then, the second-step PCR was performed for enrichment of the paternally inherited fetal mutant alleles. The reaction was performed in a 25  $\mu$ L final volume containing 2  $\mu$ L DNA product from the restriction enzyme digestion, 2  $\times$  GC buffer (TaKaRa Taq), 0.2 mM dNTPs (TaKaRa), 20 pmol of each primer and 1 unit Taq polymerase (TaKaRa). The reaction was initiated by 2 min. denaturation at 94  $^{\circ}$ C, followed by 22 cycles of 30 seconds denaturation at 94  $^{\circ}$ C, 30 seconds annealing at 56  $^{\circ}$ C and 15 seconds extension at 72  $^{\circ}$ C. Finally, the reaction was elongated at 72  $^{\circ}$ C for 5 min. After PIRA-PCR, the PCR products were analyzed by direct sequencing using their corresponding sequencing primers (P41/42-F, P17-F, P28-R and P654-F) with the 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

## Results

Primer-introduced restriction analysis-polymerase chain reactions were initially tested using gDNA samples with known genotypes to determine optimal conditions. After

Table 3. Sequences of all primers used for the primer-introduced restriction analysis-polymerase chain reaction assay.

$\beta$ Mutation	Primer	Primer Sequence (5' > 3')	Restriction Enzyme Site
Codons 41/42 (–TTCT)	<b>P41/42-F</b>	<b>CAC TGA CTC TCT CTG CCT ATT GG</b>	<i>DraI</i> (TTT↓AAA)
	P41/42-R	GGA CAG ATC CCC AAA GGA TTT AA	
Codon 17 (A>T)	<b>P17-F</b>	<b>TGC TTA CAT TTG CTT CTG ACA CAA CT</b>	<i>HindIII</i> (A↓AGCTT)
	P17-R	ACC AAC TTC ATC CAC GTT AAG CT	
–28 (A>G)	<b>P28-F</b>	<b>AGG AGC CAG GGC TGG GTT A</b>	<i>DraI</i> (TTT↓AAA)
	P28-R	<b>CAG ACT TCT CCT CAG GAG TCA G</b>	
IVS-II-654 (C>T)	<b>P654-F</b>	<b>CTT TCC CTA ATC TCT TTC TTT CAG</b>	<i>MfeI</i> (C↓AATTG)
	p654-R	TGC AGA AAT ATT TAT ATG CAG AGA TAT TGC AAT T	

Primer characters in frames are mismatched nucleotides for introducing restriction sites. Primers in bold (P41/42-F, P17-F, P28-R and P654-F) were used as sequencing primers for the corresponding PCR products.

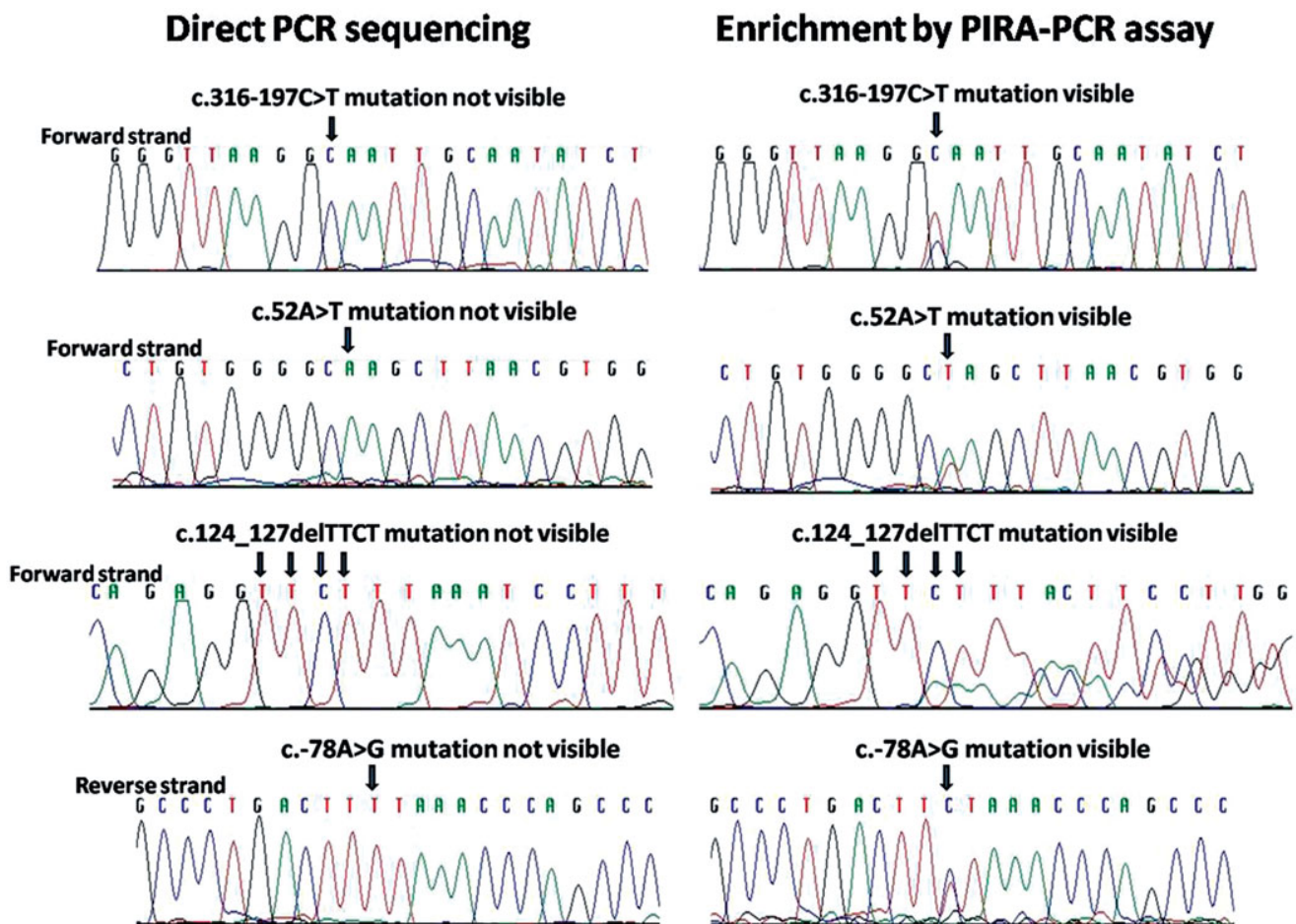


Figure 2. Sanger sequencing analyzes the ability of enrichment of PIRA-PCR. DNA containing heterozygous mutations in *HBB* genes was mixed with wild-type DNA in a ratio 3–97 and amplified by PIRA-PCR. Direct PCR-sequencing was also performed on the same samples for comparison.

optimization, we began to test the sensitivity of the PIRA-PCR assay. The initial concentration of template DNA for PCR was 20 ng/ $\mu$ L; then it was diluted from 1:10 to 1:1000. The allele of interest could be stably detected at 1:100, suggesting that the sensitivity of PIRA-PCR was about 0.2 ng/ $\mu$ L. Then, we tested the specificity of PIRA-PCR reactions using the gDNA mixtures. The DNA mixtures were diluted to 0.2 ng/ $\mu$ L with the proportion of mutant allele ranging from 1.0 to 10.0%. The mutant allele could be stably enriched and detected at the minimum proportion of 3.0%. The results suggested that the specificity of PIRA-PCR was

about 3.0%. After the enrichment of PIRA-PCR, the proportion of the mutant allele was increased from 3.0% to over 50.0%, which can be easily detected by Sanger sequencing (Figure 2).

The feasibility of the PIRA-PCR assay detecting the paternally inherited fetal mutations for  $\beta$ -thal was tested using real plasma samples. Maternal plasma samples from 10 pregnant women were collected after written consent was obtained from the participants. All pregnant women carried a different mutation from that of their husbands who carried one of the four common  $\beta$ -thal mutations. Among these cases,

one male parent carried the -28 mutation, four male parents carried the codon 17 mutation, one male parent carried the codons 41/42 deletion and four male parents carried the IVS-II-654 mutation. The PIRA-PCR detection showed that six fetuses inherited the paternal mutant allele, and four fetuses inherited the paternal wild-type allele (Table 2). The results from the PIRA-PCR analysis were confirmed by conventional PND.

## Discussion

The discovery of cfDNA in maternal plasma offered the possibility of non-invasive prenatal tests. However, cfDNA only account for 3.0-10.0% of the total cfDNA in maternal plasma, although some reported more than 10.0%, the majority of cfDNA is maternal (10,11). Removing maternal DNA background interference is important for accurate analysis of fetal DNA when studying maternal cfDNA from pregnant women. So, fetal DNA enrichment is desired (19). In this study, we developed a PIRA-PCR assay to enrich fetal DNA of paternal origin. The detection sensitivity of the PIRA-PCR assay can reach 0.2 ng/ $\mu$ L. For specificity of the assay, the mutant alleles, the proportion of which in the huge background of wild-type alleles was as low as 3.0%, could be well detected. Both sensitivity and specificity of the PIRA-PCR assay fulfill the requirement of NIPT for paternally inherited fetal-specific mutant alleles in maternal plasma (20,21). Furthermore, in our study, the accuracy of the PIRA-PCR assay for detection of paternally inherited fetal mutations in maternal plasma was 100.0% for all 10 cases. However, because our method is based on an exclusion criterion, it is necessary to incorporate a supplementary independent test to validate the presence of the fetal DNA in the maternal plasma sample to eliminate the risk of false negatives for clinical application.

Compared with other techniques (11-14) for NIPT of paternally inherited fetal mutations for  $\beta$ -thal, the PIRA-PCR assay has several advantages. First, the whole process of enriching the paternally inherited fetal mutant allele only needs two common PCRs and one enzyme digestion reaction, without the need of sophisticated and costly equipment. And it can be completed within 5 hours. Furthermore, the PIRA-PCR assay is a relatively easy and rapid method with high sensitivity and specificity. And its costs are relatively low. Therefore, it would be easily adopted by clinical diagnostic laboratories. To the best of our knowledge, this is the first report that evaluated the feasibility of PIRA-PCR used for non-invasive PND.

The major drawback of this method is that it can only be used for testing paternally inherited fetal mutations. Moreover, it was not applicable when both parents carry the same mutation which account for around 40.0% of at-risk couples. Furthermore, not every mutation could be detected using this method because of the limited kinds of restriction enzymes.

In conclusion, this study has shown that the PIRA-PCR assay is able to detect paternally inherited fetal-specific  $\beta$ -thal mutations in maternal plasma from an at-risk pregnancy. If a paternally inherited fetal mutation was detected, an invasive procedure was still required to check whether the fetus also inherited the maternal mutation. However, the clinical application of this test may potentially reduce a large number

of invasive diagnoses and avoid related miscarriages. This approach might also be applicable for other genetic disorders.

## Declaration of interest

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