Noninvasive prenatal screening test for compound heterozygous beta thalassemia using an amplification refractory mutation system real-time polymerase chain reaction technique

Narutchala Suwannakhon,[1] Tanapat Pangeson,[2] Teerapat Seeratanachot,[2] Khwanruedee Mahingsa,[3] Arunee Pingyod,[3] Wanwipa Bumrungpakdee,[3] Torpong Sanguansermsri[3]

[1]Discipline of Biology, School of Science, [2]Discipline of Biochemistry, School of Medical Science, [3]Thalassemia Unit, University of Phayao, Thailand

Abstract

We propose using a modified amplification refractory mutation system real-time polymerase chain reaction (ARMS RT-PCR) technique to exclude the invasive prenatal diagnosis for a non-paternally inherited beta thalassemia mutation in couples at-risk for having a baby with CHBT. The ARMS RT-PCR method was performed for 36 at-risk couples by using isolated fetal cell-free DNA from maternal plasma. The modified ARMS RT-PCR primers targeted one of the following paternally inherited beta thalassemia mutation: -28 A→G, CD17 A→T, CD 26 G→A, IVS1-1 G→T and CD 41-42 -CTTT. The method could be successfully employed for NIPST starting with the 7th week of gestation. The results showed that 19 pregnant women were negative for PIBTM (53%). After an on-track and on-time of one year, including postnatal thalassemia blood tests, none of the babies showed symptoms or signs of beta thalassemia disease. We concluded that the modified ARMS RT-PCR method was an accurate, cost-effective and feasible method for use as a NIPST for at-risk couples with the potential of having a baby with CHBT.

Introduction

Compound heterozygous beta thalassemia (CHBT) occurs in live births and is the most common form of severe beta thalassemia disease in Southeast Asian countries. It is a single autosomal genetic disorder with a prevalence rate of 1-9%.[1] CHBT disease originates from inheriting combination of two different mutated beta globin genes, either $\beta^0\beta^0$ or $\beta^0\beta^+$ thalassemia. Both types cause serious hereditary hematological diseases that manifest through progressive deterioration of chronic anemia and iron overload. The optimal treatments are an economic burden. Therefore, prenatal diagnosis is necessary to prevent the birth of babies with these conditions. Current prenatal diagnostics use invasive methods, such as chorionic villus sampling, amniocentesis and cordocenthesis, which have a risk of fetal loss. Fortunately, the discovery of cell-free fetal DNA in maternal plasma has provided options for noninvasive prenatal testing.[2] To date, there are many molecular methods available for the noninvasive prenatal testing of paternally inherited beta thalassemia mutation (PIBTM) in maternal plasma.[3] However, some of these methods require sophisticated biochemical techniques to obtain highly accurate results and are not readily available for use as routine screening tests. Our approach focuses on the development of a noninvasive prenatal screening test (NIPST) to reduce the use of invasive prenatal testing in the non-paternally inherited at-risk fetus.

Materials and Methods

From 2016 to 2018, 36 couples at-risk for having a baby with CHBT volunteered to participate in the NIPST program. The following paternal alleles were identified, which for both partners were one or two of the following mutations: -28 A→G, CD17 A→T, CD 41-42 CTTT, IVS1-1 G→T and CD26 G→A. The at-risk couples were from the Antenatal Care Unit, Provincial Phayao Hospital, Phayao, Thailand. The fetal age at the time of the study was 7-24 weeks. Ethical approval was obtained from the Medical Ethics Committee of Phayao University (3/011/59). Informed and written consent were collected from the pregnant women and their spouses before blood collection.

Workflow

The plasma cell-free DNA from 20 mL of fresh whole blood of each individual was isolated. The modified amplification refractory mutation system (ARMS) RT-PCR methods were validated using paternal plasma cell-free DNA for the proper fragment size and melt temperature specificity. The isolated maternal plasma cell-free DNA was tested for the presence or absence of the PIBTM using the modified ARMS RT- PCR method. All pregnant women were counselled before undergoing prenatal diagnosis.

The PIBTM negative pregnant women did not undergo invasive PND. After birth, the babies had a blood test for thalassemia. All attended a one year of clinical thalassemia follow up at the Well Baby Clinic of the Provincial Phayao Hospital.

Design of specific ARMS RT- PCR primers

All primers were designed using the Primer-Blast program (https://www.ncbi.nlm.nih.gov/tools/ primer-blast/). The U01317.1 (*HBB gene*; nucleotide number 62137-63472) DNA sequence of the *HBB* gene was derived from NCBI's GenBank. Five ARMS real-time PCR primer pairs were designed for targeting the CD41/42 –

Correspondence: Narutchala Suwannakhon, Discipline of Biology, School of Science, University of Phayao, Phayao, Thailand.
Tel.: +66(0)810296551 - Fax: +66(0)54466724.
E-mail: narutchala@gmail.com

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Contributions: NS designed the experiments, developed the methods, analyzed the data and wrote the paper. TS and TP developed the methods and analyzed the data. KM, AP, WB performed experiments. TS advised the experiments and wrote the paper. All authors reviewed and approved the final manuscript.

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CTTT, CD26 G→A (HbE trait), CD17 A→T, -28 A→G and IVS1-1 G→T and they were modified according to the allele-specific priming principle for ARMS RT-PCR.[4,5] This allowed for the detection of short DNA segments ranging between 60 and 120 base pairs.[6] All primers were purchased from the Invitrogen Corporation (Carlsbad, CA, USA). Each primer pair was validated by real-time PCR against the paternal beta thalassemia mutation in plasma cell-free DNA (Table 1).

Isolation of cell-free DNA in plasma

Twenty ml of an EDTA blood sample was separated into plasma fraction using standard procedures. Cell-free DNA was extracted using the QIAamp Circulating Nucleic Acid kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Assessing the PIBTM in maternal plasma

The ARMS RT- PCR master-mix (25 µL total reaction) contained 2.5 µL of 10x PCR buffer, 0.75 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 1.0 µL of 5 mM primer, 1.0 µL of 50 mM SYTO9, 0.2 µL of 5 units/µL Platinum *Taq*, 9.05 µL of dH_2O and 10 µL of DNA solution. The ARMS real-time PCR was performed to identify the PIBTM in maternal plasma cell-free DNA. The paternal and maternal cell-free DNA from the at-risk couples was simultaneously assessed. Thermal cycling was performed using a Bio-Rad CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA, USA) starting with an initial *Taq* DNA polymerase activation step at 94°C for 2 minutes. The complete PCR underwent 44 cycles. Each cycle consisted of DNA denaturation at 94°C for 10 seconds, annealing at 64°C for 10 seconds and extension at 72°C for 10 seconds. The fluorescence activity was measured on a SYBR Green channel (533 nm) at the end of each cycle. The amplification cycle and melt peak evaluation was performed using the Bio-Rad Precision Melt Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA). The melting program started at 95°C for 10 seconds, followed by a melting cycle from 75°C to 90°C at a transitional rate of 0.2°C every 10 seconds.

Postnatal thalassemia diagnosis

The babies of at-risk couple who participated in the NIPST program were requested for blood test to evaluate their thalassemia status and clinical physical examination at the time of the first-year compulsory immunization. Blood test for beta thalassemia diagnosis was assessed by hemoglobin separation and quantification of hemoglobin types were, according to the manufacturer's instructions using the capillary electrophoresis system (SEBIA, France).[7] The beta thalassemia mutation was carried out using high resolution DNA melting analysis and by direct DNA sequencing technique.[8]

Results

The *HBB gene, DNA* sequences and nucleotides from the five developed series of ARMS RT-PCR primers were shown in Table 1. The CD41/42 -CTTT ARMS RT-PCR primers were used to detect the paternally inherited beta thalassemia mutation from CD41/42 -CTTT carriers. The detected DNA fragment was 73 base pairs, and was identified by a specific melt peak at 82.00±0.25°C (Figure 1). The CD26 G→A ARMS real-time PCR primers were for the hemoglobin E trait, which had a 111 base pair DNA fragment and a melt peak at 84.7±0.30°C. The CD17 A→T ARMS real-time PCR primers were for the CD17 A→T carrier. The DNA fragment size was 69 base pairs and the melt peak was at 80.93±0.23°C. The -28 A→G and IVS1-1 G→T ARMS real-time PCR primers were for the -28 A→G and IVS1-1 G→T traits, respectively; these primer pairs detected 84 and 61 base pair DNA fragments, with melted peaks at 83.6±0.00 and 78.87±0.12°C, respectively. The paternal cycle threshold (Ct) for the beta thalassemia mutations in paternal plasma cell-free DNA was 30±2.80 cycles (N=10), while the cycle threshold for paternally inherited cell-free fetal DNA in maternal plasma was 35±2.10 cycles (N=17). Nineteen of the at-risk pregnancies using specific ARMS real-time PCR primers were negative for PIBTM. After birth, based on the postnatal thalassemia blood tests, the babies either inherited beta thalassemia maternally and carriers or homozygous wild type alleles (Table 2). While, there were 17 out of 36 pregnancies that were positive for PIBTM. After amniocentesis 5 fetuses were shown to have compound heterozygous beta thalassemia (Table 3). In the pregnant women, the paternal allele was detected at a fetal age of seven weeks.

Discussion

The discovery of fetal DNA in maternal plasma has allowed for many approaches for noninvasive prenatal diagnosis. There are examples showing concentrations of total plasma DNA, ranging from 3.4 to 6.2% during early and late pregnancy. Cell-free fetal DNA can be readily detected as early as the 7th week of gestation [2]. The size of cell-free fetal DNA fragments in maternal plasma had been reported as ranging from 193-313 base pairs.[6,9] All of these discoveries are very important factors to consider for the successful development of ARMS RT-PCR primers. Fortunately, the 36 at-risk couples recruited were known to be carriers of the 5 common beta thalassemia mutations, specifically CD17 A-T, CD41-42-CTTT, IVS1-1 G-T, -28 A-G and

Table 1. The DNA sequence primers, DNA fragment size and melt peak temperature of the modified ARMS RT- PCR using for the detection of the paternally inherited beta thalassemia mutations.

Primers	Nucleotide sequence (5'->3')	PCR size (base pairs)	Melt peak (°C)	NCBI GenBank (U01317.1)
F- -28	GGTTGGCCAATCTACTCCCA			62055-62074
R- -28	GTAAGCAATAGATGGCTCTGCCCTGACGTC	84	83.6±0.00	62109-62138
F-CD17	AGAAGTCTGCCGTTACTGCC			62209-62228
R-CD17	CTCACCACCAACTTCATCCACGTTCAGCTA	69	80.93±0.23	62238-62267
F-CD26	ACCATGGTGCACCTGACTC			62184-62202
R-CD26	TAACCTTGATACCAACCTGCCCAGGGCATT	111	84.70±0.30	62265-62294
F-IVSI-I	TGGATGAAGTTGGTGGTGAGG			62248-62268
R-IVSI-I	TTAAACCTGTCTTGTAACCTTGATACCGAA	61	78.87±0.12	62278-62308
F-CD41-42	TTTTCCCACCCTTAGGCTGCT			62394-62414
R-CD41-42	GAGTGGACAGATCCCCAAAGGACTCAACCT	73	82.00±0.25	62437-62470

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the hemoglobin E mutation (CD26 G-A) (Tables 2 and 3). In this study, 19 of 36 at-risk pregnancies showed negative results for PIBTM. When notified about the results of the mother's blood test, most of the mothers chose to not undergo additional checks, such as invasive prenatal diagnosis.[10-12] Currently, there are many techniques for detecting paternally inherited beta thalassemia mutations, including next-generation sequencing,[13] COLD-PCR and microarrays,[14] next-generation sequencing of SNPs,[15] TaqMan genotyping assay,[16] droplet digital PCR[17] and simple fetal DNA enrichment with allele-specific real-time PCRs.[18-22] The ARMS RT- PCR technique was simple, accurate, cost-effective, and able to detect paternally inherited beta thalassemia mutations directly from cell-free DNA in maternal plasma. The method was successfully employed for noninvasive prenatal screening tests in at-risk couples starting from the 7th gestational week. The remaining 17 pregnancies whose PIBTM was positive required further investigation of the fetus for a maternal inherited beta thalassemia mutation (Table 3). In the meantime, methods for the detection of maternally inherited beta thalassemia mutation of the at-risk fetus remained in the experimental stage. Some of these methods, namely, droplet digital PCR, may be available in the near future for use in noninvasive prenatal testing.[23,24]

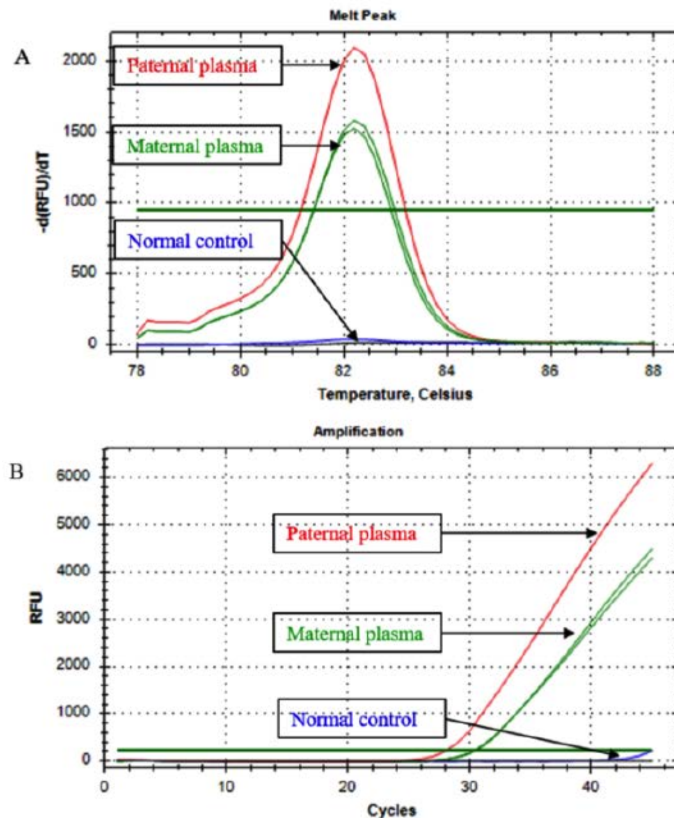

Figure 1. Image of the PIBTM melt peak and cycle threshold of the CD41/42-CTTT using specific ARMS RT- PCR primers. The paternal and the paternally inherited CD41/42-CTTT melt appearance were similar and were at the same temperature at 82.00±0.25°C (A). The paternal cycle threshold for the beta thalassemia mutations in paternal plasma and the cycle threshold of the paternally inherited cell-free fetal DNA in maternal plasma (B).

Table 2. Negative cases of the NIPST using modified ARMS RT- PCR for the detection of PIBTM in maternal plasma cell-free DNA in the at-risk couples.

Couple No.	Maternal mutation	Paternal mutation	Gestation age (week)	Paternal ARMS test	ARMS results	Postnatal diagnosis
1	CD17 A→T	CD26 G→A	16	CD26	NEG	CD17 trait
2	IVS1-1 G→T	CD26 G→A	16	CD26	NEG	Normal
3	-28 A→G	CD26 G→A	10	IVS1-1	NEG	-28 trait
4	CD17 A→T	CD26 G→A	10	CD41/42	NEG	Unaffected
5	CD26 G→A	CD41/42 –CTTT	18	CD41/42	NEG	Normal
6	CD26 G→A	CD41/42 –CTTT	11	CD41/42	NEG	Unaffected
7	CD41/42 –CTTT	-28 A→G	18	CD17	NEG	Unaffected
8	CD17 A→T	CD26 G→A	16	CD41/42	NEG	CD17 trait
9	CD17 A→T	CD26 G→A	19	CD17	NEG	Normal
10	CD41/42 –CTTT	CD17 A→T	13	-28	NEG	CD41/42 trait
11	CD26 G→A	CD41/42 –CTTT	15	CD26	NEG	CD26 trait
12	IVS1-1 G→T	CD26 G→A	16	CD26	NEG	Normal
13	CD41/42 –CTTT	CD26 G→A	16	CD26	NEG	CD41/42 trait
14	CD41/42 –CTTT	CD26 G→A	14	CD26	NEG	Normal
15	CD17 A→T	CD41/42 –CTTT	14	CD26	NEG	Normal
16	CD41/42 –CTTT	CD26 G→A	10	CD41/42	NEG	Normal
17	CD17 A→T	CD26 G→A	7	CD26	NEG	CD17 trait
18	CD26 G→A	IVS1-1 G→T	8	CD26	NEG	CD26 trait
19	CD26 G→A	CD41/42 –CTTT	10	CD26	NEG	CD26 trait

NEG, Negative result; POS, Positive results; Unaffected baby did not determine thalassemia genotype.

Table 3. The results of the cycle threshold value of positive cases of NIPST using modified ARMS RT-PCR for the detection of PIBTM in the at-risk pregnancies.

Couple No.	Maternal mutation	Paternal mutation	Gestation age (week)	Paternal ARMS test	ARMS results	Postnatal diagnosis	Invasive prenatal diagnosis results
1	CD26 G→A	CD17 A→T	21	CD17	POS	37	CD17 trait
2	CD41/42 –CTTT	CD26 G→A	17	CD26	POS	34	CD26/CD41/42
3	CD26 G→A	-28 A→G	10	-28	POS	35	CD26/-28
4	CD17 A→T	CD26 G→A	9	CD26	POS	36	CD26 trait
5	CD26 G→A	CD41/42 –CTTT	24	CD41/42	POS	30	CD41/42 trait
6	CD26 G→A	IVS1-1 G→T	19	IVS1-1	POS	33	IVS1-1 trait
7	CD26 G→A	CD41/42 –CTTT	21	CD41/42	POS	33	CD41/42 trait
8	CD26 G→A	CD41/42 –CTTT	11	CD41/42	POS	34	CD41/42 trait
9	CD26 G→A	CD41/42 –CTTT	12	CD41/42	POS	37	CD41/42 trait
10	CD17 A→T	-28 A→G	11	-28	POS	36	CD17/-28
11	CD41/42 –CTTT	Homozygous CD26 G→A	12	CD26	POS	36	CD26 trait
12	CD26 G→A	CD41/42 –CTTT	12	CD41/42	POS	37	CD26/CD41/42
13	CD17 A→T	CD26 G→A	15	CD26	POS	38	CD26 trait
14	CD26 G→A	CD41/42 –CTTT	9	CD41/42	POS	35	CD26/CD41/42
15	CD26 G→A	CD41/42 –CTTT	8	CD41/42	POS	36	CD41/42 trait
16	CD26 G→A	CD17 A→T	7	CD17	POS	35	CD17 A→T trait
17	CD26 G→A	IVS1-1 G→T	16	IVS1-1	POS	31	IVS1-1 trait

NEG, Negative result; POS, Positive results.

Conclusions

We concluded that the modified ARMS RT- PCR technique could be used as a NIPST that can allow greater than 50% of couples at risk having a baby with CMBT to omit invasive prenatal testing.

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