Fast-track strategy for the prevention of Hb Bart's hydrops fetalis syndrome

Narutchala Suwannakhon,¹
Khajohnsilp Pongsawatkul,²
Teerapat Seeratanachot,³
Wirawan Rasri,²
Khwanruedee Mahingsa,⁴
Arunee Pingyod,⁴
Wanwipa Bumrungrakdee,⁴
Torpong Sanguansermstri⁴

¹Discipline of Biology, School of Science, University of Phayao, Phayao; ²Division of Obstetrics and Gynecology, Phayao Hospital, Phayao; ³Discipline of Biochemistry, School of Medical Science, University of Phayao, Phayao; ⁴Thalassemia Unit, University of Phayao, Phayao, Thailand

Abstract

We propose a fast-track strategy [direct blood DNA analysis using a quantitative real-time polymerase chain reaction (PCR) technique] for the early risk detection and prenatal diagnosis of $\alpha(0)$ -thalassemia (SEA and Thai deletion). Blood DNA samples were obtained from a volunteer group of 1235 ANC couples. They were assessed using quantitative real-time PCR to detect carriers of $\alpha(0)$ -thalassemia (SEA and Thai deletion). At-risk couples were identified, and further prenatal diagnosis by amniocentesis was implemented. Fetal DNA was isolated from the amniotic cells and characterized by quantitative real-time PCR to detect the $\alpha(0)$ -thalassemia mutation, which was reconfirmed using the droplet digital PCR method. Fifteen at-risk couples were identified. The timing of prenatal diagnosis was appropriate for all couples and four of the fetuses were diagnosed with Bart's hydrops fetalis. The results were compatible with those calculated using the Hardy-Weinberg equation for a recessively inherited single gene disorder. The conclusion was that the fast-track strategy could shorten screening policy timelines, promoting early risk detection for couples and early prenatal diagnosis. The fast-track strategy might be beneficial for the prevention of hemoglobin Bart's hydrops fetalis syndrome.

Introduction

Hemoglobin Bart's hydrops fetalis syn-

drome is the most severe form of α -thalassemia and is almost always lethal. This syndrome is primarily associated with homozygous $\alpha(0)$ -thalassemia (α^0 -thalassemia). It is sporadically reported in all Southeast Asian countries with a prevalence of 1-3 cases per thousand pregnancies. An affected fetus succumbs within the uterus 6 to 8 month gestation, and the mother may develop fatal postpartum complications. To avoid serious and unpredicted maternal risk, early prenatal diagnosis (PND) and pregnancy interruption is often necessary.¹⁻³

In northern Thailand, the SEA type of α^0 -thalassemia deletion carrier can be detected in as much as 15% of the population, whereas the Thai type of α^0 -thalassemia deletion carrier was detected in less than 0.1%. Unfortunately, the reported success rate of the major thalassemia disease prevention strategy was less than 50%, despite the full support of the program. The clinical histories of mothers with affected fetuses with hemoglobin Bart's hydrops fetalis syndrome indicated that a major area of failure in the prevention of this disorder is due to unsatisfactory local policies.^{4,5}

To enhance and improve the process of at-risk couple identification, a quantitative real-time PCR technique (fast-track strategy) for the detection of α^0 -thalassemia (SEA and Thai deletion) carrier was applied to all pregnant women and their spouses. The modified strategy might improve the early risk detection and early PND.

Materials and Methods

After approval was received from the institutional Research Ethics Committee, blood samples were collected from the volunteer pregnant women and their spouses from the antenatal care clinic at the Phayao Provincial Hospital in Thailand. Recruitment was carried out from January 2015 to August 2016, and included 1235 pregnancies. The average fetal age at the time of registration was 10 ± 3 weeks.

Workflow

Three ml of blood was collected from each volunteer and transferred to the Thalassemia Unit, University of Phayao to detect carriers of α^0 -thalassemia (SEA and Thai deletion). Blood DNA isolation was carried out following the Chelex method with some minor modifications.⁶ Each DNA sample was tested for α^0 -thalassemia (SEA and Thai deletion) by multiplex real-time PCR. Couples at risk of having a fetus with hemoglobin Bart's hydrops fetalis syndrome were counseled and subject to prena-

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

Key words: Fast-track diagnosis; $\alpha(0)$ -thalassemia (SEA and Thai deletion) carriers; prevention of Hb Bart's hydrops fetalis syndrome.

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tal diagnosis by amniocentesis.⁷ The fetal DNA was isolated from amniotic cells. The fetal α^0 -thalassemia mutation was identified by multiplex real-time PCR and was confirmed by the droplet digital PCR (ddPCR) technique.⁸

Blood DNA extraction (Chelex method)

Blood (50 μ L) was mixed with 1 ml of 0.5% Triton X-100 in a 1.5 ml tube, vortexed and then centrifuged at 14,000 RPM for 1 min. The supernatant was removed, and a volume of approximately 20 μ L was recovered. Water (1 ml) was added, and the sample was vortexed and centrifuged, and approximately 20 μ L supernatant was recovered. Subsequently, 300 μ L 10% Chelex suspended in water (Bio-Rad Laboratories, Hercules, CA, USA) and 110 μ L of water were added to the sample. The mixture was incubated at 56°C for 1 h or overnight. After boiling for 5 min, the supernatant that contained the extracted DNA was collected and stored at -20°C until analysis.

Quantitative real-time PCR

For the diagnosis of the SEA and Thai deletions, multiplex quantitative real-time PCR primers and condition were performed in according to the previous protocol.^{7,9} In brief, the PCR mixtures (25 µL) included 5 µl of DNA, 1X PCR buffer, 1.5 µM MgCl₂, 200 µM of each dNTP, 2 µM of SYTO9, 1 unit of Platinum® *Taq* DNA polymerase, and 0.2 µM of each primer of P1, P2, P3, TF, and TR. Thermal cycling was performed on the platform of a Bio-Rad CFX96 real-time system (Bio-Rad Laboratories, California, USA) beginning at 94°C for two minutes to activate the *Taq* DNA polymerase, followed by 40 cycles of denaturing at 94°C for 15 s, annealing at 64°C for 15 s, and extension at 72°C for 20 s. Fluorescence was measured on the SYBR channel (533 nm) at the end of each cycle.

Droplet digital PCR

The method was applied to diagnose fetuses at risk for hemoglobin Bart's hydrops fetalis syndrome. Genomic DNA was isolated from the amniotic cells according to the manufacturer's protocol using the QIA-amp DNA Mini Kit (QIAGEN, Hilden, Germany). The isolated DNA was diluted with 1 x PCR buffer to 5 ng/µl and stored at -20°C until analysis. The gene quantification of the wild-type and SEA genes were analyzed according to the manufacturer's protocol using the Bio-Rad QX100 Droplet Digital PCR system. The workflow included preparation of the PCR reaction mixture (20 µl) by combining ddPCR 2X PCR Master Mix, 20X Primers (P1, P2 and P3), Taqman Probe Mix (PIP2 probe and PIP3 probe, Applied Biosystems, USA) (Table 1) and 5 µl of the DNA sample. The combination of the PCR reaction mixture and oil were generated in a droplet generator. The ddPCR droplet samples were transferred to a PCR plate and were amplified in a Bio-Rad CFX96 real-time system (Bio-Rad Laboratories, California, USA). After PCR amplification, the PCR plate was placed in a QX100 droplet reader. The droplet reader and QuantaSoft software enumerate the PCR-positive and PCR-negative droplets to provide absolute quantification of target DNA.

Results

There were 1,235 couples enrolled in the fast-track strategy to prevent α^0 -thalassemia-thalassemia. Three patterns were detected in the α^0 -thalassemia (SEA and Thai deletion) carriers by real-time PCR. The control wild-type DNA revealed a single peak with a melting point of 92.4°C,

Figure 1. Here shows chromatograms using the quantitative real-time PCR. The control wild type DNA show only an image of single melt peak with a melting point of 92.4 °C (A) while SEA and Thai trait demonstrated the double melt peaks with melting points of 87.4 °C with 92.4 °C (B) and 85.4 °C with 92.4 °C (C), respectively.

Table 1 Sequence and location of the ddPCR primers and Taqman probes.

Name	Nucleotides (5'→3')	NCBI ref. Number
Primer (P1)	GTCGTCCTCCACTGTCGTC	Z84721, 26202-19
Primer (P2)	ACGCCGTCCGACTCAAGGA	Z84721, 26334-53
Primer (P3)	GGCTTACTGCAGCCTTGAAC	Z69706, 2647-67
PIP2 probe	FAM-TCT AGC CCC TGA GCA CCG- MGBNFQ	-
PIP3 probe	VIC-CTC CAA GTG AAC CTC C-MGBNFQ	-

while the SEA and Thai traits demonstrated double peaks with melting points of 87.4°C and 92.4°C, or 85.4°C and 92.4°C, respectively (Figure 1). The melt peaks images and melting temperatures (T_m) were specifically for carriers of the α^0 -thalassemia SEA and Thai deletion. The application of the droplet digital PCR technique for the PND of α^0 -thalassemia show three types of droplets detected, namely droplets with FAM- fluorescence, droplets with VIC- fluorescence and droplets with both FAM and VIC fluorescence. The average amount of FAM (indicative of the wild-type gene) detected in a normal subject was 209 copies/ μ L, while the amount of VIC (indicative of Hb Bart's hydrops fetalis fetus) detected was 226 copy/ μ L, and the count for the SEA trait (FAM and VIC) was 237:255. The image of the two-dimension chromatogram, generated using the QuantaSoft program, revealed specific patterns for a normal fetus, α^0 -thalassemia (SEA deletion) carrier and Hb Bart's hydrops fetalis fetus (Figure 2).

Of the 1235 couples, 15 couples were at risk of having a fetus homozygous for α^0 -thalassemia (SEA deletion). All couples underwent PND by amniocentesis. The PND was assessed by the droplet digital PCR technique and revealed 4 cases that were homozygous for α^0 -thalassemia (SEA deletion). Of the other cases, 3 were normal and 8 were SEA deletion carriers. The PND diagnosed by quantitative real-time PCR of all 15 cases were concordant with droplet digital PCR analysis. The prevalence of the α^0 -thalassemia SEA deletion carrier calculated using the Hardy-Weinberg equation for a recessively inherited single gene disorder was 11%.

Discussion

The prevalence of α^0 -thalassemia deletion carriers (involving a deletion of the duplicated α -globin genes) is in the average of 5% (3 to 12 %) but may be as high as 15% in some ethnic of northern Thailand. This indicates that the 8-16 of every 1000 pregnant women are at risk of having a fetus with Hb Bart's hydrops fetalis.^{2,10-12} In this study, the prevalence of the α^0 -thalassemia SEA deletion carrier was 11%. Homozygous α^0 -thalassemia is often fatal in utero due to severe hydrops fetalis. Although in utero transfusions are increasingly used to allow fetal survival in Hb Barts hydrops fetalis syndrome, however the management of pre- and postnatal outcomes are not well established.^{13,14} To avoid the severe toxemia complications associated with hydropic fetuses, early prenatal diagnosis is necessary. Since 2005, the con-

ventional thalassemia prevention program has been fully established and supported. However, the surveillance reported after 5 years of the prevention program revealed the success rate was 50% lower than expected. Meanwhile, sporadic cases of the full-blown Hb Bart's hydrops fetalis syndrome have been reported every year. Furthermore, evidence the controlled tha-

lassemia prevention program study of the conventional thalassemia prevention program within the past year revealed the success rate was approximately 30% (one affected in 1127 expectants). There are many factors contributing to the ineffectiveness. The major factor might be related to the complexity of the program activity, which requires various groups of special-

Figure 2. Here shows the image of the two-dimension chromatograms, generated using the QuantaSoft program, revealed specific patterns for a normal fetus (A), α^0 -thalassemia (SEA deletion) carrier (B) and Hb Bart's hydrops fetalis fetus (C).

ists, including a thalassemia counselor, CBC-lab technologist, molecular technologist, a hematologist and an obstetrics/gynecology. An abnormal sample will be examined by all these specialists. The turnaround time for recruiting an at-risk couple might be prolonged by 8-10 weeks, which could affect PND by CVS or amniocentesis. Moreover, co-inheritance of β -thalassemia trait with the α^0 -thalassemia trait has a significant effect on the red cell indices, particularly the MCV and MCH, which may be normalized. In such a case, the α^0 -thalassemia trait might escape further evaluation.¹⁵

On the other hand, the fast-track strategy uses real-time PCR technology to assess α^0 -thalassemia (SEA and Thai deletion) carrier directly from the blood DNA of pregnant couples. The quantitative real-time PCR technique used for the identification of α^0 -thalassemia (SEA and Thai deletion) carriers is a simple molecular technology. The method is highly accurate and allows for the rapid detection of at-risk couples; 96 to 384 samples could be assessed within one day. Therefore, an early at-risk detection and prenatal diagnosis are possible. In this study, the fast-track strategy was applied to 1235 volunteer expectant couples. Fifteen at-risk couples were identified and were informed within one week. The strategy developed was applicable for the thalassemia counselors. All at-risk women were satisfied with the service and requested more PND.

Conclusions

The fast-track strategy could shorten screening policy timelines, promoting early at-risk detection and prenatal diagnosis. The fast-track strategy might increase the feasi-

bility of α^0 -thalassemia carrier screening, which might be beneficial in the prevention of hemoglobin Bart's hydrops fetalis syndrome.

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