Analysis of Real-Time PCR Cycle Threshold of α-Thalassemia-1 Southeast Asian Type Deletion Using Fetal Cell-Free DNA in Maternal Plasma for Noninvasive Prenatal Diagnosis of Bart's Hydrops Fetalis

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Background: *Noninvasive prenatal diagnosis based on detection of fetal cell-free DNA is hampered when mother and father are both carriers for the same autosomal recessive mutation.*
Objective: *To compare the diagnosis of Bart's hydrops fetalis using conventional Gap-PCR analysis of fetal cells/tissues with the measurement of quantitative difference (ΔC_T) between α-thalassemia-1 SEA type deletion gene ($C_{T\text{-}mutant}$) and wild type α-globin gene ($C_{T\text{-}wild\ type}$) in plasma of pregnancies by using the Taqman real-time quantitative PCR.*
Material and Method: *Plasma DNA samples were collected from three groups of pregnancies whose fetuses have known thalasemia status (7 normal, 11 heterozygote α-thalassemia-1 SEA type deletion, and 7 Bart's hydrops fetalis). The α-thalassemia-1 SEA type deletion gene and wild type α-globin gene were quantified by using Taqman real-time quantitative PCR and then the ΔC_T was analyzed by subtracting the $C_{T\text{-}mutant}$ from $C_{T\text{-}wild\ type}$.*
Results: *Mean ΔC_T values were not significantly different among the three groups. However, women whose fetuses were diagnosed as Bart's hydrops fetalis had a higher proportion (43%) of plasma DNA samples that had negative ΔC_T value than women whose fetuses were diagnosed as normal or heterozygote α-thalassemia-1 SEA type deletion (0 and 27%, respectively).*
Conclusion: *Further investigations are needed to improve the diagnosis of Bart's hydrops fetalis using fetal cell-free DNA.*

Keywords: *α-thalassemia-1 SEA type deletion, Bart's hydrops fetalis, Fetal cell-free DNA, Prenatal diagnosis, Real-time PCR*

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The α-thalassemia-1 results from the deletion of two α-globin genes on the same chromosome 16. The most common type of α-thalassemia-1 in the Asian population is the Southeast Asian type (SEA)[1]. Even though 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[2-4]. In addition, approximately 75% of mothers carrying fetuses with homozygous for the α-thalassemia-1 SEA type will develop toxemia of pregnancy[5]. The best strategy for prevention and control of the disease is

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prenatal diagnosis in the mothers at risk. The acceptable methods for prenatal diagnosis are chorionic villi sampling, amniocentesis, and cordocentesis[5-8]. However, these methods involve invasive procedures that may result in infection or abortion[9]. For noninvasive prenatal thalassemia diagnosis, fetal cells have been isolated from maternal blood and used in a single-cell polymerase chain reaction (PCR). However, the limitation of this approach is a very low number of fetal cells in maternal blood circulation during pregnancy (1 fetal cell/ml of maternal blood) and the complexity of isolation process[10]. The discovery of fetal cell-free DNA circulating in maternal blood during pregnancy has and continues to revolutionize the field of noninvasive prenatal diagnosis in research and clinical care[11]. Most studies have looked at sequences not present in the mother such as Y chromosome DNA sequences from male fetuses and fetal RhD gene in rhesus-D-negative pregnant women[12-14]. Fetal cell-free DNA has been shown to be useful for excluding the diagnosis of recessive diseases in which the paternal mutations differs from maternal one such as β-thalassemia and hemoglobinopathy[15-17]. These approaches could not be applied to situations where the mother and father are both carriers for the same autosomal recessive mutation. The digital real-time PCR have been developed to estimate the relative proportions of normal and mutant genes present in maternal plasma. This technique opens up the possibility of noninvasive prenatal diagnosis for all recessive disorders[18]. However, it is unknown whether the quantification of allelic ratio of normal and mutant genes present in maternal plasma could be used for prenatal diagnosis of Bart's hydrops fetalis in fetus born to mother and father who are both carriers for α-thalassemia-1 SEA.

Material and Method

Subject and sample processing

The present study was approved by the Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University. After informed consent, blood samples were obtained from α-thalassemia-1 SEA carriage pregnant women with risk for having Bart's hydrops fetalis. Approximately 10 ml maternal blood samples were collected into two 5 ml EDTA blood collection tubes (BD Vacutainer™, Franklin Lakes, NJ, USA). Plasma were prepared from the maternal blood samples by high-speed centrifuge as described previously[19] and stored at -20°C until use.

Plasma DNA extraction

DNA was extracted from 1 ml maternal plasma using ChargeSwitch® kit technology (Invitrogen, CA, USA). The kit was used according to the manufacturers' instructions. The plasma DNA was stored at -20°C until analysis.

Allele specific real-time PCR

The amplification was carried out in a reaction volume of 25 μl containing 12.5 μl of the 2x Absolute QPCR ROX mix (Thermo Fisher Scientific, KT, USA), 300 nM of forward and reverse primers, 200 nM of BHQ1 probes with nucleotide sequences as shown in Table 1, and 5 μl of plasma DNA sample. The final volume was adjusted to 25 μl with sterile distilled water. The real-time PCR was performed on Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia) with hot-start at 95°C for 15 min, followed by 50 cycles of denaturation at 95°C for 15 sec, annealment and extension at 60°C for 1 min. For the real-time PCR analysis, the cleaved fluorescent probes were used to monitor the PCR

Table 1. Primer and probe sequences used in quantitative real-time PCR for detection of wild type α-globin gene allele and α-thalassemia-1 SEA type deletion allele

Primer	Sequence (5' → 3')	Product length (bp)	GenBank accession no.
Common primer-FW	TCG GTC GTC CCC ACT GT	-	Z84721
Wild type primer-RW	GGA CTG CTC CGC TCC AC	107	Z84721
α-thal-1 SEA primer-RW	CAG CCT TGA ACT CCT GGA CTT AA	110	Z69706
Wild type α-globin probe	FAM-TC+T AG+C CC+C TGA G+CA CCG-BHQ1	Wild type	Z69706
α-thal-1 SEA probe	HEX-CT+C C+A+A G+TG+ AA+C C+TC C-BHQ1	Mutant	Z69706

"+" presented in probe sequences indicate the position which was added by the locked nucleic acid (LNA)

reaction. The quantitative process used by real-time PCR makes use of a defined threshold value, which is determined by the crossing of defined threshold by the accumulated PCR product, which is termed the threshold value (C_T). This value is inversely related to the amount of specific input template DNA. To measure the quantitative difference between mutant and wild type, the difference between the respective C_T values (ΔC_T) can be used and analyzed by subtracting the C_T value of the amplification of mutant allele from those of wild-type allele. The defined threshold was set at level of given ΔC_T of heterozygous a-thallassemia-1 SEA control DNA was equal to zero.

Descriptive statistics were used to summarize the data in term of mean $\pm$ standard deviation (SD), range and percentage. The ΔC_T of plasma DNA were compared among the three groups by using ANOVA which significant level was set at $p < 0.05$.

Results

Blood samples were collected from 25 α-thalassemia-1 SEA carriage pregnant women with risk for having fetuses with Bart's hydrops fetalis at mean $\pm$ SD of gestational age of 18.2 $\pm$ 5.46 weeks (range 7-31 weeks). The fetal α-globin genotypes obtained by routine Gap-PCR analysis of chorionic villi sampling, amniotic fluid, or cord blood sample indicated that seven women carrying normal fetuses, 11 women carrying fetuses with heterozygote α-thalassemia-1 SEA type deletion, and seven women carrying fetuses with Bart's hydrops fetalis. The ΔC_T of plasma DNA analysis were compared among the three groups. The women carrying fetuses with Bart's hydrops fetalis had lower mean ΔC_T than those carrying normal fetuses and fetuses with heterozygote α-thalassemia-1 SEA type deletion (Fig. 1). However, the difference was not significant. Interestingly, a higher proportion (3 of 7; 43%) of plasma DNA samples, which had a lower C_T value of α-thalassemia-1 SEA type deletion allele than those of the wild type α-globin gene allele that caused of negative ΔC_T value, was observed in women carrying fetuses with Bart's hydrops fetalis (Fig. 1). The negative ΔC_T value was not observed in plasma DNA sample of women carrying normal fetuses whereas it was found in three of 11 (27%) plasma DNA samples of woman carrying fetuses with heterozygote α-thalassemia-1 SEA type deletion (Fig. 1).

Discussion

The discovery of the presence of fetal cell-free DNA in maternal plasma has offered new approaches to noninvasive prenatal diagnosis, especially for those of paternally inherited disorders as well as fetal gender[11,20,21]. Since Bart's hydrops fetalis is occurs in a fetus born to mother and father who are both carriers for α-thalassemia-1 SEA type deletion, the qualitative molecular analysis of fetal cell-free DNA in maternal plasma could not be applied. In the present study, the quantitative real-time PCR was used to measure the quantitative difference between α-thalassemia-1 SEA allele and wild type α-globin gene allele in maternal plasma. The α-thalassemia-1 SEA allele was increased in plasma of women carrying fetuses with Bart's hydrops fetalis; it was indicated by the lower level of mean ΔC_T value when compared to the mean ΔC_T value of those carrying fetuses with heterozygote α-thalassemia-1 SEA type deletion. On the other hand, wild type α-globin gene allele was increased in women carrying normal fetuses; it was indicated by a higher level of mean ΔC_T value when compared to the mean ΔC_T value of those carrying fetuses with heterozygote α-thalassemia-1 SEA type deletion. Moreover, the presence of fetal cell-free DNA in maternal plasma was confirmed by observing Y chromosome DNA sequences in six maternal plasma samples which was 100% concordance with the fetal gender observed at delivery and obtained by PCR analysis of chorionic villi sampling, amniotic fluid or cord blood sample. The α-thalassemia-1 SEA allele of fetuses with Bart's hydrops fetalis and wild type α-globin gene allele of normal fetus was present at a low level; therefore, it was not enough to induct a significant difference of mean ΔC_T among the three

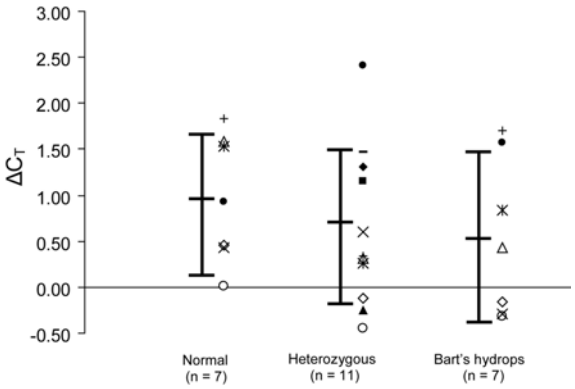

Fig. 1 Mean $\pm$ SD of ΔC_T values in each group of pregnancies whose fetuses have known thalasemia status. The symbols are represented the ΔC_T value of each plasma DNA sample

groups of pregnant women. These results were associated with previous studies showing that fetal cell-free DNA was present at 10-20% of all DNA in maternal plasma[18,20]. Chan et al[22] suggested that a low concentration of fetal cell-free DNA in maternal plasma has led to false-negative results and wrong diagnoses. Moreover, the quantitative analysis of those is also less precise at low concentration[23]. To enrich the concentration of fetal cell-free DNA, gel electrophoresis and selection of short fetal DNA molecules have been applied[22]. Furthermore, the suppression of maternal background DNA was also developed[24]. However, these techniques are too complex, labor intensive, and not sufficiently efficient for a routine clinical setting. Moreover, gel electrophoresis methods may be prone to DNA contamination. Although the mean ΔC_T of the three groups of pregnant women showed no significant difference, a higher proportion of plasma DNA samples, which had negative ΔC_T value, were observed in women carrying fetuses with Bart's hydrops fetalis. These ΔC_T values were not found in women carrying normal fetuses.

In summary, the results of the present study have demonstrated that the fetal cell-free DNA was present in maternal plasma at low concentration, which did not induce a significant difference of mean ΔC_T values among pregnant women carrying fetuses with Bart's hydrops fetalis, with heterozygote α-thalassemia-1 SEA type deletion and normal fetuses. Although the negative ΔC_T value may be used to aid in diagnosing and monitoring Bart's hydrops fetalis pregnancies, it was not found in all plasma DNA samples of women carrying fetuses with Bart's hydrops fetalis. Therefore, further analyses are needed to improve the diagnosis of Bart's hydrops fetalis using this technique.

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การวิเคราะห์ค่า cycle threshold ของ แอลฟา-ธาลัสซีเมีย-1 SEA จาก fetal cell-free DNA ที่มีอยู่ในกระแสเลือดแม่เพื่อการวินิจฉัยก่อนคลอดสำหรับ Bart's hydrops fetalis

สาคร พรประเสริฐ, กัญญากาญจน์ สุคันธมาลา, เนาวรัตน์ กันยานนท์, ศิริชัย สิทธิประเสริฐ, คะนึงนิจ ถุงคำ, สุเมธ จิโนรส, คจรศิลป์ ผ่อนสวัสดิ์กุล, วิสุทธิ์ พัฒนาภรณ์, จันทรทิพย์ จิตรวงค์, ต่อพงศ์ สงวนเสริมศรี

ภูมิหลัง: *การตรวจวินิจฉัยก่อนคลอดที่ไม่มีการรุกล้ำโดยการตรวจหา fetal cell-free DNA ที่มีอยู่ในกระแสเลือดแม่ไม่สามารถทำได้ หากทั้งแม่และพ่อเป็นพาหะของจีนด้อยบนโครโมโซมร่างกายที่ผิดปกติชนิดเดียวกัน*

วัตถุประสงค์: *เพื่อเปรียบเทียบการตรวจวินิจฉัย Bart's hydrops fetalis ด้วยวิธี Gap-PCR ซึ่งใช้ตรวจในงานประจำวันและต้องตรวจจากตัวอย่างเลือดหรือเนื้อเยื่อของทารกกับการตรวจโดยการวิเคราะห์ค่า ΔC_T ระหว่างจีนแอลฟา-ธาลัสซีเมีย-1 SEA ($C_{T\text{-mutant}}$) และจีนแอลฟาปกติ ($C_{T\text{-wild type}}$) ที่ตรวจพบในพลาสมาของหญิงตั้งครรภ์โดยวิธี Taqman real-time quantitative PCR*

วัสดุและวิธีการ: *นำตัวอย่างพลาสมาดีเอ็นเอของหญิงตั้งครรภ์ 3 กลุ่ม ซึ่งประกอบด้วย หญิงตั้งครรภ์ที่ทารกในครรภ์ได้รับการวินิจฉัยก่อนคลอดเป็นปกติจำนวน 7 ราย เป็นพาหะ แอลฟา-ธาลัสซีเมีย-1 SEA จำนวน 11 ราย และเป็น Bart's hydrops fetalis จำนวน 7 ราย มาตรวจหาปริมาณจีนแอลฟา-ธาลัสซีเมีย-1 SEA และจีนแอลฟาปกติด้วยวิธี Taqman real-time quantitative PCR จากนั้นจึงวิเคราะห์ค่า ΔC_T โดยนำค่า $C_{T\text{-mutant}}$ มาลบด้วย $C_{T\text{-wild type}}$*

ผลการศึกษา: *ค่าเฉลี่ย ΔC_T ของหญิงตั้งครรภ์ทั้งสามกลุ่มไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตามพบร้อยละ 43 ของจำนวนตัวอย่างพลาสมาดีเอ็นเอของหญิงตั้งครรภ์ที่ทารกในครรภ์เป็น Bart's hydrops fetalis มีค่า ΔC_T ที่เป็นลบ ซึ่งเป็นจำนวนมากกว่าของหญิงตั้งครรภ์คู่เสี่ยงที่ทารกในครรภ์เป็นปกติ หรือ เป็นพาหะแอลฟา-ธาลัสซีเมีย-1 SEA (ร้อยละ 0 และ 27 ตามลำดับ)*

สรุป: *เพื่อให้ได้ผลการตรวจที่มีความถูกต้อง แม่นยำมากยิ่งขึ้นการตรวจวินิจฉัย Bart's hydrops fetalis โดยใช้ตัวอย่างตรวจ ที่เป็น fetal cell-free DNA ที่มีอยู่กระแสเลือดแม่ ควรได้รับการศึกษาและพัฒนาต่อไปในอนาคต*