Semi-Nested Taqman Real-Time Quantitative PCR for Noninvasive Prenatal Diagnosis of Bart's Hydrops Fetalis

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Background: *Non-invasive prenatal diagnosis based on detection of fetal cell-free DNA is limited when mother and father are both carriers for the same autosomal recessive mutation.*
Objective: *Develop the semi-nested Taqman real-time PCR for quantification of α-thalassemia-1 SEA type deletion allele in plasma of α-thalassemia-1 SEA carriage pregnancies.*
Material and Method: *Plasma DNA was extracted from six women who carried fetuses with normal, 11 with heterozygote α-thalassemia-1 SEA type deletion and seven with Bart's hydrops fetalis. DNA was amplified using conventional PCR with the primary specific primer set for α-thalassemia-1 SEA type deletion. PCR product was then subjected to the semi-nested real-time PCR using the secondary specific primer and Taqman probe set for α-thalassemia-1 SEA type deletion. The standard curve was constructed using ten-fold serial dilutions of conventional PCR product of the heterozygote α-thalassemia-1 SEA type deletion.*
Results: *Women who carried fetuses with Bart's hydrops fetalis displayed a trend toward higher mean copy number of α-thalassemia-1 SEA type deletion allele vs. women who carried fetuses with normal and heterozygote, albeit not reaching statistical significance.*
Conclusion: *The maternally inherited fetal allele present in maternal plasma is difficult to discern the fetal cell-free DNA from a higher background DNA of the mother. Thus, further investigation is needed to improve the diagnosis of Bart's hydrops fetalis using this technique.*

Keywords: *α-thalassemia-1 SEA type deletion, Fetal cell-free DNA, Prenatal diagnosis, Semi-nested real-time PCR, Taqman probe*

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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[1-3]. Since Bart's hydrops fetalis occurred in fetuses born to mothers and fathers who are both carriers for α-thalassemia-1 Southeast Asian (SEA) type deletion, the qualitative molecular analysis of fetal cell-free DNA in maternal plasma could not be applied. Moreover, fetal cell-free DNA is present within a high background of maternal DNA (only about 10-20% of all DNA in maternal plasma)[2,4]. Previous studies demonstrated that fetal cell-free DNA in maternal plasma increases in pathological pregnancies including those with preterm labor and preeclampsia[4,5]. Furthermore, Tungwiwat et al showed that the amounts of α-thalassemia-1 SEA type deletion allele detected by the SYBR green1 semi-nested real-time quantitative PCR analysis was higher in plasma of two mothers whose fetuses were diagnosed as Bart's hydrops

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fetalis than in those of mothers whose fetuses were diagnosed as normal or heterozygous for α-thalassemia-1 SEA type deletion[6]. Therefore, the increase of α-thalassemia-1 SEA type deletion allele in maternal plasma might potentially prove useful in development of a noninvasive prenatal diagnosis of Bart's hydrops fetalis. However, the quantitative real-time PCR analysis based on SYBR green1 is less specific than the florescence probe. The present study used the Taqman probe semi-nested real-time PCR for quantification of α-thalassemia-1 SEA type deletion allele in plasma of pregnancies that had a risk for Bart's hydrops fetalis.

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, approximately 10 ml maternal blood samples were collected into EDTA blood collection tubes (BD Vacutainer™, Franklin Lakes, NJ, USA). Plasma was prepared by high-speed centrifuge as described previously[7] and stored at -20°C until use.

Plasma DNA extraction

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

Semi-nested real-time quantitative PCR analysis

The conventional PCR was carried out in a reaction volume of 25 μl containing 5 μl of plasma DNA sample, 0.3 μM of α-thalassemia-1 SEA type deletion specific primers (Forward; 5'-GCGATCTGGGCTCTGTG TTCT-32 and Reward; 5'-CAGCCTTGAACTCCTGGA CTTAA-3'), 10% DMSO, 0.2 mM dNTPs, 2.5 μl PCR buffer containing 15 mM $MgCl_2$ and 1.25 unit of Taq DNA polymerase (Qiagen GmbH, Hilden, Germany). The thermocycling condition was as follows: 94°C for 5 minutes, then 35 cycles at 94°C for 30 seconds, 58°C for 60 seconds and 72°C for 60 seconds, followed by 72°C for 7 minutes. Five μl-amplified product was then subjected to a semi-nested real-time quantitative 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), 150 nM primers (Forward; 5'-TCGGTCGTCCCCACTGT-3' and the reward primer with similar nucleotide sequences as used for conventional PCR), 100 nM of TaqMam probe (HEX-CT+CC+A+A G+TG+AA+C C+TC C-BHQ1; "+" represents the position which was added by the locked nucleic acid). 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 40 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 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 and termed the threshold value (C_T). This value is inversely related to the amount of specific input template DNA. The ten-fold dilutions of conventional PCR product of the heterozygote α-thalassemia-1 SEA type deletion were used to construct the standard curve by plotting the C_T against the number of copies of DNA template calculated by using the formulation created by Andrew Staroscik[8]. All standard and sample DNA were performed in duplicate.

Results

Blood samples were collected from 24 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 18.17 $\pm$ 5.58 weeks (range and median were 7 to 31 and 17 weeks, respectively). The fetal α-globin genotypes obtained by routine Gap-PCR analysis of chorionic villi samples, amniotic fluid samples, or cord blood samples indicated that six women carried fetuses with normal, 11 women carried fetuses with heterozygote α-thalassemia-1 SEA type deletion and seven women carried fetuses with Bart's hydrops fetalis. The women carrying fetuses with Bart's hydrops fetalis had a trend toward higher copy number of α-thalassemia-1 SEA type deletion allele than women carrying normal fetuses and fetuses with heterozygote α-thalassemia-1 SEA type deletion, albeit not reaching statistical significance (Fig. 1).

Discussion

The α-thalassemia-1 SEA type deletion allele was increased in plasma of women carrying fetuses with Bart's hydrops fetalis. It was associated with previous studies demonstrating that there are some factors that increase the quantity of fetal cell-free DNA in maternal circulation, such as

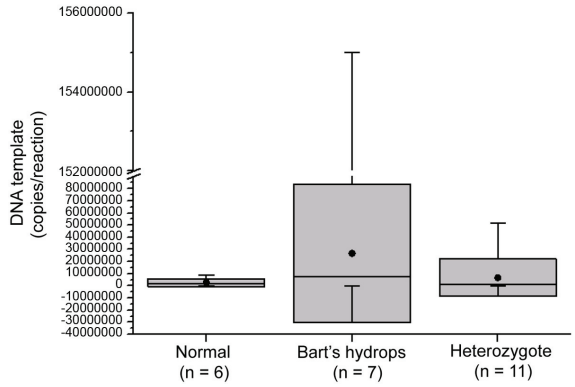

Fig. 1 The copy number of α-thalassemia-1 SEA type deletion allele in plasma of each group of pregnancy. Data are presented as median (horizontal line), mean (dot), standard deviation (box) and 1st and 99th percentile (whisker lines). The results among the three groups of pregnancies were compared using the Mann-Whitney U test. The p-value which is less than 0.05 has an important statistical significance

pathological pregnancies associated with placental abnormalities[5,9]. Bart's hydrops fetalis causes fetal growth retardation and placenta abnormality that increases the amount of fetal cell-free DNA in maternal circulation[10]. Although, a higher copy number of α-thalassemia-1 SEA type deletion was observed in women carrying fetuses with Bart's hydrops fetalis, the statistical analysis was not significantly different when compared with that of women carrying normal fetuses and fetuses with heterozygote α-thalassemia-1 SEA type deletion. Because the Bart's hydrops fetalis occurred in fetuses born to mothers and fathers who are both carriers for α-thalassemia-1 SEA type deletion, the maternally inherited fetal α-thalassemia-1 SEA type deletion allele presented in maternal plasma is therefore difficult to discern from the high background of maternal allele. Previous studies demonstrated that fetal cell-free DNA was present at a low concentration, about 10-20% of all DNA in maternal plasma[2,11]. Thus, the quantitative analysis of circulating fetal cell-free DNA is less precise at low concentration[12]. Although, the study by Tungwiwat et al showed that the semi-nested real-time quantitative PCR analysis of fetal cell-free DNA in maternal plasma is useful in development of a noninvasive prenatal diagnosis of Bart's hydrops fetalis, the data limited by the size of samples. Moreover, the use of a SYBR Green1 for semi-nested real-time quantitative PCR analysis is less specific than the use of a fluorescent probe[3].

In summary, the results of the present study have demonstrated that the α-thalassemia-1 SEA type deletion allele was at a higher level in plasma of women carrying fetuses with Bart's hydrops fetalis. However, within a high background of maternal DNA, it is difficult to use the semi-nested real-time quantitative PCR analysis for a noninvasive prenatal diagnosis of 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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Potential conflicts of interest

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References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997; 350: 485-7.
2. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998; 62: 768-75.
3. Tungwiwat W, Fucharoen G, Ratanasiri T, Sanchaisuriya K, Fucharoen S. Non-invasive fetal sex determination using a conventional nested PCR analysis of fetal DNA in maternal plasma. Clin Chim Acta 2003; 334: 173-7.
4. Leung TN, Zhang J, Lau TK, Hjelm NM, Lo YM. Maternal plasma fetal DNA as a marker for preterm labour. Lancet 1998; 352: 1904-5.
5. Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W, et al. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am J Obstet Gynecol 2001; 184: 414-9.
6. Tungwiwat W, Fucharoen S, Fucharoen G, Ratanasiri T, Sanchaisuriya K. Development and

application of a real-time quantitative PCR for prenatal detection of fetal alpha(0)-thalassemia from maternal plasma. Ann N Y Acad Sci 2006; 1075: 103-7.
7. Li Y, Zimmermann B, Rusterholz C, Kang A, Holzgreve W, Hahn S. Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. Clin Chem 2004; 50: 1002-11.
8. URI Genomics & Sequencing Center. Calculator for determining the number of copies of a template [database on the Internet]. 2004 [cited 2011 Jun 25]. Available from: http://www.uri.edu/research/gsc/resources/cndna.html.
9. Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, et al. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin Chem 1999; 45: 184-8.
10. Ko TM, Tseng LH, Hsu PM, Hwa HL, Lee TY, Chuang SM. Ultrasonographic scanning of placental thickness and the prenatal diagnosis of homozygous alpha-thalassaemia 1 in the second trimester. Prenat Diagn 1995; 15: 7-10.
11. Lun FM, Chiu RW, Allen Chan KC, Yeung LT, Kin LT, Dennis Lo YM. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008; 54: 1664-72.
12. Lo YM, Lun FM, Chan KC, Tsui NB, Chong KC, Lau TK, et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proc Natl Acad Sci U S A 2007; 104: 13116-21.

การตรวจวินิจฉัยก่อนคลอดสำหรับ Bart's hydrops fetalis แบบ noninvasive โดยวิธี Semi-nested Taqman real-time quantitative PCR

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

ภูมิหลังและวัตถุประสงค์: *การตรวจวินิจฉัยก่อนคลอดด้วยวิธี noninvasive โดยการตรวจหา fetal cell-free DNA มีข้อจำกัดคือการที่ทั้งแม่และพ่อเป็นพาหะของจีนด้อยบนโครโมโซมร่างกายที่ผ่าเหล่าชนิดเดียวกัน ดังนั้น วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อพัฒนาเทคนิค Semi-nested Taqman real-time PCR สำหรับตรวจวัดปริมาณจีนแอลฟา-ธาลัสซีเมีย-1 SEA ในพลาสมาของหญิงตั้งครรภ์ที่เป็นพาหะแอลฟา-ธาลัสซีเมีย-1 SEA*

วัสดุและวิธีการ: *สกัดดีเอ็นเอจากพลาสมาของหญิงตั้งครรภ์ที่ทารกในครรภ์เป็นปกติจำนวน 6 ราย เป็นพาหะแอลฟา-ธาลัสซีเมีย-1 SEA จำนวน 11 ราย และเป็น Bart's hydrops fetalis จำนวน 7 ราย นำตัวอย่างดีเอ็นเอมาเพิ่มปริมาณด้วยวิธี PCR ที่ใช้โดยทั่วไป และใช้ primer ชุดแรกที่มีความจำเพาะต่อจีนแอลฟา-ธาลัสซีเมีย-1 SEA จากนั้นจึงตรวจหาปริมาณดีเอ็นเอด้วยวิธี Semi-nested real-time PCR โดยใช้ primer และ Taqman probe ชุดที่สองที่มีความจำเพาะต่อ จีนแอลฟา-ธาลัสซีเมีย-1 SEA กราฟมาตรฐานเตรียมได้จากการตรวจวิเคราะห์ปริมาณดีเอ็นเอของพาหะแอลฟา-ธาลัสซีเมีย-1 SEA ภายหลังผ่านการเพิ่มจำนวนชิ้นส่วนดีเอ็นเอด้วยวิธี PCR ที่ใช้โดยทั่วไป และเจือจางเป็นลำดับในอัตราส่วน 1 ต่อ 10*

ผลการศึกษา: *หญิงตั้งครรภ์ที่ทารกในครรภ์เป็น Bart's hydrops fetalis มีแนวโน้มที่มีค่าเฉลี่ยปริมาณจีนแอลฟา-ธาลัสซีเมีย-1 SEA ในพลาสมาสูงกว่าของหญิงตั้งครรภ์ที่ทารกในครรภ์เป็นปกติ หรือเป็นพาหะแอลฟา-ธาลัสซีเมีย-1 SEA อย่างไรก็ตามปริมาณพลาสมาดีเอ็นเอที่เพิ่มขึ้นไม่ทำให้เกิดความแตกต่างอย่างมีนัยสำคัญทางสถิติ*

สรุป: *จีนที่ผิดปกติของทารกในครรภ์ที่ได้รับการถ่ายทอดจากแม่จะปรากฏในพลาสมาของแม่ได้ หากแต่การแยกจีนผิดปกติของ fetal cell-free DNA ออกมาจากดีเอ็นเอของแม่ซึ่งมีอยู่ในปริมาณที่มากกว่าทำได้ยาก ดังนั้นจึงต้องมีการศึกษาต่อไปเพื่อพัฒนาการตรวจวินิจฉัย Bart's hydrops fetalis โดยใช้เทคนิคนี้*