รายงานวิจัยฉบับสมบูรณ์

โครงการ การตรวจวินิจฉัยก่อนคลอดภาวะโฮโมซัยกัสอัลฟ่าธาลัสซีเมีย-1 โดยดีเอ็นเอของทารกที่อยู่ในเลือดมารดา

Prenatal diagnosis of homozygous alpha-thalassemia-1 by cell-free fetal DNA in maternal plasma

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พฤษภาคม พ.ศ. 2554

สัญญาเลขที่ RSA5180013

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ได้สนับสนุนทุนวิจัย เพื่อศึกษาความเป็นไปได้ในการใช้ดีเอ็นเอของทารกที่ลอยอยู่ในกระแสเลือดมารดาเพื่อการวินิจฉัยก่อนคลอดภาวะโฮโมซัยกัสอัลฟาธาลัสซีเมีย-1 ขอขอบคุณแพทย์และเจ้าหน้าที่ทุกท่านที่มีส่วนร่วมในการวิจัยนี้ และงานวิจัยนี้จะสำเร็จลุล่วงลงไม่ได้หากไม่ได้รับความร่วมมือจากสตรีตั้งครรภ์ทุกท่านที่เสียสละเวลาในการเข้าร่วมวิจัยครั้งนี้

คณะผู้วิจัย

พฤษภาคม 2554

สารบัญ

บทคัดย่อ

รหัสโครงการ : RSA5180013

ชื่อโครงการ : การตรวจวินิจฉัยก่อนคลอดภาวะโฮโมซัยกัสอัลฟ่าธาลัสซีเมีย-1 โดยดีเอ็นเอของทารกที่อยู่ในเลือดมารดา

ชื่อนักวิจัย : ร.ศ. พ.ญ. สุพัตรา ศิริโชติยะกุล
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ที่มาของปัญหา: ภาวะโฮโมซัยกัสอัลฟาธาลัสซีเมีย (ฮีโมโกลบินบาร์ท) เป็นโรคถ่ายทอดทางพันธุกรรมที่พบบ่อยที่สุดโรคหนึ่งในเอเชียตะวันออกเฉียงใต้ คู่สมรสที่เป็นพาหะของยีนอัลฟาธาลัสซีเมีย-1 ทั้งสองคนมีโอกาสทารกในครรภ์เป็นโรคดังกล่าวร้อยละ 25 ซึ่งเป็นผลจากการถ่ายทอดทางพันธุกรรมแบบยีนด้อย สตรีตั้งครรภ์ที่ทารกในครรภ์เป็นโรคฮีโมโกลบินบาร์ทมีความเสี่ยงที่จะเกิดภาวะแทรกซ้อน เช่น ครรภ์เป็นพิษ ตกเลือดหลังคลอด เพิ่มสูงขึ้น การตรวจวินิจฉัยก่อนคลอดของภาวะนี้ยังคงอาศัยการตรวจแบบรุกล้ำ เช่น การเจาะชิ้นเนื้อรก การเจาะน้ำคร่ำ หรือการเจาะเลือดจากสายสะดือทารกในครรภ์ ซึ่งการตรวจด้วยวิธีดังกล่าวมีความเสี่ยงต่อการแท้งบุตร มีรายงานการตรวจวินิจฉัยก่อนคลอดของโรคพันธุกรรมหลายโรคด้วยวิธีไม่รุกล้ำโดยใช้ cell-free DNA ของทารกที่อยู่ในพลาสมาของมารดา อย่างไรก็ดี มีเพียงไม่กี่รายงานที่ศึกษาการใช้ cell-free DNA ของทารกในพลาสมามารดาในการวินิจฉัยภาวะฮีโมโกลบินบาร์ท

วัตถุประสงค์: เพื่อศึกษาความเป็นไปได้ของการใช้ cell-free DNA ของทารกในพลาสมามารดาในการวินิจฉัยทารกในครรภ์ที่มารดามีความเสี่ยงต่อการมีลูกเป็นฮีโมโกลบินบาร์ท

วิธีดำเนินการวิจัย: คู่สมรสที่เป็นพาหะของยีนอัลฟาธาลัสซีเมียชนิด SEA deletion ได้รับการเชิญให้เข้าร่วมการวิจัยในวันที่มาขอรับการตรวจวินิจฉัยก่อนคลอด (ด้วยการเจาะชิ้นเนื้อรกหรือการเจาะเลือดจากสายสะดือทารกในครรภ์) สกัดพลาสมา DNA จากเลือดมารดา 10 มิลลิลิตร ทำ Real time quantitative PCR โดยใช้ probe ที่ติดฉลาก fluorescence ในการติดตาม wild type (wt) allele และ SEA allele ปริมาณการสร้างของแต่ละ allele จะได้รับการประเมินโดย

cycle threshold (Ct) คำนวณความแตกต่างของ Ct (delta Ct) ของอาสาสมัครแต่ละราย (โดยคำนวณจาก Ct ของ wt allele ลบด้วย Ct ของ SEA allele) อาสาสมัครได้รับการตรวจวินิจฉัยก่อนคลอดด้วยวิธีมาตรฐานเพื่อวินิจฉัยทารกในครรภ์ว่าเป็น ฮีโมโกลบินบาร์ท พาหะชนิดอัลฟา หรือทารกปกติ

ผลการศึกษา: ในการศึกษานี้มีทารกฮีโมโกลบินบาร์ท 62 ราย ทารกเป็นพาหะชนิดอัลฟา 62 ราย และทารกปกติ 25 ราย ค่าเฉลี่ยของ delta Ct เท่ากับ 1.04 +/-0.38, 0.21+/-0.37 และ 0.20+/-0.59 ในทารกฮีโมโกลบินบาร์ท, พาหะชนิดอัลฟา และทารกปกติ ตามลำดับ เมื่อใช้ Receiving Operating Curve (ROC) ค่า cut-off ที่ดีที่สุดของ delta Ct ในการทำนายทารกฮีโมโกลบินบาร์ท เท่ากับ 0.51 ซึ่งจะให้ความไวร้อยละ 98.4 และมีผลบวกลวงร้อยละ 23.0 ทารกฮีโมโกลบินบาร์ท 61 รายจาก 62 ราย (ร้อยละ 98.4) มีค่า delta Ct มากกว่า 0.51 ในขณะที่ทารกที่เป็นพาหะชนิดอัลฟาร้อยละ 74.2 และทารกปกติร้อยละ 84.0 มีค่า delta Ct ต่ำกว่า 0.51 เมื่อใช้ค่า delta Ct 0.51 เป็นค่า cut-off ในการทำนายภาวะฮีโมโกลบินบาร์ท จะมีความไวร้อยละ 98.4 ความจำเพาะร้อยละ 77.0 positive predictive value ร้อยละ 75.3 และ negative predictive value ร้อยละ 98.5

สรุป: ผลจากการศึกษานี้พบว่ามีแนวโน้มที่ดีในการใช้ cell-free DNA ของทารกในพลาสมามารดาในการตรวจวินิจฉัยก่อนคลอดสำหรับภาวะโฮโมซัยกัสอัลฟาธาลัสซีเมีย-1 (ฮีโมโกลบินบาร์ท) เมื่อใช้เทคนิคนี้ สองในสามของทารกที่ไม่เป็นโรคสามารถหลีกเลี่ยงการตรวจวินิจฉัยก่อนคลอดด้วยวิธีรุกล้ำและภาวะแทรกซ้อนที่อาจเกิดขึ้นได้

คำหลัก: ธาลัสซีเมีย, อัลฟาธาลัสซีเมีย, ฮีโมโกลบินบาร์ท, DNA ของทารก, พลาสมามารดา, การตรวจวินิจฉัยก่อนคลอด

Abstract

Project Code : RSA5180013

Project Title : Prenatal diagnosis of homozygous alpha-thalassemia-1 by cell-free fetal DNA in maternal plasma

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Project Period : 1 May 2008 – 1 May 2011 (3 years)

Background: Homozygous alpha-thalassemia (Hb Bart's) is one of the most common inherited genetic diseases in Southeast Asia. Couples who both were the carrier of alpha-thalassemia 1 gene will have a 25% risk of having affected fetuses as a result of autosomal recessive inheritance. Pregnant women who carried Hb Bart's fetuses will usually have increased risk for complications such as preeclampsia and postpartum hemorrhage. Prenatal diagnosis for this condition is still based on invasive procedures such as chorionic villus sampling (CVS), amniocentesis or fetal blood sampling (cordocentesis). These techniques accompanied with risk of fetal losses. Non-invasive prenatal diagnosis using cell-free fetal DNA (cff-DNA) in maternal plasma had been reported in various genetic diseases. However, there were a few studies reported using cff-DNA in maternal plasma for diagnosis of Hb Bart's.

Objective: To investigate the possibility of using cell-free fetal DNA (cff-DNA) to determine the fetal status in pregnant women who were at risk of having Hb Bart's fetuses.

Material and Method: Couples who both were alpha-thalassemia carrier (SEA deletion) were invited to participate in the study at the time they came for prenatal diagnosis (chorionic villi sampling or fetal blood sampling). Plasma DNA was extracted from 10 mL of maternal blood. Real time quantitative PCR was performed using fluorescence-

labeled probe to monitor wild type (wt) allele and SEA allele. The cycle threshold (Ct) was used to determine the quantity of each allele. Delta Ct (Ct of wt allele – Ct of SEA allele) was calculated from each sample. Prenatal diagnosis was performed using standard technique to determine fetal status (Hb Bart's, alpha-trait, or normal).

Result: There were 62 Hb Bart's, 62 alpha-trait and 25 normal fetuses recruited into this study. Mean delta Ct was 1.04 +/-0.38, 0.21+/-0.37 and 0.20+/-0.59 in Hb Bart's hydrops, alpha-trait and normal fetuses, respectively. Based on the Receiving Operating Curve (ROC) of delta Ct value, the best cut-off point for predicting Hb Bart's hydrops was 0.51, which gave 98.4% sensitivity and a false-positive rate of 23.0%. All but one Hb Bart's hydrops (98.4%) had delta Ct above 0.51, whereas 74.2% of alpha-trait and 84.0% of normal fetuses had delta Ct below 0.51. Using delta Ct 0.51 as a cut-off gave 98.4% sensitivity, 77.0% specificity, 75.3% positive predictive value and 98.5% negative predictive value.

Conclusion: There is a positive trend to use cff- DNA in maternal plasma for prenatal diagnosis of homozygous alpha-thalassemia-1 (Hb Bart's). With this technique, invasive prenatal testing and its complications can be avoided in approximately two-third of unaffected fetuses

Keywords: thalassemia, alpha-thalassemia, Hb Bart's, fetal DNA, maternal plasma, prenatal diagnosis

บทที่ 1

ความสำคัญและที่มาของปัญหาที่ทำการวิจัย

The prevalence of alpha-thalassemia-1 trait in the northern part of Thailand is relatively high compared to other part of the country[1;2]. It has been estimated that there will be 2 fetuses with homozygous alpha-thalassemia-1 (Hb Bart's hydrops fetalis) in every 1000 pregnant women[1]. Prenatal diagnosis is widely accepted as a part of prenatal control of severe thalassemia[3]. Each year approximately 80-100 risk couples for this condition come to Maharaj Nakorn Chiang Mai hospital for prenatal diagnosis. Currently, prenatal diagnosis methods offered are either chorionic villi sampling, amniocentesis or cordocentesis[4-9]. However, these methods carry risks of complications such as infection, leakage, and miscarriage[10].

Non-invasive prenatal diagnosis for detection of Hb Bart's hydrops has been proposed using ultrasonographic markers such as cardio-thoracic ratio, placental thickness, fetal ascites[11]. However, these markers are usually detected after mid-pregnancy or later.

In 1997, Lo et al[12] reported the presence of free fetal DNA in maternal plasma and serum, their findings gives light to a new method of prenatal diagnosis which is non-invasive and carries no risk for the fetuses. Since then, there has been many studies reported the use of free fetal DNA in maternal plasma for molecular diagnosis. In the earlier period, free fetal DNA in maternal plasma was used for detection of SRY(Y-chromosome specific sequence) and fetal RhD status[13;14]. Recently, there have been many reports in using this technique to detect other genes for prenatal diagnosis of various genes such as achondroplasia, myotonic dystrophy, beta-thalassemia gene and Hb E[15-18].

In this study we proposed to investigate the feasibility of using free fetal DNA in maternal plasma for the prenatal diagnosis in the pregnancies at risk for having homozygous alpha-thalassemia-1 fetuses. The recent advance in real-time PCR makes it possible to detect minute amount of DNA because of the high sensitivity and specificity of amplification. If this method is proved to be useful, it can be offered as a non-invasive prenatal diagnosis to prevent the complications of invasive prenatal diagnosis. This will facilitate the prevention and control program of severe thalassemia syndrome.

The objectives of this study are

1. To evaluate the feasibility of using free fetal DNA in maternal plasma for the prenatal diagnosis in the pregnancies at risk for having homozygous alpha-thalassemia-1 fetuses
2. To evaluate the sensitivity and specificity of prenatal diagnosis of alpha-thalassemia-1 gene from fetal DNA in maternal plasma using real-time PCR
3. To investigate whether the quantity of PCR product can be used to distinguish between normal, heterozygous and homozygous state of the fetuses

บทที่ 2
ผลงานวิจัยที่เกี่ยวข้อง

Thalassemia is a major problem in healthcare in Thailand due to its high prevalence of carrier states. The prevalence of alpha-thalassemia-1 trait in the northern part of Thailand is relatively high compared to other part of the country[1;2]. It has been estimated that there will be 2 fetuses with homozygous alpha-thalassemia-1 (Hb Bart's hydrops fetalis) in every 1000 pregnant women[1]. Prenatal diagnosis is widely accepted as a part of prenatal control of severe thalassemia[3]. Each year approximately 80-100 risk couples for this condition come to Maharaj Nakorn Chiang Mai hospital for prenatal diagnosis. Currently, the accepted prenatal diagnosis in Thailand is either chorionic villi sampling, amniocentesis or cordocentesis[4-9]. However, these methods carry risks of complications such as infection, leakage, and miscarriage[10].

Non-invasive prenatal diagnosis for detection of Hb Bart's hydrops has been proposed using ultrasonographic markers such as cardio-thoracic ratio, placental thickness, fetal ascites[11]. However, these markers are usually detected after mid-pregnancy or later.

In 1997, Lo et al[12] reported the presence of free fetal DNA in maternal plasma and serum. They studied 30 maternal plasma samples from women bearing male fetuses (gestational age 12-40 weeks) and found Y-specific fetal sequences in 80% of the cases. Negative control used in their study were 13 women bearing female fetuses and 10 non-pregnant women, none of them had a positive Y signal when plasma or serum DNA was amplified. Their findings gives light to a new method of prenatal diagnosis which is non-invasive and carries no risk for the fetuses.

In 1998, Lo et al[14] reported that fetal DNA can be detected in maternal serum as early as 7th week of gestation. The concentration of fetal DNA in early pregnancy was 3.4% (range 0.39-11.9%) of the total plasma DNA or 25.4 genome equivalents/mL. In late pregnancy the concentration increased to 6.2% (range 2.33-11.4%) of the total plasma DNA or 292.2 genome equivalents/mL. This concentration is high compared to the DNA derived from fetal cells in maternal plasma which found only 1.2 cells/mL[19]. Later on, Dhallan et al[20] had reported that the addition of formaldehyde along with careful sample processing can increase the proportion of free fetal DNA in maternal plasma to about 25%.

There has been many studies reported the use of free fetal DNA in maternal plasma for molecular diagnosis. In 2002, Honda et al[21] studied the SRY gene in maternal serum of pregnant women 5th-10th week gestation for fetal gender determination and reported 100% sensitivity when using real time PCR assay. Fetal sex determination from free fetal DNA in maternal plasma has been reported in many publications[21-26]. The clinical implication was to determine male fetuses in X-linked recessive disorders. Fetal RhD status was also reported to be correctly determined by real-time PCR from maternal serum[13;27;28]. Recently, there have been many reports in using this technique to detect other genes for prenatal diagnosis of various genes such as achondroplasia, myotonic dystrophy, beta-thalassemia gene and Hb E[15-18].

In 2006, Tungwiwat et al[29] reported the prenatal diagnosis of alpha-thalassemia from fetal DNA in maternal serum in 13 pregnant women. They used semi-nested real-time PCR system and detected the product with SYBR green. In their series, there were 2 normal, 8 heterozygous, 1 Hb H disease and 2 homozygous alpha-thalassemia fetuses correctly diagnosed. However, the number of the cases is limited. In this study we proposed to perform the prenatal diagnosis of this condition using maternal serum in a larger scale to prove the feasibility to implement in clinical practice.

บทที่ 3
ระเบียบวิธีวิจัย

รูปแบบการวิจัย (Research design)

Diagnostic test

ประชากรและกลุ่มตัวอย่าง (Study population)

Target population

Risk couples who seek for prenatal diagnosis of alpha-thalassemia 1 at Maharaj Nakorn Chiang Mai hospital between May 2008 and September 2010.

Inclusion criteria

1. Age 18 years old or more
2. Risk for having homozygous alpha-thalassemia 1 fetus (by history of having previous child with the disease or by carrier screening results)
3. Schedule for conventional method of prenatal diagnosis to detect whether the fetus is affected or not (either by chorionic villi sampling, amniocentesis or cordocentesis)

Exclusion criteria

1. The result of conventional method of prenatal diagnosis could not be obtained
2. Unwilling to give consent or unable to understand about the protocol

ขั้นตอนในการดำเนินการวิจัย (Conduct of study)

1. Subjects who meet the inclusion criteria will be invited to participate the study
2. After informed consent, 8-10 mL of blood from pregnant women will be collected in EDTA tube
3. Blood samples will be processed immediately for plasma DNA preparation
4. The plasma will then be collected and stored at -20^{o}C until processing
5. DNA will be extracted from the plasma and will be amplified using real-time PCR to identify alpha-thalassemia 1 gene (SEA-deletion)
6. Pregnant women will have conventional prenatal diagnosis as scheduled and will be managed accordingly

7. Demographic data and the results from conventional prenatal diagnosis will be collected and compared with the results from fetal DNA in maternal serum

Plasma DNA preparation will be processed will be process within 1-3 hour after blood collection. Blood samples will be centrifuged at 3000 g for 10 minutes. Plasma will be carefully removed and transferred to clean tubes, then recentrifuged at 8000 g for 10 minutes and transferred to fresh tubes. The supernatant was then centrifuged again at 13200 rpm for 15 min and the final plasma was stored at -20^{o}C until DNA extraction and analysis.

DNA extraction from plasma using QIAamp DNA Blood Mini Kit (QIAGEN Inc., Hilden, Germany)

Real-time quantitative Polymerase Chain Reaction (RT-qPCR) assay

RT-qPCR using gap-PCR technique to determine the amount of wild type (wt) allele and alpha-thal-1 (SEA) allele was carried out on the ABI 7500 Real-time PCR system (Applied Biosystems, Foster city, CA, USA). Two primer sets, P123F/P12R and P123F/P13R, were designed to simultaneously amplify the wt allele and SEA allele, respectively (Table 1). The PCR products were monitored and analyzed by fluorescence-labeled Taqman probes specific for each amplicon; P1P2 probe (labeled with 6-FAM) for wt allele and P1P3 probe (labeled with VIC) for SEA allele. The quantity of the PCR product was determined by cycle threshold (Ct) which is the number of PCR cycles that the amount of PCR product was enough to cross the defined threshold. If the DNA template in the sample is less, it would need more PCR cycles to get enough PCR product to the threshold level and the Ct value would be high. Conversely, if the DNA template is more, the Ct value would be low.

Table I : Sequences of the primers and probes for real time quantitative PCR (RT-qPCR) for wild type and SEA allele (GenBank accession no.Z84721 and Z69706)

P123F: TCG GTC GTC CCC ACT GT

P12R: GGA CTG CTC CGC TCC AC

P13R: CAG CCT TGA ACT CCT GGA CTT AA

P1P2 probe: 6-FAM-TCT AGC CCC TGA GCA CCG-MGBNFQ

P1P3 probe: VIC-CTC CAA GTG AAC CTC C- MGBNFQ

The 25 uL reaction mixture consisted of 5 uL of plasma DNA, 1X Taqman Universal PCR master mix, 0.3 uM of each primer and 0.1 uM of each fluorescence-labeled probe. The PCR conditions were as followed; 50^{o}C for 2 min, 95^{o}C for 10 min, then 45 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 min.

The efficacy of each primer set was tested before use in 10 heterozygous blood samples from men in which the ratio of wt allele and SEA allele was one to one. Therefore the cycle threshold (Ct) of both alleles should be closed to one another, and the delta Ct (Ct of wt allele – Ct of SEA allele) should be close to zero. The delta Ct of the designed primer sets ranged between -0.21 to 0.26, thus confirmed that both primer sets had almost equal efficacy and can be used in this study. Theoretically, delta Ct of heterozygous alpha-thalassemia (alpha-trait) should be close to zero while delta Ct of Hb Bart's, and normal genotype should be higher and lower than zero, respectively.

วิธีวิเคราะห์ข้อมูล (Method of data analysis)

SPSS will be used to analyze sensitivity, specificity, and accuracy of the test.

บทที่ 4
ผลการวิจัย

One hundred and forty nine samples were recruited into this study including 62 Hb Bart's hydrops, 62 alpha-trait and 25 genotypic normal fetuses. Mean gestational age was 17.23 +/- 3.61 weeks (range 11-28 weeks). The delta Ct of the samples from each group was shown in Figure 1. Mean delta Ct was 1.04 +/-0.38, 0.21+/-0.37 and 0.20+/-0.59 in Hb Bart's hydrops, alpha-trait and normal fetuses, respectively. Mean delta Ct of Hb Bart's hydrops was statistically different from that of alpha-trait and normal group ($p < 0.01$). However, mean delta Ct between alpha-trait and normal could not be differentiated. Based on the Receiving Operating Curve (ROC) of delta Ct value, the best cut-off point for predicting Hb Bart's hydrops was 0.51 (Figure 2), which gave 98.4% sensitivity and a false-positive rate of 23.0%. All but one Hb Bart's hydrops (98.4%) had delta Ct above 0.51, whereas most alpha-trait (74.2%) and normal (84.0%) had delta Ct below 0.51. Using delta Ct 0.51 as a cut-off would give 98.4% sensitivity, 77.0% specificity, 75.3% positive predictive value and 98.5% negative predictive value. All samples were also tested for SRY gene using RT-PCR to confirm the presence of fetal DNA in maternal plasma. There were 77 female and 72 male fetuses in this study and the results of SRY gene determination were consistent with the gender of the fetuses in all cases.

Figure 1 : Delta Ct (Ct of wt allele – Ct of SEA allele) of maternal plasma DNA analysis from pregnant women who carried Hb Bart's hydrops (N = 62), alpha-trait (N = 62) and normal fetuses (N = 25).

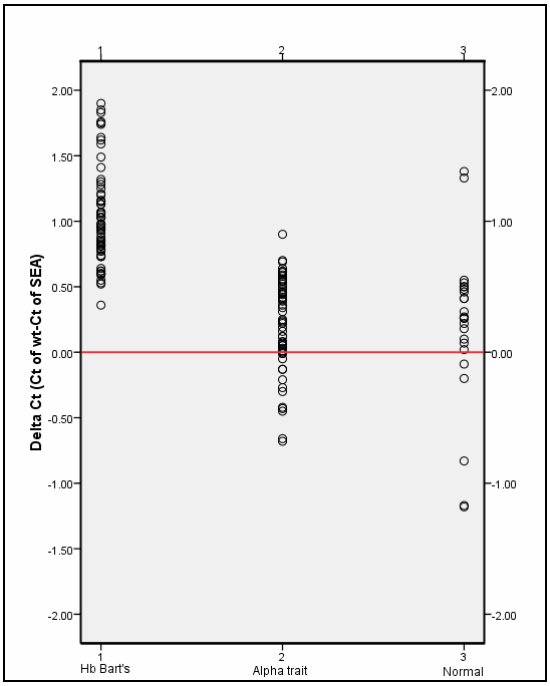
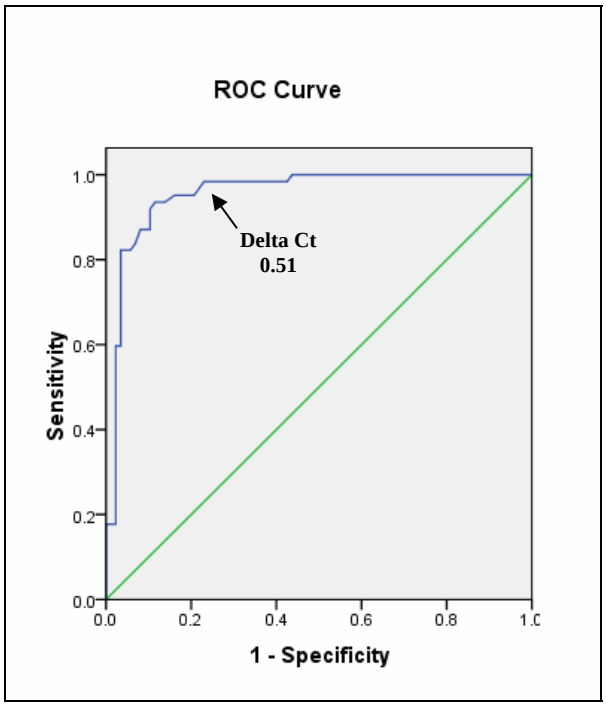


Figure 2 : ROC curve of delta Ct for predicting Hb Bart's hydrops fetus (are under the curve was 0.96 and 95% confident interval was 0.92-0.99)



บทที่ 5
สรุป อภิปรายผล และข้อเสนอแนะ

In maternal plasma, free fetal DNA is of little amount (3.4-6.2%) compared to free maternal DNA[14]. In couple at risk of having Hb Bart's fetuses, the mother is always heterozygote and carries both wt allele and SEA allele in equal amount. Fetal DNA from the affected fetus that passed into maternal plasma contained only SEA allele, resulting in increased total amount of SEA allele in the maternal plasma.

In this study, the delta Ct in Hb Bart's group was increased which indicated that there was higher amount of SEA allele (from fetal DNA) than wt allele in maternal plasma, resulted in less number of the PCR cycle required to amplify the SEA allele to reach the threshold. The delta Ct of alpha-trait fetuses was closed to zero as expected, but normal fetuses also showed delta Ct close to zero in spite of the negative value. As a result, we could not distinguish normal from alpha-trait fetuses in this study. However, in clinical practice the fetuses in both conditions are unaffected and may not be necessary to distinguish one from another.

Our finding suggested that quantitative RT-qPCR for prenatal diagnosis of Hb Bart's hydrops had good sensitivity when using the delta Ct of 0.51 as a cut-off. Although this method could not exclusively differentiate unaffected fetuses from Hb Bart's, invasive prenatal testing can be avoided in approximately one-third (77.0%) of the unaffected fetuses if this test was performed.

Previous report by Pornprasert et al[30] using RT-qPCR with cff-DNA in maternal plasma showed that delta Ct was above zero in all Hb Bart's fetuses, which was consistent with our study. However, they were unable to differentiate Hb Bart's from the unaffected fetuses possibly due to small sample size.

Ho et al[31] reported the use of microsatellite markers to detect non-deletion (wild type) paternal allele in maternal plasma in order to exclude Hb Bart's fetus. In their study only one-third of the fetuses can be excluded, these markers were uninformative in another two-third of the cases because both parents share the same markers. Therefore, to adopt this technique these markers need to be tested in advance to see whether they were informative in each couple.

The advantage of serial ultrasound to detect Hb Bart's fetuses is that it is non-invasive. The ultrasound findings of fetal anemia in these fetuses include increased

cardio-thoracic ratio, increased placental thickness, ascites, hepatomegaly and increased middle cerebral artery peak systolic velocity. Although some studies reported that ultrasonographic changes could be detect as early as 12 weeks[32;33], but the majority of the cases show such finding in midpregnancy. Furthermore, it needs to be performed by experienced sonographers and high resolution ultrasound machines. The presence of cff-DNA in maternal plasma as early as 7th weeks gestation[14] opens another possibility of early detection of Hb Bart's fetuses. However, future researches need to be explored in very early pregnancy.

In conclusion, there is a positive trend to use cff- DNA in maternal plasma for prenatal diagnosis of alpha-thalassemia-1. With this technique invasive prenatal testing and its complications can be avoided in approximately two-third of unaffected fetuses.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ชื่อผู้แต่ง : Sirichotiyakul, S., Charoenkwan, P., Sanguansermsri, T.
ชื่อเรื่อง : Prenatal diagnosis of homozygous alpha-thalassemia-1 by cell-free fetal DNA in maternal serum
ชื่อวารสาร : American Journal of Hematology

สถานะของการตีพิมพ์ : submitted

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)

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- เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)

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- เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

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- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

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3. อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

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ภาคผนวก

(Manuscript)

Prenatal diagnosis of homozygous alpha-thalassemia-1 by cell-free fetal DNA in maternal serum

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Abstract

Objective

To investigate the possibility of using cell-free fetal DNA (cff-DNA) to determine the fetal status in pregnant women who were at risk of having Hb Bart's fetuses

Material and Method

Couples who both were alpha-thalassemia carrier (SEA deletion) were invited to participate in the study at the time they came for prenatal diagnosis (chorionic villi sampling or fetal blood sampling). Plasma DNA was extracted from 10 mL of maternal blood. Real time quantitative PCR was performed using fluorescence-labeled probe to monitor wild type (wt) allele and SEA allele. The cycle threshold (Ct) was used to determine the quantity of each allele. Delta Ct (Ct of wt allele – Ct of SEA allele) was calculated from each sample. Prenatal diagnosis was performed using standard technique to determine fetal status (Hb Bart's, alpha-trait, or normal).

Result

There were 62 Hb Bart's, 62 alpha-trait and 25 normal fetuses recruited into this study. Mean delta Ct was 1.04 +/-0.38, 0.21+/-0.37 and 0.20+/-0.59 in Hb Bart's hydrops, alpha-trait and normal fetuses, respectively. Based on the Receiving Operating Curve (ROC) of delta Ct value, the best cut-off point for predicting Hb Bart's hydrops was 0.51, which gave 98.4% sensitivity and a false-positive rate of 23.0%. All but one Hb Bart's hydrops (98.4%) had delta Ct above 0.51, whereas 74.2% of alpha-trait and 84.0% of

normal fetuses had delta Ct below 0.51. Using delta Ct 0.51 as a cut-off gave 98.4% sensitivity, 77.0% specificity, 75.3% positive predictive value and 98.5% negative predictive value.

Conclusion

There is a positive trend to use cff- DNA in maternal plasma for prenatal diagnosis of alpha-thalassemia-1. With this technique, invasive prenatal testing and its complications can be avoided in approximately two-third of unaffected fetuses.

Keywords: thalassemia, alpha-thalassemia, Hb Bart's, fetal DNA, maternal plasma, prenatal diagnosis

Running Title: PND of Hb Bart's by cff-DNA in maternal serum

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Introduction

Prenatal diagnosis for genetic diseases nowadays is still based on invasive procedures such as chorionic villus sampling (CVS), amniocentesis or fetal blood sampling (cordocentesis). These techniques, however, accompanied with risk of fetal losses. Since the discovery of cell-free fetal DNA (cff-DNA) in maternal plasma by Lo et al[1], there had been huge progress in non-invasive prenatal diagnosis (NIPD). Detection of the paternal allele not present in maternal genotype using cff-DNA, such as Y-specific sequence or Rh antigen, was reported in several studies[2-6]. However, there were limited number of studies using cff-DNA for NIPD on genetic disorders where the mother and the father carried the same mutation.

Homozygous alpha-thalassemia (Hb Bart's) is one of the most common inherited genetic diseases in Southeast Asia. Couples who both were the carrier of alpha-thalassemia 1 gene will have a 25% risk of having affected fetuses as a result of autosomal recessive inheritance. Pregnant women who carried Hb Bart's fetuses will usually have increased risk for complications such as preeclampsia and postpartum hemorrhage. Therefore, prenatal diagnosis should be offered to prevent these complications. Prenatal diagnosis for this condition can either be done by invasive procedures or by serial ultrasound to detect the ultrasonograpic signs of fetal anemia which usually developed in midtrimester[7-10]. There were a few studies reported using cff-DNA for diagnosis of this condition[11-15]. Most studies had a small sample size and the results were controversial. In this study we would like to investigate the possibility of using cff-DNA to determine the fetal status in pregnant women who were at risk of having Hb Bart's fetuses in a larger scale.

Materials and Methods

Plasma DNA preparation:

This study was approved by research ethic committee of Faculty of Medicine, Chiang Mai University. During May 2008 to September 2010, pregnant women and their husbands who both were alpha-thal 1 carrier (SEA deletion) and at risk for having Hb Bart's fetuses were invited to participate in this study at the time they were seeking for prenatal diagnosis, either by CVS or cordocentesis. Ten milliliters of venous blood were obtained in EDTA tube from the pregnant women before the invasive prenatal diagnosis

procedures. Plasma was separated from maternal blood within 3 hours after the collection by 3-step centrifuge. First, EDTA blood was centrifuged at 3000 rpm for 10 min. The plasma was collected and subjected to second centrifuged at 8000 rpm for 10 min. The supernatant was then centrifuged again at 13200 rpm for 15 min and the final plasma was stored at -20oC until DNA extraction and analysis. DNA was extracted from 2400 uL of plasma according to manufacturer's protocol using QIAamp DNA Blood Mini Kit (QIAGEN Inc., Hilden, Germany) and brought up to 50 uL of plasma DNA. Prenatal diagnosis using conventional methods (CVS or cordocentesis) and DNA analysis using gap-PCR technique as described by Sanguansermsri et al[16] to confirm the diagnosis was performed in all cases.

Real time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR using gap-PCR technique to determine the amount of wild type (wt) allele and alpha-thal-1 (SEA) allele was carried out on the ABI 7500 Real-time PCR system (Applied Biosystems, Foster city, CA, USA). Two primer sets, P123F/P12R and P123F/P13R, were designed to simultaneously amplify the wt allele and SEA allele, respectively (Table 1). The PCR products were monitored and analyzed by fluorescence-labeled Taqman probes specific for each amplicon; P1P2 probe (labeled with 6-FAM) for wt allele and P1P3 probe (labeled with VIC) for SEA allele. The quantity of the PCR product was determined by cycle threshold (Ct) which is the number of PCR cycles that the amount of PCR product was enough to cross the defined threshold. If the DNA template in the sample is less, it would need more PCR cycles to get enough PCR product to the threshold level and the Ct value would be high. Conversely, if the DNA template is more, the Ct value would be low.

The 25 uL reaction mixture consisted of 5 uL of plasma DNA, 1X Taqman Universal PCR master mix, 0.3 uM of each primer and 0.1 uM of each fluorescence-labeled probe. The PCR conditions were as followed; 50oC for 2 min, 95oC for 10 min, then 45 cycles of 95oC for 15 seconds and 60oC for 1 min.

The efficacy of each primer set was tested before use in 10 heterozygous blood samples from men in which the ratio of wt allele and SEA allele was one to one. Therefore the cycle threshold (Ct) of both alleles should be closed to one another, and the delta Ct (Ct of wt allele – Ct of SEA allele) should be close to zero. The delta Ct of the designed primer sets ranged between -0.21 to 0.26, thus confirmed that both primer sets had almost equal efficacy and can be used in this study. Theoretically, delta Ct of

heterozygous alpha-thalassemia (alpha-trait) should be close to zero while delta Ct of Hb Bart's, and normal genotype should be higher and lower than zero, respectively.

Statistical analysis

Data analysis was performed using SPSS version 17.0 software. Student t-test was used to compare means between groups. P-value < 0.05 was considered statistically significant.

Results

One hundred and forty nine samples were recruited into this study including 62 Hb Bart's hydrops, 62 alpha-trait and 25 genotypic normal fetuses. Mean gestational age was 17.23 +/- 3.61 weeks (range 11-28 weeks). The delta Ct of the samples from each group was shown in Figure 1. Mean delta Ct was 1.04 +/-0.38, 0.21+/-0.37 and 0.20+/-0.59 in Hb Bart's hydrops, alpha-trait and normal fetuses, respectively. Mean delta Ct of Hb Bart's hydrops was statistically different from that of alpha-trait and normal group ($p < 0.01$). However, mean delta Ct between alpha-trait and normal could not be differentiated. Based on the Receiving Operating Curve (ROC) of delta Ct value, the best cut-off point for predicting Hb Bart's hydrops was 0.51 (Figure 2), which gave 98.4% sensitivity and a false-positive rate of 23.0%. All but one Hb Bart's hydrops (98.4%) had delta Ct above 0.51, whereas most alpha-trait (74.2%) and normal (84.0%) had delta Ct below 0.51. Using delta Ct 0.51 as a cut-off would give 98.4% sensitivity, 77.0% specificity, 75.3% positive predictive value and 98.5% negative predictive value. All samples were also tested for SRY gene using RT-PCR to confirm the presence of fetal DNA in maternal plasma. There were 77 female and 72 male fetuses in this study and the results of SRY gene determination were consistent with the gender of the fetuses in all cases.

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Acknowledgement

This project was supported by the Thailand Research Fund (grant number RSA 5180013).

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Table I : Sequences of the primers and probes for real time quantitative PCR (RT-qPCR) for wild type and SEA allele (GenBank accession no.Z84721 and Z69706)

P123F: TCG GTC GTC CCC ACT GT

P12R: GGA CTG CTC CGC TCC AC

P13R: CAG CCT TGA ACT CCT GGA CTT AA

P1P2 probe: 6-FAM-TCT AGC CCC TGA GCA CCG-MGBNFQ

P1P3 probe: VIC-CTC CAA GTG AAC CTC C- MGBNFQ

Figure 1 : Delta Ct (Ct of wt allele – Ct of SEA allele) of maternal plasma DNA analysis from pregnant women who carried Hb Bart's hydrops (N = 62), alpha-trait (N = 62) and normal fetuses (N = 25).

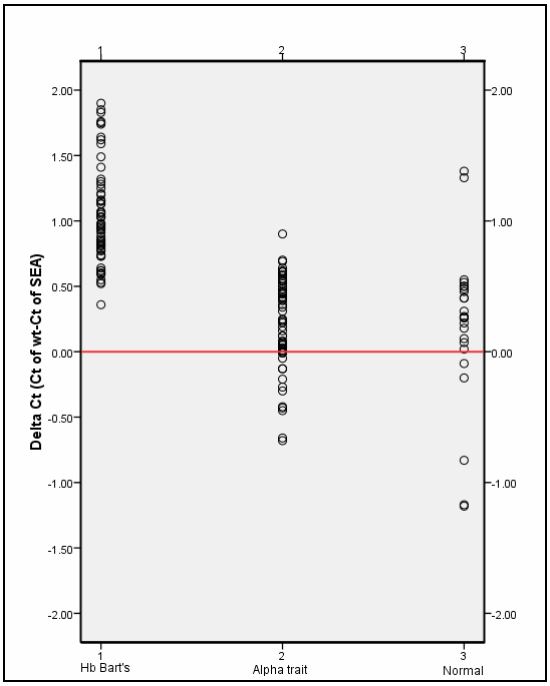

Figure 2 : ROC curve of delta Ct for predicting Hb Bart's hydrops fetus (are under the curve was 0.96 and 95% confident interval was 0.92-0.99)