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Fetal Sex Determination Using *Cell-Free Fetal Dna* (cffDNA) in Maternal Blood

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Background: Prenatal test has routinely performed in antenatal care and has become a part of the obstetric care feature in many countries. Prenatal test is divided into screening and diagnostic test. Recently, the early noninvasive method in order to found and lessen the risk factors of pregnancy loss, has been studied. One of the methods is molecular test using cffDNA which has many screening purpose such as sex determination, aneuploidy, paternal inherited genetic disorder, fetus rhesus, and performed early at 7 weeks of pregnancy. **Objective:** The purpose of this study is to measure diagnostic value of cffDNA in determining fetal sex prenatally. **Methods:** In a diagnostic test study, 18 randomized samples were selected and divided based on fetal gender confirmed at birth. The group consisted of 9 pregnant women with male babies and 9 pregnant women with female babies. CffDNA then isolated from maternal blood sample and specific region in Y chromosome termed SRY is detected by PCR and electrophoresis. The data obtained analyzed both descriptively for baseline characteristic and analytically to determine its diagnostic value. **Results:** This study found significant correlation between SRY detection in cffDNA with male fetal phenotype ($p<0.05$). The sensitivity of the method is 100% with 89% specificity. In addition, we found 9.09 values for positive likelihood ratio (LR+) and 0 for negative likelihood ratio (LR-). Moreover, the result yielded 100% positive predictive value (PPV+) and 88.8% of negative predictive value (PPV-). **Conclusion:** This study proofed that cffDNA have a great diagnostic value to determine fetal sex prenatally. However, further study with several group of gestational age mother and better matching is required to further confirm the diagnostic potential of cffDNA

Keywords: cffDNA, Fetal, Maternal.

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INTRODUCTION

Fetal sex determination is a common obstetric issue until today. For some parents' fetal sex determination had a special value either traditionally or as a means to prepare baby's needed. In medicine, fetal sex sometimes associated with related genomic disorder such as turner and klinefelter syndrome.[1]

However, current modality to detect fetal sex and associated genomic status is still limited. USG is common modality used in this field. In order to accurately detect by USG, gender detection is usually made beginning at 12 weeks of gestational age.

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However, at current sensitivity and specificity (86.5% and 90.6% respectively) USG still has an error margin.[2] Moreover, USG can only detect fetal morphological change and other genetic analysis is needed to confirm the diagnosis. Other diagnostic methods commonly used are Chorionic Villus Sampling (CVS) for 11-14 weeks' gestational age or amniocentesis after 15 weeks' gestational age. These methods have higher accuracy compare to USG but deemed to be too invasive and had risk harming developing fetus.[3,4]

In 1997, Lo et.al found fetal circulating cell free DNA (cffDNA) in maternal blood. This finding brought outstanding revolution in non-invasive prenatal diagnosis.[5] The DNA can be isolated from peripheral venous blood, a method that is risk free and highly cost effective. Combine with appropriate genomic testing, cffDNA has a huge diagnostic value to detect fetal condition related to its genome such as aneuploidy, Rh status,

chromosomal aberration, or sex determination.[6] Specifically for sex determination, several study had already confirmed usefulness of cffDNA. Honda et.al found that cffDNA had 100% sensitivity in determining male sex in 7 weeks' fetuses.[7] On the other hand, Hill et al. reported a sensitivity test to determine the sex of a fetus in the UK of 99.5% compared with the gold standard of the sex of the baby at birth.[8] This highlights the diagnostic potential of cffDNA as a non-invasive analysis technique particularly in fetal sex determination.

MATERIAL AND METHOD

Study Design and Sample Collection

A cross sectional analytic study was conducted at Obstetrics and Gynecology Department of Sanglah Hospital conducted from 2011 until 2012. Pregnant women with singleton fetus confirmed by USG and agreed to participate were selected. Patients with comorbidities such as preeclampsia, intra-uterine growth retardation, or confirmed had baby with congenital disorder were excluded. According to calculation[9], the minimum number of sample required was 16 pregnant women. For analytical purpose, the subjects will be divided into 2 groups based on fetal gender confirmed at birth. Each group consists of 9 subjects. 5 ml of peripheral venous blood were obtained from subjects and collected in EDTA tube. The sample was stored in 4^0C until processed further.

Blood Processing and Cell Free Fetal DNA Isolation

After blood samples were collected, the blood was processed to isolate cffDNA within it. The blood samples were centrifuged at 1600g for 10 min at 4^0C. The plasma portion was transferred into micro centrifuged tubes and centrifuged at 7470g for 10 min at 4^0C and the supernatants were collected into another micro centrifuged tubes. cffDNA isolation was conducted using Plasma Circulating Nucleic Acid Isolation Kit (*Promega*) based on product protocol.

Primer Construction and DNA Amplification by Polymerase Chain Reaction

In order to differentiate fetal sex based on cffDNA, we Sex Determining Region of Y (SRY), one of genes in Y-chromosome. We use pre-determined primer sequence for SRY that is 5'-CAT GAA CGC ATT CAT CGT GTG GTC-3'; and 5'-CTG CGG GAA GCA AAC TGC AAT TCT T-3'.[10] 2µL of each sample cffDNA was used as template. Then we add 1.5µL of forward and reverse primer. The mixture was incubated in *thermocycler* for denaturation process at 94^0C for 1 min and at 72^0C for 7 min. Following denaturation process, we proceed to PCR reaction for 40 cycles. Each cycle consists of incubating sample in 3 different temperatures for 30 second (94^0C, 72^0C, and 57^0C). The result was visualized by electrophoresis technique. The presence of SRY (and thus Y chromosome within fetal cells) would be marked by the appearance of 254 bp band in electrophoresis gel.

Statistical Analysis

Baseline characteristic will be analyzed descriptively and compared. Then, normality test was conducted to determine data distribution. Fisher exact test was used to analyze the association between SRY status (+/-) with baby's phenotype. To evaluate the diagnostic value of cffDNA SRY in fetal sex determination, we calculate sensitivity, specificity, likelihood ratio and predictive value of cffDNA SRY. The analysis was conducted using SPSS version 16.

RESULTS

18 subjects were enrolled in this study with 9 subjects per fetal gender. The mean age for subject was 29.73 years for male group and 27.45 years for female group. There are no significant differences in gestational age as well as educational background of the samples. Normality assessment of the data from baseline characteristic showed that the data were normally distributed.

Table 1. Baseline Characteristic of Study Subjects

Characteristic	Group		*p*
	Male	Female	
Maternal Age (years old)	29,73±1,72	27,45±1,97	**0,213**
Gestational Age	38,32±0,90	38,62±1,03	**0,372**
Gravida	0,83±0,88	0,62±0,63	**0,467**
Education			**0,135**
Junior High	**1**	**2**	
Senior High	**4**	**5**	
University	**4**	**2**	

Table 1. Baseline Characteristic of Study Subjects

	SRY Positive	SRY Negative	p
Male	9 (90%)	0 (0%)	0,000 (*Fisher-Exact*)
Female	1 (10%)	8 (100%)	
Total	10 (100%)	8 (100%)	

Cross tabulation of SRY status and gender type was conducted after confirmation of babies gender at birth. Fisher-exact test showed that SRY status of CffDNA was significantly associated with male phenotype ($p<0.05$). Diagnostic analysis of the data showed that 100% sensitivity and 89%

specificity. Calculation of positive and negative likelihood ratio (LR+ and LR-) yield 9.09 and 0. Meanwhile, predictive value calculation yield 100% for positive predictive value (PPV+) and 88.8% for negative predictive value (PPV-).

DISCUSSION

Fetal sex determination is a common obstetric issue. Common method used by obstetrician to determine fetal sex is USG imaging. However, USG had a moderate specificity and sensitivity (90.6% and 86.5%, respectively) and operator dependent which mean there is some range of error when conclusion was made.[2] Other techniques like amniocentensis and chorionic villus sampling have an excellent accuracy with sensitivity and specificity close to 100% but the procedures deemed too invasive for sex determination and usually saved for more serious cases like congenital genetic defect.[3,4]

Currently, it reveals that fetal DNA (Cell Free Fetal DNA/cffDNA) is freely circulated within maternal circulation.[5,6,7] The DNA was originated from throphoblastic cell that shed into maternal circulation and lyses. Since throphoblast is genetically similar with embryo, it can be used to analyze fetal condition genomically in which sex determination is one of them.[11]

Several study had evaluate cffDNA as a new screening and diagnostic method to determinine fetal sex. Zargari et,al reported that cffDNA can be used to detect sex phenotype of human embryo as early as 6th week of gestation.[12] They found sensitivity and specificity of the method is 95.2% and 98% respectively which is in accordance with our study. Another studies show the same range of diagnostic accuracy mainly around 80-95% in sensitivity and 87-98% for specificity.[11] However, Schefer et.al showed somewhat lower sensitivity value at 74.5%.[13] But the cffDNA was not amplified in the study which was the reason for low detection threshold. Area under the curve (AUC) and ROC analysis from several studies proof that this method is more accurate than USG (0.989-0.995) in fetal sex determination.[11]

Overall, our study proof that cffDNA can be used as accurate diagnostic test to determine fetal sex with high sensitivity and specificity. However, because we only tested one group of subjects, no range of diagnostic parameter can be made and analysis of ROC was not possible. Further study involving several gestational age groups with better subject selection and matching is needed in order to investigate the true diagnostic value cffDNA in determining fetal sex.

CONCLUSION

CffDNA is a circulating fetal DNA originating from placental trophoblastic cell shed into maternal circulation. The potency of CffDNA analysis as a means to detect several genome related condition in fetus is already studied extensively. Our study confirmed its potential in fetal sex determination with high sensitivity and specificity. High PPV+ and PPV- value that we detected was also confirm high diagnostic value of cffDNA in determining fetal sex. However, further study is needed in order to confirm it diagnostic value comprehensively.

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