Early Fetal Gender Determination Using Cell-Free Fetal DNA in Maternal Circulation

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ABSTRACT

Background: The presence of cell-free fetal DNA in the maternal circulation offers an alternative source of fetal genetic material for prenatal diagnosis.
Objective: To identify fetal gender in the first trimester by detection of sex determining region on human Y chromosome using cell-free fetal DNA.
Methods: A prospective study has been carried out at AL-Yarmouk Teaching Hospital in cooperation with Forensic DNA Research and Training center at AL-Nahrain University from February to December 2014. The study included 78 individuals, sixty eight out of them pregnant women with gestational age between 7th and 11th weeks, 8 of them were excluded. So, pregnant women were sixty. Blood sample was collected from maternal plasma and DNA analyzed by real-time polymerase chain reaction (PCR).
Results: Examinations of fetal DNA in maternal plasma were performed for 60 pregnant women. 37(97.36%) out of 38 pregnant women were gave male baby and 21(95.45%) out of 22 pregnant women were gave female baby. A considerable sensitivity (97.4%) and specificity (95.5%) were obtained, which was significant in the first trimester of pregnancy ($P<0.001$) giving the study a 96.7% accuracy.
Conclusion: Fetal gender can be determined in the first trimester with a high level of accuracy by detection of sex determining region on Y chromosome using cell-free fetal deoxyribonucleic acid.
Keywords: Cell-free fetal DNA, Fetal gender, Y-chromosome.

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Cell-free fetal deoxyribonucleic acid (cffDNA) is fetal deoxyribonucleic acid circulating freely in maternal blood stream. It can be sampled by a venipuncture on the mother[1].

Detection and quantification of circulating fetal and total nucleic acids in maternal plasma has recently emerged as an alternative method for prenatal genetic diagnosis. Cell-free fetal DNA exist in plasma and serum of pregnant women as early as sixth week of gestation, with concentration increasing during pregnancy and peaking before parturition[2].

The main technical difficulty of this type of analysis is differentiation between cell-free fetal DNA and cell-free maternal DNA, which is present in plasma at higher amounts than the former; this differentiation is only possible when fetal genes or DNA segments with sequences that are different from the maternal genotype are analyzed[3].

Detection of Y-chromosome sequences in maternal plasma indicates that the gender of the fetus is male, as normal women do not have the Y-chromosome in their genome. In pregnant women in whom these sequences are not detected, the fetus is presumed female. Fetal gender determination was the first clinically available test that used analysis of fetal nucleic acids in maternal blood[4].

Determination of fetal gender using cffDNA in maternal blood can be achieved as early as 6th week of gestation by detection of Y chromosome specific sequences which are inherited from the father and absent in the maternal genome; such as the SRY, DYS14, DAZ, AMELY and

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others that are amplified using polymerase chain reaction (PCR)[5].

Using conventional PCR amplification of Y chromosome specific DNA sequences, it was able to detect fetal DNA in most blood samples of male-bearing pregnancies. The subsequent development of PCR technique enabled us to measure the concentration of cffDNA in maternal plasma. There is an increase of concentration with gestational age from a mean of 25.4 GEq/ml in early stage of pregnancy to 292.2 GEq/ml in late stage. Theoretically, the absolute concentration of cffDNA is more in maternal plasma than in serum, this difference is due to the presence of a larger quantity of background maternal DNA in maternal serum compared with plasma. However, the percentage of cffDNA concentration in proportion to the total DNA circulating in maternal blood is about 3.4% in early pregnancy and reach to about 6.2% or even up to 10% in late pregnancy, while the rest being maternal in origin[6].

The distinguishing of cffDNA in an overwhelming background of maternal DNA is a significant technical challenge. There is a number of general problems associated with detecting cffDNA in the maternal circulation include: The concentration of all cffDNA in blood is relatively low. The total amount of cell-free DNA varies between individuals cffDNA molecules are outnumbered 20:1 by total maternal DNA molecules. The fetus inherits half its genome from the mother[7].

Therefore, a number of methods have been developed to address these problems. Once a sample of maternal blood has been taken, the plasma fraction is separated from cellular matter by centrifugation. This is followed by isolation and purification of cffDNA with different handling procedures. As the total DNA fragments have been purified, small differences between the fetal and maternal DNA that are exploited by detection of the sequences located on Y chromosome that are paternal in origin. Therefore, the only way to identify them is through male-bearing pregnancies. Also, to distinguish fetal from maternal DNA sequences, variable regions of the extracted DNA are detected which might be differ even by a single nucleotide base from maternal DNA[8].

Amplification is done by using conventional PCR or real-time PCR, the most common and efficient technique currently used for detection and identification of these small amplicons, even when the circulating fetal DNA fragments are very small. Because the fetal DNA is not immediately metabolized and is so readily amplified from plasma or serum, it has been suggested that the circulating DNA is protected within apoptotic bodies[9].

The turnover of circulating fetal DNA was subsequently studied by investigating their clearance after delivery. Most of women had undetectable levels of circulating cffDNA by 2 h postpartum. The mean half-life for fetal DNA was estimated to be 16.3 min (range 4–30 min). So it was assumed that to maintain a steady state, fetal DNA must be continuously liberated in large quantities into maternal circulation. These calculations suggested that cffDNA is liberated at a mean rate of 2.24–104 copies/min. This rapid turnover implies that fetal DNA measurement provides an almost real-time picture of fetal DNA production and clearance and thus, may be useful for monitoring feto-maternal events having rapid dynamics[10].

The most important clinical applications of using cffDNA in NIPD are based on distinct and detectable differences between fetal and maternal genomes as follow[11]:

(1) Sex determination: by detecting SRY gene sequences on Y chromosome used for single gene disorders.

(2) Rhesus gene: by detecting fetal RhD and blood group.

(3) Abnormal placentation associated with pregnancy-related disorders: by detecting the presence of an elevation in the absolute concentration of cffDNA.

(4) Aneuploidy: by detecting an abnormal concentration of a particular chromosome, potentially using cffDNA specific to the fetus and chromosome of interest.

This method can be particularly beneficial in providing special treatment on fetus in cases of genital ambiguity or metabolic conditions such as congenital adrenal hyperplasia.

Methods

This prospective study was carried out in the Department of Obstetrics and Gynecology of Al-Yarmouk Teaching Hospital in cooperation with the Forensic DNA Research and Training Center at Al-Nahrain University Baghdad-Iraq. It was conducted from February to December 2014. The study was approved by the Committee of Iraqi Scientific Council for Medical Specialization of Obstetric and Gynecology. Healthy pregnant women without pregnancy-associated complications were chosen by simple criteria. A simple questionnaire format was done to evaluate the dependent variables such as maternal age, gestational age, obstetrical history and medical history. A verbal consent was obtained from all participants before enrolling them in the study.

The study includes 78 individuals, 68 out of them pregnant women of reproductive age, with single viable pregnancy and gestational age between 7^{th} and 11^{th} week, those were collected over a period of three months from the start of the study. From the total 68 pregnant women, spontaneous miscarriage was observed in three cases, while in five cases the follow up was lost, so they were excluded from the final statistical analysis and so pregnant women were sixty.

Ultrasound was done for all the participants before withdrawal of blood sample from them to date pregnancy, ensure its viability and to exclude multiple gestations. For those whom ultrasound findings were difficult to identify the viability of their pregnancy due to early gestational age, an appointment to repeat their ultrasound after two weeks was given to them. Those with viable pregnancy were included in the study, while those 10 with non-viable pregnancy were excluded.

The follow up of the pregnant was done by taking their phone number and call them to know the outcome of their pregnancies later on.

Steps for Gender Detection: Five ml of whole peripheral blood was collected for all groups in a tube containing 200 µl of 0.5 MEDTA and immediately stored in an icebox at 4˚C. After collection between 4-6 blood samples, they were taken immediately to The Forensic DNA Research and Training Center at Al-Nahrain University. The overall time from sample collection till reaching the center usually did not exceed 2 hours. At this center, the samples were centrifuged at 3000 g for 10 min and the upper plasma layer was carefully removed without disturbing the buffy coat, transferred into a new 1.5 ml microcentrifuge tube for further processing for DNA extraction.

DNA Extraction: The genomic DNA was extracted using the QIAamp DNA Blood Mini kit (Qiagen, USA) that recommended by the manufacturer's protocol as the following steps: From QIAGEN Protease, 20µl pipetted into the bottom of a 1.5 ml Eppendorf tube, then 200µl of the plasma sample that was previously prepared added into the tube. Of Buffer AL, 200µl added into the sample and mixed by pulse-vortexing for 15 seconds. Incubated at 56^{o}C for 10 min. The tube centrifuged to remove drops from the inside lid and 200µl of ethanol added to the sample and mixed again by pulse-vortexing for 15 seconds. Then the tube centrifuged again to remove the drops.

The mixture from the last step was applied into the QIAGEN spin column, the cup closed and centrifuged at 8000 rpm for 1 min. Then the spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded.

The spin column carefully opened and 500µl of Buffer AW1 added and centrifuge at 8000 rpm for 1 min. Then the spin column placed in another clean 2 ml collection tube and the tube containing the filtrate discarded. Of Buffer AW2, 500µl added

carefully into the spin column and centrifuged at 14000 rpm for 3 min.

The spin column added in a new 1.5 ml Eppendorf tube and the collection tube containing the filtrate discarded. The spin column opened and 60µl of Buffer AE added, then incubated at room temperature for 5 min, then centrifuged at 8000 rpm for 1 min.

The filtrate in the tube now contained the extracted DNA (maternal + fetal). Then 2µl of the extracted DNA was used to measure the concentration and purity of DNA automatically using Scanning NanoDrop System.

Primers: SRY, DYS14 and DAZ genes are used for detection of sequences on the human Y-chromosome in male-bearing pregnancies. In this study, only SRY gene (Alpha DNA, Canada) was used as an internal control of gender determination. Primers are short sequences of bases made specifically to recognize and bind to the section of DNA that want to be amplified by using PCR, which are the very specific sequence of bases that are part of the gene of interest. Primers are called "forward" and "reverse" in reference to the direction that the bases within the section of the double strand DNA are copied.

DNA amplification using conventional PCR: The amplification reactions were set in a total volume of 25 µl and are ready to be amplified using PCR which was carried out on an Applied Biosystem act as a thermocycler instrument run in 40 cycles. The method of amplification performed as follow:

1. The first cycle of PCR was to separate the strands of DNA into two single strands by increasing the temperature of the sample to 95^{o}C for 5 min. This is called "DNA denaturation".

2. Once the strands separated, the sample was cooled slightly to 60^{o}C for 1 min and forward and reverse primers would be allowed to bind to the single DNA strands.

3. After the two pairs of primers had been attached to each strand of the DNA, TaqMan™ polymerase enzyme (Promega Company, USA) copied the DNA sequence on each half of the helix from the forward to the reverse primer, forming two double stranded sections of DNA, each with one original half and one new half. TaqMan™ polymerase is especially helpful for laboratory testing because (unlike many other enzymes) it does not break down at very high temperatures needed to do PCR product.

4. When heat was applied again, each of the two double strands separated to make four single strands and, when cooled, the primers and polymerase act to make four double strand sections. The four strands became eight in the next cycle, eight became sixteen, and so on.

5. Within 30 to 40 cycles, as many as a billion copies of the original DNA section can be produced and are then available to be used in numerous molecular diagnostic tests. This process had been automated so that a billion copies of the original DNA can be produced within a few hours. At the end of PCR program, the PCR product was ready and containing the amplified DNA fragments.

After electrophoresis, the molecular size of specific nucleic acids or bands on SRY gene were compared with the molecular size of DNA bands in what is known as the "DNA step ladder" (Promega Company, USA) which is a DNA marker with 25/100 bp. For our target gene, the size was 203 bp.

During Gel Documentation System which is an advanced technique worked by the principle of fluorescence, these nucleic acids and bands were stained with the fluorescent substance (ethidium bromide + loading dye) and visualized by exposure to ultraviolet light. A fluorescent light was emitted and the intensity of this fluorescence depends on the concentration and molecular weight of the nucleic acids and bands of SRY gene on Y chromosome sequences.

The aim of this study was to identify fetal gender in the first trimester by detection of

sex determining region on human Y chromosome using cell-free fetal DNA.

Statistical analysis: Each patient was assigned a serial identification number. The data were reviewed, cleaned and carried out with double check entry into the computer using Statistical Package for Social Sciences (SPSS) version 20.

Pearson's chi-square test (x2-test) was used. Independent t-test and analysis of the variance (ANOVA) tests were used for comparing between the continuous variables of the study sample.

Reliability values sensitivity, specificity, positive predictive value, negative predictive value and test accuracy were calculated.

The level of significance in this study was of p – value less than 0.05.

Results

This study was carried out on seventy eight participants. Sixty eight of them were pregnant women with gestational age between 7th and 11th week subjected to the study to identify the gender of fetus by a laboratory test through detection of SRY gene sequence on Y-chromosome in male-bearing pregnancy. Five non pregnant women and five men were included in the study as negative and positive controls, respectively.

From the total 60 pregnant women, 38(63.4%) of them gave a male outcome and the study diagnosed 37 of them correctly, while 22(36.6%) had a female outcome in which 21 of them were identified correctly by the study.

The mean of gestational age at time of blood sampling among the included pregnant women was 9.2±1.4 weeks. More than one fourth (28.3%) of the participants were in their 10th week of pregnancy being the most prevalent group, while only 5 of them (8.3%) were in their 7th week of pregnancy and considered as the least frequent gestational age, (Table 1).

There was no association between the extracted DNA concentration and the purity; with the gestational age of the pregnant women. The mean highest concentration was 56.3±17.3 ng/µl in the 9th week of gestational age, while the lowest mean concentration was (44.7±18.4) ng/µl in the 10th week of the gestational age. There was no significant statistics between the concentration (p=0.465) and purity (p=0.398) of the extracted DNA with the gestational age, (Table 2).

There was no correlation between DNA concentration and purity with the expected male or female gender that identified by the laboratory test among the included maternal samples, (Table 3).

A false positive result was observed in the present study at the age of 8+5 weeks, citing the theory of vanishing twins within the first 7 weeks of gestation as a possibility of this result. It concluded that at time of blood sampling there was a male twin that disappeared in the subsequent weeks of pregnancy and only baby girl was born. This is occurred in 0.3-0.7% of pregnancies. The other possibility of this false positive may result from the probability of maternal blood sample contamination with exogenous male DNA during time of blood sampling or during DNA extraction procedure.

The only false negative result occurred in a sample with gestational age 7+2 weeks that could be belong to the insufficient cffDNA concentration in maternal circulation at this early gestational age.

In a comparison between the laboratory test results with the outcomes at birth; the current study was highly significant in detecting the gender of the fetus in first trimester (p<0.001), (Table 4).

The main reliability values of the study in comparison to the real outcome of the gender after delivery are list in table 5. The sensitivity and the positive predictive value were 97.4%, the specificity and the negative predictive value were 95.5%, and the accuracy was 96.7%.

Table 6 demonstrated the formulas for counting the reliability values of the study for gender prediction among the included

pregnant women in comparison to the real gender after delivery.

Table 1: Frequency and percentage of the gestational age among the pregnant women group included in the study, n=60.

Gestational age (weeks)	Number	Percentage
7	8	11.8
8	12	17.6
9	16	23.5
10	18	26.5
11	14	20.6

Table 2: Association between the concentration and purity of the extracted DNA with the gestational age, n=60.

Parameter	Gestational age (weeks)	Number	Mean±(SD)	p-value†
Concentration of the extracted DNA (ng/µl)	7	12	53.2±(20.5)	0.465 (NS)
	8	8	48.6±(19.4)	
	9	16	56.3±(17.3)	
	10	18	44.7±(18.4)	
	11	14	51.0±(18.2)	
Purity of the extracted DNA	7	12	1.6±(0.15)	0.398 (NS)

ANOVA, SD: standard deviation, NS: not significant.

Table 3: Correlation between the extracted DNA concentration and purity with the expected laboratory, n=60.

Parameters	Laboratory test		p-value[a]
	Male (n=40) Mean±(SD)	Female (n=28) Mean±(SD)	
DNA concentration (ng/µl)	48.8 ±(19.4)	53.4±(17.3)	0.305 (NS)
Purity	1.7 ±(0.17)	1.6 ±(0.17)	0.448 (NS)

Independent t-test, SD: standard deviation, NS: not significant at alpha >0.05.

Table 4: Comparison between the results of the expected gender according to laboratory test and the real gender at birth, n=60.

		Gender at Birth		Total
		Male No. (%)	Female No. (%)	No. (%)
Lab. Results	Male	37 (97.4)	1 (4.5)	38 (63.3)
	Female	1 (2.6)	21 (95.5)	22 (36.7)
Total		38 (100.0)	22 (100.0)	60 (100.0)

Pearsons chi-square=55.8 p<0.001(significant<0.05)

Table 5: Values of the sensitivity, specificity, PPV, NPV, accuracy, PLR and NLR of the current study, n=60.

Test	Value
Sensitivity	97.4%

Specificity	95.5%
Positive predictive value	97.4%
Negative predictive value	95.5%
Accuracy	96.7%
Positive likelihood ratio	21.4
Negative likelihood ratio	0.03

Table 6: 2x2 table (a gold standard) calculating the reliability values of the current study for gender detection confirmation, n=60.

		Gender at birth		
		Male	Female	
Test results	Male SRY(+)	True positive (TP) = 37	False positive (FP) = 1	Positive predictive value = TP / (TP + FP) = 37 / (37 + 1) = 97.4%
	Female SRY(-)	False negative (FN) = 1	True negative (TN) = 21	Negative predictive value = TN / (FN + TN) = 21 / (1 + 21) = 95.5%
		Sensitivity = TP / (TP + FN) = 37 / (37 + 1) = 97.4%	Specificity = TN / (FP + TN) = 21 / (1 + 21) = 95.5%	Accuracy =(TP+TN)/(TP+FP+FN+TN) = (37+21)/(37+1+1+21) =96.7%

Positive likelihood ratio = Sensitivity/ (1- Specificity) = 0.974/ (1- 0.955) = 21.4 □ this means that if the women is really pregnant with male so the study has 21.4 times to detect the gender as a male (positive test) than as a female.

Negative likelihood ratio = (1- Sensitivity)/ Specificity = (1- 0.974)/0.955 = 0.03 □ this means that if the women is really pregnant with a male, then the study has a very weak chance to diagnose the pregnancy as a female (negative test).

Discussion

The findings of this study demonstrate that non-invasive prediction of fetal sex from examination of ccffDNA in maternal blood can be achieved with high accuracy in the first trimester of pregnancy.

In this study, we have shown that the increase in total cell free DNA that occurs with increasing time from blood draw can largely be prevented by taking blood into tubes which stabilize maternal cells, thus allowing the proportion of cell-free fetal DNA to remain more or less constant[12].

In current study, we show how a simple protocol that implies the study of SRY gene in maternal blood at early gestational age allows us to have 96.7% accuracy in the diagnosis meaning that this study is highly accurate and useful to be enrolled in clinical practice. The mean gestational age at time of blood sampling was (9.2 ± 1.4) weeks.

The Y-chromosome specific sequences used in most of the studies were either the single-copy SRY or the multi-copy DYS14. Two studies comparing the performance of the SRY and DYS14 sequences in a combined total of 94 pregnancies with male fetuses reported that only 62(66.0%) were

correctly predicted by prenatal testing with the SRY sequence, compared to 93(98.9%) with the DYS14 sequence, (Zimmermann et al, 2005). The problem with the DYS14 sequence is that it has considerable homology to sequences other than Y-chromosome that could falsely classify female fetuses as male[13].

Kutyavin IV study (2000) was done on 43 pregnant women which identify Y specific sequences in 24 of the 30 maternal plasma samples for women bearing male fetuses and no positive results for the reminder 13 women bearing female fetuses. In this study, the researchers used the heat extraction method as a procedure for DNA extraction, in this conventional method, the concentration and purity of the extracted DNA will be less efficient. Also, they used plasma volume 10µl for extraction, where at a similar volume when used only 17% of samples gave positive results in male bearing pregnancies. These contributing factors led to decrease the sensitivity of this study to be 80%. Whereas in the current study, QIA amp DNA Blood Mini Kit with plasma volume 200µl was used for DNA extraction giving 97.4% sensitivity[14].

Houfflin-Debarge et al study (2000) they utilized maternal serum samples for DNA extraction by using QIAamp Blood Mini Kit. The sensitivity of this study for diagnosing male-bearing pregnancies was 46%. This low diagnostic sensitivity was disagreed with sensitivity of the present study that was 97.4% which may belong to using maternal serum for DNA extraction rather than plasma that was used in present study[15].

The present study is in agreement with Rijnders RJ et al study (2001), which was done to identify fetal sex in first trimester by isolating free fetal DNA in maternal plasma in pregnancies at risk of (CAH). The SRY gene was used as a fetal marker for the fetal Y chromosome detected by quantitative polymerase chain reaction analysis. It was performed on 25 and 19 women in the first and second trimester, respectively. Fetal sex was detected correctly in 97.8% of cases that is close to the accuracy of the current study which was 96.7%[16].

Honda H et al (2002), a study was done by using a real-time quantitative PCR (RT-qPCR), the results of this study was demonstrated 100% sensitivity for fetal gender determination after 5th weeks of gestation. By comparing with sensitivity of the present study which was 97.4%, this may be due to the use of RT-PCR which accurately quantifies fetal DNA in maternal plasma and serum through identification of fetal gender by SRY sequences, while in the present study the conventional PCR was used[17].

Davalieva K et al study (2006), was carried out on 46 pregnant women prior to amniocentesis. DNA was extracted from maternal plasma using a QIAmp DNA Blood Mini Kit. The SRY gene was detected in 25 of 28 plasma samples from women with male fetuses and in none of the 18 samples from women with female fetuses giving this study a sensitivity 89.2% and specificity 100%, in comparison with the results of the present study which were 97.4% and 95.5%, respectively[18].

Khorram Khorshid HR et al study (2013), include 80 pregnant women with gestational age between 6th to 10th weeks, fetal sex was identified using SRY, DYS14 & DAZ sequences. All the obtained results were compared with the actual gender of the newborns after birth to calculate the test accuracy. By using SRY and DAZ sequences, a sensitivity of 97.3% and 94.5% respectively was obtained. While using DYS14 sequence, gender of all newborn boys was identified correctly giving 100% sensitivity. This was belong to the use of the multicopy DYS14 gene that considered to be the best approach for early fetal gender assessment because it is more sensitive, accurate, and efficient when compared to the single copy SRY gene. The PPV and NPV of this study when only SRY gene was used were 97.3% (95% CI= 0.862- 0.995) and 97.4% (95% CI=0.865-0.995), respectively. This was close to the PPV and NPV results in the present study which were 97.4% and 95.5%, respectively[19].

By Perlado-Marina S et al study (2013), two blood samples at different gestational, 2weeks apart, were be taken to identify the SRY gene by using RT- qPCR. Neither false positives nor false negatives diagnoses have been registered, thus optimizing a diagnostic accuracy of 100%. While in the present study, a single blood sample was used giving the study an accuracy of 96.7%[20].

Determination of fetal sex based on the detection of Y-chromosome specific markers in maternal plasma is prone to false-negative results because female fetuses are not detected directly but inferred by a negative result for Y-chromosome sequences, which could also caused by undetectable levels of ccffDNA.[21]

In conclusions; fetal gender can be determined in the first trimester with a high level of accuracy by detection of SRY gene sequences on Y chromosome using cell-free fetal deoxyribonucleic acid by a reliable, simple and non-invasive test.

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