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Original Article

Sex determination using free fetal DNA in early pregnancy: With the approach to sex linked recessive disorders

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Abstract
Introduction: Prenatal diagnosis is testing for detection of diseases or conditions in a fetus or embryo before it is born. Most of prenatal diagnostic (PD) techniques are invasive and done in late stages of pregnancy. Using fetal DNA in maternal blood for fetal sex determination in early pregnancy might help in management of X-linked genetic diseases. This study aimed to investigate the accuracy of sex determination using fetal DNA in maternal blood at 8-12 weeks of gestation.
Methods: In this cross-sectional study, 30 pregnant women at 8-12 weeks of gestation were enrolled. The sex-determining region Y (SRY) gene expression with the internal control (IC) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was investigated with quantitative real-time polymerase chain reaction (PCR) using specific primers and probes.
Results: Accuracy of sex determination with SRY gene expression in 8-12 weeks of pregnancy were 85%, 85%, 90% and 100% respectively.
Conclusion: It seems that fetal sex determining using fetal DNA in maternal blood is a reliable method for early stage of pregnancy.

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Introduction

The widespread use of prenatal diagnostic (PD) techniques date back to more than three decades. PD refers to the ability of diagnostic tools to detect diseases and disorders of the fetus. Also, PD focuses on detecting anatomic and physiologic problems with the zygote, embryo or fetus as early as possible, even before gestation. Many genetic, metabolic and chromosomal diseases can be diagnosed before birth, and might be prevented before delivery of a fetus with an incurable disease. PD is the process of ruling in or out fetal anomalies or genetic disorders, to provide expecting parents with the information and opportunity to modify pregnancy management and/or postnatal care.[1] Indications for PD include high risk situations for chromosomal abnormalities in the fetus, such as high maternal age, balanced translocation in one parent, history of previous child with a chromosomal abnormality, structural defects in one of the parent`s chromosomes (mosaicism, pericentric, inversion), fragile X syndrome, or family with a single gene disorders child (recessive, dominant or sex-linked, like metabolic inherited disorders, maple syrup urine disease, phenylketonuria, galactosemia, etc.) or diseases with ethnic background (Tay-Sachs, cystic fibrosis, thalassemia), history of recurrent spontaneous abortion,

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risk of neural tube defects (NTD) as well as multiple anomalies in previous newborn.[2-4]

PD techniques include amniocentesis, chorionic villus sampling (CVS), maternal serum biochemical tests including alpha-fetoprotein (AFP), estriol and beta human chorionic gonadotropin (β-hCG), ultrasonography, embryoscopy, fetoscopy, preimplantation genetic diagnosis (PGD) and isolation of fetal trophoblast cells from maternal blood.[5] However, most of these methods are high risk.[6]

Application of molecular genetic techniques as a part of PD process of genetic diseases is expanding every day. These could be implemented for detecting diseases with recognized genetic defects. In other words, any genetic disease with identified defect can be diagnosed with these techniques. Molecular analysis of genetic diseases is commonly carried out on the DNA molecule which is the same in all body cells with a nucleus such as white blood cells as well as any fetal tissues. Tissues which are commonly used for this purpose include chorionic villus tissue, amniotic fluid cells, umbilical cord blood, cells or fetal DNA in maternal blood.[5] Cell-free fetal DNA can be detected from 4-5 weeks of gestation;[7] therefore, the study of all genes in human DNA extracted from maternal plasma is possible from the beginning of the sixth week of pregnancy.[8-11] The whole fetal genome is represented in the maternal plasma. Prenatal diagnosis except for free fetal DNA requires access to the embryo and is an invasive procedure. Moreover, other techniques can be applied later in pregnancy compared to free fetal DNA, while non-invasive diagnosis, especially in the first trimester of pregnancy, is very important.[12] Fetal DNA can be detected after the 6th week of pregnancy in maternal blood, and its value significantly increases during the 8th week of gestation. It comprises 2.6% and 4.3% of the total DNA in maternal plasma in the first and third trimesters, respectively.[13]

Evaluation of fetal gender, Rh, and several autosomal dominant diseases such as achondroplasia, and autosomal recessive diseases such as Duchenne muscular dystrophy (DMD) is performed from fetal DNA in maternal blood in many laboratories. Presence of such technique with a high degree of accuracy seems necessary to achieve better results in diagnosis Labs, thus this study aims to evaluate practical approach to non-invasive diagnostic tests for sex-linked diseases. In women who are carriers of X-linked conditions, the early fetal sex determination using cell-free fetal DNA can be performed by non-invasive testing.

Methods

In this experimental study, the aim was to investigate the presence of sex-determining region Y (SRY) gene, the specific gene on Y chromosome.

Using a vacuum blood sample system, 5 ml of peripheral blood was obtained from 30 pregnant women at 8 to 12 gestational week, with intervals of 5 hours into ethylenediaminetetraacetic acid (EDTA) tubes. The samples were centrifuged at 4000 rpm, plasma was isolated under sterile conditions, and was frozen for further DNA extraction. Free DNA circulating in maternal blood was extracted using an exclusive and ultra-sensitive kit (from Qiagen Co. USA # Catalogue number: 54604 according the manufacturers recommendation). After DNA extraction, quantitative real-time polymerase chain reaction (PCR) was performed using primers specified in table 1.

Table 1. Primer sequences studied by quantitative real-time polymerase chain reaction (PCR)

Primer	Sequence
SRY-F	5`- AGATTAATCCTTGCTAAGGACTGGAT- 3`
SRY-R	5`- TCCTCAATTGAAACCGTGCAT- 3`
SRY	5`Fam- AAGAGGTTGTGGCCAGTTA-Tamara- 3`
GAPDH-F	5`- CCCCACACACATGCACTTACC- 3`
GAPDH-R	5`- CCTAGTCCCAGGGCTTTGAT- 3`
GAPDH	5`Fam- AAAGAGCTAGGAAGGACAGGCAACTT-Tamara- 3`

SRY: Sex-determining region Y; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Quality control of for the process was performed with simultaneous internal control (IC) gene of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The target gene (GOI) and IC primers were designated to be amplifying simultaneously.

PCR conditions were as follows:

1) Denaturation: 95 °C for 20 seconds, over 40 rounds (no fluorescent light absorption)

2) Annealing: 65 °C at 65 seconds, during 40 rounds (with the fluorescent light in the green channel FI = 4-8)

3) Extension: at 65 °C for 65 seconds, over 40 rounds (with the fluorescent light in the green channel FI = 4-8)

For PCR amplification, the TaqMan master mix enzyme (Takara Co., Japan # Catalogue number: RR037A) was used. PCR reaction was done with mix substances, and concentrations are presented in table 2.

Table 2. Components mixed for polymerase chain reaction (PCR)

Substance	Unit value	Total value (µl)
TaqMan master mix	-	25.0
SRY/GAPDH-F primer	5 µm/µl	0.3
SRY/GAPDH-R primer	5 µm/µl	0.3
SRY/GAPDH primer	5 µm/µl	0.4
Extracted DNA	20 ng/µl	5.0
DNAse free D.W.	-	20.0
Total volume		50.0

SRY: Sex-determining region Y; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Results obtained from these procedures were compared to the findings by ultrasonography which confirmed the gender of fetus in week 12.

Results

The clinical characteristics of the enrolled women are summarized in table 3.

The mean age of mothers was 32 years old. The results of SRY gene presence in DNA obtained from maternal blood are shown in figure 1. Number of male and female fetuses in 8 to 12 weeks and accuracy of fetal DNA evaluation compared to the results of ultrasound is shown in table 4.

With the GAPDH, SRY, and combined GAPDH + SRY analyses, we observed concordances of 85% (in 8 to 9 weeks of gestation), 85% (in 9 to 10 weeks of gestation), 90% (in 10 to 11 weeks of gestation) and 100% (in 11 to 12 weeks of gestation) respectively, with the results verified by follow-up at birth.

In figure 1, the Ct curve with respect to threshold was 0.02, and this mean was the highest efficacy of real-time PCR in specific probe.

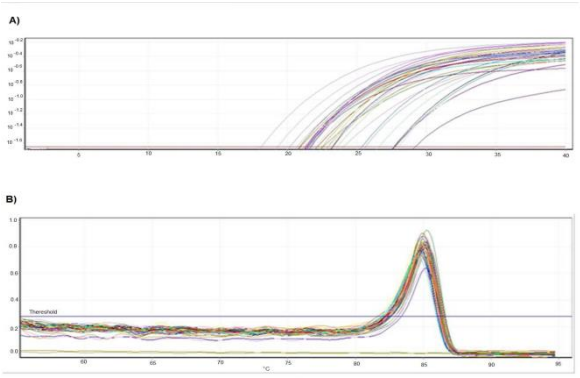

Figure 1. The Ct curve with respect to threshold = 0.02 (A) and the Melt curve analysis for specific binding of primers (B)

Discussion

PD is the most reliable way to prevent the birth of a neonate with genetic diseases in community. Today, prenatal diagnosis of diseases and termination of high risk pregnancies is one of the selected ways that has been accepted in most countries. It can prevent delivery of an infant with various congenital and genetic diseases with timely prenatal diagnosis. Indeed, PD is using different diagnostic methods for detection of fetal status during pregnancy.

Table 3. Clinical characteristics of the whole sample

Characteristic	Whole sample (n = 30)
Age (years)	32 (19–42)
Gestational age at the time of blood sampling (weeks)	9 (6-13)
Birth weight (g)	3715 (1120–4510)

Table 4. Frequency and accuracy of sex determination with evaluation of sex-determining region Y (SRY) gene expression and the internal control GAPDH during 8 to 12 weeks of gestation

	Gender	8 to 9 weeks of gestation	9 to 10 weeks of gestation	10 to 11 weeks of gestation	11 to 12 weeks of gestation
Frequency	Male	4	3	5	3
	Female	2	3	6	4
Accuracy (%)	Male	85	85	90	100
	Female	85	85	90	100

Generally, genetic diseases are not curable after birth so far. In addition to the problems of patients during their lifetime, there are huge financial burdens imposed to the patient's family and community.

This study was carried out by real-time PCR analysis in order to assess cell-free fetal DNA in maternal plasma between 8 and 12 gestational weeks, and to verify whether amplification of GAPDH and SRY genes could be technically employed in fetal gender determination, and to identify whether the combination of two Y amplification analyses could improve the performance of the test. Kim et al. carried out non-invasive prenatal determination of fetal gender using quantitative fluorescent PCR (QF-PCR) analysis of cell-free fetal DNA in maternal plasma.[14]

Many sex-linked diseases such as hemophilia could easily be detected before birth. For example, this is achievable for classic hemophilia (A) that results from defects in the gene for coagulation factor 8 (FVIII). PD offers couples the opportunity to be informed about the status of their fetus and adopt a correct decision on continuing the pregnancy. Also it provides the possibility of having a healthy child for couples who are carriers of disease genes.[1]

However, due to the extremely high costs of such tests which have to be performed in late phases of pregnancy, and the risk of fetal sampling, many families refuse to carry out such experiments.[5] Fetal sex determination in early pregnancy can be very important in high-risk families where the fetus is female and it does not need any further genetic test, so additional costs might be avoided.

The method provided in the present study could be reviewed at two perspectives: First, the issue of sex-linked inheritance of the disease is extremely important. According to our results, this technique provides acceptable results and could be a reliable guide for informing the family about further evaluations. Second, in addition to sex-linked diseases, our results suggest that these steps could be implemented for numerical abnormalities of chromosomes. Further studies about this topic might help families who suffer from delayed diagnosis of their condition.

Conclusion

Free fetal DNA from maternal blood seems to be reliable source for sex determination in early stages of pregnancy. This method could be helpful in early diagnosis of sex-linked diseases. Now, fetal sex determination is available as a clinical service in molecular genetics unites in many countries and enables accurate determination of fetal sex from 8-12 weeks of gestation. On the other hand, comparing this method to other prenatal screening methods of common single gene disorders and chromosomal aneuploidies, cell-free fetal DNA testing has the best performance.

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Authors' Contribution

Amir Monfaredan and Seyed Mahmoud Tabatabaei designed the study and collected the data. Data analysis was done by Shahrokh

Amiri. Editing the whole manuscript was done by Seyed Mahmoud Tabatabaei.

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Conflict of Interest
Authors have no conflict of interest.

Ethic Approval
The Ethics Committee of Islamic Azad University, Tabriz Branch, approved the study.