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ORIGINAL ARTICLE

Gender Determination by Isolation of Cell-free Fetal DNA from the Maternal Circulation

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ABSTRACT

Objectives: Early identification of fetal gender is important in management of X-linked and other metabolic disorders. Since ultrasound may not predict gender accurately during the first trimester, noninvasive fetal gender determination using deoxyribonucleic acid (DNA) amplification has been proposed. The aim of this study is to evaluate the feasibility of noninvasive prenatal gender determination by examining cell-free fetal DNA (cffDNA) from maternal plasma.

Materials and methods: Blood samples were collected from 49 pregnant women of gestational ages ranging from 12 to 41 weeks. Deoxyribonucleic acid was extracted from maternal plasma using a QIAamp DNA Blood Mini Kit. Real-time quantitative polymerase chain reaction (PCR) was performed to amplify the male specific DNA marker sex-determining region Y (SRY).

Results: From a total of 49 subjects, fetal gender was correctly determined in 13 out of 14 male fetuses and 32 out of 35 female fetuses, giving an overall accuracy of 92%. The sensitivity and specificity of the test to detect male fetuses was 93 and 91% respectively. There were three false-positive cases and one false-negative case.

Conclusion: Identification of fetal gender from maternal plasma using real-time PCR technique is feasible in a developing country, like Pakistan, and appears to be a promising tool for noninvasive prenatal diagnosis.

Keywords: Cell-free fetal DNA, Fetal gender determination, Maternal plasma, X-linked recessive disorders.

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INTRODUCTION

Identification of fetal gender is valuable in pregnancies at risk of X-linked genetic disorders and certain metabolic disorders like congenital adrenal hyperplasia.[1] Contemporary methods of determining fetal gender rely upon the ultrasound scan in the second trimester for the presence of penis and scrotum in the male or of the labial folds in the female. However, these physical markers are less beneficial to be used early in pregnancy; since, the development of the external genitalia is not complete. Further, fetal gender determination is incorrectly reported on ultrasound in 40% of the cases.[2] Traditionally, chorionic villus sampling and amniocentesis can be used to help identify fetal gender at around 11 weeks of gestation.[3] However, the risk of miscarriage in these procedures is around 1 to 3%.[3] Hence, pregnancies carrying female fetuses have to be subjected unnecessarily to invasive procedure and its associated risk.

In 2014, Pakistan's population stands at 184 million, ranking 6th in the world. Pakistan has a per capita income of US $1368 and per capita government expenditure on health is only US $21.78.[4] Hence, health priorities are focused to address issues, such as communicable diseases, reproductive health problems and malnutrition while genetically determined conditions are often neglected. Due to lack of a national registry database in Pakistan, true estimates of X-linked disorders are not available. According to one estimate, the incidence of severe mental retardation in Pakistan is 1.1/100 live births, 36% of which is attributable to Down syndrome.[5] According to estimates of the Pakistan Hemophilia Patients Welfare Society (2006), Pakistan harbors 9000 of the 400,000 hemophilia cases worldwide.[6] Prevalence of X-linked disorders, such as hemophilia, may be much higher due to the high rate of consanguineous marriages in Pakistan (around 60%).[7] Further, these conditions require life-long management with repeated blood transfusions and need for hospitalizations that carry cost implications for a developing country, like Pakistan, with limited healthcare resource allocation. Hence, access to safe, accurate and affordable prenatal screening and diagnosis of X-linked

disorder is essential to help to reduce the burden of disease in this part of the world.[7]

Apoptotic trophoblasts in the placenta are the source of cell-free fetal DNA (cffDNA).[8] The quantity of cffDNA increases as the gestational age progresses from the first to the third trimester (25.4 GEq/ml in early stage of pregnancy to 292.2 GEq/ml in late stage) and attains its highest concentration just before delivery.[9] Cell-free fetal DNA is rapidly cleared from the maternal circulation with a mean half-life of 16.3 minutes (4–30 minutes), hence, making it less susceptible to false-positive results from previous pregnancies.[9] Several investigators have successfully demonstrated the utilization of cffDNA for the determination of fetal gender[1] as well as for fetal abnormalities.[10]

Noninvasive determination of fetal gender uses deoxyribonucleic acid (DNA) amplification by polymerase chain reaction (PCR) and detection of Y chromosome specific sequences in the maternal plasma.[11] Initially, conventional PCR was used for such studies which were of less sensitivity and lower specificity.[1] Currently, the use of real-time PCR is the most extensively used technique and highly acclaimed as a sensitive and specific tool for noninvasive prenatal diagnosis.[1]

The aim of this study was to assess the accuracy of identifying fetal gender using cffDNA from the maternal plasma.

MATERIALS AND METHODS

Forty-nine pregnant women visiting our hospital participated and provided written consent for this validation study. Pregnant women from all the three trimesters of pregnancy were sampled during routine prenatal visits. Three nonpregnant females and two males were also considered as negative and positive controls in this study. Fetal gender was not known at the time of sample collection and was later confirmed at birth. The study protocol was approved by the Ethics Committee of our hospital.

DNA Extraction and PCR Analysis

A total of 5 to 8 ml of maternal peripheral blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes. Blood samples from EDTA tubes were transferred to clean 15 ml polypropylene centrifuge tubes (Alpha Laboratories, Hampshire, UK) and centrifuged at 2670 rpm for 15 minutes at room temperature. Plasma was carefully removed and placed into clean 1.5 ml Eppendorf tubes and re-centrifuged at 14000 rpm for 15 minutes. The supernatant (containing free-DNA) was collected in a fresh 1.5 ml Eppendorf tube. Deoxyribonucleic acid was extracted by use of a QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with minor modifications. A total of 500 µl of plasma sample was used for DNA extraction and was treated with proteinase K so as to remove contaminations.

Table 1: Primer and Probe sequences used in real-time PCR

Name	*Sequence (5'→ 3')*
SRY forward	5'-GCG ACC CAT GAA CGC ATT- 3'
SRY reverse	5'-GCC ATC TTG CGC CTC TGAT- 3'
SRY probe	5' (FAM) ACG AAT GAT TGC ATC AGT-MGB-3'
β-globin forward	5'GTG CAC CTG ACT CCT GAG GAG A 3'
β-globin reverse	5'CCT TGA TAC CAA CCT GCC CAG 3'
β-globin probe	5'(FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (BHQ) 3'

Real-time quantitative PCR analysis was performed in the Chromo[4] real-time PCR detector (BioRad, Hercules, CA), which is a combined thermal cycler/fluorescence detector. The primers and probes were obtained from BioSearch Technologies Inc, (Novato, CA). β-globin gene, a chromosome 11 locus, was used as a house-keeping gene to detect the presence of total DNA. Male and female genomic DNA was used as positive and negative controls respectively. All the reactions were performed in duplicates. Primer and probe sequences for sex-determining region Y (SRY) and β-globin gene sequences are shown in Table 1.

The probe was labeled with 6-carboxyfluorescein (FAM). A total volume of 25 µl was made for the PCR reaction that contained 5 µl of buffer, 5.5 µl of $MgCl_2$, 0.5 µl of dNTPs mixture, 1.125 µl of primer, 0.2 µl of Taq polymerase, 0.56 µl of probe, 7.1 µl of water and 5 µl of DNA template. Before the thermal cycling, the master mix was centrifuged for 2 minutes at 4ºC. Thermal cycling was initiated with a 2-minute incubation at 50ºC, followed by a first denaturation step at 95ºC for 5 minutes, and then 45 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Negative water blank, positive and negative controls were included with each sample run for real-time quantitative PCR.

The samples were processed for DNA extraction and PCR analysis by a female operator throughout the study to avoid the chance of contamination. Aerosol-resistant pipette tips (Molecular Bio-products, San Diego) were used throughout all the experimental procedures as an anticontaminant measure. All steps for the analysis were conducted in a separate area.

STATISTICAL ANALYSIS

All results were analyzed and compared with the gender of the newborn at birth. This was used to calculate the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the method. Statistical analysis was performed using SPSS version 19.

RESULTS

A total of 49 pregnant women participated in the study with gestational age ranging from 12 to 41 weeks. Among these subjects, fetal gender was correctly identified in 45 cases and the overall accuracy of the test was 92%. Out of 14 male-bearing pregnancies, correct gender was predicted by the noninvasive prenatal test in 13 subjects. The sample for real-time PCR for the false-negative case was obtained at 12 weeks of gestation. Thus, the sensitivity of the test for prediction of male fetuses was 93% (95% CI: 64%, 99.6%). Among 35 female-bearing pregnancies, correct gender identification was possible in 32 subjects only; hence, the specificity of the test was 91% (95% CI: 75.8%, 97.7%) (Table 2). The sample for the three false-positive cases was obtained at 12, 30 and 32 weeks of gestation.

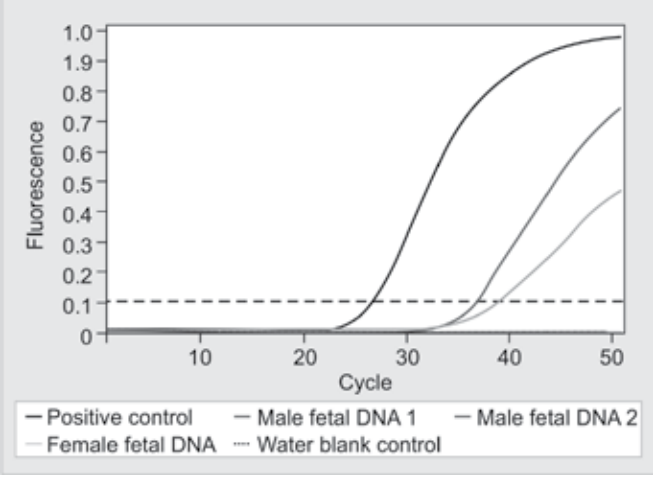
In total, noninvasive prenatal testing predicted that the fetus was male in 16 cases, 13 of which were confirmed to be males postnatally. Hence, the positive predictive value of the test was 81% (95% CI: 53.6, 95%). Among the 33 fetuses predicted to be female by the test, 32 were confirmed as females at birth. This gave a negative predictive value of 97% (95% CI: 82.5, 99.8%).

Graph 1 shows the SRY amplification curve for a male fetus. There were no samples with inconclusive results. β-globin gene was amplified in all the samples, thus, confirming the presence of DNA in all the cases.

Table 2: Gender determination using SRY sequence in real-time PCR

		Gender verified at birth		*Total*
		M	*F*	
RT-PCR	M	13	3	16
	F	1	32	33
Total		14	35	49

M: Male; F: Female



Graph 1: Sex-determining region Y amplification curve from cell-free fetal DNA enriched from maternal plasma of a male-bearing pregnancy

DISCUSSION

This pilot study reported from a tertiary care hospital in Pakistan aimed to assess the feasibility of gender identification using cffDNA from maternal plasma as a reliable and a potential clinical tool for X-linked disorders. We predicted the gender of fetuses based on amplification of the SRY sequence (male specific genetic sequence) in the samples. Real-time PCR was used in our study as this technique can detect very low copy numbers of DNA and is the method of choice in many laboratories.[12] A meta-analysis involving 90 studies with over 10,000 pregnancies by Wright et al demonstrated that the overall sensitivity of this technique to detect fetal gender is 96.6% and the overall specificity is 98.9%.[12] Further, these numbers vary very little with the age at gestation, hence, indicating the high reliability of the test.[12]

The specificity of our study was found to be 91%. Among the total male fetuses, we predicted 13 samples accurately. However, one case was found to be false negative. Female fetuses are not detected directly but are inferred by a negative result for Y chromosome sequences in the maternal plasma.[11] Hence, false-negative results could possibly be explained due to degradation of fetal DNA prior to or during the processing of blood samples. There were three (19%) maternal samples which were incorrectly predicted to have a male fetus (false positive). Even though strict anticontamination procedures were used, contamination may have occurred during sample handling and transport resulting in false-positive results. It could also be explained by vanishing (male) twin or by confined placental mosaicism, whereby cytogenetic abnormalities are confined to the placenta only.[13] If noninvasive prenatal diagnosis is to be used in clinical service, false-negative results are of greater concern than false positives.[14] This means that it is important not to miss out a male fetus which may be at risk of disease and may require further testing using CVS or amniocentesis.

Due to the high accuracy and noninvasive nature of the test, cffDNA from maternal plasma can be used as a valuable diagnostic tool and can add to timely clinical management of certain congenital disorders.[14] In Pakistan, CVS and amniocentesis are available at only few centers due to lack of expertise. Further, the cost of this technique ranges from US $100 to 300, which can be expensive for an average Pakistani family whose monthly income ranges around US $115.[4] Hence, noninvasive prenatal gender identification can reduce the need for costly, invasive procedures in pregnant women with possible X-linked disorders by 50%.[14]

Noninvasive prenatal diagnosis (NIPD) as a promising technology in fetal medicine, though on one hand, will simplify many aspects of screening for X-linked and other

genetic disorders, but, on the other hand, it raises some ethical concerns.[15] One of these concerns is the potential for termination of pregnancy of the 'unwanted' gender due to personal, cultural or economic reasons.[15] Also, as it is relatively simple to obtain maternal plasma for prenatal gender determination, this technology may become available on a commercial basis, bypassing the need for medical professionals.[15] Hence, ethical as well as regulatory issues regarding NIPD using cffDNA needs to be addressed.

CONCLUSION

This study confirms the feasibility of carrying out non-invasive prenatal testing in developing country, like Pakistan.

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