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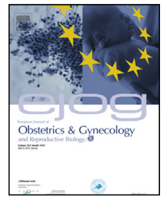

Successful early fetal sex determination using cell-free fetal DNA isolated from maternal capillary blood: A pilot study

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ABSTRACT

Objectives: The discovery of cell-free fetal DNA (cffDNA) fragments in maternal plasma made it possible to determine fetal sex at early stages of pregnancy without carrying a risk miscarriage, which is especially important for the management of X-linked genetic abnormalities. The vast majority of studies used cffDNA extracted from maternal venous blood, excluding the possibility of capillary sampling for those who cannot tolerate venipuncture. This study evaluates the possibility of fetal sex determination using cffDNA isolated from capillary blood of women with early gestational pregnancies.

Study design: Samples were obtained from 24 pregnant women from the Ukrainian population, whose gestational age varied between 5th to 10th weeks. Sex determination was performed using real-time quantitative PCR of SRY male-specific markers. Results were compared to the known fetal sex (detected by next-generation sequencing during the preimplantation genetic testing procedure) to calculate the test accuracy.

Results: Results demonstrated 85.71–100% sensitivity and 100% specificity of the test. Cohen's Kappa coefficient of agreement in sex determination test varied from 0.8 to 1.0 ($P < 0.00001$).

Conclusion: This test, which is the first known so far detailed report of successful early fetal sex determination using cffDNA isolated from maternal capillary blood, is a reliable alternative to traditional venipuncture.

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Introduction

Although the presence of circulating, cell-free nucleic acids (cfNAs) in human blood was described for the first time in 1948 (by Mandel and Métais [1]), cell-free DNA has recently gained huge research interest. The discovery of cell-free fetal DNA (cffDNA) fragments in maternal plasma [2] pushed the boundaries for non-invasive prenatal diagnosis, making it possible to determine fetal sex at early stages of pregnancy. It is especially important for the management of X-linked genetic abnormalities, such as hemophilia, fragile X-linked mental retardation, Duchenne muscular dystrophy, etc., where early diagnosis is very critical. Noninvasive prenatal diagnosis in contrast to invasive techniques, such as chorionic villus sampling (CVS) or amniocentesis, does not carry a risk of miscarriage, which is higher during the first trimester of pregnancy, and can be performed earlier.

Circulating cffDNA originates from the trophoblasts making up the placenta and accounts for about 3–13% of the total maternal DNA isolated from venous blood [3,4]. The amount of cffDNA increases with progression of the pregnancy and is already detectable from the 4-5th week of pregnancy [4,5]. The average size of cffDNA is about 150 bp [6].

A number of articles report the early fetal sex determination [7–11,5,12], however vast majority of them used cffDNA extracted from maternal venous blood, excluding the possibility of capillary sampling for those who cannot tolerate venipuncture. Capillary sampling is preferred by most patients and is simpler, less painful. The method, providing the elimination of exogenous male DNA from the collection site, could be a reliable alternative to traditional venipuncture. So far, the only mention of this analysis performed in capillary blood can be found in 69th AACC Annual Scientific Meeting Abstract eBook [13], however no details on methodology are given. Also, a study by Xiong et al. [14] describes fetal gender determination using maternal dried capillary blood spots (DYS14 marker; specificity = 91.6%) in 19 samples.

Abbreviation: AACC, The American Association for Clinical Chemistry; cffDNA, cell-free fetal DNA; cfDNA, cell-free DNA; CVS, chorionic villus sampling; DNA, deoxyribonucleic acid; ICSI, intra cytoplasmic sperm injection; NGS, next-generation sequencing; PGT-A, preimplantation genetic testing for aneuploidy.

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The aim of this study was to find out if fetal sex determination is possible using cffDNA isolated from capillary blood of pregnant women at early gestational age. Here we report successful early (5–10 weeks of pregnancy) fetal sex determination using cffDNA isolated from maternal capillary blood in a pilot study of 24 Ukrainian women pregnant with fetuses of a known sex (after ICSI + PGT-A procedure).

Materials and methods

Patients

We describe a cohort of 24 Ukrainian pregnant women (35.33 ± 5.62 years old) at early gestational age (5–10 weeks) who underwent the ICSI + PGT-A (NGS) procedure at IGR (Institute of Genetics and Reproduction) Medical Center (Kyiv, Ukraine) during 2017–2018, therefore sex of their fetuses was known. Three men and three non-pregnant women were considered as positive and negative controls, respectively. All participants signed an informed consent form before blood sampling. The protocol of the study was approved by the Ethics Committee of IGR, the study was carried out in accordance with The Code of Ethics of the Declaration of Helsinki.

Sample collection

About two milliliters of capillary blood was collected by fingertip puncture in a tube Microvette CB200 K3E (Sarstedt, Germany) and immediately delivered for further processing. Before blood collection, the skin puncture site was properly cleansed with sterile cotton and disinfected with a 70% aqueous solution of isopropanol with subsequent drying of the puncture area. The first drop of blood was wiped away with a clean gauze. Blood samples were centrifuged twice at 3000 *g* for 10 min and the upper plasma layer was carefully removed without disturbing the buffy coat and transferred into a new 1.5 ml Eppendorf tube for immediate cffDNA extraction.

DNA extraction

cffDNA was extracted from the whole volume of collected capillary blood (up to 2 ml) using the Quick-cfDNA™ Serum & Plasma Kit (Zymo Research, USA) according to the manufacturer's instructions. DNA was eluted into 30 μl of elution buffer.

Quantitative real-time PCR (qPCR)

DNA samples were subjected to quantitative real-time PCR with *GAPDH* gene as a marker for total DNA amplification, using two pairs of SRY male-specific primers (SRY1-F (forward) 5′– CCAGAAGT-GAGCCTGCCTAT –3′, SRY1-R (reverse) 5′– GCATTTAAAGTAAGCCA-CAGTGT –3′, SRY4-F 5′– GATAGAGTGAAGCGACCCATGAA – 3′, SRY4-R 5′– CTCTGAGTTTCGCATTCTGGG –3′,Thermo Fisher Scientific, USA), and GAPDH-F (forward) 5′–CCCCACACACATGCACTT–3′, and GAPDH-R (reverse) 5′–CCTAGTCCCAGGGCTTTG–3′ (Metabion international AG, Germany). QPCR was carried out in duplicates on CFX96 Touch real-time PCR system (Bio-Rad, USA) using SYBR Green I chemistry, separately for SRY and GAPDH primers in a total volume of 20 μl containing 0.25 mkM of each primer (forward and reverse), 1X of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA), and 9 μl of extracted DNA to a final volume of 20 μl, under the following cycling conditions: 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. Also, a non template control, containing DNA-free water was analyzed to recognize the contamination. In case of the absence of the *SRY* amplification, one more analysis was performed in 2 weeks after the first analysis.

Statistical analysis (tests of sensitivity, specificity and kappa coefficient of agreement in sex determination) was performed using Statistica 10 (StatSoft Inc., USA). A *P*-value less than 0.05 was considered statistically significant.

Results

Of 24 women analyzed, fourteen had a pregnancy of 5–7 gestational age and ten – of 8–10 weeks (Table 1). The *GAPDH* gene was amplified in all samples analyzed, indicating the presence of sufficient total DNA in the extracted samples (data not shown). Comparison of the obtained results with the known data using SRY markers demonstrated that fetal sex of 87.5% of samples (21 of 24, sensitivity 85.71%/ sensitivity index 0.86, specificity 100%) was correctly identified at the first amplification. While sex of three male fetus samples that failed to amplify SRY marker (two - SRY1 marker, and one – SRY4 marker) at the first amplification (6 and 6.5 gestational weeks), it was successfully identified at the second amplification (8–8.5 gestational weeks) and amplified with another SRY marker (SRY4 and SRY1, respectively), increasing the sensitivity of the method up to 100% (sensitivity index 1.0). Cohen's Kappa coefficient of agreement in sex determination test varied from 0.8 to 1.0, $P < 0.00001$. Results of both tests are presented in Fig. 1.

Comment

The discovery of cffDNA fragments in maternal plasma created new possibilities for non-invasive prenatal diagnosis and determination of fetal sex at early stages of pregnancy in the management of X-linked genetic abnormalities, in particular. Previous studies [7–11] demonstrated high sensitivity of fetal sex determination using SRY, DYS14 and DAZ markers. However, data are scarce on the use of cffDNA for the analysis of fetuses at early gestational age (since 6 weeks), especially extracted from maternal capillary blood. To the best of our knowledge, this report is the first known so far giving a detailed description of the methodology of fetal sex determination using cffDNA isolated from capillary blood of women at early gestational age pregnancy.

In this study, we successfully identified fetal sex using two SRY markers in 87.5% of early pregnancies (5–10 weeks) in the first test and in 100% pregnancies in the second test (two weeks later after the first one), obtaining sensitivity level of the analysis of 85.71–100%. In comparison, sensitivity level to identify male fetuses using several male-specific markers from cffDNA isolated from maternal venous blood, ranged from 80 to 100% (described in [8]).

The failure to identify fetal sex in three of 24 samples after the first amplification is probably related to the very low cffDNA concentration in maternal blood at weeks 6–6.5 of gestational age, because these samples demonstrated amplification of the *SRY* gene during the second test two weeks later, indicating the increase of cffDNA concentration [15] and accuracy of the test [5] with increasing of gestational age. According to Lo and co-authors [3], the level of cffDNA fragments isolated from maternal venous blood varies from 0.39% to 11.7% (starting from week 11 of gestational age) that is about 3.4% of the total maternal plasma DNA. Later studies demonstrated higher levels of the cffDNA fraction for the first trimester (ca.9.15–9.86%) [16–18]. Moreover, a lower level of cell-free DNA (cfDNA) was reported in capillary blood compared to venous blood [19,20]. Taking into account significantly earlier gestational age of these samples compared to the earliest age of samples described in other studies, e.g., Lo and co-authors (5–6 vs 11 weeks), and the tendency of lower DNA concentration in capillary compared to venous blood, we can suggest that cffDNA concentration in maternal capillary blood at

Table 1
Fetal sex determination by real-time PCR amplification of the *SRY* gene.

ID of samples	Known fetal sex	Gestational age, weeks	SRY amplification 1	SRY amplification 2[*]
C1[**]	M[#]	6	No	Yes
C2	M	8	Yes	NA
C3	M	6	Yes	NA
C4	M	7	Yes	NA
C5	M	6.5	No	Yes
C6	F	8	No	No
C7	M	6	Yes	NA
C8	M	8	Yes	NA
C9	M	8.5	Yes	NA
C10	M	8	Yes	NA
C11	M	6	Yes	NA
C12	M	7	Yes	NA
C13	M	9	Yes	NA
C14	M	7	Yes	NA
C15	M	9	Yes	NA
C16	M	9	Yes	NA
C17	M	10	Yes	NA
C18	M	9	Yes	NA
C19	M	5	Yes	NA
C20	M	7	Yes	NA
C21	M	6	Yes	NA
C22	F	6	No	No
C23	F	7	No	No
C24	M	6	No	Yes
K1+	M	NA	Yes	NA
K2+	M	NA	Yes	NA
K3+	M	NA	Yes	NA
K1-	F	NA	No	NA
K2-	F	NA	No	NA
K3-	F	NA	No	NA

[*] Second fetal sex determination (2 weeks later after the first test) in case of no SRY amplification.
[**] C1-C24 – patients (pregnant women), $K1^+$-$K3^+$ - male DNA as a positive control, K1-K3 - non-pregnant women DNA as a negative control.
[#] M – male, F – female, NA – not applicable.

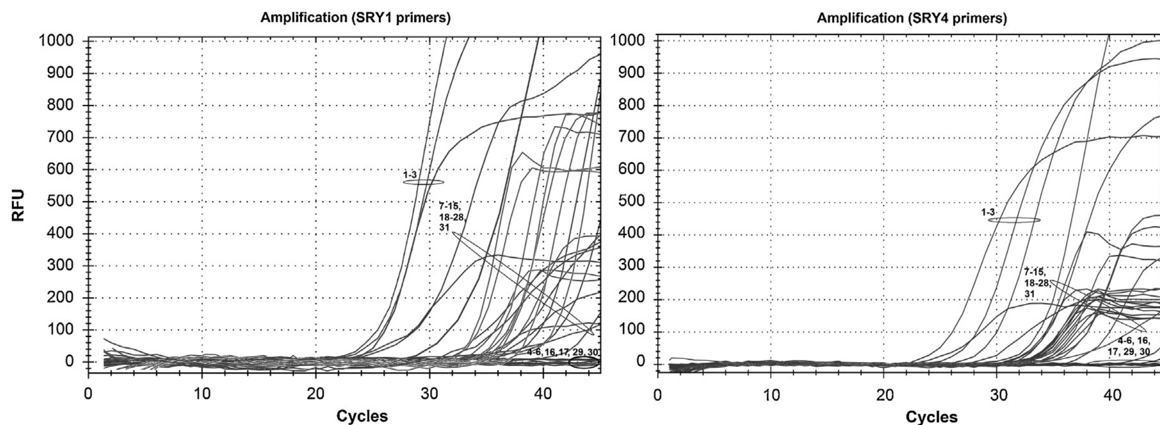

Fig. 1. Real-Time PCR Analysis of fetal DNA samples extracted from the maternal capillary blood.
A – amplification with SRY1 primers, B - amplification with SRY4 primers.
Curves 1–3 (samples $K1^+$-$K3^+$): male DNA as a positive control, Curves 4–6 (samples K1-K3): non-pregnant women's DNA as a negative control, Curves 7–16, 18-31: fetal DNA, Curve 17: PCR reaction negative Control (Water). Signals in samples C1-C5 & C7-C21, C24 (curves 7–15, 18–28, 31) indicate that fetal sex is male, and lack of amplification in samples C6, C22-C23 (curves 16, 29, 30) indicates that fetal sex is female.

weeks 5–6 of gestational age is considerably lower than that of venous blood reported for the first trimester. It could affect fetal sex identification analysis, significantly complicating it and resulting in the need of additional test at later stages of the pregnancy. Therefore, the use of several markers combination is preferable to overcome this issue.

We believe that our findings will be a valuable asset to non-invasive prenatal diagnosis, to the determination of fetal sex at early stages of pregnancy in the management of X-linked genetic abnormalities, in particular, bringing such testing to a whole new level. Such technique is simple, fast, less expensive than standard non-invasive prenatal test from the maternal venous blood, highly sensitive and it could be used as an effective tool in routine prenatal care from as early as week 6 of gestational age.

Conclusions

To the best of our knowledge, here we describe the first known so far successful pilot study on early fetal sex determination using

cffDNA isolated from maternal capillary blood in the management of X-linked genetic abnormalities.

Conflict of interest

The authors declare that they have no conflict of interest.

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