Non-invasive fetal sex determination from maternal plasma: impact on Romanian clinical practice of X-linked disorders

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Abstract

Introduction: The noninvasive prenatal diagnosis (NIPD) approach had been studied for more than a decade and successfully offered an alternative to classical invasive methods. Our aim was to develop and validate a protocol for noninvasive fetal gender determination in maternal plasma and to demonstrate its applicability in clinical practice of pregnancies at risk for X-linked disorders.

Materials and Methods: A three-step method was employed for fetal sex determination from maternal plasma using specific primers for two Y chromosome specific sequences, namely SRY and DYS14; we also included the GAPDH sequence detection as an internal control. The PCR products were automatically analyzed by high-resolution capillary electrophoresis.

Results: We validated the noninvasive prenatal detection of fetal gender by testing 75 plasma samples from pregnant women, non-pregnant women and males. We applied this noninvasive approach as a screening method in pregnancies at risk for Duchenne muscular dystrophy and we succeeded to eliminate the invasive chorionic villus sampling procedure for gender detection.

Conclusion: Our results confirmed that this non-invasive approach is feasible and accurate and would improve the management of X-linked disorders in Romanian healthcare system.

Keywords: NIPD, fetal gender determination, X-linked genetic disorder

Introduction

The fetal sex determination at an early gestational age is of most importance for pregnant women carrying an X-linked recessive chromosomal abnormality [1]. The identification of a male fetus indicates hemizygosity for the X chromosome and thus potential diseases such as Duchenne and Becker muscular dystrophies, hemophilia or fragile X-linked mental retardation [2], [3]. Although the fetal sex determination can be performed by sonography it may not be reliable until the 13th week of pregnancy because the development of external genitalia is not complete [1], [4]. Currently the pregnancies at risk for inheriting an X-linked disorder are recommended for invasive techniques, such as chorionic villus sampling (CVS) used for fetal gender determination [1]. However, this invasive procedure carries a risk of miscarriage of around 1% [5] and cannot be performed until 11 weeks of gestation [6].

The detection of cell-free fetal DNA (cffDNA) in maternal circulation by Dennis Lo in 1997 [7] opened a new, noninvasive approach for prenatal diagnosis (NIPD) and raised the challenge of finding safer methods to use in prenatal diagnosis for both the fetus and the mother. The cffDNA originates from syncytiotrophoblast cells of the chorionic villi [8], is detected starting with the 5th week of pregnancy [9], [10] and its quantity increases with gestational age reaching up from 3.4% to 6.2% of the mean total DNA concentration in
maternal plasma [11]. The cffDNA has a mean half-life of 16.3 min and in 2 hours after delivery it is cleared from the maternal circulation [12]. This is the main advantage when analyzing cffDNA because its persistence from a previous pregnancy is excluded. The main challenge is separating the fetal and the maternal DNA because of the absence of a unique fetal specific genetic marker that could identify and measure the presence of fetal DNA in both male and female fetuses. Many studies focused on the detection of paternally inherited sequences that are entirely absent or different from the maternal genotype. The first two applications of the NIPD approach in (that were translated into) routine clinical use were the fetal gender determination by analyzing the Y chromosome of male fetuses [1-12] and the fetal Rhesus D genotyping in D-negative mothers and D-positive fathers [13-15]. The NIPD targeted also paternally-inherited fetal single-gene disorder: cystic fibrosis [16, 17], thalasemia [18, 19], achondroplasia [20], and other more. In the last four years, with the advent of new NextGen sequencing tests it become possible to detect noninvasively fetal aneuploidy [21, 22, 23].

The aim of this study was to develop and validate a protocol for noninvasive fetal gender determination in maternal plasma in order to be used as a screening method in X-linked recessive disorders and potentially reduce the invasive diagnostic procedures for pregnancies with female fetuses.

Materials and Methods

Study design and the patients

We designed a two steps experiment. First, we developed a method for fetal gender determination from maternal blood and validate the protocol. Secondly, we applied this NIPD approach to pregnancies at risk for Duchenne muscular dystrophy.

To validate our protocol we tested 15 plasma samples from non-pregnant female patients, 15 plasma samples from male patients and 45 plasma samples from pregnant women. The later had gestational age between 9 and 16 weeks of pregnancy and were performing biochemical maternal serum screening tests (MSS) for the first and the second trimester of pregnancy. The participants for NIPD method validation were healthy individuals with no history of significant diseases and, when applicable, certified by ultrasound for the pregnancy. All the participants in this study were informed about the procedure and they signed an informed consent approved by the laboratory ethical committee. All DNA samples were amplified in triplicate for both Y specific sequences. We analyzed also a template-free sample containing ultrapure water to test for the contamination in the reaction.

In the second part of this study, we performed the prenatal screening for fetal gender determination in pregnancies at risk for X-linked disorders in two pregnant women with carrier status for deletions in the DMD gene.

Sample preparation

Twelve milliliters of maternal blood were collected in EDTA vacutainers from each participant of this study. The blood samples were processed within 24 hours after venipuncture by a two-steps centrifugation procedure. The first step was performed at a low speed of 2200xg for 30 min. The plasma sample was carefully removed from the tube without disturbing the buffy coat and placed into a fresh tube and centrifuged at 14000xg for 30 min. Plasma was carefully placed into fresh tubes and then stored at -80°C until DNA extraction.
DNA extraction from maternal plasma

The plasma samples were gradually defrosted from -80°C to room temperature and then centrifuged at 14,000g for 10 min to eliminate any cryoprecipitate.

The total cell-free DNA extraction protocol was optimized starting from 1ml of plasma. We used the QIAamp® DSP Virus kit (Qiagen) with some changes to the manufacturer’s protocol: the reagents volume was adjusted to the increased input of maternal plasma. In the DNA elution step we used 45µl of AVE elution buffer. Subsequent to the cell-free DNA extraction we made the target amplification.

PCR analysis

The PCR reaction was performed using a Veriti® Thermal Cycler machine. The PCR reaction targeted two commonly used sequences on the Y chromosome: the single copy SRY gene and the multi-copy sequence DYS14. We incorporated into the PCR reaction the GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) housekeeping gene; GAPDH is the reaction’s internal control and amplify both fetal and maternal DNA. We performed two distinct multiplex PCR reactions for the previously mentioned Y chromosome specific sequences. The specific primers sets for SRY [21], DYS14 [12] and GAPDH [24] were synthesized by Integrated DNA Technologies (IDT) (http://eu.idtdna.com/site). The 25µl multiplex PCR reactions were prepared on ice and included 1x multiplex PCR master mix (Qiagen Multiplex PCR kit), 480nM of each Y specific primers, 240nM of each GAPDH primer and 10µl cell-free DNA freshly extracted. The cycling conditions were: 1 x (95°C for 5min) followed by 50 or 40 cycles of (95°C for 1min, 60°C for 1min and 72°C for 1min) for the SRY gene and for the DYS14 gene, respectively. All the samples were amplified in triplicate. The PCR products were automated analyzed by high-resolution capillary electrophoresis on a QIAxcel system using QIAxcel DNA High Resolution kit. The high-resolution gel cartridge allowed a resolution of 3–5 bp and a detection sensitivity of 0,1 ng/µl DNA in undiluted amplification reactions, offering a more robust result with less sample input material.

For DMD gene analysis we used the SALSA MLPA kit P034-A2/P035-A2 DMD/BECKER (MRC Holland). The amplicons were re separated by capillary electrophoresis with CEQ™ 8800 Genetic Analysis System, Beckman Coulter.

Results

The fetal gender determination protocol was successfully performed in one day. The results were considered positive when specific DNA amplicons were detected for both SRY and DYS14 sequences, negative when no Y sequence specific amplicons were observed but with positive amplification for GAPDH and inconclusive when the multi-copy DYS14 marker was positive and the single copy SRY was negative (Figure 1). We also monitored the no-template control sample to validate the run. The results interpretation was made by a scoring model combining the results for both SRY and DYS14. Before analyzing the Y specific sequence, the PCR reaction was validated by the positive amplification of the internal control of the GAPDH and the negative non-template control. For the SRY reaction the results were considered positive when at least two out of three replicates were positive and inconclusive if just one was positive and the other two, negative. The inconclusive results were retested using another plasma aliquot. We considered a result as being negative when all three replicates were negative. For the DYS14 reaction, the results were considered positive when all three replicates were positive and inconclusive if just one was negative and the other two positive.
Figure 1. High-resolution capillary electrophoresis – electrophoregram view: A01 and A02 lanes stand for SRY and GAPDH positive results; A03, A04 and A05 lanes stand for SRY negative and GAPDH positive result; A06 lane represents the non-template control for SRY assay; A07 and A08 lanes are the DYS14 and GAPDH positive result; A09 and A10 lanes correspond to DYS14 negative and GAPDH positive result; A11 lane represents the non-template control for DYS14 assay; in A12 lane there is the pUC 18/Hae III marker.

This interpretation considered the fact that DYS14 is a multi-copy marker and its detection was most robust comparing with the SRY marker. In this specific case we recommended the collection of a fresh new sample and the repetition of the test. After establishing the result for each marker, we proceed to the final result interpretation by combining them (Table I).

Table 1. The scoring model for (the results interpretation) interpreting the results obtained by combining the results of the SRY and the DYS14 assays.

<table>
<thead>
<tr>
<th>PCR result</th>
<th>SRY</th>
<th>DYS14</th>
<th>Final result</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>positive</td>
<td>negative</td>
<td>inconclusive</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>positive</td>
<td>possible positive but repeat the test</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td></td>
</tr>
</tbody>
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The results obtained by analyzing plasma sample attained from the non-pregnant women and males were conclusive. The SRY was detected in 24 samples and the DYS14 in 26 cases. The two cases were reported as male fetuses after repeating the test from another maternal plasma aliquot. In one case we had an inconclusive result for the DYS14 and requested the collection of a new sample.

The results obtained from all 45 pregnant women were confirmed by amniocentesis followed by the rapid prenatal diagnosis of trisomy 13, 18, 21 and sex chromosome aneuploidies.

We also tested 11 blood samples of pregnant women with gestational age between 9 and 12 weeks of pregnancy who didn’t performed the invasive tests and have late confirmed NIPD results.

This NIPD protocol for fetal gender determination was applied in two cases at risk for Duchenne muscular dystrophy (DMD) with long histories regarding the DMD gene heritability. In the first case, the pregnant woman was a heterozygous carrier for the exons 51-55 deletion and her five years old sun inherited the abnormal X chromosome (figure 2).
The maternal blood collection was performed at 9 weeks of pregnancy. The fetal gender was determined as female and the mother didn’t perform the CVS. Later in pregnancy, the amniocentesis for rapid aneuploidy detection (RAD) of the 13, 18, 21, X and Y chromosomes confirmed the noninvasive result. The second pregnant woman tested had the heterozygous deletion of exons 30-43 deletion and the noninvasive fetal gender detection was positive for the presence of a Y chromosome. The villous biopsy was performed at 11 week to analyze the fetal DMD gene. The peaks heights between sex chromosomes markers were analyzed to exclude the maternal DNA.
contamination of the fetal sample. The test revealed a normal DMD gene in the male fetus without any maternal contamination (figure 3).

![Graphs showing MLPA patterns](image)

**Figure 3.** The MLPA patterns of all 79 exons and the pDP427c promoter of the DMD gene in the pregnant woman with the 30-43 exons heterozygous deletion and the male fetus with normal DMD gene

**Discussion**

The NIPD analysis of fetal DNA circulating freely in the maternal blood stream offered new possibilities of improving the management of prenatal diagnosis [7]. The new challenge was the possibility of completely eliminating the invasive trophoblast biopsy and amniocentesis and securing the health of the fetus and the mother. Since the first detection of cffDNA in maternal plasma in 1997, many studies were aiming at finding a universal marker that amplifies both genders DNA [7]. The main drawback of the NIPD when using other methods then NextGen sequencing was that only paternally inherited alleles could be detected. The main clinical applications were the fetal gender determination in pregnancies at risk for X-linked disorders and the fetal RHD genotyping in pregnant Rhesus D-negative women with Rhesus D-positive partners [2, 3, 13, 25].

With this study we aim to develop a rapid, feasible and less expensive method for cffDNA analysis isolated from maternal blood in order to determine the fetal gender from an early stage in pregnancy in cases at risk for X-linked disorders. In cases where the mother carries an X-linked mutation, the fetal gender determination is highly necessary [1, 2]. The ultrasound for sex determination is not reliable until the 13th week of gestation and it is impossible to avoid the CVS. The NIPD method for fetal sexing proposed in this paper is feasible and offers an early result in pregnancy, starting with the 9th week of gestation. The amplification of the two Y-linked sequences SRY and DYS14 ensures a feasible test result.
The DYS14 assay targeted a multi-copy sequence and therefore has a higher sensitivity than SRY [1, 2], but as mentioned in other papers, the test specificity increases by combining both markers [1]. The lack of a specific marker for female fetal DNA circulating in the maternal blood made questionable the report of a female fetus when both SRY and DYS14 are negative. The large quantity of maternal DNA in the sample renders more difficult the inclusion of satisfactory internal control to test for successful amplification of fetal DNA [25]. Although the inclusion of the GAPDH gene in the test wasn’t sufficient to provide the specific control for the presence of the fetal DNA as well as other types of housekeeping genes (β-globin, actins, HPRT), it demonstrated the success of our PCR approach.

Another important trait of our method was the analysis of PCR products using the QIAxcel high-resolution gel cartridge thus providing a high resolution separation and a detection sensitivity of 0.1 ng/µl DNA in undiluted amplification reactions. The fetal gender determination using the two assays for the SRY and DYS14 combined with the GAPDH control was reliable and accurate. Very important, the interpretation of the results based on the scoring model presented previously excluded false-negatives and false-positives results.

The introduction of this NIPD test into the clinical practice of our laboratory was performed for pregnant women carriers for heterozygous DMD mutations. The obstetrician recommended this NIPD test for fetal gender determination at 9th weeks of pregnancy and in both cases the results were conclusive. The results were confirmed by ultrasonography, fetal RAD by QF-PCR and karyotype.

**Conclusion**

The results obtained in this study confirmed that this NIPD method for fetal gender determination is feasible and accurate and can be successfully implemented as a screening test for all pregnancies at risk for X-linked disorders therefore eliminating the need for invasive sampling procedure in nearly as 50% of cases. A safe NIPD test for fetal sexing in the 9th week of pregnancy would improve the management of X-linked disorders in Romanian healthcare system.

**References**