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Research Article

Early Non-invasive Determination of Fetal Sex Using Cell-free DNA

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Abstract

The first step in the prenatal diagnosis of X-linked genetic disorders is determining fetus gender. Current invasive methods to obtain the DNA source of the fetus instead of its miscarriage risk, has harmful stress for high risk pregnancies. Cell free fetal DNA (cffDNA) circulating in the maternal blood, has now become a useful source of noninvasive prenatal diagnosis. Considering limitation of cffDNA; as its small fragment size and low concentration in maternal plasma; using this source for clinical diagnostic material, requires a high efficiency extraction method and reasonable molecular tests to lead more accurate results.In the current study, we optimized Triton/Heat/Phenol (THP) protocol for extracting cffDNA in 8 and 12 weeks gestation. Fetal sex determined for prenatal diagnosis of hemophilia using SRY gene markers and high resolution markers of sex chromosomes by QF-PCR. The results compared with genetic tests on CVS samples. We confirmed the persistence of fetal DNA in maternal blood and investigated cell-free fetal DNA as a reliable approach in prenatal diagnosis of hemophilia. High accuracy and possibility of analyzing circulating fetal DNA in maternal blood highlights this method as a reliable one to early non-invasive determination of fetal sex to avoiding problems of invasive methods.

Keywords: Fetal DNA, Hemophilia, SRY gene, Prenatal diagnosis

Introduction

Particular Mendelian inheritance manner of X-linked diseases, accursed presence and expressing of phenotype in male form a mutation in a gene on the X chromosome.

Early diagnosis of genetic diseases linked to genes on the X chromosome is so important implications, as since those diseases only affect males. In most cases today, doctors diagnose X-linked diseases prenatally with invasive tests, such as chorionic villus sampling or amniocentesis, which carry a small risk of miscarriage. However, X-linked diseases cannot diagnose by fetal gender test, but it is possible to determine whether a fetus is male before conducting more invasive testing. The ability to determine the sex of a fetus accurately and early in pregnancy could reduce the number of invasive tests for sex-specific diseases by 50% (Costa et al., 2002).

In the routine classic clinical methods, for determination of fetal sex in the X-linked genetic disorders prenatal diagnosis, Duchenne muscular dystrophy (DMD) or hemophilia, Chorionic villus sampling (CVS) and amniocentesis should collected for genetic tests base on the extracted fetus DNA. A female fetus may have a wild-type genotype or be a carrier of X-linked genetic disorders, but further genetic analysis is crucial for a male fetus because a male fetus has a 50% change of having X-linked genetic disorders. Pregnant carriers risk miscarriage when undergoing such invasive prenatal diagnosis (IPD). Noninvasive prenatal diagnosis (NIPD) is preferable for fetal sex determination during the first trimester since it avoids the unnecessary risks of IPD in pregnant female X-linked genetic disorders carriers.

Cell-free circulating DNA (cffDNA) has been studied in a wide range of physiological and pathological conditions, including pregnancy, trauma, inflammatory disorders and malignancy (Costa et al., 2002; Pietropolli et al., 2016). It is present in normal healthy individuals at low concentrations (ng/ml) (Bianchi et al., 2001). For the first time, in 1997, Cell-free fetal DNA (cffDNA) was found in maternal plasma (Xue et al., 2009) and potential form of noninvasive prenatal diagnosis considered for it due to its measurement also its early

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detection, as early as 7 weeks, in maternal plasma. cffDNA comprises about 3-6% of the total cell-free DNA in maternal plasma (Wright et al., 2012).
The first trimester of pregnancy is clearly a critical period for prenatal diagnosis using non-invasive procedures (Yang et al., 2011). A technique for non-invasive fetal sex determination during the early trimester of pregnancy is Y chromosome (SRY) or other Y chromosome-specific sequences based on cffDNA from maternal plasma (Bianchi et al., 2001; Kimura et al., 2011; Wright et al., 2012; Xue et al., 2009; Yang et al., 2011).
To address the possibility that the investigation of cell-free fetal DNA can be a useful tool for the prenatal diagnosis of hemophilia by the assessment of Y-chromosomal sequences, we have analyzed maternal plasma for determination of the fetus gender.

Materials and Methods

Various methods have been used to purify cfDNA, including using modified salting-out, chromatography resins, magnetic beads, or guanidium thiocyanate (Keshavarz et al., 2015; Mackie et al., 2016; Qi et al., 2016; Swanson et al., 2013). The most popular is the QIAamp blood kit, which binds DNA to a silica-gel membrane, providing a fast and easy way to purify total DNA for polymerase chain reaction (PCR) analysis. Because DNA is present in plasma/serum at such Low concentrations (ng/ml), it is crucial to optimize laboratory protocols for the processing and extraction of cfDNA also comparing it by some standard commercial methods. In this study, we evaluated a simple Triton/Heat/ Phenol (THP) protocol and in parallel check its yield by the Bioscience cfDNA extraction kit.

Sampling and Extraction of cffDNA In Maternal Plasma

From patients referred to our center for PND test of hemophilia, some patients at about 9 weeks of gestation 8 mL blood samples were taken on the EDTA tube. The blood samples were centrifuged twice at 3,000 *g* and then at 12,000 *g* to obtain cell free plasma. Cell-free DNA was extracted from 2 mL of maternal plasma using Triton/Heat/Phenol protocol (THP) method. DNA was eluted into 40 µl of solution buffer. However, in some studies (Keshavarz et al., 2015; Mackie et al., 2016; Pietropolli et al., 2016), it recommended to separate plasma by double centrifugation at 800 *g* for 10 min, and 1600 *g* for 10 min and then immediately freeze it at -70°C.
For the THP method, 500 µl of plasma was mixed with 5 µl Triton X-100 (Sigma-Aldrich, UK) for better mixes, we incubate plasma and triton on 37 °C for 10 min and short vortex. After that, samples heat denatured at 98°C for 5 min and were placed on ice for 5 min to be cold. An equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v: v: v) (Sigma-Aldrich, UK) added to each tube, then centrifuged for 10 min at 14,000 g.
The aqueous upper phase was separated gently in the new tube to avoid mixing with down phase. 1/10 volume of 3 M NaOAc added to each tube within 2.5 volumes of 100% ethanol. The samples precipitated overnight with at -20°C.
Samples centrifuged for 30 min at 16000 *g* and removed supernatant. The DNA pellet was washed with 1000 µl ethanol 70% and following twice with 1000 µl absolute ethanol. Final washing with 100% ethanol should followed by 5 min centrifuge at 1600 *g* and remove carefully all aqueous phase. Samples air-dried and extracted DNA re suspended in 50 µl of ddH_2O. To optimize the protocol and determining if this DNA extraction method led to loss of small DNA fragments, the 100-bp-DNA Ladder was spiked into collected plasma. After re-purification, the ladder DNA was analyzed on 1.5% agarose gels with 0.5 µg/ml ethidium bromide.

Sex Determination Using SRY Gene Markers

The sequence of sex-determining region (SRY) markers of the Y chromosome was used to identify male fetal DNA present in each sample of a pregnant woman. To detect SRY gene on the samples we prefer Sequence-Tagged-Site (STS) Markers of SRY. STS marker in this region (SY14) under Gene bank access number G38356 is a short (469 bp) location known DNA sequence which is a single occurrence in the genome. For constructing genetic and physical maps of this STS marker we designed specific primers to detect it by the polymerase chain reaction (PCR).
Primer sequences were as follows:
SRY forward primer: GAATATTCCCGCTCTCCGGA
SRY reverse primer: GCTGGTGCTCCATTCTTGAG
Reactions were set up in a 20 µl volume using 50 ng of template, 1 µM of each primer, 100 µM of dNTPs and 1 unit of Taq Polymerase. The test consisted an initial 2 min at 92°C, a denaturation step of 95°C for 10 min, and then 30 cycles of 94°C for 1 minute, 58°C for 1.5 minutes and 72°C for 2 minutes. All samples were analyzed blindly with respect to fetal

gender. This method can indicate a male genotype by the presence of the amplified product from the SRY gene, but cannot accurately indicate a female genotype.

One of the reasons for this limitation is that the amplicon may be absent due to lack/degeneration of DNA in the examined specimen. The other reason is that a technical error during the examination process might cause a false negative result.

To solve this problem, also as complementary test, we used short tandem repeats (STRs) markers included in QF-PCR kits designed for testing chromosomal aneuploidies. In this test, homologous sequences in Amelogenin genes on the X and Y chromosomes (AMELX and AMELY) are simultaneously amplified and the sample of female origin can be discriminated from a false negative by single peak derived from the X chromosome on the electropherogram (Chan et al., 2004).

We used Aneufast QF-PCR kit to check high resolution markers of XY to assess sex determining markers also aneuploidy on X and Y chromosome for samples.

Results and Discussion

Optimization of THP Protocol

In the extraction of cffDNA, the obtained yield hardly depends on methods of sample collection, storage to different parts of the extraction protocol. Thus, optimizing the process is so important.

To optimize the yield of low-level cfDNA from plasma, we considered the effects of delays in blood processing and storage temperature prior to the DNA extraction also temperature of centrifuge and different mount of initial buffers for extraction. For each blood sample, different aliquots tests under different conditions of plasma collection and extraction protocol.

Finally THP protocol has optimized for our lab and used to experiment. The obtaining of cffDNA was differ in the different conditions, but there was no significant difference in cfDNA yield for samples processed up.

We tested different times also temperatures for incubation in the THP method and finally optimized, 98°C for denature plasma proteins also inactivate inhibitors of PCR. We used Triton X-100 for solubilization of protein instead of SDS to avoiding contamination of DNA.

For plasma collection method, we tested different time of delays in separating the plasma. Our finding was under contradicts of Jung et al., 2003 reports (Jung et al., 2003).

They reported that the DNA concentration in plasma did not change when blood samples were stored at room temperature for 8 hours, or store for 24 hours at 4°C.

We optimized the collected blood samples on the EDTA tubes and held for maximum 2 hours at room temperature or below before plasma separation. This method will be useful for avoiding leukocyte lysis and contamination of the plasma with genomic DNA.

Also for avoiding leukocyte lysis, we separated the Plasma from whole blood samples by separately double centrifugation for 10 minutes at 800 *g* and then in 1600 *g*.

After plasma preparation, we tested cffDNA extraction from frozen plasma on −70 °C and freeze plasma samples. However, there were no difference on cffDNA yield, but to avoiding DNA fragmentation in the repeated freezing and thaw cycles, we preferred start extraction immediately after plasma preparation. This finding is in the agreement of Xue, et al., 2009 and Keshavarz Z et al., 2015 reports on THP protocol.

To test the efficiency of extraction of small DNA fragments in the THP protocol, we added DNA ladder (100-bp) in the collected blood before cffDNA extraction.

1.5% agarose gel used to test extracted DNA and ladder fragments. The result showed the possibility of small fragments DNA extraction. However, for finding out the presence of DNA we prefer direct standard PCR for SRY marker and quantitative fluorescence PCR test for XY markers.

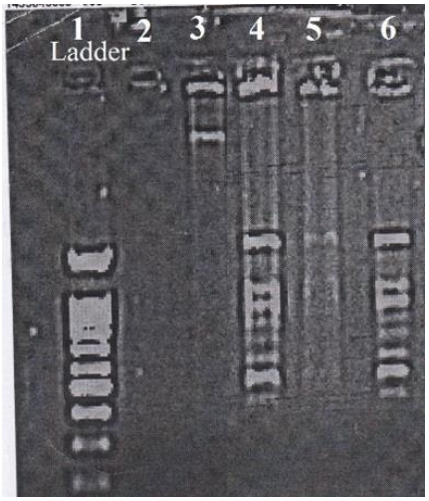

Figure 1. Detection of small DNA fragments in the THP protocol by adding ladder before extraction. 2 and 3: unsuccessful extraction sample 4,5 and 6 detected small fragments by extraction.

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Assessment of Male Fetal DNA Presence

An STS marker for the SRY region has been detected in normal male samples as positive control. It also checked in the female samples as negative sample. All extracted cffDNA tested for SRY presence as a male fetus. In different samples mount of DNA for this test optimized after the first test. We find out that gestation age has a direct effect on cffDNA yield. Gestation age 12 weeks yield more cffDNA comparing 8 weeks gestation. SRY negative samples did not lead clear result of female fetus due to the possibility of no DNA or degenerated DNA on the tested specimen.

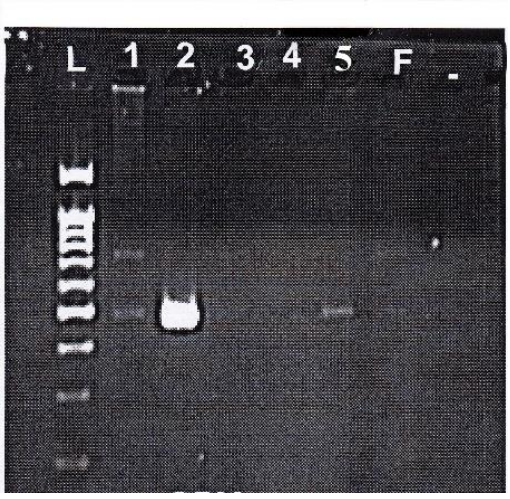

Figure 2. STS marker of SRY gene in some extracted cffDNA samples. 1 and 5: Male fetus. 3 and 4: Female fetus. 2: Control positive male sample F: Female sample as negative control.

For high accuracy of sex detection and eliminate the limitation of SRY test, all samples tested for 5 markers of sex chromosome. Figure 3 shows QF-PCR result of one of the samples. According to the kit manual, AMXY marker included two alleles with 104 and 110 bp size, which the last one belongs to the Y chromosome. The combination of this marker and SRY of the QF-PCR test is a common test to identify the sex of the sample. Which two alleles of AMXY and one allele of SRY indicates healthy X and Y chromosome in the fetus. SRY marker in healthy male should presence a 463 bp allele.

As a complementary test for aneuploidy of sex chromosomes we simultaneously tested X22, HPRT and DXYS218 marker indicating of a number of X and Y chromosome on the samples. X22 has two alleles on chromosome Xq28 and Yq by 226 and 229 bp size respectively. DXYS218 has 278 bp allele on Xp22.32 and 282bp allele on Yp11. HPRT has just allels on the Xq26.1 which number of the alleles indicate number of X chromosome on the sample. All studied markers in the sample of Fig.3 are two informative allele with a normal ratio of pick height and area except HPRT marker which has three allele. In this example, usually more high resolution markers on sex chromosomes should genotype to understand and distinguish the correct karyotype of the fetus.

Conclusion

For this study, we collected blood from 10 voluntary pregnant women in their 8 and 12 weeks of gestation age. They have applied for PND program for their hemophilia/thalassemia carrier mutations. For PND program, at the 12th week of gestation age CVS sample collecting to test the fetus genotype. We tested fetus gender from extracted cffDNA and CVS DNA in parallel for each sample. In the result we correctly detect 4 male and 6 female fetus, which results of both SRY and QF-PCR method had confirmed by CVS DNA tests.The results of this study showed that prediction accuracy of sex detection of SRY and QF-PCR method from cffDNA is high and would be useful in prenatal diagnosis as a pretest to detecting the fetus gender. Maternal plasma can use to extract cffDNA instead of unnecessary chorionic villus sampling on the pregnancies at risk of X-linked disease.

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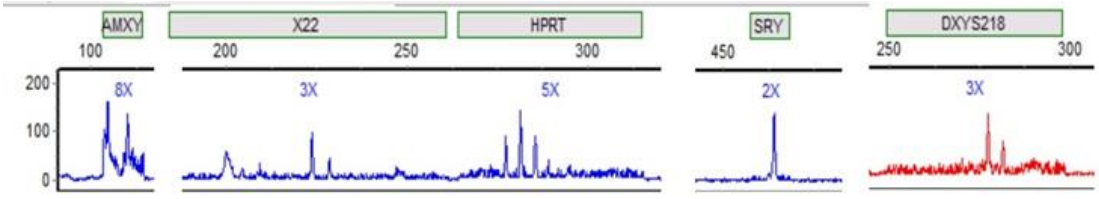

Figure 3. QF-PCR markers on sex chromosomes. A sample of extracted cffDNA, Male fetus.

References

1. Bianchi D. W., LeShane E. S. and Cowan J. M. (2001) Large amounts of cell-free fetal DNA are present in amniotic fluid. Clinical Chemistry 47:1867-1869.
2. Chan K. A., Zhang J., Hui A. B., Wong N., Lau T. K., Leung T. N., Lo K.-W., Huang D. W. and Lo Y. D. (2004) Size distributions of maternal and fetal DNA in maternal plasma. Clinical Chemistry 50:88-92.
3. Costa J.-M., Benachi A. and Gautier E. (2002) New strategy for prenatal diagnosis of X-linked disorders. New England Journal of Medicine 346:1502-1502.
4. Jung M., Klotzek S., Lewandowski M., Fleischhacker M. and Jung K. (2003) Changes in concentration of DNA in serum and plasma during storage of blood samples. Clinical Chemistry 49:1028-1029.
5. Keshavarz Z., Moezzi L., Ranjbaran R., Aboualizadeh F., Behzad-Behbahani A., Abdullahi M. and Sharifzadeh S. (2015) Evaluation of a modified DNA extraction method for isolation of cell-free fetal DNA from maternal serum. Avicenna Journal of Medical Biotechnology 7:85.
6. Kimura M., Hara M., Itakura A., Sato C., Ikebuchi K. and Ishihara O. (2011) Fragment size analysis of free fetal DNA in maternal plasma using Y-STR loci and SRY gene amplification. Nagoya Journal of Medical Science 73:129.
7. Mackie F., Hemming K., Allen S., Morris R. and Kilby M. (2016) The accuracy of cell-free fetal DNA-based non-invasive prenatal testing in singleton pregnancies: a systematic review and bivariate meta-analysis. BJOG: An International Journal of Obstetrics & Gynaecology.
8. Pietropolli A., Capogna M. V., Cascella R., Germani C., Bruno V., Strafella C., Sarta S., Ticconi C., Marmo G. and Gallaro S. (2016) Three-hour analysis of non-invasive foetal sex determination: application of Plexor chemistry. Human Genomics 10:1.
9. Qi Q., Lu S., Zhou X., Yao F., Hao N., Yin G., Li W., Bai J., Li N. and Cram D. S. (2016) Copy number variation sequencing-based prenatal diagnosis using cell-free fetal DNA in amniotic fluid. Prenatal Diagnosis 36:576-583.
10. Swanson A., Sehnert A. J. and Bhatt S. (2013) Non-invasive prenatal testing: technologies, clinical assays and implementation strategies for women's healthcare practitioners. Current Genetic Medicine Reports 1:113-121.
11. Wright C. F., Wei Y., Higgins J. P. and Sagoo G. S. (2012) Non-invasive prenatal diagnostic test accuracy for fetal sex using cell-free DNA a review and meta-analysis. BMC Research Notes 5:1.
12. Xue X., Teare M. D., Holen I., Zhu Y. M. and Woll P. J. (2009) Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. Clinica Chimica Acta 404:100-104.
13. Yang Y.-H., Han S.-H. and Lee K.-R. (2011) Noninvasive prenatal diagnosis using cell-free fetal DNA in maternal plasma: clinical applications. Journal of Genetic Medicine 8:1-16.