Detection of Fetal Nucleic Acid in Maternal Plasma: A Novel Noninvasive Prenatal Diagnostic Technique

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Abstract: *Prenatal diagnosis is routinely offered to the antenatal women seeking advice regarding genetic abnormalities in the fetus. The routine tests available for prenatal screening and diagnosis are invasive and have risk of miscarriage. The detection of fetal nucleic acid especially the cell free fetal DNA in maternal plasma is a newer non invasive prenatal diagnostic technique. Its analysis is based on distinct and detectable differences between fetal and maternal genomes thus it can be employed in the detection of aneuploidy, single gene disorders, sex linked diseases and pregnancy related disorders like preeclampsia.*

INTRODUCTION

Prenatal diagnosis is an established part of modern obstetrics. It is routinely offered to the antenatal women seeking advice regarding genetic abnormalities in the fetus or as such it can be part of routine screening offered to all antenatal women.

The tests available for prenatal screening and diagnosis involve removal of fetal cells directly from the uterus, using either chorionic villous sampling (CVS) at 11-14 weeks or amniocentesis after 15 weeks. Although this approach to the fetal testing is gold standard and gives definitive diagnosis, the chances of miscarriage (around 1%)[1] and invasiveness makes it inconvenient to pregnant women.

Thus the need for the non-invasive methods of detection of fetal cells led to detection of these fetal cells in the cervical mucus[2,3] and in maternal blood. Research has now mainly focused on strategies for detecting genetic elements from the fetus present in the maternal circulation.

It is popularly thought that placenta forms an impermeable barrier between mother and child, but on the contrary there is bidirectional traffic between the fetus and the mother during pregnancy[4]. Intact fetal cells present in maternal blood present an attractive target for non-invasive prenatal diagnosis (NIPD), particularly for the diagnosis of fetal sex and chromosomal abnormalities. In 1990, Bianchi et al first isolated intact fetal nucleated red blood cells for the purpose of prenatal diagnosis[5]. Since then, the isolation and detection of fetal cells from maternal blood has been extensively investigated by different researchers[6,7], and various methods of fetal cell enrichment were developed[8]. However, results to date have been disappointing because of:

- Scarcity of intact fetal cells in the maternal circulation (around one cell/ml of maternal blood)[9]
- Low efficiency of enrichment**.**
- Difficulties with chromosomal analysis associated with abnormally dense nuclei in some cells[10]
- Persistence of fetal cells in the maternal circulation for decades following pregnancy potentially causing false-positive results in subsequent pregnancies**.**

Therefore the majority of recent research has focused on cell-free fetal DNA (cffDNA) in the maternal blood.

Mandel and Metais were first to discover the presence of small amounts of extracellular DNA in the circulation of both healthy and diseased subjects in 1947. After 50 years the presence of fetal DNA in the maternal circulation was demonstrated by Lo et al. Fetal DNA can be detected from the 4th week of gestation, though only reliably from 7 weeks, and the concentration increases with gestational age-from the 16 fetal genomes per ml of maternal blood in the first trimester to 80 fetal genomes per ml in the third trimester, with a sharp peak during the last 8 weeks of pregnancy. Fetal DNA originates trophoblast cells and comprises around 3–6% of the total cell-free DNA in maternal circulation during early and late pregnancy, respectively (the other 94–97% being maternal cell-free DNA). Unlike cellular DNA, circulating cffDNA consists predominantly of short DNA fragments rather than whole chromosomes, of which 80% are <193 base-pairs in length. In contrast to fetal cells, cffDNA is rapidly cleared from the maternal circulation with a half life of 16 minutes and is undetectable after 2 hours of delivery.

METHODS OF DETECTING cff DNA

Isolation of fetally derived cell-free DNA in an overwhelming background of maternal cell-free DNA is a significant technical challenge. There are a number of general problems associated with detecting cffDNA in the maternal circulation:

- Concentration of all cell-free DNA in blood is relatively low.
- Total amount of cell-free DNA varies between individuals.
- cffDNA molecules are outnumbered 1:20 by maternal cell-free DNA molecules.
- Fetus inherits half its genome from the mother so that cannot be distinguished.

The basic principle in extracting the cffDNA is to take initially maternal plasma, separate cellular matter by centrifugation, followed by isolation and purification of all cell-free DNA, followed by exploiting the small differences between the fetal and maternal DNA sequences in order to make a specific fetal diagnosis.

To date, the majority of studies have focused on the detection of paternally inherited sequences that are entirely absent from the maternal genotype, such as those on the Y chromosome of male fetuses. Variable regions of repeated DNA (short tandem repeats or STRs) can be used to identify paternally inherited sequences. Importantly, all these methods rely upon the fetus inheriting a uniquely paternal sequence that is conveniently located for a particular diagnosis. The most common technique currently used for detection and identification of specific cffDNA sequences are:

Polymerase chain reaction (PCR). Most popular types of PCR used are

- real-time quantitative PCR[18]
- Nested PCR[19]
- pyrophosphorolysis-activated polymerization PCR[20]
- digital PCR which allows the exact number of original template DNA molecules to be counted.[21]

Mass spectrometry[22], in which the precise mass of each DNA fragment is analysed to determine the genetic sequence, and hence detect fetal-specific alleles that differ from the maternal sequence by as little as a single base.

Multiplexed maternal plasma DNA sequencing analysis to rule out fetal trisomy 21 among high risk pregnancies[23].

The accuracy and reliability of detection can be significantly improved by increasing the proportion of fetal DNA relative to maternal DNA in the sample thus increasing the signal-to-noise ratio. There are two alternative methods specifically aimed at this:

1. ***Selective enrichment of fetal DNA:*** It is based on a difference in the average physical length of fetal and maternal DNA fragments, which can be exploited to increase the relative amount of cffDNA. Fetal derived DNA fragments are generally smaller than those that are maternally derived (mostly less than 313 base-pairs in length). Therefore, by using standard size fractionation to select only DNA fragments <300 base-pairs, circulating cffDNA can be enriched such that it comprises around 70% of the total cell-free DNA, prior to detection and identification by either PCR or mass spectrometry.
2. ***Suppression of maternal DNA by the addition of formaldehyde[24]:*** A chemical that is thought to stabilize intact cells, thereby inhibiting further release of maternal DNA into the sample and increasing the relative proportion of fetal DNA. However, the use of formaldehyde for this purpose is controversial[25].

CLINICAL APPLICATIONS OF cff DNA ANALYSIS

cff DNA analysis is based on distinct and detectable differences between

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fetal and maternal genomes thus it can be employed in the detection of:

1. Sex determination can be done by detecting cff DNA sequences on the Y chromosome; thus mainly helpful for sex-linked disease, such as haemophilia, Duchenne muscular dystrophy, X-linked mental retardation, adrenoleukodystrophy, Alport's syndrome, X-linked severe immunodeficiency, retinitis pigmentosa, X-linked hydrocephalus, anhidrotic ectodermal dysplasia, Hunter's syndrome, Menke's syndrome and Lesch–Nyhan syndrome. Sex determination is also important in cases where development of external genitalia is ambiguous and in some endocrine disorders, such as congenital adrenal hyperplasia (CAH), where there is masculinization of the female fetus, which is preventable with antenatal treatment. It has recently been shown that some false positives are due to the presence of a vanishing (male) twin. In order to reduce this problem, it has been suggested testing for fetal sex by cffDNA should be accompanied by an ultrasound scan, which could be done early in pregnancy, as loss of the twin usually occurs in the first 7 weeks
2. Single gene disorders can be detected by identifying a paternally inherited allele in cffDNA; Huntington's disease, achondroplasia, myotonic dystrophy, fetal carrier status in cystic fibrosis, hemoglobinopathy, CAH
3. Aneuploidy can be identified by detecting an abnormal concentration of a particular chromosome, potentially using cffRNA specific to the fetus and chromosome of interest i.e. Down's syndrome (trisomy 21), Edward syndrome (trisomy 18), Patau syndrome (trisomy 13), Turner syndrome (X0) and triple X syndrome (XXX) in female births, Klinefelter syndrome (XXY) and XYY syndrome in male births.
4. Pregnancy-related disorders can be identified by detecting either the presence of a working copy of the Rhesus gene or an elevation in the absolute concentration of cffDNA in abnormal functioning of placenta (usually measuring Y chromosome DNA of male pregnancies). It is elevated by 2–3 folds before the onset of preeclampsia and 2–14 folds during preeclampsia[26]. In addition to preeclampsia, a number of other pregnancy-related disorders have been linked to an elevated concentration of cffDNA which include preterm labour, hyperemesis gravidarum, invasive placentation, intrauterine growth restriction, feto-maternal haemorrhage and polyhydramnios.

TECHNICAL DIFFICULTIES

There are a number of technical and clinical obstacles to achieving high diagnostic accuracy:

- It is important to emphasize that complete fetal genotyping is not conceivable using cffDNA in the maternal circulation and that the genetic information derived from cffDNA is entirely restricted to the specific DNA sequence (or chromosome) detected.
- False negatives can be the result of failure to extract or detect sufficient material, due to individual variability in the amount of total cell-free DNA and the small proportion of fetal versus maternal cell-free DNA.
- False positives can be the result of either technical issues, such as contamination, or clinical abnormalities such as the presence of a non-identical vanishing twin.

Therefore, extensive clinical trials will be required for each application to evaluate both the analytical and clinical validity before this technique could be used reliably in a clinical setting.

UNIVERSAL FETAL MARKERS

A major area of current research is aimed at finding universal fetal-specific markers that could be used either as diagnostic tests in their own right or to confirm and quantify the presence of fetal DNA independent of sex or other specific diagnostic tests. These could be used alongside clinically relevant diagnostic tests as a positive control for the presence of cffDNA, in order to highlight false-negative results either caused by low levels of circulating DNA below the detection limit of the test or problems with the DNA extraction process. One of the methods under investigation is the detection of specific DNA sequences located on the autosomal chromosomes that can be shown to be paternally inherited, including:

1. Single nucleotide polymorphisms (SNPs), or point mutations, which differ between the maternal and paternal genomes but may not be directly linked to a specific disease[27,28]. It relies upon selective enrichment of the cffDNA followed by analysis by a highly sensitive technique such as mass spectrometry.
2. Polymorphic segments of DNA that vary between the maternal and paternal genomes, such as STR sequences. Because of the highly variable nature of STRs, the paternally inherited fetal STR sequence will differ in the number of repeats from the maternal sequence. Amplification of these STR sequences will therefore result in two major products corresponding to the maternal alleles (and the maternally inherited fetal allele) and one minor product corresponding to the paternally inherited fetal allele. However, the technique has yet to be optimized for clinical diagnostic use and the sensitivity and specificity have not been established.

Fetal nucleic acids other than cffDNA detected in the maternal circulation which can be helpful in the prenatal diagnosis are:

- Detection of cell free fetal RNA (cffRNA): Like cffDNA, cffRNA is detectable within the maternal circulation early in the first trimester and is rapidly cleared following birth, with a half-life of 14 minutes[29]. Since the expression of certain genes is unique to pregnancy, detection of placental/fetal RNA is an extremely promising avenue for research, as it is relatively easy to isolate completely from background maternal RNA.
- Detection of proteins derived from genes that are uniquely expressed in the placenta or fetus. Placentally expressed genes may result in potentially diagnostic fetal proteins in the maternal bloodstream[30].

CONCLUSION

The study of fetal proteomics is currently still in the infancy, with the primary aim being to improve the panel of serum markers used in screening for Down syndrome and Rh D typing. It is expected that over the coming years, technological advances will make it possible to implement it for a number of other clinical conditions.

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