Review Article

Fetal Nucleic Acids in Maternal Circulation: A Genetic Resource for Noninvasive Prenatal Diagnosis

Monisha Banerjee and Deepika Misra

Molecular & Human Genetics Laboratory, Department of Zoology, University of Lucknow, Lucknow 226007, India

Correspondence should be addressed to Monisha Banerjee; mhglucknow@yahoo.com

Received 30 May 2012; Accepted 31 July 2012

Academic Editors: C. Walss-Bass, A. Yamamoto, and A. Zamborlini

Copyright © 2013 M. Banerjee and D. Misra. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Invasive prenatal diagnosis (PND) holds a multitude of psychological considerations for women, their partners, family and community as a whole. Earlier, the non-invasive screening methods for certain disorders were serum analytes or ultrasound with low sensitivity and high false positivity. The discovery of fetal DNA in maternal plasma has opened up an approach for noninvasive PND (NIPD). Presence of fetal cells and cell-free fetal DNA (cffDNA) in the blood of pregnant women has been accepted universally and constant efforts are being made to enrich fetal DNA from maternal blood/plasma. Real-time quantitative polymerase chain reaction (qrt-PCR) has enabled fetal DNA to serve as a marker for chromosomal abnormalities, for example, trisomy 21, preterm labor, and preeclampsia. In India, PND is provided in few centers since invasive methods require trained gynecologists, this limits investigation and therefore NIPD with cffDNA from mother’s blood will revolutionize fetal medicine. The present paper deals with the latest developments in procurement of cffDNA, the probable source and enrichment of fetal DNA in maternal plasma, and the current status of its detection methodologies, applications, and its potential to be used as a powerful diagnostic tool.

1. Introduction

The frequency of inherited disorders database (FIDD) contains a total of 1580 records, classified into 14 groups of inherited disorders, of which information on 280 conditions comprising of 109 autosomal dominant, 136 autosomal recessive, and 35 X-linked disorders is currently available. There are also 19 groups of less well-defined conditions such as “inherited neuromuscular disorders” or “hemophilias” [1]. The only solution to diagnose such genetic disorder is prenatal diagnosis (PND). PND had its beginning in 1966, when Steele and Breg showed that the chromosome constitution of a fetus could be determined by analysis of cultured cells from amniotic fluid [2]. Later with the advent of molecular techniques, mutation and carrier analysis was performed by obtaining fetal tissue using invasive methods such as chorionic villus sampling (CVS), placental tissue, and amniocentesis [3]. Genetic disease in a family involves a great deal of anxiety such as having an affected child, lifelong morbidity, risk of miscarriage, and dilemma of taking the correct decision (Table 1) [4]. Nucleic acids (DNA and RNA) in the blood were observed >60 years ago as early as 1948, when Mandel and Metais reported the presence of cell free DNA (cfDNA) and RNA (cfRNA) in blood plasma of healthy and sick individuals [5–7]. Unfortunately, their work went unnoticed due to lack of clear understanding about cell free nucleic acids (cfNAs). Increased quantities of cfDNA were also observed in the plasma of cancer patients; for example, pancreatic cancer [8]. The presence of fetal cells and cfDNA in the blood of pregnant women has been accepted universally. It is known that 3–6% of maternal plasma DNA is cfDNA which has 100% detection rate [9–11]. An attempt has been made to review the developments till date by various groups in obtaining cell-free fetal nucleic acids (cffNAs) from maternal blood, its probable source, methods of detection, and applications in non-invasive prenatal diagnosis (NIPD) for a variety of genetic disorders.

2. Source of Cell-Free Fetal Nucleic Acids (cffNAs) in Maternal Circulation

Circulation of cfDNAs was demonstrated within maternal plasma and serum from healthy pregnant women, in surprisingly high mean concentrations of 3.4 and 6.2% of total
plasma DNA and 0.13 and 1.0% of total serum DNA in early and late pregnancies, respectively [9, 12]. An increase in the number of fetal cells was demonstrated in the blood of pregnant women who were carrying aneuploid fetuses [13]. Since then, analysis of fetal DNA in maternal plasma has been introduced as a new method for NIPD. There are three possible sources of fetal DNA in maternal circulation: (a) direct transfer of DNA, (b) placenta, and (c) haematopoietic cells.

Placenta is no longer thought to be an impermeable membrane since fetal DNA/RNA circulates freely in maternal plasma. Since a rapidly growing fetus and placenta have "tumor-like" characteristics cfDNA of fetal origin can be expected to be found in maternal circulation [11, 14].

Presence of fetal RNA in the plasma of pregnant women was shown through the mRNA detection of a gene on the Y chromosome [15, 16]. The detection rates of plasma fetal RNA in early and late pregnancies were 22 and 63%, respectively [9, 15, 17]. Plasma fetal RNA analysis provided valuable information regarding gene expression in fetal tissues as in case of pregnancies with preeclampsia where abnormal patterns were observed [15]. Cell free fetal RNA (cffRNA) was not expected to be present in plasma due to the presence of RNAses. However, it was shown by detection of human placental lactogen (hPL) and β-hCG (human chorionic gonadotropin) mRNAs that the placenta was a major source of fetal-derived RNA [18]. A microarray-based approach was developed for systematic identification of placental mRNA markers such as the one transcribed from placental-specific 4 gene (PLAC4) located on chromosome 21, which could be detected in maternal plasma [19]. Subsequently, it was postulated that the placental-expressed mRNAs were encapsulated within a syncytiotrophoblast-derived microvesicle (STBM), which gave RNase resistance to "free" fetal RNA in maternal plasma [20]. Thus, with the development of RNA markers, analysis of maternal plasma allowed the non-invasive monitoring of fetal gene expression in a multitude of physiological and pathological conditions [21].

The various possible pathways by which cfDNA/cfRNA is released into circulation have been shown in Figure 1. Although evidence of high levels of circulating DNA and RNA in plasma of patients is increasing day by day, the actual origin of cfRNA is still not clear [22]. The cfDNA rapidly disappears from the maternal circulation following childbirth with one exception in case of maternal liver disease where significant levels of fetal DNA were seen even after parturition [16, 23]. A proper understanding of these issues will help in identifying the clinical applications of cfRNAs.

### Table 1: Risk of miscarriage in invasive methods of PND.

<table>
<thead>
<tr>
<th>Invasive test for prenatal diagnosis</th>
<th>Sample taken through</th>
<th>Gestation time at which procedure performed</th>
<th>Risk of miscarriage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chorionic villus Sampling (CVS) or chorionicentesis</td>
<td>Abdomen or vagina</td>
<td>11–13 weeks</td>
<td>&lt;1% (1.9)</td>
</tr>
<tr>
<td>Amniocentesis or amniotic fluid test (AFT)</td>
<td>Abdomen</td>
<td>9–18 weeks</td>
<td>&lt;1% (1.4)</td>
</tr>
<tr>
<td>Cordocentesis or percutaneous umbilical cord blood sampling (PUBS)</td>
<td>Abdomen</td>
<td>18–20 weeks</td>
<td>~2%</td>
</tr>
</tbody>
</table>

*Procedure risk of miscarriage increases with advancement in maternal age.

2.1. Concentration of Fetal DNA in Maternal Plasma. The fractional concentration of cfDNA in maternal plasma is determined by the ratio of absolute concentration of cfDNA to absolute concentration of total (maternal and fetal) cfDNA. The concentration of cfDNA in maternal plasma was significantly higher in pregnant women compared to controls, median genome equivalent (GE) was 3.186 and 0.595 respectively ($P = 0.001$). This increased amount of cfDNA independent of previous pregnancies could be utilized for PND [24, 25]. There is significant correlation between early gestation age and total DNA concentration with a mean increase of 29.3% fetal DNA every week [26, 27]. Selective enrichment of fetal DNA requires targeting its unique characteristics that are different from maternal DNA [28]. Thus, the fractional concentration of cfDNA can be increased through selective enrichment of fetal DNA or through suppression of the background maternal DNA [22]. The three major approaches are shown below:

(A) % of fetal DNA = fetal DNA/maternal DNA + Fetal DNA,

(B) Increased % of fetal DNA = increased Fetal DNA/ maternal DNA + Fetal DNA,

(C) Increased % of fetal DNA = fetal DNA/decreased maternal DNA + fetal DNA.

2.2. Size Distribution of Maternal and Fetal DNA. The specific characteristic of fetal DNA in maternal plasma is the size of cfDNA, which is smaller than the corresponding maternal DNA. The median percentages of plasma DNA with size >201 bp were 57, and 14% respectively, in pregnant and nonpregnant women ($P < 0.001$). The fetal-derived fragments were shorter than the maternal ones, 80% being <193 bp and 90% <313 bp [29, 30]. Size fractionation of circulating DNA indicated that major portion of cfDNA was <0.3 kb, whereas maternally derived sequences were >1 kb. Analysis of size-fractionated DNA (<0.3 kb) from maternal plasma samples facilitated the ready detection of paternally and maternally inherited microsatellite markers. The size fractionation method is based on agarose gel electrophoresis followed by DNA extraction from manually cut agarose gel slices containing factors of different sizes [28]. This method was applied for the detection of paternally inherited fetal mutations [31]. However, the need for automated size fractionation methods such as microfluidics digital PCR was felt for its practical use in NIPD [22]. It revealed ~3.1 times higher fraction of fetal DNA than conventional nondigital real-time PCR [32].
3. Detection of cfDNA in Maternal Circulation

DNA up to picogram concentrations can be detected using PCR. Earlier SRY markers were used for detection of cfDNA in maternal plasma [10]. However, with the advent of molecular techniques, various strategies have been developed, the list of fetal-specific markers has expanded from sex-dependent Y chromosomal sequences [33] and paternally inherited polymorphic markers [34–37], universal fetal markers such as placental-specific mRNA transcripts [18, 19, 37] and placental-specific epigenetic signatures [38–40]. Presently, real-time quantitative PCR is used for amplifying and quantifying the circulating DNA/RNA of interest [41, 42]. Light-cycler based real-time PCR is rapid, eliminates carry-over contamination problems and does not require post-PCR processing [43]. Total plasma circulating nucleic acids (DNA+RNA) can be detected using SYBR Green II dye [44]. The fetomaternal trafficking is a bidirectional process [45], therefore epigenetic markers may also have a role in the investigation of DNA transfer from mother to fetus [46, 47]. The measurement of plasma mRNA markers has provided a gender-independent approach for noninvasive prenatal gene expression profiling and has opened up numerous research and diagnostic possibilities.

3.1. Fetal DNA Detection by Y-Specific STR Markers. The presence of cfDNA in plasma of healthy pregnant women was showed in 1997 and confirmed by real-time PCR of Y-chromosome-specific SRY sequences [11, 48]. Several reports have confirmed positive correlation of fetal DNA concentration with gestational age. There is a slow increase in the first trimester which is raised by second and third trimesters [9, 49]. The amplification sensitivity of Y-specific STRs and DYS19 sequences was 100% in male fetal samples, while STRs DYS385 and DYS392 were 91% each [16]. However, sensitivity of STRs might be lowered with maternally inherited alleles. Therefore, cfDNA enabled the early detection of fetal sex so that further invasive tests could be avoided in case of X-linked diseases 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, Menkes syndrome, Lesch-Nyhan syndrome, and congenital adrenal hyperplasia (CAH) [10, 50].

3.2. Fetal RhCE Genotyping. Fetal Rh genotyping using cfDNA in maternal plasma was reported as a better source for prenatal diagnosis. NIPD of several conditions were achieved with this approach including fetal rhesus D status [51], myotonic dystrophy [52], achondroplasia [33], and certain chromosomal translocations [53, 54]. The flow chart in Figure 2 shows in detail the strategy to be undertaken for prenatal screening of potential fetal RH incompatibility in pregnant women [39]. The collective precision of noninvasive fetal genotype for RhC/c and RhE/e determination using maternal peripheral blood was 96.3 and 98.2%, respectively. This can safely replace the current methods used in the management of RhC/c or RhE/e alloimmunised pregnancies [55].

3.3. Differential DNA Methylation of Maternal and Fetal DNA. Epigenetics is the study of molecular phenomenon that affects gene expression, but does not involve a change in DNA sequence [22]. The best studied epigenetic phenomenon is the process of DNA methylation, which involves addition of methyl groups to cytosine residues in the DNA sequence.
It is known that if such cytosine methylation occurs in the promoters of genes, gene expression may be switched off, such as in certain tumor suppressor genes [56].

As the amount of fetal DNA is currently determined by quantifying Y-chromosome-specific sequences, alternative approaches such as the measurement of total cfDNA or the use of gender-independent fetal epigenetic markers, such as DNA methylation, offer a promising alternative [57]. The status of differentially methylated region in human $IGF2$-$H19$ locus and single nucleotide polymorphism (SNP) was maintained such that paternal and maternal alleles were methylated and unmethylated, respectively [58]. Methylation was ascertained by bisulfite conversion and PCR using methylation-specific primers (MSP) for detecting paternally and maternally inherited fetal alleles in maternal plasma [59]. Differentially methylated fetal alleles were detected in maternal plasma by direct sequencing and primer-extension assay [46]. The strategy for epigenetic detection of fetal DNA has been illustrated in Figure 3.

The feasibility of epigenetic markers for fetal DNA detection in maternal plasma opened up a new approach for the development of gender and polymorphism-independent fetal markers. The epigenetic analysis of maternal plasma has obvious applications to disorders associated with genomic imprinting such as Prader-Willi syndrome and certain chromosomal aneuploidies associated with methylation abnormalities [60–62]. Till date, the diagnosis of fetal Down syndrome (trisomy 21) was carried out by obtaining fetal tissue using invasive procedures since NIPD of chromosomal aneuploidies was complicated due to the presence of coexisting background maternal DNA [63].

The discovery of 22 CpG islands (CGIs) containing 255 CpG sites on chromosome 21 indicated that epigenetic differences could be exploited for the development of markers for circulating fetal DNA [64]. The bisulfite-based approach increased the number of applicable CpG sites by 5-folds compared to HpaII-based approach [65]. The epigenetic signatures of placental DNA have the desirable feature of being widespread in the human genome (22 of 114, 19.3% analyzed CGIs on chromosome 21). Trisomy 21 could be detected either by analysis of epigenetic allelic ratios or direct comparison with a placenta-specific DNA methylation marker on a reference chromosome [39]. A combination of markers based on both fetal RNA and DNA may pave way for the development of a NIPD test for trisomy 21 that would be applicable to almost all fetomaternal pairs in the general population [64].

Another fetal epigenetic marker, $RASSF1$ (Ras association (RalGDS/AF-6) domain family 1) was found to be hypermethylated in placenta but completely unmethylated in maternal blood cells [66]. The placental methylated form, M-$RASSF1$, was developed as a universal marker for fetal DNA in maternal plasma [40]. On the basis of these epigenetic differences, Chim et al. [64] developed 2 novel fetal-DNA markers, U-$PDE9A$ and U-$CGI137$ that were pregnancy specific in maternal plasma and rapidly cleared from the circulation upon delivery of fetus. These markers were independent of

![Figure 2: A schematic diagram showing logistics and results of prenatal screening for potential fetal RhD incompatibility in pregnant women](image-url)
Allele inherited from grandfather, thus methylated

Mother

Allele inherited from grandmother, thus unmethylated

Fetus

Allele inherited from father, thus methylated

Methylated sequences are denoted by filled bars, whereas unmethylated sequences are denoted by open bars. A/G denotes the respective allele of the SNP.

Unmethylated maternal allele
Methylated maternal allele
Methylated fetal allele

Figure 3: Strategy for the epigenetic detection of fetal DNA [46] (a) schematic diagram showing the difference in methylation status of the studied region in the IGF2-H19 locus (depending on origin of parents); (b) schematic diagram showing the detection of differentially methylated fetal alleles in maternal plasma. The region is methylated when inherited from father and unmethylated when inherited from mother. Methylated sequences are denoted by filled bars, whereas unmethylated sequences are denoted by open bars. A/G denotes the respective allele of the SNP.

3.4. Determination of Fetal Aneuploidy by Allelic Ratio Discrimination. In a series of developments since 2000, plasma RNA was been established as a prenatal diagnostic tool. The plasma RNA was surprisingly stable, possibly through protection by particulate matter [70, 71]. The comparative quantification of allelic SNPs arising from the same gene could be used to assess chromosome copy number. Candidate genes were identified on chromosome 21 of which PLAC4 was most abundant and appeared to be polymorphic. Lo et al. [37] developed the RNA-SNP allelic ratio approach for determining the dosage of chromosome 21 in maternal plasma using PLAC4 mRNA (Figure 5). It has been further proposed that the SNPs associated with such candidate genes need to be different between mother and father in order to achieve allelic discrimination [16, 37]. However, one group showed that the expression of PLAC4 is unaltered in trisomy 21 fetuses [72].

This new, noninvasive source of fetal DNA was rapidly used for prenatal determination of fetal gender in sex-linked diseases [73], congenital adrenal-hyperplasia [74], and fetal RhD status in pregnancies involving RhD-negative mothers [41]. This approach has also been used for the prenatal detection of mutations inherited from father and absent in mother, for example, beta-thalassaemia [34, 75]. The main limitation of RNA-SNP allelic ratio approach was that only the fetus heterozygous for an analysed SNP can be successfully diagnosed and approximately 45% of fetuses would be expected to be heterozygous. When this approach was used on an unknown sample, it was not known whether
the fetus was heterozygous for the analyzed SNP. In case of two SNP alleles, one could proceed to measure the SNP allelic ratio to determine whether the fetus was euploid or aneuploid [40].

The main disadvantage of the epigenetic approach over the RNA-SNP allelic ratio method was that many commonly used methods for DNA methylation analysis involved the use of bisulphite-based reagents [22, 68]. Such reagents were shown to result in DNA degradation thereby reducing the amount of target DNA available for subsequent analysis [76]. However, the main advantage of the DNA methylation-based approach was that such markers were relatively abundant in the human genome [39].

4. Applications of cfDNA

In developing countries, chromosomal anomalies form the major cause of birth defects and mental retardation and are the leading cause of maternal and fetal/neonatal mortality and morbidity. All pregnancies carry a baseline risk of 3-4% for major congenital anomalies [77]. Analysis of circulating fetal DNA in maternal plasma is useful for NIPD
of chromosomal aneuploidies [37, 41], sex-linked disorders [74], β-thalassemia [35], fetal RhD status [78], preeclampsia (PE), and detection of paternally inherited disease-causing sequences in maternal plasma [40].

It has been estimated that up to 80% of Down syndrome infants (Trisomy 21) are conceived by women under 35 years of age and is by far the most common disorder affecting approximately 1:800 pregnancies, although the probability increases with maternal age [78]. Over 95% of pregnant women choose some form of prenatal screening and most of them consider serum-based screening tests [79]. Risk can be assessed by combining several factors, including (a) elevated or depressed serum levels of several biomarker proteins including β-hCG, α-fetoprotein, PAPP-A (pregnancy associated plasma protein-A), and inhibin A, (b) maternal age and (c) increased nuchal translucency [16]. RT-PCR revealed that the concentration of fetal DNA was 97.5 GE/mL (29.2–187.0) for trisomy 13 cases and 31.5 GE/mL (18.6–77.6) for trisomy 18 in contrast to 40.3 GE/mL (3.7–127.4) for controls [80].

Preeclampsia is a multisystem disorder of pregnancy which is characterized by the onset of hypertension and proteinuria that develop after 20 weeks of gestation in previously normotensive women [81–83]. The early identification of patients with an increased risk for preeclampsia has been one of the most important goals in obstetrics [82]. Proteinuria is more closely associated with fetal growth restriction (FGR) or placental dysfunction [25]. However, the direct cause for preeclampsia is not identified and it may occur in any situation thereby complicating 3 to 5% of all pregnancies. It was found that fetal DNA concentration was elevated in the plasma of pregnant women with preeclampsia, the elevation being approximately double that of controls which regresses after delivery [84]. It was first reported that cfDNA increased approximately 5-folds in the plasma of women with preeclampsia in third trimester in a small scale study of 20 each of preeclamptic women and gestational age-matched controls [85, 86]. The same outcome was observed in second trimester with 10 preeclamptic women and 40 controls as well as in a large-scale study with 120, each of pre-eclamptic women and controls [87, 88]. The amount of cfDNA was routinely determined by quantifying Y-chromosome specific sequences [89]. Alternative approaches also showed an increase of total cfDNA in women with preeclampsia before onset and at term [90–92]. Recently, several multicentric studies are being conducted to confirm the predictive value of cfDNA for preeclampsia in combination with other potential markers, for example, P-selectin, PAPP-A, PP-13, sflt-1, sEng, PlGF. The β-globin values in maternal plasma are linked with the severity of preeclampsia as well, their levels being five-folds higher than in controls [93].

5. Conclusion

Scientific and clinical status of the use of circulating cfNA technology for NIPD is a rapidly developing and dynamic field. Quantitative changes of cfDNA in maternal plasma have been reported in different studies using real time-PCR for Y-chromosome specific sequences. There has been a constant effort to develop alternative approaches such as measurement of total cfDNA or use of gender-independent fetal epigenetic markers such as DNA methylation as well as cfRNA of placental origin. These offer a promising alternative for screening and prenatal diagnosis of genetic disorders. The presence of cfRNAs in maternal circulation is a low-cost approach for the future development of novel strategies for NIPD as compared to invasive methods used for karyotyping and fluorescent in situ hybridization (FISH). The isolation and quantification methods of plasma/serum cfNA (DNA/RNA) are very critical in analyzing data. There is an urgent need for the standardization of techniques, careful evaluation and analysis of their specificity and sensitivity. In conclusion, efforts have to be made towards the establishment of standardized and simplified protocols for the analysis of cfNA biomarkers so that they can be used for routine obstetrics care in any clinical setup.

Conflict of Interests

The authors declare no conflict of interests.

Authors’ Contribution

The authors contributed equally to this paper.

Acknowledgments

This study is dedicated to all mothers and their families. The authors are grateful to University Grant Commission, New Delhi, India for providing research grant and fellowship. Research grants from ICMR and DST to the Molecular and Human Genetics Lab, and from DST-FIST and DST-PURSE to the department are duly acknowledged.

References


