Review Article

Cell-free Fetal Nucleic Acid Identifier Markers in Maternal Circulation

Mahboubeh Ramezanzadeh, Sharifeh Khosravi[1], Rasoul Salehi[1]

From the Department of Genetics and Molecular Medicine, School of Medicine, Bushehr University of Medical Sciences, Bushehr 751463341, [1]Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan 81744-176, Iran

Abstract

From the discovery of cell-free fetal (cff)-DNA in 1997 so far, many studies have been performed on various aspects of cff-nucleic acid. It is undoubted that currently, invasive prenatal diagnosis progresses to the noninvasive test. However, there are many problems. One of the most challenging issues in this field is differentiation and detection of the small amount of cff-nucleic acid in maternal plasma. Many markers and methods have been used for this purpose. This review makes an attempt to review and compare the studies in the field. Six identifier markers including Y-specific sequence, polymorphisms, epigenetic difference, DNA size difference, fetal mRNA, and microRNA as well as the advantages and disadvantages of each marker are discussed. This review provides a relatively perfect set on cff-nucleic acid biomarkers in various physiological and pathological status of pregnancy, helping to review and compare the prior obtained results, and improving designation in future studies.

Keywords: *DNA, marker, pregnancy, prenatal diagnosis, RNA*

Introduction

Prenatal diagnosis is a critical issue of gynecological practice. To perform a genetic test in this field, it is necessary to obtain placental or fetal material. This material, currently, is achieved through invasive procedures such as chorionic villus sampling or amniocentesis. These invasive procedures carry 1% risk of miscarriage, and other maternal and fetal complications have been reported to relate to these invasive procedures.[1] In 1997, the presence of cell-free fetal DNA (cff-DNA) in the circulation of pregnant women was reported.[2] Fragmented cff-DNA originates primarily from apoptotic syncytiotrophoblasts.[3] It is demonstrated now that maternal plasma and serum contain cff-DNA, but the mean fractional concentration in plasma is higher than serum 3.4–6.2% and 0.13–1.0%, respectively depending on the gestational age.[4] It is detectable as early as 18 days following embryo transfer in *in vitro* fertilization pregnancy and is soon cleared from maternal circulation after delivery, with a mean half-life of 16 min, so it is a suitable source for noninvasive prenatal diagnosis (NIPD) in pregnancy.[5,6] Following these reports, a new area of research in the diagnostic field was opened; however, there are two problems with cff-DNA for the development of the noninvasive diagnostic test, the small proportion of cff-DNA in maternal plasma as little as ~19%, and coexistence with maternal DNA.[7] The presence of cff-DNA in maternal circulation is accepted universally, and although via the discovery of cff-DNA many potentially clinical applications have been assessed such as fetal rhesus D status, sex-linked disorders, monogenic disorders, aneuploidies, and many pregnancy complications such as preeclampsia and preterm labor,[8-14] but confirming the presence of cff-DNA in maternal plasma extracts is still a challenge in diagnostic tests. Researchers have applied many methods to differentiate the fetal-derived sequences from that of mother. This study makes an approach to cff-nucleic acids identifier markers applied by researchers of the field including Y-specific sequence, polymorphisms, epigenetic difference, DNA size difference, fetal mRNA, and microRNA (miRNA) as well as the advantages and disadvantages of each marker.

Y-Chromosomal Sequences

One of the most common targets used for the detection of cff-DNA in maternal

How to cite this article: Ramezanzadeh M, Khosravi S, Salehi R. Cell-free Fetal Nucleic Acid Identifier Markers in Maternal Circulation. Adv Biomed Res 2017;6:89.

Received: September, 2015. **Accepted:** July, 2016.

Address for correspondence:
Dr. Rasoul Salehi, Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan 81744-176, Iran.
E-mail: r_salehi@med.mui.ac.ir

Access this article online

Website: www.advbiores.net

DOI: 10.4103/2277-9175.211800

Quick Response Code:

circulation has been Y-specific sequences. If one can detect Y-specific sequences in plasma of a pregnant woman, it means that the plasma contains a male fetus cf-DNA in detectable level. These Y-specific sequences can be quantified as an index of cff-DNA in maternal circulation because they are genetic sequences of the fetus not in the maternal genome. Thus, it can be used for the evaluation of cff-DNA extraction method and detection systems. The first report of cff-DNA in maternal plasma used Y-specific sequences.[2] Since then, many groups have validated the initial finding and used Y-specific sequences to differentiate the fetal-derived sequences from that of the mother as the primary step for confirmation of cff-DNA in NIPD. The other application of prenatal sex determination is in pregnant women carriers of an X-linked disease, because identification of a male fetus indicates hemizygosity for the X chromosome and potential disease; therefore, invasive testing can be avoided in female bearing pregnancy. For some endocrine disease such as congenital adrenal hyperplasia, prenatal diagnosis can help to prevent the disease via antenatal treatment.[15] Researchers have performed fetal sex determination using various sex-specific markers through a broad range of gestational age. The most reported study on fetal sex determination has used single copy SRY gene or the multicopy DYS14 marker sequence of the TSPY gene on Y chromosome. The studies on sex determination which used sex-specific markers and their outcomes are summarized in Table 1.[15-19] Almost all studies have used polymerase chain reaction (PCR) or real-time PCR as detecting methods. All in all, the accuracy of tests increases in higher gestational age and in the use of real-time PCR than conventional PCR.[16] Many groups have used the combination or ratio of markers and obtained highly reliable results than the single marker.[15,18] In addition to some advantages, use of Y-specific sequences as cff-DNA identifier markers is associated with the number of limitations. Hence, this method is applicable only in male-bearing pregnancies, and a negative test can imply either the fetus is female, or the cff-DNA is below the detection limit of the experiment.[20]

Paternally Inherited Polymorphic Markers

The other way for differentiating fetal-derived sequences from the mother is the use of paternally inherited polymorphic markers. Some studies have used a panel of number of biallelic highly polymorphic markers to confirm the presence of fetal DNA in maternal plasma.[21,22] In some studies, the use of these polymorphic markers helps to avoid invasive procedure in more than 60% of cases.[21] Other groups have developed a detection method for determination of the presence of either free fetal DNA or fetal cells in maternal circulation using polymorphic short tandem repeats (STR), or a panel of single nucleotide polymorphisms (SNP); the data were summarized in Table 2.[23-25] The most important advantage of these markers is that they are gender independent and can potentially ascertain the presence of fetal DNA in female bearing pregnancies. However, the use of paternally inherited polymorphism is associated with many disadvantages and requires prior data on the polymorphic status of the parent; therefore, it is labor intensive and could apply only to individuals who own polymorphisms, and due to obtaining an appropriated number of informative alleles, a large number of polymorphisms need to be used.[20,26]

Epigenetic Markers

The epigenetic markers can differentiate cff-DNA independently of the gender or polymorphic status of the parents and fetus. Epigenetic modifications are molecular events that affect gene expression without changing in DNA sequences; they are stable through cell division. The best studied epigenetic event is DNA methylation which refers to the addition of methyl group to cytosine residues in DNA sequence; when this occurs in promoters of genes, gene expression may be switched off.[27,28] The specific DNA methylation signatures of tumoral DNA were detected in 1999 for the first time, and the possibility of using them as noninvasive biomarkers was examined.[29-31] After such developments, various studies were performed to detect

Table 1: Examples of studies used Y- chromosome sequences for detection of cff-DNA in maternal plasma

Y- Chromosome sequence	Method	Sensitivity	Specificity	Reference
SRY	PCR	95	99.3	16
DYS14	PCR	95	97.7	16
DYS1/DAZ	PCR	98.4	98.7	16
DYS19	PCR	100	-	17
DYS 392, DYS 385	PCR	91	-	17
DYS14/GAPDH	Real time PCR	100	100	15
DYS14, SRY, DAZ	Real time PCR	100	99.5	18
SRY	Real time PCR	100	100	19

Table 2: Examples of studies used polymorphic markers for detection of cff-DNA in maternal plasma

Polymorphic marker	Method	accuracy	Reference
4 STR	Fluorescent PCR	100%	24
9(Informative) STR	Fluorescent PCR	84%	23
10 Indels*	Real-time PCR	78%	21
9(Informative) SNP	Mass spectrometry	100%	25
24 Indels	Real-time PCR	87%	22

* Bi-allelic insertion/deletion polymorphism

cff-DNA from maternal plasma.[32,33] Detection of cff-DNA from maternal plasma using epigenetic markers has been performed based on two patterns. The first pattern is an imprinted locus, in which the DNA methylation patterns are inherited in a parent-of-origin-specific manner. The methylation status of this locus can distinguish between the fetus and the mother. In 2002, using this method for the first time, the stretch of DNA that a fetus has inherited from the mother was detected.[32] However, this method is so complicated to be used in routine tests as a fetal marker.[20] Therefore, the second pattern, placenta-specific methylation pattern has been explored. Many groups have demonstrated that the methylation pattern of human placenta is different from other somatic tissues.[34,35] Now, it is believed that cff-DNA in maternal plasma is derived from syncytiotrophoblasts, and maternal free DNA is derived from hematopoietic cells.[3,36] Therefore, these two fractions of free DNA in maternal plasma can be distinguished based on their different methylation status in a single genomic locus. The first successful study was in 2005, based on the different methylation patterns of maspin gene in the mother and fetus.[33] This was the first universal fetal marker which could be used in all pregnancies independent of gender or polymorphic status, and this is the main advantage of this epigenetic pattern. Since then, many studies were developed based on this pattern.[37-39] In general, the detection of epigenetic markers in maternal plasma requires two steps; the first is differentiation of methylated and unmethylated sequences using bisulfate modification or differential cleavage by restriction enzymes. The second is identification or quantification of fetal-specific methylation pattern via sequencing, methylation-specific PCR, or real-time PCR.[20] Many studies have used bisulfate treating method; however, the main disadvantage of this technique is that bisulfate treating is proved to degrade the huge amount of DNA template, so not suitable for cff-DNA, which is low in maternal plasma.[40] If there are recognition sites of methylation-sensitive restriction enzymes within the differentially methylated region of placenta genome, this can be the basis of cff-DNA identification in maternal plasma.[38,41] Compared with bisulfate treating, this digestion-based method brings less damage to the cell-free DNA in plasma, therefore, is more appropriate for cff-DNA.[20] Many applications for epigenetic markers have been reported in NIPD including (1) to indicate the presence of fetal DNA or as quantitative markers to quantify the amount of fetal DNA in maternal plasma, (2) to indicate the quantitative aberrations of cff-DNA in some disorders for example, in preeclampsia, (3) reports have demonstrated the feasibility of using fetal epigenetic markers for prenatal diagnosis of aneuploidy.[33,37] Another application of epigenetic factors is cff-DNA enrichment. It is now accepted that the amount of cff-DNA in maternal plasma is very little and coexists with maternal-free DNA. These two problems are the major challenges for the use of cff-DNA in the diagnostic field. Hence, researchers need an enrichment method to solve these problems, which means a selective amplification of cff-DNA to overcome scarcity and a huge amount of maternal background. One group has developed a method for preferentially amplification of cff-DNA in maternal plasma using the first universal epigenetic marker for fetal DNA, maspin [Table 3].[33,41] The other disadvantage of using epigenetic markers is that these assays are multiprocedure and relatively labor intensive.

Differential Characteristic

Fragmentation of cff-DNA in maternal plasma has been proved by many groups. It is proved that the major amount of cff-DNA is shorter than 300 bp, and maternal-free DNA molecules are obviously longer.[42,43] Although this differential characteristic is not a marker, but it has been successfully recruited by some groups as a basis of enrichment method in size separation manner on agarose gel electrophoresis. Although this method is prone to contamination, it has been solved by strict anticontamination measures used at all stages of sample preparation, and the contamination has been avoided.[44-46] Many studies have used various methods as an enrichment method for improving the results of subsequent diagnostic tests, but some are too complex, expensive in fee, and labor intensive to be applied in clinical practice [Table 4].[41,44,45,47-52] Enrichment method based on the size separation on agarose gel is easy to perform, not expensive, and available in almost all laboratories [Figure 1].

Fetal mRNA

After the discovery of fetal DNA in maternal plasma, many studies confirmed the presence of fetal DNA in

Table 3: Examples of studies used epigenetic markers for cff-DNA in maternal plasma

Epigenetic marker	Differentiation method	Indication	Accuracy	Reference
Imprinted region 1IGF2-H19 & A SNP	Bisulfate conversion	Cff-DNA detection	-	32
2maspin	Bisulfate conversion	Quantification of cff-DNA	100%	33
3RASSF1A	Enzyme digestion	Cff-DNA detection	100%	38
SERPINB5; 4maspin	Enzyme digestion	Cff-DNA detection	100%	41
5HLCS	Combined bisulfate Restriction analysis	Detection of trisomy 21	96%	37
6RASSF1A	Enzyme digestion	Cff-DNA detection	88%	39

Table 4: Summarization of cff-DNA enrichment methods applied by researchers

Enrichment method	Target	Diagnostic method	Outcome	Reference
1Formaldehyde	SRY- Cystic fibrosis gene	PCR	Increasing in the relative percentage of cff-DNA, Not reproducible	47, 52
2Digital nucleic acid size selection (NASS)	PLAC4 SNP	Digital PCR-relative mutation dosage (RMD)	Improvement of fetal allele detection	50
3PCR- SABER*	HBB mutation, SNP link to HBB	MALDI-TOF MS	All of cases detected correctly	51
4PCR	HBB mutations	Nested real-time allele specific PCR	All of cases detected correctly	48
5Size selection by gel electrophoresis with WGA**	DYS1-HBB	Real-time PCR	DYS1 sequence amplification was best observed when using the 100-300bp fragments as template	44
6Size selection by gel electrophoresis with PNA*** clamping	HBB mutation	Allele specific real-time PCR	All of cases but one detected correctly	45
7Methylation sensitive restriction endonuclease and stem-loop assay	Hypo methylated SERPINB5 on Ch18	Real time-PCR genotyping assay (PCR and Mass EXTEND)	All of cases genotyped correctly	41
8Targeted enrichment(in solution capture)	Exons on Ch X	Massively parallel sequencing	The mean sequence coverage of enriched samples was 213-fold higher than that of non-enriched samples	49

*SABER: Single allele base extension reaction, amplify only mutant allele - MS identify this products, **WGA: Whole Gene Amplification ,***PNA: Peptide Nucleic Acid clamp

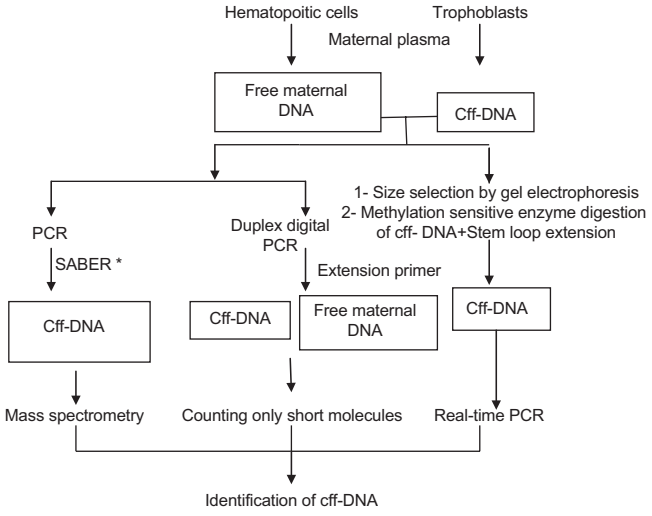

Figure 1: Schematic diagram representative various enrichment methods for cff-DNA. *Single Allele Base Extension Reaction

maternal plasma and tried to use the fetal-free DNA in many diagnostic destinations mentioned above. However, the presence of fetal RNA was not known till 2000, and one group for the first time, using the two-step reverse transcription (RT)-PCR assay, demonstrated the presence of Y chromosome-specific zinc finger protein mRNA which is a fetal-derived, male-specific mRNA in plasma of pregnant women carrying male fetuses.[53] The study also showed that the detection rate of plasma fetal RNA is lower than that of plasma fetal DNA. It is possible because of degradation nature of the fetal RNA in maternal blood. Although some groups have demonstrated that the placenta-mRNA is stable in maternal plasma, possibly it is because of the presence of microparticles that protect them from degradation. This study followed other studies which demonstrated the detectability of RNA of human placental lactogen (hPL) and the β-subunit of human chorionic gonadotropin (β-*hCG*) mRNA in pregnancy, using placental-specific mRNA analysis.[53,54] Many groups have examined the possibility of the use of fetal mRNA in maternal plasma as a marker for pregnancy complications such as fetal-maternal hemorrhage or preeclampsia. Due to significant increase in fetal *CRH* mRNA concentration in preeclamptic pregnancy rather than healthy pregnancy and rapidly clearance through 2 h postpartum, it is suggested that maternal plasma *CRH* mRNA might be a new molecular marker for preeclampsia.[55,56] In 2007, Lo *et al*., using PLAC4 mRNA-SNP on chromosome 21, determined the allelic ratio of interest SNP in heterozygote fetus and diagnosed trisomy 21 with comparable sensitivity and specificity to many other multimarker screening strategies. An additional advantage of this strategy is insensitivity to the gestational age at which sampling is performed unlike the current strategy which uses serum biochemical markers; thus, the clinical use of this strategy is relatively simple. The main drawbacks of this method are that this method is polymorphism-dependent and usable only for heterozygote fetus. PLAC4 SNP (rs8130833) has a heterozygosity rate of 45% in the studied population. Therefore, it has a low population coverage; this can be compensated by combining several SNPs of genes

transcribed from chromosome 21.[57] Some studies have attempted to systematically identify placental tissue gene expression profiling using oligonucleotide microarrays followed by real-time quantitative RT-PCR for the detection of identified expressed gene in maternal plasma. In this study, six genes could be identified: *hPL*, β-*hCG*, *CRH*, *TFPI2*, *KISS1*, and *PLAC1*, and their transcript are detected in maternal plasma [Table 5].[58] Plasma fetal RNA analysis has a number of advantages: First, these markers can provide valuable data on gene expression patterns of fetal tissues. For example, complicated pregnancies such as preeclampsia have been confirmed associated with abnormal gene expression. Thus, with the development of further RNA markers, RNA analysis of maternal plasma may lead to the noninvasive monitoring of the gene expression of an unborn fetus in many physiological and pathological statuses. The second is gender- and polymorphism-independent unlike previously described markers. Third, the use of these markers are relatively simple and inexpensive involving mRNA extraction, and RT-PCR analysis can be used for many plasma RNA markers unlike other markers such as epigenetic markers which need multi or damaging procedures such as bisulfate conversion.[54]

MicroRNA

MicroRNAs (miRNAs) are small noncoding RNAs about 20–24 nucleotides long. They are critical factors in regulating cellular gene expression, suppressing the translation of protein-coding genes at the posttranscriptional level.[59] MicroRNAs are critical in cell development, proliferation, communication, and tissue differentiation. They are involved in regulating pregnancies.[60] The miRNA expression patterns change in various pathological and physiological statuses including pregnancy. The placental development has many critical processes such as differentiation, migration, invasion, angiogenesis, proliferation, and apoptosis.[61-63] It has been shown that miRNAs regulate placental development and functions through these processes.[64-66] MicroRNAs are released from placental syncytiotrophoblasts into the maternal circulation via exosomal particles which are small vesicles secreted by many cells. Circulating miRNAs are complex with circulating ribonucleoprotein and high-density lipoproteins. This complex form of miRNAs converts them to stable forms by protection from digestion by RNase.[67,68] If miRNA biomarkers for noninvasive diagnosis in maternal circulation is to be used, first miRNA expression profile both in normal controls and patients need to be determined using microarray or next generation sequencing. Subsequently, the under-or over-regulated miRNAs are quantified by real-time PCR to validate the repeatability of the results. Candidate miRNAs can then be tested to determine their changes in maternal serum or plasma. As an alternative strategy, we can directly examine specific miRNAs with known expression patterns that are associated with the pregnancy complication of interest.[69] Due to the role of miRNAs in placental development, the aberrant expression pattern of placental miRNAs has been detected in maternal circulation with pregnancy complications or human placental diseases such as preeclampsia,[70] intrauterine growth restriction (IUGR),[71] and miscarriage.[72] Although some complications, such as preeclampsia have been well studied, the other complications such as IUGR and miscarriage, the role of these potential biomarkers need to be confirmed by further investigations. Consideration of these data including the role of miRNA in critical steps of placenta developing, founding these genetic materials in maternal circulation in a stable form, possessing specific expression patterns in various maternal and fetal status, and importantly, the demonstration of the fact that many of these miRNAs are detectable in maternal plasma during pregnancy but undetectable in postdelivery plasma which confirm their placenta origin,[69] suggest that these regulating factors can be used as a potential biomarkers

Table 5: Examples of studies used mRNA markers in maternal plasma

Target	Method	Indication	Outcome	Reference
ZFY	RT-PCR	Detection of fetal mRNA	Detection rate 22-63% in early and late pregnancy	54
CRH	RT-PCR	To determine increase in mRNA in preeclampsia	CRH- mRNA concentration was 10.5 times higher in preeclampsia than control pregnancies	56
hPL,βhCG, CRH, TFPI2, KISS1, PLAC1	RT-PCR	Detection of fetal mRNA	All of them detected in maternal plasma with specific pattern	58
DHPS-DNA CGB7-mRNA	RT-PCR	To determine the more sensitive marker for detection of fetal-maternal hemorrhage	The cff-DNA is more sensitive than mRNA	55
PLAC4 mRNA-SNP	Base extension & Mass Spectrometry	Detection of trisomy21 (Allelic ratio)	Senitivity90%, specificity96%	57

in NIPD. Although these potential markers (like mRNA) have a number of advantages such as independency on gender and polymorphism and relative simple detection assay, the clinical application of these markers need many requirements such as standardization in all steps of assay including sampling, miRNA isolation, and quantification. The obtained results should be confirmed by further analytical studies and appropriate normalization methods.[73]

Conclusion

Nowadays, it is confirmed that cff-nucleic acids are an excellent potential genetic resource for clinical NIPD due to riskless sampling, low-cost detecting test in comparison to current methods such as karyotyping and fluorescent *in situ* hybridization using invasive sampling and other appropriate characteristic such as detectability in early pregnancy.[1,5,6,54] However, due to the scarcity of cff-nucleic acid and mixing with maternal background, all stages including sampling, plasma preparation, nucleic acid extraction, cff-nucleic acid differentiation, and detection strategies need standardization before definite clinical usage. The most challenging step is differentiation and detection of cff-nucleic acid in maternal plasma. Many studies on various aspects of cff-nucleic acid have been performed, but regarding method standardization and improvement in performance and subsequent results, the prior obtained results need to be reviewed and compared. This study provided a relatively comprehensive source on cff-nucleic acid markers and the advantages and disadvantages for interested investigators. Due to potential application of cff-nucleic acid in diagnostic field, this data collection can help to develop these diagnostic tests [Figure 2].

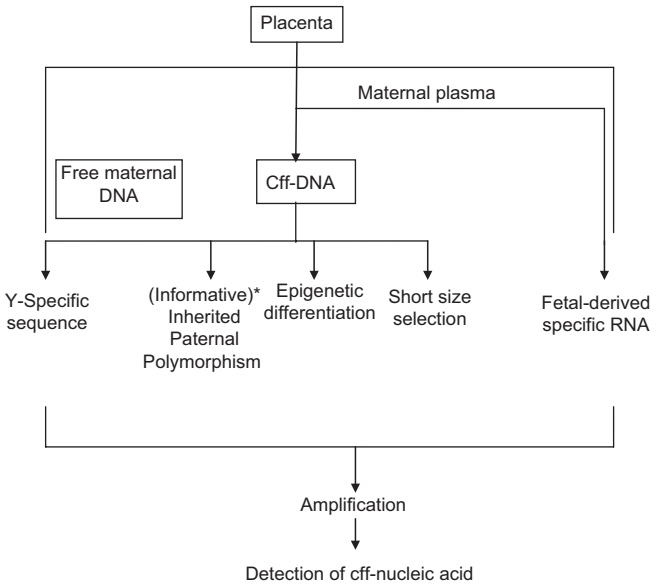

Figure 2: Schematic diagram representative various cff-nucleic acid identifier markers. *Those alleles present on the paternal genome and absent from the maternal genome could potentially serve as a marker to confirm the presence of fetal DNA

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Tounta G, Kolialexi A, Papantoniou N, Tsangaris GT, Kanavakis E, Mavrou A. Non-invasive prenatal diagnosis using cell-free fetal nucleic acids in maternal plasma: Progress overview beyond predictive and personalized diagnosis. EPMA J 2011;2:163-71.
2. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, *et al.* Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485-7.
3. Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N, *et al.* Free fetal DNA in maternal plasma in anembryonic pregnancies: Confirmation that the origin is the trophoblast. Prenat Diagn 2007;27:415-8.
4. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, *et al.* Quantitative analysis of fetal DNA in maternal plasma and serum: Implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768-75.
5. Guibert J, Benachi A, Grebille AG, Ernault P, Zorn JR, Costa JM. Kinetics of SRY gene appearance in maternal serum: Detection by real time PCR in early pregnancy after assisted reproductive technique. Hum Reprod 2003;18:1733-6.
6. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 1999;64:218-24.
7. Lun FM, Chiu RW, Chan KC, Leung TY, Lau TK, Lo YM. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008;54:1664-72.
8. Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: Introduction of a new noninvasive fetal RHD genotyping service. Transfusion 2002;42:1079-85.
9. Amicucci P, Gennarelli M, Novelli G, Dallapiccola B. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. Clin Chem 2000;46:301-2.
10. Chiu RW, Lau TK, Cheung PT, Gong ZQ, Leung TN, Lo YM. Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: A feasibility study. Clin Chem 2002;48:778-80.
11. Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. Lancet 2000;356:1170.
12. Leung TN, Zhang J, Lau TK, Chan LY, Lo YM. Increased maternal plasma fetal DNA concentrations in women who eventually develop preeclampsia. Clin Chem 2001;47:137-9.
13. Sekizawa A, Jimbo M, Saito H, Iwasaki M, Sugito Y, Yukimoto Y, *et al.* Increased cell-free fetal DNA in plasma of two women with invasive placenta. Clin Chem 2002;48:353-4.
14. Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM, *et al.* Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. Clin Chem 1999;45:1747-51.
15. Lim JH, Park SY, Kim SY, Kim do J, Choi JE, Kim MH, *et al.* Effective detection of fetal sex using circulating fetal DNA in first-trimester maternal plasma. FASEB J 2012;26:250-8.
16. Devaney SA, Palomaki GE, Scott JA, Bianchi DW. Noninvasive

fetal sex determination using cell-free fetal DNA: A systematic review and meta-analysis. JAMA 2011;306:627-36.
17. Nair SP, Peter S, Pillay VV, Remya UM, Krishnaprasad R, Rajammal B. Detection of Y STR markers of male fetal dna in maternal circulation. Indian J Hum Genet 2007;13:69-72.
18. Fernández-Martínez FJ, Galindo A, Garcia-Burguillo A, Vargas-Gallego C, Nogués N, Moreno-García M, *et al.* Noninvasive fetal sex determination in maternal plasma: A prospective feasibility study. Genet Med 2012;14:101-6.
19. Perlado-Marina S, Bustamante-Aragones A, Horcajada L, Trujillo-Tiebas MJ, Lorda-Sanchez I, Ruiz Ramos M, *et al.* Overview of five-years of experience performing non-invasive fetal sex assessment in maternal blood. Diagnostics (Basel) 2013;3:283-90.
20. Tsui DW, Chiu RW, Lo YD. Epigenetic approaches for the detection of fetal DNA in maternal plasma. Chimerism 2010;1:30-5.
21. Page-Christiaens GC, Bossers B, van der Schoot CE, DE Haas M. Use of bi-allelic insertion/deletion polymorphisms as a positive control for fetal genotyping in maternal blood: First clinical experience. Ann N Y Acad Sci 2006;1075:123-9.
22. Scheffer PG. Noninvasive fetal genotyping of paternally inherited alleles. Netherlands: Utrecht University; 2012.
23. Pertl B, Sekizawa A, Samura O, Orescovic I, Rahaim PT, Bianchi DW. Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats. Hum Genet 2000;106:45-9.
24. Samura O, Pertl B, Sohda S, Johnson KL, Sekizawa A, Falco VM, *et al.* Female fetal cells in maternal blood: Use of DNA polymorphisms to prove origin. Hum Genet 2000;107:28-32.
25. Chow KC, Chiu RW, Tsui NB, Ding C, Lau TK, Leung TN, *et al.* Mass spectrometric detection of an SNP panel as an internal positive control for fetal DNA analysis in maternal plasma. Clin Chem 2007;53:141-2.
26. Daniels G, Finning K, Martin P, Massey E. Noninvasive prenatal diagnosis of fetal blood group phenotypes: Current practice and future prospects. Prenat Diagn 2009;29:101-7.
27. Callinan PA, Feinberg AP. The emerging science of epigenomics. Hum Mol Genet 2006;15:R95-101.
28. Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet 1999;21:163-7.
29. Lo YM, Wong IH, Zhang J, Tein MS, Ng MH, Hjelm NM. Quantitative analysis of aberrant p16 methylation using real-time quantitative methylation-specific polymerase chain reaction. Cancer Res 1999;59:3899-903.
30. Wong IH, Lo YM, Zhang J, Liew CT, Ng MH, Wong N, *et al.* Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res 1999;59:71-3.
31. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res 1999;59:67-70.
32. Poon LL, Leung TN, Lau TK, Chow KC, Lo YM. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. Clin Chem 2002;48:35-41.
33. Chim SS, Tong YK, Chiu RW, Lau TK, Leung TN, Chan LY, *et al.* Detection of the placental epigenetic signature of the maspin gene in maternal plasma. Proc Natl Acad Sci U S A 2005;102:14753-8.
34. Chiu RW, Chim SS, Wong IH, Wong CS, Lee WS, To KF, *et al.* Hypermethylation of RASSF1A in human and rhesus placentas. Am J Pathol 2007;170:941-50.
35. Novakovic B, Rakyan V, Ng HK, Manuelpillai U, Dewi C, Wong NC, *et al.* Specific tumour-associated methylation in normal human term placenta and first-trimester cytotrophoblasts. Mol Hum Reprod 2008;14:547-54.
36. Lui YY, Chik KW, Chiu RW, Ho CY, Lam CW, Lo YM. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem 2002;48:421-7.
37. Tong YK, Jin S, Chiu RW, Ding C, Chan KC, Leung TY, *et al.* Noninvasive prenatal detection of trisomy 21 by an epigenetic-genetic chromosome-dosage approach. Clin Chem 2010;56:90-8.
38. Chan KC, Ding C, Gerovassili A, Yeung SW, Chiu RW, Leung TN, *et al.* Hypermethylated RASSF1A in maternal plasma: A universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. Clin Chem 2006;52:2211-8.
39. White HE, Dent CL, Hall VJ, Crolla JA, Chitty LS. Evaluation of a novel assay for detection of the fetal marker RASSF1A: Facilitating improved diagnostic reliability of noninvasive prenatal diagnosis. PLoS One 2012;7:e45073.
40. Grunau C, Clark SJ, Rosenthal A. Bisulfite genomic sequencing: Systematic investigation of critical experimental parameters. Nucleic Acids Res 2001;29:E65-5.
41. Tong YK, Chiu RW, Leung TY, Ding C, Lau TK, Leung TN, *et al.* Detection of restriction enzyme-digested target DNA by PCR amplification using a stem-loop primer: Application to the detection of hypomethylated fetal DNA in maternal plasma. Clin Chem 2007;53:1906-14.
42. Chan KC, Zhang J, Hui AB, Wong N, Lau TK, Leung TN, *et al.* Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem 2004;50:88-92.
43. Li Y, Zimmermann B, Rusterholz C, Kang A, Holzgreve W, Hahn S. Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. Clin Chem 2004;50:1002-11.
44. Jorgez CJ, Bischoff FZ. Improving enrichment of circulating fetal DNA for genetic testing: Size fractionation followed by whole gene amplification. Fetal Diagn Ther 2009;25:314-9.
45. Li Y, Di Naro E, Vitucci A, Zimmermann B, Holzgreve W, Hahn S. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. JAMA 2005;293:843-9.
46. Ramezanzadeh M, Salehi M, Farajzadegan Z, Kamali S, Salehi R. Detection of paternally inherited fetal point mutations for β-thalassemia in maternal plasma using simple fetal DNA enrichment protocol with or without whole genome amplification: An accuracy assessment. J Matern Fetal Neonatal Med, DOI: 10.3109/14767058.2015.1095883
47. Dhallan R, Au WC, Mattagajasingh S, Emche S, Bayliss P, Damewood M, *et al.* Methods to increase the percentage of free fetal DNA recovered from the maternal circulation. JAMA 2004;291:1114-9.
48. Tungwiwat W, Fucharoen G, Fucharoen S, Ratanasiri T, Sanchaisuriya K, Sae-Ung N. Application of maternal plasma DNA analysis for noninvasive prenatal diagnosis of Hb E-beta-thalassemia. Transl Res 2007;150:319-25.
49. Liao GJ, Lun FM, Zheng YW, Chan KC, Leung TY, Lau TK, *et al.* Targeted massively parallel sequencing of maternal plasma DNA permits efficient and unbiased detection of fetal alleles. Clin Chem 2011;57:92-101.
50. Lun FM, Tsui NB, Chan KC, Leung TY, Lau TK, Charoenkwan P,

et al. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. Proc Natl Acad Sci U S A 2008;105:19920-5.
51. Ding C, Chiu RW, Lau TK, Leung TN, Chan LC, Chan AY, *et al.* MS analysis of single-nucleotide differences in circulating nucleic acids: Application to noninvasive prenatal diagnosis. Proc Natl Acad Sci U S A 2004;101:10762-7.
52. Chinnapapagari SK, Holzgreve W, Lapaire O, Zimmermann B, Hahn S. Treatment of maternal blood samples with formaldehyde does not alter the proportion of circulatory fetal nucleic acids (DNA and mRNA) in maternal plasma. Clin Chem 2005;51:652-5.
53. Poon LL, Leung TN, Lau TK, Lo YM. Presence of fetal RNA in maternal plasma. Clin Chem 2000;46:1832-4.
54. Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS, *et al.* mRNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 2003;100:4748-53.
55. Miura K, Yoshiura K, Miura S, Yamasaki K, Nakayama D, Ishimaru T, *et al.* Cell-free DNA is more sensitive than cell-free mRNA as a marker for evaluation of fetal-maternal hemorrhage. Clin Chem 2006;52:2121-3.
56. Ng EK, Leung TN, Tsui NB, Lau TK, Panesar NS, Chiu RW, *et al.* The concentration of circulating corticotropin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. Clin Chem 2003;49:727-31.
57. Lo YM, Tsui NB, Chiu RW, Lau TK, Leung TN, Heung MM, *et al.* Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. Nat Med 2007;13:218-23.
58. Tsui NB, Chim SS, Chiu RW, Lau TK, Ng EK, Leung TN, *et al.* Systematic micro-array based identification of placental mRNA in maternal plasma: Towards non-invasive prenatal gene expression profiling. J Med Genet 2004;41:461-7.
59. Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A 2007;104:9667-72.
60. Morales Prieto DM, Markert UR. MicroRNAs in pregnancy. J Reprod Immunol 2011;88:106-11.
61. Fu G, Brkic J, Hayder H, Peng C. MicroRNAs in human placental development and pregnancy complications. Int J Mol Sci 2013;14:5519-44.
62. Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: Key pieces of the development puzzle. Science 1994;266:1508-18.
63. Aplin JD. Developmental cell biology of human villous trophoblast: Current research problems. Int J Dev Biol 2010;54:323-9.
64. Luo L, Ye G, Nadeem L, Fu G, Yang BB, Honarparvar E, *et al.* MicroRNA-378a-5p promotes trophoblast cell survival, migration and invasion by targeting Nodal. J Cell Sci 2012;125(Pt 13):3124-32.
65. Morales-Prieto DM, Schleussner E, Markert UR. Reduction in miR-141 is induced by leukemia inhibitory factor and inhibits proliferation in choriocarcinoma cell line JEG-3. Am J Reprod Immunol 2011;66 Suppl 1:57-62.
66. Li P, Guo W, Du L, Zhao J, Wang Y, Liu L, *et al.* microRNA-29b contributes to pre-eclampsia through its effects on apoptosis, invasion and angiogenesis of trophoblast cells. Clin Sci (Lond) 2013;124:27-40.
67. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654-9.
68. Ouyang Y, Mouillet JF, Coyne CB, Sadovsky Y. Review: Placenta-specific microRNAs in exosomes-good things come in nano-packages. Placenta 2014;35:S69-73.
69. Zhao Z, Moley KH, Gronowski AM. Diagnostic potential for miRNAs as biomarkers for pregnancy-specific diseases. Clin Biochem 2013;46:953-60.
70. Li H, Ge Q, Guo L, Lu Z. Maternal plasma miRNAs expression in preeclamptic pregnancies. Biomed Res Int 2013;2013:970265.
71. Mouillet JF, Chu T, Hubel CA, Nelson DM, Parks WT, Sadovsky Y. The levels of hypoxia-regulated microRNAs in plasma of pregnant women with fetal growth restriction. Placenta 2010;31:781-4.
72. Ventura W, Koide K, Hori K, Yotsumoto J, Sekizawa A, Saito H, *et al.* Placental expression of microRNA-17 and -19b is down-regulated in early pregnancy loss. Eur J Obstet Gynecol Reprod Biol 2013;169:28-32.
73. Tsochandaridis M, Nasca L, Toga C, Levy-Mozziconacci A. Circulating microRNAs as clinical biomarkers in the predictions of pregnancy complications. Biomed Res Int 2015;2015:294954.