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Detection of circulating fetal nucleic acids: a review of methods and applications

E C W Hung, R W K Chiu, Y M D Lo

Centre for Research into Circulating Fetal Nucleic Acids, Li Ka Shing Institute of Health Sciences, and Department of Chemical Pathology, The Chinese University of Hong Kong, Hong Kong SAR, China

Correspondence to: Professor Y M D Lo, Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Room 38023, 1/F Clinical Sciences Building, 30–32 Ngan Shing Street, Shatin, New Territories, Hong Kong Special Administrative Region, China; loym@cuhk.edu.hk

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ABSTRACT

The discovery of cell-free circulating fetal nucleic acids in maternal plasma has opened up new possibilities in non-invasive prenatal diagnosis. The rapid advancement of this field in the past decade is catalysed by the discovery of new classes of fetal nucleic acid markers and technological developments in nucleic acid detection and amplification. In this review, some of the more significant recent developments in this field will be discussed, including the detection of single molecule, chromosomal aneuploidies, single nucleotide variations and placental microRNAs in maternal plasma.

ACHIEVEMENTS IN THE PAST DECADE

Circulating nucleic acids (CNAs) refer to nucleic acids in plasma or serum. The first evidence for CNAs was provided by Mandel and Metais in 1948.¹ Their work was largely overlooked until subsequent workers detected CNAs in systemic lupus erythematosus,^{2–3} rheumatoid arthritis⁴ and cancer.^{5–11} In prenatal diagnosis, risks associated with conventional prenatal diagnostic techniques, such as chorionic villus sampling and amniocentesis, have stimulated an ongoing interest in the development of non-invasive, risk-free methods.¹² One strategy is the isolation of intact fetal cells from maternal blood for molecular cytogenetic analysis.¹³ However, fetal nucleated cells are very rare in the maternal circulation. For example, Hamada *et al* observed only 2 fetal cells in 770 000 maternal cells from maternal blood samples in early gestation.¹⁴

During fetal cell isolation, cell-free plasma/serum was routinely discarded. In 1997, Lo *et al* discovered circulating fetal DNA in this cell-free portion of maternal blood¹⁵ and further showed that the mean fetal DNA concentration was 25.4 genome-equivalents/ml in early pregnancy.¹⁶ The relative abundance of circulating fetal DNA compared to circulating fetal cells highlights the potential of using cell-free maternal plasma/serum for non-invasive prenatal diagnosis. Genetic loci with absolute fetal specificity such as Y-chromosome-specific markers¹⁶ and fetal *RhD* gene^{17–21} were subsequently shown to be feasible for non-invasive diagnosis of sex-linked disorders (eg, haemophilia A) and rhesus D incompatibility in RhD-negative pregnant women, respectively. Notably, fetal *RhD* blood group genotyping is the first plasma-based non-invasive prenatal diagnostic test translated from bench to bedside. It has been adopted by the British National Blood Service as a clinical test since 2001,²² and is now provided as part of standard obstetric care by a number of centres in

France and the Netherlands.²³ The fetal *RhD* blood group genotyping has significantly reduced the unnecessary use of anti-D immunoglobulin prophylaxis in RhD-negative pregnant women by 16-fold from 38% to 2%.²⁴

In addition to prenatal diagnosis,^{25–31} the detection of CNA has been applied in many fields such as oncology,^{32–36} transplantation medicine,³⁷ and critical care medicine^{38–46} for diagnosis, monitoring and prognostication. Comparatively, the detection of fetal nucleic acids is particularly challenging because of the predominant maternal CNA background. The relatively low level of fetal nucleic acids also underscores the potential risks of generating false negative results due to the loss of the already scarce target genetic materials during processing. The rarity of fetal CNA also undermines the detection of small sequence differences and fetal chromosomal aneuploidies in cell-free CNA. In the following sections, we shall illustrate how technical refinements in fetal CNA extraction, innovative assay designs and technological advancement have helped resolve these major challenges in fetal CNA detection and discover a novel class of pregnancy-specific microRNAs in the maternal circulation (fig 1).

PREANALYTICAL ISSUES IN FETAL CNA DETECTION

To study rare nucleic acids such as fetal CNAs, one needs to develop strategies to minimise the background, non-target DNA, and to increase the absolute amount and the relative proportion of target CNA recovered. In this regard, preanalytical factors such as the types of blood collection tubes, storage conditions and centrifugation protocols have been shown to affect the fractional concentration of fetal CNA recovered. Through the use of maternal plasma, the fractional fetal DNA concentration has been shown to be enriched 20-fold compared to the use of maternal serum for DNA extraction.¹⁶ The comparative advantage conferred by using maternal plasma is thought to be related to the absence of clotting of maternal blood cells and thus the lesser release of DNA from maternal leucocytes. A number of anticoagulants such as EDTA, citrate and heparin have therefore been evaluated for their efficacy in maintaining fetal DNA concentration.⁴⁷ It was noted that if plasma separation was delayed until 24 hours of venesection, fetal DNA concentration was better preserved by EDTA.⁴⁷ To minimise any additional release of maternal DNA, optimisation of the centrifugation is also required so that it is gentle enough to prevent lysis of maternal leucocytes but yet vigorous enough to generate cell-free plasma.⁴⁸ In

head and neck cancers,⁷¹ colon cancer⁷² and glioma⁷³ to develop cancer markers based on promoter hypermethylation. In non-invasive prenatal diagnosis, previous studies have shown that DNA methylation in placental tissues (the major source of fetal DNA in maternal plasma)⁷⁴ and maternal blood cells (the presumed major source of maternal DNA in maternal plasma)⁷⁵ are different in many genomic regions.^{76–79} These patterns allow epigenetic assays to be developed, using bisulphite conversion^{80–81} (fig 2A) or non-bisulphite conversion based methods such as restriction enzyme digestion (fig 2B). Using bisulphite sequencing, Chim *et al* demonstrated the methylation differences between the placenta and maternal blood cells in certain regions in the *SERPINB5* gene (a member of the serine proteinase inhibitor B family) and established hypomethylated *SERPINB5* as the first universal fetal-specific marker for detection in maternal plasma.

One of the caveats of bisulphite conversion is that as much as 90% of the DNA molecules are destroyed during the process.⁸² This can jeopardise the detection of the already trace fetal CNA. Therefore, a non-bisulphite conversion based approach using methylation-sensitive restriction enzyme such as *HpaII* and *BstUI* has been explored in the development of universal fetal markers. These enzymes specifically cut unmethylated sequences, leaving hypermethylated DNA intact for detection (fig 2B). Using this methylation-sensitive enzyme digestion strategy, hypermethylated *RASSF1A* was developed as another universal fetal DNA marker.⁸³ This method has also been recently shown to be useful for cancer detection.⁸⁴

This methylation-sensitive restriction enzyme digestion strategy is typically only applicable for the detection of hypermethylated targets. The situation is more complicated for hypomethylated targets. For example, the *SERPINB5* gene is hypomethylated in the placenta. Placental *SERPINB5* sequences will thus be cleaved by *HpaII* into fragments which are too short to be amplified by conventional primers. Tong *et al* explored the possibility that the use of stem-loop primers, previously developed for amplifying microRNAs,⁸⁵ might allow one to extend the restriction enzyme concept to the detection of hypomethylated targets.⁸⁶ Tong *et al* demonstrated that using stem-loop primers, restriction enzyme-digested *SERPINB5* fragments could be selectively amplified without co-amplifying the non-enzyme-digested maternal background of methylated *SERPINB5*.⁸⁶

With the availability of these universal fetal markers, maternal plasma being negative for the target fetal DNA (eg, fetal *RhD*) can be examined further if this could be explained by low fetal DNA concentrations (indicated by the absence of the universal fetal DNA marker) and hence false negative results. The incorporation of these sex- and polymorphism-independent universal DNA markers into non-invasive prenatal diagnosis could therefore allow laboratories to interpret negative results with greater certainty.⁸³

DETECTION OF CHROMOSOMAL ANEUPLOIDIES

Since fetal CNAs exist freely in the maternal circulation and not within the nuclei, the derivation of fetal chromosome dosage information for the detection of chromosomal aneuploidies has been a challenge in non-invasive prenatal diagnosis. Pregnancies affected by chromosomal aneuploidy cannot be distinguished from normal cases by simple measurement of fetal DNA levels in maternal plasma. Previous data in fetal trisomy 21 cases have shown that fetal DNA levels, albeit elevated, overlapped with the concentrations in normal pregnancies.⁶² Another option could be to measure fetal chromosome 21 sequences with reference to another chromosome in the circulation. However, the overwhelming excess of maternal DNA in maternal plasma would make it difficult for any increase in fetal signal caused by the aneuploidy to be detected.

Instead of using DNA, RNA derived from target tissues may allow the study of chromosomal aneuploidies. Cell-free circulating tumour-derived RNA was first reported by Lo *et al*⁸⁷ and Kopreski *et al*⁸⁸ in 1999, and subsequently by other researchers in various cancers.^{89–92} In 2000, fetal RNA was found in maternal plasma,⁹³ with the placenta identified as an important source.⁹⁴

One of the placental-specific transcripts, *PLAC4* mRNA, was chromosome 21 encoded.⁹⁵ Lo *et al* hypothesised that the ratio between RNA transcripts derived from alleles of *PLAC4* might be useful for fetal trisomy 21 detection. They postulated that the allelic ratio at the fetal DNA level might be preserved at the mRNA level for selected genes involved in chromosomal aneuploidies. In this RNA-SNP strategy, the percentages of the fetal alleles at a single nucleotide polymorphism (SNP) in the *PLAC4* mRNA were measured by mass spectrometry.⁶⁶ Using this *PLAC4* RNA-SNP allelic ratio analysis, fetal trisomy 21 was successfully diagnosed by using maternal plasma in 9 of 10 affected cases and confidently excluded in 55 of 57 euploid

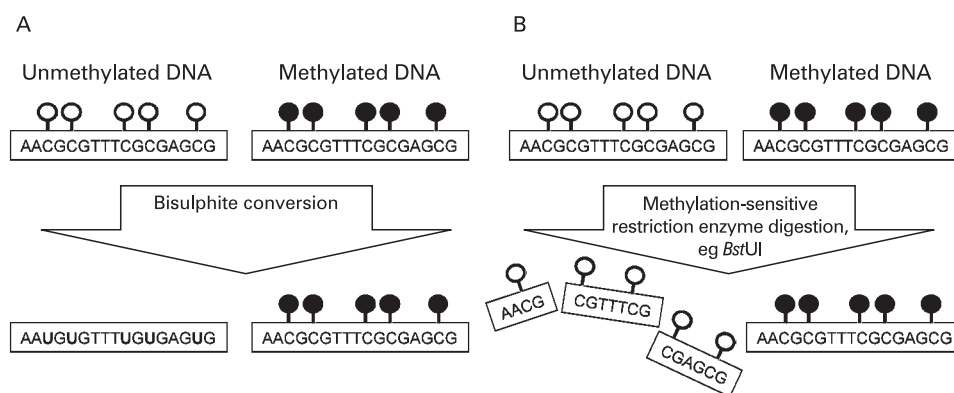


Figure 2 Schematic diagram illustrating the differences between (A) bisulphite conversion and (B) a non-bisulphite based strategy using methylation-sensitive restriction enzyme digestion in the development of universal fetal markers. The filled and unfilled lollipops denote the methylated and unmethylated cytosines, respectively. (A) On bisulphite conversion, methylated cytosines would remain as cytosines while unmethylated cytosines would be converted to uracils. (B) Methylation-sensitive restriction enzymes (eg, *BstUI*) would cut unmethylated DNA sequences at specific recognition sites (eg, 5'-CG^vCG-3' for *BstUI*) while methylated sequences would remain intact.

controls.⁶⁶ The sensitivity (90%) and specificity (96.5%) of this single marker test compares favourably to some of the best available Down syndrome screening strategies which require the measurement of 3–4 serum biochemical markers and nuchal thickness of the fetus.^{96–97} Since the technology requires the fetus to be heterozygous at the analysed SNP, the inclusion of other SNPs within *PLAC4* or other fetal-specific chromosome 21 loci^{98–99} would allow this test to be applicable to more fetuses. With the millions of pregnant women undergoing prenatal diagnosis and even invasive diagnostic procedures for fetal Down syndrome detection worldwide each year, large-scale studies to validate the utility of this promising non-invasive prenatal diagnostic test for Down syndrome are eagerly awaited.²⁵

DETECTION OF SINGLE NUCLEOTIDE CHANGES

The overwhelming maternal DNA background can also make the detection of CNAs with small genetic difference difficult. Using allelic-specific amplification,¹⁰⁰ Sorenson *et al* and Vasioukhin *et al* provided the first evidence on the detection of single point mutations in the *KRAS* gene in serum DNA from pancreatic cancer patients⁷ and in the *NRAS* gene in plasma DNA from patients with myelodysplastic syndrome and acute myelogenous leukaemia,⁸ respectively. In the prenatal diagnostic setting, the detection of β -thalassaemia¹⁰¹ is also challenging as the majority of disease-causing mutations are single nucleotide substitutions, or deletions/insertions leading to frameshift. Chiu *et al*¹⁰² were able to exclude β -thalassaemia non-invasively by using allele-specific primers to study the fetal inheritance of a common disease-causing mutation, a four-nucleotide deletion of CTTT at codons 41/42, using a RT-qPCR platform. However, real-time quantitative PCR is generally not robust enough to detect a minority nucleic acid population which differs from the majority background by a single nucleotide, as non-specific signal generation from the wild-type allele may occur.¹⁰³

SABER (single allele base extension reaction)

In 2003, a mass spectrometry-based strategy was proposed to meet this challenge. Mass spectrometry is an analytical technique which measures the mass-to-charge ratio of an ion, with resolution down to a few daltons. Target nucleic acids are first amplified by sequence-specific primers and then serve as templates for a nucleotide extension step. By using a known mixture of dideoxynucleotide (ddNTP) and dNTP, and an extension primer which anneals up to the nucleotide immediately upstream of the site of the point mutation, one or only a small number of nucleotides will be extended depending on the sequence of the alleles. The products generated from either allele will carry different mass-to-charge ratios and thus be resolvable by mass spectrometry. However, if the background allele is present at a very high proportion, preferential amplification can occur and the target allele may go undetected. In this regard, the extension step can be modified so that only the type of dNTP complementary to the target allele is added. As a result, nucleotide extension is only possible for the target allele. This so-called single allele base extension reaction (SABER) has been successfully applied to the detection of single nucleotide changes in β -thalassaemia and achondroplasia.^{104–105}

ASBER (allele-specific base extension reaction)

The SABER assay has been noted to produce false positive results in certain applications such as haemoglobin E

Take-home messages

- ▶ Circulating fetal nucleic acids are present in the plasma of pregnant women and are useful for the development of non-invasive prenatal diagnosis.
- ▶ A number of diagnostic applications using such circulating fetal nucleic acids are already in clinical applications, including fetal sex determination for sex-linked diseases and fetal *RhD* status determination.
- ▶ Ongoing work in this area includes the detection of fetal chromosomal aneuploidies, the development of new analytical strategies such as digital PCR, and the development of new markers such as plasma epigenetic and plasma microRNA markers.

genotyping.¹⁰⁶ The exact cause is currently unknown, but it could be related to the fidelity of the polymerase and the ddNTP concentration.¹⁰⁷ By introducing a 3' primer-template mismatch for the wild-type allele and competitor ddNTPs in the extension step, Tsang *et al* reduced non-specific extension and correctly identified 80% of cases with haemoglobin E mutations using this alternative assay called ASBER (allele-specific base extension reaction).¹⁰⁷

A NEW CLASS OF CNA: MICRORNAS

In addition to DNA and mRNA, small RNA molecules such as microRNAs (miRNAs) are now being investigated as novel circulating markers. MiRNAs were first discovered in 1993 in *Caenorhabditis elegans*^{108–109} and are now found in many species including humans.¹¹⁰ MiRNAs are involved in processes such as morphogenesis, organ development and possibly cancer development.^{110–112} Since miRNAs are only 18–25 nucleotides long, new assay designs such as the stem-loop based strategy^{85–113} and the polymerase A-based strategy¹¹⁴ to extend the length of cDNA for subsequent amplification are required. Using the stem-loop based TaqMan assays, Chim *et al* systematically searched for and successfully detected placental-specific miRNAs in maternal plasma samples.¹¹⁵ In oncology, Lawrie *et al* and Mitchell *et al* further showed that circulating miRNAs were detectable in patients with diffuse large B-cell lymphoma¹¹⁶ and metastatic carcinoma of the prostate.¹¹⁷ The development of miRNAs as blood-based diagnostic markers is at its infancy stage. This is an exciting area because miRNAs represent a new class of post-transcriptional regulators and the biological functions for circulating miRNAs remain to be explored. As miRNAs have recently been found to have sequence variants, particularly at the 3' end, newer assay designs will be needed for robust detection of these highly similar sequences.

CONCLUSIONS

The discovery of circulating fetal nucleic acids in maternal plasma has opened up new opportunities for non-invasive prenatal diagnosis. Optimised fetal CNA recovery protocols, new assay designs and recently developed analytical tools have enabled the study of chromosomal aneuploidies, single nucleotide variations, single molecule detection and plasma miRNA analysis. It is expected that this technology would become an increasingly important tool in the future development of non-invasive prenatal diagnosis.

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Competing interests: RWKC and YMDL have filed patent applications on aspects of circulating fetal nucleic acids in maternal plasma. YMDL is a consultant to Sequenom and has equities in Sequenom and Core Healthcare.

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