Noninvasive prenatal detection of fetal chromosomal aneuploidies by maternal plasma nucleic acid analysis: a review of the current state of the art

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Accepted 29 September 2008.

Fetal nucleic acids in maternal plasma have opened up new possibilities for noninvasive prenatal diagnosis of chromosomal aneuploidies. One approach is based on the measurement of the allelic ratio of single nucleotide polymorphisms in the coding region of placental mRNA. Another approach is through the analysis of DNA fragments with different patterns of DNA methylation between fetal and maternal DNA. One other alternative is to enrich the fractional concentration of fetal DNA in maternal plasma using physical or chemical methods. Finally, the development of more precise digital polymerase chain reaction-based methods for fetal nucleic acid analysis might further catalyse the developments in this area. It is hoped that plasma-based molecular prenatal diagnosis might ultimately make prenatal testing safer for pregnant women and their fetuses.

Keywords Plasma DNA, plasma RNA, Down syndrome, trisomy 21, prenatal diagnosis

Introduction

Noninvasive prenatal diagnosis is an actively researched area in prenatal medicine. Much work has been carried out on the development of a variety of screening methods for fetal chromosomal aneuploidies, including ultrasonography and maternal serum biochemical screening. However, it should be borne in mind that these approaches are essentially measuring epiphenomena, which are associated with the chromosomal aneuploidies, rather than detecting the core pathology directly. Because of this indirect nature, such methods have a number of limitations, including a relatively narrow gestational window in which a particular test can be used, and the limited sensitivity and specificity if a single marker is used. Nonetheless, through the use of combinations of markers, impressively high sensitivity and specificity have been achieved.

Nonetheless, over the past several decades, many workers have explored the possibility of developing noninvasive prenatal diagnostic methods that would allow the direct analysis of fetal genetic materials. From the 1960s to 1990s, many workers have investigated a variety of approaches for the isolation of fetal nucleated cells from maternal blood. However, the rarity of circulating fetal nucleated cells in maternal blood has limited the practicality of this approach and the eventual diagnostic sensitivity, specificity and reproducibility. For example, in a multicentre trial reported in 2002, using possibly the best technology for the isolation of fetal nucleated red blood cells from the maternal circulation available at the time, a sensitivity of 41.4% was reported for the detection of male fetal cells in maternal blood with a false-positive rate of 11.1%. Thus, it was clear from these figures that an alternative approach for the direct detection of fetal genetic material from maternal blood, rather than through the isolation of fetal nucleated cells in maternal blood, was needed.

Between the 1970s and 1990s, a parallel group of investigators were studying the intriguing phenomenon of cell-free DNA in the plasma of human subjects. One particularly interesting line of research has been the detection of tumour-derived DNA in the plasma of women suffering from a variety of cancer. Hence, tumour-associated oncogene mutations and microsatellite alterations have been detected in the plasma...
DNA of women with cancer. Inspired by such work, Lo et al. hypothesised in 1997 that a fetus might also release its DNA in cell-free form into the plasma of its mother. This hypothesis has led to the direct experimental demonstration of cell-free male fetal DNA in the plasma and serum of women carrying male fetuses. This discovery has thus opened up new possibilities for noninvasive prenatal diagnosis.

**Biology and diagnostic applications of fetal DNA in maternal plasma**

Over the past decade, much has been found out about the fundamental parameters governing cell-free fetal DNA in maternal plasma. Thus, it is known that cell-free fetal DNA represents a mean of 3–6% of the DNA that is present in maternal plasma. Following delivery, fetal DNA has been found to be cleared from maternal plasma with a half-life in the order of 16 minutes. This new, noninvasive source of fetal DNA was rapidly used for prenatal diagnostic purposes. Thus, this method has been used for the prenatal determination of fetal gender in sex-linked diseases and in congenital adrenal hyperplasia, and in fetal RhD status determination in pregnancies involving RhD-negative pregnant women. This approach has also been used for the prenatal detection of mutations that the fetus has inherited from the father, but which are absent in its mother, for example in beta-thalassaemia when the father and mother carry different mutations.

**Detection of fetal chromosomal aneuploidies from plasma nucleic acids: the challenge**

Compared with the above conditions, the detection of fetal chromosomal aneuploidies has offered considerable additional technical challenge. Early work has shown that cell-free fetal DNA in maternal plasma/serum is elevated in pregnancies complicated by fetal trisomy 21 and trisomy 13 but not by trisomy 18. However, the use of such fetal DNA measurement for the noninvasive screening of fetal chromosomal aneuploidies would have been an underutilisation of this technology because when used in such a fashion, fetal DNA measurement would just represent yet another maternal serum biochemical marker. The real promise of circulating fetal nucleic acids for fetal chromosomal aneuploidy detection lies in its potential for the direct detection of the trisomy status, provided that an appropriate detection strategy can be developed. The latter, however, requires considerable technical ingenuity. The main reason is that as discussed above, fetal DNA only represents a mean of some 3–6% of the DNA in maternal plasma, while the bulk of the DNA in maternal plasma is derived from the mother herself. Thus, a simple measurement of chromosome dosage in maternal plasma DNA, for example using conventional quantitative polymerase chain reaction (PCR) technique, would have yielded chromosome dosage information for the mother but not that of the fetus.

Thus, the first challenge in the use of maternal plasma nucleic acid for fetal chromosomal aneuploidy detection is the development of an approach for the selective targeting of a subset of nucleic acid in maternal plasma that is completely fetal specific. The second challenge is the development of a method that would allow the chromosomal dosage information to be determined from this subset. As will be described in the next section, the placental RNA-single nucleotide polymorphism (SNP) allelic ratio method is probably the most promising approach at this stage, which illustrates the feasibility of this strategy.

**The placental RNA-SNP allelic ratio method**

Each nucleated cell in the human body contains approximately 25,000 genes. However, not all genes are switched on in a particular cell. In active genes, the DNA will be copied into mRNA. It is well known that each tissue in the body contains a characteristic profile of mRNA. Thus, if one can find a tissue type that is only present in the fetus, then one might be able to find an mRNA that is completely fetal specific. The placenta is an obvious example of a tissue type that is only present in the fetus. Provided that one can develop a systematic method to identify placental-specific mRNA, then one could theoretically generate tens or even hundreds of new markers for prenatal investigation. Another advantage of an RNA-based method is that each active gene will produce many mRNA molecules, thus providing an intrinsic amplification effect.

In a series of developments since 2000, the basis for plasma RNA as a prenatal diagnostic tool has been established. In 2000, Poon et al. showed that mRNA transcribed from the Y chromosome could be detected in the plasma of women carrying male fetuses. In 2002, plasma RNA was shown to be surprisingly stable, possibly through the protection by particulate matter. In 2003, it was shown that the placenta was a major source of fetal-derived RNA in maternal plasma using human placental lactogen mRNA and mRNA coding for the beta subunit of human chorionic gonadotrophin as examples. In 2004, a microarray-based approach was developed for the systematic identification of placental mRNA markers, which can be detected in maternal plasma. In 2007, a placental-specific mRNA that is transcribed from a gene located on chromosome 21, PLAC4 (placenta-specific 4), was identified using the microarray-based approach and was shown to be detectable in maternal plasma and cleared following delivery of the fetus.

The next technical hurdle would be to develop a method to determine the dosage of chromosome 21 using PLAC4 mRNA in maternal plasma. Lo et al. developed the RNA-SNP allelic ratio approach for this purpose (Figure 1). Let us consider an
SNP that is present in the coding region of the \textit{PLAC4} gene. If a fetus is heterozygous for this SNP, it would possess two alleles that are distinguishable by DNA sequence. If the fetus is euploid, that is containing two copies of chromosome 21 and thus two copies of the \textit{PLAC4} gene is switched on in the placenta, and if there is no allele-specific preference in gene expression, mRNA transcribed from each of the two alleles would be identical. When the placenta releases its mRNA into maternal plasma, the ratio of placental mRNA in maternal plasma that is transcribed from each of these two alleles would also be 1:1. Conversely, if the fetus has trisomy 21, then the mRNA-SNP allelic ratio would become 1:2 or 2:1. Through the use of a mass spectrometry-based method for measuring the mRNA-SNP allelic ratio precisely, Lo et al. have demonstrated that this strategy does in fact work with a diagnostic sensitivity and specificity for trisomy 21 of 90 and 96.5%, respectively. The latter figures suggest that this RNA-SNP allelic ratio approach is probably the most accurate single marker approach for the noninvasive prenatal detection of trisomy 21 yet described.

While these early results are already promising, the sensitivity and specificity figures can probably be further increased. The initial definition of the ‘normal range’ in the original report is based on the mean mRNA-SNP allelic ratio ±1.96 SD. By definition, this method would result in a fixed specificity for the method. With large-scale clinical trials, it might be possible to further refine the definition of the reference interval. It has also been shown that the accurate measurement of the RNA-SNP allelic ratio requires a minimum number of target mRNA molecules. Thus, further development of more efficient protocols for plasma RNA processing and extraction might result in improved yield of the target mRNA molecules for analysis. Development of alternative methods, such as by digital PCR technology, in which individual target molecules are amplified and the positive reactions counted, might further improve the precision in which one might be able to measure the allelic ratio. Indeed, Lo et al. demonstrated that this digital RNA-SNP approach could yield a diagnostic result with as little as a couple hundred of target RNA molecules extracted from maternal plasma. This is also the demonstration that digital PCR can be used for the detection of fetal-derived mRNA species in maternal plasma. The application of digital PCR to the analysis of DNA materials will be discussed in a later section.

The main limitation of the RNA-SNP allelic ratio approach is that only fetuses heterozygous for the analysed SNP can be successfully diagnosed. For example, with the use of the single SNP in the \textit{PLAC4} gene in the first description of the method, approximately 45% of fetuses would be expected to be heterozygous and thus diagnosable using this approach. When this approach is used on an unknown sample, one would not know beforehand whether the fetus is going to be heterozygous for the analysed SNP. However, this information would be available just from maternal plasma RNA analysis alone. For example, if one could only see one placental mRNA SNP allele from the analysis of maternal plasma RNA, then the fetus is homozygous and thus the method is not informative for this case. However, if one could see two SNP alleles, then one could proceed to measure the SNP allelic ratio to determine if the fetus is euploid or aneuploid.

Since the first description of the RNA-SNP allelic ratio method, a number of investigators have described new markers or polymorphisms that can be analysed using this approach. One preliminary report describes ten markers with a combined heterozygosity rate that covers up to 95% of the US general population. The evaluation of these markers in large-scale clinical trials is expected over the next few years.

**Other approaches**

Apart from the RNA-SNP allelic ratio approach, a number of other methods have also been proposed in the literature. These methods are briefly discussed below.

**Epigenetic markers**

One key feature of the RNA-SNP allelic ratio method is the use of placental mRNA, which is completely fetal specific. An alternative to the use of placental mRNA for developing fetal-specific markers is to use epigenetic markers. Epigenetics is the study of molecular phenomena that affect gene expression, but which do not involve a change in DNA sequence. As an illustration, a gene mutation that switches off the
expression of a gene is not an epigenetic phenomenon because it involves a change in DNA sequence. The best studied epigenetic phenomenon is the process of DNA methylation, which involves the addition of a methyl group to the cytosine residues of a DNA sequence. It is known that if such cytosine methylation occurs in the promoters of genes, the gene expression may be switched off, such as in certain tumor suppressor genes in cancer. Furthermore, different tissues may have a tissue-specific pattern of DNA methylation.

In 2002, Poon et al. demonstrated that it was possible to develop fetal DNA markers based on differential DNA methylation patterns between maternal and fetal tissues. This strategy has led to the identification that the SERPINB5 gene, coding for maspin, is hypomethylated in the placenta but hypermethylated in maternal blood cells. As the SERPINB5 gene is located on chromosome 18, this characteristic has allowed the development of a strategy that is analogous to the RNA-SNP allelic ratio approach, the so-called epigenetic allelic ratio approach (Figure 1). Thus, for a fetus heterozygous for an SNP on the SERPINB5 gene, through the measurement of the ratio of the SNP alleles in the hypomethylated version of the gene, the fetus’s trisomy 18 status can be ascertained.

Such developments have led to a search for fetal-specific epigenetic markers on chromosome 21. Chim et al. have performed a systematic search for such markers on chromosome 21. Of the 114 studied genomic regions, 22 (19%) are differentially methylated between the maternal and fetal (placental) tissues. These figures therefore suggest that DNA methylation markers may offer an abundant source of markers for developing noninvasive prenatal diagnosis of trisomy 21.

The main disadvantage of the epigenetic approach over the RNA-SNP allelic ratio method lies in the fact that many commonly used methods for DNA methylation analysis involves the use of bisulphite-based reagents. Such reagents have been shown to result in DNA degradation that would reduce the amount of target DNA available for subsequent analysis. The development of assays that would avoid the use of bisulphite is in progress in a number of laboratories and might allow DNA methylation markers to be more easily used for diagnostic purposes. Another relative disadvantage of the DNA methylation approach when compared with the RNA-SNP allelic ratio approach is the fact that the DNA-based approach cannot benefit from the ‘biological amplification’ following the transcription of multiple copies of mRNA from a single genomic DNA locus. However, the main advantage of the DNA methylation-based approach is that recent data suggest that such markers are likely to be relatively abundant in the human genome. Nonetheless, the use of the RNA-SNP allelic ratio and DNA methylation-based approaches is not mutually exclusive, as it should be possible to develop sample collection and nucleic acid extraction procedures, which would allow both types of approaches to be used on the same maternal blood sample.

Fetal DNA enrichment

The RNA-SNP allelic ratio approach and the DNA methylation approach target subsets of nucleic acid molecules that are present in maternal plasma in a molecular fashion. An alternative to these approaches is the use of physical methods that would result in the relative enrichment of fetal DNA in maternal plasma.

In 2004, it was shown that the length of fetal DNA in maternal plasma was generally shorter than the maternally derived DNA present in maternal plasma. As a consequence, it has been shown that the use of gel electrophoresis could allow one to size-fractionate plasma DNA and to specifically isolate the shorter DNA fragments. This approach has been used successfully to enrich for circulating fetal DNA. While this approach has been shown empirically to be useful for the qualitative detection of disease-causing mutations, for example those causing beta-thalassaemia, it is yet unknown whether the degree of enrichment might be sufficient for fetal chromosomal aneuploidy detection, which requires the quantitative measurement of chromosome dosage. In addition, the first reported method for size fractionation requires the use of gel electrophoresis, which might create opportunities for contamination.

Dhallan et al. reported another approach for the enrichment of fetal DNA in maternal plasma. Dhallan et al. hypothesised that a significant source of maternal DNA in maternal plasma is that released by maternal white blood cells following venesection. The authors proposed that if such maternal nucleated blood cells could be fixed using formaldehyde, then this dilution of fetal DNA in maternal plasma could be avoided. Dhallan et al. then went on to demonstrate the utility of this approach for the noninvasive prenatal diagnosis of trisomy 21. However, the beneficial effects of formaldehyde treatment could not be replicated by a number of groups. Furthermore, methodological and statistical concerns with regard to the original reports on formaldehyde treatment have been raised. Nonetheless, it is possible that the future evaluation of other chemical treatment methods might allow further progress in this area.

Relative chromosome dosage by digital PCR

The above-mentioned approaches, namely the RNA-SNP allelic ratio method, DNA methylation markers and fetal DNA enrichment approaches, work on the assumption that the low fractional concentration of fetal DNA in maternal plasma makes it challenging to pursue the direct detection of fetal chromosomal aneuploidies. This assumption is based on the limited precision of conventional methods for circulating fetal DNA detection, for example by real-time PCR. In 2007, Lo et al. after having demonstrated that digital PCR could be used to measure the RNA-SNP allelic ratio in
maternal plasma (see above), extended the use of this strategy to the discrimination of trisomy 21 placental DNA samples from euploid ones. Digital PCR refers to the performance of multiple PCRs in parallel in which each PCR would typically contain either a single or no target molecule. Through the counting of the number of positive reactions at the end of amplification, one could arrive at an estimation of the number of input target molecules. In this section, digital PCR is discussed in the context of analysing fetal DNA in maternal plasma. Lo et al. have further demonstrated that the aneuploidy status detection is still possible even when the trisomic DNA is present as a minor fraction and have tabulated the fractional fetal DNA concentrations. The latter is essentially the specifications for the construction of a test for detecting fetal trisomy 21 in maternal plasma. Fan and Quake have subsequently published data largely supportive of those of Lo et al. using DNA mixtures from two commercially available cell lines.

The main limitation of digital PCR-based approaches at the moment concerns the labour intensiveness for conducting the hundreds or thousands of digital PCR assays that are needed for each analysis. However, the development of automated platforms for conducting digital PCR, for example using microfluidics, might allow this approach to be used in routine clinical diagnosis in the future.

Conclusions

In the first decade since the discovery of circulating cell-free fetal nucleic acids in maternal plasma, much has been revealed with regard to the biology of this phenomenon and a number of diagnostic applications have been developed. Of note, a number of applications have already reached clinical practice. The use of this technology for the prenatal diagnosis of fetal chromosomal aneuploidies has taken more time to develop, possibly because of the technical challenge that is involved. Nonetheless, the past few years have seen the publication of a number of alternative strategies for this goal to be accomplished. Over the next few years, large-scale clinical trials of some of these strategies would be expected. It is hoped that when this is completed, we would have new and safer choices for the millions of pregnant women worldwide who are considering prenatal diagnosis each year.

Disclosure of interests

The author holds patents and has filed patent applications on aspects of the use of cell-free fetal nucleic acids in maternal plasma for noninvasive prenatal diagnosis. Part of this patent portfolio has been licensed to Sequenom, Institut Jacques Boyd and Core Healthcare. The author is a consultant to, is supported by a sponsored research agreement by, and holds equities in Sequenom.

Funding

The author is supported by the Areas of Excellence Scheme of the University Grants Committee of the Government of the Hong Kong Special Administration Region (AoE/M-04/06) and the Li Ka Shing Foundation.

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