Review

Noninvasive Prenatal Diagnosis of Fetal Chromosomal Aneuploidies by Maternal Plasma Nucleic Acid Analysis

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BACKGROUND: The discovery of circulating cell-free fetal nucleic acids in maternal plasma has opened up new possibilities for noninvasive prenatal diagnosis. The potential application of this technology for the noninvasive prenatal detection of fetal chromosomal aneuploidies is an aspect of this field that is being actively investigated. The main challenge of work in this area is the fact that cell-free fetal nucleic acids represent only a minor fraction of the total nucleic acids in maternal plasma.

METHODS AND RESULTS: We performed a review of the literature, which revealed that investigators have applied methods based on the physical and molecular enrichment of fetal nucleic acid targets from maternal plasma. The former includes the use of size fractionation of plasma DNA and the use of the controversial formaldehyde treatment method. The latter has been achieved through the development of fetal epigenetic and fetal RNA markers. The aneuploidy status of the fetus has been explored through the use of allelic ratio analysis of plasma fetal epigenetic and RNA markers. Digital PCR has been shown to offer high precision for allelic ratio and relative chromosome dosage analyses.

CONCLUSIONS: After a decade of work, the theoretical and practical feasibility of prenatal fetal chromosomal aneuploidy detection by plasma nucleic acid analysis has been demonstrated in studies using small sample sets. Larger scale independent studies will be needed to validate these initial observations. If these larger scale studies prove successful, it is expected that with further development of new fetal DNA/RNA markers and new analytical methods, molecular noninvasive prenatal diagnosis of the major chromosomal aneuploidies could become a routine practice in the near future.

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Prenatal diagnosis is now an established part of modern obstetrics care. The detection of fetal chromosomal aneuploidies is a major reason why many pregnant women go for prenatal diagnosis. Many conventional methods for prenatal diagnosis, however, require obtaining fetal materials for analysis through procedures such as amniocentesis and chorionic villus sampling. These methods are invasive and constitute a finite risk to the fetus. To stratify pregnant women according to their risk of carrying a fetus affected by chromosomal aneuploidy, several screening methods have been developed, including ultrasonography and maternal serum biochemistry *(1)*. These methods, however, are targeted at epiphenomena associated with the chromosomal aneuploidies, rather than at the core molecular abnormalities, and have limited sensitivities and specificities, with strictly defined gestational age windows that must be used for specific tests. To circumvent such limitations, there is a need for the development of a new generation of noninvasive tests that target the core molecular pathology of such fetal chromosomal abnormalities.

The discovery of circulating cell-free fetal DNA in maternal plasma in 1997 has offered a new approach for noninvasive prenatal diagnosis *(1, 2)*. Although this method can be readily applied for the detection of unique fetal genetic targets, e.g., the Y chromosome from a male fetus *(3)* and the Rh blood group, D antigen (*RHD*)[2] gene of an RhD-positive fetus *(4)*, the development of tests enabling the use of this approach for fetal chromosomal aneuploidies has been challenging. One fundamental technical challenge is a consequence of the fact that between weeks 11 and 17 weeks of gestation, fetal DNA constitutes only a mean of approximately 3% of total cell-free DNA in maternal plasma *(5)*, with the obvious implication that most of the DNA

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[2] Human genes: *RHD*, Rh blood group, D antigen; placenta-specific 4, *PLAC4*.

in the plasma is maternal in origin. Thus, most molecular methods for aneuploidy detection, if applied to maternal plasma, would essentially be measuring the chromosome status of the mother. In addition, because cell-free nucleic acids are circulating in an extracellular form in maternal plasma, all of the cell-based methods for aneuploidy detection, e.g., fluorescence in situ hybridization, would not be applicable. This review summarizes the current developments in the use of circulating cell-free nucleic acids in maternal plasma for the noninvasive prenatal detection of fetal chromosomal aneuploidies.

APPROACHES TO SOLVE THE PROBLEMS ASSOCIATED WITH THE LOW FRACTIONAL CONCENTRATION OF FETAL NUCLEIC ACIDS

The fractional concentration of cell-free fetal DNA in maternal plasma is determined by the ratio of the absolute concentration of cell-free fetal DNA to the absolute concentration of total (maternal and fetal) cell-free DNA. Thus, the fractional concentration of cell-free fetal DNA can be increased through the selective enrichment of fetal DNA, or through the suppression of the background maternal DNA. A schematic illustration of the major approaches is shown in Fig. 1.

SIZE FRACTIONATION OF PLASMA DNA

Selective enrichment of fetal DNA requires the targeting of fetal DNA characteristics that are different from those of maternal DNA in maternal plasma. Chan et al. have demonstrated that circulating fetal DNA molecules are generally shorter than the circulating maternal DNA molecules (6). Li et al. have shown that enrichment of fetal DNA can be achieved by the selective targeting of the shorter plasma DNA molecules (7). This method has been applied to enhance the detection of paternally inherited fetal mutations in maternal plasma (8). However, whether the degree of enrichment achieved might allow the direct detection of fetal chromosomal aneuploidies is currently unknown. Furthermore, the reported method for such size fractionation is based on the size separation of plasma DNA using agarose gel electrophoresis, followed by the extraction of DNA from manually cut agarose gel slices containing different size fractions (7). Apart from the labor-intensiveness of this procedure, it is prone to contamination. Thus, new and potentially automatable methods for the size fractionation of plasma DNA must be developed before this approach can become practical for noninvasive prenatal diagnosis.

SUPPRESSION OF THE MATERNAL DNA BACKGROUND

With regard to the suppression of the maternal DNA background, Lui et al. have demonstrated in a sex-mismatched bone marrow transplantation model that
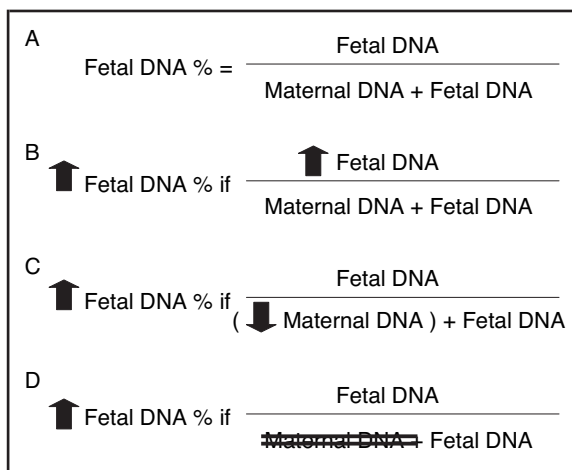

Fig. 1. Schematic illustration of the major approaches to increasing the fractional concentration of cell-free fetal DNA.

The fractional concentration of fetal DNA in maternal plasma is given by the ratio of the absolute concentration of cell-free fetal DNA to that of the total cell-free DNA in maternal plasma (A). Approaches to increase the fractional concentration of fetal nucleic acids in maternal plasma may involve selective enrichment of fetal DNA (B), suppression of maternal background DNA (C), or elimination of the maternal nucleic acid background by detecting nucleic acids that are virtually completely fetus specific, such as fetal epigenetic or fetal RNA markers (D).

most of the DNA in plasma is hematopoietic in origin (9). It has been postulated that maternal hematopoietic cells might also be the major cell types contributing to the maternal nucleic acid background in maternal plasma (10, 11). Dhallan et al. hypothesized that the addition of formaldehyde might result in the stabilization of the maternal leukocytes following venesection, thus reducing the liberation of maternal DNA by such cells into the plasma, a process that might result in the dilution of the fetal DNA in maternal plasma (12). Although the initial data presented by Dhallan et al. were impressive (12), these results have not been reproduced by a number of independent groups (13, 14). One possible explanation for these discrepant results is the use by Dhallan et al. of an imprecise analytical method that might overestimate the fractional fetal DNA concentration in a proportion of formaldehyde-treated samples (15). Nonetheless, it would be interesting to test preservatives other than formaldehyde for their ability to suppress the maternal DNA background.

MOLECULAR ENRICHMENT STRATEGIES: FETAL EPIGENETIC MARKERS AND FETAL RNA MARKERS

Another approach to address the low fractional concentration of fetal DNA is the targeting of selected subsets of nucleic acids in maternal plasma that are virtually completely fetus specific. One approach is to identify loci that exhibit fetus-specific epigenetic signatures. Epigenetics is a field that studies molecular processes that affect gene expression without altering DNA sequences *(16)*. One of the best studied epigenetic processes is DNA methylation. In 2002, Poon et al. postulated that loci exhibiting differential DNA methylation patterns between fetal and maternal tissues might be used to develop fetal epigenetic markers for detection in maternal plasma *(17)*. Chim et al. have reported that the *SERPINB5* gene, coding for maspin, is hypomethylated in placental tissues and hypermethylated in maternal blood cells *(11)*. Because the placenta is likely to be the major source of fetal DNA in maternal plasma *(18, 19)*, and as discussed above, the maternal hematopoietic cells are likely to be a major source of maternal DNA in maternal plasma, hypomethylated *SERPINB5* sequences may serve as a marker for placental DNA in maternal plasma. The feasibility of this epigenetic approach for noninvasive prenatal diagnosis has been demonstrated by the good correlation between the concentrations of hypomethylated *SERPINB5* sequences and *SRY* sequences from male fetuses in maternal plasma, and the clearance of *SERPINB5* sequences from maternal plasma following delivery *(11)*. The location of *SERPINB5* on chromosome 18 has also provided a valuable opportunity to test the application of this epigenetic approach for the prenatal detection of fetal chromosomal aneuploidy, using trisomy 18 as a model system (see later sections) *(20)*.

Since the development of the *SERPINB5* marker, many other fetal epigenetic markers suitable for detection in maternal plasma have been described, including the *RASSF1A* gene on chromosome 3 *(21, 22)* and numerous markers on chromosome 21 *(23, 24)*. Through the targeting of such fetus-specific DNA methylation markers in maternal plasma, the detected signal is virtually completely fetal in origin. Thus, the number of fetal chromosomes on which the epigenetic marker is located can be ascertained (see later sections). From the systematic survey of chromosome 21 *(24)*, it appears that markers informative for noninvasive prenatal diagnosis are relatively plentiful. The main limitation of this DNA methylation approach is that many of the commonly used methods for detecting DNA methylation markers, e.g., methylation-specific PCR *(25)*, are based on bisulfite conversion. Bisulfite conversion has been shown to result in a massive degradation of the input DNA *(26)*. This undesirable characteristic is det-

rimental for the detection of circulating fetal DNA markers in which a relatively limited number of target molecules are present in a particular sample. In this regard, markers that are hypermethylated in the placenta and hypomethylated in maternal blood cells are particularly valuable because methylation-sensitive restriction enzymes that cut hypomethylated sequences but leave hypermethylated sequences intact can be used for the selective destruction of the maternal sequences in maternal plasma *(22)*. The extension of such a restriction enzyme–based approach to markers exhibiting a reverse pattern of differential DNA methylation, namely hypomethylated in the placenta and hypermethylated in maternal blood cells, will require methods that allow the selective detection of the restricted (i.e., hypomethylated) sequences. One such approach has recently been described, which is based on the use of a stem-loop primer *(27)*. Another possible approach is through the development of new enzymes that would selectively restrict methylated (i.e., maternal) DNA, while leaving hypomethylated (i.e., fetal) DNA intact.

RNA molecules represent another nucleic acid subset in maternal plasma that can be targeted for fetal-specific molecules. Fetal RNA was first detected in maternal plasma in 2000 *(28)*. This finding was followed by work demonstrating the extraordinary stability of plasma RNA molecules *(29)*, possibly through their association with particulate matter *(30)*, a phenomenon that has been postulated to protect plasma RNA against degradation by plasma RNases *(31)*. One important development is the demonstration that placental mRNA represents an important source of fetal RNA in maternal plasma *(32)*. This latter discovery and the above-mentioned realization that maternal hematopoietic cells are likely to be the major contributor of maternal-derived nucleic acids in maternal plasma have led to the development of a systematic microarray-based approach for the identification of new placental mRNA markers suitable for maternal plasma detection *(10)*. This series of developments has led to the demonstration that mRNA from genes located on chromosome 21 can be detected in maternal plasma *(33, 34)*. One such gene for which mRNA in maternal plasma has been shown to be completely fetal-specific is placenta-specific 4 (*PLAC4*) *(34)*. Thus, with the identification of fetus-specific mRNA markers in maternal plasma from a chromosome involved in a chromosomal aneuploidy, all that remains would be to develop a technology for obtaining chromosome copy number information from such a marker (see later sections).

One key advantage of plasma RNA markers is that there is an intrinsic amplification process in which a

gene is transcribed into multiple mRNA copies. Another advantage is that plasma RNA markers can be readily detected by reverse transcriptase PCR or other amplification technologies. The main disadvantage is that in many reported procedures TRIZOL-treated plasma was used to stabilize plasma RNA during storage *(34)*, and thus archival plasma samples not been treated in this manner might not be suitable for plasma RNA analysis.

APPROACHES FOR DETERMINING FETAL CHROMOSOME DOSE IN CELL-FREE NUCLEIC ACIDS IN MATERNAL PLASMA

Although physical or molecular approaches for fetal nucleic acid enrichment may render the fetal proportion of nucleic acids to be more readily detectable in maternal plasma, methods are needed to allow assessment of the number of potentially aneuploid chromosomes in the fetal genome. Previous studies have shown that the fetal DNA concentration in maternal plasma is increased in pregnancies with certain fetal aneuploidies, such as trisomy 21 *(35)*. Large interindividual variations in maternal plasma fetal DNA and *PLAC4* mRNA concentrations, however, have precluded the use of their mere quantification as a robust means for identifying fetal aneuploidies *(34)*. Thus, strategies that allow the objective determination of fetal chromosome dose are needed, and the main approaches are shown in Fig. 2.

ALLELIC RATIO ANALYSIS

One approach for determining fetal chromosome dose is via the analysis of allelic ratio of genetic variations at the detected locus. This approach can be used only if the fetus is heterozygous at the detected locus. This approach can be applied in combination with the approaches described in the previous section, when the detected molecules are sufficiently enriched for fetal-derived targets. The simplest illustration of this concept is the molecular targeting of fetus-specific targets using DNA methylation markers or placental mRNA markers. For DNA methylation markers, the first demonstration is the use of hypomethylated *SERPINB5* sequences as a fetus-specific target on chromosome 18 *(11)*. Through the determination of the allelic ratio of a single nucleotide polymorphism (SNP) in the hypomethylated *SERPINB5* promoter in heterozygous fetuses, trisomy 18 can be detected *(20)*. This approach is called the epigenetic allelic ratio approach. With the recognition of fetal DNA methylation markers on chromosome 21 *(24)*, this approach can potentially be applied for the noninvasive prenatal detection of trisomy 21.

For RNA markers, the first demonstrated application of the allelic ratio approach was for an SNP in the expressed region of *PLAC4* *(34)*. This approach is
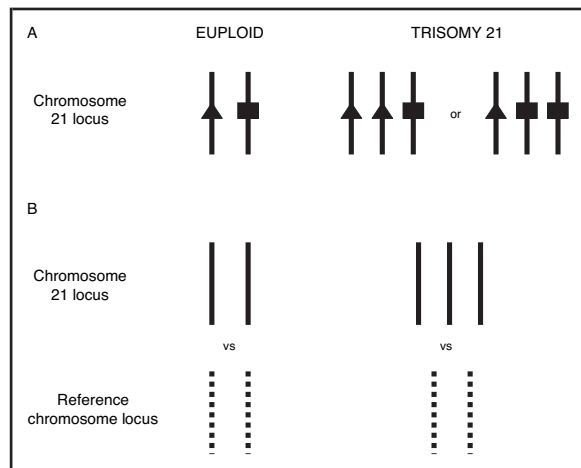

Fig. 2. Approaches for determining fetal chromosome dosage in cell-free nucleic acids in maternal plasma using trisomy 21 as an example.

(A), allelic ratio analysis involves the assessment of the ratio between alleles at a heterozygous locus located on chromosome 21. The allelic ratio for such a locus would be expected to be 1:1 for a euploid fetus but 2:1 or 1:2 for a trisomic fetus. (B), relative chromosome dosage analysis involves the assessment of the ratio between a chromosome 21 locus and a nonchromosome 21 reference locus. The ratio among fetal-derived nucleic acid molecules would be expected to be 2:2 for a euploid fetus but 3:2 for a trisomic fetus.

called the RNA-SNP approach. This first series using the RNA-SNP approach established a sensitivity of 90% and a specificity of 96.5% for the detection of fetal trisomy 21 from maternal plasma *(34)*. These results suggest that for informative (i.e., heterozygous) cases, the RNA-SNP approach is the most accurate single-marker approach for the noninvasive prenatal detection of fetal trisomy 21.

Approaches for improving the diagnostic sensitivity and specificity of the RNA-SNP approach may be on the horizon. It has been shown that the robust measurement of the RNA-SNP allelic ratio requires at least 1000 molecules per reaction *(34)*. It has recently been shown that through the use of digital PCR, it is possible to reduce the number of input molecules *(36)*. Digital PCR is a method for molecular analysis in which multiple PCRs are carried out on a sample diluted to a concentration such that on average each reaction will contain ≤ 1 target molecule *(37)*. In this manner, a proportion of the reactions will be negative, owing to the absence of the target molecule in a given reaction. For the subset of the reactions giving a positive detec-

tion result, most of the reactions will be positive because of the presence of a single target molecule. By use of this method, the number of target molecules can be accurately counted. In the context of the digital version of RNA-SNP allelic ratio analysis, the number of each RNA allele will be counted. Fetal aneuploidy can thus be detected through the use of appropriate statistical analysis to determine the probability that one of the alleles is overrepresented, i.e., when trisomy is present (36). With this digital PCR-based approach fetal aneuploidy status can be determined very accurately with just a few hundred molecules, opening the possibility for the application of this assay method even very early on in gestation, at a time when the circulating *PLAC4* mRNA concentration in maternal plasma is relatively low (34). The main disadvantage of digital PCR-based techniques is their requirement for the performance of multiple PCR analyses per tested sample, a process that is tedious if carried out manually. However, with the development of automated strategies for digital PCR analysis, such as microfluidics (38), variants of emulsion PCR (39, 40), and various approaches for massively parallel sequence analysis (41), it is likely that such a system will eventually be practical for use in routine diagnosis.

RELATIVE CHROMOSOME DOSAGE ANALYSIS

The main disadvantage of the allelic ratio–based approach is the requirement that the fetus be heterozygous for the analyzed genetic polymorphisms. To overcome the requirement for heterozygosity, methods that directly measure the relative dose of different chromosomes are required. However, conventional methods for direct measurement of chromosome dose [e.g., real-time PCR (42) and paralogous sequence quantification (43)] cannot be directly used, because of the low fractional concentration of circulating fetal DNA. One potential solution to this limitation is to combine such conventional methods for relative chromosome dose analysis with methods for the physical [e.g., size fractionation of plasma DNA (7) and suppression of maternal DNA background (44)] or molecular enrichment of fetal DNA [e.g., fetus-specific DNA methylation (23, 24)] as discussed earlier in this report.

An alternative solution is the development of analytical strategies that can perform relative chromosome analysis despite the low fractional concentration of circulating fetal DNA in maternal plasma. In this regard, the digital PCR-based approach, which has previously been discussed in this review in the context of allelic ratio analysis, can also be applied for chromosome dose analysis, the so-called digital relative chromosome dose approach (36). In this method, digital PCR analysis is carried out for 1 or more targets located on a chromosome involved in a trisomy (e.g., chromosome 21) and for 1 or more targets located on a reference chromosome not involved in the trisomy. Through the use of appropriate statistical procedures for testing the presence of overrepresentation of reactions involving the potentially aneuploid chromosome, digital relative chromosome dose measurement has been demonstrated to possess the discrimination power to detect the presence of DNA from a trisomic fetus, even if the trisomic DNA constitutes only some 10% of the tested sample (36). It is envisioned that the digital relative chromosome dosage approach can also be combined with the physical (7, 12) and molecular methods (11, 23, 24) for fetal DNA enrichment described above. The superior discrimination power of digital PCR can be expected to allow an aneuploidy detection system to be based on a degree of fetal DNA enrichment that would otherwise be insufficient for conventional nondigital PCR systems.

Conclusions

Following a decade of development, it has been demonstrated that noninvasive prenatal detection of fetal chromosomal aneuploidies can be achieved by analysis of cell-free fetal nucleic acids in maternal plasma. Thus far, feasibility studies have been carried out using a relatively small sample set. Over the next few years, larger-scale independent studies are needed to validate these initial observations. Further development of new fetal DNA/RNA markers and new analytical methods is expected to allow molecular noninvasive prenatal diagnosis of the major chromosomal aneuploidies to become a routinely practiced reality in the near future. Such a development would ultimately make prenatal testing safer and less stressful for the millions of pregnant women each year who undergo such testing.

Review

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