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COMMENTARY

A Proof-of-Concept Case Study for Personalized Noninvasive Prenatal Diagnosis

Can We Put It to Work?

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In this issue of *The Journal of Molecular Diagnostics*, van den Oever et al[1] present an alternative method of noninvasive prenatal diagnosis (NIPD) for monogenic disorders using maternal plasma samples. This approach uses locked nucleic acid (LNA) technology to block amplification of maternal sequences and high-resolution melting curve analysis (HR-MCA) to identify nonmaternal alleles.

The ability to diagnose monogenic disorders during the prenatal period using maternal plasma is an extremely appealing possibility for families and physicians alike, promising early diagnosis for devastating genetic conditions and providing information that can be useful in medical decision-making, planning, and perhaps even early intervention.

The recent increase in demand for prenatal testing of common aneuploidies and even microdeletions and duplications using cell-free trophoblastic DNA in maternal plasma has revealed the utility and pitfalls of using this sample source. The primary issue is the overwhelming excess of maternal DNA in the maternal plasma compared with nonmaternal (ie, paternal or fetal) DNA, making it difficult to detect variants present at low levels.

Massively parallel sequencing (MPS) has typically been the method of choice for detecting low-level variants in many laboratories; however, it is out of reach for other laboratories and is not necessarily the most efficient approach in detecting known familial mutations.[2] van den Oever et al[1] describe an alternative method of diagnosing single-gene disorders prenatally using preferential amplification of the nonmaternal allele and analysis by HR-MCA. Furthermore, they present two cases as proof-of-concept for this method in either autosomal dominant or recessive conditions.

Prenatal genetic diagnosis has, until recently, been limited to testing that could be performed on amniotic fluid or chorionic villi. Indications for such testing include advanced maternal age, positive serum screening, a previous affected child, a parental chromosome rearrangement, or an ultrasonography-identified anomaly. A variety of diagnostic tests can be performed on these samples, including fetal karyotyping, chromosomal microarray, fluorescence *in situ* hybridization, and even DNA-based molecular testing for inherited disorders for which there is a family history. However, both of these approaches involve invasive procedures that pose inherent risks to the fetus. Amniocentesis involves sampling the amniotic fluid and culturing the amniocytes for further testing or isolating DNA from the amniotic fluid directly. Although rare, complications from

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the procedure include vaginal bleeding, membrane rupture, chorioamnionitis, and fetal loss. Although these complications also occur in pregnant women not undergoing amniocentesis, there is an increased risk in pregnant women undergoing the procedure. Patients are counseled that the additional risk of spontaneous abortion from amniocentesis is 0.5% to 1%. Improvements in the sampling procedure and amniocyte culturing techniques have made it possible to perform amniocentesis before 14 weeks' gestational age. However, early amniocentesis poses a significantly increased risk of fetal deformities and pregnancy loss.[3–5] Studies on the risks of chorionic villus sampling (CVS) reveal a miscarriage rate of 2% to 3% between the date of the procedure to 28 weeks' gestation at experienced centers. In addition, CVS poses an increased risk of procedure-induced limb defects. The incidence of severe limb reduction defects could be as high as 1% to 2% in sampling performed before 10 weeks' gestational age[6]; however, this risk is greatly reduced when CVS is performed after 70 days of gestation, with minimal to no risk.[4–6] Amniocentesis and CVS remain the gold standard for prenatal diagnosis; however, many women decide not to undergo invasive testing because of the associated discomfort, the small but significant risk of miscarriage, or the fact that they would not terminate the pregnancy regardless of the results.

Current Approaches to NIPD

Alternative methods for NIPD have long been sought to reduce this risk of miscarriage and allow earlier testing. The discovery of the presence of fetal cells in maternal circulation and cervical mucus occurred more than a century ago but yielded few clinical applications that have primarily been limited to fetal sex determination, Rh factor diagnosis, and detection of aneuploidy.[7–9] More recent approaches to NIPD have focused on fetal DNA (and also RNA) present in the maternal blood.[10,11] This so-called cell-free fetal DNA (cffDNA) present in the maternal serum can be used as a source of DNA for diagnostic testing. In 1948, Mandel and Metais[12] discovered the presence of small amounts of extracellular DNA in the circulation of both healthy and diseased subjects. Leon et al[13] found an increased concentration of circulating cell-free DNA in the serum of patients with cancer. Twenty years later, Lo et al[14] found the presence of fetal DNA in the maternal circulation. Fetal DNA is produced from apoptotic placental cells (trophoblasts)[15,16] and comprises approximately 3% to 6% of the total cell-free DNA in maternal circulation.[10] Unlike cellular DNA, circulating cffDNA consists predominantly of short DNA fragments (<193 bp).[17] The concentration of fetal DNA increases throughout the pregnancy and can be detected reliably as early as 7 weeks' gestational age.[18]

Because cffDNA represents only a small fraction of the circulating DNA, methods with high levels of sensitivity are required to detect low-level variants. Enrichment for fetal DNA has been achieved by size-based selection, suppression of maternal DNA, and the use of fetal DNA markers, such as single-nucleotide variants (SNVs) or short tandem repeats. This enriched population of nucleic acids can be used in sensitive assays, such PCR-based assays, mass spectrometry, and MPS. Clinical applications of cffDNA testing have included sex determination, pregnancy-related disorders (specifically Rh factor status), aneuploidy, and even monogenic disorders. Applying these same techniques to the diagnosis of monogenic disorders is still in its infancy and is currently limited to paternally inherited dominant disorders or autosomal recessive disorders in which the mother and father carry different variants. The following monogenic disorders have been detected using cffDNA[8]: Huntington disease [autosomal dominant (AD), *HD*]; achondroplasia and thanatophoric dysplasia (AD, *FGFR3*); myotonic dystrophy (AD, *DMPK*); MEN2A (AD, *RET*); cystic fibrosis [autosomal recessive (AR), *CFTR*]; hemoglobinopathies, including sickle cell disease and hydrops fetalis (AR, various globin genes); and congenital adrenal hyperplasia (AR, *CYP21*).

Advantages of the LNA/HR-MCA Method

van den Oever et al[1] describe a method for detecting non-maternal alleles in maternal plasma that involves three basic steps. First, the parental alleles are characterized and allele-specific probes are designed. Second, DNA extracted from the maternal plasma is amplified by PCR using LNA probes targeted to the maternal allele. Third, the PCR products are analyzed using HR-MCA to detect the presence or absence of a paternal allele. This method enables detection of low-level variants within the maternal plasma and is ideal for diagnostic testing for known familial mutations or even for a panel of well-characterized mutations (eg, mutations common to a particular ethnic group). This approach might also be useful in cancer detection and for assessment of minimal residual disease, allowing for sensitive detection of SNV common to a particular tumor type. Furthermore, this method offers laboratories an alternative to MPS, which is not the most cost-effective approach for analyzing an SNV despite its ability to provide great depth of coverage. Additional advantages of this method include the following: avoidance of detection of variants of uncertain significance because the testing is mutation specific, simplification of interpretation of heterozygous variants because the phenotype can be inferred from the heterozygous carrier parent given that they have had a complete clinical evaluation, and reduction of the possibility that low-level variants detected in the maternal plasma DNA are from confounding nonfetal sources (eg, maternal tumors or allogeneic transplants).

Limitations

Despite these advantages, the method described by van den Oever et al[1] has some limitations that must be considered. Logistically, this assay requires a tremendous amount of care in probe design and optimization for reliable results. Furthermore, the assay hinges on the ability to differentiate