REVIEW ARTICLE

Noninvasive Prenatal Testing Using Cell-free DNA in Maternal Circulation

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Abstract Noninvasive prenatal testing (NIPT) is revolutionizing prenatal screening and diagnosis. Through the analysis of cell free DNA in maternal plasma, it is possible to screen for trisomies 21, 18, and 13, sex chromosome aneuploidies, triploidy and some microdeletion syndromes. For aneuploidies the sensitivity and specificity approaches, but does not meet, that of diagnostic testing. It is estimated that the positive predictive value for NIPT for the identification of trisomy 21 is approximately 90 % compared to 4–6 % for conventional prenatal screening tests. Depending on the NIPT technology used, discordancy between NIPT and the true fetal karyotype can be due to low fetal DNA concentration, presence of a maternal chromosome abnormality, undetected vanishing twin, or mosaicism (including apparent confined placental mosaicism). NIPT can be expanded to include additional cytogenetic abnormalities such as other small copy number variations and will be increasingly used for monogenic disorders. The testing poses a number of ethical challenges including potentially increased pregnancy termination rates for affected pregnancies, the detection of milder or late onset disorders, and identification of fetal sex. Developing nations such as India have very high rates of birth defects and advanced molecular genetic technologies such as NIPT are needed to help reduce neonatal morbidity and mortality. It is important that the implementation of the Pre-Conception and Pre-Natal Diagnostic Techniques Act that prohibits disclosure of fetal sex does not prevent access to this extraordinarily powerful new technology that offers substantial benefits to the Indian population.

Keywords Prenatal · Ultrasound · Screening · Diagnosis · Trisomy 21 · Aneuploidy

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Introduction

Noninvasive prenatal testing (NIPT) for fetal aneuploidy was first introduced into China in early 2011, and the USA in October, 2011. Uptake was unprecedented; it is likely that in the USA alone in excess of 500,000 NIPT studies were performed in 2013. Although not fully diagnostic, the testing has substantially closed the gap between the performance of conventional screening tests (maternal serum markers and ultrasound) and diagnostic testing (karyotyping or chromosome microarray on chorionic villus samples (CVS) or amniotic fluid cells). Currently, NIPT is available for the detection of the common autosomal trisomies seen at birth (21, 18 and 13), sex chromosome aneuploidies, triploidy and some microdeletion syndromes. The advantage of NIPT is clear; by providing more effective prenatal screening, the number of invasive tests is reduced and therefore the number of procedure-related pregnancy losses is minimized.

Technology

NIPT for fetal aneuploidies is based on the analysis of "fetal" cell-free DNA (cf-DNA) in maternal plasma. First discovered in 1997, cf-DNA consists of short sequences approximately
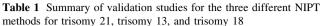
150 base-pairs in length [1, 2]. By 9–10 weeks of pregnancy, on average 10 % of the cf-DNA is derived from apoptotic trophoblasts (loosely described as "fetal"), the remainder being maternal in origin [3]. The fetal component of cf-DNA does not persist in the maternal circulation from one pregnancy to the next and therefore testing of this material should accurately reflect a current pregnancy. Maternal plasma also contains an abundance of fetal RNA [4].

Although a broad range of strategies have been proposed to diagnose fetal aneuploidy taking advantage of fetal nucleic acids in maternal circulation, current methods now in clinical practice are based on the quantification or properties of cf-DNA. One approach, shotgun massively parallel sequencing (s-MPS) is based on sequencing and counting large numbers of unique (single locus) DNA fragments in the plasma and assigning them to the chromosome from which they originated [5, 6]. A relative excess, or deficiency, of DNA sequences from any one particular chromosome compared to that expected provides evidence for trisomy, or monosomy, respectively. Large numbers of fragments need to be counted because the difference between aneuploidy and euploidy will be small especially when the fetal fraction is low [7]. A variation on this method, referred to as targeted massively parallel sequencing (t-MPS), includes an enrichment of the plasma-derived DNA for the chromosomes of interest (e.g., chromosomes 21, 18, 13, X, and Y) before sequencing [8]. The advantage of the enrichment is that it allows counting of higher numbers of sequences for the chromosomes of interest and/or reducing sequencing costs.

Another approach relies on analyzing single nucleotide polymorphisms (SNPs) and determining the relative contributions of maternal and fetal DNA in the plasma. A multiplex PCR amplification on the plasma DNA involving nearly 20,000 SNP sequences in a single reaction is carried out followed by sequencing to determine which SNPs are present and their relative amounts [9, 10]. Each product is evaluated based on the hypothesis that the fetus is monosomic, disomic, or trisomic. After considering the positions of the SNPs on the chromosomes and the possibility that there may have been recombination, a maximum likelihood is calculated that the DNA is from a pregnancy that is normal, aneuploid or triploid. The method can provide information about the presence of consanguinity or uniparental disomy. For SNP-based NIPT, a paternal blood or saliva sample can improve test success rate but this is not essential.

Efficacy of NIPT

Table 1 summarizes a meta-analysis of clinical validation studies for NIPT for fetal trisomy 21, 18, and 13. For all methods combined, for these three trisomies the overall

Table 1 Summary of validation studies for the three different NIPT methods for trisomy 21, trisomy 13, and trisomy 18

Method	Trisomy	Detection rate (%)	False positive rate (%)
s-MPS	21	515/520 (99.0)	13/6,881 (0.19)
	18	120/123 (97.6)	10/6,665 (0.15)
	13	33/37 (89.2)	18/5,695 (0.32)
	21, 18, & 13	668/680 (98.2)	41/19,241 (0.21)
t-MPS	21	167/168 (99.4)	1/6,840 (0.01)
	18	93/95 (97.9)	5/6,350 (0.08)
	13	9/11 (81.8)	2/3,179 (0.06)
	21, 18, & 13	269/274 (98.2)	8/16,369 (0.05)
SNP-based	21	83/83 (100)	0/1,104 (0.00)
	18	27/28 (96.4)	1/1,167 (0.09)
	13	13/13 (100)	0/1,181 (0.00)
	21, 18, & 13	123/124 (99.2)	1/3,452 (0.03)

Based on meta-analysis data. Individual study results and references are summarized elsewhere [11]

detection rate was 98.3 % and the false-positive rate 0.13 % [11]. Table 2 compares NIPT with conventional screening for the identification of trisomy 21. For illustrative purposes, data is presented for a population referred for testing with a trisomy 21 prevalence of 1/100 (high risk) and 1/500 (low risk). The positive predictive value for both high- and low-risk populations is dramatically higher for NIPT compared with the best of the conventional approaches. However, even for a population with high a priori risks, it should be recognized that approximately one in ten referrals will be a false-positive. Positive predictive values are lower for the other aneuploidies because the prevalences are lower and/or the test performance is lower. Confirmation of positive results is essential regardless of the a priori risk and the NIPT result.

All methods for NIPT do require that there is sufficient fetal DNA in the maternal plasma specimen for analysis and most laboratories do not provide results if the fetal fraction is less than a specific level, typically 4 %. Fetal digynic triploidy, trisomy 18, and trisomy 13 are associated with a small placental volume [13] and consistent with this cf-DNA, fetal fraction is low in cases of fetal trisomies 18 and 13, digynic triploidy and also monosomy X [14–16]. Inability to obtain a result due to a low fetal fraction may therefore be associated with a somewhat increased risk for these fetal karyotypes.

Reasons for Discordant Results

Methods based on counting the number of DNA fragments present (s-MPS and t-MPS based testing) require a high fetal fraction or deeper sequencing to obtain clear distinction between affected and unaffected pregnancies [7]. For

Table 2 Comparison of the detection rates, false-positive rates, and positive predictive values (PPV) for Down syndrome screening using conventional approaches (combined, quad, and sequential) and NIPT

Test	Detection rate (%)	False positive rate (%)	PPV high-risk population (1/100) (%)	PPV low-risk population (1/500) (%)
Combined (NT, PAPPA, hCG)	80	3	21	5
Quad (AFP, uE3, hCG, INH-A)	60	3	17	4
Sequential (combined & quad)	93	3	24	6
NIPT (composite of all methods)	99.3	0.1	91	67

Conventional screening based on efficacy assessments in ref. [12]

NIPT efficacy based on data in Table 1

example, Allen et al. [17] described a trisomy 21 NIPT false-negative result that appeared to be attributable to low fetal fraction. Since counting methods do not distinguish between fetal and maternal DNA fragments, a cytogenetic abnormality that is present in the mother can be misinterpreted as a fetal karyotype abnormality. This is particularly important for X-chromosome testing because somatic cell variation in X-chromosome copy number is a common age-related finding in adult female lymphocytes and some women may have a true constitutional maternal X-chromosome aneuploidy mosaicism present in multiple tissues. Wang et al. [18] reported evidence for maternal X-chromosome abnormality in 16 of 181 (8.6 %) cases with an NIPT result positive for a sex chromosome aneuploidy (SCA). DNA reflecting other maternal cytogenetic abnormalities is also possible including the presence of DNA derived from a malignancy [19]. Another source for potential mis-interpretation can arise if there is an undetected vanishing twin that has a karyotype discordant to that of the surviving twin [20]. The SNP-based method for NIPT should potentially distinguish maternal aneuploidy or vanishing twin discordancy because it analyzes allele polymorphisms that can be specifically attributed to the mother and fetus.

All NIPT methods are susceptible to failure to detect fetal or placental mosaicism. This includes apparent confined placental mosaicism in which a cell line appears to be substantially restricted to the placental cell lineages and not the fetus. There have been numerous instances of this described and this can potentially cause both false-positive and false-negative NIPT results [21]. In some instances, an abnormal NIPT result has been confirmed in a subsequent analysis of CVS or placental tissue but not in amniotic fluid cells or in the liveborn infant. This observation raises questions about the suitability of CVS as a confirmatory test following a positive NIPT result.

Additional Chromosomal Imbalances Detectable by NIPT

NIPT analyses involving X- and Y-chromosomes will also identify other sex chromosome abnormalities in addition to

45, X. Specifically, XXX, XXY and XYY will be found. Robust estimates for the detection rates and false-positive rates for these karyotypes are not available [11]. SNP-based NIPT will detect diandric triploidy [15]. In theory, NIPT could be extended to any aneuploidy. One provider of NIPT offers testing for trisomy 16 and 22 but the value of this is questionable because first trimester pregnancies with these karyotypes will mostly be non-mosaic and will spontaneously abort while second trimester cases are mosaic and are associated with a highly variable outcome [22]. It is also possible to expand NIPT to other very rare aneuploidies that are generally only encountered in a mosaic state with normal cells [23]. This includes identification of situations where there is a risk for a clinically significant uniparental disomy (chromosomes 6, 7, 11, 14, 15, and 20) following correction of trisomy to disomy. One NIPT laboratory offers this approach [23, Conrad, B. Genesupport, Switzerland, unpublished]. However, the number of such cases that might be identified needs to be considered in the context of the increased false-positive rates as well as the considerable clinical uncertainty associated with detection of mosaicism for these chromosomes.

NIPT can also be applied to microdeletion and microduplication syndromes. Detecting these reliably by counting methods appears to be limited by the need for deeper sequencing [7]. However, proof-of-principle studies have been carried out and clinically significant small imbalances have been detected [24]. Microdeletions can also be detected using SNP-based NIPT where absence of paternal or maternal alleles provides the basis for an extremely valuable screening test [25].

Monogenic Disorders

NIPT can be used for the detection of fetal blood group antigens, notably Rhesus-D genotyping, to avoid fetal hemolytic disease [26]. The cf-DNA is tested for the presence or absence of the paternally derived DNA sequence that confers the Rh positive allele. Similarly, for autosomal dominant disorders carried by a father, cf-DNA can be

analyzed to determine if the mutation (or a closely-linked polymorphism) is present. This approach has been used in the diagnosis of Huntington's disease, myotonic dystrophy and early onset primary dystonia I [11]. There are also autosomal dominant disorders such as achondroplasia and thanotrophic dysplasia where highly specific mutations arise de novo, and where cf-DNA can be analyzed once the disorder is suspected based on ultrasound findings.

Although technically more challenging, it is also possible to provide testing for mutations that are maternally inherited, including autosomal recessive disorders where both mother and father are carriers of an identical mutation. The approach has recently been illustrated in a prenatal diagnosis of a fetus with the autosomal disorder methyl-malonic acidemia [27] where the mother and father were both carriers for the same mutation. Digital PCR droplet technology was used to quantify the fetal DNA fraction (using counts of paternally inherited SNPs) and also to count the mutation and wild type DNA fragments. A generalized approach has been proposed that could identify any genetic disorder through the detection of paternally inherited mutations and SNPs in combination with assessments of the relative dosage of maternally inherited mutations and linked SNPs in maternal plasma [28]. A logical extension of this (although currently impractical) would be to construct essentially a full map of the fetal genome through noninvasive methods [29].

Ethical and Social Considerations

The introduction of NIPT offers the possibility of greatly increased ability to prenatally detect a broad range of genetic abnormalities and conditions. Concerns include the possibility that there will be many more terminations of affective pregnancies which would lead to a greater emphasis on prevention and less on the accommodation of those with handicaps [30]. Much of the new technology is in the hands of commercial companies and the commercial promotion of testing implicitly emphasizes the desirability of testing and diagnosis. The potential to detect milder disorders, paternity, late onset conditions, and the protection of the future child from physiological damage and the "right not to know" are important additional considerations.

Special Considerations for India

India is the world's second most populous nation and its population is expected to exceed that of China by 2010. There are 2.7 million births per year in India and the birth defect rate is thought to be 6–7 %. This high rate can be attributed to poor maternal nutrition, lack of early antenatal

care, consanguinity, high rates of carriers for hemoglobinopathy mutations, and environmental factors [31]. Improved nutrition, prenatal care, patient education, and the application of advanced molecular genetic tests such as NIPT are all needed if neonatal morbidity and mortality are to be reduced.

An important aspect of NIPT for India is the fact that its application for the detection of sex chromosome aneuploidy necessarily identifies fetal sex. It is extremely important that the implementation of the Pre-Conception and Pre-Natal Diagnostic Techniques (PCPNDT) Act that prohibits disclosure of fetal sex does not prevent access to this extraordinarily powerful new technology that offers substantial benefits to the Indian population.

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