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Advantages of the single nucleotide polymorphism-based noninvasive prenatal test

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Down syndrome screening with cell-free DNA (cfDNA) in the maternal plasma has recently received much attention in the prenatal diagnostic field. Indeed, a large amount of evidence has already accumulated to show that screening tests with cfDNA are more sensitive and specific than conventional maternal serum and/or ultrasound screening. Globally, more than 1,000,000 of these noninvasive prenatal tests (NIPTs) have been performed to date. There are several different methods for NIPTs that are currently commercially available, including shotgun massively parallel sequencing, targeted massively parallel sequencing, and single nucleotide polymorphism (SNP)-based methods. All of these methods have their own advantages and disadvantages. In this review, I will focus specifically on the SNP-based NIPT.

Key words: Prenatal diagnosis, Single nucleotide polymorphism, High-throughput nucleotide sequencing, Noninvasive prenatal test, Cell free DNA.

Introduction

The past 20 years has seen remarkable development in the field of prenatal diagnosis, especially with respect to screening for trisomy 21 (Down syndrome). Despite advances in screening tests, the low positive predictive value (PPV <5%) of the conventional screening test (i.e., use of maternal serum analytes and ultrasound markers, including nuchal translucency) is problematic [1,2]. The low PPV is associated with the slew of unnecessary invasive tests performed, including amniocentesis and chorionic villi sampling, which carry a small but significant risk of fetal loss. In 1997, Lo et al. [3] reported the presence of a DNA fragment from the Y-chromosome in maternal plasma, which was found to be derived from pregnant women carrying a male fetus. This fetal cell-free DNA (cfDNA) is thought to originate from the placental trophoblast and then cross from the placenta into the maternal circulation as the cells break down (apoptosis) and the DNA becomes fragmented [4,5]. Circulating fetal cfDNA comprises approximately 3-13% of the total maternal cfDNA. These DNA fragments can be detected as early as 4 weeks of gestation and are generally cleared out within 2 hours after childbirth [6-8]. A noninvasive prenatal test (NIPT) using cfDNA has proven to be highly sensitive and specific for detecting trisomy 21 in both high- and low-risk groups [1,2,9,10]. At present, there are about five major companies providing commercial NIPT services globally (Fig. 1). NIPT using cfDNA is now available through these providers in more than 60 countries. According to business reports, over 500,000 NIPT studies on women with a high risk for fetal aneuploidy were performed in the United States in 2013 [11]. These companies have published several articles showing that their own method is very effective for screening for trisomy 21, 18, and 13

[1,2,10,12-18]. However, there has been no well-controlled, large-scale study comparing the products of all 5 companies in terms of test accuracy and efficiency. In this article, I review the differences and advantages of single nucleotide polymorphism (SNP)-based NIPTs in comparison to other methods.

Fig. 1. Classification of different noninvasive prenatal test (NIPT) methods. cfDNA, cell-free DNA; S-MPS, shotgun massively parallel sequencing; T-MPS, targeted massively parallel sequencing; SNP, single nucleotide polymorphism. Sequenom: San Diego, USA; Illumina: San Diego, USA; BGI: Beijing Genomics Institute, Shenzhen, China; Ariosa: San Jose, USA; Natera: San Carlos, USA.

Counting Methods of the NIPT Using cfDNA [11]

1. Shotgun massively parallel sequencing

After extraction and amplification of whole cfDNA from the maternal plasma, shotgun massively parallel sequencing (S-MPS) is conducted based on the sequencing and subsequent counting of large numbers of DNA fragments in the plasma while allocating them to their chromosome of origin. With this method, evidence of aneuploidy is obvious when there is a relative excess (trisomy) or deficit (monosomy) in any particular chromosome of interest compared to the number expected.

2. Targeted massively parallel sequencing

Targeted massively parallel sequencing (T-MPS) is similar to S-MPS but includes an extra step that selectively amplifies only the chromosomal regions of interest (for example, chromosomes 21, 18, and 13), and then calculates whether there is an excess for one particular chromosome relative to another. A benefit of the T-MPS methodology is the lower sequencing cost because this method avoids having to sequence all regions.
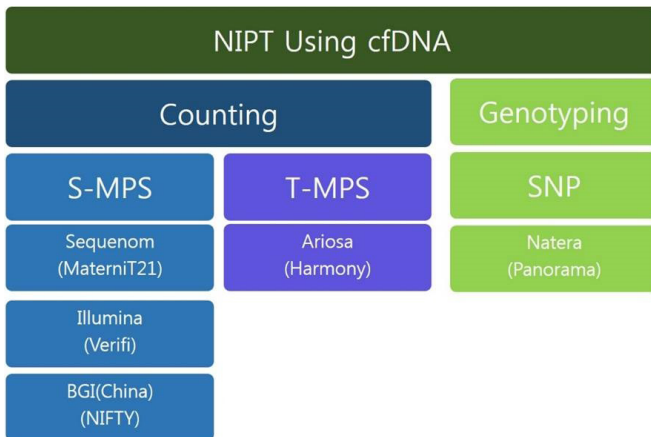
Principle of the SNP–based NIPT [19,20]

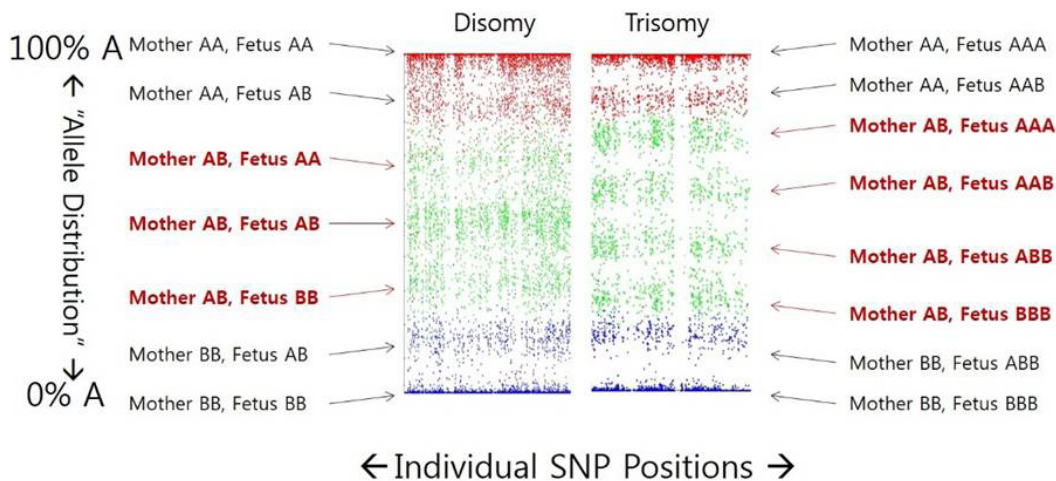SNPs are normal genetic variations that can be used to

Fig. 2. Simplified principle of the single nucleotide polymorphism (SNP)-based noninvasive prenatal test. Each dot represents one SNP and the sum of both the maternal and fetal contribution. SNPs are biallelic and thus have only 1 of 2 DNA bases possible (designated as A and B). There is only one SNP per location going from left to right (as though the chromosome is lying on its side), and there is a total of ~3,000 SNPs per chromosome (this could be visualized if the image were spread out). The top of the profile represents a situation of 100% A and 0% B. Continuing down the profile, the frequency of A decreases and that of B increases until reaching the case of 100% B and 0% A at the bottom. SNPs in red represent those for which the mother's genotype is AA. The top line is the case in which the baby is also AA (thus, the case of 100% A). The second line shows the case of 75% A and 25% B (the baby genotype is AB). Green SNPs are those for which the mother's genotype is AB. Again, the percentage of B increases from the first to the third line. Blue SNPs are those for which the mother's genotype is BB. Again, the percentage of B increases until the final line, where both the baby and mother are 100% B. When the mother's SNP is heterozygous (green SNPs), an extra band appears when the fetus is trisomic. However, the band positions are dependent on the allele frequency and fetal fraction. When the mother's SNP is homozygous (red and blue SNPs), one band is shifted when the fetus is trisomic. The extent of this shift is dependent on the allele frequency and fetal fraction. Not all SNP profile results are visually conclusive, which is why an algorithm is needed for interpretation [21].

distinguish between any two individuals. Applying SNP analysis to the NIPT helps to determine the difference between parent and child DNA as well as variations in copy number (Fig. 2) [21]. The currently available SNP-based NIPT evaluates 19,488 SNPs and then determines the relative quantitative contributions of maternal and fetal DNA in the maternal plasma. Multiplex polymerase chain reaction amplification of plasma DNA (comprising a mixture of maternal and fetal DNA) and the buffy coat (only maternal DNA) for SNP sequences is followed by direct sequencing to identify which of the amplified products are present. Given that a mother passes on one of each chromosome pair to her children, the chromosomal condition can be classified as monosomy (only one copy from the mother or the father), normal (one copy from each parent), trisomy (an extra copy from either parent), or uniparental disomy (UPD, both copies received from the mother or the father). The Next-generation Aneuploidy Test Using SNPs (NATUS) algorithm uses the sequencing data from the mother and father (note that an optional paternal buccal mucosa swab for SNP analysis can improve the reporting rate but does not affect accuracy). This test also incorporates data from the Human Genome Project, which can reveal where crossovers are most likely to occur, since these are region-specific events. Multiple hypotheses for a chromosome of interest are possible: monosomy (a copy from either parent), disomy (one copy from each parent or UPD), or trisomy (with the extra chromosome being either maternal or paternal in origin). Then, millions of sub-hypotheses are established based on all of the potential crossover points. The fetal/maternal DNA pattern is compared to the expectations of each of these sub-hypotheses to find the closest match using a Bayesian-based maximum likelihood statistical method.

Advantages of the SNP-based NIPT

1. Less dependency on the fetal fraction

The fetal fraction represents the proportion of cfDNA in the maternal plasma that is of fetal origin [22], and is one of the most important components of the NIPT. The fetal fraction varies among individuals and factors; for example, it tends to be lower earlier in pregnancy, and can vary with maternal weight [23,24]. In the counting methods of the NIPT, the test accuracy is highly dependent on the fetal fraction. If the fetal fraction in maternal plasma is less than 8%, the detection rate of trisomy 21 is about 75% [25,26]. Thus, the main cause of a false-negative result with the counting methods is a low fetal fraction. Although the fetal fraction is the most critical part of an NIPT,

similar to cell counting in fluorescence *in situ* hybridization, most of the counting-based NIPTs, except for the Harmony™ test (Ariosa Diagnostics, Inc., San Jose, CA, USA), do not report the fetal fraction. By contrast, the SNP-based NIPT is less dependent on the fetal fraction. Indeed, the rate of trisomy 21 detection is not reduced even if the fetal fraction is 4-8%. Given that approximately 1 out 3 commercial samples have a fetal fraction less than 8%, this distinction is important. The counting methods tend to produce incorrect results from samples with a very low fetal fraction (<4%), whereas the SNP-based method tends to make no call in such cases. No call is preferred to a false-negative result, as a no-call result simply requires a redraw and retest, whereas a miscall can result in serious problems and missed diagnoses.

2. Reduced effect of GC amplification bias

GC pairs have 3 bonds between them, whereas A/T pairs only have 2 bonds. When there are fewer or more GC pairs than average, the chromosomes do not amplify in the expected manner during the amplification/sequencing process. The reason is that a variable frequency of the GC pairs can create more or fewer hairpin loops in the DNA. Thus, when the DNA polymerase attempts to bind to the sequence for amplification, it is prevented from binding at the regions with hairpin loops. This is problematic for the counting method, which relies on comparison to reference chromosomes, and therefore any variation in amplification reduces the detection rates. Chromosome 21 contains the optimum median amount of GC pairs. The GC content of chromosome 18 is lower than but similar to that of chromosome 21. Nevertheless, both of these chromosomes tend to amplify close to the expected amount of DNA. By contrast, chromosome 13 has the second-lowest GC content in the genome (after chromosome 4, and with a similar amount as the X chromosome) [27]. Consequently, amplification is variable, thus throwing off the ratios from expectations by amplifying either more or less than the true amount of chromosome 13. This situation decreases the sensitivity of detection for trisomy 13 or monosomy X compared to that for trisomy 21 or trisomy 18 with the counting method [25,28]. However, the SNP-based method is not affected by this amplification bias, since it does not require comparison to a reference chromosome; thus, the results are not dependent on the amount of DNA in a chromosome but rather on the specific sequence of the DNA [13,19].

3. Effective differentiation between maternal and fetal cfDNA

The SNP method can readily distinguish between maternal and fetal free-floating DNA by analyzing the buffy coat that contains purely maternal DNA. This allows for establishment of a maternal-only SNP profile. In contrast, the counting method amplifies and sequences both the maternal and fetal DNA, without differentiating between them. This distinction is particularly important for conditions such as monosomy X; for example, when it is difficult to determine whether the mother is a mosaic for XO. X chromosome loss (XCL) is dependent on maternal age; XCL is present in less than 1% of women below the age of 25, and the rate of incidence increases with age after this point in a logistic quadratic manner [29]. Therefore, in such cases, the counting method could yield a false-positive result. One study demonstrated that 8.56% of the positive results for sex chromosome aneuploidies determined with the counting method were revealed to in fact be false positives due to maternal mosaicism [30]. Therefore, without analyzing maternal DNA separately, the counting methods may conclude a fetal sex anomaly. The SNP method analyzes the specific maternal DNA contribution, which helps to decrease the rate of false positives related to maternal mosaicism and copy number variants [31].

4. Detects a vanishing twin

Although cfDNA generally goes away within 2 hours after delivery, if a fetus remains in the uterus due to co-twin demise, apoptosis of the placenta occurs, which releases cfDNA from the vanishing twin into the maternal circulation. Even up to 8 weeks post-demise, this vanishing twin DNA can be detected and potentially lead to false-positive results with the counting method. The SNP method can detect the possibility of vanishing twin/triploidy, which would alert the doctor to perform an ultrasound. Studies have indicated that presence of a vanishing twin is one of the major causes of false-positive results given that the demised twin is more likely to be chromosomally abnormal. One study showed that more than 15% of the false-positive results were associated with the presence of a vanishing twin [15]. The SNP method is the only method that can pick up this additional haplotype, and thus prevent false positives [32].

5. Detects triploidy

One major disadvantage of the counting method is that it cannot detect triploidy. Two studies with the counting method confirmed that 8 cases of triploidy were called as 'normal', and thus had a 100% false negative rate [15,28]. As mentioned above, the SNP method does not require comparison to a reference chromosome. Indeed, it was the only test to correctly identify 4/4 cases of paternal triploidy in two blinded studies [33]. Even though most cases of triploidy will miscarry spontaneously, the incidence is 1/1,000 at 10 weeks. In particular, paternal triploidy carries a risk for a partial molar pregnancy. Early diagnosis of triploidy could be helpful for managing these pregnant women [34].

Limitations of the SNP-based NIPT

The SNP-based method might not be applicable to cases of multiple pregnancy. However, a new algorithm for handling multiple pregnancies is under development. Furthermore, this method is not currently applicable to ovum-donated pregnancy. The NATUS algorithm is too computationally complex to validate other algorithms. Therefore, to add a new target would require intensive work on the SNP method (i.e., addition of new SNPs, primers, interpretations).

Conclusion

The well-known causes of false positives in an NIPT are a vanishing twin, maternal chromosomal abnormalities (especially sex chromosome mosaicism), and confined placental mosaicism. Of these causes, only the SNP-based NIPT can detect a vanishing twin and maternal abnormalities. Moreover, because the

Table 1. Comparison of test performance with different noninvasive pretatal test methods [11]

	S-MPS		T-MPS		SNP	
	DR	FPR	DR	FPR	DR	FPR
Trisomy 21	99.0 (97.7-99.7)	0.19 (0.11-0.33)	99.4 (96.4-100)	0.01 (0-0.09)	100 (96.8-100)	0 (0-0.29)
Trisomy 18	97.6 (92.8-99.5)	0.15 (0.08-0.28)	97.9 (92.2-99.9)	0.08 (0.03-0.19)	96.4 (80.8-100)	0.09 (0-0.54)
Trisomy 13	89.2 (74.7-96.3)	0.32 (0.20-0.51)	81.8 (51.2-96.0)	0.06 (0-0.25)	100 (79.7-100)	0 (0-0.28)
All above aneuploidies	98.2 (96.9-99.0)	0.21 (0.16-0.29)	98.2 (95.7-99.9)	0.05 (0.02-0.10)	99.2 (95.1-100)	0.03 (0-0.18)

Values are presented as a percentage (95% confidence interval).
S-MPS, shotgun massively parallel sequencing; T-MPS, targeted massively parallel sequencing; DR, detection rate; FPR, false positive rate.

SNP-based NIPT is less dependent on the fetal fraction, lower false-negative and false-positive rates are expected with the SNP method compared with the counting method (Table 1). However, it is important to note that NIPTs are not direct diagnostic tests but rather advanced screening tests. Although generally designated as fetal cfDNA, the primary source of the cfDNA tested is in fact placental trophoblast cells. Therefore, confined placental mosaicism, which is estimated in 1-2% of all 10-12 week gestations, impacts all NIPTs [35].

It is clear that when an NIPT is implemented in a clinical setting, the influence of mosaicism cannot be ignored, and its impact on false-positive and false-negative results should be addressed. Therefore, pre-test counseling and post-test counseling by experts is the most important process when performing an NIPT. Because false-positive test results can occur, confirmation with amniocentesis or chorionic villi sampling is recommended. Patients also need to be aware that a negative test result does not ensure an unaffected pregnancy; false-negative test results can also occur. In the US, approximately 6.2% of women with high-risk results chose to terminate the pregnancy without invasive test confirmation [10].

I hope that in the future, decisions to terminate a pregnancy will not be based on only NIPT results without a confirmation test. Given a normal NIPT result, maternal serum alpha-fetoprotein screening or ultrasonographic assessment for open neural tube defects should continue to be offered.

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