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Non-invasive prenatal testing for fetal aneuploidy by massively parallel DNA sequencing of maternal plasma: the future has arrived today

Nicht-invasiver Pränatal-Test auf fetale Aneuploidie mittels massiv-paralleler DNA-Sequenzanalyse im mütterlichen Plasma: in der Zukunft angekommen

Abstract: After decades of research, non-invasive prenatal testing (NIPT) using maternal blood to determine fetal chromosome status has found its way from the research laboratory into clinical practice, triggering a long-awaited paradigm shift in prenatal care. A variety of methods using sequencing of maternal cell-free DNA (cfDNA) have now been studied, primarily demonstrating their ability to detect the most common fetal aneuploidy, trisomy 21 (T21). The focus of this article is on massively parallel sequencing (MPS) with optimized sequence tag mapping and chromosome quantification, which accurately detects T21 as well as multiple other aneuploidies across the genome. The power of this technique resides in its high precision and reduction of variation within and between sequencing runs. Using MPS, classification of aneuploidy status for a given sample can be reliably assigned from the genetic information alone without the need to factor in other maternal pre-test risk or other clinical variables. Performance of this method has been prospectively demonstrated in a rigorous, blinded, multi-center study in the United States. The findings suggest that MPS can be incorporated into existing prenatal screening algorithms to reduce unnecessary invasive procedures. This technology and key considerations for clinical implementation are discussed.

Keywords: cell-free DNA (cfDNA); fetal aneuploidy; massively parallel sequencing (MPS); maternal plasma; non-invasive prenatal testing (NIPT).

Zusammenfassung: Nach jahrzehntelanger Forschung hat der Nicht-invasive Pränatal-Test (NIPT) des fetalen Chromosomenstatus im mütterlichen Plasma seinen Weg aus den Forschungslaboren in die klinische Praxis gefunden und löst einen lange herbeigesehnten Paradigmenwechsel in der pränatalen Diagnostik aus. Es wurde bereits eine Vielzahl von Methoden, welche die Sequenzanalyse von mütterlicher zell-freier DNA (cfDNA) verwenden, untersucht. Dabei zeigte sich, dass dieser Ansatz für den Nachweis der häufigsten fetalen Aneuploidie, der Trisomie 21 (T21) genutzt werden kann. Der Fokus dieses Artikels liegt auf der massiv-parallelen Sequenzanalyse (MPS) mit optimiertem „Sequence Tag Mapping“ und Chromosomenquantifizierung, wodurch T21 und zahlreiche andere Aneuploidie im Genom exakt nachgewiesen werden können. Der Vorteil dieser Methode liegt in ihrer hohen Präzision und der Reduzierung der Variation innerhalb eines sowie zwischen mehreren Sequenzierungsläufen. Durch die Verwendung von MPS kann der Aneuploidie-Status einer Probe zuverlässig allein aus der genetischen Information ohne die Fakturierung in anderen mütterlichen Prätest-Risiko oder anderen klinischen Variablen ermittelt werden. Die Eignung dieser Methode konnte prospektiv in einer streng verblindeten, multizentrischen Studie in den USA demonstriert werden. Die Ergebnisse deuten darauf hin, dass MPS in existierende pränatale Screening-Algorithmen integriert werden kann und somit unnötige invasive Eingriffe reduziert werden können. Die Technologie und ihre klinische Implementierung werden diskutiert.

Schlüsselwörter: fetale Aneuploidie; massiv-parallele Sequenzanalyse (MPS); mütterliches Plasma; Nicht-invasiver Pränatal-Test (NIPT); zellfreie DNA (cfDNA).

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Introduction

Fetal chromosome abnormalities are a major contributor to miscarriages, congenital anomalies and perinatal deaths [1, 2]. Since the 1970s, when amniocentesis was first introduced, followed by the introduction of chorionic villus sampling (CVS) in the 1980s, pregnant women have had options to obtain information about fetal chromosome status [3]. Cytogenetic karyotyping of fetal cells or chorionic villi obtained from these procedures leads to diagnosis in the vast majority of cases with very high sensitivity and specificity (~99%) when adequate tissue is obtained [4, 5]. However, these procedures also pose risks to the fetus and pregnant woman [6, 7]. To mitigate these risks, a series of prenatal screening algorithms have been developed to stratify women for their likelihood of the most common fetal trisomies, trisomy 21 (T21, Down syndrome), trisomy 18 (T18, Edwards syndrome) and, to a lesser extent, trisomy 13 (T13, Patau syndrome). The screens involve measurement of multiple biochemical analytes in the maternal serum at different time points combined with ultrasonographic measurement of the fetal nuchal translucency (NT) and incorporation of other maternal factors, such as age, to generate a risk score. Based on their development and refinement over the years and depending on when the screening is administered (first or second trimester only, sequential, or fully integrated) and how the screening is administered (serum-only or serum combined with NT), a menu of options has evolved with variable detection rates (65%–90%) and high screen positive rates (5%) [3]. For patients, following this multi-step process, the resultant non-definitive "risk score" can be confusing and anxiety provoking, particularly in the absence of comprehensive counseling [8]. Ultimately, the results are weighed against the risks for miscarriage from an invasive procedure in a woman's decision-making. For decades, beginning with the pursuit of fetal cells in the maternal circulation, a better non-invasive means to obtain more definitive information on fetal chromosomal status had been sought [9].

Massively parallel sequencing of maternal plasma DNA

Fan et al. were the first to demonstrate counting chromosomes by mapping sequence tags generated by massively parallel sequencing (MPS) as a potential quantification method for detecting fetal aneuploidy from total cell-free DNA (cfDNA) in maternal plasma [10]. Since this seminal paper, this method has been optimized and proven to be robust for detecting multiple chromosome abnormalities in hundreds of samples from two independent studies [11, 12]. Other studies have also demonstrated performance of the method for detection of T21 [13–15]. Maternal plasma isolated from a single 10 mL blood tube provides sufficient cfDNA for random sequencing analysis, generating tens of millions of sequence tags across the entire genome that can be uniquely aligned and counted [11]. The plasma is a mixture of maternal and fetal cfDNA, and the percent contributed by the fetus is referred to as the fetal fraction. In the presence of fetal aneuploidy, sequencing produces an increase or decrease in the relative number of tags on the affected chromosome compared to the euploid chromosomes. Calculating and comparing the relative number of tags between affected and unaffected chromosomes as described below leads to accurate classification of aneuploidy status. Analysis methods have approximately 10 million tags per sample to maintain high precision down to low levels of fetal fraction (approximately 3%–4%) [16]. Importantly, improvements in MPS efficiencies have increased sequencing depth such that multiple samples can now be analyzed per lane (e.g., 6–12 samples per lane, referred to as a 6-plex or 12-plex), while preserving the necessary counting statistics to accurately detect fetal aneuploidy [12]. These efficiencies are expected to continue, along with decreasing sequencing costs and shorter run times, keeping MPS on the forefront as a robust and cost-effective platform for non-invasive prenatal testing (NIPT) with genome-wide capability [17].

No enrichment strategies (with their concomitant increase in error) are required to produce adequate statistics. When compared to current chromosome-selective sequencing methods [18], MPS offers several advantages, including removal of inherent bias created by selective primers, avoidance of the need for target enrichment strategies to produce adequate allelic counts, no requirement to incorporate the fetal DNA fraction in the final diagnostic algorithms and higher precision allowing definitive aneuploidy classification vs. "risk score" assignment [19].

Optimized chromosome quantification

Intra-run and inter-run sequencing variation in the chromosomal distribution of sequence reads can obscure the effects of fetal aneuploidy on the distribution of mapped sequence sites. Several publications in 2011 noted this difficulty in determining aneuploidy in chromosomes 18 and 13, for example [13, 14]. To correct for such variation, we have utilized a chromosome ratio, where the count of mapped sites for a given chromosome of interest (e.g., chromosome 21) is normalized to cumulative counts observed on a predetermined set of custom chromosomes. We refer to this predetermined set of chromosomes as the "reference chromosomes", and they are used in the denominator for the ratio calculations. This approach mitigates the need to perform additional corrections on the data [e.g., correction for guanine-cytosine (GC) content used by others] (Figure 1) [20, 21]. The optimal set of reference chromosomes for each chromosome of interest is determined from results of data on a training set that includes only euploid samples (diploid karyotype). Any combination of autosomes other than chromosomes 21, 18 and 13 are considered as potential denominators in a ratio of counts with the chromosomes of interest. To achieve the highest precision, denominator chromosomes were determined that minimize the variation of the chromosome ratios within and between sequencing runs [11, 12]. Using this approach, any chromosome can be analyzed in the numerator as the "chromosome of interest", allowing whole genome interrogation.

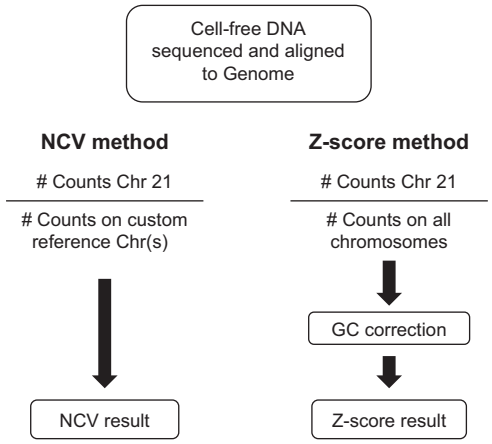

Figure 1 Differences between sequencing analysis approaches for the normalized chromosome value (NCV) method vs. Z-score method with guanine-cytosine (GC) correction.

The NCV method uses reference chromosomes to reduce variability and maximize precision and dynamic range between affected and unaffected samples.

To begin to test samples for aneuploidy status by this method, the following steps are taken. The averages and standard deviations of chromosome ratios for the unaffected samples in the training set are determined, and for each test sample, a normalized chromosome value (NCV) is calculated using the equation:

$$NCV_{ij} = \frac{x_{ij} - \hat{\mu}_j}{\hat{\sigma}_j}$$

where $\hat{\mu}_j$ and $\hat{\sigma}_j$ are the estimated mean and standard deviation of the ratios in the training set, respectively, for the *j*-th chromosome ratio, and x_{ij} is the observed *j*-th chromosome ratio for the test sample, *i*. When chromosome ratios are normally distributed, the NCV is equivalent to a statistical Z-score for the ratios. Moving from NCV score to classification of the autosomes' aneuploidy state, we require an NCV>4.0 to classify the chromosome as affected (i.e., aneuploidy detected for that chromosome) and an NCV<2.5 to classify a chromosome as unaffected (aneuploidy not detected). Samples with autosomes that have an NCV between 2.5 and 4.0 are unclassifiable. We intentionally maintain the "unclassifiable" zone to ensure a safe and effective test result. In other words, if there is an equivocal value from MPS, we want to be able to identify it as such. At the same time, this also allows greater confidence in sample results that lie in the "detected" (NCV>4) and "not detected" (NCV<2.5) zones.

The classification strategy for sex chromosomes is somewhat more complex and described in detail by Bianchi et al. [12]. In addition to the high sensitivity and specificity for detection of T21, T18 and T13, the NCV method also demonstrates high performance to detect monosomy X (45,X, Turner syndrome). This condition, which is not included in current conventional prenatal screens, results in a high miscarriage rate and can present at birth with life-threatening cardiac defects and other significant medical issues. The demonstration of MPS to detect monosomy X offers a potential new avenue for early prenatal diagnosis of this condition, which occurs even more frequently than T13. And when used in cases where fetal cystic hygroma is seen by ultrasound, monosomy X is more commonly detected than T18.

Importance of study design

Early studies in the field led to some urgency to perform large-scale properly conducted clinical evaluation studies of NIPT [22]. Since then, the results of several independent large studies have now been reported [12, 15, 20, 23].

When comparing results from these studies, it is not only important to understand their underlying technical differences, but to also recognize the value of study design on the strength of the results before transitioning to clinical use. The MatErnal BLood IS Source to Accurately Diagnose Fetal Aneuploidy (MELISSA) study, reported by Bianchi et al., was a prospective, blinded study designed to emulate real-world testing in which fetal chromosome status is not known a priori [12]. In this study, all samples with any abnormal karyotype were included for analysis, sequenced and assigned a ploidy status (affected, unaffected or unclassified) for each of six independent categories: chromosome (Chr) 21, Chr 18, Chr 13, male, female and monosomy X. The ratio and frequency of abnormal to normal samples was unknown, and no maternal clinical factors were used in the classification of samples. The importance of this design is that for each of the six independent categories analyzed, all samples that did not have the test condition (e.g., aneuploidy 18 for Chr 18), served as controls in the analysis. For example, after unblinding, it was shown that a very diverse set of karyotypes was represented in 35% of the "non-aneuploidy 18" population. In other words, non-aneuploidy 18s included samples with other major aneuploidies (e.g., T21) and other chromosome abnormalities as would be expected to be encountered in real-world testing (Figure 2). This is an important distinction that has not been demonstrated in any other studies to date. Under these conditions, the test achieved 100% specificity, no false-positives, with a narrow 95% confidence interval range (99.1%–100%) and very high sensitivity (Chr 21 100%, 95% CI 95.9–100; Chr 18 97.2%, 95% CI 85.5–99.9 and Chr 13 78.6%, 95% CI 49.2–95.3). For monosomy X, 15 of 16 cases were detected (sensitivity 93.8%, 95% CI 69.8–99.8). All results showed superior sensitivity and specificity compared to serum analytes and ultrasound.

Another distinction of this study was that all chromosomes were examined for each sample. This analysis led to the correct identification of two cases of other autosomal aneuploidies (T20 and T16) and several cases of sex chromosome aneuploidies (XXX, XXY and XYY). Thus, MPS offers a whole-genome approach without having to design specific primers – as would be required with chromosome-selective sequencing analysis. From a clinical standpoint, this is very important and offers future development of the technology to meet a broader number of conditions.

Lastly, the MELISSA study also included samples from pregnant women who conceived by in vitro fertilization, some of whom had pregnancies affected by aneuploidy. These results suggest that NIPT by MPS is also accurate in this group of women who may particularly wish to avoid an invasive procedure if possible.

Clinical use

The overall goal of NIPT is to minimize anxiety surrounding multi-step screening and reduce false-positive results, thereby reducing exposure to invasive procedural risks. This goal is finally being realized through the introduction of laboratory developed prenatal MPS testing. In response to these offerings, the International Society of Prenatal Diagnosis (ISPD) and the National Society of Genetic Counselors (NSGC, USA) have issued rapid response and position statements on the topic [24, 25]. ISPD accepts that with suitable genetic counseling MPS can be helpful for

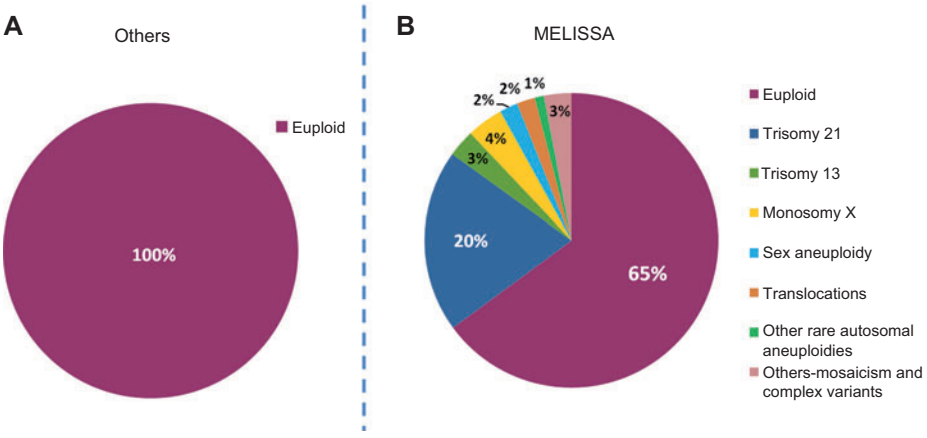

Figure 2 Differences in control populations used for specificity analysis.
Panel A shows a fully euploid population used in all prior studies. Panel B shows diverse non-T18 karyotypes included in Chr 18 analysis by Bianchi et al. [12].

women who may have been determined to be high-risk by one of the previously recommended screening strategies. Based on the peer-reviewed evidence, NSGC also recognizes and supports NIPT as an option for aneuploidy assessment of chromosomes 21, 18 and 13 in pregnancy for high-risk women. They suggest that any abnormal results should be confirmed through a conventional prenatal diagnostic procedure. NSGC also recommends both pre- and post-test genetic counseling to provide up-to-date information and ensure patient understanding of the limitations of the test and implications of the results (see below). Neither ISPD nor NSGC currently supports NIPT as a routine, first-tier aneuploidy screening test in low-risk populations until further evidence is provided.

While these initial steps of implementation and introduction of NIPT into clinical practice have quickly taken place, it is clear that ongoing education is needed for healthcare providers and patients alike to fully understand all the ramifications of testing. In individual situations, there may be certain scenarios where a pregnant woman may request NIPT as an alternative to conventional screening, an invasive prenatal procedure or both. It is particularly important in these cases that comprehensive counseling be provided.

Genetic counseling considerations

By offering NIPT as a secondary screen to those women with a positive conventional screen (or other a priori risk), the number of unnecessary amniocentesis and CVS procedures are expected to decrease. Conversely, the need for genetic counseling will increase, as informed consent is a critical component of NIPT. Resource limitations to meet this demand need to be considered within practices to ensure that a qualified healthcare provider (if not a certified genetic counselor) is available to provide non-directive pre-test counseling for all women considering the test. As a positive NIPT result is more similar to a positive result from amniocentesis or CVS, women should be given the opportunity prior to this testing to decide whether they desire this degree of information. Pre-test genetic counseling for NIPT should also include discussion of the recommendation for confirmation of abnormal test results via CVS or amniocentesis (depending upon gestational age) so that appropriate consideration can be given to the expected timing of results for post-test planning. Per the NSGC statement, because NIPT does not currently screen for all chromosomal or genetic conditions, it does not replace standard risk assessment and prenatal diagnosis. Patients with other factors (e.g., abnormal ultrasound findings) suggestive of a chromosome abnormality should receive genetic counseling and have the option of conventional diagnostic testing, regardless of NIPT results. Women should also be made aware that for some patients an NIPT result may not be informative.

Biological considerations

NIPT is perhaps more similar to CVS than amniocentesis, in that detection of aneuploidy is typically representative of the chromosomal constitution of the fetus, but in some instances may be representative of confined placental aneuploidy or confined placental mosaicism (CPM). CPM occurs in approximately 1%–2% of cases of CVS results today, and some women undergo an amniocentesis at a later gestational age after CVS to make the distinction between apparently isolated placental aneuploidy vs. fetal aneuploidy. As NIPT is implemented more widely, cases of CPM are expected to cause some number of positive NIPT results that may not be subsequently confirmed by an invasive procedure, particularly amniocentesis. Further investigation of the pregnancy course and outcomes as well as placental pathology will be important in these cases. Similarly, NIPT also has the potential to identify maternal full or mosaic aneuploidy given that total cfDNA is a mixture of maternal and fetal DNA. These and other conditions, such as low-level fetal mosaicism and undetected multiples or demised co-twins, need to be considered in the overall clinical context.

Clinical test

Verinata Health, Inc. (Redwood City, CA, USA), began offering the verifi® prenatal test in early 2012 in the US for detection of aneuploidies in chromosomes 21, 18 and 13 from a single maternal blood draw as early as 10-weeks' gestation. The test is currently indicated for women with singleton pregnancies and high-risk indications for fetal aneuploidy. The test is available through Verinata's College of American Pathologists (CAP) accredited clinical laboratory. Results are reported as "Aneuploidy Detected", "No Aneuploidy Detected", or "Unclassifiable" for each of the three chromosomes evaluated. An option for monosomy X detection in patients with findings of fetal cystic hygroma or increased nuchal translucency has recently been added. Physician signature and patient informed consent are required for testing.

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