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Non-invasive prenatal testing for the diagnosis of congenital abnormalities: Insights from a large multicenter study in southern China

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Abstract

Although non-invasive prenatal testing (NIPT) is widely used to detect fetal abnormalities, the results of NIPT vary by population, and data for the screening efficiency of NIPT positive predictive value (PPV) from different populations is limited. Herein, we retrospectively analyzed the NIPT results in a large multicenter study involving 52,855 pregnant women. Depending on gestational age, amniotic fluid or umbilical cord blood was extracted for karyotype and/or chromosome microarray analysis (CMA) in NIPT-positive patients, and the PPV and follow-up data were evaluated to determine its clinical value. Among the 52,855 cases, 754 were NIPT-positive, with a positivity rate of 1.4%. Karyotype analysis and/or CMA confirmed 323 chromosomal abnormalities, with a PPV of 45.1%. PPV for trisomy 21 (T21), trisomy 18 (T18), trisomy 13 (T13), sex chromosomal aneuploidies (SCAs), and copy number variations (CNVs) were 78.9, 35.3, 22.2, 36.9, and 32.9%, respectively. The PPVs for T21, T18, and T13 increased with age, whereas the PPVs for SCAs and CNVs had little correlation with age. The PPV was significantly higher in patients with advanced age and abnormal ultrasound. The NIPT results are affected by population characteristics. NIPT had a high PPV for T21 and a low PPV for T13 and T18, and screening for SCAs and CNVs showed clinical significance in southern China.

Key words: NIPT; Trisomy; Positive predictive value; Karyotyping; Chromosomal abnormalities

Introduction

Approximately 3–5% of pregnancies are complicated by birth defects or genetic disorders (1). Chromosomal abnormalities are present in about 1 in 150 live births, and congenital malformations remain the leading cause of infant and child mortality (2). Despite the rapid advancements in prenatal screening, diagnosis, and fetal therapy, chromosomal abnormalities remain the most common cause of congenital disabilities, with no effective treatment (3,4). Therefore, developing a safe, simple, economical, and practical prenatal screening procedure to detect congenital disabilities is essential. Lo et al. (5) discovered cell-free fetal DNA (cffDNA) in pregnant women's plasma for the first time in 1997, and non-invasive prenatal testing (NIPT) to detect fetal chromosomal abnormalities has rapidly gained much attention (6,7). cffDNA in the mother originates from apoptotic placental cells and apoptotic fetal nucleated red cells and enters the maternal blood circulation through the placenta. However, it is removed quickly after childbirth (8). In NIPT, cffDNA fragments in maternal peripheral blood are amplified and analyzed using high-throughput sequencing technology and bioinformatics tools to determine the risk of fetal chromosomal aneuploidy (9). Compared to traditional serological screening, NIPT demonstrates improved sensitivity and specificity for screening of trisomy 21 (T21), trisomy 18 (T18), and trisomy 13 (T13) syndromes (10,11). NIPT can also detect sex chromosomal aneuploidies (SCAs) and copy number variations (CNVs) (12,13) and has been widely employed and promoted as an accurate, safe, and fast method to detect fetal chromosomal aneuploidy. Moreover, with NIPT, the risks of abortion, infection, and injury are minimal compared with those of conventional

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interventional prenatal testing for pregnant women, and NIPT can significantly reduce the rate of congenital disabilities (14).

However, NIPT has certain limitations. A positive predictive value (PPV) refers to the proportion of patients with actual disease in the screening-positive population, reflects the diagnostic benefit of screening, and holds critical reference value for clinical consultation and follow-up treatment. The PPV of NIPT is affected by population characteristics; data for the screening efficiency of NIPT PPV from different populations is limited. In this study, we retrospectively analyzed the results of NIPT in 52,855 pregnant women in a large multicenter study in southern China.

Material and Methods

Ethical approval and consent to participate

The study was approved by the Ethics Committee of the Fujian Provincial Maternal and Children Health Hospital (2014-042), and written informed consent was obtained from all participants. All patients consented to participate. All methods were carried out according to relevant guidelines and regulations. All data used and provided were deidentified and anonymized.

Participants

A large multicenter study was conducted in southern China from January 2014 to February 2021, wherein 52,855 pregnant women who underwent NIPT were enrolled. The women were aged 15 to 49 years, with an average age of 29.5 ± 4.7 years, and the gestational age ranged from 11 to 36 weeks, with an average of 22.2 ± 2.4 weeks. Inclusion criteria for NIPT were as follows: single pregnancy, advanced age (expected age $\geqslant$35 years), serologically screened as high-risk group (risk value of T21 $\geqslant$1/270 or T18 $\geqslant$1/350), abnormality based on ultrasonography, previous miscarriages, and no indication (Figure 1). Exclusion criteria for NIPT were as follows: presence of malignant tumors or chromosomal abnormalities and blood transfusion or stem cell therapy or transplantation surgery within one year before the study.

Laboratory methods for NIPT

About 10 mL of peripheral blood was collected from participants by routine venous sampling. Blood samples were then transferred to tubes containing EDTA anticoagulant and DNA preservation agent, which were agitated 8 to 10 times to mix with the stabilizer. The Streck (USA) tube was centrifuged at 1600 *g* at 4°C for 10 min. The supernatant was collected, transferred, and

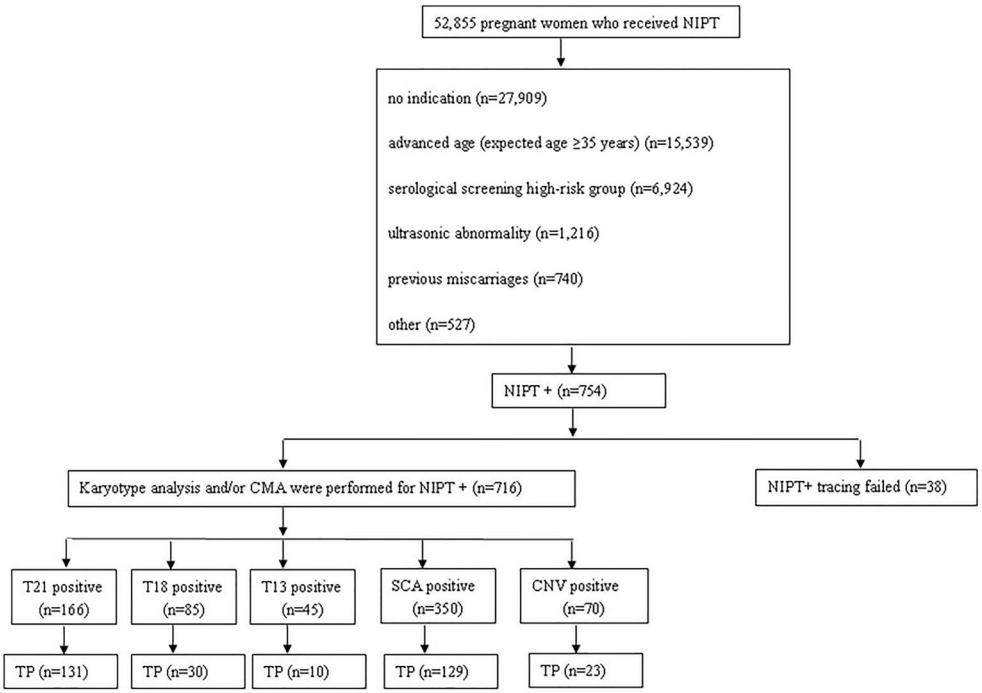

Figure 1. Flowchart showing the number of patients at different stages of NIPT screening for fetal chromosomal aneuploidy. NIPT: non-invasive prenatal testing; CMA: chromosomal microarray analysis; T21: trisomy 21; T18: trisomy 18; T13: trisomy 13; SCA: sex chromosomal aneuploidy; CNV: copy number variety; TP: test positive.

labeled. The supernatant was centrifuged at 16,000 *g* at 24°C for 10 min to remove the remaining white blood cells or cell debris. Plasma was separated within 72 h of collection and stored at –20°C. The Plasma Cell-Free DNA Isolation Kit (Berry Genomics Corporation, China) was used to extract plasma cffDNA from isolated plasma samples. The Qubit® fluorometer (Thermo Fisher Scientific, Inc., USA) was used for quantification of the extracted cffDNA. Reextraction was carried out when the resulting concentration was <0.75 ng/μL to exclude genomic DNA contamination. Construction and purification of a cffDNA library for sequencing was performed using the StepOnePlus™ real-time PCR system (Thermo Fisher Scientific). The cffDNA fragments in maternal peripheral blood were amplified, sequenced using high-throughput sequencing technology, and analyzed and processed using bioinformatics tools. The risk rates of fetal T21, T18, and T13 and sex chromosome abnormalities were obtained. A library was constructed using the kits produced by Hangzhou Berui Hekang Gene Co., Ltd. (China). The NextSeq CN500 sequencer was used to calculate the proportion of each chromosome read (% ChrN) and the Z value of each chromosome (cut-off: | Z | = 3).

Chromosome karyotype analysis

Depending on the gestational age of the pregnant woman, transabdominal amniocentesis was performed, and 20–30 mL of amniotic fluid or 1.0–2.5 mL of umbilical cord blood was extracted under ultrasonic guidance. The collected samples were cultured, treated with colchicine, and stained with Giemsa stain following the laboratory standard operating procedures for G-banding karyotype analysis. Karyotypes were labeled according to the 2016 International System for Human Cytogenetic Nomenclature; 40 karyotypes were counted in each case, and five karyotypes were analyzed.

Chromosomal microarray analysis

The standard operating procedures provided by Affymetrix were followed for all the experiments: DNA extraction and preparation, DNA digestion, ligation, amplification, purification, fragmentation, labeling, hybridization, washing, staining, and scanning. Data were analyzed using the Chas 2.0 software. The CMA structure was analyzed in combination with the relevant database to determine the nature of CNVs. According to the American College of Medical Genetics and Genomics (ACMG) guidelines (15), CNVs are divided into five categories: pathogenic, benign, and variation of uncertain clinical significance (VUS). For VUS, it is recommended that the pregnant woman and her spouse undergo parental testing for verification. According to the ACMG guidelines, if the CNVs are inherited from a parent with a normal phenotype, VUS might be benign; if the variation is a new mutation, VUS is likely pathogenic.

Pregnancy outcome and postnatal follow-up

Pregnant women who were NIPT-positive and confirmed by amniocentesis were followed up with a call. Pregnancy outcomes and the growth and development of the fetus after birth were recorded.

Statistical analysis

Microsoft Office Excel (USA) was used for data input, and SPSS 21.0 (IBM, USA) statistical software package was used for data analysis. The results were compared using the chi-squared test or Fisher's exact probability method. All statistical tests were conducted by bilateral testing. The level of significance was set at α=0.05, and $P<0.05$ was considered statistically significant.

Results

NIPT results

Among the 52,855 pregnant women, 754 were NIPT-positive, accounting for 1.4% (754/52,855) of the total sample. Of the 754 NIPT-positive cases, T21, T18, T13, SCAs, and CNVs were identified in 171, 89, 48, 372, and 74 cases, respectively.

Karyotype analysis and/or chromosome microarray analysis

Among the 754 NIPT-positive pregnant women, 716 were tested for chromosome karyotype analysis and 312 for chromosome microarray analysis (CMA) simultaneously, and together, 323 cases of chromosome abnormality were confirmed with a total PPV of 45.1% (323/716). The confirmed 312 chromosome abnormalities included 131, 30, 10, 129, and 23 cases of T21, T18, T13, SCAs, and CNVs, respectively. PPVs for T21, T18, T13, SCAs, and CNVs were 78.9, 35.3, 22.2, 36.9, and 32.9%, respectively (Figure 1). Karyotype analysis and CMA were performed simultaneously in 312 NIPT-positive cases. Due to the technical limitations of karyotype analysis and CMA, the test results were not exactly the same. The results were different in 20 cases, including 14 cases of microduplication or microdeletion, two cases of balance translocation, and four cases of low-proportion chimerism of sex chromosomes. Balanced translocations and low-proportion chimerism of the sex chromosomes were normal with CMA. Chromosome microduplication or microdeletions were normal by karyotype analysis.

Maternal age and chromosomal abnormalities

Pregnant women (52,855) were divided by age into a young group ($\leqslant$34 years old, 37,316 cases) and an advanced-age group ($\geqslant$35 years old, 15,539 cases). According to the type of chromosome abnormality, they were divided into five groups: T21, T18, T13, SCAs, and CNVs. The ratios of NIPT positivity and PPV for different types of chromosomal abnormalities in the two maternal age groups are shown in Table 1. The PPV for T21, T18,

Table 1. Distribution of chromosomal abnormalities, NIPT-positive, and PPV in different maternal age groups.

Group	NIPT	T21		T18		T13		SCA		CNV	
		NIPT+	TP (PPV%)	NIPT+	TP (PPV%)	NIPT+	TP (PPV%)	NIPT+	TP (PPV%)	NIPT+	TP (PPV%)
⩽34 y	37,316	92	66 (71.7)	50	12 (24.0)	33	4 (12.1)	256	97 (37.9)	49	16 (32.7)
⩾35 y	15,539	74	65 (87.8)	35	18 (51.4)	12	6 (50.0)	94	32 (34.0)	21	7 (33.3)
Total	52,855	166	131 (78.9)	85	30 (35.3)	45	10 (22.2)	350	129 (36.9)	70	23 (32.9)

NIPT+: non-invasive prenatal testing-positive; T21: trisomy 21 syndrome; T18: trisomy 18 syndrome; T13: trisomy 13 syndrome; SCA: sex chromosomal aneuploidy; CNV: copy number variation; TP: true positive; PPV: positive predictive value.

and T13 increased with maternal age and showed statistically significant differences between the advanced and young groups (P values of 0.011, 0.009, and 0.007, respectively). In contrast, the difference in SCAs and CNVs between the advanced and young groups was not statistically significant (P values of 0.508 and 0.956, respectively) (Table 1).

NIPT-positive indications and PPV comparison

According to NIPT-positive indications, 716 NIPT-positive pregnant women were divided into five groups: among 370 cases in the no-indication group, 145 were diagnosed with a fetal chromosomal abnormality (PPV=39.2%); among 51 cases in the serological screening high-risk group, 19 cases of fetal chromosomal abnormality were confirmed (PPV= 37.3%); among 213 cases in the advanced age group, 107 cases were diagnosed with fetal chromosomal abnormalities (PPV=50.2%); in the 58 abnormal ultrasound cases, 36 fetal chromosomal abnormalities were confirmed (PPV=62.1%); and among 24 cases in the group containing two or more of the above indications, 16 cases were diagnosed with a fetal chromosomal abnormality (PPV=66.7%). The PPV difference of various NIPT-positive indications was statistically significant ($P<0.05$).

Obstetric follow-up

Among the 716 NIPT-positive cases, 678 cases were followed up successfully (follow-up rate: 94.7%) and 38 cases were lost to follow-up. Of the 678 cases, term delivery occurred in 374 cases (52.2%), the pregnancy was terminated in 289 cases (40.4%), and spontaneous abortion or stillbirth were noted in 14 cases (2.0%). Among confirmed cases with SCAs, 15 cases had a normal delivery following genetic counseling, including nine cases with 47,XYY, four with low-proportion chimerism, and two with balanced chromosomal translocation. In 18 of the confirmed CNV cases, the pregnancy was terminated.

Discussion

In traditional serological screening, the accuracy rate is 66–80%, and the rate of missed detection is 20–34%, demonstrating relatively high false-positive and misdiagnosis rates (16). Despite invasive prenatal diagnosis being considered the gold standard, the risks of miscarriage and infection significantly reduce the compliance of pregnant women to these procedures. NIPT shows high sensitivity and specificity for T21, T18, and T13, and its screening accuracy is significantly higher than that of traditional serological screening methods, reducing the number of invasive prenatal diagnoses (17). Therefore, the development and application of NIPT plays a vital role in diagnosing fetal chromosomal abnormalities. In this study, 754 of 52,855 cases were NIPT-positive, accounting for 1.4% of the total sample, similar to the results of Sago et al. (18)

We observed lower PPVs for T21, T18, and T13 in our study compared to those in previous reports (19–21), possibly due to the difference in demography. However, some studies have reported a higher PPV for T13 (22–24). Specifically, the PPV for T13 was significantly low. This could be explained by the likely occurrence of a trisomy self-rescue mechanism during meiosis in the event of T13 abnormal fertilization, leading to a normal fetal karyotype. The placenta comprises normal and abnormal chromosomal chimeras, referred to as confined placental mosaicism (CPM) (25,26). In addition, cffDNA detected by NIPT is mostly from placental trophoblast cells; hence, false-negative results of T13 are easily detected during NIPT (27). Therefore, strategies to improve the accuracy of T13 detection are to be further studied.

In this study, the PPVs for T21, T18, and T13 in the advanced age group (⩾35 years old) were significantly higher than those in the young group (⩽34 years old). The content of fetal fraction is positively correlated with gestational age. However, a woman's ovarian function is more prone to decline with maternal age, resulting in an aging ovum and increasing the probability of chromosomal variation in the resulting embryo. Therefore, gestation at an advanced age is an independent risk factor for fetal chromosomal aneuploidy (28,29). However, there was no difference between the PPV for SCAs and CNVs in the advanced and young age groups, indicating that SCAs and CNVs have little correlation with maternal age. Among the NIPT-positive indications, the PPV of the group containing two or more indications, the group with abnormal ultrasound, and the advanced age group were the highest: 66.7, 62.1, and 50.2%, respectively.

Ultrasonographic changes in young patients always have higher PPV than that in patients of an advanced age without ultrasonographic changes. Abnormal fetal ultrasound is closely related to chromosomal aneuploidy (30), and patients with abnormal fetal ultrasounds should be prioritized for NIPT. For cases with fetal ultrasound abnormalities and advanced age, direct extraction of amniotic fluid during the second trimester is recommended for prenatal diagnosis. We also found that PPV was 39.2% for groups with no indication of voluntary requirements and 37.3% for groups at high risk of serological screening. Before NIPT, serological screening for high-risk pregnant women was generally recommended for invasive prenatal diagnosis to determine the fetal chromosomal status. As the most sensitive screening method available, NIPT can effectively reduce the puncture rate (31,32), and many pregnant women with abnormal serological screening choose NIPT to avoid unnecessary invasive prenatal diagnoses (33).

Karyotype analysis detects chromosomal aneuploidy abnormalities and obvious chromosomal structural abnormalities that can be observed under a microscope. CMA is a molecular genetic technique developed in recent years (34) and has significant advantages, as it can detect chromosome microdeletions or microduplications that cannot be detected by conventional karyotype analysis at the genome level (35,36). For the detection of rare autosomal abnormalities, CMA has its advantages (37). However, CMA cannot detect balanced structural translocation and low-proportion chimerism; karyotype analysis is recommended for such abnormalities. In this study, balanced translocations and low-proportion chimerism of the sex chromosomes were normal with CMA. Thus, to confirm the PPV for SCAs and CNVs, the two methods should be used simultaneously to provide a scientific and accurate molecular genetic diagnostic basis for targeted pre-pregnancy eugenics in subsequent pregnancies.

With a sizeable NIPT test population, this study obtained reliable PPVs for T21, T18, T13, SCAs, and CNVs, providing critical reference for clinical genetic counseling and management. However, the limitation of this study was the failure to complete the follow-up of all the cases after birth and the failure to conduct the placenta test for all the NIPT false-positive cases, especially the false-positive cases of SCAs. Hence, the presence of CPM or the influence of maternal DNA cannot be excluded (38,39). Concomitantly, no NIPT-negative cases were followed up in this study; hence, the sensitivity and false-negative rate of NIPT could not be accurately calculated.

The results of NIPT are affected by maternal age and patient history in the post-test clinical evaluation. NIPT demonstrated a high PPV for T21 and low PPVs for T13 and T18, and the screening of CNVs and SCAs was found to be significant in southern China. NIPT is used in older pregnant women and those with an abnormal ultrasound with better PPV; however, an invasive prenatal diagnosis is warranted for NIPT-positive cases to avoid false positives or unnecessary termination of pregnancy.

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