Application of High-Throughput Sequencing Technology in Prenatal Screening and Diagnosis

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Introduction. This study aims to evaluate the feasibility and effectiveness of high-throughput sequence-based non-invasive prenatal screening (NIPT) for fetal chromosomal aneuploidy in high-risk populations.
Methods. A total of 163 pregnant women with positive NIPT and high risk factors who underwent NIPT in the prenatal diagnosis center of our hospital from June 2020 to June 2023 were selected. Amniotic fluid was collected to detect fetal chromosomes by karyotyping and CNV-seq technology, and the types and proportions of chromosomal abnormalities were statistically analyzed.
Results. After NIPT, 82 pregnant women with trisomy 21, 11 with trisomy 13, 25 with trisomy 18 and 45 with sex chromosome abnormalities were found. All patients underwent prenatal diagnosis and amniocentesis, and 74 cases of trisomy 21, 2 cases of trisomy 13, 20 cases of trisomy 18 and 27 cases of sex chromosome abnormalities were finally diagnosed. The results showed that the positive predictive rates of this method were 90.2%, 18.2%, 80.0% and 66.7%, respectively.
Conclusions. NIPT has a high positive predictive value for trisomy 21 and trisomy 18, but a relatively low accuracy for the detection of other chromosomal abnormalities. Therefore, in order to confirm the diagnosis, further interventional examination is still needed. NIPT combined with prenatal diagnosis can effectively improve the prenatal diagnosis rate and reduce the incidence of birth defects.
Keywords. High-throughput sequencing; Chromosomal aneuploidy abnormality; Prenatal diagnosis

INTRODUCTION

Chromosomal abnormalities refer to numerical and structural abnormalities of chromosomes. Common numerical abnormalities include 21, 18, 13 and sex chromosome abnormalities, which account for 30% to 35% of chromosomal diseases. Chromosomal abnormalities are one of the main causes of birth defects, but there is no effective treatment. Therefore, effective prenatal screening and diagnosis has become an important means to reduce birth defects. In recent years, the rapid development of high-throughput sequencing technology has led to the application of non-invasive prenatal screening (NIPT) in clinical practice [1-2]. NIPT can effectively predict the risk of aneuploidies of chromosome 21, 18, 13 and sex chromosomes by detecting the concentration of free fetal DNA in maternal peripheral blood. It has become a new prenatal screening technology. Foreign studies have shown that the clinical application of NIPT technology has significantly reduced the need for invasive prenatal diagnosis. However, further analysis of the detection rate of fetal 21, 18, 13 and sex chromosome aneuploidies is still lacking [3]. Therefore, the aim of this study is to verify the results of NIPT screening in the prenatal diagnosis center of our hospital, compare the coincidence rate of NIPT and invasive prenatal diagnosis, and explore the efficiency and feasibility of NIPT in the diagnosis of fetal chromosomal aneuploidies in the high-risk

population. The goal of this study is to provide a theoretical basis for the rational application of NIPT in prenatal diagnosis.

1 MATERIALS AND METHODS

1.1 Data sources

A retrospective analysis was performed on 9398 pregnant women with high risk factors who underwent NIPT screening in the prenatal diagnosis center of our hospital from June, 2020 to June, 2023. The screening gestational age was 12-23 weeks. A total of 163 high-risk pregnant women with positive screening results underwent amniocentesis, all of them were singleton pregnancies, and the amniocentesis time was 18-25 weeks. The indications of NIPT screening in pregnant women included advanced age (pre-delivery age ≥35 years), high risk and borderline risk of Down's screening, abnormal ultrasound soft markers, and adverse pregnancy and childbirth history. The cut-off value of trisomy 21 and trisomy 18 was 1:380 and 1:334, respectively. Down's critical risk: pregnant women with a high risk cut value of 1 in 1000; Abnormal ultrasound findings included nuchal translucency ≥2.5mm < 3.0mm at 11-13 +6 weeks or nuchal skin ≥6 mm at 14-22 weeks, choroid plexus cyst, mild dilatation of lateral ventricle, ventricular hyperechogenic spots, and bowel echo enhancement. Exclusion criteria: multiple births; The delivery history of the fetus with chromosomal abnormalities or one of the couples with definite chromosomal abnormalities; Fetal ultrasound showed obvious structural malformations. Received allogeneic blood transfusion, transplantation surgery or immunotherapy within 1 year; Pregnancy complicated with malignant tumor. All pregnant women were counseled and informed before testing and signed an informed consent.

1.2 Methods

1.2.1 NIPT TESTING

① Using EDTA-coated tubes, 10 ml of maternal venous blood was drawn.

② The blood sample tubes were placed in a centrifuge and centrifuged at 1600 rpm per minute for 10 minutes. The aim of this step is to allow the hemocytes in the blood to precipitate at the bottom of the tube, while the plasma is retained in the upper layer.

③ Using a pipettor or similar instrument, carefully aspirate the upper plasma to avoid simultaneous aspiration of blood cells."

④ The extracted plasma sample tubes were put back into the centrifuge and centrifuged at 16000 rpm per minute for 10 minutes. The aim of this step is to further precipitate the residual blood cells at the bottom of the tube to obtain a purer plasma.

⑤ Carefully remove the plasma from the centrifuge to avoid bringing blood cells into the bottom of the centrifuge tube.

1.2.2 Plasma DNA extraction

Follow the steps of Berry-SOP-02 V1.0 procedure for plasma DNA extraction as follows:

① 15 mL BD tubes were added with 15μL Reagent II, 30μL magnetic beads, 100μL Reagent I and 2 mL catalytic buffer, respectively. The 15 mL BD tubes were numbered one by one and checked for correctness. Remove 1.2 mL of plasma from the centrifuge tube in which plasma is stored and tighten the tube cap immediately.

② A BD tube at 12 RPM was used for stirring for 20 min, and then the magnetic beads were adsorbed with a magnetic holder and the supernatant was aspirated.

③ Add 550μL Buffer AL, mix thoroughly, absorb and discard the supernatant.

④ Add 550μL of wash bufferII, mix thoroughly, and discard the supernatant.

⑤ Add 500μL of wash bufferIII and discard the supernatant.

⑥ Instant separation (4000-5000 grams), the residual liquid is extracted.

⑦ Add 42μL elution buffer, mix thoroughly, and then proceed in 55℃ water bath for 10 minutes.

⑧ Replace the centrifuge tube on the magnetic holder and transfer the supernatant to a 1.5-ml centrifuge tube.

⑨ Detection of DNA concentration.

1.2.3 Library establishment

① Modification of cell-free fetal DNA in maternal plasma by adding an "A" base at the 3' end so that the DNA fragment does not contain a "T" base and is linked to a specific linker. ② Using PCR method, modified DNA fragments containing specific sequence tags are introduced into the PCR reaction. ③ The PCR reaction was carried out and the products of each step were purified. ④ The reverse transcription fluorescence quantitative reaction was designed by mixing the reaction mixture including KAPA (2×), reverse transcription (50×), dUTP (10 mM), ROX correction dye, template DNA, primer and probe completely. ⑤ The mixture was dispensed into the PCR tube and gently shaken and centrifuged. ⑥ Using C-bot bridge amplification technology, Qrt-PCR was used as a template for PCR quantitative detection. ⑦ High-throughput sequencing technology (e.g. NextSeq CN500) was used to sequence PCR products and obtain raw data. ⑧ The raw data were aligned with the human genome reference sequence in the Ensembl database to identify each reads sequenced.

1.2.4 Sampling of amniotic fluid and preparation of chromosome karyotype analysis

At 18-23 gestational weeks, amniocentesis was performed under the guidance of B-ultrasound, and 25 ml of amniotic fluid was extracted, 5 ml of which was sent for CNV-seq, and the other 20 ml was injected into a sterile disposable centrifuge tube. Next, amniotic cells from these amniotic fluid samples were isolated and cultured together with other amniotic cells. The culture conditions were 37 ° C, 5% CO2, and a constant temperature incubator was used for timely cultivation. At the end of the culture period, the amniotic cells were routinely prepared and stained with G belt. This procedure describes an ultrasound-guided amniocentesis performed between 18 and 23 weeks of gestation, with amniocytes obtained from the amniocentesis. These cells were subjected to appropriate culture conditions and cultured together with other amniotic cells in a constant temperature incubator. Finally, these cells were prepared into regular slides and subjected to G belt staining.

1.2.5 CNV-seq test

① All samples were shaken and mixed for 5 s, briefly centrifuged for 3 s, then 200 uL buffer GB was added, shaken and mixed for 5 s, briefly centrifuged for 3 s, and put on a constant temperature oscillation mixer, and the reaction was carried out at 70℃ and 500 rpm for 10 min. ② The treated samples were cooled at room temperature for 1 min, and then the samples were spread out at intervals, and 200 uL of absolute ethanol was added in suspension. The samples were shaken thoroughly for 15 s and centrifuged for 3 s. ③ Prepare the corresponding number of adsorption column and casing, and mark the column cover with serial number. All solutions and flocculent precipitates were transferred to the corresponding adsorption column according to the flow number

and centrifuged at 13400×g for 30 s at room temperature. ④ Prepare absorbent paper folded in half three times (8 layers) and lay it on the experimental table. The waste liquid in the casing was poured out and stamped twice on absorbent paper to blot out the residual liquid and put the adsorption column back into the casing. (Note: Each sample must be stamped twice on absorbent paper separately from other samples to avoid cross contamination). ⑤ Add 500 uL buffer GD to the adsorption column (please check whether absolute ethanol has been added before use), and centrifuge at 13400×g for 30 s. At the end of centrifugation, operation d was repeated. 600 uL of the rinse solution PW was suspended into the adsorption column (please check whether absolute ethanol has been added before use) and centrifuged at 13400×g for 30 s. After the end of centrifugation, the operation was repeated ④. The previous step ⑥ was repeated once. ⑥ The adsorption column was placed into the cannula and centrifuged at 13400×g for 2 min. Prepare the same amount of clean 1.5 mL centrifuge tubes, and the tube body and lid should be marked with the serial number. After centrifugation, 10 uL suction head ring with filter element was used to absorb the pink plastic ring in the adsorption column, then the residual liquid and tissue residue were sucked out and put into the prepared 1.5 mL centrifuge tube one by one (as shown in Figure 2), and the tube cover of the adsorption column was opened to dry at room temperature for 3 min. ⑦ The sterilized ddH2O preheated at 70℃ (tissue elution volume 150 uL, other samples 50 uL) was added to the middle of the adsorption membrane, then the lid was covered and left at room temperature for 5 minutes. ⑧ After the end of standing, the 1.5 mL centrifuge tube equipped with the adsorption column was centrifuged at 13400×g for 2 min, and the obtained solution was the extracted DNA.

1.2.6 Data Analysis

The positive range of NIPT screening was ≥3 or ≤--3 for trisomy 21, 18, or 13. The resolution of karyotyping was 320 bands.

Count data were expressed as percentage (%), and positive predictive value (PPV) was calculated using the following formula: PPV = number of true positive cases/(number of true positive cases + number of false positive cases).

2 RESULTS

2.1 The results of chromosome abnormality detection after NIPT positive prenatal diagnosis were analyzed

A total of 9398 pregnant women with high risk factors who underwent NIPT in the prenatal diagnosis center of our hospital from June, 2020 to June, 2023 were screened, of which 163 were screened positive, including 82 with high risk of trisomy 21, 11 without high risk of trisomy 13, 25 with high risk of trisomy 18 and 45 with sex chromosome abnormalities. All the pregnant women underwent amniocentesis, and their amniotic fluid was collected for chromosome karyotype analysis and CNV-seq, respectively. Among the positive results of CNV-seq, only chromosome aneuploidy was selected, and chromosome microdeletion, microduplication and results of unknown significance were excluded. A total of 126 positive cases were finally diagnosed, with a positive predictive value of 75.5%, including 74 cases of trisomy 21, 2 cases of trisomy 13, 20 cases of trisomy 18 and 30 cases of sex chromosome abnormality. The detailed data of chromosomal abnormalities after prenatal diagnosis with positive NIPT screening for different high-risk factors are shown in Table 1.

Table 1 Diagnosis by karyotype analysis of positive results of NIPT screening for different high-risk factors

	Trisomy 21		Trisomy 18		Trisomy 13		45,X0		47,XXY		47,XXX		47,XYY	
	Screening Positive	Diagnosis. Positive	Screening Positive	diagnosis. Positive	Screening Positive	diagnosis. Positive	Screening Positive	diagnosis Positive	Screening Positive	diagnosis. Positive	Screening Positive	diagnosis. Positive	Screening Positive	diagnosis. Positive
Advanced age	44+16①	43+14①	9+4①	6+4①	6+1①	1+1①	6+1①	1	7	6	4	4	5	2
Abnormality of Tang Sieve	1	0	0	0	0	0	0	0	0	0	0	0	0	0
History of adverse pregnancy and childbirth	4	2	0	0	0+1②	0	2	0	0	0	1	1	0	0
Ultrasound	17+16①	15+14①	12+4①	10+4①	3+1①+1②	0+1①	6+1①	4	7	7	2	1	4	4
Sum up	66+16①	60+14①	21+4①	16+4①	9+1①+1②	1+1①	14+1①	5	14	13	7	6	9	6

Among them, ① denotes the number of people with both advanced age and ultrasound soft markers, and ② denotes the number of people with both advanced age and adverse pregnancy and childbirth history

2.2 Positive predictive value of NIPT test for different risk factors

According to the data in Table 2, the positive predictive values of NIPT for trisomy 21, 18, 13, and sex chromosome abnormalities were 90.2%, 80.0%, 18.2%, and 66.7%, respectively. And it can be seen that the correlation between advanced age and ultrasound soft markers is higher in high-risk factors.

Table 2 Positive predictive values of NIPT testing for different risk factors

	Trisomy 21	Trisomy 18	Trisomy 13	Sex chromosomes				
				45，XO	46,XXY	46,XXX	47,XXY	Sum up
Advanced age	95%	76.9%	28.6%	14.3%	85.7%	100%	40%	56.5%
Abnormality of Tang sieve	0	0	0	0	0	0	0	0
History of adverse pregnancy and childbirth	50%	0	0	0	0	0	0	33.3%
Ultrasound	87.9%	87.5%	20%	57.1%	100%	50%	100%	80%
Sum up	90.2%	80.0%	18.2%	33.3%	92.9%	85.7%	66.7%	66.7%

3 DISCUSSION

3.1 To evaluate the efficiency of prenatal diagnosis combined with NIPT

Traditional prenatal diagnosis methods are usually invasive, have a potential risk of abortion, and require a long time of cell culture and detection cycle, so this is an extremely important issue for high-risk groups [4]. In recent years, NIPT technology has made great progress, which is favored by pregnant women for rapid, noninvasive and accurate detection of aneuploidies of 21, 18 and 13 and sex chromosomes. Based on this, we selected 9398 pregnant women with high risk factors and performed prenatal diagnosis in those with positive 163NIPT screening. The results showed that NIPT showed good efficacy in trisomy 21, trisomy 18 and sex chromosome abnormalities, which was in line with the results of foreign studies [5-6]. The results showed that NIPT showed high sensitivity and specificity in the detection of 21, 18, 13 and sex chromosome abnormalities. However, there were still 37 cases with false positive results of NIPT screening requiring amniocentesis, suggesting that invasive prenatal diagnosis is needed after positive NIPT screening. Therefore, NIPT technology has certain applicability in prenatal screening.

According to domestic studies, in prenatal screening for trisomy, the incidence of trisomy 21 is the highest in pregnant women with advanced maternal age, followed by trisomy 18 and trisomy 13. For women over 35 years old, according to the Maternal and Child Health Law of China, it is clearly recommended to use the traditional "intervention" prenatal diagnosis method. However, a number of clinical data have shown that the positive rate of chromosome in this population has not been significantly improved, and there is a risk of abortion. Therefore, the 2018 edition of the Preconception and antenatal Care Guidelines proposed "35-39 years old" and "age alone" as high risk factors for prenatal screening and diagnosis in women with advanced maternal age. With written informed consent, screening for fetal chromosomal abnormalities by NIPT can reduce the number of invasive tests such as amniocentesis in high-risk populations. With the liberalization of

the birth policy, the number of pregnant women with advanced maternal age has been increasing. As a non-invasive prenatal screening method, NIPT has been widely recognized for its accuracy and specificity. The results of large-scale epidemiological studies have shown that as the population of pregnant women with advanced maternal age increases, the proportion of pregnant women receiving prenatal screening also increases, a trend consistent with Western countries.

In this study, we tested 163 patients with high NIPT risk for the seven common aneuploids. We found that NIPT was the most accurate in detecting trisomy 21 with a positive predictive value of 90.2%, while NIPT was less accurate in detecting trisomy 13 with a positive predictive value of 18.2%. Some studies have suggested that screening for trisomy 13 May not be accurate because of sequencing bias due to the low content of guanine deoxyribonucleotide and cytosine deoxyribonucleotide on chromosome 13 or because of the small size of the placenta in women with trisomy 13, which releases a relatively small amount of free fetal DNA into the maternal blood. Meanwhile, we found that NIPT had the lowest positive predictive value for 45,X chromosome, which might be related to restricted placental mosaicism, low maternal serum free fetal DNA, maternal mosaicism, potential maternal tumor and non-random inactivation of X chromosome. The positive predictive value of 45, XO was the lowest (33.3%), and the positive predictive value of the other three sex chromosome abnormalities were all above 65% (46,XXY 92.9%, 46,XXX 85.7%, 47,XXY 66.7%, respectively). The causes may be related to restricted placental mosaicism, low content of cell-free fetal DNA in maternal serum, maternal chromosomal mosaicism, maternal tumor, and non-random inactivation of the X chromosome.

3.2 Combined NIPT program for the reduction of birth defects

In early studies, some pregnant women found abnormal ultrasound soft markers during prenatal examination, or had abnormal Down screening or adverse pregnancy history, because they could not accept invasive prenatal diagnosis, they refused to undergo further prenatal diagnosis, which may lead to birth defects. This study found that the positive predictive value of high-risk pregnant women combined with NIPT screening can reach 75.5%, which can effectively reduce the occurrence of birth defects. With the development of NIPT technology and its unique advantages, more and more pregnant women are willing to actively participate in prenatal screening. At the same time, through continuous and meticulous ultrasound detection, doctors can more accurately determine whether to perform invasive prenatal diagnosis. This method not only significantly reduces the psychological stress of pregnant women, but also reduces the related risks such as miscarriage, thus effectively reducing the incidence of birth defects.

3.3 Advantages of high-throughput sequencing technology and limitations of clinical application

As the current "gold standard", karyotype analysis can detect the number of chromosomes and large fragment abnormalities. However, this method has limitations in terms of time consuming, high requirement of amniotic fluid, high requirement of amniotic fluid cell culture, subjective resolution, and detection of minor chromosomal loss or duplication less than 10 Mb [7]. Therefore, the use of high-throughput sequencing technology CNV-seq combined with karyotype analysis to diagnose fetal chromosomes in this study can reduce the anxiety of pregnant women waiting for the results, and can further diagnose fetal chromosome microdeletions and microduplications, providing more accurate and comprehensive information for clinical practice. High-throughput sequencing technology has become a research hotspot due to its advantages of high throughput, speed and economy. Compared with the first-generation Sanger sequencing, high-throughput sequencing can

simultaneously detect hundreds of thousands to millions of DNA molecular sequences.

Since 2003, the Human Genome Project has spent $3 billion and has finally mapped the complete human genome for several years. However, by 2014, Illumina's HiSeqX next-generation high-throughput sequencing technology could obtain massive data equivalent to 16 times the whole human genome in only 3 days [8]. These results show that high-throughput sequencing technology has significant advantages in terms of speed, sensitivity, and information acquisition. Therefore, in the field of prenatal screening and prenatal diagnosis in the future, high-throughput sequencing technology will continue to play an important role in providing more accurate and comprehensive information for clinical medicine, promoting early intervention and treatment, and improving the prognosis and quality of life of patients. However, it should be noted that there are some limitations of high-throughput sequencing technology. For example, in the samples of chromosome balanced heterotopia and pericentric inversion, high-throughput sequencing methods cannot detect any abnormality because there is no chromosome gain or loss, and other detection methods such as karyotype analysis are needed to confirm this situation [9]. In addition, the high cost of equipment and reagents, and the complexity of data processing and analysis lead to the relatively high cost of using high-throughput sequencing in prenatal screening. However, it is still difficult to determine the functional impact and pathogenicity of some genetic variants due to its inability to cover all variant types and the complexity of the results, which may lead to the risk of misinterpretation and misinterpretation. Although there are still some limitations of current high-throughput sequencing technology, with the continuous progress of technology and the deepening of research, these limitations are expected to be gradually overcome and improved, and its potential and application prospects will become increasingly apparent.

4 Outlook

In summary, high-throughput sequencing, as a molecular biology technology of great significance, has an innovative impact on the development of precision medicine and the field of prenatal screening and prenatal diagnosis. With the continuous progress and widespread application of high-throughput sequencing technology, its cost will also gradually decrease, which will enable more and more patients to benefit from prenatal screening and prenatal diagnosis through high-throughput sequencing [10]. However, the current problem is that the increasing amount of sequencing data has exceeded the human ability to interpret various genetic variants and chromosomal abnormalities. Therefore, we need to further improve the level of analysis, mining, verification and update of the huge sequencing data, so as to be more effectively applied in the field of prenatal screening and diagnosis, so as to reduce the incidence of birth defects and improve the quality of the population.

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