High-Throughput Sequencing of Plasma Free DNA In the Second Trimester for Non-Invasive Prenatal Testing of 21-Trisomy Syndrome

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Abstract. Objective: To investigate the application of fetal free DNA (cfDNA) in the peripheral blood of pregnant women to the diagnosis of non-invasive prenatal bowel syndrome (21-trisomy). Methods: 1735 cases of single-pregnant pregnant women with 12-24 weeks of pregnancy were collected, and peripheral blood DNA was extracted. The new generation high-throughput sequencing technology was used for detection and bioinformatics analysis, and amniotic fluid or umbilical cord was selected for 21-three high-risk pregnant women. Blood karyotype analysis was performed. Result: In the 1735 specimens, 12 cases of 21-trisomy positive samples were detected by the new non-invasive prenatal technique, and 12 cases were confirmed by karyotype analysis. The sensitivity and specificity of the non-invasive prenatal technique for the 21-trisomy detection were 100%. Conclusion: The clinical non-invasive prenatal 21-trisomy diagnosis of cfDNA has broad application prospects in prenatal screening.

1. Introduction
In China, the birth defects of newborns are as high as 800,000 to 1.2 million per year, and the incidence of birth defects is about 4% to 6%, which brings heavy mental and economic burden to families and society. At present, the most effective response to neonatal birth defects is to conduct screening and diagnosis as soon as possible. The timely termination of pregnancy after discovery is an effective way to reduce the incidence of neonatal birth defects. Invasive prenatal diagnosis methods such as amniocentesis, villous cell method and fetal cord blood method are commonly used in the fetus and pregnant women, and non-invasive prenatal testing methods such as ultrasound and serological screening. Sensitivity and accuracy are poor. Therefore, the development of new non-invasive prenatal testing methods has important clinical significance and has been one of the focuses of the current international medical genetics and reproductive medicine community.

According to statistics [1], China is a high-incidence country with birth defects, accounting for 4% to 6% of the national birth population. At the same time, according to research reports by Feng Xinglin [2], 21-trisomy syndrome, 18-trisomy syndrome and 13-trisomy syndrome are the most common chromosomal diseases in clinical practice. Among them, 21-trisomy syndrome is also called Down's syndrome, and the cause is due to the addition of a chromosome 21 in the fetal somatic cells. Some studies [3,4] have shown that 21-trisomy syndrome is more common in older women, the incidence rate is about 0.13% to 0.17%, and it is pointed out that children may have more serious mental retardation, accompanied by congenital heart disease. Other types of malformations, in
addition to this increase the probability of suffering from leukemia. To date, there is no effective treatment for such diseases. In this regard, Wang Yan [5] and other believe that prenatal screening, non-invasive DNA testing and prenatal diagnosis can be detected early, and timely intervention is an effective means to prevent the birth of children. Among them, non-invasive DNA prenatal testing is used as a new screening technique in clinical practice. Clinical applications such as Yang Xingkun [6] and Bian Xuming [7] have shown that the non-invasive DNA prenatal testing technology has compared the positive detection rate of fetal 21-trisomy syndrome with the level of invasive interventional diagnosis, and has the advantage of non-invasive. In view of this, in order to further enhance the accuracy and reliability of existing clinical research data, this paper will focus on the application value of non-invasive DNA prenatal testing in high-risk pregnant women with 21-trisomy syndrome. In this study, we used the high-throughput sequencing technology of pregnant mother plasma free DNA to explore its effect and feasibility for non-invasive prenatal diagnosis of T-21S.

2. Information and methods

2.1. General information

1735 pregnant women who received non-invasive prenatal testing in our hospital from October 2017 to September 2018 were all singletons, aged 19-40 years, and gestational weeks 12-24w. 5 mL of venous blood of pregnant women was collected, EDTA anticoagulated, centrifuged at 4 °C, 1600 × g for 10 min, plasma was collected, centrifuged at 4 °C, 16000 × g for 10 min, the upper plasma was aspirated, and stored at -80 °C. The above operation was completed within 8 hours of blood drawing. Specimen collection was informed by the subjects and approved by the ethics committee of the hospital.

2.2. Plasma DNA purification and high-throughput sequencing library preparation

2.2.1. Plasma DNA extraction. The DNA in each of the 600 μL plasma samples was extracted according to the QI Aamp DNA MicroKit (Qiagen, Germany) kit instructions. PCR was used to detect whether DNA extraction was successful. GAPDH (GenBank SEQ ID NO: 163954974) gene fragment was selected as the detection target fragment, designed with Oligo6.0 software, upstream primer sequence: 5'-CAATGA CCCCTTCATTGACCT-3', downstream primer sequence: 5'-AGCATCGCCCCACTTGATT-3'; PCR reaction system 15 μL, including: 1.5 μL of 10×r Taq DNA buffer, 1.5 μL of each of 4 dNTP mixtures, 3 pmol of upstream and downstream primers, 5 μL of plasma DNA template, 0.5 μL of r Taq DNA polymerase, and 15 μL of ddH2O. Cycle parameters: 98 °C 2 min; 98 °C 30 s, 54 °C 30 s, 72 °C 30 s, a total of 35 cycles; 72 °C 5 min. The product was electrophoresed on a 20 g/L agarose gel, stained with ethidium bromide (EB) and observed under UV light.

2.2.2. High-throughput sequencing library preparation. The library was prepared to add a DNA fragment for sequencing to both ends of the DNA molecule [8]. The main process is as follows: (1) End repair. T4 DNA polymerase, Klenow DNA polymerase, and T4 polynucleotide kinase repair the extracted plasma DNA fragments into blunt ends; (2) 3' plus base "A". The 3' end of Klenow DNA polymerase adds base "A" to allow the DNA fragment to be ligated to a specific linker with a "T" base at the 3' end; (3) A linker. T4 DNA ligase ligates the linker to the DNA fragment; (4) PCR amplification. The DNA of the ligation reaction was completed, and PCR amplification was carried out according to the instructions of the Multiplexing Sample Preparation Oligonude Tit Kit (Illumina), and the DNA molecule of the ligated linker sequence was enriched by PCR and the content was adjusted to the concentration of Illumina. The PCR amplification products were purified according to the Ampure Beads kit instructions to remove the mixed primer dimers in the amplification product mixture.
2.2.3. **Quality Control of High-throughput Sequencing Libraries.** The prepared library was analyzed according to the Agilent Bioanalyzer 2100 Biochemical Analyzer's operating manual, and the DNA fragment size and concentration were measured, and the prepared library concentration was accurately quantified by Real-time quantitative PCR.

2.3. **High-throughput sequencing reactions**

Using a one-way sequencing strategy, the sequencing length was 36 bp. According to the literature [9], multiple samples were simultaneously sequenced in one reaction using the sequence tag method to reduce the cost of sequencing and detection of each sample.

2.4. **Sequencing data processing and quality control standards**

The raw data obtained by sequencing is not fault-tolerantly aligned by ELAND software (Efficient Large-Scale Alignment of Nucleotide Databases), and a DNA sequence that can be aligned to a unique position on the reference sequence of the de-repetitive sequence is obtained as a unique sequence (Unique reads). The raw data of each sample shall not be <5×10⁶ reads, and the unique sequence obtained after the comparison shall not be <2×10⁶ reads.

2.5. **Sequencing and data analysis**

Illumina's next-generation sequencing technology is sequenced, using patented chips, preparing DNA clusters, sequencing while synthesizing, automatically reading bases, and transferring data to an automated analysis channel for secondary analysis, with large-scale, high-throughput, parallel sequencing Unique advantages. All free DNA in the blood of pregnant women, regardless of their source, is sequenced to obtain the actual number of nucleic acid fragments distributed on each chromosome. According to bioinformatics analysis, the corresponding depth of coverage of each chromosome is calculated and converted into a risk. The risk index determines the risk of fetal suffering from Down's syndrome. The proportion of total DNA of chromosome NDNA in the test sample can be expressed as: coverage depth Cov-chrN. The GC-associated T-test method independently developed by Huada Gene was used to detect whether the coverage depth Cov-chrN of...
the test sample and the standard reference sample coverage depth Cov-chrN were significantly
different to determine whether the fetus was Down's syndrome. The value of the statistical test was T.
Value, calculation of T value: coverage depth Cov-chrN = total number of valid sequencing fragments
on sample chromosome N / total number of valid sequencing fragments on reference sequence
chromosome N; correlation of test sample ChrN T-Score = (cover depth of sample - reference sample
Correct coverage depth) / standard deviation of reference samples. Compared with the T-mean and
standard deviation of chr21 in the maternal plasma of gestational fetuses, the T value of chr21 in the
plasma of pregnant women with 21-trisomal fetus is higher, and the T value of chr21 of the test
sample is ≥ 3 down syndromes is at high risk.

Fig. 2 Sequencing and data analysis

3. Result

3.1. Non-invasive and amniotic fluid test results
A total of 1735 eligible clinical samples were collected in this study, of which 632 were high-risk
screening for serology (risk value ≥ 1/270) and 620 were critical for serological screening (1/270 to
1/1000). Serum screening was not performed in 483 patients. After non-invasive genetic testing, 12
cases of 21-trisomy positive samples were detected, of which 7 were high-risk screening for serology,
3 were critical for serological screening, and 2 were not screened for serology. Twelve cases of 21-
trisomy positive samples were all diagnosed by karyotype analysis. The results are shown in Table 1.

| Prenatal screening risk value during routine pregnancy | Number of cases | 21-three-body non-invasive detection number (n) | 21-trisomy type confirmed number (n) | Positive rate (%) |
|-------------------------------------------------------|----------------|---------------------------------------------|---------------------------------|------------------|
| ≥1/270                                                 | 632            | 7                                          | 7                              | 1.11             |
| 1/270~1/1000                                          | 620            | 3                                          | 3                              | 0.48             |
| Unscreened pregnant women                              | 483            | 2                                          | 2                              | 0.41             |
3.2. 21-trisomy distribution of different age groups

In the 1735 clinical samples of this trial, the age distribution was 19-40 years old, and 3 of the 12 21-trisomy positive samples were older than 36 years old, and 4 were aged 26-30 years old. Table 2 shows the 21-trisomy detection at each age.

| Generation | Total (n) | 21-three-body non-invasive detection number | 21-three-body karyotype diagnosis | Positive rate (%) |
|------------|----------|--------------------------------------------|-----------------------------------|------------------|
| <20        | 59       | 1                                          | 1                                 | 1.69             |
| 21-25      | 401      | 2                                          | 2                                 | 0.50             |
| 26-30      | 655      | 4                                          | 4                                 | 0.61             |
| 31-35      | 371      | 2                                          | 2                                 | 0.54             |
| ≥36        | 249      | 3                                          | 3                                 | 1.20             |

3.3. Authenticity evaluation

At present, the karyotype analysis method is the gold standard for prenatal diagnosis of DS. In this experiment, 12 cases of 21-trisomy positive samples detected by non-invasive samples were all analyzed for amniotic fluid or umbilical cord blood karyotype, and the remaining 1723 cases were confirmed by follow-up. For trisomy patients, the karyotype results are shown in Table 3. In this experiment, the sensitivity of non-invasive prenatal genetic testing to DS detection was 100% and the specificity was 100%.

| Serial number | Karyotype analysis result       |
|---------------|--------------------------------|
| 1-10          | 47, XN, +21                    |
| 11            | 47, XN, 15p+, +21              |
| 12            | 46, NX, der (15; 21) (q10; q10), +21 |

4. Discussing

According to the literature [10], the incidence of 21-trisomy syndrome in live infants is 1/600 to 1/800, which is a common chromosomal disease in newborns. At the same time, relevant data [11] showed that about 60% of the children died in the early pregnancy, while the surviving children showed severe congenital mental retardation, abnormal face, growth and development disorders, and more with congenital heart disease. And so on. It can be seen that prenatal screening or even diagnosis of 21-trisomy syndrome is of great significance. In the past, the accuracy of the results was only 70% to 80% due to the use of screening for Down's syndrome in the second trimester or a few hospitals using Down's syndrome screening in early pregnancy [12]. In addition, according to Ye Yuhua [13] and other studies, the traditional clinical screening of 21-trisomy syndrome mainly uses invasive methods, which have different degrees of damage to pregnant women and fetuses, resulting in adverse consequences such as miscarriage and intrauterine infection. Therefore, how to reduce the false positive rate of 21-trisomy syndrome, and thus reduce the pain and intrauterine infection that may be caused by interventional prenatal diagnosis is a serious and difficult problem.

In this study, 1735 cases of pregnant women's peripheral blood free DNA were detected by high-throughput sequencing technology of Huada Gene, and 12 cases of 21-trisomy high risk were detected. The positive rate was 0.7%, which was lower than the high-risk group of serum screening in mid-pregnancy for amniocentesis. The incidence of chromosomal abnormalities was 2.14%, which may be related to the selection of the population in this study and only the 21-trisomy abnormalities. In this study, 3 cases of 21-trisomy were detected as the critical risk of serological screening (1/270~1/1000), and the positive rate was 0.48%, which was higher than the incidence rate of 1/600~1/800, suggesting that pregnant women with a critical risk of serological screening (1/270~1/1000) need
to pay attention to the follow-up prenatal examination, the conditions allow for further non-invasive genetic testing. The 12 cases of 21-trisomy pregnant women detected in this study were between 19-36 years old, with an average age of 27 years, of which 9 cases were <35 years old, indicating that the incidence of 21-trisomy syndrome was younger. Trends, this result is consistent with previous reports. Twelve cases of 21-trisomy high-risk samples were diagnosed by karyotype analysis, and the remaining 1723 cases were all non-trisomy 21 patients. The sensitivity and specificity of the test results were 100% and 100%, respectively. Kappa test for the control showed that the consistency of the two methods was good. Therefore, choosing a rapid, effective, non-invasive detection method to expand the scope of prenatal diagnosis is of great significance in reducing the birth rate of DS patients.

Of course, Illumina sequencing technology also has certain limitations. The free DNA in the peripheral blood of pregnant women with NIPT sequencing includes both the free DNA of the pregnant woman and the free DNA of the fetus. Therefore, the interference of the maternal background cannot be excluded, and there will be some false positives and false negatives. For pregnant women with fraternal twins, the NIPT results suggest a high risk. The abnormal chromosome belongs to which of the twins or the chromosomes of the twins are abnormal and indistinguishable. The concentration of fetal free DNA in the peripheral blood of twin pregnant women is also different from that of singleton, and the test conditions need to be changed. Since non-invasive DNA detects fragments of base sequences in free DNA, chromosomal aneuploidy abnormalities can be detected, but balanced translocations and chimeras, circular chromosomes, etc., which do not increase or decrease the number of baseless sequences, cannot be accurately detected. Chromosomal abnormalities. For pregnant women who have received stem cell therapy, transplant surgery, and allogeneic blood transfusion, the introduction of exogenous DNA may also lead to false positive or false negative results. Although the current study shows that NIPT has a very high detection rate in twin-sex screening, and the false positive rate is extremely low, the clinical situation of twin pregnancy is more complicated, and more evidence is needed for verification.

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