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ORIGINAL ARTICLE

Application of Non-Invasive Prenatal Tests in Serological Preclinical Screening for Women with Critical-Risk and Low-Risk Pregnancies but Abnormal Multiple of the Median Values

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SUMMARY

Background: **To explore the clinical value of secondary screening using noninvasive prenatal testing (NIPT) for women with critical-risk and low-risk pregnancies who had multiple of the median (MoM) abnormalities in serological screening.**
Methods: **NIPT was used to analyze fetal free DNA in the peripheral blood of 2,325 women with critical-risk pregnancies and 239 women with low-risk pregnancies with MoM abnormalities in serological screening. Based on NIPT results, women with high-risk pregnancies were recommended for amniocentesis for fetal karyotype analysis.**
Results: **Among 2,325 women with critical-risk pregnancies as determined by serological screening, NIPT indicated 15 high-risk pregnancies (11 cases of trisomy 21 and 4 cases of trisomy 18). Of the 15 patients, 1 case refused prenatal diagnosis. The other 14 cases underwent invasive amniocentesis for fetal karyotype analysis, and 13 cases of fetal chromosomal abnormalities were diagnosed, including ten cases of trisomy 21 and three cases of trisomy 18. NIPT of 239 patients with low-risk pregnancies but abnormal MoM showed one case with a high risk of trisomy 21, which was diagnosed as a false positive by amniotic fluid karyotype analysis, and one case with a high risk of trisomy 13 (stillbirth) that was diagnosed by karyotype analysis. A case of gender chromosome abnormality was diagnosed as aneuploidy by karyotype analysis.**
Conclusions: **The application of NIPT as a secondary screening for women with low- and critical-risk pregnancies as determined by serological screening but with MoM abnormalities will greatly reduce the number of invasive prenatal diagnosis procedures, significantly improve the rate and accuracy of fetal chromosomal abnormality detection.**
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KEY WORDS

NIPT, serological screening, critical risk, chromosome abnormalities, MoM

INTRODUCTION

Fetal chromosomal abnormalities are important causes of neonatal birth defects. Throughout the world, about five million children with birth defects are born every year. Genetic factors cause about 70% of major birth defects [1]. Chromosome abnormalities and gene mutations are the main causes of these hereditary diseases. At present, there are no effective methods to treat chromosomal diseases. Prenatal screening is the main way to screen women with high-risk pregnancies to make prenatal diagnoses and allow early detection and termination of pregnancies, to prevent the birth of children with chromosomal diseases. The traditional prenatal screening method is serological screening. At present, maternal serum markers including pregnancy associated plasma protein-A (PAPP-A), free β-human chorionic gonadotropin (β-hCG), and fetal nuchal translucency (NT) in the first trimester, or alpha fetoprotein (AFP), free β-hCG, and unconjugated-estriol (μE3) in the second trimester are used as first-line serological screening programs. The serological screening method is simple and low-cost, but the rates of false positives are high and the detection rate is low [2]. Higher false-positive rates and lower detection rates are bound to cause many unnecessary prenatal diagnosis procedures using invasive amniocentesis and the birth of children with Down syndrome (DS) or Edwards syndrome (ES). At the same time, they increase the risk of amniocentesis abortion. Women with critical-risk or low-risk pregnancies and abnormal multiple of the median (MoM) values identified by serological screening are generally not considered to have high-risk pregnancies, and they are often reluctant to have chromosome karyotype analysis of the fetuses by invasive amniocentesis. Compared with traditional serological screening, noninvasive prenatal testing (NIPT) has been welcomed by pregnant women and clinicians for its high detection rate, low false-positive rate and non-invasiveness [3,4]. However, the price of the technology for NIPT is still on the high side, and there are still problems in promoting NIPT without the support of the government and management departments. The purpose of this study was to explore the clinical value of NIPT in secondary screening of pregnant women with critical-risk or low-risk pregnancies but abnormal MoM values after serological screening.

MATERIALS AND METHODS

Subjects

Subjects included pregnant women who were selected for serological screening in early pregnancy (11 - 13^{+6} weeks) or mid-pregnancy (15 - 20^{+6} weeks) from April 2017 to July 2019. A total of 2,325 cases were women with serological screening results of critical-risk pregnancies and another 239 cases were women identified with low-risk pregnancies but abnormal MoM. Women were excluded if they had a family history of congenital malformations or if abnormal results of the fetus were found by ultrasound. The NIPT test was conducted according to the informed voluntary principle. The karyotype analysis of amniocentesis was performed on women with high-risk pregnancies based on NIPT results. The women with low-risk pregnancies continued their pregnancies without amniocentesis, and all pregnant women were followed up for the duration of their pregnancies. Follow-up methods included telephone follow-up and tracking of local health information systems. This study was reviewed by the Hospital Ethics Committee and informed consent of the subjects was obtained.

Serological screening

Two to three milliliters of fasting venous blood was collected from each pregnant woman, using a non-anticoagulated vacuum tube, and naturally solidified at room temperature. Samples were centrifuged (1,600 g for 10 minutes) within two hours and the serum was collected and stored at -20°C. The serum markers for women who were 11 - 13^{+6} weeks pregnant were PAPP-A and free β-hCG. AFP, free β-hCG, and μE3 were detected in serum of women who were 15 - 20^{+6} weeks pregnant. Time-resolved fluorescent immunoassay (TRFIA) was used to detect the levels of PAPP-A, AFP, β-hCG, and μE3 in strict accordance with the instructions of the kit (PerkinElmer, Turku, Finland). Each batch of experiments used Bio-Rad (CA, USA) products for high-, medium-, and low-quality control. The instruments used and the kits tested were from PerkinElmer. Using combined clinical data of the pregnant women, including age, weight on the day of the blood draw, the gestational weeks, and the number of fetuses indicated by ultrasonography, the risk assessment and MoM correction of trisomy 21 (T-21) and trisomy (T-18) were carried out using LifeCycle 4.0, a data management software for prenatal screening. MoM value refers to the test of serum markers in pregnant women in prenatal screening, which is the number of times the concentration median of serum markers in normal pregnant women during the gestational age. The high-risk cutoff value of T-21 was ≥ 1/270, and the critical-risk cutoff value was 1/1,000 ≤ T-21 risk value < 1/270. The high-risk cutoff value of T-18 was ≥ 1/350, and the critical-risk cutoff value was defined as 1/1000 ≤ T-18 risk value < 1/350. PAPP-A-MoM and β-hCG-MoM values < 0.5 or > 2.5 were judged as abnormal. AFP-MoM values of < 0.7 or > 2.5 were judged as abnormal, and μE3-MoM values of < 0.65 were judged as abnormal. In addition, the fetal nuchal translucency (NT) was measured by ultrasonography in women who were 11 - 13^{+6} weeks pregnant. The NT measurement method is the fetal NT measured by ultra-

sound when the fetal crown-rump length (CRL) is 45 - 84 mm.

Noninvasive prenatal testing (NIPT)

Five milliliters of peripheral blood was collected from each subject and anticoagulated with EDTA. After centrifugation at 1,600 g for 10 minutes at 4°C, the resulting plasma was transferred to a new 2.0 mL centrifuge tube on ice and stored at -80°C. The above steps were completed within 8 hours. DNA was extracted using a FlexiGene DNA kit (QIAGEN, NY, USA). The obtained free DNA was end-filled, added with "A," ligated with a linker, and PCR-enriched to obtain a DNA library of the corresponding samples. Several libraries were mixed with a mass ratio of 1:1 to establish a pooling library. The obtained pooling library was amplified by C-bot bridge reaction and detected by the Ion Proton sequencing system (Life Technologies, CA, USA). The chromosomal location of each sequencing read was determined by aligning the human genome reference sequence to the GRCH37/hg19 database. The results were interpreted with reference to public databases (Decipher, OMIM, Clinva, ISCA) and analyzed for genotype-non-type correlation. Using the instrument pairing software "Noninvasive prenatal data analysis software system," the Z value was automatically calculated as the difference between the ratio of the test chromosomes to the population of the test population divided by the variance of the chromosomes to be tested in the control group. A result of $Z > 3$ was judged to be high-risk (positive). Amniotic fluid puncture was recommended to confirm the diagnosis. For the critical values $1.96 < Z < 3$, blood samples were taken again. The Z value < 1.96 was judged to be low-risk (negative), and subjects with these values entered the routine pregnancy examination process.

Karyotype analysis

Patients with high risk of NIPT underwent amniocentesis at 16 - 24 weeks of pregnancy. Following informed consent, 15 - 20 mL of amniotic fluid were taken for karyotype analysis. After centrifugation, 0.5 mL of precipitate was taken, mixed, transferred to a flask supplemented with amniotic fluid medium, and placed in an incubator at 37°C and 5% carbon dioxide for open culture. After changing the medium for a suitable number of days, culture was carried out to observe cells from the amniotic fluid. At this time, a certain amount of colchicine was added to the culture flask, so that growth of the amniotic cells was stopped in the middle stage of cell division, and then the amniotic cells were fixed, produced, banded, and stained after incubation at 37°C in hypotonic solution. The amniotic fluid cell specimens were visually examined microscopically for karyotype analysis.

Statistical methods

Statistical analyses were performed using SPSS 20.0 statistical software. The measurement data were expressed by mean and standard deviation ($x \pm s$); the count data were expressed by case (%).

RESULTS

The screening results and analysis of each age group are shown in Table 1. Among 2,325 pregnant women deemed as critical risk by serological screening and 239 women with low-risk MoM abnormalities, NIPT results showed that the high-risk positive rates of T-21 and T-18 screening showed an increasing trend with increasing expected age at delivery. Pregnant women with fetal chromosomal abnormalities confirmed by subsequent karyotype analysis of invasive amniotic fluid cells were between 25 and 35 years old. Among them, the number of high-risk positives for T-21 and T-18 increased for pregnant women over 30 years old. In this study, the positive rate of high risk of fetal chromosomal aneuploidy in pregnant women younger than 20 years old was 0.00%.

The NIPT screening results are shown in Table 2. NIPT of 2,325 pregnant women serologically screened as critical risk indicated a total of 15 high-risk pregnant women, including 11 cases of T-21 and 4 cases of T-18. The positive rate was 0.65% (15/2,325, see Table 2). One case with high risk of T-21 indicated by NIPT refused prenatal diagnosis. This woman was 35 years old, had five previous pregnancies and three births, with three normal live female infants. She was followed up continuously with standard care. The other 14 of the 15 high-risk cases underwent invasive amniocentesis for fetal karyotype analysis. Thirteen cases of fetal chromosomal abnormalities were diagnosed, including ten cases of T-21 and three cases of T-18. The positive predictive values were 100% and 75%, respectively. Among 239 pregnant women with low-risk MoM abnormalities, NIPT indicated one case with high risk of T-21 had a low PAPP-A-MoM value abnormality, which was a false positive by amniotic fluid cell karyotype analysis, and one case with high-risk of trisomy 13 (stillbirth induction) also had a low PAPP-A-MoM value abnormality. Another case of NIPT suggesting gender chromosome aneuploidy was diagnosed as having a low AFP-MoM value. The positive rate for the women with low-risk MoM abnormalities was 1.26%, and the detection rate was 0.84% by the karyotype analysis of invasive amniotic fluid cells. There were no abnormalities in the follow-up of other low-risk pregnant women. Among all the subjects, NIPT identified a total of 18 high-risk cases, with a positive rate of 0.70% (18/2,564), and 17 of these cases were diagnosed by amniotic fluid cell karyotype analysis (see Table 3), with a consistency of 88.24% (15/17) and a detection rate of 0.59% (15/2,564).

Table 1. Distribution of fetal chromosomal abnormalities.

Age (years)	21-trisomy (n)		18-trisomy (n)		13-trisomy (n)		Gender chromosome (n)	
	NIPT high-risk	Karyotype analysis	NIPT high-risk	Karyotype analysis	NIPT high-risk	Karyotype analysis	NIPT high-risk	Karyotype analysis
17 - 19	0	0	0	0	0	0	0	0
20 - 24	1	1	1	0	0	0	0	0
25 - 29	4	4	1	1	1	1 [b]	0	0
30 - 35	7 [a]	5	2	2	0	0	1	1
Total	12 [a]	10	4	3	1	1 [b]	1	1

[a] - 1 case refused amniocentesis, [b] - stillbirth, NIPT - noninvasive prenatal testing, n - number.

Table 2. Distribution of NIPT screening and confirmed pregnant women.

Abnormal group	Gestational weeks	Number	NIPT		Confirmed (n)
			Positive (n)	Positive rate (%)	
Critical-risk	11 - 20^{+6}	2,325	15 [a]	0.65	13
PAPP-A MoM	11 - 13^{+6}	69	2	2.9	1 [b]
Free β-hCG MoM	11 - 20^{+6}	75	0	0	0
AFP MoM	15 - 20^{+6}	61	1	1.64	1
μE3 MoM	15 - 20^{+6}	34	0	0	0
Total		2,564	18 [a]	0.7	15

[a] - 1 case refused amniocentesis, [b] - stillbirth, MoM - multiple of the median, PAPP-A - pregnancy associated plasma protein-A, β-hCG - β-human chorionic gonadotropin, AFP - alpha fetoprotein, μE3 - unconjugated estriol.

DISCUSSION

At present, fetal birth defects are still a worldwide problem, with many countries having high incidences of birth defects. The extensive development of traditional serological screening has greatly contributed to the reduction of the birth of infants with chromosomal abnormalities such as T-21 and T-18. The most common and effective early pregnancy screening program of the combined screening of PAPP-A, free β-HCG, and NT [5] and the combined screening of AFP and β-HCG [6] are mostly used between 15 - 20 weeks of pregnancy. However, even if all pregnant women with high-risk pregnancies follow traditional serological screening with invasive amniocentesis for fetal karyotype analysis, there is still a 5% to 50% missed diagnosis rate [7]. Some scholars have suggested that missed diagnosis occurs mostly in pregnant women whose serum screening results are at critical risk. Lewis C et al. [8] found that 20.1% of the pregnant women with critical-risk pregnancies felt anxious and hoped to obtain safe and accurate further examinations. If all pregnant women with critical-risk pregnancies undergo fetal karyotype analysis through invasive amniocentesis, there will be many unnecessary invasive prenatal procedures. There are also risks of fetal abortion and maternal complications in amniocentesis. Therefore, many pregnant women do not consider invasive prenatal diagnosis as their primary choice. In some areas, the acceptance rate of amniocentesis in pregnant women is less than 50% [9].

NIPT can detect fetal chromosomes through maternal cell-free fetal DNA (cffDNA). Because of its high sensitivity and specificity, NIPT is widely used in clinical detection of common aneuploidies [10-12]. As opposed to invasive prenatal diagnosis, NIPT can avoid the risk of fetal loss and infection caused by puncture [13,14]. In this study, 2,325 pregnant women with critical-risk pregnancies as determined by serological screenings had a secondary screening by NIPT of peripheral blood during early- and mid-pregnancy. The results showed that 15 pregnant women had high-risk pregnancies. Among them, 11 cases were T-21 (1 case refused amniocentesis) and 4 cases were T-18. Fourteen pregnant women underwent invasive amniocentesis for fetal kar-

Table 3. Results of abnormal karyotype analysis of fetal chromosomes.

Number	Pregnancy (days)	Age (years)	Group	Risk assessment	NIPT (high-risk)	Karyotype analysis	Pregnancy outcome
1	90	28.3	Critical risk	1:417 (T-21)	T-21	47, XN, 21	Termination
2	92	25.7	Critical risk	1:320 (T-21)	T-21	47, XN, 21	Termination
3	89	34.7	Critical risk	1:637 (T-21)	T-21	47, XN, 21	Termination
4	91	32.8	Critical risk	1:297 (T-21)	T-21	47, XN, 21	Termination
5	88	24.1	Critical risk	1:497 (T-21)	T-21	47, XN, 21	Termination
6	86	30.2	Critical risk	1:717 (T-21)	T-21	47, XN, 21	Termination
7	90	32.3	Critical risk	1:552 (T-21)	T-21	47, XN, 21	Termination
8	91	27.6	Critical risk	1:637 (T-21)	T-21	47, XN, 21	Termination
9	123	28.3	Critical risk	1:835 (T-21)	T-21	47, XN, 21	Termination
10	120	31.2	Critical risk	1:473 (T-21)	T-21	47, XN, 21	Termination
11	127	35.2	Critical risk	1:905 (T-21)	T-21	Refused amniocentesis	Follow-up
12	130	23.6	Critical risk	1:573 (T-18)	T-18	No abnormalities	Continued
13	133	27.6	Critical risk	1:657 (T-18)	T-18	47, XN, 18	Termination
14	118	35.2	Critical risk	1:451 (T-18)	T-18	47, XN, 18	Termination
15	90	34.5	Critical risk	1:533 (T-18)	T-18	47, XN, 18	Termination
16	89	28.6	PAPP-A MoM	Low-risk (MoM:0.32)	T-13	47, XN, 13	Stillbirth
17	90	33.4	PAPP-A MoM	Low-risk (MoM:0.43)	T-21	No abnormalities	Continued
18	136	33.2	AFP MoM	Low-risk (MoM:0.47)	Gender chromosome	47, XXY	Termination

T-21 - 21-trisomy, T-18 - 18-trisomy, T-13 - 13-trisomy, MoM - multiple of the median, PAPP-A - pregnancy associated plasma protein-A, β-hCG - β-human chorionic gonadotropin, AFP - alpha fetoprotein, μE3 - unconjugated estriol.

yotype analysis and 13 cases were confirmed, of which 10 cases were T-21 and three cases were T-18. There was one false positive with low risk but abnormal MoM in the group of 239 pregnant women (NIPT of a low-risk PAPP-A-MoM pregnant woman indicating a T-21 high risk was a false positive, as determined by amniotic fluid karyotype analysis). The coincidence rate of T-21 was 90.9% (10/11), and the coincidence rate of T-18 was 75% (3/4), which were similar to those reported by other scholars [15], lower than the 99% reported by Bianchi DW [16] and Liao C [17]. Possibly because of the small number of positive results in this study, there were no T-21 false negative cases and fewer NIPT false negative cases.

This study confirmed that NIPT can be used for further and more accurate screening beyond serological screening. With a very low false-positive rate, almost all fetuses with T-21 and most of those with T-18 can be identified. Use of NIPT can greatly reduce the rate of amniocentesis, improve the accuracy of diagnoses, and significantly reduce the rate of missed diagnoses. It is worth noting that there was still one case of NIPT T-18-positive determined to be a false positive by amniotic fluid karyotype analysis. It is speculated that the cffDNA in the peripheral blood of pregnant women is mainly derived from placental tissue [18].

In addition, in this study, 239 pregnant women with low-risk but abnormal MoM values were also screened by NIPT. The results showed that the concentration of PAPP-A in the serum of a woman diagnosed with of a fetus with trisomy 13 was lower than that of normal pregnant women at the same gestational age. The PAPP-A-MoM value was also abnormally low, and the fetus died in the later stage. The MoM value of the AFP of a woman diagnosed with a potential fetal gender chromosome abnormality was also abnormally low. It has been suggested that the low MoM values of PAPP-A and AFP may be used as a reference indicator for detecting adverse pregnancy, which is consistent with the report by Ozkan S et al. [19]. Fetal PAPP-A or AFP levels are lower than those of pregnant women at the same gestational week. These may be due to birth defects and fetal liver dysplasia, resulting in a decrease in the synthesis of PAPP-A and AFP levels, thus reducing mater-

nal blood levels of PAPP-A and AFP. It is noteworthy that the probability of chromosomal diseases in the fetus, especially T-21 and T-18, increases with the age of the pregnant women. Especially for pregnant women over 30 years old, the incidence increases. Studies have shown that pregnant women over 35 years of age have a significantly higher risk of having children with DS than do pregnant women between 25 and 29 years of age [20]. In this study, the positive rate of pregnant women over 30 years old for either T-21 or T-18 was higher than that of pregnant women under 30 years old. Most of the chromosomal abnormalities identified by amniocentesis karyotype analysis were found for older pregnant women. The reason may be that the proportion of older pregnant women is increasing. With increasing age, the oocytes are aging. The age of the pregnant woman with a potential fetal gender chromosome abnormality in this study was also over 30 years. Therefore, we should pay attention not only to the risk value of screening, but to pregnant women with low-risk pregnancies but abnormal MoM values, so as to avoid missed diagnoses. Sometimes, clinicians tend to pay more attention to the risk of increased MoM values, but because of the low risk of serological screening, some pregnant women with low MoM values are neglected, especially if they are older. Because low-risk MoM abnormalities seem to be more sensitive to other chromosomal abnormalities except trisomy 21 and trisomy 18, while serological screening risk values target trisomy 21 and trisomy 18. Therefore, these pregnant women may not be at high risk. If these women have no further NIPT test, fetal chromosomal abnormalities will likely be missed.

In summary, women with critical-risk pregnancies are the main group with missed diagnoses, indicating the necessity of using NIPT technology for secondary screening for pregnant women whose serological screening indicates a critical-risk or low-risk MoM value. NIPT not only improves the detection rate of fetal birth defects but also greatly improves the accuracy of detection. In the final analysis, 17 cases in this study required amniocentesis, giving a greatly reduced puncture rate of only 0.66% (17/2564).

Limitations of this study

This study failed to confirm amniocentesis for all women with high-risk pregnancies by NIPT, and the results were not completely correct. Another factor is that NIPT has limitations on the detection of abnormal chromosome structure. There may be some missed diagnoses or misdiagnoses, and the diagnosis by invasive prenatal procedures is still needed [21].

CONCLUSION

The application of NIPT as a secondary screening for women with low-risk pregnancies but low PAPP-A-MoM or AFP-MoM abnormalities will greatly reduce the number of invasive prenatal diagnosis procedures and significantly improve the detection rate of fetal chromosomal abnormalities.

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Declaration of Interest:
The author has no conflict of interest.

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