Original Article
Non-invasive prenatal DNA testing for genomic copy number variations

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Abstract: Objective: To investigate the clinical significance of non-invasive prenatal testing (NIPT) of genomic copy number variation (CNV) for screening chromosomal micro-deletion/micro-duplication syndromes. Methods: 14235 pregnant women were enrolled in the NIPT. The NIPT detection results revealed the presence of genomic CNV in pregnant women who were informed of the detection results and performed confirmatory amniocentesis after they had provided informed content. The CNV results were further confirmed by the G-band karyotyping and Chromosomal Microarray Analysis (CMA), followed by routine follow-ups to all the cases undergone the NIPT. Results: Among the total 14235 NIPT-detected samples, 24 cases were detected in the presence of CNVs, 15 cases (4 cases with micro-deletion and 11 cases with micro-duplication) were required to undergo further prenatal diagnosis, in which 13 cases were concordant with the CMA findings, with a positive concordant rate of 86.7%. Only 7 of 13 cases were positive for chromosomal karyotyping, with a missed diagnosis rate of 46.2%. Besides, one case was confirmed with partial inversion of chromosome 9 by the G-band karyotyping. Conclusion: The high accuracy of positive CNV results by NIPT screening indicates complementary function of the NIPT.

Keywords: Non-invasive prenatal testing, copy number variation, prenatal screening, chromosome from amniotic fluid, chromosome microarray analysis

Introduction

Previous studies have revealed that genomic copy number variation (CNV) plays an important role in human phenotypic variation or diseases [4]. Partial chromosome micro-deletion or micro-duplication can cause some syndromes whose degree of disability is similar to that of Down syndrome. The common relevant syndromes include Williams syndrome, DiGeorge/velocardiofacial syndrome (VCFS), Prader-Willi syndrome, etc [5, 6]; some are even more severe, like Smith-Magenis syndrome, Miller-Dieker syndrome, and other syndromes caused by 1p and 4p micro-deletion [7]. Recently, chromosome microarray analysis (CMA) is a main diagnostic method and the "gold standard" for chromosome micro-deletion or micro-duplication diagnosis. It can not only provide a high resolution genome-wide screening for the CNV diagnosis but also detect the increase or decrease of the unknown genomic DNA fragments [8]. Thus it is of significance to discovery

and identification of gene-related diseases. However, as a conventional prenatal procedure, CMA findings must be confirmed by invasive procedures, such as the chorionic vellus sampling (CVS), amniocentesis or percutaneous umbilical cord blood sampling (PUBS) to collect fetal cells for analysis [9], which may cause abortion, infection or other complications, reducing the patients' compliance.

Non-invasive prenatal testing (NIPT) is a non-invasive prenatal screening technique, targeting at trisomy 21, 18 and 13, with an accuracy rate of approximate 100% and false positive rates < 0.1% [1-3]. Clinical findings have showed that the NIPT for fetal cell-free DNA (cfDNA) from maternal plasma could be used for genome-wide screening and partial chromosomal micro-deletion/micro-duplication (genomic copy number variation) detection. This study was designed to investigate the accuracy of NIPT in CNV screening and its clinical significance in the prevention and reduction of neo-

nates with chromosome micro-deletion/micro-duplication syndromes.

Material and methods

Subjects

During the period from October 2014 to October 2015, upon the approval of Hospital Ethics Committee on Prenatal Screening, a total of 14235 pregnant women (five patients were lost in follow-ups), ranging in age from 21 to 42 years old, (mean 31.5 years old), were enrolled to undergo the NIPT procedure for CNV detection in the Second people's Hospital of Shenzhen City. The pregnant women who suffered from chromosome abnormalities and underwent transplantation, stem cell therapy, immunotherapy within 4 weeks or infusion of allogeneic blood products within one year were excluded from the study. The included women were at the gestational age of 12 to 24 weeks, each having singleton fetus. All the subjects gave informed consent for participation in the study.

Methods

5 mL peripheral blood drawn from the pregnant women was detected by the NIPT. The pregnant women who were detected with positive chromosome micro-deletion/micro-duplication performed amniocentesis and further analyzed the results by G-band karyotyping and Chromosomal Microarray Analysis (CMA). If necessary, the pregnant women and their husbands were also required to undergo chromosome G-band karyotyping and CMA for further identifying the sources of CNVs and comprehensively evaluating the pathogenicity of CNVs, so as to assess fetal prognosis. In addition, autopsy was performed on the labor-induced fetuses and follow-ups were conducted on the women who continued their pregnancies, tracking their gestation and neonatal outcomes.

NIPT detection

5 mL peripheral blood drawn from the included pregnant women was infused into a blood collection tube containing EDTA dipotassium salt (EDTA-2K). The maternal plasma samples were centrifuged at 1600 rpm for 10 minutes, and then the centrifuged products were isolated and transferred to a new tube. The supernatant was transferred to an aseptic tube and centrifuged at 14000 rpm for 10 minutes, and the plasma was graded and stored at -80°C for future treatment. Subsequent standard methods, including non-cellular DNA detection, library construction and sequencing isolation, were performed in the clinical laboratory in BGI Shenzhen, China. The bioinformatic method was combined with the local weighted polynomial regression to eliminate the GC-bias and the binary hypothesis to obtain a higher accuracy for aneuploidy detection. In particular, a fetal copy-number analysis through maternal plasma sequencing (FCAPS) was performed to analyze fetal genomic copy numbers for micro-deletion and micro-duplication detection.

G-band karyotyping analysis

Under the supervision of ultrasonic tomography equipment, 20~30 mL amniotic fluid was extracted in an aseptic context. The peripheral blood samples were seeded in 1640 medium while the amniotic fluid samples were cultured in AmnioMAX-II medium for 9 d to 10 d. The cultured cells were collected for G-band karyotyping analysis: each specimen was counted as 25 division phases, and 5 karyotypes were analyzed. If there was a chimera, it was counted as 100 division phases. And the Leica chromosome analysis system was used to take photographs which were stored. The chromosome analyzer was a MetaSystems chromosome auto scanning and analyzing system (German ZEISS). Chromosome karyotypes were named with reference to the international system for human gytogenetic nomenclature (ISCN 2009).

Chromosomal microarray analysis (CMA)

Genomic DNAs were extracted from peripheral blood and amniotic fluid samples (Qiangen Kit) respectively. Intact genomic DNA 500 ng in each sample was equally split into two fragments which were digested by restrictive endonuclease respectively. DNA ligases were used for ligating corresponding joints to perform polymerase chain reaction (PCR) amplification, followed by purification of PCR productions. The purified products were digested into segments (50 bp). Terminal transferases were utilized to label at DNA terminals while DNA samples were denatured. The labeled samples (70 μL) and the newly-formulated hybridization solution (190 μL), totaling 260 μL, were mixed

Table 1. CNV positive results of NIPT screening and G-banding karyotyping analysis, CMA results for validation

| Number	Maternal age (years old)	Gestational age (week)	NIPT	G-banding karyotyping analysis (Amnioticfluid)	CMA	Fetal outcomes
1	38	13⁺⁵ w	46, XX, dup (18p11.3→p11.21, 13 M)	47, XX, 18p+	46, XX, dup (18p11.32→p11.21)	Induced fetus without abnormalities at autopsy
2	32	14	46, XY, dup (9p24.39 →p11.2, 46 M)	46, XY, add (9) (p24?), inv (9) (p11q13)	46, XY, dup (9p24.1→p11, 50 M)	Induced fetus with atrial septal defect at autopsy
3	36	19	46, XY, dup (5q14.2, 10 M)	46, XY, add (5) (p14?)	46, XY, dup (5q14.2, 5 M)	Normal pregnancy
4	37	21	46, XY, dup (13q12.11→13q12.13, 3.3 M)	Normal	46, XY, dup (13q12.11→q12.13)	Normal pregnancy
5	34	13	46, XX, dup (13q31.1, 3 M)	Normal	46, XX, dup (13q31.1, 3 M)	Normal pregnancy
6	24	13⁺⁶ w	46, XX, dup (9) (9p13→q13, 30 M)	46, XX, add (9) (p13?), pat	46, XX, dup (9p13→q13)	Paternal origins, normal pregnancy
7	28	26	46, XY, dup (18p16.1, 5 M)	Normal	Normal	Normal pregnancy
8	35	18⁺¹ w	46, XX, dup (3p26.3→p22.3, 35 M)	46, XX, add (3) (p22?)	46, XX, dup (3p26.3→p22.3, 35 M)	Normal pregnancy
9	32	23⁺⁴ w	46, XY, dup (2q34→2q37.2, 27.08 M)	46, XY, add (2) (q34?)	46, XY, dup (2q34→q37, 28 M)	Maternal origins, normal pregnancy
10	22	20	46, XY, dup (13q12.11→q12.13, 3 M)	Normal	46, XY, dup (13q12.11→q12.13, 3 M)	Maternal origins, normal pregnancy
11	29	19⁺⁴ w	46, XY, dup (17)(q21.31→q31.32, 3.05 M)	Normal	46, XY, dup (17q21, 380 Kb)	Paternal origins, normal pregnancy
12	34	22	46, XY, del (18q11.2→q12.1, 6 M)	Normal	46, XY, del (18q11.2→q12.1, 4.5 M)	Maternal origins, normal newborn
13	25	17⁺³ w	46, XX, del (20q11.23→20q13.31, 20 M)	Normal	Normal	Normal pregnancy
14	36	14⁺³ W	46, XX, del (13q31.1, 3.71 M)	Normal	46, XX, del (13q31.1, 3.05 M)	Maternal origins, normal newborn
15	33	14	46, XX, del (5q14.1→5q34, 80 M)	46, XX, del (5) (q14?)	46, XX, del (5q14.1→q34, 80 M)	Rejection of autopsy on the induced fetus
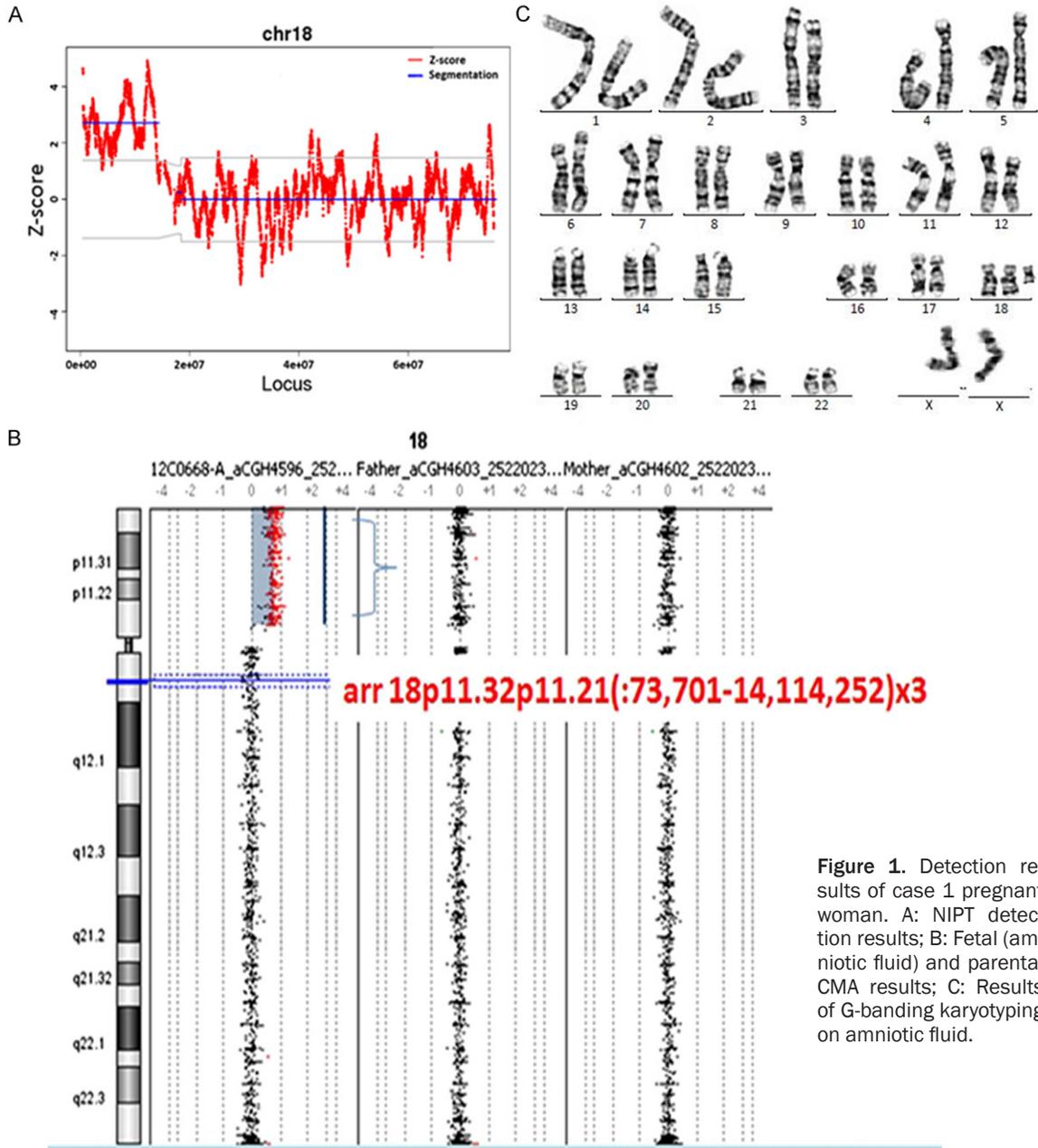
Figure 1. Detection results of case 1 pregnant woman. A: NIPT detection results; B: Fetal (amniotic fluid) and parental CMA results; C: Results of G-banding karyotyping on amniotic fluid.

and denatured before being hybridized for 16-18 h. After the hybridized chips were eluted at the Affymetrix fluid working station, they combined with the vectors of biotin-labeled goat-anti streptavidin. After the elution and dyeing processes were over, the chips automatically scanned in fluid working station (Affymetrix 30007 G scanner, with imagine resolution of 0.7 μm). The scanned photos were analyzed with the CHas software of Affymetrix, then the genotypes or the relative intensity of signals in each site were counted to discover the CNVs.

Results

CNV results by NIPT screening

In the 14235 samples, apart from 18 cases of Down Syndrome, 4 cases of trisomy 18, 2 cases of trisomy 13, 24 cases of CNVs were also detected by the NIPT screening, among which, 15 cases (including 4 cases of micro-deletion, and 11 cases of micro-duplication) agreed to undergo G-banding karyotyping and CMA for validation after amniocentesis.
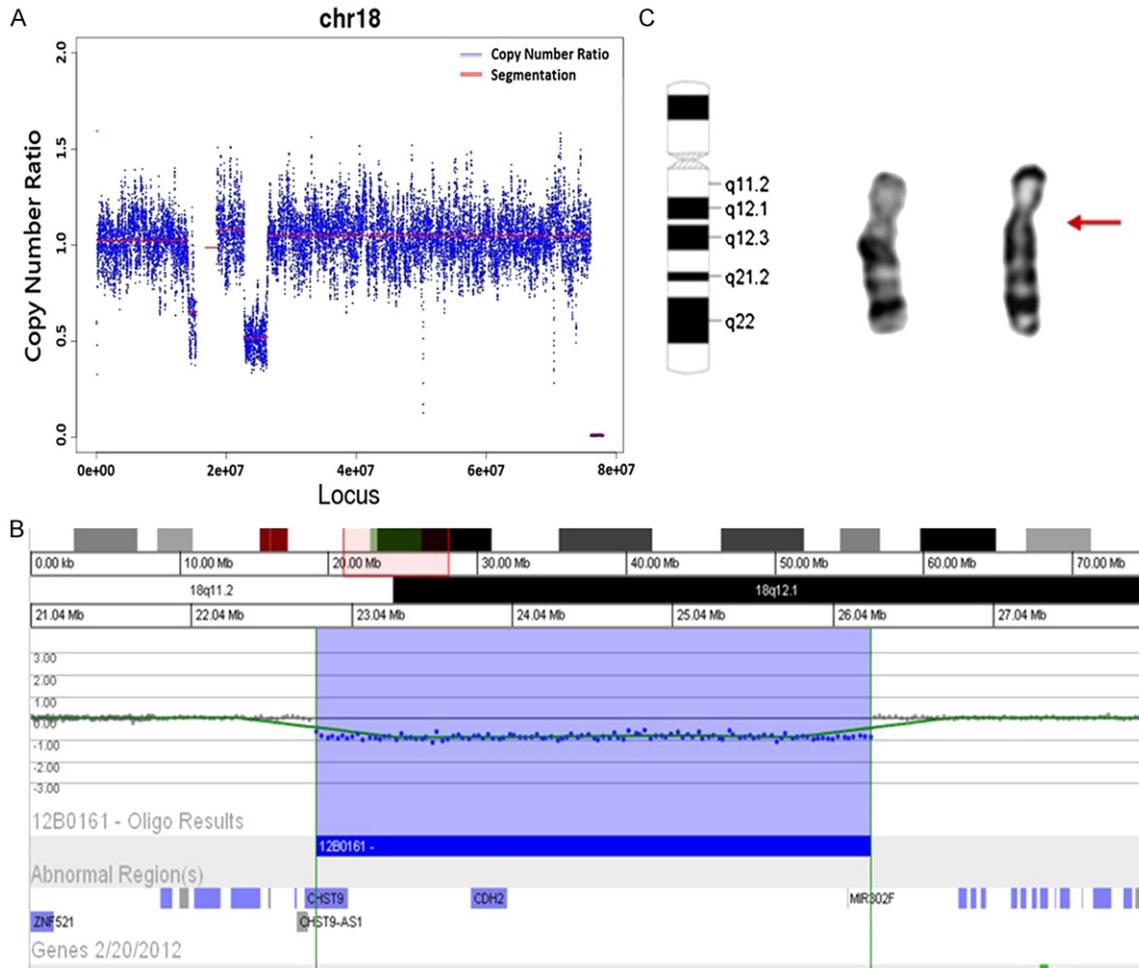
Figure 2. Detection results of case 12. A: NIPT detection results; B: Amniotic fluid CMA detection results; C: Results of G-banding karyotyping on amniotic fluid showed that chromosome 18 and pattern charts, no significant deletion-ate in 18q11.2-18q12.1 (arrow).

Comparison of positive CNV results from the NIPT screening and validation results from G-banding and CMA assays

The findings of detection on 15 cases of pregnant women were shown in **Table 1**, in which case 7 and case 13 had high positive CNV by NIPT screening, but they were confirmed as false positive by G-banding karyotyping and CMA validation. Seven cases of pregnant women (case 1, 2, 3, 6, 8, 9, 15) were validated as positive by the G-banding karyotype and CMA. For example, the NIPT screening findings of case 1 showed a 13 Mb fragment micro-duplication on 18p11.3→p11.21. The results were consistent with those of G-banding and CMA validation, and no CNVs were found in the same region in the fetus' parents, confirming that the

micro-duplication of fetus was a novel mutation (**Figure 1**). Six cases (case 4, 5, 10, 11, 12, 14) were validated to be positive by the CMA but no chromosome abnormalities were found by the G-banding karyotyping. For instance, the NIPT screening findings of case 12 revealed a 6Mb fragment micro-deletion on 18q11.2→q12.1, consistent with the findings of CMA validation, but no chromosome abnormalities were found by the G-banding karyotyping, as shown in **Figure 2**. The CNVs of six cases (case 6, 9, 10, 11, 12, 14) were originated from their parents; the CNVs of seven cases (case 1, 2, 3, 4, 5, 8, 15) were novel mutations. 3 cases (case 1, 2, 15) performed induction of labor respectively and the findings of autopsy revealed case 2 had atrial septal defect.

Table 2. CNV fragment size and proportion by NIPT screening

	Micro-duplication n (%)	Micro-deletion n (%)	Total n (%)
< 5 M	4 (36.4%)	1 (25.0%)	5 (33.3%)
< 10 M	5 (45.5%)	2 (50.0%)	7 (46.7%)
≥ 10 M	6 (54.5%)	2 (50.0%)	8 (53.3%)

CNV fragments sizes by NIPT screening

NIPT screening detected 5 cases of CNV < 5 Mb (33.3%), 7 cases of CNV < 10 Mb (46.7%) and 8 cases of CNV ≥ 10 Mb (53.3%), as shown in **Table 2**.

Discussion

Studies have found that the incidence rate of chromosomal micro-deletion or micro-duplication in the common population was 1‰-3‰ [10]. In this study, a total of 24 cases of CNV were found in the 14235 samples, with an incidence of about 1.69‰ (close to those in literature reports).

Chromosomal micro-deletion/micro-duplication syndromes are harmful, but the performance of prenatal diagnosis is rather difficult due to current paucity of simple and effective screening way [11]. The method of fetal copy-number analysis via maternal plasma sequencing (FCAPS) for detection of fetal aneuploidy with low-coverage may potentiate to detect fetal large deletions/duplications (> 10 Mb) [12]. Currently, studies have also proved that it is feasible to detect fetal fragment deletion/duplication from maternal plasma sequencing. Peters reported that the presence of 4 MB deletion in fetus (gestation age of 35 weeks) could be identified by read counts of maternal plasma 243 M sequencing [13]. Jensen [14] et al. also developed the detection strategy of 22q11.2 micro-deletion syndrome by using maternal plasma sequencing data. Theoretically, the information derived from the NIPT genome-wide sequencing can be used to identify additional chromosomal copy number abnormalities, including structurally chromosomal deletions and duplications [15]. The NIPT detection findings of case 1 in this study showed a 13 Mb duplication fragment in the short arm of chromosome 18, and the findings of G-banding karyotyping also indicated a par-

tial trisomy of chromosome 18. And the CMA validation results confirmed that there were duplication segments on 18p11.32→p11.21. The findings of 13 cases among the 15 CNV cases by the NIPT screening were basically consistent with those of the CMA validation, with a positive concordance rate of 86.7% and a false positive rate of 13.3%. Only 7 cases by the G-banding karyotyping were positive, indicating a missed diagnosis rate of 46.2%. For example, the findings of case 12 by the NIPT screening showed a 6 MB deletion fragment in the long arm of chromosome 18, which was further validated by CMA, but the G-banding karyotyping finding showed no abnormalities. The reason is that there are evident limitations in the G-banding karyotyping that almost all subtly unbalanced chromosome aberrations < 5 Mb could not be identified by it [16]. Moreover, it will take long time for the cytogenetic analysis. Therefore, the positive CNV by the NIPT screening is relatively more accurate, close to that of the "gold standard" CMA for CNV diagnosis, and it is more effective than the traditional G-banding karyotyping. With the advances in bioinformatics, NIPT can also be used to detect the deletions and duplications < 5 Mb. NIPT screened out 5 cases of CNV fragments < 5 Mb (33.3%) and 7 cases of CNV fragments < 10 Mb (46.7%). We are convinced of the prospect of universal and practical noninvasive screening for deletion/duplication in fetal genome, which can be incorporated into the current program of noninvasive prenatal detection of fetal aneuploidy without increasing sequencing depth nor requiring 50-time sequencing data for deletion/duplication detection as reported. As a result, such extra cost analyses and report time are negligible.

Two cases of the NIPT screening results in this study were confirmed as false positive by the G-banding karyotyping and the CMA. The possible reasons are as follows: the positive results by NIPT might be originated from the placental abnormalities. The incidence of placental mosaicism was far larger than what we had previously recognized, as the incidence in full-term placenta was at least 4.8% [17]. In addition, the results of molecular genetic testing showed the gene dosage of average genetic material in an individual cell sample. This is applicable to most people whose chromosomes in all cells are same, but in the case of chimerism, it may

occasionally lead to wrong results. In view of the potential limitations, G-banding karyotyping and CMA should be performed when the NIPT shows positive results, especially when the rare abnormalities have been detected, or when the fetus ultrasonography-based abnormalities are undetectable. However, just like CMA, NIPT is ineffective in detecting balanced chromosomal abnormalities without complications of DNA copy number variations, including Robertson translocation, balanced reciprocal translocations, balanced insertion and inversion. For example, in case 2 of this study, both CMA and NIPT failed to detect the partial inversion in chromosome 9, but G-banding karyotyping could overcome such limitations. Therefore, in this study we combined G-banding karyotyping with CMA to further validate the positive results of the NIPT. Attention should also be paid to the patients with high risk of chromosomal aberrations in clinical practice.

NIPT is a novel technology with a high sensitivity which can be used to detect common aneuploidies and other chromosome abnormalities in fetuses. This study confirmed that NIPT has a high accuracy on the screening of positive CNV results. Therefore, more attention should be paid to screened abnormalities, which need further confirmatory prenatal procedures. Most of the CNV reveal polymorphic, but only a few are pathogenic [19, 20]. In addition, the positive results of CNV by NIPT screening may also be derived from fetal parents' abnormities. In this study, after comparing the results of G-banding karyotyping and CMA in parents to those of in fetuses, we found that 4 cases of CNVs originated from the mothers showing abnormal phenotypes and 2 cases of CNVs originated from the fathers showing abnormal phenotypes, and 6 cases of CNVs whose parents' phenotypes are normal. The pregnant women were confirmed with CNV by fetus screening continued their pregnancy, but no abnormalities were found in follow-ups. The autopsy findings showed no abnormalities in the labor-induced fetus of case 1. Consequently, the results of these tests should be interpreted with caution. In addition, as there were a large number of cases screened in this study, we can't further confirm all the negative cases, which is expected to be improved in the future tests.

In conclusion, NIPT as a screening tool is of high accuracy in detecting the positive results of CNVs, so it can act as supplementary screening.

Disclosure of conflict of interest

None.

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