Research Article

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Comparison of noninvasive prenatal screening for defined pathogenic microdeletion/microduplication syndromes and nonsyndromic copy number variations: a large multicenter study

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Background: This retrospective study assessed the precision of noninvasive prenatal testing (NIPT) in detecting microdeletion/microduplication syndromes (MMSs) and nonsyndromic copy number variations (CNVs). **Methods:** The study included 19,086 singleton pregnancies screened on NIPT using high-throughput sequencing. Pregnancies with CNVs on NIPT underwent amniocentesis for karyotyping and CNV sequencing (CNV-seq). We analyzed pathogenic MMSs and nonsyndromic CNVs separately, dividing the CNVs into subgroups based on fragment size and fetal ultrasound findings. **Results:** A total of 170 abnormalities were detected by NIPT, of which 113 (66.5%) underwent invasive testing. The positive predictive value (PPV) of CNV-seq for all types of CNV detected by NIPT was 35.4%, with PPVs of 61.5 and 27.6% for pathogenic MMSs and nonsyndromic CNVs, respectively. PPVs for NIPT showed different values depending on gestational characteristics, with the highest PPV for NIPT in the group with increased nuchal thickness (66.7%) and for the abnormal ultrasound group (57.1%). CNVs ≤5 Mb with normal ultrasound findings were generally associated with a healthy fetus. **Conclusion:** NIPT can detect chromosomal aberrations in the first trimester, with high performance for MMSs. However, due to the low PPV for nonsyndromic CNVs, and the good pregnancy outcome in most cases, the introduction of expanded NIPT would cause an increase in unnecessary invasive procedures and inappropriate terminations of pregnancy.

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Keywords: CNV • microdeletion/microduplication syndromes • MMS • next-generation sequencing • NIPT • noninvasive prenatal testing • nonsyndromic copy number variation

Noninvasive prenatal testing (NIPT) is rapidly becoming a primary screening test in clinical practice [1] and is widely used in clinical practice. NIPT is highly accurate, sensitive and specific in detecting fetal trisomy 21, 18 and 13, all with predictive values greater than 99% [2,3]. A growing number of studies have shown that NIPT can reduce the incidence of nonessential invasive procedures and medically induced fetal deaths [4].

Currently, prenatal diagnostic methods associated with genetic testing include genome-wide or targeted genetic testing [5], and the use of NIPT is rapidly evolving and widely used beyond genome-wide chromosomes, which also includes the identification of subchromosomal aberrations known as copy number variants (CNVs). NIPT focuses on additional analysis of CNVs, including microdeletion/microduplication syndromes (MMSs) [6]. Although MMSs are relatively rare, overall they represent an important group of chromosomal disorders, accounting for 1–2% of all congenital chromosomal abnormalities in newborns and placing a heavy burden on families and society [7]. In contrast to the common trisomies, the prevalence of CNV is independent of maternal age [8]. The rate of prenatal testing for pathogenic CNVs is 1.0–1.7%, with many prenatal tests taking place in the context of normal maternal ultrasound. Therefore, NIPT testing is beneficial for all pregnant women [9].

Table 1. Characteristics of study subjects.

Clinical characteristic	Nonsyndromic CNVs	MMS	p-value
Samples	87	26	
Age (years)	30.35 (20–32)	30.77 (25–43)	0.648
BMI (kg/m^2)	24.57 (19.2–29.8)	24.84 (19.1–27.5)	0.641
Gestational age (days)	146.38 (126–189)	144.93 (119–159)	0.622
cffDNA concentration (%)	8.34 (5.6–10.2)	7.89 (7.2–12)	0.129
Unique mapped reads (M)	15.42 (11–19)	15.56 (12–19)	0.696
CNV type			
Duplications	12	58	0.051
Deletions	14	29	0.205

CNV: Copy number variant; cffDNA: Cell-free fetal DNA; M: Million; MMS: Microdeletion/microduplication syndrome.

The utility of using NIPT to detect CNVs has now been studied by many institutions [8,9], demonstrating that NIPT performs well for certain types of MMS. As of June 2016, 66 diseases with clearly annotated CNVs have been included in the decrypted database [10], including named types of chromosomal MMS syndromes. We found that many studies cursorily assessed the effect of CNVs in general or were limited to the effectiveness for detecting specific pathogenic MMSs, while some additional reports on NIPT suggest some nonsyndromic CNVs which are either of variants of unknown significance (VUS) or are currently undetected. The validity of this aspect of NIPT remains to be proven. The American College of Obstetrics and Gynecology recommends that "*routine cell-free DNA screening for microdeletion syndromes should not be performed*" [11], while the American Society of Human Genetics and the European Society of Human Genetics agree that "*routine cell-free DNA screening for microdeletion syndromes is not recommended at this time*" because extensive screening via NIPT which includes the detection of subchromosomal aneuploidy may result in reduced diagnostic specificity [12]. Although the International Society for Prenatal Diagnosis does not explicitly oppose testing, it states that "" [1].

For nonsyndromic CNVs, the lack of sensitivity and specificity of accurate information, prevalence and pathogenicity are not defined, and it is difficult to accurately estimate the pregnancy outcome. The objective of this retrospective, multicenter study is to review the efficacy of NIPT as a screening test for defined pathogenic MMSs and nonsyndromic CNVs in single pregnancies.

Patients & methods

Participant recruitment

Due to the rarity of screening for CNVs, it is difficult to adequately validate CNV screening using traditional prospective randomized trials. Therefore we used a retrospective, multicenter clinical study with a large sample size. From 13 January 2018 to 30 September 2021, a total of 19,086 pregnant women were studied from 13 hospitals: the First Affiliated Hospital of Xinjiang Medical University, the Second Affiliated Hospital of Xinjiang Medical University, the Fifth Affiliated Hospital of Xinjiang Medical University, Chang ji People's Hospital, Fuyu County Maternal and Child Health Care and Family Planning Service Centre, Kurla Second People's Hospital, Kurla County Maternal and Child Health Care Hospital, Ba Zhou City People's Hospital, Bole County Maternal and Child Health and Family Planning Service Center, Ha Ba he Maternal and Child Health and Family Planning Service Center, Brotula Mongolian Autonomous Prefecture People's Hospital, To li Maternal and Child Health and Family Planning Service Center and Qing he Maternal and Child Health and Family Planning Service Center. Maternal characteristics that are important risk indicators for fetal CNV include advanced maternal age, BMI, gestational age and abnormal fetal ultrasound findings [2,3,5,11]. Clinical characteristics of the study subjects are shown in Table 1. If NIPT shows an abnormal CNV signal, further antenatal diagnosis should be made. If the fetal CNV sequencing (CNV-seq) result is abnormal, a peripheral blood sample from the parents should be collected for further testing to determine inheritance.

Process design

Under existing procedures previously developed for NIPT, the pregnant women involved will be registered at a prenatal diagnostic center accredited and authorized by the provincial health authorities and will enter into a consent process specifically designed for NIPT, which includes notification of the type of sample, test method, diseases covered by the screening, limitations and risks. The consent form also includes other agreements such as

information about the insurance plan offered and a statement about the study in accordance with hospital policies, laws and national ethical guidelines. In terms of insurance, all women are registered with China Life under a specific insurance plan that covers specific criteria and diagnosed conditions.

Sample preparation & NIPT

For each patient, 5 ml of venous blood was collected using potassium–ethylenediaminetetraacetic acid tubes and centrifuged for 10 min at 1600 × g at 4°C within 8 h of blood collection. The plasma was then centrifuged at 4°C and 16,000 × g for 10 min to obtain cell-free plasma, which was stored at -80°C. Plasma circulating cell-free DNA (cfDNA) was extracted using the Circulating Nucleic Acid kit (Berry Genomics,Changping District, Beijing, China) from 700 μl of stored plasma. cfDNA was extracted from the maternal plasma according to standard operating procedures and the DNA library was constructed using enzymatic reactions, molecular labeling and PCR. DNA fragments were subjected to end repair and linker ligation. After PCR amplification and pooling, single-strand cyclization and DNA nanosphere preparation were carried out to construct a library for sequencing. Each sample was sequenced using the BGISEQ-500 platform and a combinatorial probe-anchored polymer sequencing method, and bioinformatics analysis was performed using BGI(Shenzhen, Guangdong, China) Halos software. Quality control parameters were: DNA library concentration above 4 ng/μl, GC content of 38–42% and fetal DNA percentage above 3.5%.

Bioinformatics analysis was performed as follows. First, the reference genome window division was used for sequence alignment correction, so as to reduce the sequencing depth and determine the CNV breakpoint position more accurately. Then, the normal control reference data were used to correct the GC content between batches and within batches of samples, and conduct regional correction for the regions with uneven sequencing data on the genome, so as to effectively improve the detection accuracy. Finally, the position and size of CNVs was determined by binary segmentation and z-test.

Karyotype analysis

Chromosomal karyotyping of cultured amniotic cells and lymphocytes was performed under sterile conditions according to standard protocols. Amniocentesis is performed under ultrasound guidance and the cells collected by centrifugation, inoculated into the culture medium and incubated at 37°C. When many round, translucent dividing cells appear, colchicine is added and the cultures are incubated for a further hour. When the number of round, translucent cells increases, the cells are collected for chromosome preparation. In this study 60 dividing cells were counted using the Artificial Intelligence Chromosome Image Analysis System and described according to the principles of the International Code of Human Cytogenetic Nomenclature ISCN 2016; 20 cells were analyzed and four fully karyotyped.

CNV-seq

Genomic DNA was extracted from amniotic fluid or fetal tissue using the Genomic DNA Extraction Kit (Qiagen, NY, USA) and then purified using the Purified DNA Kit (Zyme Research, CA, USA). DNA was quantified using Invitrogen Qubit™ 2.0 (Thermo Fisher Scientific, MA, USA) at >8 ng/l. Quantification of DNA concentrations was performed. Using the same procedure, libraries were constructed and purified in maternal plasma. DNA libraries were then quantified at >25 nmol/l using the Kapa Biosystems (Shanghai, China) Kapa SYBR® Rapid QPCR Kit. The quantified DNA libraries were combined and loaded into an Illumina NextSeq CN500 flow cell, then sequenced using a single-end 36-bp sequencing protocol and finally analyzed using software provided by Berry Genomics(Beijing, China).

Statistics

Statistical analysis of the data was carried out using Excel and R. Continuous variables were expressed as median and interquartile range, and categorical variables were expressed as n (%). A total of 113 samples that showed CNV by NIPT were divided into groups based on age, fetal free DNA concentration, number of uniquely mapped reads, CNV size and CNV type. The positive predictive value (PPV) of CNV detected by NIPT was calculated from the CNV-seq results. Fisher's exact probability test was used to compare the CNV PPV of NIPT between groups. Results with p-values less than 0.05 were statistically significant.

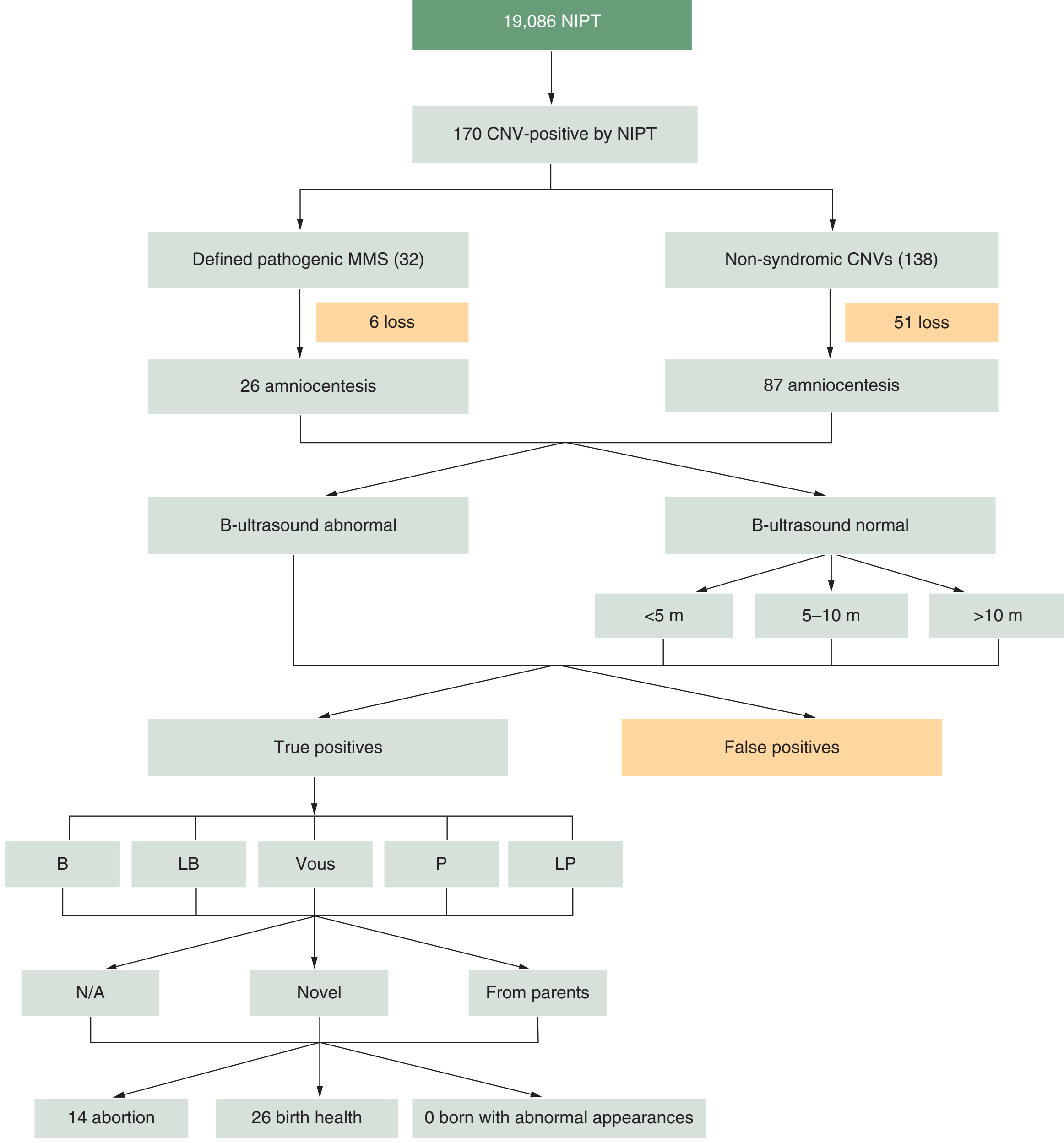

Figure 1. Noninvasive prenatal testing results and clinical outcomes.
B: Benign; B-ultrasound; Brightness-mode ultrasound; CNV-seq: Copy number variant sequencing; LB: Likely benign; LP: Likely pathogenic; MMS: Microdeletion/microduplication syndrome; N/A: Not available; NIPT: Noninvasive prenatal testing; P: Pathogenic; VUS: Variant of uncertain significance.

Results

Population profiles

The complete study cohort included 19,086 women from Xinjiang whose singleton pregnancies were analyzed by NIPT for the detection of CNVs (Figure 1). A total of 32 fetuses (0.20%) were suspected to have pathogenic MMSs, and 138 (0.72%) were suspected to have nonsyndromic CNVs. In total, 57 cases were lost to follow-up due to refusal of further prenatal diagnosis (six MMSs and 51 nonsyndromic CNVs); thus 113 patients (66.5% of the total with abnormal NIPT findings: 26 MMSs and 87 nonsyndromic CNVs) underwent amniocentesis to obtain amniotic fluid after signing an informed consent form and were followed up with CNV-seq and karyotype analysis. The enrollment, outcome classification and follow-up of pregnant women who participated in this study

Table 2. Efficiency of noninvasive prenatal testing for detecting copy number variants and association with various factors.

Factor	NIPT positive	TP	PPV	p-value
Age				
≥35 years	19	6	47.3	0.452
<35 years	94	34	36.2	
cffDNA				
≥8%	65	23	35.3	0.538
<8%	48	17	35.4	
Unique mapped reads (M)				
≥15	98	42	42.9	0.066
<15	15	8	53.3	
Type				
Duplication	70	23	32.9	0.755
Deletion	43	16	37.2	

cffDNA: Cell-free fetal DNA; M: Million; NIPT: Noninvasive prenatal testing; PPV: Positive predictive value; TP: True positive.

are shown in Figure 1. The pregnancy outcomes were 14 abortions and 26 normal pregnancies. Table 1 shows the two groups' statistical characteristics; there were no significant differences at baseline.

Efficiency of NIPT for detecting CNVs & association with various factors

First, the PPV of each group of CNVs detected by NIPT was calculated as described above, based on maternal age, cffDNA concentration, number of clearly mapped reads and CNV type. The results showed no statistically significant differences in PPV between the different factors (Table 2).

Fetuses with suspected MMSs

The 26 detected cases of MMSs in the 113 patients who underwent follow-up invasive testing were associated with classical chromosomal abnormalities (Table 3). These included five cases of 22q microduplication syndrome (three true positives and two false positives; PPV: 60%), four cases of high-risk DiGeorge syndrome (two true positives and two false positives; PPV: 40%), three cases of cri-du-chat syndrome (one true positive and two false positives; PPV: 33.3%) and two cases of Xp deletion syndrome (both true positives; PPV: 100%). Combined with the ultrasound results, we found that the PPV of fetal structural abnormalities in the abnormal ultrasound group was 100%, which was much higher than that in the normal ultrasound group (47.4%).

Fetuses with suspected nonsyndromic CNVs

Comparison of NIPT, karyotyping & CNV-seq results

Of the 113 fetal CNVs, 87 were classified as nonsyndromic CNVs. Following amniocentesis, CNV-seq confirmed the presence of CNVs in 24 cases and identified a total of 27 CNVs, with a PPV of 27.6% (Table 3). The 13 positive karyotype analyses included seven polymorphisms, two balanced translocations, one sex chromosome abnormality, and three karyotypes consistent with CNV-seq abnormalities but in which the karyotype failed to identify chromosomal sub-bands (cases 22, 25 and 44) (Table 4). Thus, the misdiagnosis rate for karyotype analysis of CNVs was 88.9% (24/27). Of the 27 CNVs detected by NIPT and CNV-seq, 48.1% (13/27) had fragment length differences within the 1-Mb range reported by the two methods (Tables 3–5).

Predictive value of NIPT in cases with no syndromic CNV combined with ultrasound results

Based on the ultrasound findings shown, the different PPVs for NIPT are shown in Table 5. The highest PPV for NIPT was 66.7% for the elevated NT group and 57.1% for the other ultrasound abnormalities group. Notably, the PPV for fetal structural abnormalities was higher in the ultrasound group than in the normal ultrasound group.

Predictive value of NIPT in cases with nonsyndromic CNV combined with the size of CNV fragment

Based on CNV sequence validation, the most common CNV fragment size was ≤5 Mb and its PPV was the highest (30.00%), with 21 true positives and 49 false positives. There were 12 CNVs in the 5–10 Mb range, with two true

Table 3. The results of fetuses with suspected microdeletion/microduplication syndromes.

Case	Age (years)	Gestational weeks	Ultrasound	NIPT results	Karyotype	CNV-seq results	ACMG classification	Source	Fetal outcome
1	26	19 + 1	Normal	dup(22)(q11.2), 8 Mb	46,XN	chr22q11.21(18,648,855_21,800,471)×3 (3.1 Mb)	P 22q dup syndrome	Novel	TOP
2	30	22 + 5	Normal	High risk of CDC	46,XN	Negative			Normal
3	29	22	Normal	del(Xp), 50Mb	46,X.i(X)(q10)[32]/46,X, del(X)(p10)[6]	46,XN,chrXp22.33p11.21(2710001_56160001)×1 (53.45 Mb) [70%]/46,XN, chrXp11.1q28(56210000_155260000)×3 (99.05 Mb) [30%]	P Xp del syndrome Xq26.3 dup syndrome Xq27.3q28 dup syndrome	NA	TOP
4	30	20 + 3	NT 3.8 mm	High risk of 7dup syndrome	46,XN,der(13)?t(7;13)	chr7q33q36.3(134950001_159125000)×3 (24.175 Mb) chr13q34q34(112120001_115070000)×1 (2.95 Mb)	P 7q partial trisomy syndrome 13q del syndrome	NA	TOP
5	29	22	NT 2.4–2.8 mm	High risk of 9pter del syndrome	46,XN,del(9)(p23)	chr9p24.3p22.3(210001_15110000)×1 (14.9 Mb)	P 9pter del syndrome	NA	Normal
6	28	19 + 5	Normal	High risk of DGS	46,XN	Negative			Normal
7	27	22 + 6	Normal	dup(X)(q28q28.3), 2.15 Mb	46,XN	chrXq28q28(151310001_154960000)×3 (3.65 Mb)	P *MECP2* syndrome	Maternal	TOP
8	28	22 + 4	VSD	del(22)(q11.21q11.21), 2.88 Mb	46,XN	chr22q11.2122q11.21(18688433 21608132)×1 (2.9 Mb)	P DGS	NA	TOP
9	26	17	Normal	del(X)(p22.13), 1.68 Mb	46,XN	chrXp22.31p22.31(6410001_8160000)×1 (1.75 Mb)	P X-linked ichthyosis	NA	TOP
10	29	20 + 2	Normal	High risk of DGS	46,XN	Negative			Normal
11	28	22	Normal	del(17)(q12q12), 1.45 Mb	46,XN	chr17q12q12(34800001_36250000)×1 (1.45 Mb)	LP 17q12 del syndrome	Maternal	Normal
12	38	19 + 5	Normal	dup(22)(q11.21), 2 Mb	46,XN	Negative			Normal
13	31	19 + 5	Normal	dup(22)(q11.1q11.21), 3.6 Mb	46,XN	chr22q11.1q11.21(17850001_21450004)×3 (3.6 Mb)	P 22q11 dup syndrome	Paternal	Normal
14	36	18	Normal	dup(22)(q11.2), 3.5 Mb	46,XN	Negative			Normal
15	35	20 + 5	Normal	del(1)(q43q44), 39.2 Mb	46,XN	Negative			Normal

ACMG: American College of Medical Genetics; ASD: Atrial septal defect; B: Benign; CDC: Cri-du-chat syndrome; CNV-seq: Copy number variant sequencing; DGS: DiGeorge syndrome; del: Deletion; dup: Duplication; FGR: Fetal growth restriction; LB: Likely benign; LP: Likely pathogenic; NA: Not available; NIPT: Noninvasive prenatal testing; NT: Nuchal thickness; P: Pathogenic; SUA: Single umbilical artery; TOP: Termination of pregnancy; VSD: Ventricular septal defect; VUS: Variant of uncertain significance.

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Table 3. The results of fetuses with suspected microdeletion/microduplication syndromes (cont.).

Case	Age (years)	Gestational weeks	Ultrasound	NIPT results	Karyotype	CNV-seq results	ACMG classification	Source	Fetal outcome
16	33	21 + 3	Normal	High risk of CDC	46,XN,16qh+	Negative			Normal
17	26	20 + 5	Normal	High risk of Xq23–qter del syndrome	46,XN	chr14q21.1q21.3(38653727_50191149)×2 hmz (11.5 Mb)	VUS	?	Normal
18	25	23 + 2	Intrauterine growth restriction	High risk of CDC	46,XN,del(5)(p15)	chr5p15.33p15.1(1_17926930)×1 (17.9 Mb)	P CDC syndrome		TOP
19	32	24 + 3	Normal	High risk of 15q26–qter del syndrome	46,XN	Negative			Normal
20	37	19 + 5	Normal	High risk of Xp dup syndrome	46,X,dup(X)(p11.23p21.1), 9qh+	chr4p16.3p11(1_49587838)×2∼3 (49.5 Mb)chrXp21.1p11.23(34535958_48811462)×3 (14.2 Mb)	P Xp dup syndrome Wolf–Hirschhorn syndrome	Novel	TOP
21	34	20 + 3	Normal	dup(12)(q24.21q24.35), 8 Mb	46,XN,der(12)	chr12q24.23q24.32(119565250_127308116)×3 (7.7 Mb)	LP	Novel	Normal
22	25	22 + 3	Normal	High risk of 16p del syndrome	46,XN	Negative			Normal
23	31	21 + 1	Persistent arterial trunk	High risk of DGS	46,XN	chr22q11.21(18884003_21571177)×1 (2.6 Mb)	P DGS	NA	TOP
24	32	21 + 5	Normal	High risk of 15q22–qter dup syndrome	46,XN	Negative			Normal
25	34	20 + 1	NT 2.8 mm Bilateral renal sinus separation	dup(12)(p13p11), 3 Mb	46,XN,der(12)	chr12p13.33p11.1(152728_34828126)×3∼4 (34.6 Mb)	P Pallister–Killian syndrome	NA	TOP
26	32	19 + 5	ASD	chr22q11.1-q11.21 ×3, 4.1 Mb	47,XY,+mar	chr22q11.1-q11.21(16840001_21460000)×3 (4.62 Mb)	P 22q11 dup syndrome	NA	TOP

ACMG: American College of Medical Genetics; ASD: Atrial septal defect; B: Benign; CDC: Cri-du-chat syndrome; CNV-seq: Copy number variant sequencing; DGS: DiGeorge syndrome; del: Deletion; dup: Duplication; FGR: Fetal growth restriction; LB: Likely benign; LP: Likely pathogenic; NA: Not available; NIPT: Noninvasive prenatal testing; NT: Nuchal thickness; P: Pathogenic; SUA: Single umbilical artery; TOP: Termination of pregnancy; VSD: Ventricular septal defect; VUS: Variant of uncertain significance.

Table 4. The results of fetuses with suspected nonsyndromic copy number variants.

Case	Age (years)	Gestational weeks	Ultrasound	NIPT results	Karyotype	CNV-seq results	ACMG classification	Source	Fetal outcome
1	33	20 + 5	Normal	dup(3)(p12.2p12.1), 4.35Mb	46,XN	Negative			Normal
2	37	22	Normal	del(15), 5.1 Mb	46,XN	Negative			Normal
3	32	20 + 2	Normal	dup(5)(q34q34), 3.85 Mb	46,XN,15p+	chr5q34q34(162760001_166210000)×3 (3.45 Mb)	VUS	Paternal	Normal
4	28	22 + 1	Normal	del(5q), 73.75 Mb	46,XN	Negative			Normal
5	31	18 + 6	Fetal cerebral ventriculomegaly	dup(13)(q31.1q31.2), 5 Mb	46,XN	chr13q31.1q31.2(84470001_88270000)×3 (3.8 Mb)	VUS	NA	Normal
6	27	21 + 3	Normal	dup(1)(q43q44), 9.35 Mb	46,XN	Negative			Normal
7	31	22	SUA	del(3q), 3 Mb	46,XN,?t(3; 20)	chr3q26.31q26.32(172360005_175960004)×1 (3.6 Mb)	VUS	NA	Normal
8	32	20 + 1	Normal	del(16p), 3.35 Mb	46,XN	Negative			Normal
9	34	22 + 1	NT 3.0 mm	dup(4)(p15), 4.35 Mb	46,XN	chr4p15.1p14(33557001_37707000)×3 (4.15 Mb)	VUS	Paternal	Normal
10	33	20 + 3	Normal	dup(21)(q21), 5.85 Mb	46,XN	chr21q21.1q21.1(19211194_21311193)×3; (2.1Mb)	VUS	Maternal	Normal
11	30	20 + 6	Normal	dup(8q), 5.7 Mb	46,XN	Negative			Normal
12	32	20 + 3	Normal	dup(16p), 1.0 Mb	46,XN	chr16p12.3p12.3(16910001_18310000)×3 (1.4Mb)	VUS	Maternal	Normal
13	30	20 + 4	Normal	dup(13q), 0.6 Mb	46,XN	Negative			Normal
14	28	19 + 3	Normal	del(10q), 3.35 Mb	46,XN,14pstk+[mat]	chr10q21.1-q21.1 ((53,929,790_50947080)×1 (3.1Mb)	VUS	NA	Normal
15	39	19 + 5	Normal	del(4)(q34.3q34.3), 4.6 Mb	46,XN	chr4q34.3q34.3(178232601_182732600)×1 (4.5Mb)	VUS	Paternal	Normal
16	25	21 + 1	Normal	del(17)(p13.3p13.2), 4 Mb	46,XN	Negative			Normal
17	35	18 + 6	Normal	del(X)(p11), 3.0 Mb	46,XN	46,XN[71%]/46,XN,chrXp11.1q12(58510001_65860000)×3[29%] (7.35 Mb)	VUS	Maternal	Normal
18	33	22 + 2	Ventricular bright spot	dup(10)(q11.22q11.23), 4.55 Mb	46,XN	chr10q11.22q11.23(46760001_10260000)×3, 3.65 Mb	VUS	NA	Normal
19	38	26 + 1	NT 2.8 mm	dup(14p), 1.75 Mb	47,XN,+14[2]/46,XN,t(6;14)(p21q32)[2]/46,XN[96]	chr18p11.31p11.23(5610001_7360000)×1 (1.75 Mb)	VUS	Paternal	Normal
20	37	22 + 5	Normal	dup(7), 1.0 Mb	46,XN	Negative			Normal
21	26	22	Ventricular bright spot	dup(7), 3.5 Mb	46,XN	Negative			Normal
22	32	18	Normal	dup(1)(q22.3q24.2), 6.8 Mb	46,XN,dup(1)(q22q24)	chr1q23.3q24.2(161710001_169510000)×3 (7.8 Mb)	VUS	Novel	TOP
23	32	21 + 1	Normal	del(5)(p15.33p14.3), 20.56 Mb	46,XN	Negative			Normal
24	30	22 + 5	Normal	del(5p), 19.4 Mb	46,XN	Negative			Normal
25	39	20 + 1	Normal	dup(9p), 7.2 Mb	47,XN,+mar[47]/46,XN[53]	46,XN[42%]/46,XN,chr9p21.2p13.1(26060001_38960000)×3 (12.9 Mb) [58%]	LP	Novel	TOP
26	27	21 + 4	Normal	dup(17p), 3.35 Mb	46,XN,9qh+	chr17p12p12(14200001_15500000)×3 (1.3 Mb)	LP	Paternal	Normal

ACMG: American College of Medical Genetics; CNV-seq: Copy number variant sequencing; del: Deletion; dup: Duplication; LP: Likely pathogenic; NA: Not available; NIPT: Noninvasive prenatal testing; NT: Nuchal thickness; P: Pathogenic; SUA: Single umbilical artery; VUS: Variant of uncertain significance.

Table 4. The results of fetuses with suspected nonsyndromic copy number variants (cont.).

Case	Age (years)	Gestational weeks	Ultrasound	NIPT results	Karyotype	CNV-seq results	ACMG classification	Source	Fetal outcome
27	31	19 + 2	Normal	del(16)(p13.12p12.3), 4.0 Mb	46,XN	Negative			Normal
28	28	20 + 2	Normal	del(4)(p16), 2.0 Mb	46,XN	chr4p16.3p16.1(3315501_6115500)×1 (2.8 Mb)	VUS	NA	TOP
29	33	18 + 5	Normal	dup(1p), 4.35 Mb	46,XN	Negative			Normal
30	27	18	Left ventricular bright spot	del(3)(q25.33q26.1), 4.10 Mb	46,XN	chr3q25.33q26.1(160310005_164360004)×1 (4.05 Mb)	VUS	Maternal	Normal
31	34	20 + 2	Normal	del(9)(p24.3p24.2), 3.35 Mb	46,XN	Negative			Normal
32	30	22 + 1	Normal	del(12)(p13.31p13.1), 6.10 Mb	46,XN	Negative			Normal
33	30	23	NT 2.5 mm	dup(7p), 3.5 Mb	46,XN	Negative			Normal
34	34	22 + 1	Normal	dup(7), 3 Mb	46,XN,15pstk+	Negative			Normal
35	29	20 + 5	Normal	del(4p), 0.5 Mb	46,XN	Negative			Normal
36	30	19	Normal	del(17)(q22q23.2), 7.0 Mb	46,XN	Negative			Normal
37	29	20	Normal	del(20q), 5.5 Mb	46,XN	Negative			Normal
38	40	19 + 6	Bilateral renal sinus separation	dup(20), 1.0 Mb	46,XN,9qh+	Negative			Normal
39	28	19 + 4	Normal	del(14), 0.45 Mb	46,XN	Negative			Normal
40	38	19	Normal	dup(4), 1.2 Mb	46,XN	Negative			Normal
41	33	19 + 6	Echogenic bowel	dup(1p), 3.1 Mb	46,XN	Negative			Normal
42	28	21 + 6	Normal	dup(7), 0.4 Mb	46,XN	Negative			Normal
43	31	19 + 4		dup(7), 1.25 Mb	46,XN	Negative			Normal
44	25	22	Agenesis of corpus callosum	dup(Xq), 20 Mb	45,X[58]/46,X,+mar[42]	chrXp22.33p11.21(1_56589229)×1 (56.5 Mb) chrXq11.21q22.1(56597980_100845680)×1~2 (44.2 Mb) chrXq22.1q28(100862630_155270560)×1 (54.4 Mb)	P	NA	TOP
45	25	19 + 2	Normal	del(18q), 3.15 Mb	46,XN	Negative			Normal
46	20	23 + 4	Normal	dup(3q), 47.61 Mb	46,XN	Negative			Normal
47	37	19 + 4	SUA	dup(13q), 5.85 Mb	46,XN	Negative			Normal
48	25	20	Normal	del(16q), 3.85 Mb	46,XN	Negative			Normal
49	35	20 + 4	Normal	del(3q), 3.6 Mb	46,XN	Negative			Normal
50	30	20 + 1	Fetal cerebral ventriculomegaly	dup(20q), 0.65 Mb	46,XN	Negative			Normal
51	27	21 + 5	Normal	dup(8q), 1.2 Mb	46,XN	Negative			Normal
52	29	20 + 3	Normal	dup(6), 1.35 Mb	46,XN	Negative			Normal
3	30	20 + 1	Normal	dup(20p), 0.56 Mb	46,XN	Negative			Normal
54	27	17 + 4	NT 2.8 mm	dup(16)(p11.2), 4.1 Mb del(Y), 4.5 Mb	45,X[33]/46,XY[49]	45,X[31%]/46,XY[69%]	P	NA	TOP
55	24	18	Normal	dup(9q), 1.0 Mb	46,XN	chr9q21.13(77238728_78549914)× 3 (1.3Mb)	VUS	NA	Normal
56	27	19 + 6	Normal	dup(8q), 1.2 Mb	46,XN	Negative			Normal
57	32	21 + 2	Normal	dup(9p), 5.35 Mb	46,XN	Negative			Normal

ACMG: American College of Medical Genetics; CNV-seq: Copy number variant sequencing; del: Deletion; dup: Duplication; LP: Likely pathogenic; NA: Not available; NIPT: Noninvasive prenatal testing; NT: Nuchal thickness; P: Pathogenic; SUA: Single umbilical artery; VUS: Variant of uncertain significance.

Table 4. The results of fetuses with suspected nonsyndromic copy number variants (cont.).

Case	Age (years)	Gestational weeks	Ultrasound	NIPT results	Karyotype	CNV-seq results	ACMG classification	Source	Fetal outcome
58	36	18	Normal	dup(22p), 0.53 Mb	46,XN	Negative			Normal
59	31	19 + 5	Normal	dup(7p), 1.3 Mb	46,XN,9qh+	Negative			Normal
60	22	25 + 2	Normal	dup(5q), 0.56 Mb	46,XN	Negative			Normal
61	32	18 + 3	Normal	dup(1p), 4.1 Mb	46,XN	chr5q13.3(74892336_76034753)×3 (1.1 Mb)	VUS	NA	TOP
62	30	22	Bilateral renal sinus separation	dup(20q), 0.77 Mb	46,XN	Negative			Normal
63	31	18 + 5	Normal	dup(7), 0.45 Mb	46,XN,1qh+,9qh+	Negative			Normal
64	31	19	Normal	dup(9), 0.78 Mb	46,XN	Negative			Normal
65	31	21 + 4	Normal	del(10q), 16.55 Mb	46,XN	Negative			Normal
66	35	19	Normal	dup(22), 3.0 Mb	46,XN	chr14q23.2q24.2(62613542_72909710)×2 hmz (10.2 Mb)	VUS	Paternal	Normal
67	29	19	Normal	dup(11), 1.42 Mb	46,XN	Negative			Normal
68	30	21	Normal	dup(7p), 1.42 Mb	46,XN	Negative			Normal
69	28	21 + 2	Normal	del(8p), 3.6 Mb	46,XN	Negative			Normal
70	31	18 + 5	Normal	del(11)(q22q23.1), 5 Mb	46,XN	chr11q23.3(121042408_121156707)×1 (114.3 Kb)	VUS	Maternal	Normal
71	27	19	SUA	dup(7), 0.15 Mb	46,XN	Negative			Normal
72	24	20 + 4	Normal	del(1)(q43q43), 5.25 Mb	46,XN	Negative			Normal
73	32	19 + 4	Bilateral renal sinus separation	del(1q), 1.2 Mb	46,XN	Negative			Normal
74	22	27	Normal	dup(7), 0.88 Mb	46,XN	Negative			Normal
75	43	23 + 4	Normal	dup(14), 0.35 Mb	46,XN	Negative			Normal
76	26	23	Normal	dup(3), 2.1 Mb	46,XN	Negative			Normal
77	27	19 + 5	Normal	dup(7), 1.0 Mb	46,XN	Negative			Normal
78	34	21	Left ventricular bright spot	dup(3)(q29), 4.36 Mb	46,XN	chr3q29(193281915_198022430)×3 (4.7 Mb) chr7p22.3p22.2(1_3115830)×1 (3.1 Mb)	VUS VUS	Novel	Normal
79	30	19 + 3	Normal	dup(7), 0.98 Mb	46,XN	Negative			Normal
80	29	24 + 5	Normal	dup(14), 0.55 Mb	46,XN	chr14q31.3(88906320-89219419)×1 (313.1 Kb)	VUS	NA	Normal
81	33	21 + 3	Normal	dup(18), 0.48 Mb	46,XN	Negative			Normal
82	30	25 + 5	Normal	dup(17)(q23.1q25.3), 4 Mb	46,XN	Negative			Normal
83	38	23 + 2	Fetal septum pellucidum 2 mm	dup(20), 0.35 Mb	46,XN	Negative			Normal
84	30	19 + 5	Normal	dup(20), 0.44 Mb	46,XN	Negative			Normal
85	36	18	Normal	dup(15), 1.1 Mb	46,XN	Negative			Normal
86	31	20	NT 2.6 mm	dup(14), 0.98 Mb	46,XN	Negative			Normal
87	31	18 + 3	Normal	dup(7), 1.1 Mb	46,XN	Negative			Normal

ACMG: American College of Medical Genetics; CNV-seq: Copy number variant sequencing; del: Deletion; dup: Duplication; LP: Likely pathogenic; NA: Not available; NIPT: Noninvasive prenatal testing; NT: Nuchal thickness; P: Pathogenic; SUA: Single umbilical artery; VUS: Variant of uncertain significance.

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Table 5. Performance parameters of noninvasive prenatal testing for detection of microdeletion/microduplication syndromes and nonsyndromic copy number variants in 94,085 pregnancies.

	NIPT positive	TP	FP	PPV
CNVs				
Total	113	40	73	35.4%
MMSs				
Total	26	16	10	61.5%
Fetal ultrasound				
Normal	19	9	10	47.4%
Abnormal	7	7	0	100%
Classical MMS				
22q dup syndrome	5	3	2	60%
DGS	4	4	2	50%
CDC	3	1	2	33.3%
Xp del syndrome	2	2	0	100%
Nonsyndromic CNVs				
Total	87	24	63	27.6%
Fetal ultrasound				
Normal	68	14	55	20.6%
Abnormal	19	10	7	52.6%
Increased NT	6	4	2	66.7%
Other	13	6	7	57.1%
CNV size				
≤5 Mb	70	21	49	30%
Within 5–10 Mb	12	2	10	16.7%
>10 Mb	6	1	5	16.7%

CDC: Cri-du-chat syndrome; CNV: Copy number variant; del: Deletion; DGS: DiGeorge syndrome; dup: Duplication; FP: False positive; MMS: Microdeletion/microduplication syndrome; NT: Nuchal thickness; PPV: Positive predictive value; TP: True positive.

positives and ten false positives (PPV: 16.7%). Among the CNVs over 10 Mb, there was one true positives and five false positives (PPV: 16.7%). Combined with normal fetal ultrasound findings, CNV-seq confirmed a diagnosis of nonsyndromic CNV in only 11 cases, for which eight follow-up samples were received from parents and three were without comparison. All fetuses were healthy, except for two cases of spontaneous premature labor.

Clinical outcomes of confirmed positive fetuses

In the MMS cohort, there was a high prevalence of termination of pregnancy (TOP) in confirmed MMS pregnancies, including 22q duplication syndrome (66.7%), cri-du-chat (CDC) syndrome (100%) and Xp deletion syndrome (100%) (Table 1). A total of 24 CNVs were confirmed in the nonsyndromic CNV cohort, including two pathogenic CNVs, two likely pathogenic CNVs and 20 variants of uncertain significance (VUSs). To verify the origin of the CNVs, we further analyzed CNVs in the parents' peripheral blood and followed up pregnancy outcomes. The results showed that the two pathogenic CNVs eventually aborted and two progressed to potentially pathogenic CNV pregnancies, one of which (case 25) was diagnosed as novel and therefore selected for abortion. Another (case26) was identified as paternally inherited; the pregnancy proceeded normally pregnancy and the fetus was healthy at follow-up. Among the 20 VUS pregnancies, eight refused further detection; the rest were compared with their parents' peripheral blood CNV-seq. It was found that two were novel variants (one abortion and one normal pregnancy); five cases were inherited from the mother and five from the father. All cases resulted in normal pregnancies and the fetuses were healthy during follow-up.

Discussion

The American College of Obstetrics and Gynecology and the 2011 Society for Obstetrics and Gynecology recommended cffDNA NIPT as a highly accurate method for detecting fetal aneuploidy in high-risk pregnancies [11].

However, the clinical added value of CNV testing remains controversial [13,14]. MMSs are clinical syndromes caused by CNVs in specific chromosomal regions. Similar to chromosomal aneuploidy, a MMS can lead to congenital malformation and intellectual disability. Nonsyndromic CNVs include some genome-wide segmental CNVs with unclear clinical significance, because the specific syndromes associated with these changes cannot be identified in any existing database, so their pathogenicity is unknown. Opponents of genome-wide testing argue that the lower PPV and uncertainty about the pathogenicity of many CNVs complicates genetic counseling for high-risk outcomes and adds unnecessary invasive procedures. However, proponents argue that the goal of any prenatal diagnosis is to prevent the birth of children with severe chromosomal disorders, even if the PPV is low or moderate, and NIPT is sensitive enough to identify a large number of fetuses with rare chromosomal syndromes that cannot be detected by conventional ultrasound [15]. Recently, many studies have been devoted to the diagnostic accuracy of NIPT for CNVs [16,17]. Most studies have focused on CNVs and chromosomal disorders associated with classical MMSs [18], and the value of NIPT in MMSs as a whole and for nonsyndromic CNVs remains to be explored. The aim of this study was to assess the diagnostic efficacy of NIPT in fetal pathogenic MMSs and nonsyndromic CNVs by high-throughput sequencing.

In the present study, we first assessed the efficiency of NIPT in detecting CNVs in association with different factors. The PPV was used to assess NIPT, and we found no significant difference in PPV for the detection of CNVs between the 35 and <35-year-old groups. We conclude that, unlike aneuploidy, CNVs are independent of maternal age and that even young women are susceptible to fetal chromosomal microdeletions. The concentration of cffDNA and the number of clearly assigned reads directly affect the efficiency of NIPT testing [19,20]. We used 8% as the threshold group to determine the PPV of CNVs detected by NIPT in different cffDNA concentration groups and found no significant differences. We used 15% as the threshold to determine the PPV of CNVs detected by NIPT in different effective read arrays and found no significant differences between the two groups. Interestingly, however, we found significantly higher PPVs for CNVs smaller than 15 Mb than for CNVs larger than 15 Mb detected by NIPT; this finding may be related to the shallow depth of sequencing and limited data volume of NIPT, a finding similar to that of Pei *et al.* [21].

More recently, broader guidelines have been proposed for routine screening of young women for MMSs, in whose pregnancies microdeletions are more common than aneuploidy [22]. Based on our retrospective study of 19,086 pregnant women, NIPT showed characteristics of a more suitable screening method for MMSs with CNVs, with a PPV of 61.5% for MMSs. Previous clinical validation studies have reported differential performance in detecting specific MMSs, detecting only low-to-moderate PPV [7]. A large study showed a known positive PPV of 40.8% for MMSs [15], similar to our findings. Wapner *et al.* developed a targeted single-nucleotide polymorphism-based sequencing approach to detect large deletions associated with five microdeletion syndromes and achieved detection rates of >97%, much higher than the current study [8].

The overall PPV of DiGeorge syndrome has changed significantly in the last few studies, with the PPV in this study being 50%. This PPV is significantly higher than that currently achieved using other methods, with reported PPVs ranging from a relatively low 16–21% in three studies [23–25] to 93% in one study [15] and 71% in another study [26]. 22q11.2 deletion syndrome is a well-defined and severe condition, with prenatal diagnosis showing improved clinical outcomes [27]. The data presented in this study add to the growing body of evidence that CNV-seq-based NIPT can detect 22q11.2 deletions with high PPV. For 22q duplication syndrome, CDC and Xp deletion syndrome, PPV was 60, 33.3 and 100%, respectively, and a similarly low PPV has been reported in other studies for CDC and 22q duplication syndromes, respectively [24]. There are no other reports of NIPT for Xp deletion syndrome in the literature, but the 100% PPV shown in this study may be due to the sample size.

Among the nonsyndromic CNVs, 27 CNVs detected by NIPT and confirmed by CNV-seq were found without fetal karyotype analysis, of which three had abnormal karyotypes, but the karyotype did not identify chromosomal sub-bands. Karyotyping is considered the 'gold standard' for prenatal diagnosis of chromosomal disorders. However, it is often difficult to identify chromosomal aberrations smaller than 10 Mb [21]. In the present study, of the 27 CNVs detected by NIPT and CNV-seq, 48.1% (13/27) had fragment length differences of ±1 Mb or less reported by the two methods. Thus, NIPT appears to be superior to karyotyping for the detection of CNVs. In addition, we found the highest PPV for NIPT in the increased NT group (66.7%), and a higher PPV for fetal structural ultrasound abnormalities (57.1%) than in the ultrasound normal group. Therefore the PPV of fetal structural abnormalities on ultrasound is an important reference in predicting fetal CNV. The PPV of CNV ≤5 Mb was the highest (30.0%), and the PPVs of CNVs 5–10 and >10 Mb were equal (16.7%). In Chen's study [7] the PPV for CNVs <10 Mb was 31%, which is similar to the results in this paper, while in Liang's paper [15], the PPV

was lower for CNVs >10 Mb (32%) and CNVs <10 Mb (19%). In addition, in CNVs ≤5 MB combined with normal fetal ultrasound findings, 11 cases of abnormal CNV-seq resulted in healthy fetuses at follow-up, with no abnormal appearance in all; there were two cases of spontaneous induction of labor. Based on these results, we suggest that CNVs detected by NIPT should be used to determine fetal outcome in combination with ultrasound findings, karyotype analysis and CNV-seq.

A previous study reported a PPV of 9.2% for overall CNVs [15], but the PPV in our study was much higher. These false-positive CNVs may be due to placental mosaicism or maternal abnormalities [16,28]. Chromosomal abnormalities that occur only in the placenta and not in the fetus are known as confined placental mosaicism [29], which occurs at a rate of approximately 1–2% [30]. With the increased use of NIPT in prenatal diagnosis and the current shift to CNV reporting, it is expected that there will be more false-positive results for NIPT due to abnormal maternal/placental origins. Yatsenko *et al.* emphasized that NIPT is a screening test and further diagnostic studies are needed to determine the precise genomic coordinates of chromosomal structural abnormalities and their parental origin, which is essential for determining the affected gene content as well as providing optimal prenatal management and accurate genetic counseling [31]. Therefore, positive NIPT reports of CNVs must be interpreted with caution. With further validation studies, a clear distinction should be made between chromosomal structural abnormalities in the fetus, mother and placenta.

It is noteworthy that only 47.5% of the deletions and duplications in our study were associated with known abnormalities. Many of the abnormalities are likely to be normal genetic mutations of no clinical significance. Unfortunately, among the 40 samples with abnormal CNVs, only 13 pregnant couples were willing to undergo peripheral blood CNV-seq testing to determine whether the fetal CNV was inherited from a parent. We attended a genetic counseling session for all women with CNVs to discuss the eventual management of the pregnancy. Unfortunately, two pregnant women with CNVs diagnosed as VUSs were informed of the CNV and chose to terminate their pregnancies. More work is needed to enhance educational programs to raise awareness of genetic disorders among pregnant women. On 10 April 2019, an expert consensus on the use of low- and high-depth whole genome sequencing technologies in prenatal diagnosis established that CNV-seq should be considered as a first-line prenatal diagnostic technique for pregnancies with suspected fetal chromosomal abnormalities [32]. However, CNV-seq has many limitations. As sampling requires invasive testing, such as for *Bifidobacterium abortus* miscarriage and intrauterine infection, and the opportunity to identify variants of uncertain importance, some women may decline.

Conclusion

In summary, this was a retrospective study of a large sample of pregnant women with different CNV types. Based on our extended high performance of screening for MMS, we propose that NIPT is a candidate primary screening method for all pregnancies, including for MMS. However, due to the low PPV associated with nonsyndromic CNV testing and the fact that most CNVs are associated with good pregnancy outcomes, particularly in fetuses with normal ultrasound findings, the introduction of expanded NIPT would lead to an increase in unnecessary invasive procedures, as well as unnecessary anxiety and inappropriate terminations of pregnancy. Consideration should also be given to whether to respect the availability of clinical resources to provide pre-test counseling and manage women with positive screening results. However, as NIPT technology improves and data related to the pathogenicity of CNVs accumulate, the effectiveness of NIPT for CNV detection in general risk populations needs to be further evaluated in future studies.

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Ethical conduct of research

The study was approved by the local research ethics committee (K202203-08).

Summary points

- Microdeletion/microduplication syndromes (MMSs) are clinical syndromes caused by copy number variants (CNVs) in specific chromosomal regions.
- Nonsyndromic CNVs include some genome-wide segmental CNVs with unclear clinical significance (i.e., their pathogenicity is unknown).
- With the development of high-throughput sequencing technology, research has become focused on the feasibility of noninvasive prenatal testing (NIPT) for fetal chromosome CNVs beyond the common aneuploidies.However, debate still exists as to the added clinical value of testing for CNVs.
- The value of NIPT in overall MMS and nonsyndromic CNVs is still unknown.
- This study quantified multiple factors of different clinical indicators, combined with pregnancy outcomes, to evaluate the clinical efficiency of NIPT in detecting MMSs and nonsyndromic CNVs.
- This study provides important evidence that NIPT displays the hallmarks of a screening method suitable for MMSs caused by CNVs. especially in the context of fetal ultrasound structural abnormalities.
- However, the study identified moderate-to-low performance for the detection of nonsyndromic CNVs, most of which have good pregnancy outcomes, especially for fetuses with normal ultrasound.
- Introducing expanded NIPT will result in an increase in unnecessary invasive procedures and increased cost, as well as unnecessary anxiety and inappropriate terminations of pregnancy.
- We propose that our NIPT method, combined with ultrasound as an independent screening system, may eventually have clinical application as the new standard of care for routine screening of pregnancies for fetal pathogenic CNVs associated with chromosomal syndromes.
- We suggest strongly that all women carrying a fetus suspected of having a CNV by NIPT should accept a genetic counseling session to discuss pregnancy management options.

Data sharing statement

The datasets presented in this study can be found in online repositories.

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