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ORIGINAL ARTICLE

Systematic analysis of the causes of NIPS sex chromosome aneuploidy false-positive results

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Abstract

Objective: To investigate the underlying causes of false positives in NIPT of fetal sex chromosomal aneuploidies using fetal cell-free DNA from maternal plasma.

Methods: In the present study, we focus on a cohort of 23,984 pregnancy cases with NIPT. Karyotyping and FISH analysis were employed to verify the NIPT detected false-positive results of fetal sex chromosomal aneuploidies, and a comparative CNV sequencing on positive and negative NIPT cases was uniquely performed to elucidate the underlying causes.

Results: A total of 166 cases (0.69%) were identified as fetal sex chromosomal abnormalities, while 84 cases were found to be false-positive results possibly associated with maternal X chromosomal aneuploidies ($n = 8$), maternal X chromosomal structural abnormalities ($n = 1$), maternal CNVs ($n = 4$) as well as known placental mosaicism ($n = 1$). Furthermore, our study showed that the maternal chromosome CNV between 1–1.6 Mb was associated with false-positive NIPT results in sex chromosomal abnormalities.

Conclusion: Our research demonstrated the spectrum of factors causing false positives in NIPT of fetal sex chromosomal abnormalities based on a large cohort. The effective maternal CNV size cut-off identified in our study could integrate into bioinformatics algorithms for reducing the false-positive rate, however, further investigation is necessary to confirm this.

KEYWORDS

false positives, maternal CNV, NIPT, X chromosomal abnormalities, *Z* score

1 | INTRODUCTION

Chromosomal aneuploidies are one of the most serious types of birth defects. Trisomy 21 (Down syndrome), trisomy 18 (Edward syndrome), trisomy 13 (Patau syndrome), and sex chromosome aneuploidies (SCAs) are known as the most common chromosomal aneuploidies (Everest et al., 2015). Fetal SCAs are caused by the presence of an abnormal number of sex chromosomes (X, or Y) in a cell, and 45,X (Turner syndrome); 47,XXX (Triple X syndrome); 47,XYY (Klinefelter syndrome); 47,XXY are among those recognized (Visootsak & Graham Jr, 2009).

Recently, non-invasive prenatal testing (NIPT) for fetal aneuploidies based on circulated fetal DNA in the maternal

plasma has gained popularity due to its features of non-invasiveness and high positive predictive values (PPV) in T21 (65%–94%), T13 (47%–85%) and T18 (12%–62%) (Neofytou et al., 2017; Quezada et al., 2015; Yaron et al., 2015). However, researches involved in NIPT for fetal SCAs are limited and these reports likewise suggested a significant false-positive rate in SCAs (approximately 53%) (Suo et al., 2018).

In order to make NIPT a reliable method for the clinical screening of fetal SCAs, understanding the biological causes for these discordant positives becomes crucial. This discovery is important in optimizing the statistical approach to decrease the false-positive rates of NIPT (Zhang et al., 2015). Commercially available tests are based on counting statistics following the massive parallel sequencing of total cfDNA in maternal plasma, a minority of which are feto-placentally derived. The obtained sequence reads are converted to a normal distribution and compared to a reference distribution, generating a *Z* score and estimating the likelihood of fetal aneuploidy (Nygren et al., 2010). This approach assumes that every woman carries the same proportion of genetic material on a given chromosome. As the majority of cfDNA is maternally determined, it is clear that maternal copy number variants can alter the interpretation of NIPT results. In cases of a diploid pregnancy in which the mother carries a duplication, the *Z* value can cause similar false-positive results. A relationship between maternal CNVs (mCNVs) and false-positive results of NIPT was proposed for fetal T13, 18, and 21 by Snyder et al., which employed a small cohort to prove the concept (Snyder et al., 2015). The constraint of their exploration and the shortage of research accessible to elucidate the underlying mechanism have instigated our group's desire to carry out this research focusing on fetal sex chromosomal abnormalities, whose relationships with maternal chromosomal abnormalities were not elucidated.

In this study, we utilize NIPT to investigate a large Chinese cohort to examine its specificity, sensitivity, and the false-positive rate of the SCAs. The underlying biological causes of the false-positive results were assessed and illustrated to provide tools to improve the application of NIPT in fetal SCAs. We also provide new insight into the mCNVs associated with false positives in the NIPT of fetal SCAs.

2 | MATERIALS AND METHODS

2.1 | Patient and sample processing

From November, 2016 to March, 2018, NIPT was performed on 23,984 pregnant women at the Genetic Testing Center in Qingdao Women's and Children's Hospital, of which 17,077 pregnant women (71.2%) were determined by serological screening to be at high risk (risk of 21 trisomies was higher than 1/270, or risk of 18 trisomies was higher than 1/350). Their gestation periods were distributed between 12 and 34 weeks, and the average gestational age was 19.6 weeks. All the patients signed the informed consent form. This study was approved by the ethics board with approval #QD20180927.

2.2 | NIPT detection

Eight milliliters of peripheral blood was withdrawn from each of the pregnant women into an EDTA tube. Plasma and leukocytes were separated at 1600 rpm (10 min, 4°C) and 16,000 rpm (10 min), respectively, and stored at −80°C until further use. The extracted DNA from the plasma was subjected to DNA library construction and the genome sequencing was performed using the NextSeq CN500 gene sequencer on the Illumina sequencing platform. The sequencing data was compared with the human genome reference sequence, hg19, and the *Z* score was calculated (the *Z* score of the chrN = (% chrN of the sample- Reference mean of % chrN)/Standard deviation of % chrN; % chrN = (the number of unique reads of chromosome N/the total number of unique reads) × 100%, $N = 1, 2, 3, \ldots 22, X, Y$).

2.3 | Prenatal diagnosis

Among the 166 cases of NIPT SCA high risk, 107 patients accepted prenatal diagnosis after clinical counseling. Through amniocentesis, cells from the fetal amniotic fluid were used for karyotype analysis, fluorescent in situ hybridization (FISH), QF-PCR, and chromosome microarrays. Sixteen newborns of the women who had refused prenatal diagnosis received only peripheral karyotype analysis.

2.4 | Karyotyping analysis

Among the 84 false-positive cases of sex chromosome aneuploidy indicated by NIPT and newborn blood studies, the maternal peripheral blood karyotype analysis was performed. The pregnant women's peripheral blood was taken for cell culture, chromosome preparation, and G-banding. For each subject, 100 cells at metaphase were counted and 20 were karyotyped.

2.5 | FISH analysis

After the NIPT "false-positive SCA result" was confirmed by amniocentesis, pregnant women were followed up to

delivery. Among them, 21 women had voluntarily donated their placenta for FISH analysis. Each placenta was selected from the fetal and maternal surfaces of the central and marginal parts of the placenta, and also from the root and distal ends of the umbilical cord. A total of six tissues were used for hypotonic, fixation, sampling on treated slides, pre-treatment, probe denaturation, hybridization, and DAPI staining. The hybridization signal was observed under a fluorescent microscope. The probe was selected as the centromere probe CSP18/CSPX/CSPY on the 18 and X, Y chromosomes (Beijing GP Medical Technologies Ltd., Beijing, China). Around 100 cells were counted per slide. The calling parameters were as follows: XX or YY ≥90%, normal disomy; XO or YO ≥10%, mosaic monosomy; XXX or XXY or XYY ≥10%, mosaic trisomy. The images were taken by Cytovision software, an AI FISH analysis workstation, and the results were recorded.

2.6 | CNVseq analysis of maternal CNVs

To further evaluate the impact of mCNVs on false positives, we recruited pair-matched controls from the 23,818 NIPT negative women to compare with the NIPT SCA false-positive women. The inclusive criteria were as the following: NIPT negative cases, BMI ± 5, and at the same gestational age. The maternal peripheral blood samples of the two cohorts were used to perform CNV sequencing (CNV-seq) and analysis (Figure 1).

The genomic DNA in the peripheral blood leukocytes of the pregnant women was extracted with a QIAamp DNA Mini kit (Qiagen, Germany). The DNA concentration was determined, and its integrity was detected with 1% agarose gel electrophoresis. A 50 ng genomic DNA sample was used to construct a sequencing library. The BGI-seq500 platform was used for single-end sequencing of the whole genome. The sequencing read length was 35 bp and each sample produced 35–40 M clean reads. The population-scale CNV calling was used for bioinformatics analysis to detect deletions and duplications above 100 kb in X chromosomes.

2.7 | Statistical analysis

SPSS 22.0 statistical software and the pair test analysis (McNemer χ^2 test) were used to compare the CNV-seq results between the 76 NIPT SCA false-positive pregnant women and the pair-matched NIPT negative women. Linear regression analysis was applied to obtain the linear relationships between the *Z*-score of X chromosomes revealed by NIPT and the size of the mCNVs.

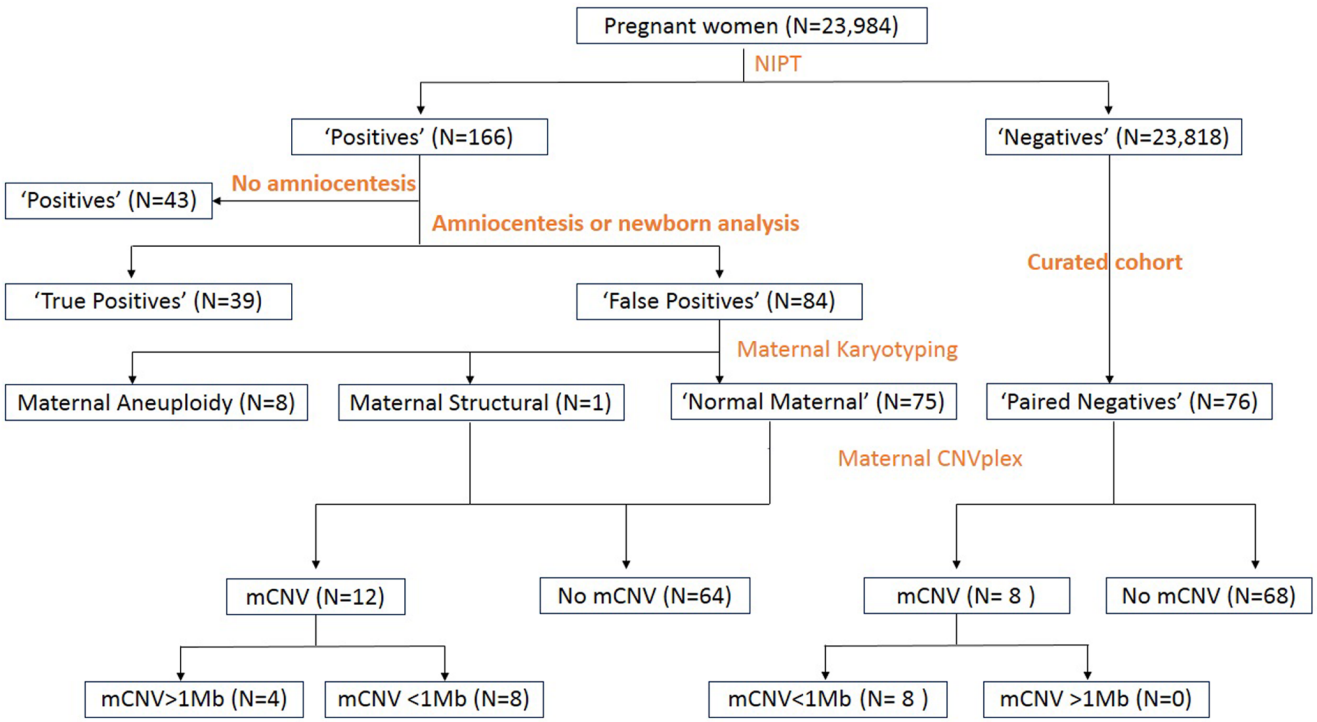

FIGURE 1 Study design

3 | RESULTS

3.1 | PPV of fetal SCA with NIPT

As shown in Figure 1, among the 23,984 singleton pregnancies tested by NIPT at the Genetic testing center, 166 cases (0.69%) showed sex chromosome abnormalities, of which 107 (64.5%) pregnant women chose amniocentesis for prenatal diagnosis and 16 (9.6%) chose karyotype analysis of the neonatal peripheral blood following delivery. The remaining 43 (25.9%) pregnant women declined further testing.

In the 123 (107 + 16) cases of NIPT SCA positive pregnancies, 31.1% (390/123) of pregnancies were confirmed to be true positive. The PPV of NIPT for fetal SCAs was 31.7%. The PPV of different sex chromosome aneuploidy abnormalities such as 45,X, 47,XXX, 47,XXY, 47,XXY, and 45,Y were 17.5%, 60.0%, 56.5%, 100%, and 0%, respectively, as shown in Table 1. In addition, one case of abnormal fetal sex chromosome structure was detected, whose karyotype was 46,X, der(X)del(X) (p21.3) dup(X) (q26.3).

3.2 | Analysis of the causes of false positives in sex chromosome abnormalities

The pregnant women of the 84 false-positive cases of sex chromosome aneuploidy were analyzed by karyotypes of peripheral blood chromosomes. Eight cases of aneuploidy abnormalities were detected. The abnormal rate was 9.5% (8/84), of which 5 cases were 47, XXX; 3 cases were 45, XO/46, XX/47, XXX involved mosaicism, as displayed in Table 2.

In addition, one case of chromosome structural abnormality was detected, of whom the karyotype was 46, X, der(X)del(X) (p21.3) dup(X)(q26.3). To further determine the CNV size of the fragment involved in structural abnormalities, the maternal DNA was also subjected to low-coverage whole-genome sequencing using the CNV-seq method.

Postpartum follow-up of the 75 pregnant women with discordant fetal SCAs with NIPT and normal karyotypes was carried out. Twenty-one cases of voluntarily donated placenta were obtained, and tissues from six locations were taken for sampling. A CSP18/CSPX/CSPY probe was used to perform a FISH test. One case of confined placental mosaicism was detected, and details of the NIPT and FISH tests are shown in Table 3.

A 76 women control cohort was recruited to pair-match with the above 76 women, and a CNV-seq analysis was performed. As shown in Figure 1, 0.1-11 Mb CNVs were found in 12 cases. In the control cohort, curated from 23,818 cases composing the low-risk group, based on stringent criteria, 0.1–0.8 Mb CNVs are identified in 8 cases (12/76 vs. 8/76, $p = .64$).

A detailed comparison of the results is displayed in Table 4. According to ACMG standards (Riggs et al., 2020), only 4 CNVs that were larger than 1 Mb could be classified as Pathogenic. This made the "false positive cases" of clinical significance to 4. The four cases were selected subsequently for comparative analysis. When we plotted the corrected CNV sizes (>1 Mb) and NIPT Z score, their linear regression became statistically significant ($R^2 = 0.977$; $p < .05$), as shown in Figure 2. On the contrary, random distribution was found when we plotted the relationship between CNV <1 Mb and the Z score, as shown in Figure 3. Whereas mCNV of 1.69 Mb in size is shown as the minimal mCNV size associated with false-positive results via linear regression analysis. These data lead us to deduce a mCNV size of 1–1.6 Mb as the cut-off to be associated with false positives in fetal SCA NIPT.

4 | DISCUSSION

Our study explored the utilization of NIPT for fetal SCAs employing a large cohort of the Chinese population.

TABLE 1 NIPT detection of aneuploidy abnormalities in sex chromosomes

Types	Abnormal NIPT cases	Prenatal diagnosis	Postnatal diagnosis	No follow-up	Confirmed positives	Abnormal karyotypes	PPV (%)
45,X	78	52	5	21	10	45,X ($N = 3$), 45,X/46, XX/47,XXX mosaic ($N = 7$)	17.5
47,XXX	23	13	2	8	9	47,XXX ($N = 9$)	60.0
47,XXY	30	18	5	7	13	47,XXY ($N = 12$) 48,XXYY ($N = 1$)	56.5
47,XYY	10	6	1	3	7	47,XYY ($N = 7$)	100.0
45,Y	25	18	3	4	0	–	0
Total	166	107	16	43	39	–	31.7

Abbreviation: PPV, positive predictive value.

TABLE 2 Details of cases with maternal aneuploidy

No.	Gestational week	ChrX (*Z* score)	Karyotype
1	15^{+5}	62.68	47,XXX
2	19^{+0}	−69.29	45,X[32]/46,XX[68]
3	20^{+1}	51.44	47,XXX
4	14^{+1}	24.04	47,XXX
5	18^{+4}	−30.82	45,X[63]/46,XX[21]/47,XXX[16]
6	18^{+4}	76.6	47,XXX
7	19^{+4}	48.79	47,XXX
8	21^{+4}	23.36	47,XXX[72]/45,X[20]/46,XX[8]

The positive screening rate of fetal SCAs in this study was 0.69% (166/23,984), which was similar to 0.42% (34/8152) and 0.55% as reported in a previous report (Hu et al., 2019; Zhang et al., 2017). This positive screening rate in the Asian cohort was lower than that in a Western cohort, in that Kornman et al. reported a 2.3% of SCA-positive screening rate in an Australian cohort of 5267 singleton pregnancies (Kornman et al., 2018). The difference in these frequencies is likely to be associated with how the referrals were ascertained, the average maternal age of the tested population, and NIPT methodological differences in calling SCAs. In the 166 NIPT for fetal SCAs positive cases, 123 (74.1%) cases underwent amniocentesis or

TABLE 3 Confined placental mosaicism case

NIPT result		FISH test result					
		Middle placenta		Placental margin		Umbilical cord	
Type	*Z*-score	Fetus surface	Maternal surface	Fetus surface	Maternal surface	Root	Remote
45,X	−9.76	45,X (92%)	45,X (91%)	45,X (15%)	45,X (12%)	46,XX	46,XX

TABLE 4 Comparative analysis of mCNVs with a false-positive and true negative results

Case	Gestation	*Z* score	Cytogenetic location	Hg19 coordinates	Copy ratio	Copy number	Size in Mb	Classifications
False positive cohort								
P-1	20	−4.01	Xp11.3p11.4	37,870,222–44,410,784	0.51	1	6.54	Pathogenic
P-11	18	−6.63	Xq27.2	140,348,537–140,777,701	0.47	1	0.43	Benign
P-12	20	−3.43	Xp11.21	56,265,333–56,472,269	1.44	3	0.21	VOUS
P-21	18	−4.55	Xq27.3	143,675,241–143,845,227	0.57	1	0.17	Probably Benign
P-23	19	−13.16	Xq25	128,053,813–128,187,652	0.44	1	0.13	Probably Benign
P-36	17	−3.06	Xq11.2	63,716,235–63,913,270	1.44	3	0.2	Probably Benign
P-42	15	−3.76	Xp22.31	7,800,601–8,454,726	1.47	3	0.65	Benign
P-58	19	10.612	Xq21.1	77,165,280–77,703,455	1.4	3	0.54	VOUS
P-74	18	3.18	Xp22.31	6,440,776–8,135,053	1.45	3	1.69	VOUS
P-75	20	−3.44	Xp21.2p21.1	30,602,789–37,472,435	0.53	1	6.87	Pathogenic
P-76	18	−6.62	Xp22.33p21.3	168,551–28,447,436	0.5	1	10.9	Pathogenic
			Xq26.3q28	137,837,884–155,233,098	1.48	3		Pathogenic
True negative cohort								
C-5	21	−2.69	Xp11.23	47,883,618–47,996,008	0.57	1	0.11	Probably Benign
C-8	18	0.12	Xp21.1	35,915,918–36,643,075	1.6	3	0.73	Probably Benign
C-12	20	−0.92	Xp11.23	47,871,701–48,008,640	1.4	3	0.14	Probably Benign
C-14	19	0.29	Xq27.1	140,030,891–140,152,079	1.4	3	0.12	Probably Benign
C-17	21	0.86	Xq11.1	61,938,980–62,345,557	1.54	3	0.41	Probably Benign
C-18	20	1.4	Xp11.4	42,118,475–42,249,899	1.41	3	0.13	Probably Benign
C-25	16	0.45	Xq12	67,147,431–67,281,795	1.43	3	0.13	Probably Benign
C-33	16	−1.2	Xp22.33	3,007,138–3,223,696	1.49	3	0.22	Benign

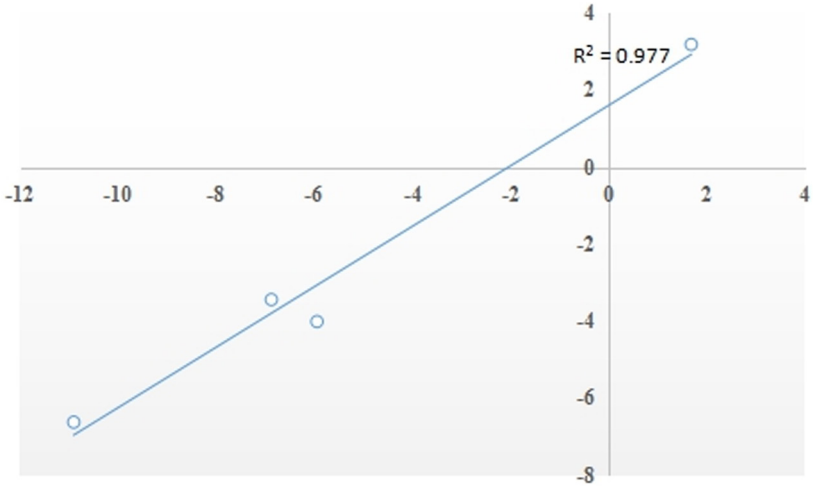

FIGURE 2 Graph of fetal *Z*-scores for ChrX mCNV sizes (>1 Mb)

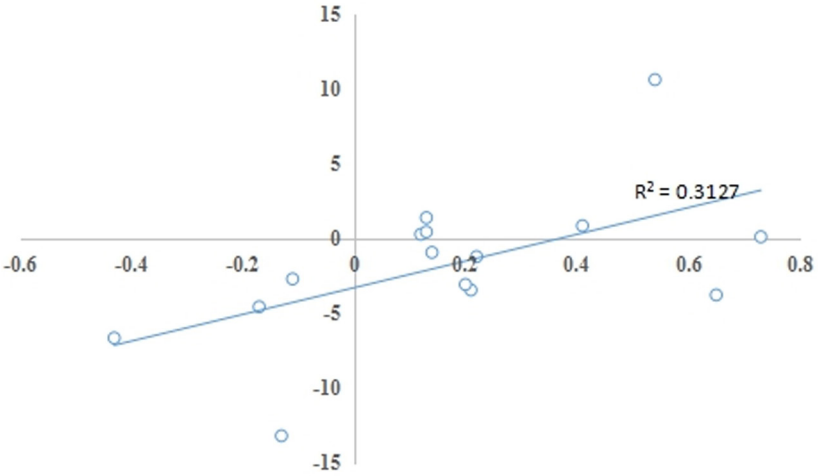

FIGURE 3 Graph of fetal *Z*-scores for ChrX mCNV sizes (<1 Mb)

newborn chromosome karyotype analysis. Among them, 39 cases of fetuses or newborns were diagnosed with sex chromosome aneuploidy with an overall positive predictive value of 31.7%. Specifically, the NIPT positive predictive value of 47,XXX (60%), 47,XXY (56.5%), and 47,XYY (100%) were higher than that of 45,X (17.5%), 45,Y(0%), suggesting that NIPT might have more advantages in screening for sex chromosomes trisomy than monosomy. The PPV of NIPT in sex chromosomes trisomy was consistent with the report of Suo et al. (2018) on 47,XXX, 47,XXY, and 47,XYY. The PPV of 45,X was consistent with the results reported by Kornman et al. (2018), and was lower than those of which reported by Suo et al. (2018), Persico et al. (2016), Porreco et al. (2014), Song et al. (2013), and Luo et al. (2021). The lower PPV of 45,X might also be related to the fact that a greater number of 45,X fetuses have abnormal ultrasonic structures than other types of SCA fetuses, so their parents tend to forgo diagnosis and proceed to the termination. In our study, 21 cases of pregnant women carrying fetal 45,X detected with NIPT declined further fetal chromosomal analysis. Among them, 6 were diagnosed with fetal ultrasonic anomalies, though none had gone through karyotype verification. In addition, for the cases of NIPT suggested 45,Y, although no abnormal fetus was found in the prenatal diagnosis, the possibility of 45,X/46,XY mosaicism still exists (Mao et al., 2014).

In the current study, the causes of false-positive NIPT chromosome aneuploidy were investigated from the perspectives of maternal sex chromosome abnormalities and fetal placenta. Among 84 false-positive cases proceeding with the chromosome karyotype analysis, there were 5 cases of 47,XXX and 3 cases as 45,XO / 46,XX / 47,XXX mosaicism (9.52%). As women with 47,XXX or mosaicisms of 45, XO / 46, XX / 47, XXX is often healthy, fertile, and have no specific abnormal clinical manifestations, maternal SCAs significantly contribute toward false positives of fetal SCAs. Wang et al. found that 8.6% of false positives in fetal SCA are related to maternal SCA (Wang et al., 2015).

Assessment of confined placental mosaicism was undertaken in a subgroup through FISH evaluation. The collected false-positive placenta cases were further verified with FISH. One case (1/21, 4.76%) of which was found to

exhibit 45,X/46,XX mosaicism in the placenta, with the proportion of abnormal karyotype mosaicism in the placental tissue ranging from 12% to 92%, while the karyotype types of the umbilical cord and fetal amniotic cells were normal, as shown in Table 3. As the majority of fetal-free DNA in the maternal plasma derives from placental trophoblastic cells (Chim et al., 2005; Ramdaney et al., 2018; Taglauer et al., 2014), confined placental mosaicism can affect NIPT detection and cause false positives. Similar conditions have been reported in chromosomes 22 (Chen et al., 2017), 13 (Hall et al., 2013), 18, and 21 (Crooks et al., 2015).

This study provided a unique assessment of the contribution of X chromosome CNV to the technical accuracy of the cfDNA. In general, NIPT can estimate the risk of fetal chromosome aneuploidy by detecting free DNA in the peripheral blood of pregnant women and calculating the relative ratio of read numbers belonging to each chromosome, without distinguishing between the maternal and fetal DNA. CNVs are common in human chromosomes and vary in size. Therefore, when CNVs exist in the chromosomes of pregnant women, NIPT detection results will theoretically be affected. It has been confirmed that CNVs on chromosomes 21, 18, and 13 in pregnant women would affect the results of NIPT detection (Snyder et al., 2015). To study the effects of maternal X chromosome CNV and its fragment size on the detection of fetal chromosome aneuploidy abnormalities by NIPT, a 1:1 pair-matched design method was adopted and a CNV-seq technique was used to detect the presence of CNV on the X chromosome. Eight pregnant women in the matched group were detected to have CNVs on their X chromosomes, all of which were less than 1 Mb in size. Conversely, in the false-positive group, a total of 11 pregnant women were detected to have CNV on their X chromosomes, among which four pregnant women had X chromosome CNVs of greater than 1 Mb. No statistically significant difference was found between the false-positive and matched group with mCNV (12/76 vs. 8/76, $p = .64$). This observation was in line with the previous maternal NIPT reanalysis report that 0.42 Mb microduplication in maternal Xq27.2 and 1.32 Mb microdeletion in maternal Xp22.31 did not cause false positives in fetal SCA (Zhang et al., 2020). However, most importantly, linear regression analysis of the *Z* value and copy number variation of four cases with CNV > 1 Mb in our study showed a statistically significant positive correlation ($R^2 = 0.99$, $p < .05$). The CNVseq study suggested that copy number variation in the sex chromosomes of pregnant women could be one of the causes of NIPT SCA false-positive results and that when mCNV is larger than 1 Mb, it could lead to false positives. However, as our study only found a very small amount of positive cases, further investigation is required to support this.

Snyder et al. (2015) utilized a modeling strategy based on a European cohort to define the relationship between mCNVs and false positives NIPT in T13, 18, and 21, presenting that mCNV sizes of 487 kb and 1.15 Mb were associated with the increased rate in false positives in fetal T18. The authors acknowledged the limitation of their studies and several assumptions fundamental to their model, which were supported by a small cohort. The current study also faced the same limitation of sample size, with only 4 mCNV of clinical significance from a total of 23,984 pregnant women. Kaseniit et al. focus on optimizing bioinformatics algorithms to minimize the false positives of NIPT in autosomal fetal chromosomal abnormalities, which laid out the framework in the strategy (Kaseniit et al., 2018). To follow this strategy, our study would present only a path that may be pursued but more data on X chromosome CNV size is needed.

In a summary, as reported by several centers, the positive predictive value of NIPT for screening sex chromosome aneuploidy is lower than that for trisomy 21, 18, and 13, while the false-positive rate was higher. Maternal sex chromosomal aneuploidy, maternal sex chromosomal structural abnormalities, mCNV, and placental confined mosaicism were important factors leading toward false-positive NIPT SCAs. Novel to this study was the finding of X chromosome mCNV >1.0 Mb in 5.33% of positive SCA NIPT with 1.00–1.69 MB significantly positively correlated to X chromosome *Z* score in 4 individuals. These initial findings deserve replication and expansion in a larger cohort if the application to algorithm changes is to be considered.

5 | CONCLUSIONS

Taking advantage of a large cohort, our group systematically analyzed the multiple causes of NIPT SCA false positive cases, explaining the 16.7% (14/84) false-positive rate of fetal SCAs in this cohort. This study complement previous findings in somatic chromosomal abnormalities, refine, and improve the application of the NIPT in prenatal diagnosis.

AUTHOR CONTRIBUTIONS

Chunhong Huang conceived and designed the experiments. Zhaoru Lyu performed the experiments and analyzed the data. Chunhong Huang and Zhaoru Lyu worked together in writing the paper.

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CONFLICT OF INTEREST

The authors have no conflicts of interest relevant to this article to disclose.

ETHICAL APPROVAL

The study was approved by the Ethic Committee of Qingdao Women and Children's hospital. All patients provided written informed consent.

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