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RECEIVED 25 October 2024

ACCEPTED 27 December 2024

PUBLISHED 13 January 2025

## CITATION

Liu J-p, Wang S-B, Luo L and Guo Y-m (2025)  
Improving prenatal diagnosis with combined  
karyotyping, CNV-seq and QF-PCR: a  
comprehensive analysis of chromosomal  
abnormalities in high-risk pregnancies.  
*Front. Genet.* 15:1517270.  
doi: 10.3389/fgene.2024.1517270

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# Improving prenatal diagnosis with combined karyotyping, CNV-seq and QF-PCR: a comprehensive analysis of chromosomal abnormalities in high-risk pregnancies

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**Objective:** This study aims to assess the diagnostic efficacy of a combined approach integrating chromosomal karyotyping, copy number variation sequencing (CNV-seq), and quantitative fluorescence polymerase chain reaction (QF-PCR) in detecting chromosomal abnormalities in high-risk pregnancies.

**Methods:** This retrospective study analyzed 617 high-risk pregnancies undergoing prenatal diagnosis from February 2023 to August 2024, with amniotic fluid samples concurrently analyzed using karyotyping, CNV-seq, and QF-PCR. We evaluated clinical characteristics, diagnostic yields, and inter-method concordance rates. Longitudinal follow-up assessed pregnancy outcomes and neonatal phenotypes, with particular emphasis on cases demonstrating diagnostic discrepancies or variants of uncertain clinical significance.

**Results:** The integrated approach detected chromosomal abnormalities in 12.5% (77/617) of cases, significantly higher than the rates achieved by karyotyping alone (9.7%) and CNV-seq/QF-PCR alone (8.3%) ( $p < 0.05$ ). Karyotyping showed full concordance with CNV-seq and QF-PCR in detecting major chromosomal aneuploidies, identifying 21 cases of trisomy 21 and 4 cases of trisomy 18. CNV-seq uniquely identified additional pathogenic copy number variations in 2.1% of cases and variants of uncertain significance (VUS) in 3.2% of cases, both undetectable by conventional karyotyping. Subjects with high-risk non-invasive prenatal testing (NIPT) results had the highest abnormality detection rate (57.6%,  $p < 0.05$ ). Follow-up data revealed pregnancy termination in 44 of 97 cases with chromosomal abnormalities. Notably, neonates carrying pathogenic CNVs inherited from asymptomatic parents demonstrated normal phenotypes.

**Conclusion:** The integration of karyotyping, CNV-seq, and QF-PCR provides superior diagnostic yield compared to individual testing strategies in high-risk pregnancies. Although karyotyping remains the gold standard for

detecting major chromosomal aberrations, CNV-seq and QF-PCR enhance diagnostic precision through detection of submicroscopic variations. Multi-center studies with larger cohorts are needed to confirm these findings and clarify the clinical significance of uncertain variants.

#### KEYWORDS

chromosomal abnormalities, prenatal diagnosis, karyotyping, CNV-seq, QF-PCR, high-risk pregnancy, copy number variations

## Introduction

In China, congenital anomalies occur at a prevalence of approximately 5.6% (Xu et al., 2020). These developmental defects result from aberrant fetal morphogenesis *in utero*, potentially affecting the fetus's anatomy, physiology, and cognitive development (Zhou et al., 2024). Consequences range from intrauterine fetal demise to congenital malformations and neurodevelopmental disorders. Chromosomal abnormalities, such as aneuploidy, triploidy, and large-scale deletions or duplications, are the primary etiological factors (Kagan et al., 2022). Moreover, submicroscopic chromosomal aberrations and copy number variations (CNVs) contribute significantly to the pathogenesis of congenital anomalies (Cao et al., 2023; Chen et al., 2023). Currently, pregnancy termination following prenatal diagnosis of fetal abnormalities remains the predominant management strategy (Graf et al., 2023).

Amniotic fluid karyotyping has long been regarded as the gold standard for diagnosing chromosomal abnormalities. For 5 decades, this technique has been widely utilized to identify conditions such as aneuploidy, polyploidy, balanced structural rearrangements, and large chromosomal segment abnormalities (Luo et al., 2023). However, this method has several limitations: it requires amniotic fluid cell culture, requires skilled technicians, is time-consuming, and lacks the resolution to detect copy number variations smaller than 5 Mb (Saldarriaga et al., 2015).

To address these limitations, genome-wide copy number variation sequencing (CNV-seq) based on low-depth whole-genome sequencing, combined with quantitative fluorescent PCR (QF-PCR) targeting short tandem repeats and chromosomal microarray analysis (CMA), has been recommended in China for prenatal diagnosis (Clinical et al., 2019; Prenatal et al., 2023). These advanced methodologies offers high-throughput capabilities, rapid turnaround times, and the ability to identify small CNVs (Chen et al., 2024; Qiao et al., 2022; Cai et al., 2023; Santirocco et al., 2021). Notably, CNV-seq demonstrates higher sensitivity, improved resolution, and greater robustness with suboptimal specimens compared to CMA (Cohen et al., 2011; Cohen et al., 2015; Ma et al., 2021). Nevertheless, these methods cannot detect balanced chromosomal translocations (Qiao et al., 2022). Therefore, integrating karyotyping with CNV-seq and QF-PCR may provide a more comprehensive assessment of chromosomal abnormalities.

In this study retrospectively analyzes data from 617 cases that underwent combined testing, aiming to evaluate the clinical utility of

integrating karyotyping, CNV-seq, and QF-PCR in prenatal diagnosis.

## Methods

### Study population

This retrospective study analyzed 617 high-risk pregnant women who underwent prenatal diagnostic testing at our institution between February 2023 and August 2024. Patients were eligible if they underwent amniocentesis with subsequent chromosomal karyotyping, copy number variation sequencing (CNV-seq), and quantitative fluorescence polymerase chain reaction (QF-PCR) testing. Indications for prenatal diagnosis included ultrasonographically detected fetal structural anomalies, elevated risk on non-invasive prenatal testing (NIPT), family history of genetic disorders, previous adverse pregnancy outcomes, or other high-risk factors. Cases with insufficient sample volume, contamination, or technical failure were excluded. All participants provided written informed consent prior to procedures. The study protocol was approved by our institutional ethics committee.

### Sample collection and processing

Amniocentesis of 20 mL were obtained from each patient, equally divided for conventional karyotyping (10 mL) and combined CNV-seq/QF-PCR analysis (10 mL). The latter analyses were conducted at West China Second Hospital. Sample processing and cell culture were performed in our institutional genetics laboratory following standardized sterile protocols.

### Chromosomal karyotyping

For karyotyping, the amniotic fluid samples were centrifuged (1800 rpm, 10 min), and the pelleted cells were resuspended in 3 mL culture medium. Cultures were maintained at 37°C with 5% CO<sub>2</sub> for 11–14 days. Following harvest, G-banded chromosome preparations were analyzed using standard protocols. Two certified cytogeneticists (>5 years experience) independently evaluated 20 metaphase spreads per case, with detailed structural analysis of five randomly selected spreads. Karyotypes were reported according to the International System for Human Cytogenomic Nomenclature (ISCN, 2020),

with final review by a senior cytogeneticist (associate professor or higher rank).

## Molecular genetic analysis

### CNV-seq analysis

Genomic DNA was extracted from 4-mL aliquots of amniotic fluid specimens using standardized extraction protocols. To minimize PCR-induced artifacts, we implemented a PCR-free library preparation methodology (Berry Genomics Co., Ltd.). DNA library quantification was performed using Qubit 1X dsDNA High Sensitivity and Broad Range assay kits (Thermo Fisher Scientific) to determine precise DNA concentrations. Sequencing was conducted on the NextSeq CN500 platform using 36-bp single-end reads at approximately 0.1x coverage depth. Sequence reads were aligned to the GRCh37 human reference genome using the BWA-MEM algorithm, which enables high-fidelity mapping of short-read sequences. Copy number variation (CNV) analysis was performed using a read-depth approach, wherein significant deviations from expected genomic coverage were identified as putative CNVs. All detected variants were filtered and classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines into five categories: pathogenic, likely pathogenic, variants of uncertain significance (VUS), likely benign, and benign. Rigorous quality control measures were implemented throughout the workflow, encompassing library preparation, sequencing metrics, and variant interpretation to ensure robust detection of clinically relevant CNVs. The final diagnostic report included only pathogenic and likely pathogenic variants.

TABLE 1 Clinical characteristics.

Characteristic	Study population (617)
Maternal age (years)	30.5 ± 6.5
<35	412 (66.8%)
≥35	205 (33.2%)
<b>Nation</b>	
Han	563 (91.2%)
Miao	54 (8.8%)
Gestational age (weeks)	20.1 ± 2.2
<b>Indications for invasive prenatal diagnosis</b>	
High risk of Down's syndrome screening	294 (47.6%)
Advanced maternal age	205 (33.2%)
NIPT high-risk	33 (5.3%)
Dverse pregnancy history	26 (4.2%)
Abnormal B-ultrasound	21 (3.4%)
Family genetic history	8 (1.3%)
Other	30 (4.9%)

### QF-PCR analysis

DNA extracted from 4 mL amniotic fluid (TIANamp Genomic DNA Kit) was quantified by Qubit analysis and appropriately diluted. PCR amplification targeted 20 polymorphic short tandem repeat (STR) loci across chromosomes 13, 18, 21, and sex chromosomes using the Bio-Rad PTC 200 system. Analyzed markers included D13S628, D13S742, D13S634, D13S305, D18S1002, D18S391, D18S535, D18S386, D21S1433, 21q11.2, D21S1411, D21S1414, D21S1412, D21S1445, AMXY, DXS1187, DXS8377, DXS6809, DXS981, and SRY. PCR products were analyzed using an ABI 3500 genetic analyzer and GeneMapper 5.0 software.

Results from both molecular analyses were interpreted using the GRCh37 genome build and multiple reference databases (DGV, ClinGen, DECIPHER, GeneReviews, OMIM, UCSC, PubMed, gnomAD, and ClinVar).

### Follow-up and statistical analysis

Pregnancy outcomes were tracked through telephone follow-up or medical record review, with particular emphasis on cases showing discordant results between karyotype and CNV-seq analyses, or those with VUS findings. Statistical analysis employed SPSS version 19.0. Categorical variables were expressed as percentages and compared using chi-square tests; continuous variables were expressed as mean ± standard deviation and analyzed using t-tests. Statistical significance was defined as  $P < 0.05$ .

## Results

### Study population and clinical characteristics

This study analyzed 617 pregnant women with a mean age of  $30.5 \pm 6.5$  years and mean gestational age of  $20.1 \pm 2.2$  weeks. Among the participants ( $N = 617$ ), the primary indications for prenatal diagnosis were abnormal Down syndrome screening (47.6%,  $n = 294$ ), advanced maternal age (33.2%,  $n = 205$ ), high-risk non-invasive prenatal testing (NIPT) (5.3%,  $n = 33$ ), adverse obstetric history (4.2%,  $n = 26$ ), fetal structural anomalies on ultrasound (3.4%,  $n = 21$ ), family history of genetic disorders (1.3%,  $n = 8$ ), and other risk factors (4.9%,  $n = 30$ ) (Table 1).

### Conventional karyotype analysis

Chromosomal abnormalities were identified in 60 cases (9.7%) through cytogenetic analysis of amniotic fluid samples. These comprised 19 cases of trisomy 21, 4 cases of trisomy 18, and 9 cases of sex chromosome anomalies. The sex chromosome abnormalities included four cases of 45,X/46,XX/XY mosaicism, and single cases of 47,XXX, 47,XYY, 47,XXX/45,X/46,XX mosaicism, 46,X,psu idic(X) (p11.2), and X duplication (dup(X) (q26q28)). Additional findings included one case of low-level mosaic trisomy 20 (47,XX,+20 [4]/46,XX [43]), two autosomal structural abnormalities (46,XY,del (18) (p11.2) and 46,XN,der (4)t (4; 10) (p15.3; q24)), two balanced translocations (46,XX,t (3; 18) (q26; q22) and 46,XY,der (13; 14) (q10; q10)), and 23 cases of chromosomal polymorphisms.

TABLE 2 Pathogenic information of CNVs detected by CNV-seq.

No.	Karyotype	CNVs	Fragment size of CNVs	Clinical manifestations
1	46,XX	sseq [GRCh37]22q11.21 (18,880,000–20300,000)×3	1.42 Mb (microduplications)	22q11 duplication syndrome
2	46,XX	sseq [GRCh37]15q11.2q13.3 (27,620,000–32,460,000)×3	4.84 Mb (microduplications)	Autism
3	46,XY	sseq [GRCh37]22q11.21 (18,880,000–20300,000)×3	1.42 Mb (microduplications)	22q11 duplication syndrome
3	46,XX	sseq [GRCh37]16p11.2 (29,640,000–30200,000)×1	0.56 Mb (microdeletions)	microdeletion syndrome
4	46,XX	sseq [GRCh37]18p11.32p11.21 (11,320,000–14,980,000)×1	3.66 Mb (microdeletions)	Facial dysmorphism
5	46,XX	sseq [GRCh37]2q11.1q11.2 (96200000–97680000)×1	1.48 Mb (microdeletions)	Intellectual disability
6	46,XX	sseq [GRCh37]22q11.21 (18,880,000–21,460,000)×3	2.58 Mb (microduplications)	22q11 duplication syndrome
7	46,XX	sseq [GRCh37]Xq28 (153,560,000–153,840,000)×3	0.28 Mb (microduplications)	22q11 duplication syndrome
8	46,XY	sseq [GRCh37]16p11.2 (23,640,000–30200,000)×3	1.72 Mb (microduplications)	Developmental delay, autism
9	46,XY	sseq [GRCh37]22q11.21 (18,880,000–21,480,000)×3	2.6 Mb (microduplications)	22q11 duplication syndrome
10	46,XX	sseq [GRCh37]Xp22.31 (6,460,000–8140,000)×0	1.68 Mb	Kallmann syndrome
11	46,XY	sseq [GRCh37]22q11.21 (18,880,000–21,460,000)×1	2.58 Mb (microdeletions)	22q11 duplication syndrome
12	46,XX	sseq [GRCh37]Xq23q24 (114,480,000–119,180,000)×1	4.7 Mb (microdeletions)	Intellectual disability
13	46,XY	sseq [GRCh37]10q26.2q26.3 (129,760,000–135,440,000)×1	5.6 Mb (microdeletions)	Intellectual disability

TABLE 3 Comparison of numerical chromosomal abnormalities detected in the karyotyping, CNV-seq and QF-PCR groups.

	Karyotype	CNV-seq and QF-PCR
Trisomy 21	19	19
Trisomy 18	4	4
Sex chromosome aneuploid	9	12
Low-percentage trisomy 20 mosaicism	1	0
Autosomal structural abnormalities	2	2
Balanced translocations	2	0
Chromosomal polymorphisms	23	0
Low-percentage trisomy 13 mosaicism	0	1
Pathogenic CNVs	0	13
Variants of uncertain significance CNVs	0	20
Total	60	71

## Combined CNV-seq and QF-PCR analysis

The integrated CNV-seq and QF-PCR approach identified chromosomal abnormalities in 51 cases (8.3%). These included 19 cases of trisomy 21, 4 cases of trisomy 18, and 12 cases of sex chromosome abnormalities (4 cases of X/XX mosaicism, 2 cases of X/XY mosaicism, 1 case of XXX/X mosaicism, 1 case of XXY/XY mosaicism, 1 case of 47,XXX, 1 case of 47,YYY, 1 case of X duplication at Xq26.3q28, and 1 case of Xq duplication with partial deletion at Xq22.33p11.1).

Additional findings included one case of low-level mosaic trisomy 12, 13 clinically significant pathogenic CNVs or gene mutations (Table 2), and 20 variants of uncertain significance (VUS). The combined molecular approach achieved a significantly higher detection rate (12.5%, 77/617) compared to either method alone ( $p < 0.05$ ).

## Concordance analysis

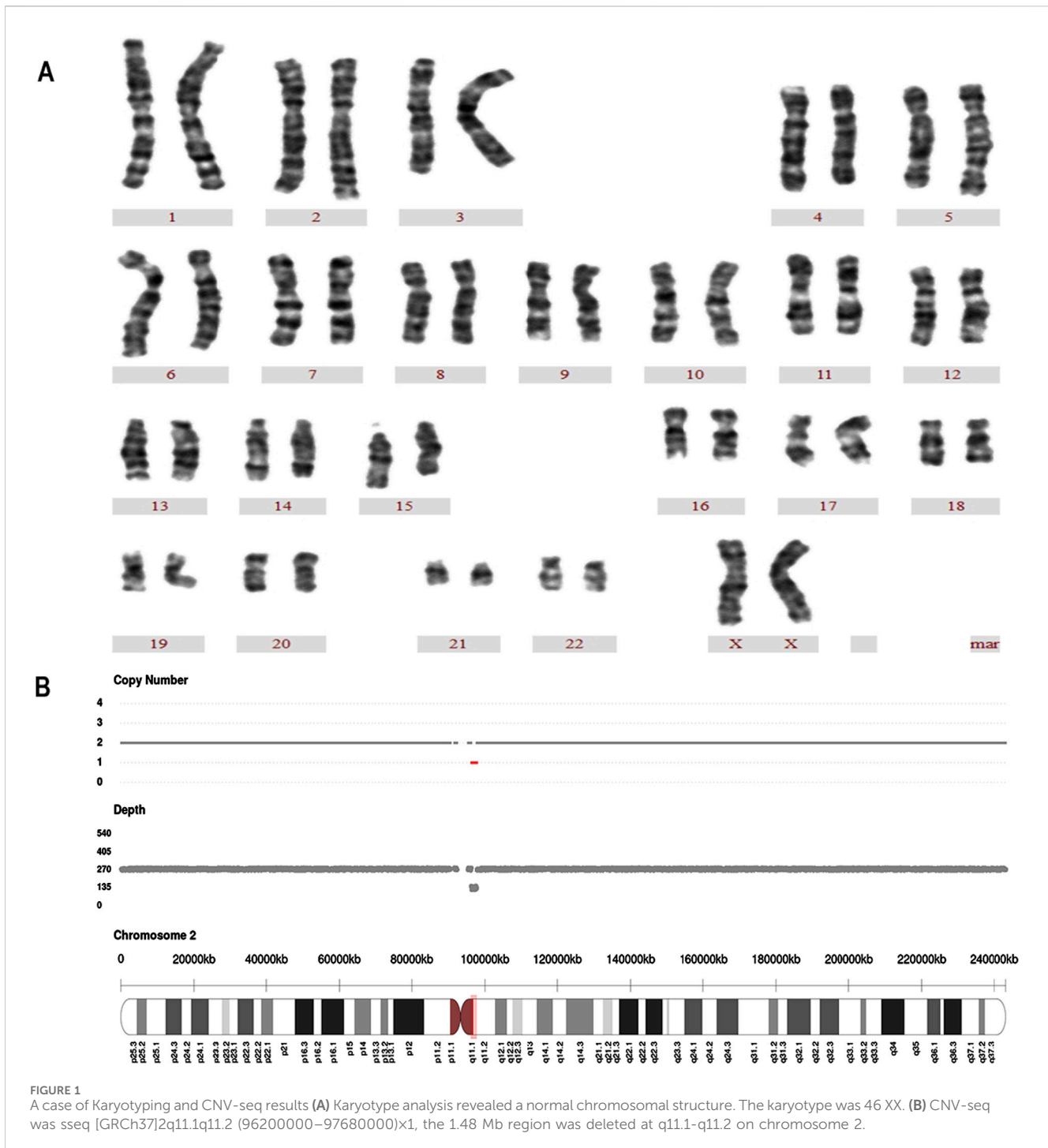
Karyotyping and combined CNV-seq/QF-PCR demonstrated 94.0% concordance (580/617 cases). Perfect concordance was observed for trisomy 21 and trisomy 18 detection. However, the molecular methods failed to detect chromosomal polymorphisms and balanced translocations identified by conventional karyotyping (Table 3). The results of karyotype analysis combined with CNV-seq and QF-PCR are presented in Figure 1.

## Risk-stratified detection rates

Detection rates varied significantly among risk groups using the combined approach: high-risk NIPT (57.6%, 19/33), advanced maternal age (11.2%, 23/205), abnormal Down syndrome screening (9.5%, 28/294), family history (12.5%, 1/8), adverse obstetric history (11.5%, 3/26), other risk factors (6.7%, 2/30), and fetal ultrasound abnormalities (4.8%, 1/21). The high-risk NIPT group demonstrated significantly higher detection rates compared to all other groups ( $p < 0.05$ ) (Table 4).

## Pregnancy outcomes and follow-up

Complete follow-up data were available for all 617 cases through 21 September 2024. Among the 97 cases



with abnormal findings, 44 pregnancies were terminated, including cases of trisomy 21 (n = 19), trisomy 18 (n = 4), trisomy 20 (n = 1), sex chromosome abnormalities (n = 9), pathogenic CNVs (n = 10), and VUS (n = 1). Of the remaining cases, 482 resulted in live births, with 89 pregnancies ongoing at the time of analysis. Two live-born infants carrying maternally inherited pathogenic CNVs were healthy at birth. All 18 infants with VUS showed normal phenotypes postnatally. Inheritance patterns were established for 25 CNV cases: among pathogenic

variants, 12 had confirmed parental origin (3 paternal, 7 maternal, 2 *de novo*), while VUS showed predominantly paternal inheritance (8 paternal, 4 maternal, 1 *de novo*).

## Discussion

Our integrated approach combining conventional karyotyping with CNV-seq and QF-PCR yielded a chromosomal abnormality



TABLE 4 Chromosomal abnormalities in pregnancies with different indications.

Indicators for prenatal diagnosis	Karyotype	CNV-seq and QF-PCR	Combined
High risk of Down's syndrome screening	7.1% (21/294)	6.8% (19/294)	9.5% (28/294)
Advanced maternal age	9.2 (19/205)	5.4% (11/205)	11.2% (23/205)
NIPT high-risk	45.5% (15/33)	51.5% (17/33)	57.6% (19/33)
Diverse pregnancy history	3.8% (1/26)	7.7% (2/26)	11.5% (3/26)
Abnormal B-ultrasound	4.8% (1/21)	4.8% (1/21)	4.8% (1/21)
Family genetic history	12.5% (1/8)	12.5% (1/8)	12.5% (1/8)
Other	6.7% (2/30)	0% (0/30)	6.7% (2/30)

detection rate of 12.5% (77/617), surpassing both isolated karyotyping (9.7% [60/617]) and CNV-seq/QF-PCR alone (8.3% [51/617]). This enhanced detection rate surpasses previously reported rates for CNV-seq as a standalone method and demonstrates comparable efficacy to the combined use of karyotyping and CNV-seq described in the literature (Wang et al., 2018; Zhang et al., 2022; Zhang et al., 2023; Ge et al., 2024).

Karyotyping remains the gold standard for chromosomal analysis, effectively detecting structural aberrations larger than 5–10 MB, aneuploidies, and balanced translocations. Our findings corroborate previous studies (Tang et al., 2024) demonstrating comparable efficacy between karyotyping and CNV-seq/QF-PCR in detecting major structural and numerical abnormalities, including trisomy 21 and 18. Notably, karyotyping identified chromosomal polymorphisms in 3.7% (23/617) of cases, two cases of balanced translocations, and one case of trisomy 20, underscoring its utility in detecting morphological chromosomal variants (Jing et al., 2021). While chromosomal polymorphisms and balanced translocations are not typically considered direct causative factors for congenital anomalies, emerging evidence suggests potential associations with subsequent infertility and oncological developments (Liehr, 2022; Pei et al., 2021; Ralapanawe et al., 2023; Verdoni et al., 2021). Thus, early identification of these variants provides valuable prognostic information for clinical surveillance and management strategies.

Our analysis detected CNVs in 5.3% (33/617) of cases, comprising 2.1% (13/617) pathogenic CNVs and 3.2% (20/617) variants of uncertain significance (VUS), consistent with published literature (Han et al., 2024; Xu et al., 2020; Zhang et al., 2022). These CNVs remained undetectable by conventional karyotyping. Notably, most CNV-carrying fetuses demonstrate no overt ultrasonographic abnormalities during gestation (Hilger et al., 2020; Jiang et al., 2024; Xue et al., 2021), underscoring the limitations of traditional prenatal screening methods and the value of incorporating molecular diagnostic techniques. However, the clinical interpretation of CNVs presents ongoing challenges. We observed two cases where fetuses carrying pathogenic CNVs (22q11.21 and 15q11.2 microduplications) exhibited normal phenotypes postnatally, with asymptomatic maternal carriers. These observations align with growing evidence of incomplete penetrance and variable expressivity in certain pathogenic CNVs (Hilger et al., 2020; Hu et al., 2017; Ramalingam et al., 2011), challenging the traditional paradigm of universal pregnancy termination for all pathogenic variants. Moreover, the

detection of VUS poses significant counseling challenges, potentially inducing unwarranted anxiety and precipitous decision-making. In one instance, pregnancy termination was pursued due to family anxiety despite the absence of parental comparative analysis. This highlights the importance of exercising caution when CNVs are detected. Physicians and families should consider parental origin testing to inform decision-making and guide appropriate clinical management.

Although the mechanistic underpinnings and preferential genomic locations of CNVs remain not fully understood, our cohort showed a predominance of 22q11.21 microduplications (6/13) and 16p11.2 microduplications (2/13) among pathogenic CNVs. This distribution differs from previous reports by Zhang et al. (2023), who identified 15q11.2 microdeletions and 22q11.21 microduplications as the most prevalent pathogenic variants. VUS demonstrated no discernible pattern of occurrence. Intriguingly, we observed a preponderance of maternal inheritance for pathogenic CNVs, while VUS showed predominantly paternal inheritance. However, these findings warrant validation in larger cohorts.

Our data demonstrated that the detection rates of chromosomal abnormalities was significantly higher in high-risk NIPT results (57.6%) compared to other risk categories, validating NIPT's utility as a screening modality. Nevertheless, these findings reinforce that NIPT should not be employed as a diagnostic tool in isolation.

In conclusion, our findings validate the enhanced diagnostic yield of integrating karyotyping with CNV-seq and QF-PCR. However, the single-center, retrospective nature of this study necessitates further validation in larger, multi-center cohorts.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## Author contributions

J-pL: Conceptualization, Resources, Writing—original draft, Writing—review and editing, Investigation, Methodology. S-BW:

Writing—original draft, Writing—review and editing, Conceptualization, Investigation, Methodology, Resources. LL: Conceptualization, Data curation, Investigation, Methodology, Writing—original draft, Writing—review and editing. Y-mG: Conceptualization, Investigation, Methodology, Writing—original draft, Writing—review and editing.

## Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by the Sichuan Medical Association youth innovation project (No. Q21022).

## Acknowledgments

The authors gratefully acknowledge the assistance of the patients who participated in this study.

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## Conflict of interest

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