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RESEARCH ARTICLE

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Copy number variation characterization and possible candidate genes in miscarriage and stillbirth by next-generation sequencing analysis

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Abstract

Background: The present study aimed to explore the etiological relationship between miscarriage and stillbirth and copy number variations (CNVs), as well as provide useful genetic guidance for high-risk pregnancy.

Methods: In total, 659 fetal samples were recruited and subjected to DNA extraction and CNV sequencing (CNV-seq), relevant medical records were collected.

Results: There were 322 cases (48.86%) with chromosomal abnormalities, including 230 with numerical abnormalities and 92 with structural abnormalities. Chromosomal monosomy variations mainly occurred on sex chromosomes and trisomy variations mainly occurred on chromosomes 16, 22, 21, 18, 13 and 15. In total, 41 pathogenic CNVs (23 microdeletions and 18 microduplications) were detected in 27 fetal tissues. The rates of numerical chromosomal abnormalities were 29.30% (109/372), 32.39% (57/176) and 57.66% (64/111) in < 30-year-old, 30–34-year-old and ≥ 35-year-old age pregnant women, respectively, and increased with an increasing age ($p < 0.001$). There was statistically significant difference ($\chi^2 = 7.595$, $p = 0.022$) in the rates of structural chromosomal abnormalities in these groups (13.71%, 18.75% and 7.21%, respectively). The rates of numerical chromosomal abnormalities were 45.44% (219/482), 7.80% (11/141) and 0% (0/36) in the ≤ 13 gestational weeks, 14–27 weeks and ≥ 28 weeks groups, respectively, and decreased with respect to the increasing gestational age of the fetuses ($p < 0.001$).

Conclusions: The present study has obtained useful and accurate genetic etiology information that will provide useful genetic guidance for high-risk pregnancies.

KEYWORDS
copy number variation sequencing, microdeletion, microduplication, miscarriage and stillbirth fetus

Xia Zhang and Qingyan Huang contributed equally to this work.

1 | INTRODUCTION

Miscarriage is the spontaneous loss of a pregnancy occurring before 28 weeks. The spontaneous loss of the fetus with a weight less than 1000 g occurring before 12 gestational weeks is called early miscarriage, and that occurring from 12 to 28 gestational weeks is called late miscarriage.[1] Stillbirth involves a fetus that dies in the uterus after 20 weeks of gestation.[2] The incidence of miscarriage is about 15–20% and there is an increasing trend year by year.[3] The incidence of stillbirth is about 0.5–0.6%.[4] The causes of miscarriage and stillbirth including environmental factors, endocrine diseases, immune diseases and genetic factors. A related study had shown that genetic factors play a leading role in early miscarriage and stillbirth.[5] Some 6–13% of stillbirths were associated with abnormal karyotype,[6] and 5–40% of stillbirths with an abnormal anatomical structure were associated with abnormal karyotype.[7] Genetic analysis of miscarriage and stillbirth tissue is of great value with respect to the analysis of the causes of miscarriage and stillbirth, assessing the risk of recurrence and prenatal diagnosis.[8]

Chromosomal abnormalities include numerical chromosomal abnormalities (such as monosomy, trisomy and polyploidy) and structural chromosomal abnormalities (such as deletion, duplication, insertion, inversion, cross-displacement, ring chromosome and translocation).[9,10] Around the beginning of the 21st Century, scientists began to recognize an intermediate size variation. Copy number variants (CNVs) are copy number changes of the genome, with variants ranging in size from several dozens of bases (> 50 bp) to megabases.[11,12] CNVs have been shown to affect gene function by changing coding sequences and regulatory elements, and thus they have association with the susceptibility to diseases such as genetic diseases, cancer, infections and metabolic disorders.[13,14] An increasing number of studies had shown that pathogenic copy number variations (pCNVs) account for a certain percentage of the fetuses in older pregnant women and with abnormal ultrasound.[15,16]

Karyotype analysis is one of the main detection techniques for chromosomal abnormality. However, karyotype analysis has some limitations. First, it has a long experimental period, high technical requirements and a high risk of failure. Second, it can only diagnose chromosomal deletion and duplication with more than 5–10 Mb, and minor structural changes may be missed.[17] With the rapid development of high-throughput sequencing technology, the advantage of sequencing technology in the detection of CNV is becoming more and more obvious. Copy number variation sequencing (CNV-seq) based on next generation sequencing (NGS) technology was used for sequencing analysis of samples, and the sequencing results were compared with the human reference genome and CNV was found through bioinformatics analysis. CNV-seq can detect chromosome aneuploidies, chromosome CNVs and polyploidies, and can also detect microdeletions and microduplications with < 5 Mb.[18–20] CNV-seq can detect DNA extracted directly from uncultured tissues, which greatly improves the success rate of detection. Second, it has high resolution and can detect CNVs that cannot be detected by karyotype analysis. At the same time, it can detect unknown variations and reveal new genetic information related to the disease.

In recent years, studies using CNV-seq to analyze the relationship between CNV and miscarriage and stillbirth have been reported. The prevalence of aneuploidy and pathogenicity-associated CNV in aborted fetal tissue was associated with an increased risk of miscarriage in advanced maternal age pregnant women.[21–23] Numerical chromosomal abnormality was the most important reason for embryo termination in early and middle pregnancy, followed by pCNVs.[24] Dai *et al*.[25] found that the fetal chromosomal abnormality rate in first-trimester spontaneous abortion was significantly higher than the second-trimester spontaneous abortion. Wang *et al*.[26] reported that 309 genes were identified as potential miscarriage candidate genes by analyzing 5,003 miscarriage specimens. These studies found that chromosomal aneuploidy was one of the main genetic factors for abortion and also that some pCNVs were associated with miscarriage and stillbirth. The number of cases with miscarriage and stillbirth studied so far is still too small to allow identification of specific variations or genes for miscarriage and stillbirth, and some of the relevant biological processes are not emerging.

To investigate the differences in the incidence and distribution of chromosomal abnormalities of miscarriage and stillbirth systematically and investigate the role of CNV with respect to genetic etiology in miscarriage and stillbirth, samples of miscarriage and stillbirth fetuses were analyzed by CNV-seq in the present study. We analyzed the genomic regions of detected CNVs aiming to identify potential miscarriage and stillbirth candidate genes, and analyzed gene functions using enrichment and signaling pathways analysis. The results of this study may help to establish population-based genetic markers for miscarriage and stillbirth screening and provide useful genetic guidance for high-risk pregnancy.

2 | MATERIALS AND METHODS

2.1 | Participants

Miscarriage and stillbirth fetal tissue samples were collected from the Department of Obstetrics, Meizhou People's Hospital, China, from 2017 to 2020. Inclusion criteria: (1) miscarriages and stillbirths without any specific causes; (2) the recruited pregnant women had no significant immunological or endocrinal abnormalities and no anatomical abnormalities of the reproductive organs (including the uterus) were found by ultrasound analysis; and (3) pregnant women without mental diseases who are able to cooperate with treatment independently. Exclusion criteria: (1) pregnant women with structural abnormalities of the genital organs and major diseases such as immunological or endocrinal abnormalities and (2) the normal fetus has been aborted. The parents were informed about the advantages and limitations of CNV-seq and consented to test and written informed consent was obtained from all participants. The flow chart for this study is shown in Figure 1.

The chorionic villus or fetal tissue was removed from the uterine cavity by the operation of clearing uterus, and the blood on the tissue surface was washed with sterile normal saline. About 100 mg of the villus or fetal tissue of miscarriages and stillbirths were cut. Next,

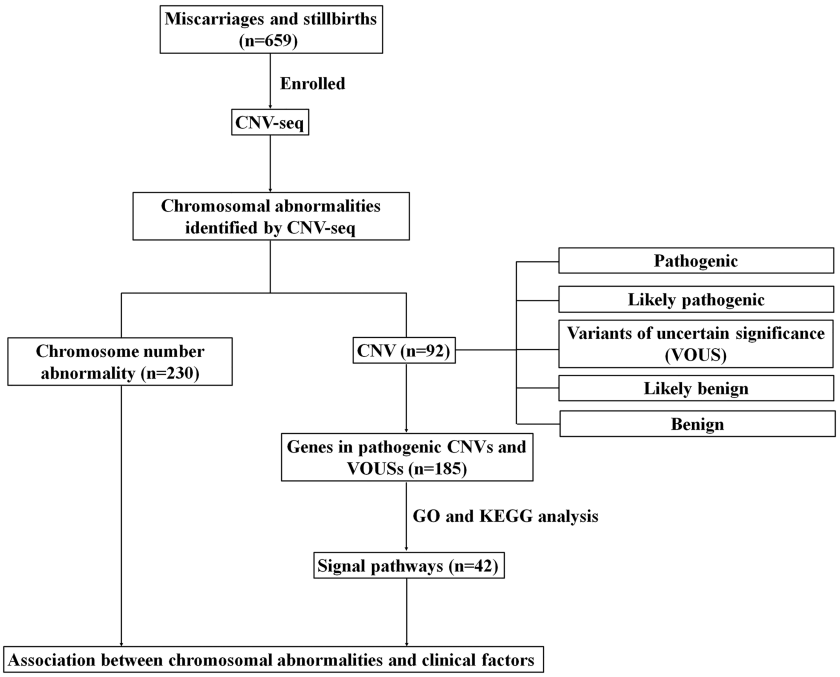

FIGURE 1 Flow diagram of the CNV-seq of miscarriage and stillbirth, as well as the analytical strategies used in the present study

peripheral blood samples (3 ml) (ethylenediaminetetraacetic acid for anticoagulation) were obtained from both parents of each fetus to identify the maternal cell contamination (MCC) of fetal samples and the genetic characteristics of CNVs. The study was performed under the guidance of the Declaration of Helsinki and approved by the Ethics Committee of Meizhou People's Hospital (Clearance No. 2016-A-45).

2.2 | Short tandem repeats (STR) analysis

Genomic DNAs were extracted from fetal tissue samples using DNA extraction kit (Tiangen Biotech Co., Ltd, Beijing, China). Fetal tissue samples may be contaminated by maternal cell, and so STR analysis was conducted before CNV-seq of the samples. The STR analysis was conducted with markers including D19S433, D5S818, D21S11, D18S51, D6S1043, AMEL, D3S1358, D13S317, D7S820, D16S539, CSF1PO, Penta D, D2S441, vWA, D8S1179, TPOX, Penta E, TH01, D12S391, D2S1338 and FGA (Microread Genetics Technology Co., Ltd, Beijing, China)[27] using an ABI 3500xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). When all the polymorphic alleles of the mother were absent from fetal sample, the fetal sample was considered free from MCC.

2.3 | CNV-seq

Genomic DNAs were extracted using DNAeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) and their integrity were tested. Accordingly, 50 ng of genomic DNA was used for the template to construct a sequencing library. Finally, sequencing was performed on BioelectronSeq 4,000 Platform (Thermo Fisher, Waltham, MA, USA). The Burrows–Wheeler algorithm was applied to calculate the change of copy number of each sequencing sequence, obtain the copy number value of each chromosome with HG19 genome sequence as reference, determine the duplication or deletion of chromosome fragments and, finally, draw the detection results map. Clinical significance of the CNVs was analyzed according to Database of Genomic Variants (DGV) (http://dgv.tcag.ca/dgv/app/homr), Database of Genomic Variation and Phenotype in Humans using Ensembl Resources (DECIPHER) (http://decipher.sanger.ac.uk) and Online Mendelian Inheritance in Man (OMIM) database (http://www.omim.org), and their pathogenicities were evaluated. There are five grades according to the American College of Medical Genetics and Genomics guidelines[28,29]: (1) pathogenic CNVs (pathological CNVs, pCNVs); (2) likely pathogenic CNVs; (3) variants of uncertain significance (VOUS) CNVs; (4) likely benign CNVs; and (5) benign CNVs.

2.4 | Statistical analysis

SPSS, version 21.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. Data were reported with the descriptive statistics method and measurement data are expressed as the mean ± SD. A chi-square test was used to analyze the difference among the groups. $p < 0.05$ was considered statistically significant.

2.5 | Functional enrichment analysis

The genes located in the pathogenic CNVs, likely pathogenic CNVs and VOUS regions were referred to in the DECIPHER database

(http://decipher.sanger.ac.uk). Enrichment analysis was tested for the functional categories defined in Gene Ontology (GO) (http://geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg) using the clusterProfiler package in R, version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). In the present study, $p < 0.05$ was considered as statistically significant enrichment.

3 | RESULTS

3.1 | Characteristics of subjects and detection rate of CNV abnormalities

The average age of the pregnant women was 29.45 ± 4.95 years and the average gestational duration was 12.83 ± 7.19 weeks. Among these pregnant women, 372 (56.45%) were under 30 years old, 176 (26.71%) were between 30 and 34 years old, 85 (12.90%) were between 35 and 39 years old, and 26 (3.95%) were over 40 years old. Among these miscarriage and stillbirth fetuses, 482 cases (73.14%) were less than 13 weeks gestational age, 141 cases (21.40%) were 14–27 weeks gestational age, and 36 cases (5.46%) were ≥ 28 weeks gestational age. The rate of chromosomal abnormalities was 48.86% (322/659), whereas no abnormal CNV was found in 337 cases (337/659; 51.14%). There were 230 cases (34.90%) with numerical chromosomal abnormality, including 165 cases (25.04%) with autosomal trisomy, 35 cases (5.31%) with sex chromosome monosomy, two cases (0.30%) with autosomal monosomy, one case (0.15%) with sex chromosome trisomy, one case (0.15%) with autosomal tetrasomy and 26 cases (3.95%) with chimera. There were 92 cases (13.96%) with structural chromosomal abnormality, including 62 cases (9.41%) with VOUS, 27 cases (4.10%) with pCNVs and three cases (0.46%) with benign CNVs (Table 1).

TABLE 1 Demographic variables and baseline characteristics of mothers who suffer from miscarriages

Characteristics	The number of cases	Proportion (%)
Age of mothers who had miscarriages (29.45 ± 4.95 years)		
< 30	372	56.45
30–34	176	26.71
35–39	85	12.90
≥ 40	26	3.95
Gestational week of fetuses (12.83 ± 7.19 weeks)		
≤ 13	482	73.14
14–27	141	21.40
≥ 28	36	5.46
Induced labor causes		
Missed abortion	570	86.49
Fetal abnormalities	89	13.51
CNV result		
Numerical chromosomal abnormality	230	34.90
Autosomal trisomy	165	25.04
Sex chromosome monosomy	35	5.31
Autosomal monosomy	2	0.30
Sex chromosome trisomy	1	0.15
Autosomal tetrasomy	1	0.15
Chimera	26	3.95
Structural chromosomal abnormality	92	13.96
VOUS CNV	62	9.41
pCNV	27	4.10
Benign variation	3	0.46
Normal	337	51.14

VOUS, variants of unknown significance; pCNV, pathogenic CNV.

3.2 | Detection results of fetuses with chromosome number abnormality and pCNVs

In the present study, there were 230 cases with numerical chromosomal abnormality. The numerical chromosomal abnormality mainly occurred in chromosomes 13, 15, 16, 18, 21 and 22, as well as the sex chromosomes. Chromosome monosomy variation mainly occurred on the sex chromosomes, whereas a few instances occurred on chromosomes 18 and 21. The trisomy variation mainly occurred on chromosomes 16, 22, 21, 13, 18 and 15. In addition, one fetus with chromosome 7 tetrasomy was found (Figure 2).

Twenty-seven (4.10%) fetal tissues with pCNVs were detected (Table 2). 23 microdeletions and 18 microduplications were detected, for which two or more microdeletions/microduplications were detected in 12 fetal tissues. There are some syndromes were found, including Wolf–Hirschhorn syndrome, 3q29 microdeletion syndrome, Trisomy 8p syndrome, 2p25.3 microdeletion syndrome, 3q29 microduplication syndrome, 15q11.2 microdeletion syndrome, Trisomy 1q syndrome, Cri du chat syndrome, hereditary neuropathy with liability to pressure palsies, 1p36 deletion syndrome, distal monosomy 14q syndrome, partial monosomy 7p, distal monosomy 13q syndrome, distal trisomy 4q syndrome, distal trisomy 11q syndrome, 19p13.3 microduplication syndrome, 16p11.2 deletion syndrome, Velocardiofacial syndrome and DiGeorge syndrome. In addition, there were 185 genes were involved in the detected deletions and duplications, including 81 genes in pCNVs and 104 genes in VOUS CNVs.

3.3 | Identification of miscarriage candidate genes

To identify the critical genes and related signaling pathways associated with miscarriage and stillbirth, the genes in the pCNVs and VOUS

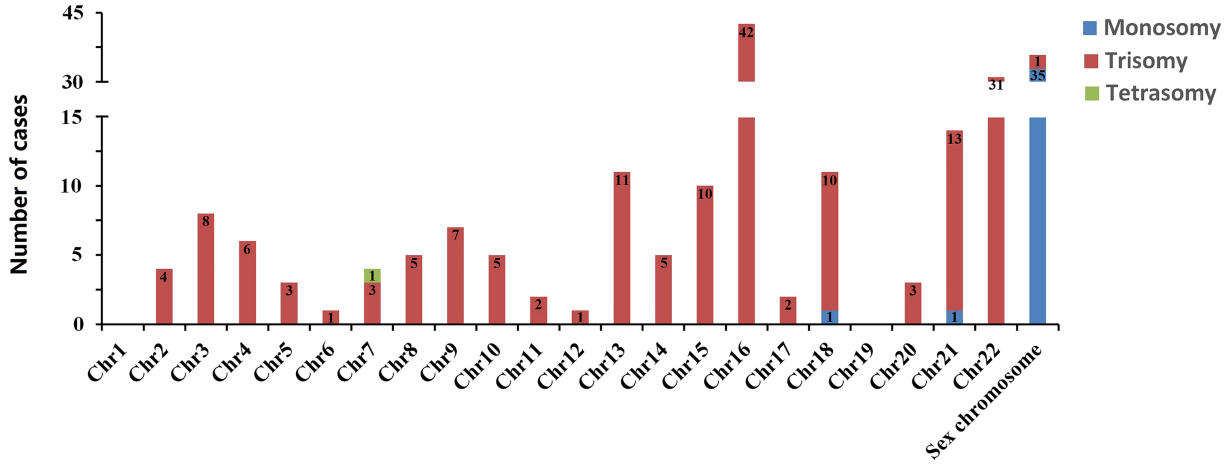

FIGURE 2 Frequency of numerical chromosomal abnormalities detected on different chromosomes

CNVs were examined using GO analysis and KEGG analysis. GO analysis showed that the 185 genes were significantly enriched in 42 different functions ($p < 0.05$). There were 37 enriched GO biological process terms, three enriched GO cellular component terms and two enriched GO molecular function. The most significant of which was "serine-type endopeptidase inhibitor activity" ($p = 0.011$), followed by "axonogenesis" ($p = 0.012$), "neuron projection guidance" ($p = 0.021$) and "forebrain development" ($p = 0.021$). These genes were mainly concentrated in the biological processes of organ development and nervous system development, transmembrane transport, molecular functions of endopeptidase inhibitor activity, and cellular component of nerve synapses (Figure 3A). KEGG analysis results showed that no significant signaling pathways were enriched.

According to the GO analysis results mentioned above, the gene functions were divided into seven functional categories: development of the brain and nervous system, heart formation and development, embryo development and organ formation, cell structure and function, respiratory system development, regulation of ion channels, and regulation of endopeptidase activity (Figure 3B). These genes were mainly enriched in functional categories: development of the brain and nervous system (40 genes) and heart formation and development (9 genes).

3.4 | Comparison of CNV results of fetuses according to different age of pregnant women and gestational week

There were 372, 176 and 111 pregnant women who were < 30 years old, 30–34 years old and ≥ 35 years old, respectively. The rates of chromosomal abnormality in these groups were 43.10% (160/372), 51.14% (90/176) and 64.86% (72/111), respectively, showing an increasing trend with an increasing age of the pregnant women ($\chi^2 = 16.838$, $p < 0.001$). The rates of numerical chromosomal abnormality in these groups were 29.30% (109/372), 32.39% (57/176) and 57.66% (64/111), respectively, showing an increasing trend with an increasing age of the pregnant women ($\chi^2 = 30.925$, $p < 0.001$). The results showed that, among the fetuses with miscarriage and stillbirth, fetuses carried by ≥ 35-year-old pregnant women were more likely to have numerical chromosomal abnormality. The rates of structural chromosomal abnormality in different age pregnant women groups were 13.71% (51/372), 18.75% (33/176) and 7.21% (8/111), respectively. The difference was statistically significant ($\chi^2 = 7.595$, $p = 0.022$). The highest rate of structural chromosomal abnormality was found in the 30–34 years old age group (18.75%), whereas the lowest rate was found in the ≥ 35 years old age group (7.21%). The proportions of VOUS CNVs in cases with structural chromosomal abnormalities were 58.82% (30/51), 78.79% (26/33) and 75.00% (6/8), respectively, and the differences were not statistically significant ($p = 0.151$). The proportions of pCNVs were 37.25% (19/51), 18.18% (6/33) and 25.00% (2/8), respectively, and the differences were not statistically significant ($p = 0.184$) (Table 3).

There were 482, 141 and 36 fetuses the ≤ 13 gestational weeks, 14–27 weeks and ≥ 28 weeks groups, respectively. The rates of chromosomal abnormality in these groups were 59.13% (285/482), 21.28% (30/141) and 19.44% (7/36), respectively, showing a decreasing trend with respect to the increasing gestational age of the fetuses ($\chi^2 = 75.741$, $p < 0.001$). The rates of numerical chromosomal abnormality in these groups were 45.44% (219/482), 7.80% (11/141) and 0% (0/36), respectively, showing a decreasing trend with respect to the increasing gestational age of the fetuses ($\chi^2 = 88.419$, $p < 0.001$). The rates of structural chromosomal abnormality in these different gestational week of fetuses were 13.69% (66/482), 13.48% (19/141) and 19.44% (7/36), respectively. There were no statistically significant differences in the rates of structural chromosomal abnormality ($p = 0.665$), VOUS ($p = 0.362$) and pCNV ($p = 0.254$) among these groups (Table 3).

TABLE 2 Clinical data of fetuses with pathogenic CNVs detected

Num.	Maternal age (years)	Weeks of gestation	CNV result	Location of the fragments	Syndrome/genes involved (genetic pattern of pathogenic variation)	Clinical feature
1	30	29	Chr18 5.06 Mb del (VOUS)	0–5,060,000		Multiple fetal malformations, ventricular septal defect, left renal polycystic dysplasia
			Chr20 13.66 Mb dup (VOUS)	0–13,660,000		
			ChrX 0.42 Mb del	30,900,000–31,320,000	DMD (XL), BMD (XL)	
2	25	19^{+6}	Chr8p 23.18 Mb del	100,000–23,180,000	GATA4 (AD)	Missed abortion
			Chr8q 76.86 Mb dup (VOUS)	69,500,000–146,360,000		
3	33	10	Chr4p16.3-p15.1 32.88 Mb del	100,000–32,880,000	Wolf-Hirschhorn syndrome	Fetal arrest
4	27	9	Chr15q26.1-q26.3 11.32 Mb del	91,200,000–102,520,000		Missed abortion
5	25	6^{+5}	Chr8p23.3-p22 18.22 Mb del	100,000–18,220,000	GATA4 (AD)	Missed abortion
6	29	10	Chr2p24.3 0.8 Mb dup	13,520,000–14,320,000	•	Missed abortion
7	28	8	Chr8q24.23-qter 28.56 Mb dup	100,000–28,660,000	PUF60 (AD), TRAPPC9 (AR)	
			Chr13 q13.3-q14.11 7.82 Mb del	36,180,000–44,000,000	MADH9 (AD)	Fetal arrest
			Chr13 q22.3-q33.1 25.06 Mb dup	77,800,000–102,860,000	MIR17HG (AD), SLITRK6 (AR)	
			Chr13 q34 4.52 Mb dup	110,640,000–115,160,000	COL4A2 (AD)	
8	27	13	Chr1 q24.3 0.26 Mb dup (VOUS)	171,720,000–171,980,000		Fetal edema syndrome with neck lymphatic hydrocystic tumor formation
			Chr6 p21.2 0.42 Mb dup	41,840,000–42,260,000	GUCA1A (AD), GUCA1B (AD)	
9	29	23^{+4}	Chr2pterp25.3 3.38 Mb del	20,000–3,400,000	MYT1L (AD)	Multiple fetal malformations, abnormal cerebellar vermis development, ventricular septal defect, femur length smaller than gestational age, NT thickening
			Chr3qter 68.92 Mb dup (VOUS)	129,100,000–198,020,000		
10	34	14^{+2}	Chr8pterq11.23 53.9 Mb dup	100,000–54,000,000	Trisomy 8p syndrome	•Multiple fetal malformations, left heart dysplasia, cleft palate, single umbilical artery, Tang sieving 21 critical risk 1/20
			Chr21q11.2-q21.2 9.6 Mb del	15,040,000–24,640,000	TMPRSS15 (AR), LIPI (AD)	
11	35	39^{+6}	Chr2pterp25.3 3.34 Mb del		MYT1L (AD)	Stillbirth
			Chr3q21.3qter 69.02 Mb dup		3q29 microduplication syndrome	
12	25	16	Chr15q11.2 0.34 Mb del		15q11.2 microdeletion syndrome; TUBGCP2 (AR), NIPA1 (AD), NIPA2 (AD), CYFIP1 (AD)	Missed abortion
			Chr17q22 0.48 Mb dup (VOUS)		ANKFN1, PCTP	

TABLE 2 (Continued)

Num.	Maternal age (years)	Weeks of gestation	CNV result	Location of the fragments	Syndrome/genes involved (genetic pattern of pathogenic variation)	Clinical feature
13	28	7	Chr1q41qter 26.2 Mb dup	223,000,000–249,200,000	Trisomy 1q syndrome, CFAP298 (AR), CLDN14 (AR), SYNJ1 (AR), DONSON (AR), HLCS (AR), IFNAR2 (AR), IFNGR2 (AR), IL10RB (AR), JAM2 (AR), KCNE1 (AD,AR), TMPRSS15 (AR), PIGP (AR), KCNE2 (AD), KCNJ6 (AD), MRAP (AR), NRIP1 (AD), RUNX1 (AD), SOD1 (AR), SON (AD), APP (AD), DYRK1A (AD)	Fetal arrest
			Chr21q11.2q22.2 25.36 Mb dup	14,600,000–39,960,000		
14	42	7	Chr8q24.12 0.46 Mb dup	120,720,000–121,180,000	TAF2 (AR)	Missed abortion
15	29	5	Chr5pterp14.1 28.06 Mb del	20,000–28,080,000	Cri du chat syndrome	Missed abortion
16	25	34^{+1}	Chr5q23.3 0.60 Mb dup (VOUS)	127,660,000–128,260,000	FBN2 (AD)	Stillbirth
			Chr17p12 1.52 Mb del	14,100,000–15,620,000	PMP22 (AD)	
17	26	9^{+5}	Chr9q34.2qter 4.78 Mb dup;	136,240,000–141,020,000	ABCA2 (AR), ADAMTS13 (AR), ADAMTSL2 (AR), AGPAT2 (AR), TPRN (AR), CACNA1B (AR), CARD9 (AR), DBH (AR), INPP5E (AR), LHX3 (AR), MAN1B1 (AR), MRPS2 (AR), MYMK (AR), PMPCA (AR), SARDH (AR), GRIN1 (AD,AR), SOHLH1 (AD,AR), TUBB4B (AD), NSMF (AD), EHMT1 (AD), COL5A1 (AD), KCNT1 (AD), NOTCH1 (AD)	Missed abortion
			ChrXp22.33p11.22 49.38 Mb del	2,720,000–52,100,000	Turner syndrome; X-linked ichthyosis (XL)	
18	26	7	Chr7p22.3 1.86 Mb del	60,000–1,920,000	AHR (AR), AQP1 (AR), CRPPA (AR), DNAH11 (AR), FAM126A (AR), SNX10 (AR), FKBP14 (AR), GHRHR (AR), GPNMB (AR), HOXA1 (AR), HOXA13 (AD), HOXA2 (AD,AR), IL6 (AD), KLHL7 (AD,AR), PDE1C (AD), PPP1R17 (AD), TWIST1 (AD), CYCS (AD), GARS1 (AD), GSDME (AD), HNRNPA2B1 (AD), HOXA11 (AD), MAD1L1 (AD), FAM20C (AR), HEATR2 (AD), ACTB (AD), AIMP2 (AR), AP5Z1 (AR), BRAT1 (AR), CARD11 (AD,AR), EIF2AK1 (AD), IQCE (AR), KDELR2 (AR), LFNG (AR), MAD1L1 (AD), MRM2 (AR), PMS2 (AD,AR), RAC1 (AD), RNF216 (AR), TMEM106B (AD), WIPI2 (AR)	Missed abortion
			Chr7p22.3p21.3 11.06 Mb dup	2,000,000–13,060,000		
			Chr7p21.3p14.3 18.82 Mb dup	13,080,000–31,900,000		

(Continues)

TABLE 2 (Continued)

Num.	Maternal age (years)	Weeks of gestation	CNV result	Location of the fragments	Syndrome/genes involved (genetic pattern of pathogenic variation)	Clinical feature
19	32	13	Chr1p36.33p34.1 43.62 Mb dup	780,000–44,400,000	Chromosome 1p36 deletion syndrome	Missed abortion
20	27	16^{+5}	Chr4q32.3q34.3 13.58 Mb del	166,420,000–180,000,000	AGA (AR), HPGD (AR), NEK1 (AD), PALLD (AD), TLL1 (AD), VEGFC (AD)	Fetal arrest
21	31	7	Chr8p22 0.44 Mb del	15,860,000–16,300,000	MSR1 (AD)	Missed abortion
22	30	9^{+2}	Chr14q24.3qter 29.78 Mb del	77,500,000–107,280,000	Distal monosomy 14q	Missed abortion
23	27	5	Chr7p12.2qter 109.68 Mb del	49,440,000–159,120,000	Partial monosomy 7p	Missed abortion
			Chr13q21.33qter 42.60 Mb del	72,500,000–115,100,000	Distal monosomy 13q	
24	28	11^{+5}	Chr4q32.1q35.2 31.18 Mb dup	157,960,000–189,140,000	Distal trisomy 4q	Missed abortion
			Chr11 q23.3qter 16.94 Mb dup	118,000,000–134,940,000	Distal trisomy 11q	
25	27	12	Chr9p13.3 5.82 Mb dup	280,000–6,100,000	19p13.3 microduplication syndrome	Missed abortion
26	24	28	Chr16p11.2 0.56 Mb del	29,640,000–30,200,000	16p11.2 deletion syndrome (AD)	Fetal ultrasound showed fetal thoracic vertebral abnormality
27	27	24	Chr22q11.21 2.56 Mb del	18,900,000–21,460,000	Velocardiofacial syndrome; DiGeorge syndrome (AD)	Fetal ultrasound demonstrated a ventricular septal defect and right aortic arch

VOUS, variants of unknown significance; XL, X-linked inheritance; XR, X-linked recessive inheritance; AD, autosomal dominant inheritance; AR, autosomal recessive inheritance.

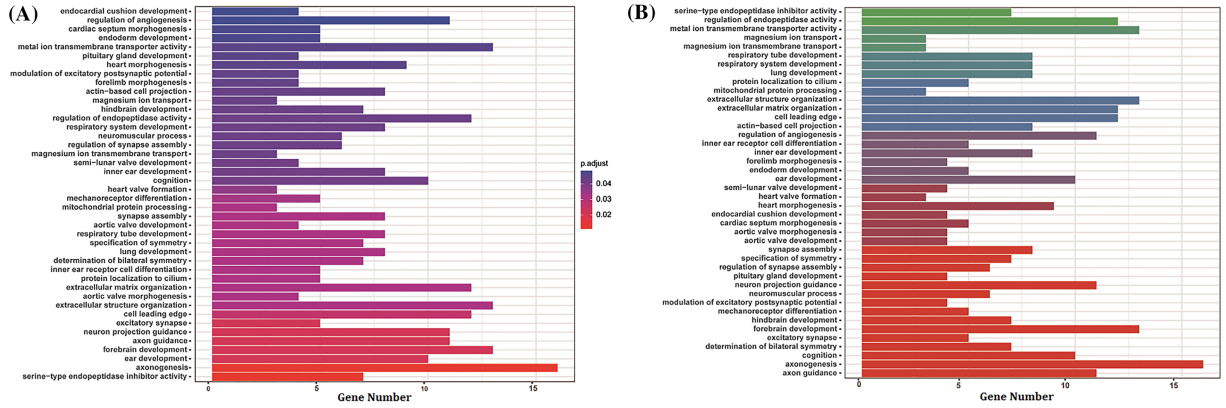

FIGURE 3 Enriched pathway results with adjusted $p < 0.05$ by Gene Ontology analysis. (A)The enriched 42 different functions with $p < 0.05$ of Gene Ontology analysis.(B) The enriched Gene Ontology functions were divided into 7 functionalcategories (different color)

4 | DISCUSSION

In recent years, the incidence of miscarriage and stillbirth has been increasing. Miscarriage and stillbirth of unknown causes pose a great psychological burden to patients and their families. The causes of miscarriage and stillbirth are complex and genetic factors are a main cause.[30] Fetal chromosome abnormality is an important genetic etiological factor with respect to fetal miscarriage and stillbirth. NGS technology can detect CNVs in the whole genome and identify chromosomal abnormalities, without the need for cell culture and in a short time. Many studies have confirmed that NGS technology can improve the diagnosis of chromosomal abnormalities.[31,32] CNV-seq is a genomic copy number variation detection technology based on low-depth whole-genome sequencing. CNV-seq can detect CNVs of different sizes by adjusting the sequencing depth and changing the resolution. Wang *et al*.[33] reported that the detection rate of pathogenic and potentially pathogenic variants increased from 1.8% to 2.8% using CNV-seq compared to karyotype analysis. There are an increasing number studies on the pathogenesis of some diseases using CNV-seq.[19,34] Recently, CNVs have also been observed in miscarriage samples.[35]

In the present study, the rates of chromosomal abnormalities and numerical chromosomal abnormalities were 48.86% and 34.90%, respectively. The results are similar to those of other studies.[25,36] Trisomy variation mainly occurred on chromosomes 16, 22, 21, 18, 13 and 15. The results in the present study are similar to those of other studies.[37–39] Some genes in chromosome 16 have been associated with diseases such as thalassemia,[40] prenatal growth retardation,[41] abnormal fetal head circumference[42] and autism.[43] One study found that CNVs on chromosome 16 play an important role in the determination of developmental delay.[44] Trisomy 16 is the most common cause of early miscarriage, accounting for about 6% of early miscarriages.[45] The results in the present study also confirmed this conclusion. In the present study, numerical chromosomal abnormality was not detected on chromosomes 1 and 19. This may be a result of the insufficient sample size to detect the variation. Several studies have found that numerical chromosomal abnormalities on chromosomes 1 and 19 were associated with some diseases and miscarriage.[46–48] The incidence of chromosomal abnormalities and numerical chromosomal abnormalities increased with an increasing age of the pregnant women, with the lowest incidence being in individuals < 30 years old. This result is in line with a previous study.[49] The causes of fetal chromosomal aneuploidy related to maternal age may involve some functional changes or degeneration of oocytes in elderly pregnant women, such as meiotic recombination failure, deterioration of chromosome cohesion, spindle assembly checkpoint dysfunction, altered post-translational modifications, and mitochondrial dysfunction.[50] In addition, the incidences of chromosomal abnormalities and numerical chromosomal abnormalities decreased with an increasing gestational age of fetuses, whereas the lowest incidence was in the individuals ≥ 28 weeks. These results are consistent with those of previous study.[17] The chromosomal abnormalities of aborted fetuses in early pregnancy may involve any one chromosome or more chromosomes, although the chromosomal abnormalities of aborted fetuses in middle and late pregnancy may be trisomy 13, 18 and 21, sex chromosome aneuploidy and CNVs, which are similar to the types of chromosomal aberrations detected in live births.[51] However another study has found that the incidences of fetal abnormality and growth restriction increase as gestation proceeds.[52] The inconsistency of these results may be a result of differences in population, sample size and detection methods in the various studies.

Chromosomal structural variation is also an important factor in miscarriage and stillbirth, except chromosome aneuploidy. In the present study, there were 23 pathogenic microdeletions and 18 pathogenic microduplications were detected in 27 aborted fetal tissues. In total, 185 genes are involved in the detected deletions and duplication. Forty-two functions were enriched by GO analysis. These functions were mainly divided into some functional categories: development of the brain and nervous system, heart formation and development, and embryo development and organ formation. There have been some studies investigating the association between genes in these functional categories and genetic diseases. One study reported that growth hormone-releasing hormone receptor gene (*GHRHR*) is associated with growth hormone deficiency, dwarfism and

TABLE 3 Comparison of CNV results of fetuses according to different age of pregnant women and gestational week

CNV result	Age of pregnant women					Gestational week of fetuses				
	< 30 years (*n*, %)	30–34 years (*n*, %)	≥ 35 years (*n*, %)	χ^2	p	≤ 13 weeks (*n*, %)	14–27 weeks (*n*, %)	≥ 28 weeks (*n*, %)	χ^2	p
Number	372	176	111			482	141	36		
Chromosomal abnormality	160 (43.10)	90 (51.14)	72 (64.86)	16.838	< 0.001	285 (59.13)	30 (21.28)	7 (19.44)	75.741	< 0.001
Numerical chromosomal abnormality	109 (29.30)	57 (32.39)	64 (57.66)	30.925	< 0.001	219 (45.44)	11 (7.80)	0 (0)	88.419	< 0.001
Structural chromosomal abnormality	51 (13.71)	33 (18.75)	8 (7.21)	7.595	0.022	66 (13.69)	19 (13.48)	7 (19.44)	0.958	0.665
VOUS CNV	30 (58.82)	26 (78.79)	6 (75.00)	3.748	0.151	46 (69.70)	13 (68.42)	3 (42.86)	2.103	0.362
pCNV	19 (37.25)	6 (18.18)	2 (25.00)	3.532	0.184	17 (25.76)	6 (31.58)	4 (57.14)	3.033	0.254

VOUS, variants of unknown significance.

congenital hypopituitarism in children.[53] Variants in the aldehyde dehydrogenase 1 family member A2 (*ALDH1A2*) gene cause lethal multiple congenital anomaly syndrome.[54] ATPase copper transporting alpha (ATP7A) is a critical copper transporter involved in some X linked genetic disorders, such as Menkes disease, occipital horn syndrome and type 3 X-linked distal spinal muscular atrophy.[55] Protocadherin related 15 (*PCDH15*) is associated with nonsyndromic deafness and type 1 Usher syndrome.[56] Muscle skeletal receptor tyrosine kinase (*MUSK*) is the pathogenic gene of congenital myasthenic syndrome.[57] Variants in the GATA binding protein 4 (*GATA4*) gene cause congenital heart disease.[58] Variants in the serine peptidase inhibitor Kazal type 5 (*SPINK5*) gene are involved in the molecular etiology of congenital ichthyosis.[59] Microdeletions in neuroligin 4 X-linked (*NLGN4X*) gene can affect neurodevelopment.[60] Microduplications and microdeletions in the par-3 family cell polarity regulator (*PARD3*) gene are known to be related to neural tube defects.[61] The functions of other genes need further investigation. Because the number of CNVs in the genome is so large, it is a challenge to identify the specific genes associated with miscarriage.

The present study has several limitations. First, the sample size was not sufficently large to identify all miscarriage- and stillbirth-associated CNVs. Second, although CNV-seq based on NGS technology has obvious advantages with respect to detecting chromosomal abnormalities, it is unable to detect chromosomal rearrangements such as translocation, inversion and loss of heterozygosity. The NGS technique also failed to detect low rates of chimerism. Third, the enrichment analysis of gene function conducted in the present study was not systematic and sufficiently in-depth. The detected genes were enriched in some functions but not significantly enriched in some specific signal pathways, and so the clinical significance was limited. Therefore, future studies need larger cohorts and more systematic and detailed information. Further functional analyses and research to validate the predicted gene functions and signaling pathways in the pathogenesis of miscarriage and stillbirth are necessary. At the same time, basic experiments are needed to validate the results of these clinical studies.

5 | CONCLUSIONS

In conclusion, CNV-seq can be used as an effective method for chromosomal CNVs analysis of fetal tissues in miscarriage and stillbirth. The results of the present study show that CNVs are a genetic etiological factor with respect to miscarriage and stillbirth. Some useful and accurate genetic etiological information regarding miscarriage and stillbirth has been obtained that provides useful genetic guidance for high-risk pregnancy. This may open up new avenues for studies on the prevention, diagnosis and treatment of miscarriage and stillbirth.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVAL

This study was conducted on the basis of the Declaration of Helsinki and was supported by the Ethics Committee of the Meizhou People's Hospital.

AUTHOR CONTRIBUTIONS

HW and XZ conceived and designed the experiments. XZ and ZY recruited subjects and collected clinical data. XZ conducted the laboratory testing. HW and QH analyzed the data. HW prepared the manuscript. All authors reviewed the manuscript submitted for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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