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

Xia Zhang1,2 | Qingyan Huang2,3 | Zhikang Yu2,3 | Heming Wu2,3

1Center for Prenatal Disgnosis, Meizhou People’s Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, Meizhou, China
2Guangdong Provincial Key Laboratory of Precision Medicine and Clinical Translational Research of Hakka Population, Meizhou People’s Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, Meizhou, China
3Center for Precision Medicine, Meizhou People’s Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, Meizhou, China

Correspondence
Heming Wu, Center for Precision Medicine, Meizhou People’s Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, No 63 Huangtang Road, Meijiang District, Meizhou 514031, P. R. China. Email: wuheming1986@126.com; wuheming@mzrmyy.com

Funding information
Guangdong Provincial Key Laboratory of Precision Medicine and Clinical Translation Research of Hakka Population, Grant/Award Number: 2018B030322003; Science and Technology Program of Meizhou, Grant/Award Number: 2019B02002001; Scientific Research Cultivation Project of Meizhou People’s Hospital, Grant/Award Number: PY-C2020031

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).

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
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. Stillbirth involves a fetus that dies in the uterus after 20 weeks of gestation. The incidence of miscarriage is about 15–20% and there is an increasing trend year by year. The incidence of stillbirth is about 0.5–0.6%. 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. Some 6–13% of stillbirths were associated with abnormal karyotype, and 5–40% of stillbirths with an abnormal anatomical structure were associated with abnormal karyotype. Genetic analysis of miscarriage and stillbirth is of great value with respect to the analysis of the causes of miscarriage and stillbirth, assessing the risk of recurrence and prenatal diagnosis.

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). 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. 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. 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.

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. 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. 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. Numerical chromosomal abnormality was the most important reason for embryo termination in early and middle pregnancy, followed by pCNVs. Dai et al. found that the fetal chromosomal abnormality rate in first-trimester spontaneous abortion was significantly higher than the second-trimester spontaneous abortion. Wang et al. 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 endocrinial 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 endocrinial 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,
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) 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: (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.
Enrichment analysis was tested for the functional categories defined in Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (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).

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, 1p13.3 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

| 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.
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 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).
| 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 | DMD (XL), BMD (XL) | 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 | |
| 2    | 25                   | 19                | 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 | |
| 5    | 25                   | 6                | 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-pter 28.56 Mb dup | 100,000–28,660,000 | PUF60 (AD), TRAPPC9 (AR) | Fetal arrest |
|      |                      |                    | Chr13 q13.3-q14.11 7.82 Mb del | 36,180,000–44,000,000 | MADH9 (AD) | |
|      |                      |                    | 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 | GUCA1A (AD), GUCA1B (AD) | Fetal edema syndrome with neck lymphatic hydrocystic tumor formation |
|      |                      |                    | Chr6 p21.2 0.42 Mb dup | 41,840,000–42,260,000 | |
| 9    | 29                   | 23                | Chr2pter25.3 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                | Chr8pter11.23 53.9 Mb dup | 100,000–54,000,000 | Trisomy 8p syndrome | Multiple fetal malformations, left heart dysplasia, deformed 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                | Chr2pter25.3 3.34 Mb del | 15,040,000–24,640,000 | 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) | ANKFN1, PCTP | Missed abortion |
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), TMFRSS15 (AR), PIGP (AR), KCNE2 (AD), KCNN6 (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 | TAF2 (AR) | Missed abortion |
| 14   | 42                   | 7                  | Chr8q24.12 0.46 Mb dup | 120,720,000–121,180,000 | TAF2 (AR) | Missed abortion |
| 15   | 29                   | 5                  | Chr5pter14.1 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 | FBN2 (AD) | Stillbirth |
| 17   | 26                   | 9⁻⁵               | Chr9q34.2qter 4.78 Mb dup; ChrXp22.33p11.22 49.38 Mb del | 136,240,000–141,020,000 | ABCA2 (AR), ADAMTS13 (AR), ADAMTS12 (AR), AGBAT2 (AR), TPRN (AR), CACNA1B (AR), CARD19 (AR), DBH (AR), INPP5E (AR), LHX3 (AR), MAN1B1 (AR), MRPS2 (AR), MYMK (AR), MPTEC (AR), SARDH (AR), GRN (AD,AR), SOHLH1 (AD,AR), TUBB4B (AD), NSMF (AD), EMT1 (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), DNACH1 (AR), FAM126A (AR), SNX10 (AR), FKBPL14 (AR), GHRHR (AR), GPNNMB (AR), HOXA1 (AR), HOXA13 (AD), HOXA2 (AD,AR), IL6 (AD), KHL7 (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), APSZ1 (AR), BRAT1 (AR), CARD11 (AD,AR), EIF2AK1 (AD), IQCE (AR), KDELRL2 (AR), LFNG (AR), MAD1L1 (AD), MRM2 (AR), PM2 (AD,AR), RAC1 (AD), RNF216 (AR), TMEM106B (AD), WIP1 (AR) | Missed abortion |
| 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                 | 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                  | 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 |
| 24   | 28                  | 11                 | Chr4q32.1q35.2 31.18 Mb dup | 157,960,000–189,140,000 | Distal trisomy 4q | Missed abortion |
| 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.
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. 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 duplications. 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...
congenital hypopituitarism in children. Variants in the aldehyde dehydrogenase 1 family member A2 (ALDH1A2) gene cause lethal multiple congenital anomaly syndrome. 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. Protocadherin related 15 (PCDH15) is associated with nonsyndromic deafness and type 1 Usher syndrome. Muscle skeletal receptor tyrosine kinase (MUSK) is the pathogenic gene of congenital myasthenic syndrome. Variants in the GATA binding protein 4 (GATA4) gene cause congenital heart disease. Variants in the serine peptidase inhibitor Kazal type 5 (SPINK5) gene are involved in the molecular etiology of congenital ichthyosis. Microdeletions in neulin (NLGN4X) gene can affect neurodevelopment. Microduplications and microdeletions in the par-3 family cell polarity regulator (PARD3) gene are known to be related to neural tube defects. 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 sufficiently 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.

ACKNOWLEDGEMENTS

We thank all our colleagues who were not listed in the authorship from the Center for Prenatal Diagnosis and Center for Precision Medicine, Meizhou People’s Hospital (Huangtang Hospital), Meizhou...
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How to cite this article: Zhang X, Huang Q, Yu Z, Wu H. Copy number variation characterization and possible candidate genes in miscarriage and stillbirth by next-generation sequencing analysis. J Gene Med. 2021;23(12):e3383. doi: 10.1002/jgm.3383