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Analysis of Copy Number Variations by Low-Depth Whole-Genome Sequencing in Fetuses with Congenital Cardiovascular Malformations

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Keywords
Congenital cardiovascular malformations · Prenatal diagnosis · Copy number variation · Chromosomal aberration · Low-depth whole-genome sequencing

Abstract
Congenital cardiovascular malformations (CVMs) due to genomic mutations bring a greater risk of morbidity and comorbidity and increase the risks related to heart surgery. However, reports on CVMs induced by genomic mutations based on actual clinical data are still limited. In this study, 181 fetuses were screened by fetal echocardiography for prenatal diagnosis of congenital heart disease, including 146 cases without ultrasound extracardiac findings (Group A) and 35 cases with ultrasound extracardiac findings (Group B). All cases were analyzed by clinical data, karyotyping, and low-depth whole-genome sequencing. The rates of chromosomal abnormalities in Groups A and B were 4.8% (7/146) and 37.1% (13/35), respectively. There was a significant difference in the incidence of chromosomal abnormalities between Groups A and B ($p < 0.001$). In Group A, CNV-seq identified copy number variations (CNVs) in an additional 9.6% (14/146) of cases with normal karyotypes, including 7 pathogenic CNVs and 7 variations of uncertain clinical significance. In Group B, one pathogenic CNV was identified in a case with normal karyotype. Chromosomal abnormality is one of the most common causes of CVM with extracardiac defects. Low-depth whole-genome sequencing could effectively become a first approach for CNV diagnosis in fetuses with CVMs.

Introduction

Congenital cardiovascular malformations (CVMs) affect 1–2% of newborn children and are the leading cause of death in infants under 1 year of age [Nemer, 2008]. CVMs consist of a wide variety of anomalies and malformations involving the heart and great vessels that develop in utero during the development of the cardiovascular system [Sun et al., 2015]. The defects may be limited to the cardiovascular system (isolated) or occur in association with anomalies of other systems as part of defined syndromes (syndromic) [Sifrim et al., 2016]. With improvements in cardiac surgical techniques and postoperative intensive care over the last 2 decades, most babies

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with CVMs today will survive. However, a significant percentage of these infants may have multiple handicaps, either related to associated birth defects or to neurodevelopmental compromise [Bensemlali et al., 2016]. Extracardiac malformations are observed in 7–50% of the patients with CVMs, bringing a greater risk of comorbidity and mortality and increasing the risks related to heart surgery [Rosa et al., 2013; Serinelli et al., 2018]. CVMs may be induced by genetic mutations, excessive alcohol consumption during pregnancy, use of medications, and maternal viral infection [Van Zutphen et al., 2015; Wen et al., 2016; Zaidi and Brueckner, 2017; Saliba et al., 2020]. Recent advances in genomic technologies have led to the identification of copy number variations (CNVs) and mutations together accounting for up to 15% of CVMs [Yuan et al., 2013]. The standard metaphase karyotype analysis is typically used in individuals with congenital malformations to identify chromosome defects, including aneuploidy, polyploidy, and translocations. Trisomy 18, trisomy 21, and 45,X are the most common aneuploidies identified in CVM fetuses [Bao et al., 2013; Pierpont et al., 2018]. Karyotype analysis revealed chromosomal aberrations in 8–13% of neonates with CVM [Ferencz et al., 1989]. Recurrent chromosomal microdeletions or microduplications can cause CNVs that affect genes involved in cardiac development. Genome-wide copy number analysis can be used to identify chromosomal microdeletions or microduplications in a range of target genetic loci. CNV discoveries in CVM help to boost clinical management, prognostication, and genetic counseling. The convergence of findings at the individual gene and by pathway levels is shedding light on the mechanisms that govern human cardiac morphogenesis. SNP arrays have been employed in the past to identify small- and large-scale chromosomal alterations. However, its device application is still restricted by the high cost and low efficiency. Low-depth whole-genome sequencing (CNV-seq) is a simple, rapid, and cost-effective methodology for prenatal diagnosis of fetal chromosomal disease syndromes. In this study, we performed CNV-seq to investigate the incidence of chromosomal abnormalities and CNVs responsible for fetal CVMs.

Table 1. Phenotypic characteristics of 181 CVM fetuses

CVM classification	Number of fetuses
Septal defects (n = 89)	
Ventricular septal defect	79
Atrial septal defect	10
Conotruncal defects (n = 22)	
Tetralogy of Fallot	7
Transposition of the great arteries	3
Double outlet right ventricle	5
Pulmonary valve stenosis	5
Persistent truncus arteriosus	2
Single atrium and single ventricle	4
Tricuspid valve dysplasia	7
Partial anomalous pulmonary venous return	3
Coarctation of the aorta	3
Pulmonary atresia	1
Persistent left superior vena cava	7
Multiple cardiac malformations	10
Congenital heart disease with extracardiac defects	35
Total	181

Materials and Methods

Patient Data

A retrospective study was performed on 181 fetal CVM cases after an ultrasound diagnosis from January 2017 to May 2019 at the Prenatal Diagnosis Center of the Liuzhou Maternal and Child Healthcare Hospital. These included 146 cases without extracardiac findings (Group A) and 35 cases with extracardiac findings (Group B). Fetal biometry was assessed at a median gestational age of 23.7 ± 2.9 weeks (range 17+2 to 34+3 weeks). One hundred and forty amniotic fluid samples were collected by amniocentesis at the 16th to 24th gestational week. Forty-one cord blood samples were collected after the 24th gestational week.

Karyotype Analysis

Amniocytes were cultured with BIO-AMF™-2 medium (Biological Industries, Kibbutz Beit-Haemek, Israel) and Chang Medium® D (Irvine Scientific, Santa Ana, CA, USA). Cord blood cells were cultivated with peripheral blood lymphocyte medium (Xiangya Gene Technology, Hunan, China). At least 20 G-banded metaphases from each sample were analyzed using the Wright's staining method at a resolution of 320–400 bands. Karyograms were interpreted by at least 2 medical technologists/cytogeneticists and diagnoses reported following the International System for Human Cytogenomic Nomenclature [ISCN, 2016].

Low-Depth Whole-Genome Sequencing (CNV-Seq)

CNV-Seq was performed with next-generation sequencing as previously described [Zhu et al., 2016]. We constructed DNA libraries by end ligation of oligonucleotide adaptors to 50 ng of fragmented DNA and PCR-amplified molecules. Adaptors included a 9-bp barcode, and libraries were subjected to massive parallel sequencing using the HiSeq2500 platform (Illumina, San Diego, CA, USA). Unmapped reads, duplicate reads, and reads with low mapping scores were filtered out by the Wheel-Burrows algorithm, allowing perfect and unique mapping of high quality reads (2.8–3.2 million) against the hg19 reference genome. Mapped reads were progressively allocated along the length of each chromosome to 20-kb sequencing bins, and copy number data analysis was per-

Table 2. CNV results of 20 fetuses with an abnormal karyotype

Karyotype	*N*	CNV result	Size, Mb	Extracardiac defect, *n*
47,XN,+21	4[a]	seq[hg19] dup(21)(q11.2q22.3) chr21:g.14300000_48129895dup	33.8	1
47,XN,+18	6	seq[hg19] dup(18)(p11.32q23) chr18:g.1_78077248dup	78.1	6
47,XN,+13	2[b]	seq[hg19] dup(13)(q12.11q34) chr13:g.19500000_115169878dup	95.7	1
47,XXX	1	seq[hg19] dup(X)(p22.33q28) chrX:g.1_155270560dup	155.3	1
45,X	1	seq[hg19] del(X)(p22.33q28) chrX:g.1_155270560del	155.3	1
47,XN,+9	1	seq[hg19] dup(9)(p24.3q34.3) chr9:g.1_141213431dup	141.2	1
47,XN,+22[2]/46,XN[98]	1[a]	seq[hg19] dup(22)(q11.2q13.33) chr22:g.17900000_51304566dup[0.4]	33.4	0
46,XN,add(9)(p24)	1[a]	seq[hg19] dup(9)(p24.3p21.3) chr9:g.560001_24240000dup	23.7	0
46,XN,del(10)(p14)	1[a]	seq[hg19] del(10)(10p15.3p14) chr10:g.120001_11000000del	10.9	0
46,XN,add(15)(q26.3)	1	seq[hg19] der(15)t(15;6)(q26.3;q23.2) g.[chr15:100540001_102400000delinschr6:133380001_170920000]	1.9 37.5	1
46,XN,der(15)t(2;15)(q35;q26.3)	1	seq[hg19] der(15)t(15;2)(q26.3;q35) g.[chr15:99680001_102400000delinschr2:215660001_243020000]	2.7 27.4	1

[a] No extracardiac defect was found in these cases by prenatal ultrasound diagnosis, no abnormal appearance was found after induced labor without autopsies. [b] In one case, prenatal ultrasound diagnosis showed no extracardiac defect, mild omphalocele was found after induced labor.

formed using previously described algorithms. For reporting CNVs, stringent copy number ranges of 2.9–3.1 for a duplication and 0.9–1.1 for a deletion were applied.

CNV Callings

For the interpretation of CNV disease phenotypes, databases of genomic variants including DGV (http://projects.tcag.ca/variation), Online Mendelian Inheritance in Man (http://www.omim.org), DECIPHER (https://decipher.sanger.ac.uk/), PubMed (http://www.ncbi.nlm.nih.gov/pubmed), UCSC (http://genome.ucsc.edu/, hg19), and Clingene (https://www.clinicalgenome.org/) were used as reference sources of published data.

Statistical Analysis

SPSS Statistics 19 software (IBM, Armonk, NY) was used for statistical analysis. Detection rates of pathogenic results were compared between Group A and Group B. $p < 0.05$ indicated statistical significance.

Results

Of the 181 cases, 146 cases (80.7%) belonged to Group A and 35 cases (19.3%) to Group B. In Group A, 89 cases showed septal defects (79 cases with ventricular septal defect and 10 with atrial septal defect), 22 cases had conotruncal defects (7 with tetralogy of Fallot, 3 with transposition of the great arteries, 5 with double outlet right ventricle, 5 with pulmonary valve stenosis, and 2 with pulmonary atresia), 4 had single atrium and single ventricle, 7 tricuspid valve dysplasia, 3 partial anomalous pulmonary venous return, 3 coarctation of the aorta, 1 pulmonary atresia, 7 persistent left superior vena cava, and 10 multiple cardiac malformations (Table 1).

Chromosomal abnormalities were identified in 20/181 cases (11%) by karyotype analysis. In Group A ($n = 146$),

Table 3. Pathogenic CNVs detected in 8 fetuses with normal karyotype

Case	Karyotype	CNV result	Size, Mb	Cardiac and extracardiac defects	Pathogenicity
013	46,XX	seq[hg19] del(22)(q11.21) chr22:g.18900001_21800000del	2.9	TOF	DiGeorge syndrome
071	46,XY	seq[hg19] del(22)(q11.21) chr22:g.18900001_21480000del	2.6	VSD, PLSVC	DiGeorge syndrome
041	46,XX	seq[hg19] dup(16)(p13.3p13.13) chr16:g.80001_11500000dup	11.4	VSD	16p13.3 microduplication
050	46,XX	seq[hg19] del(1)(q43q44) chr1:g.243360001_249220000del	5.9	PVS	1q43q44 microdeletion
078	46,XY	seq[hg19] del(7)(q11.23) chr7:g.72720001_74420000del	1.7	VSD	Williams-Beuren syndrome
137	46,XX	seq[hg19] del(22)(q13.2q13.33) chr22:g.43240001_51180000del	7.9	PLSVC	Phelan-McDermid syndrome
144	46,XY	seq[hg19] del(5)(p15.33) chr5:g.20001_4440000del	4.4	PLSVC	Cri-du-chat syndrome
114	46,XY	seq[hg19] del(2)(p16.1p14) chr2:g.61260001_65740000del	4.5	TOF Duodenal ileus	2p16.1p14 microdeletion syndrome

TOF, tetralogy of Fallot; VSD, ventricular septal defect; PLSVC, persistent left superior vena cava; PVS, pulmonary valve stenosis.

karyotype analysis identified 7 cases (4.8%) with clinically significant chromosomal abnormalities, including 5 aneuploids (3 cases of trisomy 21; 1 case each of trisomy 13 and mosaic trisomy 22), and 1 case each of 10p14 deletion and 9p24.3p21.3 duplication. Furthermore, we detected 1 case with chromosome polymorphism of inv(9) p13q13 that was not detected by the CNV-seq. In Group B (n = 35), karyotype analysis identified chromosomal abnormalities in 13 cases (37.1%), including 11 aneuploids (1 case each of trisomy 21, trisomy 9, trisomy 13, Turner syndrome, XXX syndrome, and 6 cases of trisomy 18), and 1 case each of 46,XX,add(15)(q26.3) and 46,XY,der(15)t(2;15)(q35;q26.3) (Table 2).

CNV-seq was performed for all samples successfully. Overall, CNV-seq revealed that 28 samples contained clinically significant CNVs (15.5%). In addition to the 20 cases of chromosome abnormalities, consistent with the results of the karyotype analysis, the CNV-seq analysis identified 8 pathogenic CNVs (Table 3) and 7 variants of unknown significance (VOUS) (Table 4). The 8 pathogenic CNVs were related to known chromosomal disorders, including 2 cases of 22q11 deletion (DiGeorge syndrome, OMIM 188400), 1 case each of 16p13.3 duplication (Rubinstein-Taybi syndrome, OMIM 180849), 1q43q44 deletion (mental retardation, OMIM 612337), 7q11.23 deletion (Williams-Beuren syndrome, OMIM 194050), 22q13 deletion (Phelan-McDermid syndrome, OMIM 606232), 5p15.33 deletion (cri-du-chat syndrome, OMIM 123450), and 2p16.1p14 deletion (mental retardation, OMIM 612513).

Discussion

CVMs are marked by an etiologically heterogeneous and not well understood group of lesions. Various genetic abnormalities have been linked to congenital cardiac disease [Muntean et al., 2017]. Studies have evaluated the presence of chromosomal abnormalities detected by karyotyping in patients with CVMs [Dykes et al., 2016]. In the prenatal setting, the incidence of chromosomal anomalies in fetuses with CVM is reportedly as high as 8–10%, with most anomalies being trisomies 21, 18, and 13 [van der Bom et al., 2011]. In this study, karyotype analysis identified 16 chromosomal aneuploidies (16/181, 8.8%) associated with trisomy 21, trisomy 18, trisomy 13, Turner syndrome, trisomy 9, and XXX syndrome, and 4 chromosomal structural abnormalities. Our results reveal

Table 4. Uncertain clinical significance of CNVs

Case	Karyotype	CNV result	Size, Mb	Phenotype	Pathogenicity
030	46,XY,22p+	seq[hg19] dup(11)(p11.2p11.12) chr11:g.47900001_48900000dup	1.0	TOF	LB (DECIPHER patients 285181, 250532)
104	46,XX	seq[hg19] del(2)(q37.1q37.3) chr2:g.232640001_238140000del	5.5	TOF	VOUS (LP) (PMID: 23805197)
119	46,XX	seq[hg19] dup(8)(p23.2) chr8:g.2320001_3540000dup	1.22	PVS	VOUS
138	46,XY	seq[hg19] del(15)(q24.1) chr15:g.74440001_74540000del	0.1	VSD	VOUS
147	46,XX	seq[hg19] del(16)(p13.1p12.3) chr16:g.15480000_18180000del	2.7	PLSVC	VOUS (LP) (PMID: 24105370)
173	46,XY	seq[hg19] del(17)(q23.1q23.2) chr17:g.58100001_60300000del	2.2	Complex CHD	VOUS (LP) (PMID: 20206336)
177	46,XX	seq[hg19] dup(5)(q15) chr5:g.96440001_97460000dup	1.02	TVD	VOUS

TOF, tetralogy of Fallot; PVS, pulmonary valve stenosis; VSD, ventricular septal defect; PLSVC, persistent left superior vena cava; CHD, congenital heart defect; TVD, tricuspid valve dysplasia; LB, likely benign; LP, likely pathogenic; VOUS, variation of unknown significance.

that the detection rates of chromosomal aneuploidies differed significantly between cases of Group A (5/146, 3.4%) and Group B (11/35, 31.4%), suggesting that CVM with extracardiac defects can increase aneuploidy detection rates. This conclusion is consistent with a previous study [Xia et al., 2018]. Furthermore, in our study, trisomy 18 was more commonly associated with CVM plus extracardiac defects.

Clinical genetic testing using genome-wide technologies is increasingly employed in prenatal, pediatric, and adult settings. In the prenatal setting, strong evidence has accumulated that genome-wide CNVs represent a considerable source of the genetic variation contributing to CVM susceptibility [Zhu et al., 2016]. These findings include both known associations with chromosome aneuploidies, microdeletions/-duplications, and new discoveries across the genome. Several studies have reported that the incidence of pathogenic CNVs in fetuses with CVM in prenatal evaluation ranges from 15 to 20% [Zhu et al., 2016; Xia et al., 2018]. In our study, the detection rates for pathogenic variations and VOUS were 15.5% (28/181) and 3.9% (7/181), respectively. The detection rate is significantly high in CVM with extracardiac defects suggesting that a CVM is more likely to be related to genetic disorders if it is detected in the presence of other structural anomalies.

In addition to trisomies and structural abnormalities revealed by karyotyping, CNV-seq identified the following rare chromosomal diseases: 2 cases of 22q11 microdeletions (DiGeorge syndrome), 1 case each of 16p13.3 microduplication (Rubinstein-Taybi syndrome), 1q43q44 microdeletion, 7q11.2 microdeletion (Williams-Beuren syndrome), 10p15 microdeletion (neurooculocardiogenitourinary syndrome), 22q13 microdeletion (Phelan-McDermid syndrome), 5p15 microdeletion (cri-du-chat syndrome), and 2p16.1p15 microdeletion syndrome. These syndromes are linked to a range of physical and mental disabilities, as well as congenital organ malformations, including CVM. In the 146 fetuses of Group A, where no other structural abnormalities were detected, we identified 7 CNVs associated with chromosomal syndromes, where symptoms such as mental disability and developmental delay manifest with variable penetrance after birth. CNV-seq effectively improved the positive detection rate in Group A with normal karyotypes ($p < 0.001$). Thus, in these cases, genetic screening and prenatal diagnosis provided valuable information for clinical management, prognosis, and genetic counseling.

Cytogenetic G-banding analysis only achieves the same results as chromosomal microarray analysis for deletion regions >5–10 Mb (depending on the resolution), chromosomal trisomies, and chromosomal mosaics [Berisha et al., 2020]. We found 2 cases with additional

material of unknown origin, 1 case with an unbalanced deletion, and 1 case with an unbalanced chromosome translocation inherited from his healthy father (balanced translocation carrier) by cytogenetic G-band analysis. CNV-seq revealed the origin of the additional material in chromosomes 9 and 15. In addition, CNV-seq identified the breakpoint regions of the cases with structural abnormalities. Taken together, these results suggest that CNV-seq can improve the detection rate of chromosomal aberrations in CVM fetuses and provide clinically more accurate and comprehensive results for prenatal diagnosis. We suggest that CNV-seq could effectively become a first approach for CNV diagnosis in fetuses with CVMs.

VOUS were detected at a rate of 3.9% (7/181) in our analysis. This is not substantially different from the rates reported previously [Jansen et al., 2015]. Among the 181 cases tested by karyotype analysis and CNV-seq analysis, 3 were considered likely pathogenic based on current knowledge (Table 4). The deletion 2q37.1q37.3, involving the *NPPC* gene, has been associated with a microdeletion syndrome characterized by short stature and endochondral ossification [Tassano et al., 2013]. 16p13.11 microdeletions are pleiotropic genomic variants with broad phenotypic manifestations (involving the *NDE1* gene), including neurodevelopmental phenotypes such as autism, mental retardation, epilepsy and learning difficulties, and congenital anomalies [Tropeano et al., 2014]. Microdeletions at 17q23.1q23.2 involve *TBX2* and *TBX4*, transcription factors belonging to a family of genes implicated in a variety of developmental pathways including those of heart and limb [Ballif et al., 2010].

In our study, the rates of chromosomal anomalies and pathogenic CNVs detected by CNV-seq differed significantly between cases without ultrasound extracardiac findings and cases with extracardiac defects (9.6 vs. 40.0%, $p < 0.001$). Nonetheless, 80.7% (146/181) of the cases remained elusive, suggesting that gene mutations or other factors may be implicated in the etiology of CVMs. Besides that, there were also limitations in this study. First, in this study, samples were also tested by cytogenetic analysis; chromosome aneuploidy and large-scale chromosomal alterations were consistent in CNV-seq results. But microdeletions/-duplications detected by CNV-seq in this study were not verified by commonly used techniques (such as SNP-array). Second, a larger number of cases is needed to increase the sample size and reduce the large confidence intervals for the detection rates of abnormal results. Third, we did not have access to information regarding CNVs in the parents of fetuses. In addition, an important component of prenatal consultation for the families is the long-term physical, speech, and behavioral development associated with CVM, as well as clinical symptoms, which could not be obtained in this study.

Conclusion

In summary, chromosomal abnormality is one of the most common causes of CVM with extracardiac defect, and karyotype analysis is a suitable prenatal diagnostic test in these cases. CNV-seq is particularly effective for identifying chromosomal abnormalities and CNVs in fetuses diagnosed with CVM without ultrasound extracardiac findings, which might be related to associated birth defects or to neurodevelopmental compromise.

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Statement of Ethics

Our study was approved by the ethics committee of the Liuzhou Maternal and Child Healthcare Hospital. The parents signed the written informed consent for the permission of blood sampling as well as the use of chorionic villi or amniotic fluid for further analysis.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.H. conceptualized and designed the study, carried out the analysis, drafted the initial manuscript, and revised the manuscript. X.D. collected the data, drafted the initial manuscript, and carried out the analysis. Y.L. collected the data and revised the manuscript. N.T. carried out the analysis and revised the manuscript. D.Z. designed the study, coordinated data collection, and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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