

## Detection of Copy Number Variants by Next-Generation Sequencing in Fetuses with Congenital Heart Disease

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### Abstract

**Objective:** To report the molecular findings of 89 fetuses with prenatal ultrasound diagnosis of congenital heart disease (CHD) and a normal karyotype through next-generation sequencing (NGS) in an attempt to improve our understanding of submicroscopic abnormalities present in malformed fetuses.

**Method:** High-throughput NGS was carried out in fetal cord blood samples. All potential cytogenetic alterations detected on NGS platforms were matched against the known copy number variant (CNV) database.

**Results:** A total of 204 CNVs were identified in the entire population of 89 fetuses with CHD. Eleven cases had no deletions or duplications, five cases (5.6%) had pathogenic CNVs, 13 cases had CNVs that were likely to be pathogenic, 42 cases had CNVs of uncertain significance, and 18 cases had benign and/or likely benign CNVs. All pathogenic CNVs were found only in fetuses with conotruncal heart defects.

**Conclusion:** NGS can facilitate etiological diagnosis in a high proportion of fetuses with CHD and a normal karyotype and can be implemented as a diagnostic tool in the prenatal setting to complement karyotyping for evaluation of genomic defects in fetuses with CHD.

**Keywords:** Congenital heart disease; Next-generation sequencing; Copy number variants; Prenatal diagnosis; Molecular cytogenetic analysis

### Introduction

Congenital heart disease (CHD) is the most common type of birth defect, and evidence suggests that CHD has a strong genetic component [1]. The concept that submicroscopic aberrations may contribute to the etiology of CHD has been supported by the development of methodologies to define subchromosomal changes in genome structure, called copy number variants (CNVs) [2]. CNVs embedded within the regions of chromosome imbalance may affect clinical outcomes by altering the local copy numbers of important genes or regulatory regions that can alleviate or exacerbate certain phenotypes [3]. However, determining the diagnostic and prognostic value of CNVs in fetuses with complex or serious CHD is a challenge.

Recently, as the costs of DNA sequencing have decreased and strategies for data analysis have improved, next-generation sequencing (NGS) has gradually become applicable for clinical diagnosis, including the detection of copy number variations (CNVs). Therefore, in this study, we used NGS technology to evaluate the disease risk associated with the global burden of CNVs of more than 100 kb in a case population of fetuses having sporadic nonsyndromic CHD with a normal karyotype. We aimed to improve our understanding of the submicroscopic abnormalities present in these malformed fetuses.

### Methods

#### Institution and patients

The Prenatal Diagnosis Center of Xiamen Maternal and Child Health Care Hospital is a regional tertiary referral center for expectant mothers whose fetuses have suspected anomalies and/or genetic syndromes. This center provides prenatal services for a considerable percentage of suspected anomalous pregnancies in the southwest area of Fujian of mainland China. In China, pregnancy can be terminated in any trimester if the fetus has severe malformations.

This was a prospective study. From January 2011 to December 2013, we collected consecutive singleton pregnancies with fetal CHD, which was identified by prenatal ultrasound screening. In these cases, pregnancy was terminated in case of complex or serious cardiac anomalies, among which some cases had severe extracardiac malformation. Before termination of pregnancy, cordocentesis was performed for fetal karyotyping and molecular analysis. If the fetal karyotype was normal on conventional G-band karyotype analysis, NGS was performed to detect CNVs. A total of 89 cases of fetal CHD were included in this study, and NGS analysis and karyotyping were carried out for all cases. All cases were from fetuses that were between 21 and 27 gestational weeks, and the maternal age was 19-33 years. For all cases, no parents had been diagnosed with CHD, and there were no other maternal or familial risks. No cases had a history of a previous child with CHD. Informed consent was obtained from all parents. The study was approved by the Ethics Committee of Xiamen Maternal and Child Health Care Hospital.

#### Classification of CHD

Postmortem necropsies were performed for all cases to obtain a definitive diagnosis. A fetus with more than one defect was included only in the category of the most serious defect. All cases were divided into two categories: 1) conotruncal defects, which included tetralogy of Fallot (TOF), truncus arteriosus communis (TAC), transposition of the

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great arteries (TGA), double outlet right ventricle (DORV), interrupted aortic arch (IAA), aortic atresia/stenosis, and pulmonary stenosis (PS); and 2) abnormal atrioventricular junctions, which included atrioventricular septal defects (AVSDs) and hearts with univentricular atrioventricular connections (UVHs).

### Molecular analysis

Genomic DNA was extracted and purified from blood using a Qiagen QIAamp DNA Mini kit according to the manufacturer's protocol. Qualified genomic DNA samples were randomly fragmented using a Covaris shearing system into fragments of 250 bp, and adapters were then ligated to both ends of the resulting fragments. Finally, the DNA strand was cyclized and separated into a single strand. During the entire process of library construction, we performed strict quality control testing using an Agilent 2100 Bioanalyzer and quantitative polymerase chain reaction (qPCR). Each captured library was then loaded on a HiSeq 2000 platform to perform high-throughput sequencing using paired-end 100 base pair reads on the Illumina HiSeq platform. The bioinformatics analysis utilized sequencing data that was generated from the complete Genomics' Sequencing platform. First, the base-calling software received data from the imager after each reaction cycle to form raw read data. Second, Teramap was used to perform the alignment. The regions of the genome deemed likely to differ from the reference genome were identified using the alignment data. All detected copy number gains or losses were compared with known CNVs listed in publically available databases, i.e., GRCh37/hg19 (<http://www.genome.ucsc.edu>), Online Mendelian Inheritance in Man (OMIM, <http://omim.org>), and the Database of Genomic Variants (DGV, <http://www.ncbi.nlm.nih.gov/dbvar/>).

### Variant classification

Each variant was classified according to recommendation by the American College of Medical Genetics and Genomics (ACMG) [4]. We used the specific standard terminology recommended by ACMG for describing the CNVs identified in the study as follows: pathogenic, CNVs identified in the GRCh37/hg19 pathogenic chromosomal database; benign, CNVs described in a CNV database for healthy individuals (DGV); likely benign, de novo or rare CNVs (<1% population frequency) without OMIM genes or other important functional genes; likely pathogenic, de novo or rare CNVs (<1% population frequency) containing OMIM genes or other important functional genes and/or CNVs that may result in clinical disorders found in patients with a similar chromosomal imbalance; and uncertain significance, CNVs

described in a CNV database for healthy individuals (DGV), but with OMIM genes and/or CNVs that may result in clinical disorders found in patients with a similar chromosomal imbalance.

### Statistical analysis

Statistical analysis of data was performed using the statistical software package SPSS version 15.0 (SPSS Statistical for Windows). Bivariate analysis was performed using Mann-Whitney U-tests for associations between categorical variables. Differences or associations with *P* values of 0.05 or less were considered statistically significant.

### Results

In this study, 89 cases with CHD and a normal karyotype were screened for CNVs by NGS. We identified 204 CNVs in the entire population of 89 fetuses with CHD, 11 of which had no deletions or duplications. According to the variant classification criteria used in this study, there were 88 CNVs interpreted as benign, 15 CNVs described as likely benign, five CNVs (5/89, 5.6%) interpreted as pathogenic, 18 CNVs interpreted as likely pathogenic, and 78 CNVs described as having uncertain significance. Gains were nearly 2-fold more common than losses in the total number of CNVs. Details of the phenotypes of the cases and overall CNV burden are summarized in Table 1. The analysis results revealed that all pathogenic CNVs were found only in the category of conotruncal defects; the differences in CNV distributions among pathogenic, likely pathogenic, benign and likely benign, and uncertain significance between cases of conotruncal defects and cases of abnormal atrioventricular junction were significant (Mann-Whitney U-test, *P*<0.01). The number of CNVs in the category of conotruncal defects ( $2.39 \pm 1.63$  CNVs/subject) was similar to that of the category of abnormal atrioventricular junction ( $1.86 \pm 1.23$  CNVs/subject; *p*>0.05).

The analysis results of the five pathogenic CNVs are shown in Table 2. Pathogenic CNVs were identified in two fetuses with TOF. Case A8 involved a 22.91-Mb deletion in chromosome 5q33.2q35.3, which included genes NKX2-5; these genes have been linked to atrial septal defect 7, with or without AV conduction defects and Sotos syndrome 1. Case A9 involved a 4.04-Mb deletion in chromosome 1p36.32p36.33, which has been found to be associated with chromosome 1p36 deletion syndrome. Case C4 involved a 1.71-Mb deletion in chromosome 1q21.1q21.2 in a fetus with TAC and UVH; the deletion included genes GJA5 and GJA8, which have been shown to be associated with chromosome 1q21.1 deletion syndrome. Case F2 involved a 2.53-Mb

Category	Cardiac diagnosis	Subjects	CNVs analysis results				Total
			pathogenic	Likely pathogenic	Benign and likely benign	Uncertain significance	
Conotruncal defects	TOF	9	2	2	18	8	30
	TAC	13	1	3	13	15	32
	DORV	13	0	1	13	14	28
	TGA	17	0	2	15	15	32
	Aortia atresia/stenosis	12	1	5	14	10	30
Abnormal atrioventricular junction	Pulmonary atresia/stenosis	11	1	1	11	13	26
	AVSD	6	0	1	6	2	9
total	UVH	8	0	3	13	1	17
		89	5	18	103	78	204

204 CNVs were identified in the entire population of 89 fetuses with CHD, there were 88 CNVs interpreted as benign and 15 CNVs were described as likely benign, 5 CNVs (5/89, 5.6%) were determined to be pathogenic and 18 CNVs were interpreted as likely pathogenic, 78 CNVs were described as uncertain significance.

TOF: tetralogy of Fallot, TAC: truncus arteriosus communis, DORV: double outlet right ventricle, TGA: transposition of the great arteries, AVSD: atrioventricular septal defect, UVH: heart with univentricular atrioventricular connection.

**Table 1:** Phenotype of CHD study subjects and overall CNV burden.

Case	Cardiac defects	Associated anomalies	NGS result	Size of CNV	CNV type	OMIM gene	Corresponding disorder
A8	TOF	polycystic kidney, cleft lips/palate	seq [GRCh37/hg19] (154,387,290-177,298,737) ×1	22.91 Mbp	del (5q33.2q35.3)	NKX2-5	Atrial septal defect 7, with or without AV conduction defects", "Sotos syndrome 1"
A9	TOF	Polycystic kidney, limb anomalies	seq [GRCh37/hg19] (63,300-4,104,361) ×1	4.04 Mbp	del (1p36.32p36.33)		Chromosome 1p36 deletion syndrome
C4	TAC+UVH	Asplenia, partial inversus viscerum	seq[GRCh37/hg19] (146,164,990-147,872,398) ×1	1.71 Mb	del (1q21.1q21.2)	GJA5 GJA8	Chromosome 1q21.1 deletion syndrome
F2	Pulmonary stenosis+VSD	Club foot	seq[GRCh37/hg19] (18,981,842-21,507,679) ×1	2.53 Mb	del (22q11.21)	TBX1 CRKL	22q11 deletion syndrome (Velocardiofacial / DiGeorge syndrome)
F13	Coarctation of the aortic arch+VSD	-	seq[GRCh37/hg19] (18,894,169-21,458,897) ×1	2.45 Mb	del (22q11.21)	TBX1, CRKL	22q11 deletion syndrome (Velocardiofacial / DiGeorge syndrome)

All pathogenic CNVs were found only in the category of conotruncal defects, among the 5 cases with pathogenic CNVs, 4 cases were found contain genes NKX2-5, GJA5, GJA8, TBX1 and CRKL which were previously described as CHD risk gene.

**Table 2:** Characteristics of fetuses with pathogenic CNVs detected by next-generation sequencing (NGS).

deletion in chromosome 22q11.21 in a fetus with pulmonary stenosis and VSD, and case F13 involved a 2.45-Mb deletion in chromosome 22q11.21 in a fetus with coarctation of the aortic arch and VSD; these two cases having the same deletion in chromosome 22q11.21 included the *TBX1* and *CRKL* genes, which have been linked to 22q11 deletion syndrome (velocardiofacial DiGeorge syndrome).

In this study, 18 CNVs were interpreted as likely pathogenic. We developed a method for reading pairs of 100 kb to identify these 18 CNVs, which had not been previously reported at more than 1% frequency in control populations described in the DGV. Case A9 was identified as involving pathogenic CNVs and had 12.07-Mb duplication in chromosome 9q33.3q33.4, which included 41 OMIM genes. The remaining 17 CNVs were identified from 13 cases and were analyzed to determine whether they represented chromosomal abnormalities that may be associated with CHD. Detailed results are summarized in Table 3. These data showed that cases A7 and B5 both lacked OMIM genes. Case A7 was a fetus with TOF involving a 101.73-kb deletion in chromosome 18q22.1; this overlapped with chromosome 18q deletion syndrome, which is known to cause CHD. Case B5 was a fetus with pulmonary stenosis involving 290.65-kb duplication in chromosome 12p12.2; this duplication has been shown to cause Pallister-Killian syndrome, which can lead to CHD. The OMIM genes detected in these cases are outlined in Table 3. Notably, these OMIM genes were not all CHD risk genes, as supported by published observations in human studies; only some OMIM genes detected in cases C6, C12, E13, and F3 were previously described as CHD risk genes [5]. Additionally, cases C6, C12, and F18 involved rare and large CNVs that were hypothesized to confer higher disease risk as entire genes were deleted or duplicated. Case C6 involved an 18.93-Mb deletion in chromosomal 7q34q36.3 and 14.51-Mb duplication in chromosome 20q13.13q13.33 in a fetus with TAC and UVH. The deletion included the *EZH2* gene and 20 OMIM genes, which have been found to be associated with myelodysplasia and leukemia syndrome with monosomy 7, whereas the duplication included 22 OMIM genes. Case C12 involved 2.51-Mb duplication in chromosome 9q34.3 in a fetus with TAC and VSD; this duplication included 13 OMIM genes. Case F18 involved a 15.68-Mb deletion in chromosome 8q21.11q21.3 in a fetus with aortic stenosis and VSD; this deletion included the *NBN*, *CNGB3*, *CA2*, and *PEX2* genes, which have been linked to chromosome 8q21.11 deletion syndrome and Bor-Duane hydrocephalus contiguous gene syndrome.

According to statistical analysis, CNVs that were interpreted as benign and/or likely benign were found in 18 cases with CHD.

Additionally, five cases had pathogenic CNVs, 13 cases had CNVs that were likely to be pathogenic, and 42 cases had CNVs of uncertain significance. The CNVs of uncertain significance were described in DGV at more than 1% frequency. Among these 42 cases with CNVs of uncertain significance, 18 cases were found to include OMIM genes. However, only case A5 with TOF defects included the *CTNNA3* gene, which was previously shown to confer risk of CHD [6]. Most CNVs of uncertain significance detected in these 42 cases were found to be associated with some clinical disorders that have been described in patients with similar chromosomal imbalances. A few corresponding disorders, such as chromosome 1q21.1 duplication syndrome, Pallister-Killian syndrome, chromosome 22q11.2 microduplication syndrome, and cat eye syndrome, were found to be associated with cardiac defects. Chromosome 22q11.2 microduplication syndrome and cat eye syndrome were found to have the highest frequencies among these 42 cases with CNVs of uncertain significance. The microduplication of 22q11.2 appeared to be a new syndrome, which has been recognized on the basis of common disorders such as DiGeorge/velocardiofacial syndrome and cat eye syndrome [7].

## Discussion

In this study, we used NGS technology to evaluate the disease risk associated with CNVs of more than 100 kb in a case population of fetuses having sporadic nonsyndromic CHD with a normal karyotype. Our data showed that NGS could detect CNVs classified according to risk of pathogenicity in fetuses with CHD. These data provide important insights into the submicroscopic abnormalities present in these malformed fetuses.

CHD accounts for one-third of all major congenital anomalies [8]. The incidence of CHD with intrauterine diagnosis ranges from 2.4% to 54% [9]. There has been a recent increase in abnormal cardiac findings during obstetric ultrasonography screenings in mainland China, suggesting that prenatal diagnosis may have a significant effect on the incidence of complex or serious congenital cardiac malformations. A detail evaluation of the fetal heart is important for improvement of prenatal care and providing options of termination or continuation of the pregnancy. As such, identification of the causes of CHD is critical, and an improved understanding of the genetic component and pathogenesis of CHD is required for appropriate diagnosis, determination of prognosis, and assessment of risk for patients and their families. This would further facilitate appropriate prenatal and

Case	Cardiac defect	Associated anomalies	Sequencing result	Size of CNVs	CNVs type	OMIM gene	Corresponding disorder
A9	TOF	polycystic kidney, limb anomalies	seq[GRCh37/hg19] (128,940,945-141,011,202) ×3	12.07 Mb	dup (9q33.3q34.3)	LMX1B, ABL1, SPTAN1, LRRC8A 41 genes	
C6	TAC+UVH	facial anomalies	seq[GRCh37/hg19](140,676,451-159,068,966) ×1	18.39 Mb	del (7q34q36.3)	EZH2,AGK, PRSS1, CLCN1 20 genes	Myelodysplasia and leukemia syndrome with monosomy 7
			seq[GRCh37/hg19] (48,368,806-62,877,501) ×3	14.51 Mb	dup( 20q13.13q13.33)	PTPN1, ADNP, DPM1 22 genes	
C12	TAC+VSD		seq[GRCh37/hg19] (138,552,089-141,066,206) ×4	2.51 Mb	dup (9q34.3)	NOTCH1, CARD9, LHX3 13 genes	
F18	Aortic stenosis+VSD		seq[GRCh37/hg19] (77,193,273-92,869,223) ×1	15.68 Mb	del (8q21.11q21.3)	NBN, CNGB3, CA2, PEX2;	Chromosome 8q21.11 deletion syndrome Bor-Duane hydrocephalus contiguos gene syndrome
A7	TOF		seq[GRCh37/hg19] (62,205,494-62,307,222) ×1	101.73 Kb	del (18q22.1)		Chromosome 18q deletion syndrome
B1	TGA		seq[GRCh37/hg19] (6,998,870-7,138,966) ×4	140.10 Kb	dup (Xp22.31)	STS	
B5	PS	Limb anomalies, renal agenesis	seq[GRCh37/hg19] (20,140,280-20,430,927) ×3	290.65 Kb	dup (12p12.2)		Pallister-Killian syndrome
D8	DORV+ AVSD+PS	limb anomalies	seq[GRCh37/hg19] (39,219,157-39,324,648) ×3	105.49 Kb	dup (19q13.2)	ACTN4	
E9	TGA+ VSD+PS		seq[GRCh37/hg19] (31,691,248-31,951,210) ×3	259.96 Kb	dup (7p14.3)	PPP1R17	
E13	UVH+PS +tricuspid atresia	diaphragmatic hernia, genital system anomalies	seq[GRCh37/hg19] (74,492,043-74,715,406) ×3	223.36 Kb	dup (1p31.1)	TNNI3K	
			seq[GRCh37/hg19] (135,622,032-135,796,086) ×4	174.05 Kb	dup (8q24.22)	ZFAT	
F3	Aortic atresia+ mitral atresia+ HLHS	Genital system anomalies	seq[GRCh37/hg19] (73,654,210-74,311,417) ×3	657.21 Kb	dup (14q24.2q24.3)	PSEN1, DNAL1	
F20	Aortic atresia+ VSD		seq[GRCh37/hg19] (64,588,140-64,737,188) ×1	149.05 Kb	del (6q12)	EYS	Chromosome 6q11-q14 deletion syndrome
			seq[GRCh37/hg19] (27,828,068-28,255,174) ×3	427.11 Kb	dup (10p12.1)	ARMC4, RAB18	
			seq[GRCh37/hg19] (52,100,357-52,694,536) ×3	594.18 Kb	dup (Xp11.22)		Chromosome Xp11.23-p11.22 duplication syndrome Xp11.22 microduplication syndrome
G1	UVH+ mitral dysplasia	Cleft lips/palate, asplenia	seq[GRCh37/hg19] (189,682,000-190,326,015) ×1	644.01 Kb	del (3q28)	LEPREL1, CLDN1, CLDN16	
G5	AVSD		seq[GRCh37/hg19] (220,079,872-220,421,148) ×3	341.28 Kb	dup (1q41)	SLC30A10, IARS2 RAB3GAP2	

These 18 CNVs were interpreted as likely pathogenic because had not been previously reported at >1% frequency in control populations described in the Database of Genomic Variants (DGV). Most CNVs contain OMIM genes, and some CNVs may result in clinically corresponding disorders which has been described in patients with a similar chromosomal imbalance.

**Table 3:** Characteristics of fetuses with likely pathogenic CNVs detected by next-generation sequencing (NGS).

postnatal planning, enabling improvements in neonatal morbidity and surgical outcomes.

CHD is a disorder resulting from abnormal heart development; therefore, it is likely that defects in the genetic control of cardiac

development underlie the majority of cases of CHD [10]. CHD can occur as a component of a large number of chromosomal and Mendelian malformation syndromes. However, in 80% of cases, CHD occurs as a sporadic condition that exhibits high heritability [11]. Karyotyping has been the cornerstone for prenatal genetic diagnosis

in the last few decades. Unfortunately, genomic defects that cannot be readily detected with karyotyping have been shown to have roles in CHD. This first became apparent in the 1990s with the discovery of 22q11.2 deletions, which were found to underlie roughly 2% of CHD and more than 50% of specific conotruncal lesions [12]. Indeed, as methodologies are developed to define subchromosomal changes in genome structure, studies are showing that submicroscopic aberrations may contribute to the etiology of CHD.

CNVs are common in the human genome and are found within many regions. These CNVs may involve overlap of thousands of genes, which may be deleted or amplified. CNVs are deletions or amplifications of DNA segments that arise primarily from inappropriate recombination due to region-specific repeat sequences or from highly homologous genes that misalign during meiosis [13]. Advanced molecular cytogenetic techniques enable whole genome screening for chromosomal imbalances at much higher resolution than does conventional karyotyping. Within the past decade, sequencing technology has evolved rapidly with the advent of high-throughput NGS. NGS technologies are characterized by impressive throughput and allow for simultaneous sequencing of thousands to millions of relatively short nucleic acid sequences [14]. NGS produces massive amounts of sequencing data, which must be analyzed, filtered, interpreted, and reported. Interpreting the clinical significance of CNVs in CHD continues to be a challenge. Although the previous American College of Medical Genetics and Genomics (ACMG) recommendations provided interpretative categories for sequence variants and an algorithm for interpretation, the recommendations did not provide defined terms or detailed variant classification guidance [15].

Our results indicated that NGS could provide an etiological diagnosis in a high proportion of fetuses with complex or serious CHD suggestive of a chromosomal aberration. Five cases with pathogenic CNVs were identified, indicating that there was a 5.6% chance of finding causal submicroscopic CNVs in fetuses with complex or serious structural anomalies in the heart, as identified by ultrasound, and a normal karyotype. Thus, for one in every 20 fetuses with CHD, submicroscopic CNVs may explain the phenotype and could provide prognostic information. Therefore, we suggest that NGS can be implemented, in addition to karyotyping, as a diagnostic tool in the prenatal setting to identify genomic defects in fetuses with CHD.

Although CNVs involving genes may confer disease risk, relationships between gene dosage and phenotype are still being defined [16]. Currently, the interpretation of a detected imbalance is not always straightforward, given the presence of a high level of CNVs within the human genome. The clinical significance of a variant in relation to a disease or phenotype can be determined traditionally using several logical arguments, such as identification of CNVs (deletions or duplications) affecting a gene known to cause CHD through a dosage effect or for cases in which a patient with a similar chromosomal imbalance and a similar phenotype has previously been described [17]. In our study, among the five cases with pathogenic CNVs, four cases were found contain the *NKX2-5*, *GJA5*, *GJA8*, *TBX1*, and *CRKL* genes, which were previously described as CHD risk genes. When loss of function or gain of function of a gene located within the chromosomal imbalance is known to cause a distinct phenotype, the presence of this phenotype in the patient is a very strong argument in favor of a causal aberration. Although case A9 harbored a deletion in chromosome 1p36.32p36.33, which did not contain a risk gene, this deletion had been previously been described as chromosome 1p36 deletion syndrome and was associated with cardiac defects. Thus,

our results suggested that NGS facilitated the precise identification of chromosomal aberrations and their relationships with the etiology of CHD in fetuses.

Recent analyses have identified multiple CNVs that contribute to nonsyndromic CHD [18]. These studies estimate that 5-10% of sporadic nonsyndromic CHD in patients with a normal karyotype is due to rare and de novo variants [19]. In the human genome, rare CNVs, generally considered to be those found in less than 1% of the population, have recently been investigated as potential causative factors of complex diseases [20]. In our study, 33 rare or de novo CNVs were identified by NGS; according to ACMG criteria, 15 CNVs were interpreted as likely benign because these CNVs did not overlap with OMIM genes and were not related to clinical disorders found in patients with a similar chromosomal imbalance. Another 18 CNVs were interpreted as likely pathogenic because these CNVs contained OMIM genes or were associated with causal CHD syndrome. Our data showed that rare deletions or duplications spanning a larger number of genes conferred higher risk of CHD. However, despite this evidence, the rare or de novo nature of an imbalance can no longer be sufficient for considering an aberration as causal. Further studies with larger groups of patients and controls are necessary to establish causality and penetrance and determine the extent of the phenotypic spectrum of the imbalance.

In our study population, fetuses with complex or serious CHD were classified as having conotruncal defects or abnormal atrioventricular junctions. Using NGS, we examined the disease risk associated with the global burden of CNVs of more than 100 kb in fetuses with sporadic CHD. Our results demonstrated that the frequency of pathogenic CNVs was higher in cases of conotruncal defects than that in cases of abnormal atrioventricular junctions; however, the number of CNVs did not differ between these two categories, consistent with previous observations [21].

We identified 42 cases having CNVs of uncertain significance in our study. These CNVs with uncertain significance were described in the DGV at a frequency of more than 1%, but involving OMIM genes. Moreover, some of these CNVs may result in clinical disorders found in patients with a similar chromosomal imbalance. In these CNVs of uncertain significance, the OMIM genes involved in the CNVs included a few CHD risk genes, and a few of these genes were related to cardiac defects. The contribution of the global CNV burden to the risk of sporadic CHD remains incompletely defined, and considerable investments in sample collection and processing, computational analysis, and bioinformatic interpretation of results are needed for further clarification [22].

## Conclusion

In summary, to the best of our knowledge, this is the first report of molecular characterization of fetuses with CHD through NGS. Our results supported the potential effectiveness and applicability of NGS as a prenatal diagnostic tool and provide important insights into submicroscopic genomic instabilities in fetuses with CHD.

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