RESEARCH ARTICLE

Copy number variations in the *GATA4*, *NKX2-5*, *TBX5*, *BMP4*, *CRELD1*, and 22q11.2 gene regions in Chinese children with sporadic congenital heart disease

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Background: Congenital heart disease (CHD) is a common birth defect originating from both environmental and genetic factors. An overabundance of copy number variations (CNVs) affecting cardiac-related genes has previously been detected in individuals with CHD.

Objective: To evaluate if the presence of CNVs in the 22q11.2 region, and to determine whether *GATA4*, *NKX2-5*, *TBX5*, *BMP4*, and *CRELD1* genes contributed toward the pathogenesis of isolated incidences of CHDs in southwest China.

Methods: In total 167 patients from southwest China with sporadic CHD were studied, including 121 patients with ventricular septal defect (VSD), 24 with atrial septal defect (ASD), 12 with tetralogy of fallot (TOF), six VSD cases with TOF, two cases with patent ductus arteriosus (PDA), and two VSD cases with ASD. 22q11.2, *GATA4*, *NKX2-5*, *TBX5*, *BMP4*, and *CRELD1* regions were screened using MLPA and copy number variation sequencing (CNV-Seq).

Results: A 2.5-2.8 Mb deletion in the 22q11.2 region was identified in 5 patients with CHD. Two of these patients were diagnosed with VSD, while two had VSD and ASD, and the other had TOF. 5 patients correspond to the same classical DiGeorge syndrome. A 0.86 Mb duplication in the 22q11.2 region was identified in a PDA patient, whom was without extracardiac symptoms.

Conclusion: These data suggest that copy number variation in the 22q11.2 region is common in CHD patients in southwest China. Regardless of the presence or absence of extracardiac symptoms, results also indicate that it is necessary to perform prenatal screening for CHD.

KEYWORDS

22q11 region, congenital heart disease, copy number variations, MLPA

1 | INTRODUCTION

Congenital heart disease (CHD) is a common birth defect resulting from both environmental and genetic causes, which affects

0.85%-1% newborns¹ globally. Studies have revealed that 11.9% and 7.4% of cases are associated with monogenetic and cytogenetic anomalies, respectively. Collectively, these account for an estimated 20% of all CHD cases,² while the etiology for the remaining 80% is still unclear. Recently, copy number variations (CNVs), which constitute either gross DNA deletions or duplications, were

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identified as being critical dosage-sensitive genome for cardiac development and have also emerged as important contributors to CHDs. Examples of this include the *TBX1* deletion at 22q11.2, the *GATA4* deletion at 8p23.1, and the *NKX2-5* deletion at 5q35.1.^{3,4} An increasing assortment of CNVs affecting cardiac-related genes have been detected in individuals with CHDs. Recent population studies indicate that the 22q11.2 deletion is the most common microdeletion syndrome, occurring in 1 per 5950 live births and nearly 0.5%-1.9% of all CHDs,⁵ usually arising de novo, which accounts for 34% of truncus arteriosus and 16% of tetralogy of fallot (TOF) cases.⁶ Previous studies have identified that *GATA4*, *NKX2.5*, and *TBX5* contribute to monogenic forms of CHD,^{7,8} while *BMP4* and *CRELD1* are closely related to the development of CHD. Inactivation of *BMP4* within the myocardium results in neonatal lethality results in severe defects in septation and valve diseases such as ASD and VSD.⁹ *CRELD1* gene can cause CHD in Down syndrome-atrioventricular septal defect (AVSD) and also in sporadic forms of AVSD without any syndromic features.¹⁰ However, combined mutation screening revealed that congenital heart defects are rarely caused by mutations in these genes.¹¹ Thus far, no study has been conducted to investigate the role of *NKX2.5*, *TBX5*, *BMP4*, and *CRELD1* CNVs in Chinese children with sporadic congenital heart disease, we analyzed a cohort of 167 patients with sporadic cardiac anomalies using multiplex ligation-dependent probe amplification (MLPA) and copy number variation sequencing (CNV-Seq).

2 | METHODS

2.1 | Materials and methods

The study cohort enrolled 167 patients after an ultrasound diagnosis with unselected CHD at the Liuzhou Maternal and Child Healthcare Hospitals, Guangxi, southwest China between 2012 and 2016. All the patients in this study had no history of CHD in first-degree relatives. Congenital cardiac malformations were diagnosed by echocardiography and subsequently confirmed during surgery when performed. Extra-cardiac findings were extracted from medical records and included dysmorphic features, major anomalies, non-cardiac medical problems, and deficiencies in growth or developmental delay. This study was approved by the Liuzhou maternal and children healthcare hospital ethics review board on human subjects. All the patients were assessed by MLPA and CNV-seq.

2.2 | MLPA analysis

Genomic DNA was extracted from peripheral blood by phenol/chloroform extraction and quality determined with a NanoDrop 2000 UV-vis spectrophotometer (ThermoFischer Scientific, Waltham, MA, USA). MLPA reactions were performed with a SALSA-P311A kit (MRC Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions on an Applied Biosystems Veriti PCR system

(ThermoFischer Scientific). The P311-A1 kit includes probes for all seven *GATA4* exons; upstream and downstream regions of *GATA4*' the two *NKX2-5* exons; eight of the ten *TBX5* exons; the five *BMP4* exons; three of the 11 exons (exons 1, 3 and 10) of *CRELD1*; and three probes for chromosomal region 22q11 (DiGeorge syndrome). The MLPA products were analyzed on an ABI 3500Dx sequencer (ThermoFischer Scientific). MLPA data were collected with Gene Mapper (ThermoFischer Scientific) and analyzed with Coffalyser software (MRC Holland), as per manufacturer's recommendations. Sequence deletion or duplication was considered when a 35%-50% variation in the relative peak area of the amplification product of the respective probe was obtained.

2.3 | Next generation sequencing

Copy number variation sequencing was performed with next generation sequencing (NGS) as previously described.¹² 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 a 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 (CN) data analysis performed using previously described algorithms. For reporting CNVs, stringent CN ranges of 2.9-3.1 for a duplication and 0.9-1.1 for a deletion were applied.

3 | RESULTS

A cohort of 167 patients with sporadic CHD were enrolled. CHD phenotypes included ventricular septal defect (VSD, *n* = 121), atrial septal defect (ASD, *n* = 24), TOF, (*n* = 12), VSD combined with TOF (*n* = 6), patent ductus arteriosus (PDA, *n* = 2), and VSD combined with ASD (*n* = 2). The ages of the patients ranged from 2 months to 17 years, while the mean age was 2.8 years.

Among these 167 CHD patients, no imbalances were detected in any of the *GATA4*, *NKX2-5*, *TBX5*, *BMP4*, or *CRELD1* exons. Associated with the 22q11 region, the MLPA assay detected a heterozygous deletion in the *CDC45*, *GP1BB*, and *DGCR8* genes (Figure 1). Of the 5 patients with a 22q11 region deletion, two were diagnosed with VSD and ASD, one had VSD, and one other was diagnosed with TOF. The specific type of cardiac defect and hemodynamic status identified in the 6 patients is shown in Table 1. ASD in Patient 1 and 2 was Ostium secundum type, while VSD in Patient 1-4 was perimembranous type.

MLPA results from each of these 5 patients were confirmed as all patient samples were sequenced on a NGS platform. However, NGS results of one of the 167 patients indicated the presence of a duplication of the 22q11 region in which MLPA results for

					SpO ₂ (%)	
No.	Gender/age	Cardiac phenotype	Specific type	Defect size (mm)	Preoperation	Postoperation
Patient 1	F/7 y	ASD	Ostium secundum	2 × 2	100	100
		VSD	Perimembranous	6 × 6		
Patient 2	F/8 m	ASD	Ostium secundum	6 × 5	95	99
		VSD	Perimembranous	8 × 8		
Patient 3	F/8 y	VSD	Perimembranous	13 × 13	98	98
Patient 4	F/6 y	VSD	Perimembranous	12 × 12	98	98
Patient 5	M/6 m	TOF	–	–	97	99
Patient 6	F/6 y	PDA	–	–	98	99

the same patient were reported as normal. Estimated break-points in the 22q11.21 region of GRCh37/hg19 were 18900001 and 21400000 for the 2.5 Mb interstitial deletion in Patient 1 (Figure 2A), 18880001 and 21460000 for the 2.58 Mb interstitial deletion in Patient 2 (Figure 2B), 18900001 and 21460000 for the 2.56 Mb interstitial deletion in Patient 3 (Figure 2C), 18920001 and 21800000 for the 2.88 Mb interstitial deletion in Patient 4 (Figure 2D), 18900001 and 21460000 for the 2.56 Mb interstitial deletion in Patient 5 (Figure 2E), and 20940001 and 21800000 for the 0.86 Mb interstitial duplication in Patient 6 (Figure 2F). The five low copy repeats (LCR) A-D involved in the deletions were detected in Patients 1-5, which were responsible for the majority of 22q11.21

deletion syndromes, while the Patient 6 had a LCR C-D duplication on chromosome 22q11.21, overrides about 20% of the 22q11 duplication syndrome, and this CNV has not been previously described in DECIPHER, UCSC, and DGV database. The genes listed below are located within the duplication, and their positions in detail are shown in Table S1. Further study indicated that the CNV of 0.86 Mb duplication was de novo. These six CHD patients, Patients 1-5 correspond to the same classical DiGeorge syndrome, and involved the *TBX1* gene, and the Patient 6 had not previously presented with extracardiac symptoms. Results are shown in Table 2. First-degree relatives of the patients in this study had no history of CHD and negative for the abnormal CNV.

FIGURE 2 Next generation sequencing analysis on chromosome 22. A, The 2.5 Mb 22q11 deletion (Patient 1); B, The 2.58 Mb 22q11 deletion (Patient 2); C, The 2.56 Mb 22q11 deletion (Patient 3); D, The 2.88 Mb 22q11 deletion (Patient 4); E, The 2.56 Mb 22q11 deletion (Patient 5); F, The 0.86 Mb 22q11 duplication (Patient 6)

TABLE 2 Distribution of 6 patients regarding the type of CHD and the results detected by CNV-seq

	Gender/age	Cardiac phenotypes	Other anomalies	CNV-Seq (size)	LCR	Inheritance
Patient 1	F/7 y	VSD/ASD	Developmental delay and learning disability	22q11.2 deletion (2.5 Mb)	A-D	De novo
Patient 2	F/8 m	VSD/ASD	Microcephalus	22q11.2 deletion (2.58 Mb)	A-D	De novo
Patient 3	F/8 y	VSD	Short stature	22q11.2 deletion (2.56 Mb)	A-D	De novo
Patient 4	F/6 y	VSD	Delayed independent walking	22q11.2 deletion (2.88 Mb)	A-D	De novo
Patient 5	M/6 m	TOF	Cleft palate	22q11.2 deletion (2.56 Mb)	A-D	De novo
Patient 6	F/6 y	PDA	None	22q11.2 duplication (0.86 Mb)	C-D	De novo

ASD, atrial septal defect; CNV-Seq, copy number variation sequencing; F, female; M, male; PDA, patent ductus arteriosus; TOF, tetralogy of fallot; VSD, ventricular septal defect.

4 | DISCUSSION

Genetic causes of CHDs include structural abnormalities of the chromosome and gene dosage disorders. There is accumulating evidence indicating that a number of genomic disorders are strongly associated with gene dosage and the development of CHDs.¹³ Determining which CNVs contribute toward disease is an issue that is worth studying. With the development of genetic testing for CHDs, CNVs play an important role in diagnosis and gene discovery.¹⁴ Characteristics of a pathogenic CNV may include abnormalities in a known disease-associated regions, or gene dosage irregularities.¹⁵ These may therefore be observed in individuals with cardiovascular malformations.

As molecular targets for CHD, MLPA was used to screen the 22q11.2 region, and *GATA4*, *NKX2-5*, *TBX5*, *BMP4*, and *CRELD1* genes. The presence of CNVs was confirmed by NGS sequencing. In the 167 patients with isolated incidences of CHD, normalized single probes for the *GATA4*, *NKX2-5*, *TBX5*, *BMP4*, and *CRELD1* genes fell within the standard range, while six CNVs were identified. Among the CNVs, five 22q11.2 deletions and one 22q11.2 duplication was observed.

The 22q11.2 deletion syndrome is the second most common cause of CHD globally¹⁶ and was the most prevalent abnormality in this study having been found in ~3.0% (5/167) of all samples. This is close to the 2.56% found by Campos et al.¹⁷ An abundance

of LCRs present in the 22q11 region caused non-allelic homologous recombination during meiosis, which resulted in the observed deletions and duplications.¹⁸ There were eight LCR blocks (LCR22-A to LCR22-H) in the 22q11 region, but only LCR22-A to LCR22-D were implicated in the 22q11.2 deletion syndrome. Over 90% of the patients in this study shared a deletion between the LCR22-A and LCR22-D blocks.¹⁹ In the present study, Patients 2-5 possessed a 22q11.2 rearrangement and carried the ~2.5 Mb LCR22A-D deletion. According to literature, VSD, ASD, and TOF are the most common heart defects associated with chromosomal abnormalities.²⁰ Interestingly, our results corroborate this observation as Patients 2-5 presented with VSD. Patient 2 additionally presented with ASD. Patient 1 carried the 2.5 Mb LCR22A-C deletion in the 22q11 region, and presented with VSD and ASD.

Unlike the 22q11 deletion, duplications in this region are rarely reported.²¹ Less is known about the highly variable phenotype linked to these duplications,²² but it appears to be associated with elevated rates of ASD and delays in language and psychomotor development.²³ In the present study, we detected a 0.86 Mb de novo duplication in the 22q11.2 region (chr22: 20940001-21800000), in a 6-year-old girl (Patient 6) with PDA. The duplication's range from LCR22-C to LCR22-D was identified by CNV-seq. This is the first report of a patient with PDA presenting with a duplication in the 22q11.2 region. However, some limitations must be noted. Firstly, language spoken by the patient was not assessed. Secondly, this duplication is frequently inherited and we failed to verify its presence in the family. Further investigation into this finding is thus required.

The 167 patients used in this study were initially characterized by CNV-seq and MLPA. Using MLPA, five of the patients (Table 1) had 22q11 deletion, and the other 162 patients were negative. Further detection of CNV-seq verified the accuracy of the results. Five of the previously identified 22q11 deletion were detected by this method, and no false-positive results were yielded in the remaining 162 samples, indicating 100% sensitivity and specificity. And MLPA could become a commonly worldwide used methodology for diagnosis of several genomic disorders as the DiGeorge syndrome.

5 | CONCLUSION

In summary, our results suggest that deletions in the 22q11.2 region are common in CHD cases in southwest China. Regardless of the presence or absence of extracardiac symptoms, results also indicate that it is necessary to perform prenatal screening for CHD. Although limited in sample size, there is no evidence from this study that CNVs in the GATA4, NKX2-5, TBX5, BMP4, and CRELD1 genes play a role in fetal CHD in southwest China.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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