

Rare Copy Number Variation Analysis in Chinese Children of Complete Atrioventricular Canal and Single Ventricle

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Abstract

Background: Congenital heart disease (CHD) is the most common birth defects. Copy number variations (CNVs) have been proved to be important genetic factors that contribute to CHD. Here, we screened pathogenic CNVs in Chinese children with two rare types of CHD, complete atrioventricular canal (CAVC) and single ventricle (SV) .

Methods: We screened CNVs in 262 sporadic CAVC cases and 259 sporadic SV cases respectively, using a customized SNP array. The detected CNVs were annotated and filtered using available databases.

Results: Among 262 CAVC patients, we identified 44 rare CNVs in 43 individuals (16.4 %, 43/262), including 2 syndrome-related CNVs (7q11.23 and 8q24.3 deletion). Surprisingly, 88.6% rare CNVs (39/44) were duplications of 21q11.2-21q22.3, which were categorized as trisomy 21 (Down syndrome, DS). In CAVC with DS patients, the female to male ratio was 1.6:1 (24:15), and the rate of pulmonary hypertension (PH) was 41% (16/39). Additionally, 6 rare CNVs were identified in the SV patients (2.3%, 6/259), and none of them was trisomy 21.

Conclusions: Our study identified 50 rare CNVs in 262 CAVC and 259 SV patients, representing the largest cohort of these two rare CHD types in Chinese population. The results provided strong correlation between CAVC and DS, which also showed sex difference and higher incidence of PH. The presence of rare CNVs suggests the etiology of complex CHD is incredibly diverse, and CHD candidate genes remain to be discovered.

Background

Congenital heart disease (CHD) is the most common birth defect with an incidence of 1 ~ 1.2% in live births [1, 2]. Due to disrupted early-stage development, CHD consists of many structural malformations of the cardiovascular system, ranging from simple lesions such as atrial septal defects (ASD) and ventricular septal defects (VSD), to complex lesions such as tetralogy of Fallot (ToF), complete atrioventricular canal (CAVC) and single ventricle (SV). Although clinical treatment have significantly improved, complex CHD still remains to be a leading cause of newborn-related mortality [3].

Consistent with the complexity of early heart development, the etiology of CHD is multifactorial. To date, only about 20%-30% of CHD cases could be attributed to a genetic or environmental cause based on available technologies [4–6]. The incidence of some specific CHD types has been revealed with gender or race biases. The recurrence risk of CHD in the offspring of an affected parent, as well as in the siblings of a CHD child, has been reported to be higher than the general population. The evidences emphasize that genetics plays an important role in the pathogenesis of CHD [7].

Both small genetic variations and large genetic variations could contribute to CHD. Small genetic variations, usually referred to single nucleotide variations (SNVs) and small insertions/deletions (INDELs), are typically detected by sequencing technologies. Putative deleterious small variants in single

genes could cause both syndromic and isolated CHD. For instance, Alagille syndrome is a condition with CHD, hepatic complications, skeletal and ophthalmologic anomalies, and the most commonly pathogenetic genes have been identified as *JAG1* and *NOTCH2*. Holt-Oram syndrome, characterized by CHD and upper limb anomaly, is mostly caused by mutations in cardiac transcription factor *TBX5* [8]. In addition to syndromic CHD, an increasing number of genes have been identified in individuals with isolated CHD, including genes encoding transcription factors, cell signaling and adhesion molecules, structural proteins, and histone modifiers [7]. Whole exome sequencing (WES) and whole genome sequencing (WGS) are able to effectively identify small variants associated with CHD. Since abundant genes are involved in CHD, gene panels have not been routinely applied for clinical testing.

Large genetic variants, including aneuploidies, chromosomal rearrangements and copy number variations (CNVs), are also a group of important sources of CHD genetic causes. CNVs can range in size from single genes to large contiguous deletions or duplications of millions of base pairs. Pathogenic CNVs tend to be large, de novo and disrupting coding regions, which are more frequently identified in CHD patients [9, 10]. CNVs are not only one important cause of CHD, but have also been revealed to impact clinical outcomes [11, 12]. Although recent advances in NGS showed its potential in CNVs detection, chromosome microarray, either array comparative genomic hybridization (a-CGH) or single nucleotide polymorphism (SNP) array, is still the gold standard for CNVs detection and validation.

Nowadays, investigation of genes in overlapping CNV regions can probably identify relevant genes or refined intervals for certain CHD. Considering the heterogeneity of CHD etiology, a large number of CNVs associated with CHD have been identified over the past decades. However, a systematic CNV analysis of specific CHD types from different populations remains to be unclear, which also is important for clarification of CHD genetics. In this study, we identified 44 rare CNVs in 262 Chinese CAVC patients and 6 rare CNVs in 259 Chinese SV patients using a customized Affymetrix SNP array. Based on the detected CNVs, we further identified four known CHD genes (*ELN*, *LEFTY1*, *LEFTY2* and *ZBTB10*), and eight potential candidate genes for CHD.

Methods

Study subjects

We obtained a cohort of 528 children with sporadic CHD diagnosed as CAVC or SV by echocardiography from the Shanghai Children's Medical Center between November 2010 and August 2019. The patients had an average age at 8.77 ± 2.77 (mean \pm SD) years. The phenotypic details of this cohort were summarized in **Supplementary Table S1**. The Ethics Committee of the Shanghai Children's Medical Center reviewed and approved this study (SCMCIRB-K2017009).

DNA extraction

Genomic DNA was isolated from peripheral blood samples of all patients using Gentra Puregene Blood Kit (QIAGEN, Hilden, Germany) according to manufacturers' instructions. NanoDrop2000

spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to check the quantity and quality of the DNA samples. The samples with the OD260/OD280 ratio between 1.8 and 2.0 and the OD260/OD230 ratio greater than 1.5 were selected for investigation.

Microarrays

Microarrays were designed based on the Affymetrix Arrays platforms (Thermo Fisher Scientific, Waltham, MA), namely the CytoScan 750K arrays. We deleted probes with high population frequency and added probes particularly designed for sites marked with two stars in Clinvar as well as pathogenic variants in HGMD. In the meantime, design of probes was also based on clinical data of high-morbidity diseases in newborns, which was applied to screen for CHD, especially CHD patients accompanied by extra-cardiac anomalies. Genomic DNA samples were amplified, fragmented and stringently hybridized onto arrays according to manufacturers' instructions. Microarrays were automatically processed by GeneTitan Multi-Channel instruments together with Affymetrix Command Console (AGCC) Software for instruments control and production of probe cell intensity data (CEL file).

Data analysis

Microarray data processing were implemented using the Affymetrix Chromosome Analysis Suite v2.0 (ChAS) Software, and CNVs as well as SNVs were called and based on human assembly GRCh38 (hg38). There were 521 patients passed the QC tests finally.

The CNVs calls involved at least 50 probes for deletion and 50 for duplication were analyzed. In the meantime, the size of deletion or duplication was also confined to greater than 0.2 Mb. The variants having $\geq 70\%$ overlap with variants reported in DGV were considered as common CNVs, otherwise the CNVs were identified as rare ones. The detected CNVs calls were identified by public databases and websites: Database of Genomic Variants (DGV, <http://dgv.tcag.ca/dgv/app/home>), Online Mendelian Inheritance in Man (OMIM, <https://omim.org/>), UCSC Genome Browser (<https://genome.ucsc.edu/>), the Clinical Genome Resource (ClinGen, <https://www.clinicalgenome.org/>), Database of Chromosomal Imbalance, PubMed (<https://www.ncbi.nlm.nih.gov/pubmed/>), Phenotype of Humans using Ensemble Resources (DECIPHER, <http://decipher.sanger.ac.uk/>), SCAN (<http://www.scandb.org/newinterface/about.html>), ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>).

Results

Clinical characteristics of the 528 patients

For the 264 children with CAVC, the sex ratio (Males to Females) was 0.98 (131:133) and the average age was 8.80 ± 3.76 (mean \pm SD) years; For the 264 children with SV, the sex ratio was 1.34 (151:113) and the average age was 7.04 ± 3.57 years. The results were summarized in **Table 1**.

CNV detection in CHD cases

In this study, there were seven patients failed the QC tests (CAVC209, CAVC211, SV134, SV145, SV177, SV181, SV254). Among the 521 patients who passed the QC tests, 3465 variants were detected with a median size of 922.3 kb (max 23.9 Mb, min 51.5 kb).

47 CNVs (according to the filtering criteria) were identified in 44 CAVC cases (16.8%, 44/262), which consists of 42 duplication CNVs involved 406 genes and 5 deletion CNVs affecting 452 genes (see **Table 2**). We also identified 18 CNVs in 16 SV cases (6.1%, 16/264), including 13 duplications and 5 deletions (see **Table 3**).

There were 3 common CNVs (6.4%, 3/47) identified in CAVC samples (Yp11.2, 17q21.2-17q21.31, Yq11.221-11.222), and 12 common CNVs (66.7%, 12/18) were identified in SV patients (14q23.1, Xq28, 22q13.1, 2q35, 2q13, 2q14.2, 22q13.1, 7p14.3, 5q13.2, 5q23.2-5q23.3, 17q12, Xp22.2). The rest CNVs were considered as rare CNVs.

Rare CNVs in CAVC cases

In 262 CAVC patients, 44 rare CNVs were identified (see **Table 2**). Surprisingly, 88.6% (39/44) CAVC patients were found to carry duplication of 21q11.2-21q22.3. Among them, there was one patient (CAVC274) simultaneously had a ~0.8 Mb duplication at 3q12.1-3q12.2 encompassing *COL8A1*, *HP09053*, *FILIP1L*, *CMSS1* and *TBC1D23*. For the deletion CNVs, we had identified isolated deletions of 7q11.23 (CAVC162), 8q21.13-8q21.2 (CAVC102) and 8q24.3 (CAVC145) separately.

Additionally, we also consulted the DECIPHER and ISCA databases for evidences of clinical relevance. Duplication of 9p24.3-9p13.11 (CAVC207) has been reported to associate with ToF/TGA/CoA phenotype (TGA, Transposition of Great Arteries; CoA, Coarctation of the Aorta).

Rare CNVs in SV cases

Totally, 6 rare CNVs were identified in 259 SV patients (see **Table 3**), and none of them are identified as trisomy 21. Except deletion of 8q21.13-8q21.3, the rest were all duplication CNVs (5q34-5q35.1, Xq22.1, 1q42.12-1q42.13, 2p13.2, 7p14.1). These CNVs have been reported to associate with VSD, ToF and CoA in DECIPHER and ISCA.

Additionally, we noticed two patients were simultaneously identified with two CNVs, composed of one common and one rare CNV. One patient (SV143) was identified with 1 common CNV (2q14.2, dup) and 1 rare CNV (5q34-5q35.1, dup), while the other patient (SV261) owned 1 common CNV (Xp22.2, dup) as well as 1 rare CNV (7p14.1, dup).

Discussion

CAVC, accounts for ~ 4% of CHD, is a complex cardiac malformation characterized by a variable deficiency of the atrioventricular area in the developing heart [13, 14]. SV, one of the most common forms

of severe CHD, comprises a spectrum of congenital cardiac malformations defined by severe underdevelopment of one ventricle [15].

In 262 CAVC patients reported here, 88.6% (39/44) carried the duplication of 21q11.2-21q22.3, which could be diagnosed as trisomy 21, namely Down Syndrome (DS). Based on the results, a striking association of CAVC with DS was found in this study (14.8%, 39/262). All DS patients had the same ~ 3.3 Mb duplication at 21q11.2-21q22.3, and a systematic reanalysis indicated that 21q22.13 was the minimal critical region to the DS phenotype [16]. Additionally, another study detected 57.6% cardiac malformations in 500 patients with DS, and it also suggested CAVC (35.1%) was the most frequent heart anomaly [17]. It is putative that CAVC is the most frequent type of CHD in DS patients, and our study also provide strong evidence for this correlation in Chinese population. Additionally, CAVC also referred to as complete atrioventricular septal defect, and it has been reported that AVSD (atrioventricular septal defects) are more common in the female gender of DS patients [18]. In our study, the female/male ratio of CAVC with DS patients was 1.6 (24:15), which suggest that potential gender differences existed in the prevalence of CAVC in DS patients. Besides, we also noticed that rates of pulmonary hypertension (PH) in DS patients with CAVC was 41% (16/39), which was higher than previous report (28%, 364/1242) [19]. In fact, it was well known that PH is common in children with DS, and our study intensely proved this correlation.

Nowadays, several genes located in the “CHD critical region” on chromosome 21 have been proved to be associated with CAVC, including *DSCAM*, *COL6A1*, *COL6A2*, and *DSCR1* [20]. However, there were three DS patients simultaneously had another CNV located at different chromosome in our cohort, and one of the CNVs (3q12.1-3q12.2 dup) has been reported to associate with VSD in Decipher database. Additionally, several DS patients showed not only CAVC (4/27), but also other cardiac anomalies, such as ToF, ASD, patent foramen oval (PFO) and patent ductus arteriosus (PDA). Although the above-mentioned genes can explain partial cardiac phenotypes in DS patients, the genetic causes still were difficult to clarify especially when DS probands accompanied with multiple CNVs and diverse CHD phenotypes.

In our results, two rare CNVs have been identified as main causes of certain syndromes with heart anomalies. The microdeletion on 7q11.23 caused Williams-Beuren Syndrome (WBS; OMIM 194050), which is a multisystemic developmental disorder mostly accompanied with CHD. More than 90% of WBS patients have the ~ 1.55 Mb pair deletion extending from *FKBP6* to *GTF2I*, and it has been widely accepted that the deletion or mutation of an *elastin* (*ELN*) allele is a major cause of WBS [21]. One patient in this study (CAVC162) had a ~ 1.52 Mb deletion at 7q11.23 extending from *NCF1B* to *GTF2I*, encompassing the *ELN* gene. The other microdeletion on 8q24.3 have been recognized as associated with Verheij syndrome (OMIM 615583), which is characterized by growth retardation, developmental delay (DD), microcephaly, vertebral anomalies, dysmorphic features, cardiac and renal defects. Poly(U) Binding Splicing Factor 60 (*PUF60*) were suggested as the main cause for heart defects in the syndrome, since knockdown of *Puf60* alone resulted in cardiac structural defects [22]. The patient (CAVC145) reported here had a ~ 2.5 Mb deletion of 8q24.3, representing with growth retardation and heart anomalies.

Among the rest rare CNVs identified in the CAVC patients, CNVs located at 8q21.13-8q21.2, 9p24.3-9p13.1 and 3q12.1-3q12.2 have been seldom reported. The detected rare deletion CNV, 8q21.13-8q21.2, encompasses Zinc Finger and BTB Domain Containing 10 (*ZBTB10*), which has been known as a CHD gene. *ZBTB10* encodes a telomere-associated protein [23]. Lately, a GWAS involving 4,000 unrelated Caucasian patients diagnosed with CHD indicated that *ZBTB10* was associated with TGA, since two highly significant SNPs (rs148563140 and rs143638934) closely located to this gene [24]. Furthermore, they suggested strong cell-type specificity in murine cardiac development for *Zbtb10*. Except the known CHD gene *ZBTB10*, this CNV region in the patient (CAVC102) also included *STMN2* related to abnormality of the cardiovascular system. *STMN2* encodes a member of the stathmin family of phosphoproteins, functioning in microtubule dynamics and signal transduction [25]. Compared with controls, methylation of *STMN2* significantly increased (FDR p-value = 4.27×10^{-51}) in VSD cases [26]. Besides, It has been shown that *Stmn2* expresses in atrioventricular node, endocardium and outflow tract in mouse according to the LifeMap Discovery database. Among the rest 2 duplication CNVs, duplication of 9p24.3-9p13.1 (CAVC207) has been reported as VSD or TOF in DECIPHER. In this region, only *Rfx3* gene was in “ventricular septal defect” derived from the MGI (mouse genome informatics) database. For the duplicated region of 3q12.1-3q12.2, a report had shown a VSD patient had a ~ 116 kb duplication of this region, and *TBC1D23* has been identified as the major candidate gene [27]. In our study, the patient (CAVC274) had a ~ 0.8 Mb duplication at 3q12.1-3q12.2, encompassing this CHD candidate gene *TBC1D23*.

In 259 SV patients, 66.7% CNVs (12/18) has been identified as common CNVs. Among them, we found three duplication CNVs (Xq28, 2q13, Xp22.2) had already been reported as novel CNVs in another studies with 223 SV patients by microarray analysis [28]. Compared with this previous report, we noticed that only several genes overlapped in regions of these three CNVs, namely *HSFX1*, *MAGEA11*, *TMEM185A*, *CXorf40B* for Xq28, *BUB1* for 2q13, and *MID1* for Xp22.2. In our study, these CNVs were all identified as common CNVs.

The rest 6 CNVs (8q21.13-8q21.3, 1q42.12-1q42.13, Xq22.1, 5q34-5q35.1, 2p13.2, 7p14.1) were rarely reported, and the deleted region of 8q21.13-8q21.3 (SV007) overlapped with the above-mentioned CNVs in the CAVC patient (CAVC102). Furthermore, we also noticed that duplication of 1q42.12-1q42.13 (SV163) included 2 known CHD risk genes, *LEFTY1* and *LEFTY2* [29]. Especially, It has been reported that the SNP rs2295418 in the *LEFTY2* gene is associated with CHD in Chinese Han populations[30]. For other detected CNVs, only several genes were included in the deleted or duplicated regions, namely Xq22.1 (SV147; *PCDH19*, *TNMD*), 2p13.1 (SV195; *DYSF*, *CYP26B1*, *EXOC6B*) and 7p14.1 (SV176, *INHBA* and *SUGCT*). There were no related reports between CHD and these detected CNVs, so we focused on three genes (*PCDH19*, *CYP26B1* and *INHBA*) with a significantly higher pLI score, which reflects the intolerance to the loss of function mutations. *PCDH19* (Protocadherin 19) is a member of the delta-2 protocadherin subclass of the cadherin superfamily. *CYP26B1* (cytochrome P450 family 26 subfamily B member 1) involves in limiting retinoic acid (RA) levels within vertebrate embryos, which facilitate RA degradation [31]. It has been well-known that RA is important for the development of the heart. *INHBA* (Inhibin Subunit

Beta A) encodes a member of the TGF-beta (transforming growth factor-beta) superfamily of proteins, and it has been shown as a candidate gene for cardiac development [32].

As for the proband (SV143) with a ~ 1.6 Mb duplication at 5q34-5q35.1, we found that this region encompasses 2 genes (*SLIT3* and *TENM2*) related to septal defects of heart. *SLIT3* (Slit Guidance Ligand 3) expressed in cardiomyocyte-like progenitor cells [33], and membranous ventricular septum defects as well as atrioventricular and aortic valve abnormalities are exhibited in *SLIT3*-mutant mice [34]. Recently, *SLIT3* variants in humans has shown association with CHD involving in ToF and septal and outflow tract defects [35]. *TENM2* (Teneurin Transmembrane Protein 2) expresses abundantly in human fetal heart. Moreover, patients with loss of *TENM2* presented ASD in Decipher database, but gain of *TENM2* didn't show any type of CHD yet.

Conclusion

In conclusion, we identified 50 rare CNVs of CAVC and SV in 521 Chinese population, which represented the largest cohort of two rare CHD types in China. In this study, Chinese CAVC patients were mostly 21 trisomy with DS, which was consistent with the previous reports. Furthermore, it also suggested that there was no race difference in the close correlation between CAVC and DS patients. Combined with the present CNVs reports of CHD and the intolerance of genes within the CNVs regions, our results provided novel genetic evidences that could help clarify the etiology of CHD. Additionally, the rare CNVs we detected were seldom overlapped with known CHD loci, which implicated that abundant genes involved in heart development and diverse genetic causes of CHD.

Abbreviations

CAVC Complete Atrioventricular Canal

SV Single Ventricle

ToF tetralogy of Fallot

ASD Atrial septal defect

CoA Coarctation of the Aorta

VSD Ventricular Septal Defect

TGA Transposition of Great Arteries

PFO patent foramen oval

PDA patent ductus arteriosus

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Shanghai Children's Medical Center (No.SCMCIRB-K2017009). All written informed consents were obtained from the parents or guardian for participants under 16 years old.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

None declared.

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Authors' contributions

Conceptualization: QHF & YGY; Investigation: XYZ, XQZ, BW, GLY, YX; Writing of the original draft: XYZ, XQZ; Samples: XYZ; Preparation: XYZ; Review and editing: QHF,YGY& XQZ. All authors have read and approved the manuscript.

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Tables

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