Search for Rare Copy-Number Variants in Congenital Heart Defects Identifies Novel Candidate Genes and a Potential Role for FOXC1 in Patients With Coarctation of the Aorta

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Background—Congenital heart defects are the most frequent malformations among newborns and a frequent cause of morbidity and mortality. Although genetic variation contributes to congenital heart defects, their precise molecular bases remain unknown in the majority of patients.

Methods and Results—We analyzed, by high-resolution array comparative genomic hybridization, 316 children with sporadic, nonsyndromic congenital heart defects, including 76 coarctation of the aorta, 159 transposition of the great arteries, and 81 tetralogy of Fallot, as well as their unaffected parents. We identified by array comparative genomic hybridization, and validated by quantitative real-time polymerase chain reaction, 71 rare de novo (n=8) or inherited (n=63) copy-number variants (CNVs; 50 duplications and 21 deletions) in patients. We identified 113 candidate genes for congenital heart defects within these CNVs, including BTRC, CHRNB3, CSRP2BP, ERBB2, ERMARD, GLIS3, PLN, PTPRJ, RLN3, and TCTE3. No de novo CNVs were identified in patients with transposition of the great arteries in contrast to coarctation of the aorta and tetralogy of Fallot (P=0.002; Fisher exact test). A search for transcription factor binding sites showed that 93% of the rare CNVs identified in patients with coarctation of the aorta contained at least 1 gene with FOXC1-binding sites. This significant enrichment (P<0.0001; permutation test) was not observed for the CNVs identified in patients with transposition of the great arteries and tetralogy of Fallot. We hypothesize that these CNVs may alter the expression of genes regulated by FOXC1. Foxc1 belongs to the forkhead transcription factors family, which plays a critical role in cardiovascular development in mice.

Conclusions—These data suggest that deregulation of FOXC1 or its downstream genes play a major role in the pathogenesis of coarctation of the aorta in humans. (Circ Cardiovasc Genet. 2016;9:86-94. DOI: 10.1161/CIRCGENETICS.115.001213.)

Key Words: coarctation of the aorta • comparative genomic hybridization • congenital heart disease • copy-number variants • forkhead transcription factors • humans • tetralogy of Fallot

Congenital heart defects (CHD) are the most common congenital malformations with an incidence of 0.5% to 1% of live births.1 They also are the first cause of mortality during the first year of life of newborns in developed countries.2 Despite therapeutic advances, CHD are associated with a high proportion of long term morbidity. Among CHD, a large subset involves the outflow tract (OFT). This heterogeneous group of malformations represents 20% to 30% of the CHD diagnosed in newborns.3 Transposition of the great arteries (TGA) accounts for 5% to 7% of all CHD and is one of the most common cyanotic disorders diagnosed in the neonatal period with a prevalence of 0.2 per 1000 live births. The most common form of TGA is the dextro-looped type, which consists in a discordant ventriculoarterial connection implying that the aorta incorrectly arises from the right ventricle in an anterior and right-sided position, whereas the pulmonary artery incorrectly arises from the left ventricle in a posterior and left-sided position. By contrast to the normal heart in which both OFTs and great vessels show a dextral (right handed) spiralization, the great vessels in TGA present with a parallel course lacking normal spiralization. Coarctation...
of the aorta (CoA) is an OFT defect by which the aorta narrows in the area where the ductus arteriosus inserts. This is a relatively common defect that accounts for around 7% of all CHD.5 Tetralogy of Fallot (ToF) is defined by a combination of malpositioned aorta that overrides both ventricles, ventricular septal defect, pulmonary stenosis obstructing the blood flow into the lungs and right ventricular hypertrophy. ToF is the most common cyanotic congenital cardiac disease in humans with an occurrence of 1 per 3000 live births and accounts for 10% of all CHD.6

Although most of the patients undergo successful surgery in developed countries, the risk of cardiac malformation in their offspring is significantly higher than in the general population, suggesting genetic defects. Despite the high incidence of CHD, the pathogenesis of these malformations remains largely unknown. About 20% of CHD can be attributed to known causes such as chromosomal abnormalities, single gene disorders, or exposure to teratogens, whereas no causes is identified in ≈80% of the patients. A multifactorial origin associating environmental and genetic factors seems to be the usual mode of inheritance. The identification of new genes involved in nonsyndromic forms of CHD would help to better understand the molecular mechanisms leading to these malformations and to improve genetic counseling and disease prevention for couples having an affected child as well as adult patients willing to reproduce.

Array comparative genomic hybridization (aCGH) is a method allowing to detect copy-number variants (CNVs; ie, deletions and duplications) at a genome-wide level. The study of sporadic patients with nonsyndromic CHD by aCGH is an alternative to classic family studies for the identification of new genes implicated in these pathologies. A few studies have evidenced rare CNVs in patients with nonsyndromic CHD using this method.8–16 Here, we report a study performed in 316 children with nonsyndromic CHD and their normal parents. Our data show a high contribution of rare inherited but also de novo CNVs to human CHD and suggest a major role of Forkhead Box C1 (FOXC1) in the pathogenesis of CoA.

Materials and Methods

Patients

Informed consent for genetic analyses was obtained from all individuals participating in the study. The protocol was approved by the ethics committee of the University Hospital of Nantes (BRD 09/3A). Children from 468 families (85 with CoA, 291 with TGA, and 92 with ToF) presenting with a sporadic and nonsyndromic OFT defect were referred to the University Hospitals of Nantes, Tours, Angers, and Necker-Enfants Malades in Paris. In all included families except 1 (no. 417, 2 siblings with TGA), a single child was affected. A male:female sex ratio bias of 2.2:1 was observed in our series of patients with TGA, which is similar to that previously published in the literature. Both parents were available for 316 families (ie, 76 with CoA, 159 with TGA, and 81 with ToF), which were retained for the study. Because only 1 parent was available for the remaining 152 families, the latter were not studied. Patients with extracardiac features, such as learning disability, brain, craniofacial or renal anomalies, or carrying a clinically recognizable microdeletion/microduplication syndrome or a monogenic disorder had been excluded from the cohort. The patients for whom one of the parents or another relative was known to present a CHD were also excluded from the study. None of the included parents were symptomatic for any heart disease or underwent cardiac surgery. If a parent reported a symptom that could suggest any CHD, echocardiography was performed to exclude a minor anomaly.

DNA Extraction

DNA from all probands and their normal parents was extracted from whole peripheral blood using NucleoSpin Blood XL (Macherey Nagel). Illustra DNA Extraction Kit BACC2 (GE Healthcare) or UltraPure Phenol:Chloroform:ISOAMYL Alcohol (25:24:1, v/v; Life Technologies) according to manufacturers’ instructions.

aCGH Analysis

aCGH experiments were performed on 316 family trios using 2*400K Agilent custom-designed arrays (024825_D_F_20090731). According to our ethical rules and to minimize the detection of unsolicited findings (ie, detection of genomic imbalances unrelated to CHD), ≈8000 OMIM genes responsible for X-linked, autosomal dominant, or recessive genetic disorders were excluded from the design of the array (list of genes available on request). Microarrays contained 300000 probes located exclusively in exonic sequences with at least 1 probe in each exon of ≈19000 genes. In addition, 100000 probes covered with high-density 297 candidate genes known or suspected to play a role in heart development in humans or animal models (Table I in the Data Supplement). These probes were located in the exonic and intronic sequences, 10-kb upstream and downstream of the coding regions. Digestion, labeling, and hybridization were performed according to the protocols provided by Agilent. Children’s DNA were hybridized twice, once with that of the father and once with that of the mother (Figure 1). The arrays were analyzed with the Agilent scanner and the Feature Extraction software (v.9.1.3). Graphical overview was obtained using the customized SigmFrame software (https://github.com/lindenb/varykit/wiki/SigFrame). All genomic coordinates were based on the February 2009 assembly of the reference genome (GRCh37/hg19).

CNVs Detection

Rare CNVs were selected according to the following criteria: (1) CNVs containing at least part of an exon; (2) CNVs absent or with a frequency <1% from the CNV consensus reference set version 2.1 (42 million probes study,18 WTCCC study (http://www.wtccc.org.uk), 1000 genomes project (http://www.1000genomes.org/), and DDD controls project (http://www.ddduk.org/), integrating several high-quality copy-number variants (CNV) studies; and (3) CNVs present <4x in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home).19

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Validation

The CNVs fulfilling selection criteria were subjected to validation by qPCR. At least 1 pair of primers was designed in each selected CNV (Table II in the Data Supplement). All qPCR reactions were carried out in a 10-μL reaction volume containing 5 ng of genomic DNA, 0.5 μmol/L each primer, and 5 μL of SYBRPremix Ex Taq (Takara Bio Inc, Shiga, Japan). qPCR conditions comprised an initial denaturation at 95°C for 4 minutes, followed by 40 cycles at 95°C for 15 s, 60°C for 10 s, and 72°C for 10 s. qPCR reactions were carried out in a LightCycler480 System (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Amplification products were analyzed using LightCycler480 software version 1.5.0 (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). The ΔΔCt method was used, as previously described, to quantify the number of DNA copies. ΔΔCt were calculated with the ALB and TNNI3K genes for normalization. The validated CNVs were uploaded in the LOVD v.3.0 Leiden Open Variation Database (http://www.lovd.nl/; Data Supplement: Accession numbers).
Transcription Factor Binding Sites Enrichment Analyses

Sixty-nine of 71 qPCR-validated CNVs identified in affected children were used for transcription factor binding sites (TFBS) enrichment analyses. Two validated CNVs (7.9 and 14.5 Mb) were removed from the analyses because they could induce bias because of their large size. To identify the predicted transcription factors binding the genes included in the 69 CNVs present in the 316 patients and in each subgroup of CHD (ie, CoA, TGA, and ToF), we used the HMR Conserved TFBS track of UCSC, which contains the location and score of TFBS conserved in the human/mouse/rat alignment. A TFBS was retained when it was partially or entirely included in 1 of the 69 rare CNVs. The score and threshold were computed with the Transfac Matrix Database (v7.0) created by Biobase (http://www.biobase-international.com/). Overrepresentation of the TFBS in the observed data (ie, 69 rare CNVs in the patients) was assessed through random permutations. We simulated 10,000 data sets with 69 chromosomal regions randomly picked on the genome (a chromosomal region had a chance to be picked as location for a CNV proportional to its length). This assignation was repeated until the CNV was positioned in a region spanning at least 1 gene and where no polymorphic CNV exists. This procedure allowed mimicking the original experiment and provided a distribution of any enrichment statistics, which was really tailored for this experiment. Therefore, random regions were equivalent in size to the 69 CNVs, and 90% of their length had to overlap real rare CNVs. For each permutation, the number of random regions overlapping at least 1 binding site for the selected transcription factor was recorded, and the P value was computed as the number of permutations where this number exceeded the number observed in the real CNVs, divided by the total number of permutations. We used the rare validated CNVs (n=78) present in unaffected parents but absent in affected children as a negative control group.

Results

Rare CNVs Detected in Affected Children

The 316 family trios (affected children and both unaffected parents) retained and analyzed by aCGH led to the identification of 152 rare CNVs that fulfilled selection criteria. They were 38 in trios with CoA, 72 in trios with TGA, and 42 in trios with ToF. qPCR analysis performed in the children and their parents showed that 71 CNVs (50 duplications and 21 deletions) were present in affected children (15 in CoA, 29 in TGA, and 27 in ToF; Table III in the Data Supplement). Moreover, 78 CNVs were present in one of the parents (Table IV in the Data Supplement) and 3 CNVs were aCGH false-positive results (<2%). Seventy-one of 149 rare qPCR-validated CNVs were present in children with congenital heart defects (CHD). Eight CNVs were de novo and 63 inherited from an unaffected parent.
Table 1. Inheritance Pattern of Patients' qPCR-Validated CNVs

| No. of Patients | de novo CNVs | Inherited CNVs |
|-----------------|--------------|----------------|
|                 | n (%)*       | n (%)*         |
| 85 CoA          | 76           | 3 (4.1)†       | 12 (18.7) |
| 291 TGA         | 159          | 0 (0)          | 29 (22.3) |
| 92 ToF          | 81           | 5 (6.6)†       | 22 (27.3) |
| Total           | 316          | 8 (2.6)        | 63 (24.9) |

CNVs indicates copy-number variants; CoA, coarctation of the aorta; qPCR, quantitative polymerase chain reaction; TGA, transposition of the great arteries; and ToF, tetralogy of Fallot.

*De novo and inherited CNVs percentages were calculated regarding the total number of trios, for each type of congenital heart defects, submitted to comparative genomic hybridization array.

†P=0.002, Fisher exact test.

Table 1 shows the inheritance pattern of patients' qPCR-validated CNVs. The table includes the number of patients, the number of de novo CNVs, and the number of inherited CNVs for each type of congenital heart defect. The differences in the arrays and analysis pipelines between the recurrent CNVs were observed for 3 genomic regions, respectively at 10q24.32, 11p11.2, and 20p11.23. An overlapping duplication at 10q24.32 was identified in 2 unrelated children with TGA (patients no. 351 and no. 222). In both these cases, the CNVs were inherited and included a portion of the BTRC gene (Figure 2A and 2B). The second overlapping region at 11p11.2 was observed in 2 unrelated children presenting with 2 different forms of CHD. One patient presented with CoA (patient no. 174) and carried a paternally inherited duplication, including the entire coding sequence of the PTPRJ gene. The other patient presented with ToF (patient no. 153) and carried a paternally inherited deletion, including a portion of the PTPRJ gene (Figure 2C and 2D). The third overlapping region at 20p11.23 was detected in 2 unrelated children presenting with 2 different forms of CHD. One patient presented with ToF (patient no. 42) and carried a de novo CNV duplication. The other patient presented with TGA (patient no. 341) and carried a maternally inherited duplication. Both CNVs included the ZNF133, POLR3F, RBBP9, and DZANK1 entire gene coding sequences (Figure 2E and 2F).

In 1 family (Figure 3A), we identified a homozygous deletion in the affected child. The deletion was inherited from both consanguineous parents, each being heterozygous, and contained a portion of the CHRNB3 gene (Figure 3B). In another family (Figure 3C), we identified a rare duplication in 2 siblings. The duplication was inherited from a phenotypically normal mother and contained a portion of the TCTE3 gene and the ERNARD gene (Figure 3D).

Finally, we identified a 1q21 duplication in a patient with ToF (patient no. 43). The duplication was inherited from a phenotypically normal mother.

Discussion

This study reports a family trio-based study performed to identify rare CNVs in patients with sporadic, nonsyndromic cardiac OFT defects of 3 different types, respectively CoA, TGA, and ToF. The family trio design allowed us to identify 8 (11.3%) de novo rare CNVs and 63 (88.7%) inherited ones. De novo CNVs were significantly more frequent in patients with CoA (4.1%) and ToF (6.6%) compared with patients with TGA (no CNV in 159 patients; P=0.002). This difference indicates that novel genetic events are less frequent in the pathogenesis of TGA than in that of ToF and CoA. Thus, TGA could result from a genetic predisposition related to many low impact, mostly inherited, variants associated to environmental factors. The frequency of de novo CNVs identified in patients with ToF (6.6%) is broadly similar to previously reported frequencies considering the differences in the arrays and analysis pipelines between the studies. For example, Greenway et al reported 10% of de novo CNVs in their ToF patients’ cohort, which is slightly more than what we observed. This slight difference might also be because of the fact that some of the de novo CNVs described by Greenway et al are usually considered as syndromic, such as the 22q11.2 microdeletion syndrome. Our stringent clinical selection criteria may explain the absence of detection of such genomic disorders in our cohort.

The proportion of inherited CNVs that we detected was similar between the 3 types of CHD. The fact that a majority of the rare CNVs were inherited from a phenotypically healthy parent suggests that they contribute to the CHD but are not sufficient by themselves to cause the disease. Such variable expressivity and incomplete penetrance are again observed in such genomic disorders as the 22q11.2 microdeletion syndrome that can be inherited from a healthy parent.

Point mutations or CNVs involving binding sites located in regulatory regions of genes may cause developmental defects, such as SHH and polydactyly or PAX6 and aniridia. About CHD, Smemo et al published an elegant study showing that regulatory variation in a TBX5 enhancer leads to isolated congenital...
heart disease. We also previously showed that deletions upstream of \textit{SOX9} containing regulatory elements are likely responsible for isolated CHD.\textsuperscript{24} We performed here a computational approach to search for an enrichment of binding sites of transcription factor genes within our rare CNVs data set that might have altered the expression of genes and thus contributed to the CHD. Our TFBS approach led us to identify a significant enrichment of FOXC1-binding sites in the rare CNVs present in affected children (Table V in the Data Supplement). The strongest enrichment was observed for CoA (\textit{P}<0.0001) compared with TGA (\textit{P}=0.023) and ToF (\textit{P}=0.057). No enrichment in FOXC1-binding sites was observed in the rare CNVs identified in unaffected parents and it was absent in children. FOXC1 belongs to the forkhead family of transcription factors and plays an essential role in the regulation of embryonic development in different model organisms.\textsuperscript{25} It is notably involved in cardiovascular development and in particular in the morphogenesis of the cardiac OFT.\textsuperscript{26–28} Human \textit{FOXC1} heterozygous mutations are responsible for the Axenfeld–Rieger syndrome, a developmental disorder affecting structures in the anterior segment of the eye. Mutations in \textit{FOXC1} have been identified in a few patients presenting CHD in addition to Axenfeld–Rieger syndrome.\textsuperscript{29,30} Interestingly, a de novo deletion of \approx 45 kb including \textit{FOXC1} has been reported in a patient with atrial septal defect in addition to bilateral congenital glaucoma, partial aniridia, and club feet.\textsuperscript{31} A recent study\textsuperscript{32} has reported mutations in \textit{FOXC1} that affect gene transactivation in patients presenting with nonsyndromic TOF. Taken together, these previously published data and our results strongly suggest that a dysregulation of \textit{FOXC1} or its downstream regulated genes may contribute to the pathogenesis of CHD and in particular CoA. Nevertheless, further experimental analyses of putative sites need to be performed to strengthen this conclusion.
We compared our data to CNVs from patients with CHD downloaded from DECIPHER (https://decipher.sanger.ac.uk/) and ISCA (International Standards for Cytogenomic Array consortium; https://www.iscaconsortium.org/) public databases and from the existing literature. After exclusion of the patients with the largest CNVs, for whom genotype–phenotype correlations were not consistent, we identified 7 patients from the public databases and the literature carrying a CNV partially overlapping with a CNV identified in 4 patients of our cohort (Table 2). (1) Three patients from the public databases presenting with patent ductus arteriosus or CoA (DECIPHER 1578, ISCA nssv706487, and ISCA nssv706596) carried deletions of variable sizes, including GLIS3. These deletions partially overlapped with a duplication identified in one of our patients (no. 437). GLIS3 plays a role in the regulation of a variety of cellular processes during development, such as cell migration. (2) Two duplications, 1 identified in a patient with TGA (DECIPHER 250627) and 1 in a patient with ToF from the literature, overlapped with the duplication identified in one of our TGA patients (no. 172). All the 3 duplications included the entire PLN coding sequence gene. PLN is a membrane protein that regulates the Ca\(^2+\) pump in cardiac and skeletal muscle.

Table 2. Comparison Between CNVs From This Study Data and ISCA and DECIPHER Databases

| Trio ID | Type of CHD | Parental Inheritance | Deletion/Duplication (coordinates hg19) | Chr. Region (Mb) | CNV Size (Mb) | ISCA/DECIPHER ID | Type of CHD | Parental Inheritance | Deletion/Duplication (coordinates hg19) | CNV Size (Mb) | Candidate Gene |
|---------|-------------|----------------------|----------------------------------------|------------------|--------------|------------------|-------------|---------------------|----------------------------------------|--------------|----------------|}
| 437     | CoA         | Father               | Duplication                            | chr9:3781683–4161396 | 9p24.2        | 0.38             | 1578         | PDA                 | Unknown                               | 11.34        | GLIS3          |
|         |             |                      |                                        |                   |              |                  | nssv706487   | CoA                 | Unknown                               | 11.1         |                 |
|         |             |                      |                                        |                   |              |                  | nssv706596   | CoA                 | Unknown                               | 16.7         |                 |
| 335     | TGA         | Father               | Duplication                            | chr18:14138589–1415986B | 19p13.12     | 0.021           | 5680437      | Dextrocardia | Unknown                               | 0.7          | RLN3           |
| 172     | TGA         | Mother               | Duplication                            | chr6:118771597–119031236 | 6q22.31     | 0.26            | 250627       | TGA                 | Unknown                               | 0.85         | PLN            |
| 188     | ToF de novo |                      | Duplication                            | chr17:37813254–38033098 | 17q12       | 0.22            | nssv578742   | TAPVR               | Unknown                               | 6.4          | ERBB2          |

CHD indicates congenital heart defects; CNVs, copy-number variants; CoA, coarctation of the aorta; PDA, patent ductus arteriosus; TGA, transposition of the great arteries; and TAPVR, total anomalous pulmonary venous return; ToF, tetralogy of Fallot.
cells. Mutations in this gene cause dilated cardiomyopathy or arrhythmogenic right ventricular cardiomyopathy.13 (3) A deletion present in a patient with dextrocardia (ISCA nssv580437) overlapped with a duplication identified in one of our TGA patients (no. 335). Both genomic imbalances included the entire RLN3 gene. RLN3 plays a role in regulating blood pressure, controlling heart rate, and releasing oxytocin and vasopressin. Moreover, relaxins stimulate angiogenesis via the induction of vascular endothelial growth factor.42 (4) Finally, a duplication including the entire ERBB2 coding sequence gene was identified in a patient with total anomalous pulmonary venous return (ISCA nssv 578742) and in a de novo duplication in one of our ToF patients (no. 188). ERBB2 encodes a member of the epidermal growth factor receptor family. ErbB2 signaling is essential for heart development and function in mice.35 In 1 patient with ToF (no. 43), we identified 1q21 duplication. Recurrent deletions and duplications at this locus have been associated with both syndromic and nonsyndromic forms of CHD, including ToF.39,40,41 Because the GJA5 mutant mice exhibit a wide range of CHD, among them conotruncal defects,37 the gene seems to be a good candidate for the cardiac malformations, although no point mutations have been identified in patients yet.

Most of the rare CNVs identified in our study show a genome-wide distribution and a single occurrence (Figure I in the Data Supplement). Only 3 chromosomal regions with a variable number of copies were identified in >1 patient (Figure I in the Data Supplement): at 10q24.32 in 2 patients with TGA (no. 351 and 222), 11p11.2 in 1 patient with CoA (no. 174), and 1 ToF patient (no. 153) and at 20p11.23 in 1 TGA patient (no. 341) and 1 ToF patient (no. 42). These CNVs encompass several genes among, which BTRC, PTTPR1, and CSR2BP2 are good candidates for CHD. BTRC has been related to the Wnt signaling pathway,38 which regulates diverse cellular processes, such as gene transcription and cell proliferation, migration, polarity, and division.39 Wnt2 and Wnt11 mutations are responsible for CHD in mice.40,41 PTTPR1 is critical for embryonic heart development and vasculogenesis in mice.42 CSR2BP2 is a component of the ATAC (Ada Two-A Containing) complex, a complex with histone acetyltransferase activity on histones H3 and H4. Of note, mutations in histone-modifying genes have recently been related to CHD.43 Moreover, the double–histone–acetyltransferase complex ATAC is essential for mammalian development.44 Thereby, these genes are strong candidates for CHD in our patients.

Two additional rare CNVs were of special interest (Figure 3). A 3.7-kb homozygous deletion encompassing a portion of CHRN3 was detected in a child with TGA (no. 342; Figure 3A and 3B) issued from consanguineous heterozygous parents. CHRN3 belongs to the nicotinic acetylcholine family of receptors expressed in the neural tube during embryonic development.45 Neural tube signals are critical during heart formation and differentiation in chicken and quail embryos.46 The second CNV of interest was observed in 2 TGA siblings (patient no. 417). Both children carried a duplication including ERMARD, which was inherited from their phenotypically normal mother (Figure 3C and 3D). The exact function of ERMARD remains unknown but it has been recently shown to play a major role in the control of neuronal migration. Haploinsufficiency of ERMARD causes periventricular nodular heterotopia.47 Because periventricular nodular heterotopia has been related to cardiovascular defects, it is conceivable that duplication of ERMARD plays a role in CHD.48

By combining genes present in de novo CNVs, in overlapping CNVs (between our patients or public databases), and from TFBS analyses (http://hgdownload.soe.ucsc.edu/ goldenPath/hg19/database/tfbsConsSites.txt.gz), we identified a set of 113 candidate genes for CHD (Table VI in the Data Supplement) and a short list of 10 top-candidate genes (Table 3). Developmental anomalies resulting in conotruncal defects have
been associated with distinct changes in gene expression,\textsuperscript{49} describing a pattern of expression of developmentally important networks. This supports the hypothesis that converging and accumulating rare genomic and epigenetic variants may disrupt regulatory networks during heart development, ultimately leading to CHD.\textsuperscript{12,13,15} Because we excluded the noncoding regions of our custom array, we were not able to detect chromosomal imbalances involving regulatory elements, which is a limitation of our study. According to the multifactorial origin of CHD, environmental factors during embryo development may also be considered as contributing factors to CHD in addition to genetic variations. Further work needs to be done to determine more precisely the origin of nonsyndromic CHD and consequently helping in their diagnosis and management.

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**Disclosures**

None.

**Appendix**

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**References**

1. Öyen N, Poulsen G, Boyd HA, Wohlfihr J, Jensen PK, Melbye M. Recurrence of congenital heart defects in families. *Circulation*. 2009;120:295–301. doi: 10.1161/CIRCULATIONAHA.109.857987.

2. Gillum RF. Epidemiology of congenital heart disease in the United States. *Am Heart J*. 1994;127(4 Pt 1):919–927.

3. Hoffman JJ, Kaplan S. The incidence of congenital heart disease. *J Am Coll Cardiol*. 2002;39:1890–1900.

4. D’Antiga F, Case B, Toscana A, Calabrò R, Pacileo G, Marasini M, et al. Complete transposition of the great arteries: patterns of congenital heart disease in familial precurrence. *Circulation*. 2001;104:2809–2814.

5. Rosenthal E. Coarctation of the aorta from fetus to adult: curable condition or life long disease process? *Heart*. 2005;91:1495–1502. doi: 10.1136/hrt.2004.057182.

6. Ferenèz C, Rubín JD, McCarrier RJ, Brenner JL, Neill CA, Perry LW, et al. Congenital heart disease: prevalence at livebirth. *The Baltimore-Washington Infant Study. Am J Epidemiol*. 1985;121:31–36.

7. Burn J, Brennan P, Little J, Holloway S, Coffey R, Somerville J, et al. Recurrence risks in offspring of adults with major heart defects: results from first cohort of British collaborative study. *Lancet*. 1998;351:311–316.

8. Blue GM, Kirk EP, Sholler GF, Harvey RP, Winlaw DS. Congenital heart disease: current knowledge about causes and inheritance. *Med J Aust*. 2010;197:155–159.

9. Breckpot J, Thienpont B, Peeters H, de Ravel T, Singer A, Rayyan M, et al. Array comparative genomic hybridization as a diagnostic tool for syndromic heart defects. *J Pediatr*. 2010;156:810–817.e1. doi: 10.1016/j.jpeds.2009.11.049.

10. Derwińska K, Bartnik M, Wiśniowiecka-Kowalik B, Jagla M, Rudziński A, Pietryzk J, et al. Assessment of the role of copy-number variants in 150 patients with congenital heart defects. *Med Wieku Rosw*. 2012;16:175–182.

11. Greenway SC, Pereira AC, Lin C, DePalma SR, Israel SJ, Mesquita SM, et al. de novo copy number variants identify new genes and loci in isolated spordadic tetralogy of Fallot. *Nat Genet*. 2009;41:931–935. doi: 10.1038/ng.415.

12. Serra-Jühli C, Rodríguez-Santiago B, Cuscó I, Vendrell T, Camats N, Torán N, et al. Contribution of rare copy number variants to isolated human malformations. *PLoS One*. 2012;7:e45530. doi: 10.1371/journal.pone.0045530.

13. Hitz MP, Lemieux-Pereault LP, Marshall C, Ferzoz-Zada Y, Davies R, Yang SW, et al. Rare copy number variants contribute to congenital left-sided heart disease. *PLoS Genet*. 2012;8:e1002903. doi: 10.1371/journal.pgen.1002903.

14. Glessner JT, Bick AG, Ito K, Homsy JG, Rodríguez-Murillo L, Fromer M, et al. Increased frequency of de novo copy number variants in congenital heart disease by integrative analysis of polyorphism array data and exome sequence data. *Circ Res*. 2014;115:838–896. doi: 10.1161/CIRCRESAHA.115.304458.

15. Bittel DC, Zhou XG, Kibiryeva N, Friedler S, O’Brien JE Jr, Marshall J, et al. Ultra high-resolution centric genomic structural analysis of a non-syndromic congenital heart defect, Tetralogy of Fallot. *PLoS One*. 2014;9:e87472. doi: 10.1371/journal.pone.0087472.

16. Warburton D, Rosennus M, Kline J, Johnaputra V, Williams I, Anyane-Yenyeo K, et al. The contribution of de novo and rare inherited copy number changes to congenital heart disease in an unscreened sample of children with conotruncal defects or hypoplastic left heart disease. *Hum Genet*. 2014;133:11–27. doi: 10.1007/s00431-013-1353-9.

17. Bianca S, Ettore G. Sex ratio imbalance in transposition of the great arteries and possible agricultural environmental risk factors. *Images Paediatri Cardiol*. 2001;3:10–14.

18. Conrad DF, Pinto D, Redon R, Feuk L, Gokcumen O, Zhang Y, et al. Wellcome Trust Case Control Consortium. Origins and functional impact of copy number variation in the human genome. *Nature*. 2009;464:93–96. doi: 10.1038/nature08516.

19. Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, et al. Detection of large-scale variation in the human genome. *Nat Genet*. 2004;36:949–951. doi: 10.1038/ng1146.

20. Hinrichs AS, Karolchik D, Baertsch R, Barber GP, Bejerano G, Clawson H, et al. UCSC Genome Browser Database: update 2006. *Nucleic Acids Res*. 2006;34(Database issue):D590–D598. doi: 10.1093/nar/gkj144.

21. Soemedi R, Wilson JJ, Bentham J, Darlay R, Tió F, Zelenika D, et al. Contribution of global rare copy-number variants to the risk of sporadic congenital heart disease. *Am J Hum Genet*. 2014;94:489–501. doi: 10.1016/j.ajhg.2012.08.003.

22. Bhatia S, Kleinjan DA. Disruption of long-range gene regulation in human genetic disease: a kaleidoscope of general principles, diverse mechanisms and unique phenotypic consequences. *Hum Genet*. 2014;133:815–845. doi: 10.1007/s00439-014-1424-6.

23. Snemo S, Campos LC, Moskwitz IP, Krieger JE, Pereira AC, Nobrega MA. Regulatory variation in a TBX5 enhancer leads to isolated congenital...
heart disease. *Hum Mol Genet.* 2012;21:3255–3263. doi: 10.1093/hmg/dds165.

24. Sanchez-Castro M, Gordon CT, Petit F, Nord AS, Callier P, Andrieux J, et al. Congenital heart defects in patients with deletions upstream of SOX9. *Hum Mutat.* 2013;34:1628–1631. doi: 10.1002/humu.22449.

25. Kune T, Deng K, Hogan BL. Murine forkhead/winged helix genes Foxc1 (Mf1) and Foxc2 (Mfhl) are required for the early organogenesis of the kidney and urinary tract. *Development.* 2000;127:1387–1395.

26. Kune T. The cooperative roles of Foxc1 and Foxc2 in cardiovascular development. *Adv Exp Med Biol.* 2009;665:63–77.

27. Kune T, Jiang H, Topczewska JM, Hogan BL. The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes Dev.* 2001;15:2470–2482. doi: 10.1101/gad.907301.

28. Seo S, Kune T. Forkhead transcription factors, Foxc1 and Foxc2, are required for the morphogenesis of the cardiac outflow tract. *Dev Biol.* 2006;296:421–436. doi: 10.1016/j.ydbio.2006.06.012.

29. Do R-F, Huang H, Fan L-L, Li X-P, Xia K, Xiang R. A Novel Mutation of FOXC1 (R127L) in an Axenfeld-Rieger Syndrome Family with Glaucoma and Multiple Congenital Heart Diseases. *Ophthalmic Genet.* 2014;1–5.

30. Gripp KW, Hopkins E, Jenny K, Thacker D, Salvin J. Cardiac anomalies in Axenfeld-Rieger syndrome due to a novel FOXC1 mutation. *Am J Med Genet A.* 2013;161A:114–119. doi: 10.1002/ajmg.a.35697.

31. Töpf A, Griffin HR, Glen E, Soemedi R, Brown DL, Hall D, et al. Functionally significant, rare transcription factor variants in tetradactyly of Fallot. *PLoS One.* 2014;9:e95453. doi: 10.1371/journal.pone.0095453.

32. Kim YS, Nakanishi G, Lewandoski M, Jetten AM. GLIS3, a novel member of the GLIS subfamily of Krüppel-like zinc finger proteins with regulatory significance, is required for murine embryonic development. *Dev Cell* 2009;29:1176–1188. doi: 10.1128/MCB.01599-08.

33. Rogers SW, Tvrdik P, Capecci MR, Gahring LC. Prenatal ablation of the nicotinic receptor alpha7 cell lineages produces lumbosacral spina bifida the severity of which is modified by choline and nicotine exposure. *Am J Med Genet A.* 2012;158A:1135–1144. doi: 10.1002/ajmg.a.35372.

34. Reiner J, Rozen S. Congenital heart defects in patients with deletions upstream of SOX9. *Adv Exp Med Biol.* 2009;665:63–77.

35. Climent S, Sarasa M, Villar JM, Murillo-Ferrol NL. Neurogenic cells inhibit the differentiation of cardiogenic cells. *Dev Biol.* 1995;171:130–148. doi: 10.1006/dbio.1995.1266.

36. Conti V, Carabalona A, Pallesi-Pocachard E, Parrini E, Leventer RJ, Buhler E, et al. Periventricular heterotopia in 6q terminal deletion syndrome: role of the Chr6:q70 gene. *Brain.* 2013;136 (Pt 11):3378–3394. doi: 10.1093/brain/awt249.

37. Lee CH, Wai YY, Wu T. Periventricular nodular heterotopia and cortical malformations in connexin40-deficient mice. *Circ Res.* 2003;93:201–206. doi: 10.1161/01.RES.0000084852.65396.70.

38. Maniatis T. A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways. *Genes Dev.* 1999;13:505–510.

39. Gessert S, Kühler M. The multiple phases and faces of wnt signaling during cardiac differentiation and development. *Circ Res.* 2010;107:186–199. doi: 10.1161/CIRCRESAHA.110.221531.

40. Tian Y, Yuan L, Goss AM, Wang T, Yang J, Lepore JJ, et al. Characterization and in vivo pharmacological rescue of a Wnt2-Gata6 pathway required for cardiac inflow tract development. *Dev Cell.* 2010;18:275–287. doi: 10.1016/j.devcel.2010.01.008.

41. Zhou W, Lin L, Majumdar A, Li X, Zhang X, Liu W, et al. Modulation of morphogenesis by noncanonical Wnt signaling requires ATF/CREB family-mediated transcriptional activation of TGFbeta2. *Nat Genet.* 2007;39:1225–1234. doi: 10.1038/ng2112.

42. Argraves WS, Drake CJ. Genes critical to vasculogenesis as defined by systematic analysis of vascular defects in knockout mice. *Am Rev A Disc Mol Cell Evol Biol.* 2005;286:875–884. doi: 10.1029/2002232.

43. Zaidi S, Choi M, Wakimoto H, Ma L, Jiang J, Overton JD, et al. De novo mutations in histone-modifying genes in congenital heart disease. *Nature.* 2013;498:220–223. doi: 10.1038/nature12141.

44. Guelman S, Kozuka K, Mao Y, Pham V, Solloway MJ, Wang J, et al. The double-histone-acetyltransferase complex ATAC is essential for mammalian development. *Mol Cell Biol.* 2009;29:1176–1188. doi: 10.1128/MCB.01599-08.

45. Rogers SW, Tvrdik P, Capecci MR, Gahring LC. Prenatal ablation of the nicotinic receptor alpha7 cell lineages produces lumbosacral spina bifida the severity of which is modified by choline and nicotine exposure. *Am J Med Genet A.* 2012;158A:1135–1144. doi: 10.1002/ajmg.a.35372.

46. Climent S, Sarasa M, Villar JM, Murillo-Ferrol NL. Neurogenic cells inhibit the differentiation of cardiogenic cells. *Dev Biol.* 1995;171:130–148. doi: 10.1006/dbio.1995.1266.

47. Conti V, Carabalona A, Pallesi-Pocachard E, Parrini E, Leventer RJ, Buhler E, et al. Periventricular heterotopia in 6q terminal deletion syndrome: role of the Chr6:q70 gene. *Brain.* 2013;136 (Pt 11):3378–3394. doi: 10.1093/brain/awt249.

48. Devoto MC.01599-08.

49. Guo L, Smith FC, Taffet SM, Delmar M. High incidence of cardiac malformations in connexin40-deficient mice. *Circ Res.* 2003;93:201–206. doi: 10.1161/01.RES.0000084852.65396.70.

50. Li Y, Klena NT, Gabriel GC, Liu X, Kim AJ, Lemke K, et al. Global genetic analysis in mice unveils central role for cilia in congenital heart disease. *Nature.* 2015;521:520–524. doi: 10.1038/nature14269.

**CLINICAL PERSPECTIVE**

Congenital heart disease (CHD) is the most frequent birth defect, affecting 0.5% to 1% of live births, and it is a significant cause of morbidity and mortality. Chromosomal abnormalities, single gene disorders, or exposure to teratogens are known to explain a subset of CHD cases, but no pathogenesis is identified in >80% of the patients. The application of evolving technologies that detect structural variation throughout the genome, such as array comparative genomic hybridization, has demonstrated a significant contribution of copy-number variants (CNVs) to congenital heart disease. In this study, we analyzed by array comparative genomic hybridization method in 316 children with sporadic, nonsyndromic CHD, including 76 coarctation of the aorta, 159 transposition of the great arteries, and 81 tetralogy of Fallot, as well as their unaffected parents. Rare de novo or inherited CNVs were detected in the patients highlighting 113 candidate genes for CHD. Uniquely, this study showed that most of the rare CNVs identified in patients with coarctation of the aorta contained at least 1 gene with FOXC1-binding sites. These CNVs may alter the expression of genes regulated by FOXC1, a gene that plays a critical role in cardiovascular development in mice. Our data show a high contribution of rare inherited but also de novo CNVs to human CHD and suggest a major role of FOXC1 or its downstream genes in the pathogenesis of coarctation of the aorta.