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Duplication and Deletion of *CFC1* Associated with Heterotaxy Syndrome

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Heterotaxy syndrome, which causes significant morbidity and mortality, is a class of congenital disorders, in which normal left–right asymmetry cannot be properly established. To explore the role of copy number variants (CNVs) in the occurrence of heterotaxy syndrome, we recruited 93 heterotaxy patients and studied 12 of them by the Affymetrix Genome-Wide Human SNP 6.0 Array. The results were confirmed in the remaining 81 patients and 500 healthy children by quantitative real-time polymerase chain reaction (qPCR). The analysis of the SNP6.0 array showed a duplication of chromosome 2q21.1, which was verified by qPCR. The result of qPCR in the other 81 patients showed that 8/81 patients had the CNVs of 2q21.1 and the only overlapping gene in these patients is *CFC1*. However, in the 500 healthy children, only one carried the duplication of *CFC1* ($p = 3.5 \times 10^{-7}$). The duplication and deletion of *CFC1* may play key roles in the occurrence of heterotaxy syndrome. Moreover, the transposed great arteries, double outlet right ventricle, single atrium, and single ventricle may share a common genetic etiology with the heterotaxy syndrome.

Introduction

Heterotaxy syndrome is characterized by the malformation of organs along the left–right (L-R) axis, which results from failure to establish normal L-R asymmetry during embryonic development. The incidence of heterotaxy syndrome varies from 1/10,000 to 1.5/60,000 of live births (Gottschalk *et al.,* 2012). Approximately 90% of heterotaxy syndrome patients have a spectrum of complex congenital heart defects (CHD), including systemic or pulmonary venous return abnormalities, malposition of great arteries (MGA), and abnormal septation (Zhu *et al.,* 2006; Fakhro *et al.,* 2011; Ma *et al.,* 2012). Despite the development of surgical treatment, patients with heterotaxy syndrome still have relatively poor survival rates.

Recent researches have shown that heterotaxy syndrome is a clinically and genetically heterogeneous disorder, which may be associated with chromosome abnormalities such as single gene mutations, balanced translocations, microdeletions, and duplications (Ma *et al.,* 2012). The development of the L-R axis is controlled by a series of highly conserved cross-species genes, which is also present in the chick (Levin *et al.,* 1995), the xenopus (Hyatt *et al.,* 1996), the mouse (Nonaka *et al.,* 1998), and the zebrafish (Essner *et al.,* 2005; Matsui and Bessho, 2012). Several genes have been identified in patients with heterotaxy syndrome, including *CFC1*, *ZIC3*, *LEFTYA*, and *ACVR2B* (Gebbia *et al.,* 1997; Kosaki *et al.,* 1999; Bamford *et al.,* 2000; Ware *et al.,* 2004; Lenhart *et al.,* 2011; Haaning *et al.,* 2013). Mutations in *CRELD1* and *NKX2.5* also have been reported in heterotaxy syndrome patients, whereas they are not thought to play a direct role in L-R patterning or in the transfer of L-R axis information during organogenesis (Watanabe *et al.,* 2002; Robinson *et al.,* 2003; Zhian *et al.,* 2012).

Although many genes have been discovered to be associated with heterotaxy syndrome, mutations in these genes account for less than 10% of affected heterotaxy syndrome subjects (Zhu *et al.,* 2006). With the development of quantitative interrogation of dense set of single nucleotide polymorphism (SNP), the ability to detect small copy number variants (CNVs) has improved dramatically. By high-resolution genotyping of 262 heterotaxy syndrome subjects and 991 controls, Khalid A. Fakhro confirmed that five genes (*NEK2*, *ROCK2*, *TGFBR2*, *GALNT11*, and *NUP188*) were strongly associated with the L-R patterning (Fakhro *et al.,* 2011). Therefore, it is significant to research the roles of CNV in the occurrence of heterotaxy syndrome.

To study the probable pathogenesis of this disease, we analyzed the CNVs in 12 heterotaxy syndrome patients by the Affymetrix Genome-Wide Human SNP 6.0 Array. However,

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we did not find any causal CNV larger than 100 kb. Then, we evaluated CNVs of smaller sizes, trying to identify single genes with great effect on heterotaxy risk. Fortunately, we found a gene, *CFC1*, which had been reported in human heterotaxy syndrome. The CNV, including *CFC1*, was further studied in the other 81 patients by quantitative real-time polymerase chain reaction (qPCR). The results showed that eight patients carried the CNVs of 2q21.1, while no healthy child had this CNV. It is surprising to find that *CFC1* is the only overlapping gene in this region. Then, we investigated the copy number (CN) of *CFC1* in the 500 healthy children, and only one child was discovered to carry the duplication of *CFC1*.

Materials and Methods

Ethics statement

The ethics committee of Xinhua Hospital and Shanghai Children's Medical Center (SCMC) specifically approved of this study and written informed consents were obtained from the participants or their parents. The individuals in this article have provided written informed consent to publishing these case details.

Patient ascertainment

We initially identified 93 heterotaxy syndrome children from the electronic medical record. Diagnoses were confirmed by transthoracic echocardiography or other operative report. Extracardiac anomalies were also evaluated. None of them had a definite pathogenesis. Five hundred unrelated healthy Chinese children (of Han ethnic group, like the patients) were collected as normal controls. All of them were checked to exclude CHD by transthoracic echocardiography. Each patient was labeled with a unique identifier and the laboratory was blinded to all data, including cardiac diagnosis, sex, ethnicity, and year of birth. The patients were divided into two groups at random. The first group of 12 patients was studied by the Affymetrix Genome-Wide Human SNP 6.0 Array. The second group of 81 patients was used to verify the results of SNP 6.0 array and to identify the overlapping region of these CNVs by qPCR. Finally, the overlapping region was confirmed in the 500 healthy children. All samples were collected in Xinhua Hospital and SCMC from July 2011 to June 2013.

Affymetrix Genome-Wide Human SNP 6.0 Array

Peripheral blood samples were exsanguinated into an EDTA anticoagulant tube. DNA was extracted with the QIAamp DNA Blood Midi Kit (Qiagen) following the manufacturer's instructions. Purified genomic DNA was resuspended in ddH_2O for SNP-array analysis or in Tris-EDTA for other experiments, and DNA stocks were stored at $-80°C$. The SNP-array was performed on the patients' blood using the Affymetrix Genome-Wide Human SNP 6.0 Array, which has 906,600 SNP probes and 946,000 CNV probes across the entire genome according to the manufacturer's instruction. The samples that passed initial quality control were submitted for CNV discovery. High-confidence CNVs were detected by ADM-2 with the minimum marker 5 and threshold 6.0. The data were filtered and those regions larger than 100 kb comprising at least 50 contiguous markers were first analyzed. The CNVs of smaller sizes were evaluated when we did not find any causal CNV larger than 100 kb. Finally, the results were compared with known CNVs in the Database of Genomic Variants (DGV, http://dgvbeta.tcag.ca/dgv/app/home) and Online Mendelian Inheritance in Man (OMIM, www.ncbi.nlm.nih.gov/omim) to distinguish common CNVs from CNVs likely to be causal.

qPCR validation

Segments that have a strong association with heterotaxy were confirmed by qPCR. qPCR was performed according to the SYBR® Premix Ex Taq™ protocol (Applied TaKaRa). We used 50 ng/μL of genomic DNA in a 20 μL reaction, consisting of 10 μL of 2× SYBR Premix Ex Taq, 0.4 μL of 50× ROX Reference Dye II, 0.3 μL of Forward primer, 0.3 μL of Reverse primer, 8.0 μL of ddH_2O, and 1 μL of DNA. For each tested CNV, at least three primer pairs were designed within the boundaries of the CNV region (Table 1). Small portions of genomic DNA extracted from healthy people were mixed to form a DNA pool serving as the normal control, while the gene *GAPDH*, which has few variations, was used as the control gene in qPCR. Reactions were performed thrice. The cycle threshold (CT) values of qPCR were analyzed by comparative CT ($\Delta\Delta$CT) relative quantitation analysis. These genes, as well as pivotal genes nearby, were quantified to determine the CN. When results were different from SNP-array, the SNP-array results were not used. When the results were consistent, the segments were further validated in another 81 heterotaxy patients and 100 healthy children. The CNVs were considered to be common CNVs if detected in the normal controls. Otherwise, they were defined as causal CNVs. The overlapping region of these CNVs would be further identified in another 400 healthy children.

Mutation detection

Information about *CFC1* was searched from Build 37, which was released by the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). Exons 1–6

Table 1. Primer for Quantitative Real-Time Polymerase Chain Reaction

Gene	*Primer-F (5′–3′)*	*Primer-R (5′–3′)*
SMPD4	TTTCAGAGAGAGCCATCGGG	GTGGTGACTGGGCTGAGGA
PTPN18	CACTGGCTTTGTAGCCTGC	TAGCGGTTCTTCCTCACG
POTEI	GACCAAGGTGTCCATCATT	GCTTCCATTTCCATCCAG
CFC1	TCTTCTGTACTCGTCCATTCTGTG	CTTGTAGGTTTTTGTTAATTTTGCT
POTEJ	AGGGGTGTGTGATTTACGG	TTCGTGAGAATGAAGCCCT
GAPDH	TGCCTTCTTGCCTCTTGTCTCT	TTTCTTCCATTCTGTCTTCCACTC

of the *CFC1* gene were amplified by PCR, using the following four primers: exon 1–3F 5′-GATTGGTCACTCCTG CTGCT-3′ and exon 1–3R 5′- GCAGACTGAG ATGACG CCC-3′; exon 4F 5′-AGCAAACACACTTGGCTGGAG-3′ and exon 4R 5′-CAGACCCGCAGGTCCCTCAC-3′; exon 5F 5′-CCACCGCATTGATGCAGGTC-3′ and exon 5R 5′-GCACTGTGGATCGGTATGGAGG-3′; exon 6F 5′-TATT GCA CTCTCTCAAACCGAG-3′ and 6R 5′-TGTCCCTC TCCCAAGGATCTG-3′. PCR products were sequenced by the Sanger method on an ABI 3130 sequencer (Applied Biosystems). The sequence traces were aligned with the reference sequence in NCBI BLAST.

Results

Clinical data

We recruited 93 patients of heterotaxy syndrome, including 55 males and 38 females. The ratio of males to females was 1.4. All of them were sporadic and came from the Chinese Han ethnic group. All of them had the ectopic of heart, spleen, liver, or lungs and met the diagnostic criteria for heterotaxy syndrome. Most patients were accompanied with complex CHD; 16 patients had mesocardiac and 51 patients had dextrodiac. Outflow tract obstruction was discovered in 76 patients, atrioventricular septal defect in 54 patients, and single atrium (SA) or single ventricle (SV) in 49 patients. Twenty-seven patients had transposed or malposed great arteries (TGA/MGA). Anomalous systemic venous connections were present in 15 patients. Table 2 lists the cardiac and extracardiac manifestations of the patients in the series. Among the 16 patients with mesocardiac, 13 patients had TGA and only 2 patients had double outlet of right ventricle (DORV). Among the 51 patients with dextrodiac, 28 patients had SA/SV, 16 were accompanied with DORV, and only 5 had TGA.

Molecular and cytogenetic data

We did not find any causal CNV larger than 100 kb. Then, we evaluated the CNVs of smaller sizes. It is very difficult to correlate the phenotypes to specific genes in CNVs. Keeping it in mind, we first studied CNVs containing genes that had been previously reported in human heterotaxy or any model of L-R patterning. Only one CNV was confirmed. The duplication region of chromosome 2q21.1:130554259–131012743 encompassed the gene *CFC1*, which had been confirmed in heterotaxy syndrome (Fig. 1). Also, the results of the SNP array were verified by qPCR.

Table 2. Cardiac Phenotypes of Heterotaxy Patients

Cardiac and extracardiac manifestations	*Frequency of cases*
PS/PA	76/93 (81.72%)
ASD/VSD	54/93 (58.06%)
SA/SV	49/93 (52.69%)
DORV	27/93 (29.03%)
MGA/TGA	28/93 (30.11%)
Anomalous systemic venous connections	
IIVC	8/93 (8.60%)
BSVC	7/93 (7.93%)
Anomalous pulmonary venous connections	
TAPVR	3/93 (3.23%)
PAPVR	1/93 (1.08%)
Asplenia	7/93 (7.53%)
Polysplenia	3/93 (3.23%)

ASD, atrial septal defect; DORV, double outlet of right ventricle; IIVC, interrupted inferior vena cava; MGA, malposed great arteries; PA, pulmonary atresia; PAPVR, partial anomalous pulmonary venous return; PS, pulmonary stenosis; SA, single atrium; SV, single ventricle; TAPVR, total anomalous pulmonary venous return; TGA, transposed great arteries; VSD, ventricular septal defect.

CN determination

Further studies in the other 81 heterotaxy syndrome patients by qPCR showed that there were 4 duplication regions and 4 deletion regions overlapped with chromosome 2:130554259–131012743 (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/dna). In the 100 healthy children, we did not find the CNV of 2q21.1. We also analyzed the candidate genes in these nine patients and found that there was only one gene, *CFC1*, in common (Supplementary Figs. S2–S6). Then, we analyzed the CN of *CFC1* in another 400 healthy children, and only one child carried the duplication of *CFC1*, the CN is 3. The frequency of *CFC1* deletion ($p = 5.72 \times 10^{-4}$) and duplication ($p = 4.5 \times 10^{-4}$) in our patients was higher than that in the healthy population. Moreover, the result of sequencing in patients with the CNVs of 2q21.1 revealed no functional mutations in the coding sequences of *CFC1*.

Discussion

Heterotaxy syndrome is a disease characterized by the malformation of organs along the L-R axis, which causes significant morbidity and mortality. In 12 heterotaxy patients, only one CNV carried a gene reported to be associated with heterotaxy syndrome. The results of qPCR in the other 81 heterotaxy patients showed that there were 8 patients carrying the duplication or deletion of 2q21.1 and the only common gene was *CFC1*. As the mutation of *CFC1* had been reported in many heterotaxy patients, we thought that maybe the duplication or deletion of *CFC1* played essential roles in the pathogenesis of heterotaxy.

The function of CFC1 *mutation in heterotaxy patients*

Mutations of *CFC1*, a gene associated with heterotaxy syndrome, such as R78W, R112C, R189C, and G174del1, have been discovered in ~2.2% of heterotaxy syndrome patients with or without CHD (Ma *et al.,* 2012). Although the mutations of R78W and R189C may be benign, the pathogenicity of R112C and G174del1 had been identified in both zebrafish and mice (Yan *et al.,* 1999, 2002; Bamford *et al.,* 2000; Matsui and Bessho, 2012). These researches indicated that the correct establishment of L-R asymmetry in vertebrates required the evolutionarily conserved activity of the *CFC1*. To exclude the effect of *CFC1* mutations, we sequenced the coding sequences of *CFC1* in our patients with the CNV of 2q21.1 and no functional mutation was discovered.

The role of the duplication or deletion of CFC1 *in heterotaxy syndrome*

Not only the gene mutation but also the deletion and duplication of genes play important roles in the occurrence of diseases. Deletion of *CFC1* can lead to heterotaxy

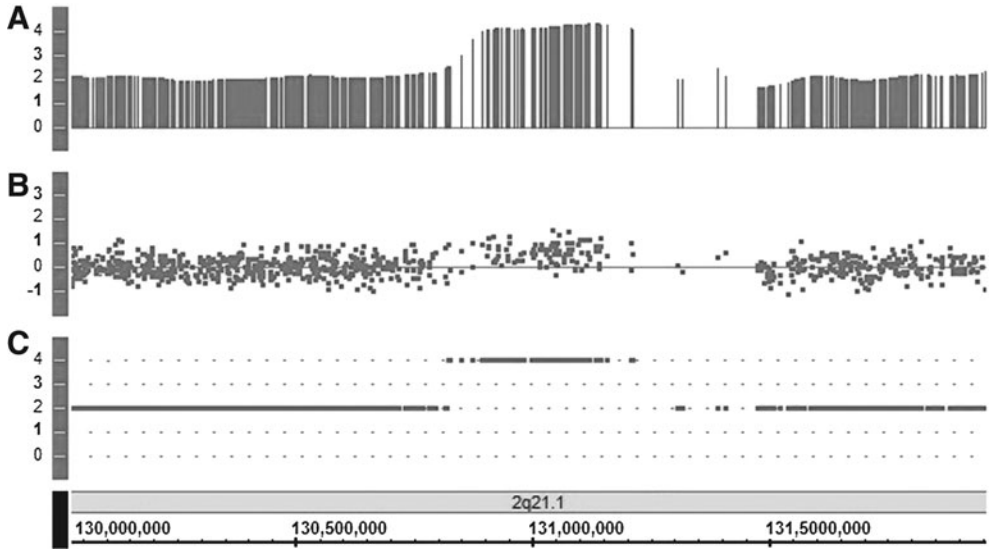

FIG. 1. Chromosomal view of the 58.4-Kb duplication at chromosome bands 2q21.1 (arr 2q21.1 [130,554,259–131,012,743]×4) (NCBI build 37). **(A)** The smooth signal of the duplication of 2q21.1. **(B)** The Log_2 ratio of the duplication of 2q21.1. **(C)** The copy number of the duplication of 2q21.1, the copy number is 4.

syndrome through reducing the translation of *CFC1* protein. The murine with target disrupted *CFC1* showed L-R laterality defects, including randomization of abdominal situs, randomized embryo turning, and cardiac looping and pulmonary right isomerism (Yan *et al.,* 1999).

However, reports about the duplication of *CFC1* are rare and only two articles have been published in medical literature. One report showed that overexpression of *Oep* RNA had no influence on the development of wild-type zebrafish (Zhang *et al.,* 1998). The other report showed that when gene overexpressed in Xenopus and Zebrafish, *Oep* elicited profound phenotypic effects, including axis disruption and anteriorization (Kiecker *et al.,* 2000). By comparing these two researches, we found that the injection dose of *Oep* RNA in the latter experiment was much higher than that in the former. Thus, we proposed that there might be a threshold value that the phenotypes of heterotaxy could be observed only when a certain amount of *CFC1* RNA was expressed.

In our study, 4/93 (4.3%) heterotaxy syndrome patients carry the deletion of *CFC1* and 5/93 (5.3%) carry the deletion of *CFC1*. Our study first discovered the deletion and duplication of *CFC1* in human beings. The high frequency of *CFC1* deletion and duplication ($p=3.5\times10^{-7}$) in our patients provides a new evidence for the idea that CNV of *CFC1* is responsible for the occurrence of heterotaxy syndrome.

TGA, DORV and SA/SV share a common genetic etiology with heterotaxy syndrome

We first analyzed the phenotypes between patients with *CFC1* duplication and with *CFC1* deletion. There is no significant difference between them (Table 3). However, neither asplenia nor polysplenia was found in patients with *CFC1* duplication or deletion. Then, we analyzed the clinical features of these 93 heterotaxy patients. It is noteworthy that patients with mesocardia are always accompanied with TGA, while patients with dextrodiac are usually accompanied with DORV or SA/SV. Previous studies have shown that some cases of TGA and DORV can share a common genetic etiology with heterotaxy syndrome in the pathogenetic group of laterality defects (Goldmuntz *et al.,* 2002; Unolt *et al.,* 2013). We propose that large vascular and atrioventricular structures may depend on the pattern of the L-R axis. The initial bending of the heart tube occurs in accordance with the L-R asymmetry formation at the beginning in the embryo when the heart tube experiences a leftward shift before looping (Srivastava *et al.,* 1997; Harvey, 1998, 2002; Wagner and Siddiqui, 2007). When the

Table 3. Clinical Feature of Patients with *CFC1* Duplications or Deletions

Patients	*CN*	*Gender*	*Age*	*Phenotypes*
3	4	Female	3 years	Dextrodiac SA
20	1	Male	6 months	Dextrodiac SA SV BSVC PS
23	3	Male	6 months	Dextrodiac SA SV BSVC PS
47	4	Male	4 years	Mesocardia TGA VSD ASD PS
52	3	Female	9 months	Mesocardia TGA VSD PS
53	1	Male	6 months	Mesocardia TGA VSD ASD PS
66	1	Male	1 year	Mesocardia TGA VSD PS
84	3	Female	2 years	Dextrodiac DORV PS
90	1	Female	1 years	Dextrodiac SV SA PA PDA

CN, copy number.

L-R axis is set up symmetrically, the heart is more likely to be located in the middle of the thoracic cavity and the cross of the aorta and pulmonary artery will be more likely to be established unsuccessfully. Ventricles, on the other hand, are oriented along the L-R axis by virtue of heart looping in the later development of heart (Unolt *et al.,* 2013). In *Lefty* mutant mice, *Pitx2* is expressed bilaterally in the LPM leading to left atrial isomerism and formation of DORV (Meno *et al.,* 1998). When the L-R asymmetry is formed incorrectly, it would more probably have dexiocardia accompanied with DORV or SV/SA.

Conclusion

In our study, we discovered the duplication and deletion of *CFC1* in 9/93 (9.67%) heterotaxy syndrome patients. To the best of our knowledge, we provide the first published evidence that the duplication and deletion of *CFC1* have a strong association with heterotaxy syndrome using clinical specimens. Moreover, the relationship between mesocardia and TGA, and dextrodiac and DORV or SA/SV also supports the idea that the TGA, DORV, and SA/SV may depend on the initial pattern of L-R asymmetry. However, we only analyzed the function of *CFC1* in heterotaxy syndrome. It is interesting to investigate whether CNVs of other known L-R axis-related genes such as *ZIC3*, *LEFTYA*, and *ACVR2B* are responsible for heterotaxy syndrome.

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Disclosure Statement

No competing financial interests exist.

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