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Mutations in ZIC3 and ACVR2B are a common cause of heterotaxy and associated cardiovascular anomalies

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Abstract

Background—Heterotaxy syndrome is caused by left-right asymmetry disturbances and is associated with abnormal lateralization of the abdominal and thoracic organs. The heart is frequently involved and the severity of the abnormality usually determines the outcome.

Methods—Direct sequence analysis of the coding sequence of genes including *Zinc Finger Protein of the Cerebellum 3, Left Right Determine Factor 2, Activin A Receptor Type IIB, and Cryptic* was performed in 47 subjects with laterality defects and congenital cardiac disease.

Results—31 (66%) of these subjects had atrioventricular septal defects, 34 (72%) had abnormal systemic venous return, 25 (53%) had transposed or malposed great arteries, and 20 (43%) had pulmonary venous abnormalities. Two novel genetic changes were identified in zinc finger protein of the cerebellum 3 and these variants were not presented in 100 ethnically matched control samples. One previously reported missense mutation in activin A receptor type IIB was identified in 2 unrelated subjects. The genetic changes identified in this study are all located in conserved regions and are predicted to affect protein function in left-right axis formation and cardiovascular development.

Conclusions—Mutations in *Zinc Finger Protein of the Cerebellum 3* and *Activin A Receptor Type IIB* were identified in 4/47 subjects with heterotaxy syndrome for a yield of approximately 8.5%. Our results expand the mutation spectrum of monogenic heterotaxy syndrome with associated cardiac anomalies and suggest that there are other causes of heterotaxy yet to be identified.

Keywords

left-right asymmetry; congenital heart defect; *LEFTYA*; *CFC1*

Introduction

The exterior body plan of vertebrates is essentially symmetric along its medial lateral axis while the formation of internal organs displays numerous left-right differences. Left right axis is the third body axis to form, and alteration of the normal pattern of asymmetry can manifest either in mirror-image reversal of all asymmetrical organs or randomized placement of organs.[1] The heart is the first organ to develop asymmetrically, and cardiac

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morphogenesis is sensitive to aberrations in left right positional information.[2] Heterotaxy syndrome is often associated with cardiac malformations which cause significant morbidity and mortality. In heterotaxy, the heart is normally patterned and formed in relation to anterior posterior and dorsal ventral axis, indicating that anterior posterior and dorsal ventral specification can act independently from left right specification. Patients often have malposition of the great arteries, systemic or pulmonary venous return abnormalities and abnormal septation such as atrioventricular septal defects.

As a clinically and genetically heterogeneous disorder, heterotaxy syndrome can be associated with chromosome abnormalities such as balanced translocations,[3,4] microdeletions or duplications,[5] single gene mutations, epigenetic factors, or environment teratogens.[6] Monogenetic causes of heterotaxy syndrome segregate as autosomal recessive,[7] autosomal dominant [8,9] and X-linked recessive disorders.[10] Highly conserved genetic pathways control determination of the left right axis[11,12] across species from xenopus,[13] zebrafish,[14] chick [15] to mouse.[16,17] It is suggested that the initial breaking of left right asymmetry occurs in the node, and the nodal flow generated by the monocilia in the node leads to establishment of morphogen gradient including *Nodal*, *Lefty*, and *Acvr2b* in either the left or right side of the body to pattern the internal organs. zinc finger protein of the cerebellum 3 (*ZIC3*) is a zinc finger transcription factor. Mutations of *ZIC3* cause X-linked heterotaxy (HTX1) and isolated congenital heart disease.[18–21] Z*ic3* knockout mice recapitulate human HTX1, and fifty percent of *Zic3* null embryos have randomized internal organs and abnormal cardiac looping.[22] Mouse homologues of left right determine factor 2 (*LEFTYA*) (homologue of mouse *Lefty-2)* and left right determine factor 2 (*LEFTYB)* (homologue of mouse *Lefty-1)* mediate Nodal signaling from the node to lateral plate mesoderm. A null mutation of *Lefty-2* in mice shows left isomerism.[23] Mutations of *LEFTYA* have been identified in heterotaxy patients as well.[24] Activin receptor IIB (*Acvr2b*) is expressed asymmetrically along left-right axis in mouse and chick. A null mutation of *Acvr2b* in mice results in situs ambiguous, atrial septal defect, ventricular septal defect and splenic hypoplasia.[25] Based on the murine phenotype, 112 sporadic and 14 familial cases of left right axis malformation were analyzed for *ACVR2B* mutations, and two missense mutations were identified leading to conclusion that *ACVR2B* mutations are rare causes of human left right axis malformation.[26] Cryptic (*CFC1*) belongs to the EGF (epidermal growth factor)-CFC1 family and encodes extracellular protein that plays a key role in intercellular signaling pathways. *CFC1* is expressed around the node and is later found in the intermediate and lateral plate mesoderm during gastrulation in mouse embryo.[27] Homozygous *Cryptic* knockout mice presented laterality defects and complex cardiac malformations. The phenotypes resemble those of mice lacking the type IIB activin receptor or the homeobox-containing factor Pitx2.[28] *CFC1* mutations and missense variants have been identified in patients with heterotaxy syndrome associated with congenital heart diseases.[29–32]

To expand the human genetic studies of heterotaxy syndrome with associated congenital heart disease, we screened 47 patients for mutations in *ZIC3*, *LEFTYA*, *ACVR2B* and *CFC1* to determine the frequency of mutations, mutation spectrum, and penetrance of the identified mutations, in order to provide data to guide development of future genetic testing for heterotaxy syndrome.

Patient and Methods

Clinical Materials

Subjects included fetuses and children diagnosed with heterotaxy syndrome. Subjects included fetuses and children diagnosed with heterotaxy syndrome defined as segmental discordances of the thoraco-abdominal organs along the left-right axis. All patients seen at

the Columbia University Medical Center were offered study entry. The study was approved by the Institutional Review Board at Columbia University Medical Center. Informed consent was obtained from parents or the patients, depending on the age of the patient. Among them, 46 patients had normal karyotypes while one patient had an extra X chromosome (47, XXX). Genomic DNA was extracted from blood, tissue, or amniocytes from each subject using Puregene reagents (Gentra Systems Inc., Minnesota, USA). Clinical data about the type of congenital heart disease and extra-cardiac phenotype was extracted from the medical record. Clinical cardiac information was reviewed by a single cardiologist (SST).

Genotyping Genetic Variations Using PCR Amplification

Primers were designed to amplify all coding regions and at least 20 bp of adjacent intronic sequence to capture the splice junctions for the four genes *ZIC3*, *LEFTYA, ACVR2B* and *CFC1.* Primer sequences are available upon request. In brief, 20ng of genomic DNA from each patient was amplified in a 20 μl volume containing 1xPCR buffer, 40ng of each oligonucleotide primers, 200uM dNTPs and 1.2 U Taq polymerase (Promega). PCR products were purified using ExoSAP-It kit (USB Scientific, OH) and sequenced by Sanger dideoxy sequencing on an ABI 3730xl genetic analyzer according to the manufacturer's instructions (ABI, CA) using one of the amplification primers. Genetic variants were confirmed on a second PCR reaction with bi-directional sequencing, and all available family members were sequenced. Sequences are analyzed with Sequencher software (Gene Codes, MI). Genetic variants were compared to all reported genetic variants in the literature, in dbSNP, at www.ncbi.nlm.nih.gov/snp and at www.genome.ucsc.edu. All novel genetic variants were independently confirmed using restriction digests. Confirmed novel genetic variants were then genotyped in 100 unrelated normal individuals without congenital heart disease of the same ethnicity.

Results

Clinical Data

The patients consisted of 23 males and 24 females. The ethnicity of the patients was 45% (21/47) Caucasian, 23% (11/47) Hispanic, 20% (9/47) African American, 6% (3/47) Asian and Southeast Asian, and 6% (3/47) other. Seven were fetal cases with prenatal terminations. 44 cases were sporadic and 3 cases were familial. Associated congenital heart defects included atrioventricular septal defect (AVSD) in 31/47 patients, systemic venous anomalies in 34/47 patients, 25/47 patients had transposed or malposed great arteries, outflow tract obstruction were presented in 23/47 patients and pulmonary venous anomalies in 20/47 patients (Supplemental Table 1). Table 1 lists all the cardiac and extracardiac manifestations of the patients in the series.

Mutation Screening

ZIC3—Sequencing of *ZIC3* demonstrated two novel genetic variants. Patient CHD32 was a male with a c.148delG resulting in a frameshift and putative truncated protein p.A50PfsX8 (Figure 1). CHD32 had right ventricle dominant AVSD, double outlet right ventricle, mitral atresia and total anomalous pulmonary venous return (Table 2). The hepatic veins connected directly into the right atrium. Extracardiac manifestations included malrotation, transverse liver and asplenia. The patient was adopted, and no family history or parental samples were available.

Analysis of *ZIC3* in CHD186 identified a heterozygous c.1204T>C transversion which results in substitution of p.S402P (Figure 2). The proband is a female patient with AVSD, pulmonary atresia and total anomalous pulmonary venous return. She had bilateral superior

vena cava with hepatic veins connect to the midline of right-sided atrium. She had abdominal malrotation. (Table 2).

The brother of CHD 186 (CHD218) also had the c.1204T>C transversion in *ZIC3* and presented with complex cardiac disease including double inlet and double outlet single left ventricle, pulmonary stenosis and a right-sided aortic arch. He had abdominal situs inversus and midline bony malformations including hypoplasia of the posterior elements of C1, rotational scoliosis of the upper cervical spine involving C2 and C3, fusion of multiple vertebral bodies including C2-C3 and two lower cervical vertebral bodies.

Analysis of the parents revealed that the mother is heterozygous for the c.1204T>C variant while the father is normal. The mother is clinically unaffected. Because this is X-linked recessive heterotaxy, we performed X chromosome inactivation assay and revealed that the mother had skewed X inactivation while there was random inactivation of X chromosome in the proband (data not shown). Further studies showed that the proband's maternal grandparents do not carry the c.1204T>C variant which indicates that the amino acid substitution p.S402P in *ZIC3* was de novo in proband's mother. (Figure. 2). The amino acid serine at position 402 lies within the fifth zinc finger domain and is highly conserved among human, mouse and zebrafish (Figure 3).

ACVR2B—Sequencing of *ACVR2B* identified two patients (CHD 141 and CHD 1067) with an identical heterozygous c.119G>A variant (p.R40H) (Figure 4). This variant has previously been reported to be a pathogenic mutation (Kosaki R et al., 1999). CHD141 had right ventricle dominant atrioventricular septal defect, pulmonary atresia, ipsilateral pulmonary venous return, inferior vena cava connecting to the base of common atrium, and bilateral superior vena cava (Table 2). CHD1067 was a fetus with atrioventricular septal defect, transposition of great arteries, interrupted inferior vena cava and a right sided stomach (Table 2). The unaffected mothers of both subjects carried the same heterozygous c. 119G>A (p.R40H).

Controls—The novel variants in *ZIC3* were genotyped in 100 ethnically matched normal controls, and were not identified in any of the normal subjects. The mutation R40H in *ACVR2B* was genotyped in 100 randomly selected individuals in the previous report and has not been identified in normal population (Kosaki R et al., 1999).

Sequencing of *CFC1* was previously performed on a portion of these subjects (25 patients) (Selamet Tierney ES et al., 2007) and completed in the remaining 22 subjects in this cohort. No novel mutations were identified. No mutations were identified in *LEFTYA.*

Discussion

Sequencing of coding regions of *ZIC3, LEFTYA, ACVR2B,* and *CFC1* in 47 heterotaxy patients with associated cardiovascular anomalies identified two novel genetic changes in two patients for *ZIC3* and a previously reported mutation in *ACVR2B* in two unrelated subjects for a total yield of 4/47 positive cases (8.5%). In the family with the S402P variant in *ZIC3*, S402P segregated with heterotaxy within the family and was associated with skewed X inactivation in the carrier mother. The mother's mutation was de novo. These data suggested that the S402P variant is pathogenic. All of the novel genetic variants we report are highly conserved across species, are located in functionally important domains of the proteins (Figure 3), and none of the variants were present in 100 randomly selected normal individuals. These data suggested that the two novel variants in *ZIC3* and the one previously reported R40H variant in *ACVR2B* are likely to be pathogenic mutations.

ZIC3 functions in early stages of embryonic development to regulate left right axis formation. *ZIC3* p.S402 is located in the α-Helical structure of zinc finger (ZF) 5 for DNA binding. The amino acid is highly conserved among species as well as among Zic/Gli/Glis zinc finger protein superfamily, [33] suggesting that this amino acid maybe important for the ZIC3-DNA complex formation. ZIC3 has been reported to interact with GLI3 through GLI consensus binding site (GLIBS) to regulate multiple aspects of neural and skeletal development.[34,35] It is speculated that S402P in *ZIC3* may account for the skeletal anomalies in CHD218. CHD186 and CHD218 had different cardiac phenotypes and extracardiac manifestations that could be due to the hemizygous state of the male compared to the heterozygous female with one copy of the wild type allele. CHD32 is a male patient who had a hemizygous c.148delG in *ZIC3*. This mutation is predicted to produce truncated protein p.A50PfsX8 without formation of Zinc finger domains. CHD32 had multiple complex cardiovascular abnormalities and cardiac transplant was performed at age 10. CHD32 also had extra cardiac manifestations. To date, eleven nonsense, frameshift and missense mutations in *ZIC3* gene have been reported among 241 sporadic and familial heterotaxy cases with 85% of mutations being maternally inherited, usually from unaffected mothers.[19] The ratio of clinically affected male to female patients with *ZIC3* mutations is 2.5:1 and may be explained by skewed X-inactivation.

The frequency of *LEFTYA* mutations in heterotaxy is low. Only one study identified two missense mutations among 126 patients to date.[24] In our current study, no novel mutations were identified in our heterotaxy patients.

One study identified two missense mutations: p.V419I and p.R40H in *ACVR2B* in 112 sporadic and 14 familial cases.[26] Our study identified the R40H mutation in 2/47 cases, both of whom inherited the mutation from an unaffected mother. R40 in *ACVR2B* is located in the region of extracellular domain within the region of ligand binding and is conserved across human, mouse, dog and elephant. [26] While the patients who carry R40H mutation in *ACVR2B* had distinct clinical presentations, all exhibited abnormal systemic venous return.

Single genes have been screened in cohorts of patients with heterotaxy, but our study is the most comprehensive genetic study that analyzed *ZIC3*, *LEFTYA*, *ACVR2B,* and *CFC1*, as a panel in all heterotaxy patients with cardiac manifestations. The total yield for all four genes was 8.5% (4/47). A comparison of the yield for each gene in previous and current studies is provided in Table 3. While this yield from these four genes is modest, it suggests that develop panels of genes in genetic study to evaluate convergent phenotypes in heart that occur in the context of inherited or sporadic diseases such as heterotaxy may be useful for diagnosis and evaluation. Our data also suggests that mutations in any one gene may account for a small percentage of cases and may be incompletely penetrant, making strategies of linkage analysis and association studies less powerful. Our study expands the mutation spectrum in heterotaxy and also suggests that additional genes are likely to be involved in the remaining 90% of cases for whom genetic changes were not identified, such as mutations in *NODAL*,[36–38] *NKX2.5* [39,40] and *CRELD1*.[41] Our study as well as the results from previous reports [42,43] suggest that genetic changes in more than one gene in the same pathway can converge to produce the same phenotype of heterotaxy with associated congenital heart disease. Patients without identified mutations will provide the substrate for identification of novel genes associated with heterotaxy using high throughput methods of genomic analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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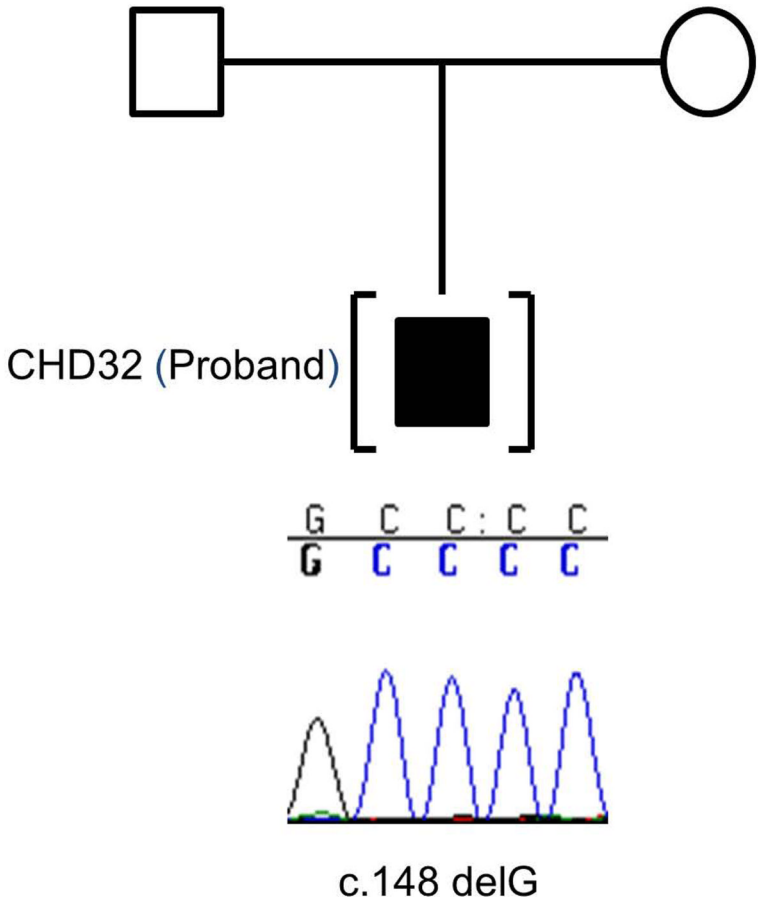


Figure 1.
Mutation of *ZIC3* exon 1 in CHD32. Sequence of *ZIC3* c.148delG in proband was shown. Brackets symbolize adoption.

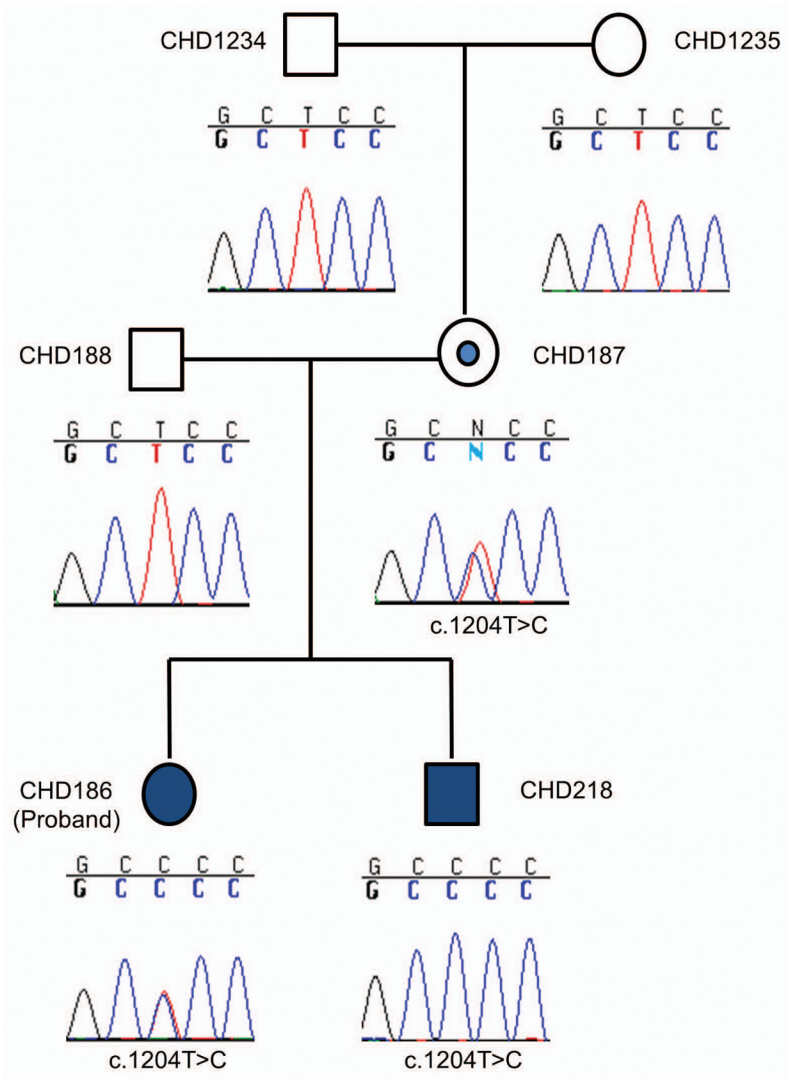


Figure 2.

ZIC3 c.1204T>C in CHD186 and the family. Pedigree indicating that the mother (CHD187) is a carrier and the two children (CHD186 and CHD218) are affected. Electropherograms below each family member show that CHD187 (unaffected mother) and CHD186 (proband, female) are heterozygous for c.1204T>C while CHD218 (affected male sibling) carries the hemizygous c.1204T>C transversion resulting in a p.S402P missense mutation. The father (CHD188) and maternal parents (CHD1234 and CHD1235) are normal. The point (CHD187) denotes carrier status.

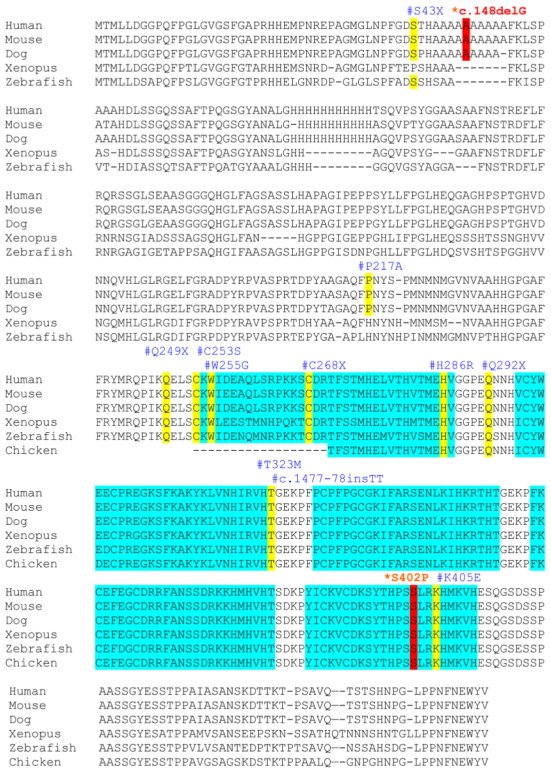
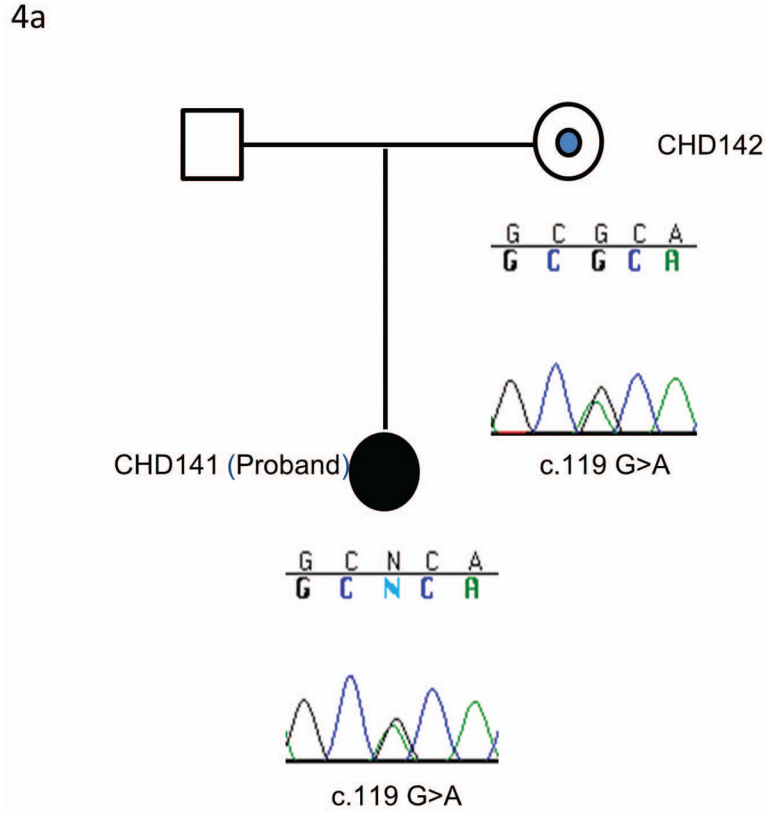


Figure 3.

Cross species comparison of *ZIC3* amino acid and locations of putative mutations. Solid rectangles in blue indicate the five C2H2 zinc finger domains. The amino acid positions for each mutation are boxed in red and types of mutations are labeled above each box. The c. 148delG mutation causes a frameshift and is predicted to form truncated protein with only 57 amino acids. A missense mutation S402P was detected in the fifth zinc finger domain. Previously reported mutations are boxed in yellow and the amino acid or nucleotide positions are labeled above.

4a



4b

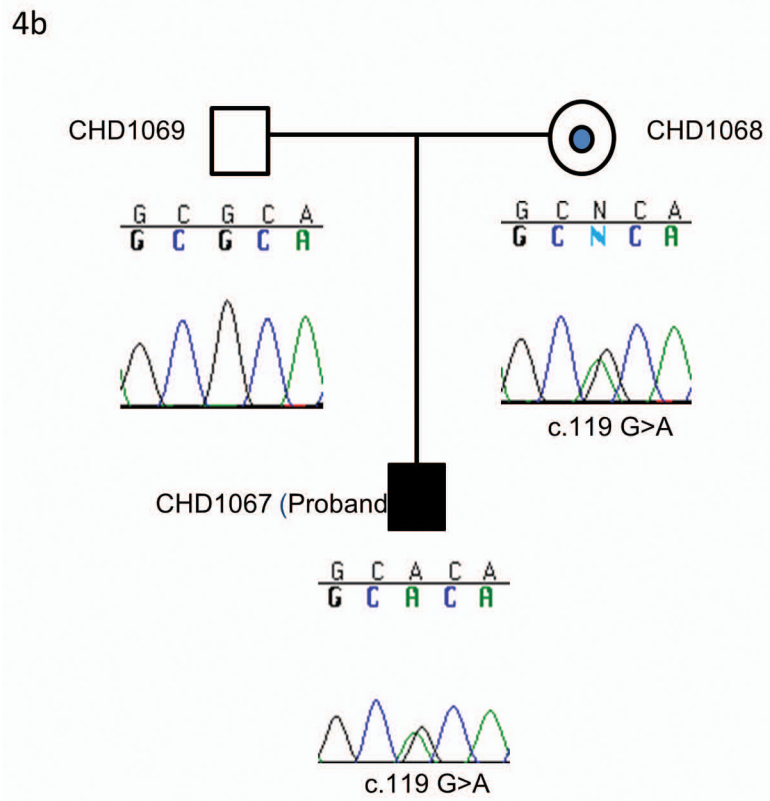


Figure 4.

An identical mutation of *ACVR2B* exon 2 in the two independent families. 4a) Pedigree and sequencing results of CHD 141 and the family. The same mutation was identified in an unrelated family and the pedigree and sequencing results were shown in 4b. In both families the unaffected mothers (CHD142 and CHD1068) are heterozygous for the c.119G>A transversion carried by the affected probands (CHD141 and CHD1067). The c.119G>A transversion results in a p.R40H missense mutation. The dot in the center of the pedigree symbol denotes carrier status.

Table 1

Cardiac Phenotypes of Heterotaxy Patients

Cardiac and Extracardiac Manifestatations	Number of Cases
Atrioventricular septal defect	31/47
Anomalous systemic venous connections	
Interrupted inferior vena cava	22/47
Bilateral superior vena cava	12/47
Malposed/transposed great arteries	25/47
Outflow tract obstruction	
Pulmonary stenosis/atresia	21/47
Aortic stenosis/atresia	2/47
Anomalous pulmonary venous connections	
Total anomalous pulmonary venous return	11/47
Ipsilateral or partial pulmonary venous return	9/47
Visceral organs	
Intestinal malrotation	16/47
Transverse liver	14/47
Right-sided stomach	11/47
Asplenia	10/47
Polysplenia	5/47

Table 2

Patients with *ZIC3* and *ACVR2B* Mutations

Patients	Gender	Ethnicity	Mutation	Cardiac Phenotype	Visceral Organs
CHD32	Male	Caucasian	*ZIC3* c.148delG	Right ventricle dominant atrioventricular septal defect, double outlet right ventricle, mitral atresia, total anomalous pulmonary venous return, hepatic veins drain directly into the right atrium	Malrotation, transverse liver and asplenia
CHD186	Female	Caucasian	*ZIC3* p.S402P	atrioventricular septal defect, pulmonary atresia, total anomalous pulmonary venous return, hepatic venous drainage to midline of right sided atrium, bilateral superior vena cava	Malrotation
CHD218	Male	Caucasian	*ZIC3* p.S402P	Double inlet double outlet single left ventricle, pulmonary stenosis, right sided aortic arch	Situs inversus
CHD141	Female	Hispanic	*ACVR2B* p.R40H	Common atrium, right ventricular dominant atrioventricular septal defect, pulmonary atresia, ipsilateral pulmonary venous return, inferior vena cava to base of common atrium, bilateral superior vena cava	Not known
CHD1067	Male	African American	*ACVR2B* p. R40H	atrioventricular septal defect, transposition of the great arteries, interrupted inferior vena cava	Right-sided stomach

Table 3

Comparison of Frequency of *ZIC3, LEFTYA*, *ACVR2B* and *CFC1* Mutations across Studies

Gene	Previous Studies	Frequency of Mutations in Previous Studies	Frequency of Mutations in Current Studies
ZIC3	69 heterotaxy with congenital heart disease	5.8–7.1%	4.7%
LEFTYA	126 heterotaxy	1.6%	0%
ACVR2B	112 sporadic and 14 familial heterotaxy	2.4%	4.7%
CFC1	257 heterotaxy with or without congenital heart disease	2.2%	0%