NPHP4 Variants Are Associated With Pleiotropic Heart Malformations

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NPHP4 Variants Are Associated With Pleiotropic Heart Malformations


**Rationale:** Congenital heart malformations are a major cause of morbidity and mortality, especially in young children. Failure to establish normal left-right (L-R) asymmetry often results in cardiovascular malformations and other laterality defects of visceral organs.

**Objective:** To identify genetic mutations causing cardiac laterality defects.

**Methods and Results:** We performed a genome-wide linkage analysis in patients with cardiac laterality defects from a consanguineous family. The patients had combinations of defects that included dextrocardia, transposition of great arteries, double-outlet right ventricle, atrioventricular septal defects, and caval vein abnormalities. Sequencing of positional candidate genes identified mutations in NPHP4. We performed mutation analysis of NPHP4 in 146 unrelated patients with similar cardiac laterality defects. Forty-one percent of these patients also had laterality defects of the abdominal organs. We identified 8 additional missense variants that were absent or very rare in control subjects. To study the role of nphp4 in establishing L-R asymmetry, we used antisense morpholinos to knockdown nphp4 expression in zebrafish. Depletion of nphp4 disrupted L-R patterning as well as cardiac and gut laterality. Cardiac laterality defects were partially rescued by human NPHP4 mRNA, whereas mutant NPHP4 containing genetic variants found in patients failed to rescue. We show that nphp4 is involved in the formation of motile cilia in Kupffer vesicle, which generate asymmetrical fluid flow necessary for normal L-R asymmetry.

**Conclusions:** NPHP4 mutations are associated with cardiac laterality defects and heterotaxy. In zebrafish, nphp4 is essential for the development and function of Kupffer vesicle cilia and is required for global L-R patterning. (Circ Res. 2012;110:00-00.)

**Key Words:** congenital heart malformations • heterotaxy • nphp4 • cilia • zebrafish

Laterality defects refer to a broad group of disorders caused by the disruption of normal left-right (L-R) asymmetry of the thoracic or abdominal visceral organs.\textsuperscript{1} Situs inversus totalis is the mirror image reversal of all visceral organs, whereas heterotaxy is the abnormal orientation of one or more organs along the L-R axis.\textsuperscript{2} In heterotaxy, congenital heart malformations result in major morbidity and mortality.\textsuperscript{3} Although heterotaxy most often occurs as a...
sporadic condition, familial clustering has been documented with pedigrees suggesting autosomal recessive, autosomal dominant and X-linked inheritance.4–7

L-R patterning of vertebrate embryos occurs before organ formation and is conducted by a conserved signaling cascade that includes asymmetrical expression of the NODAL, LEFTY, and PITX2 genes in left lateral plate mesoderm (LPM).8 Motile cilia are involved in establishing this L-R asymmetrical signaling. Laterality defects have been linked to ciliary motility by the observation that 48% of individuals with primary ciliary dyskinesia also had situs inversus totalis and 6% had heterotaxy.8

Animal models have assisted our understanding of L-R patterning and the role of cilia. The *inversus viscerum* (iv) mouse has a mutation in the ciliary *left-right dynein (lrd)* gene and often develops laterality defects.10 *Lrd* was found to be required for normal motility of monocilia on an embryonic structure called the node. These node cilia generate a leftward fluid flow that is necessary for normal asymmetrical Nodal-Lefty-Pitx2 signaling.11 In zebrafish, Kupffer vesicle (KV) is a ciliated organ analogous to the mouse node that is essential for normal L-R patterning.12 Asymmetrical fluid flow generated by the monocilia may move signaling factors11,13 and/or bend mechanosensory cilia14 to initiate asymmetrical signaling.

Dysfunction of ciliary proteins gives rise to a wide range of human disorders known as ciliopathies. They can lead to a variety of defects including craniofacial, skeletal, respiratory, reproductive, renal, visual, olfactory, and auditory abnormalities.15–17 The nephronophthisis (NPHP) and associated ciliopathies—Senior-Loken syndrome (SLSN), Joubert syndrome, and Meckel-Gruber syndrome—are characterized by cilia-related defects, including cystic kidney disease, retinal degeneration, liver fibrosis, and brain malformations.18,19 Mutations in 18 genes are known to cause nephronophthisis and associated ciliopathies.20,21 Interestingly, mutations in NPHP2/INVS and NPHP3 can also lead to heterotaxy, *situs inversus*, and isolated congenital heart malformations.22–24

Protein network analysis has shown that several of these proteins form an interaction network organized in at least 3 connected modules: NPHP1–4 to 8, NPHP5–6, and MKS.25 Ciliary localization analysis of eight nephrocystins (NPHP1–6, 9, and 10) indicates that they are present in the primary cilia, the basal body and/or the centrioles and suggest that they participate in ciliary assembly and trafficking.25–28

In this study, a genome-wide linkage analysis identified nephronophthisis-4 (NPHP4) variants in patients with cardiac laterality defects. Functional studies indicated that loss of zebrafish *nphp4* resulted in cardiac laterality defects. In addition, *nphp4* depletion disrupted asymmetrical nodal expression in the LPM, indicating *nphp4* is required for global L-R patterning of the embryo. Analysis of cilia in KV revealed that loss of *nphp4* reduced cilia length and disrupted asymmetrical fluid flow. Our results establish the importance of *nphp4* in cilia development and function. Furthermore, our findings suggest that malfunction of *NPHP4* contributes to a wide range of congenital heart malformations and more complex defects within the heterotaxy spectrum.

**Methods**

**Patients**

We studied a large consanguineous family of Iranian origin (Figure 1A) with complex consanguinity loops. This family was followed by us during many years. The healthy parents of branch 1 and 2 are double first-degree cousins; the mothers of these parents are sisters and the fathers are brothers. The healthy parents of branch 3 are first-degree cousins (their mothers are sisters) and related to the other 2 branches via the mothers (all mothers are sisters). In this family, 5 patients were born with congenital cardiac defects, of which 3 had cardiac laterality defects (IV-1, 4, 8, 12).

A total of 146 DNA samples from patient cohorts with heart (and other organs) laterality defects were collected. Patient samples were collected at the Erasmus Medical Center, Rotterdam, The Netherlands (15 samples), the Department of Clinical Genetics, University Hospital Leuven, Belgium (36 samples), and from the Baylor College of Medicine, Houston, TX (95 samples).

An expanded Methods section describing all procedures is available in the Online Data Supplement.

**Results**

**Clinical Studies**

We identified a consanguineous family including 5 patients with congenital heart malformations. Three patients (IV-1, IV-8, and IV-12; Figure 1A) were born with similar cardiac laterality defects (Table). Patient IV-1 had dextrocardia, atrial situs solitus, complete atrioventricular septal defect, and discordant ventriculoarterial connection with dextro-transposition of the great arteries (d-TGA). In addition, he had an interrupted inferior caval vein and a severe pulmonary valve stenosis (PS). He had no surgical correction and died suddenly at the age of 22 years. No autopsy was performed. Patient IV-8 had dextrocardia, dextrotorsation, and atrial situs solitus. She had an azygos continuation of the right infrahepatic part of the inferior caval vein draining into the right superior caval vein and the suprhepatic part of the inferior caval vein draining into the right atrium. She had a cor triatriatum with the right pulmonary veins draining into the
Figure 1. Genome-wide Linkage Analysis (GWLA) and NPHP4 variants identification. 

A, Simplified genealogical tree of the index family. A horizontal line above the symbol indicates medical examination. Open symbols indicate normal individuals, solid black symbols indicate patients with cardiac laterality defects, and quarter-filled symbols indicate presence of other congenital heart malformations. The double line between individuals indicates consanguinity and the diagonal line through a symbol is a deceased family member. Individuals labeled with an asterisk were included in the GWLA. For the genetic analysis, all individuals in whom medical examination was not possible were considered as “phenotype unknown.”

B, Multipoint LOD scores: X-axis represents all human autosomes and Y-axis corresponds to the LOD scores. Chromosomal regions with LOD scores above 2.5 (red horizontal line) were further investigated.

C, Summary of the NPHP4 variations identified in patients of the index family, the Dutch and the American cohorts. White and peach-colored boxes represent exons; yellow boxes represent untranslated regions. Red-boxed variants were previously described in SLSN4 or NPHP4 patients.

D, Alignment of NPHP4 protein with several species. The illustrated protein segments were derived from Ensembl reference sequences. Red letters indicate amino acid residues identical to those of human.

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Table. *NPHP4* Variants Found in Patients With Cardiovascular Malformations With or Without Laterality Defects of Other Thoracic or Abdominal Organs

<table>
<thead>
<tr>
<th>Family</th>
<th>Origin</th>
<th>Patient</th>
<th>Sex</th>
<th>Cardiovascular Abnormalities</th>
<th>Other Organs Asymmetry</th>
<th>Other Features</th>
<th>Coding Variant</th>
<th>Protein Effect</th>
<th>Doses</th>
<th>Prediction</th>
<th>Frequency in Control Subjects†§¶</th>
<th>Patient Sex</th>
<th>Cardiovascular Abnormalities</th>
<th>Other Organs Asymmetry</th>
<th>Other Features</th>
<th>Coding Variant</th>
<th>Protein Effect</th>
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<th>Prediction</th>
<th>Frequency in Control Subjects†§¶</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Iranian</td>
<td>IV-1</td>
<td>M</td>
<td>Dextrocardia, D-TGA, ASD, AVSD, PS, interrupted ICV</td>
<td>...</td>
<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>M</td>
<td>Single ventricle, MV atresia, VSD, ASD, subvalvular AS, PS, L-TGA, DORV, PDA</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
<td>p.Ala1110Val</td>
<td>HetNeutral</td>
<td>0.1% (1/6952)</td>
<td>0.1% (1/6887)</td>
</tr>
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<td>1</td>
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<td>IV-8</td>
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<td>Dextrocardia, ASD, VSD, interrupted ICV with persistent left SCV and ICV, cor triatriatum</td>
<td>Left lung isomerism</td>
<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>F</td>
<td>Dextrocardia, AVSD, PS, L-TGA, DORV</td>
<td>Right lung isomerism, abdominal situs inversus, large splenic cyst</td>
<td>...</td>
<td>c.3329C&gt;T</td>
<td>p.Ala1110Val</td>
<td>HetNeutral</td>
<td>0.2% (2/1232)</td>
<td>0.2% (2/1232)</td>
</tr>
<tr>
<td>2</td>
<td>Dutch, Chinese</td>
<td>2D3049</td>
<td>F</td>
<td>VSD, PA</td>
<td>...</td>
<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>F</td>
<td>VSD, PA</td>
<td>Right lung isomerism, abdominal situs inversus, large splenic cyst</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
<td>p.Ala1110Val</td>
<td>HetNeutral</td>
<td>0.2% (2/1232)</td>
</tr>
<tr>
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<td>Dutch, Cape Verde</td>
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<td>F</td>
<td>VSD, PA</td>
<td>...</td>
<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>F</td>
<td>VSD, PA</td>
<td>Right lung isomerism, abdominal situs inversus, large splenic cyst</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
<td>p.Ala1110Val</td>
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<td>Mesocardia</td>
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<td>...</td>
<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>M</td>
<td>Mesocardia</td>
<td>Abdominal situs inversus, midline liver and intestinal malformation</td>
<td>Cholelithiasis, azygous continuation to right SCV</td>
<td>...</td>
<td>...</td>
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<td>p.Ala1110Val</td>
</tr>
<tr>
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<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>F</td>
<td>Mesocardia, atrial isomerism, ventricular septal defect, PA, D-TGA, AVSD, PS</td>
<td>Abdominal situs inversus, midline liver</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
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<td>HetNeutral</td>
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<td>...</td>
<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>M</td>
<td>HLHS, AVSD, PS</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
<td>p.Ala1110Val</td>
<td>HetNeutral</td>
<td>0.2% (2/1232)</td>
<td>0.2% (2/1232)</td>
</tr>
<tr>
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<td>Common atrium, right atrial isomerism, single ventricle, AVSD, PS, AVSD, PA, supracardiac TAPVR</td>
<td>...</td>
<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>M</td>
<td>Common atrium, right atrial isomerism, single ventricle, AVSD, PS, AVSD, PA, supracardiac TAPVR</td>
<td>Abdominal situs inversus</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
<td>p.Ala1110Val</td>
<td>HetNeutral</td>
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<tr>
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<td>...</td>
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<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>M</td>
<td>Right atrial isomerism, single ventricle, AVSD, PS, AVSD, PA, supracardiac TAPVR</td>
<td>Abdominal situs inversus</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
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</tr>
<tr>
<td>9</td>
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<td>LAT0145</td>
<td>M</td>
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<td>...</td>
<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>M</td>
<td>HLHS, AVSD, D-TGA, DORV, PS</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
<td>p.Ala1110Val</td>
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<td>0.2% (2/1232)</td>
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</tr>
<tr>
<td>10</td>
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<td>LAT0343</td>
<td>M</td>
<td>Single ventricle, MV atresia, VSD, ASD, AVSD, PS</td>
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<td>...</td>
<td>c.3131C&gt;G</td>
<td>p.Arg1044His</td>
<td>Hom†</td>
<td>Neutral</td>
<td>0.2% (2/1232)</td>
<td>M</td>
<td>Single ventricle, MV atresia, VSD, ASD, AVSD, PS</td>
<td>...</td>
<td>...</td>
<td>c.3329C&gt;T</td>
<td>p.Ala1110Val</td>
<td>HetNeutral</td>
<td>0.2% (2/1232)</td>
<td>0.2% (2/1232)</td>
</tr>
</tbody>
</table>

ASD indicates atrial septal defect; VSD, ventricular septal defect; AVSD, atroventricular septal defect; MV, mitral valve; TGA, transposition of great arteries (dextro or levo); DORV, double-outlet right ventricle; PDA, persistent ductus arteriosus; BAV, bicuspid aortic valve; AS, aortic stenosis; PA, pulmonary atresia; PV, pulmonary valve; PS, pulmonary valve stenosis; HLHS, hypoplastic left heart syndrome; CoA, coarctation of the aorta; TAPVR, total anomalous pulmonary venous return; ICV, inferior caval vein; SCV, superior caval vein; Het, heterozygous; and Hom, homozygous.

All variants are absent or rare (<1%) in control populations.

*Prediction of the genetic variant effect on protein level (Pmut, SNPs3D, SIFT, PolyPhen, HOPE).
†Based on ethnically matched (in house) control chromosomes and the frequencies reported by the NHLBI Exome Sequencing Project.
‡Reported in patients with SLSN4.
§Inherited from father.
¶Inherited from mother.

**Total allele counts include European and African American population.
right part of the left atrium and the left pulmonary veins into the left part of the left atrium. A persistent left inferior and superior caval vein also drained into the left part of the left atrium. She had a secundum atrial septal defect (ASD) and perimembranous ventricular septal defect (VSD). She had also left bronchial isomerism. Patient IV-12 had atrial situs solitus, atrioventricular concordance, and ventriculoarterial discordance namely, double-outlet right ventricle and d-TGA. He also had a subpulmonary VSD, patent foramen ovale, and patent ductus arteriosus (PDA).

In addition, patient IV-7 died shortly after birth due to an unspecified congenital heart malformation (Figure 1A). The fifth patient (IV-16) had mild congenital heart malformations consisting of a small VSD, PS, and PDA, which was ligated at 1 year of age (Table).

Physical examination revealed no dysmorphisms and all patients had normal psychomotor development. CT/MRI or ultrasound of the abdomen revealed no kidney cysts and all individuals have reached adulthood at the time of their last examinations. No abdominal laterality defects such as asplenia or polysplenia, malrotation of the gut, or midline liver were detected in any of these cases. None of the patients had signs of abnormal mucociliary clearance. No visual problems or night blindness were reported.

**Genome-Wide Linkage Analysis**

The genome-wide linkage analysis was performed using Affymetrix SNP arrays. Two unaffected parents, 3 patients and 1 healthy sibling (Figure 1A) were included in the analysis. Multipoint linkage analysis revealed 5 regions on chromosomes 1, 2, 3, 9, and 11 with logarithm of the odds (LOD) scores above 2.5 (Figure 1B). The maxLOD scores (2.7) were located on chromosome 1p36 and 11p15. Subsequently, microsatellite markers mapping to all candidate regions were tested. The loci on chromosomes 1, 2, 3, and 9 were excluded, based on heterozygosity observed in the patients (data not shown).

On the chromosome 1p36 locus, all three patients with cardiac laterality defects (IV-1, IV-8, and IV-12) shared a homozygous region covered by 59 SNPs from rs4845835 to rs1203695. Haplotype analysis showed recombinations that delimited the borders of the region from rs2722782 (5.26 Mb) to rs1203696 (14.21 Mb) (Online Figure I, A). Thus, the candidate region spanned 9 Mb and contained 152 genes (NCBI build 37.1). These patients also showed homozygous genotypes for 49 consecutive SNPs on chromosome 11, from rs16905816 to rs10500752. Further fine mapping in the 11p region delineated the borders of the linkage region between markers D11S4188 (telomeric) and rs2896598 (centromeric) (Online Figure I, B). The chromosome 11 locus extended only 3 Mb (9.1–12.1 Mb), containing 34 genes (NCBI build 37.1).

Haplotypes from both loci were examined in all available family members. A normal person (IV-14, with normal MRI of the thorax/abdomen) had homozygous haplotypes on the chromosome 1 locus (Online Figure I, A). In addition, individuals IV-3 and IV-4 were homozygotes for the chromosome 11 locus (Online Figure I, B); both persons were reported as unaffected, but medical examinations could not be performed. Patient IV-16, exhibiting a mild cardiac phenotype and no laterality defects, carried heterozygous haplotypes at both loci (data not shown). Only the 3 patients with laterality heart defects had homozygous haplotypes on both loci. Since these were the only genomic regions where the 3 patients showed extended homozygosity, we further investigated these loci.

**Sequence Analysis**

A total of 109 genes on the chromosome 1p36 locus had a known reference sequence (NCBI build 37.1). Selection of genes for sequence analysis was based on available expression and/or functional information. The data were analyzed through the use of Ingenuity pathway analysis (Ingenuity Systems).

Thirty-six candidate genes were selected from the chromosome 1 locus. Direct sequencing of their coding regions identified 2 homozygous missense variants in the *NPHP4* gene present in three patients from the index family: c.3131G>A (p.Arg1044His) and c.3706G>A (p.Val1236Met) (Online Table I). These nonsynonymous variants are extremely rare in the Iranian (Kurdish) population (allele frequency 0.2% and 0.1% in 1232 control chromosomes). Moreover, the variants were absent in 270 Caucasian/Dutch and 178 Hispanic control chromosomes.

From the 34 genes mapping to the chromosome 11 locus, 19 genes had a well-annotated reference sequence. Sequence analysis of their coding regions and exon-intron boundaries revealed only 1 novel DNA missense variant (Online Table I). In the *AMPD3* gene (*adenosine monophosphate deaminase*), the homozygous c.2240 G>A (p.Arg747Gln) variant was found. This variant was not present in 626 control chromosomes. Mutations in the *AMPD3* gene lead to (asymptomatic) deficiency of erythrocyte AMP deaminase (OMIM 612874).

**NPHP4 Variants in Patients With Laterality Defects (Heterotaxy)**

We sequenced all 30 exons of *NPHP4* in 3 cohorts of patients with cardiac laterality defects—with or without other situs abnormalities. Patient samples were collected at the Erasmus Medical Center, Rotterdam, the Department of Clinical Genetics, Leuven, and from Baylor College of Medicine, Houston, TX. All 146 patients had a variety of cardiac laterality defects. Transposition of the great arteries was the most frequently found (49% of the patients). In addition, complete atrioventricular septal defect, double-outlet right ventricle, and abnormal pulmonary venous return were often reported. Dextrocardia was present in 33% of patients. Moreover, 41% had documented laterality defects of the abdominal organs, including abdominal situs inversus, asplenia or polysplenia, midline liver, and intestinal malrotation.

Nine missense variants were found in 10 patients (Figure 1C and 1D and Table). The population frequency of each allele was tested by sequencing ethnically matched control subjects. A variant was considered as likely nonpathogenic if the allele frequency in healthy individuals was higher than 1%. Thus, p.Pro1160Leu with a frequency of 2.1% in control chromosomes was excluded from further analysis. In addition, we investigated the frequency of these variants in available databases (dbSNP135, 1000Genomes, NHLBI exome project). All variants were very rare or absent in control subjects (allele frequency ≤0.8%; Table).
These rare NPHP4 variants were significantly more frequent in heterotaxy cases (6%, 9 of 146 cases) than in control subjects (1.2%, 3 of 250, Fisher exact test $P=0.006$). In silico evaluation was performed using five prediction computer programs. This assessment predicted the impact of amino acid substitutions on the structure and function of human proteins. The variants were classified as probably pathogenic if at least 3 programs considered them as damaging (Table). Seven variants satisfied this criterion. Interestingly, p.Phe91Leu, p.Arg961His, and p.Arg1192Trp have been reported in patients with SLSN type 4 or nephroptosis type 4.30

Identification of Zebrafish nphp4 and Characterization of Its Expression During Embryogenesis

A zebrafish nphp4 ortholog was taken from the Ensembl database (Online Figure II). To determine the pattern of nphp4 expression during embryogenesis, we performed reverse transcription PCR (RT-PCR) and RNA in situ hybridization experiments at several developmental stages. Consistent with a recent report31 we found nphp4 expression was maternally supplied and ubiquitously expressed during the first 24 hours of zebrafish development (Online Figure III, B through G). RT-PCR detected nphp4 expression at all stages tested between 4-cell stage and 100 hours postfertilization (hpf) (Online Figure III, A). This early and ubiquitous expression pattern suggested a role for nphp4 during early development.

nphp4 Is Required for Normal Cardiac Laterality in Zebrafish

To assess the function of nphp4 during embryonic development, we used antisense morpholino oligonucleotides (MO) to knockdown expression of zebrafish Nphp4 protein. Embryos injected with a MO designed to block nphp4 mRNA translation (nphp4 TB-MO) developed dose-dependent morphological abnormalities reminiscent of embryos with cilia defects,32–34 including a curved body axis (Online Figure IV, D) and otolith formation defects at 2 days postfertilization (dpf) (Online Figure V, A and B).

In addition, RNA in situ hybridization staining of the heart-specific marker cmlc2 revealed heart laterality defects. Uninjected controls and embryos injected with a standard control MO showed normal rightward looping of the heart at 2 dpf (Figure 2A and 2B). However, heart looping in nphp4 TB-MO injected embryos was significantly altered, as the heart often looped in the reverse orientation or failed to loop (Figure 2A and 2B).

To test whether heart laterality phenotypes were specific to knockdown of nphp4, we designed 2 additional MOs to interfere with nphp4 mRNA splicing at exon 4 (nphp4 SB-MO1) or exon 9 (nphp4 SB-MO2) (Online Figure IV, A and B). Quantitative RT-PCR analysis indicated nphp4 SB-MO1 reduced nphp4 mRNA levels by 90% (Online Figure IV, C) and caused heart laterality defects without inducing body axis defects (Figure 2A and 2B and Online Figure IV, D). This indicates heart L-R phenotypes are separable from axial defects. nphp4 SB-MO2 reduced the amount of normally spliced nphp4 mRNA by 50% (Online Figure IV, C) and resulted in curved body axis and heart looping defects (Online Figure IV, D, and Figure 2A and 2B, respectively), similar to nphp4 TB-MO injected embryos. Injecting a lower dose of nphp4 SB-MO2 (0.4 ng) also altered heart looping, but with reduced penetrance (Figure 2B), suggesting partial loss of nphp4 can cause cardiac laterality defects.

Other abnormalities such as hydrocephalus or gross eye defects were not observed. At 5 dpf, pronephric cysts were observed with a low penetrance in embryos injected with TB-MO (11%) or SB-MO2 (8%) (Online Figure V, C and D). No pronephric cysts were observed in SB-MO1–injected embryos. Our results using 3 independent MOs suggested a role for nphp4 that is required for normal heart laterality in zebrafish.

To further confirm that defects observed in zebrafish embryos were specifically due to nphp4 depletion, we conducted rescue experiments using human wild-type (wt) NPHP4 mRNA. Coinjecting nphp4 TB-MO with wt NPHP4 mRNA resulted in a partial, but significant, rescue of heart looping defects (percentage of normal embryos improved from 43% to 60%, $P=0.03$; Figure 2C). Next, we coinjected nphp4 TB-MO with human NPHP4 mRNA containing either the c.3131G>A (p.Arg1044His) or c.3706G>A (p.Val1236Met) missense variant identified in the index family. In contrast to wt NPHP4, these NPHP4 variants failed to rescue heart looping defects (Figure 2C). These results suggest that these variants are pathogenic and are involved in human laterality defects.

nphp4 Controls Global L-R Patterning of the Zebrafish Embryo

To determine whether nphp4 plays a role in heart laterality specifically or is involved in establishing global L-R patterning of the embryo, we analyzed additional markers of L-R asymmetry. RNA in situ hybridization, using foxa1 probes to label the embryonic gut, showed that nphp4 knockdown significantly altered laterality of the liver and pancreas in nphp4 MO injected embryos (Figure 3A and 3B). We next analyzed expression of the Nodal-related gene spaw (spaw), the earliest asymetrically expressed gene in LPM in zebrafish.35 Control embryos exhibited normal left-sided spaw expression (Figure 3C and 3D). In contrast, nphp4 MO injected embryos showed a significant disruption of spaw expression, which was often reversed, bilateral, or absent (Figure 3C and 3D). Altered asymmetrical gene expression can result from defects in the embryonic midline.36 However, analysis of the midline markers no tail and sonic hedgehog revealed that midline structures were intact in nphp4 MO–injected embryos (Online Figure VI). These results indicate nphp4 functions independent of midline development to control spaw expression and global L-R patterning of the embryo.

nphp4 Is Required for Normal Cilia Length and Directional Fluid Flow in KV

In zebrafish, KV is a transient organ that generates cilia-driven asymmetrical fluid flow necessary to bias spaw expression to the left LPM. Examination of live embryos at the 8 somite stage showed that the KV organ appeared normal in control MO (Figure 4A) and nphp4 MO–injected embryos.
However, analysis of cilia in KV by fluorescent immunostaining with acetylated Tubulin antibodies revealed that the cilia were significantly shorter in nphp4 MO–injected embryos (Figure 4E through 4G) as compared with controls (Figure 4D and 4G). We did not observe a significant difference of KV cilia number between control and nphp4 MO–injected embryos (Figure 4H). To analyze KV cilia function, we injected fluorescent beads into KV of live embryos and used video microscopy to record fluid flow.12 Most control embryos showed strong counterclockwise asymmetrical fluid flow (Figure 4I and Online Movie I). In contrast, flow was often absent (Figure 4J and 4L and Online Movie II) or reduced (Figure 4K and 4L and Online Movie III) in nphp4 MO–injected embryos. Consistent with dose-dependent effects of nphp4 SB-MO2 on heart looping (Figure 2B), we observed more severe flow defects in embryos injected with a higher nphp4 SB-MO2 dose (Figure 4L). Together, these results show that nphp4 knockdown results in short KV cilia and compromises asymmetrical fluid flow that is necessary for normal L-R patterning.

Discussion

We found homozygous missense NPHP4 variants in a consanguineous family containing 3 patients with cardiac laterality defects, bronchial isomerism, and normal abdominal situs. Interestingly, though NPHP4 is a cilia related gene that is mutated in patients with autosomal recessive juvenile nephronophthisis (NPHP type 4, OMIM 606966)37 and SLSN (SLSN4, OMIM 606996),38 our patients did not show signs of nephronophthisis or retinitis pigmentosa, which are distinctive features of these diseases.

Because of the known interaction between NPHP1, NPHP2/INVS, NPHP3, and NPHP4 proteins,23–24,37 it is obvious that mutations in one or more of these genes disrupt the same pathway(s) and can lead to similar phenotypes (ie, nephronophthisis). Conversely, mutations within the same gene can lead to various phenotypic outcomes in different patients. Mutations in NPHP2 result in nephronophthisis with or without situs inversus and mild cardiac defects,23 whereas NPHP3 mutations lead to isolated nephronophthisis or retinal
degeneration.39 Alternately, NPHP3 mutations can cause a broad clinical spectrum of early embryonic patterning defects comprising situs inversus, congenital heart defects, central nervous system malformations, and renal-hepatic-pancreatic dysplasia.24 The NPHP6 gene (CEP290) is another good example. The phenotypic spectrum of the mutations ranges from isolated blindness, SLSN, nephronophthisis, Joubert syndrome, Bardet-Biedl syndrome, to the lethal Meckel-Gru¨ber syndrome.40

We investigated the presence of NPHP4 variants in 146 sporadic patients having cardiac laterality defects, with or without involvement of other thoracic or abdominal organs. In 6% of the patients, we identified heterozygous missense variants compared with 1.2% of the ethnically matched control subjects, indicating mutation excess in the patients (P/H110030.006). No compound heterozygous or homozygous variants were detected in these sporadic cases. Similarly, single heterozygous NPHP4 variants were found in the majority of patients with autosomal recessive nephronophthisis type 4.30 A second mutation might be located in an area not covered by exon sequencing or in another (cilia-related) gene. The latest, a complex genetic model with combined effects of multiple genes, seems to be the most plausible explanation. In fact, di- or oligogenic inheritance have been demonstrated in several ciliopathies, including the nephronophthisis,21,41 Joubert syndrome,42 and Bardet-Biedl syndrome.43–44

The findings in our study are entirely consistent with a complex, oligogenic disease model. The rare heterozygous variants identified in the sporadic cases have probably an epistatic effect with additional genetic modifiers. Even in the index consanguineous family, we cannot exclude the existence of other genetic variants that explain the complex cardiovascular malformations and heterotaxy and the lack of renal/visual disease.

In congenital heart malformations and heterotaxy, the NODAL signaling pathway is a paradigm for oligogenic inheritance. Some patients with heterotaxy and/or conotruncal defects such as double-outlet right ventricle and TGA, show several mutations in genes belonging to the NODAL signaling pathway.55,46 As functional significance of mutations in these genes were demonstrated, the cumulative effects of multiple mutations may lead to reduced NODAL signaling eventually resulting in congenital heart malformations. In addition, a combinatorial role between the NODAL...
signaling pathway and ZIC3 gene has been demonstrated in familial TGA patients. These studies support the notion that genetic variants or susceptibility alleles within 1 or more developmental pathways may dysregulate signaling in a synergistic fashion and cause congenital heart malformations or heterotaxy.

Studies in humans, zebrafish, and mice indicate that NPHP2 and NPHP3 play a role in L-R axis determination. To investigate the role of NPHP4 in establishing L-R asymmetry, we used antisense MOs to knockdown expression of zebrafish nphp4. Depletion of nphp4 in zebrafish resulted in abnormal heart and gut orientation, closely resembling the (cardiac) laterality defects observed in the patients. Coinjection of nphp4 TB-MO and human wt NPHP4 mRNA significantly ameliorated the phenotypic spectrum due to nphp4 depletion. In contrast, coinjection of nphp4 TB-MO and human NPHP4 mRNA containing genetic variants found in patients failed to rescue the laterality defects suggesting that these variants are pathogenic. Furthermore, analysis of asymmetrical gene expression revealed that nphp4 knockdown...
alters asymmetrical Nodal expression in the LPM without affecting expression of midline markers.

Our analyses in zebrafish have confirmed that knockdown of nphp4 results in shortened motile cilia.48 For first time, we show that nphp4 depletion leads to disruption of cilia-driven fluid flow within KV, which probably causes laterality defects. Similarly, nphp3 knockdown in zebrafish leads to situs inversus and heterotaxy due to defective (fewer and shorter) KV cilia.49

In conclusion, we identified NPHP4 mutations in patients with cardiac laterality defects and other malformations within the heterotaxy spectrum. In zebrafish, our results demonstrate that nphp4 is required for global L-R patterning of the embryo via regulation of Nodal signaling and plays a role that is essential for the development and function of KV cilia.

The linking of NPHP4 to L-R axis determination and laterality defects will help dissect the complex genetic composition of heterotaxy and related cardiovascular malformations.

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Disclosures

None.

References

Novelty and Significance

What Is Known?

- Failure to establish normal left-right (L-R) organ orientation during embryonic development results in congenital heart defects and other malformations of thoracic and abdominal organs (laterality defects).
- Congenital heart malformations are genetically heterogeneous and complex disorders.
- The zebrafish provides a useful animal model to investigate genes involved in establishing L-R asymmetry.

What New Information Does This Article Contribute?

- NPHP4 genetic variants were identified for the first time in patients with cardiac malformations with or without laterality defects of other visceral organs.
- In zebrafish embryos, deletion of nphp4 expression leads to abnormal L-R organ patterning with abnormal heart looping and gut orientation.

- Nphp4 is involved in the formation of motile cilia in Kupffer’s vesicle, which generate directional fluid flow necessary for normal L-R axis determination.

This study describes the identification of NPHP4 variants in patients with congenital heart malformations using a genome wide linkage analysis and gene sequencing in a consanguineous family. Subsequent NPHP4 screening of sporadic cases with similar cardiac defects with or without malformations of other organs identified novel or rare variants in 6% of these patients. Deletion of nphp4 expression in zebrafish embryos leads to abnormal heart and gut orientation resembling the phenotype observed in the patients. For the first time, we show that nphp4 depletion leads to disruption of cilia-driven fluid flow, which probably causes laterality defects in zebrafish. Integration of the human genetics data and the functional work on zebrafish indicates that NPHP4 malformation contributes to a wide range of laterality defects, including complex heart malformations.
SUPPLEMENTAL MATERIAL

NPHP4 Variants are Associated with Pleiotropic Heart Malformations

METHODS

Ethics Statement
Patients and relatives from the index family gave and signed informed consent for participation in the research. DNA samples collection has been performed in accordance with the guidelines of the institutional review boards; the link to patient identifiers is retained by the host institution. Animal work (zebrafish) was conducted according to national (USA and The Netherlands) and international guidelines.

Patients and controls
We studied a large consanguineous family of Iranian origin (Figure 1a) with complex consanguinity loops. This family was personally followed by us during many years. The healthy parents of branch 1 and 2 are double first degree cousins; the mothers of these parents are sisters and the fathers are brothers. The healthy parents of branch 3 are first degree cousins (their mothers are sisters) and related to the other two branches via the mothers (all mothers are sisters). In this family five patients were born with congenital cardiac defects of which three had cardiac laterality defects (IV-1, IV-8, IV-12). All patients had extensive cardiologic examinations including electrocardiograms, echocardiograms and cardiac catheterizations. In addition, three patients (IV-1, IV-8, IV-12) with cardiac laterality defects had ultrasound of the abdomen, X-rays and/or MRI. Karyotyping was performed on peripheral blood lymphocytes from two parents and two patients with normal results (III-3, III-4, IV-8, IV-12). A fluorescent in situ hybridisation (FISH) of chromosome 22q11 was carried out on one patient (IV-8) which showed no deletion. The asymptomatic siblings and both parents from branch 2 had cardiologic examination (8 individuals), ultrasound of the abdomen and chest X-ray, all with normal results. Two of these siblings (IV-11, IV-14) also had MRI scans of the thorax and abdomen with normal results. A total of 146 DNA samples from patient cohorts with heart (and other organs) laterality defects were collected. These patients had extensive cardiological evaluation. Clinical evaluation included also X-rays of the thorax, ultrasounds, MRI and CT scans of the thorax and abdomen. Dextrocardia, mesocardia, isomerism, TGA, DORV, caval vein abnormalities, abnormal venous return among others were commonly observed. These malformations occurred in combination or not with other thoracic or abdominal laterality defects (e.g. situs inversus, bronchial isomerism, polysplenia, asplenia, tranverse liver). Patient samples were collected at the Erasmus Medical Center, Rotterdam, The Netherlands (15 samples), the Department of Clinical Genetics, University Hospital Leuven, Belgium (36 samples) and from the Baylor College of Medicine, Houston, USA (95 samples).

Control DNA samples from each of the patient populations were collected. The Iranian control samples consisted of 532 Kurdish individuals originating from the same province in Iran as the index family. Also, 90 DNA samples (controls with mixed ethnicity) from Iran were included. A total of 180 Dutch control samples were available. The US cohort consisted of 90 Caucasian and 89 Hispanic samples.

Genome-wide scan
The genome-wide search was conducted using DNA samples from six members of the index family including parents, three patients and one unaffected sibling (Figure 1a). Affymetrix GeneChip Mapping 50K HindIII Arrays containing 57,244 SNP markers were used. Samples were processed according to the manufacturer’s instructions (Affymetrix GeneChip Mapping Assay).

Linkage analysis and loci identification
For the genetic analysis, all individuals in whom medical examination was not possible were considered as “phenotype unknown”. Linkage analysis was performed with Allegro v1.2c (incorporated in the EasyLinkage Plus v5.08 package) \(^1\). LOD scores were obtained using a recessive
model of inheritance, with a penetrance of 90%. Map order and genetic inter-SNPs distances were taken from the Affymetrix website (Marshfield sex averaged genetic map) and co-dominant allele frequencies were used.

Since closely spaced SNP markers were used for the linkage analysis, the genome analyses were performed with predefined spacing of 0.1 to 0.4 cM. Single chromosomes showing positive linkage signals were independently analyzed under the same conditions and haplotypes were constructed. Genomic regions with LOD scores above 2.5 were considered as candidate intervals. To facilitate inspection and analyses, graphic visualizations were performed with HaploPainter v029.5.

Microsatellite markers mapping to the identified genomic regions were selected based on their information content. DNAs from all available individuals in the index family were genotyped. PCR products were run on an ABI Prism 3100 genetic sequencer (Applied Biosystems) and analyzed using the GeneMapper software v.3.0 (Applied Biosystems). Haplotypes were constructed based on the minimum number of recombination.

Sequencing analysis

Direct bidirectional sequencing of the entire coding region and the exon-intron boundaries of positional candidate genes was undertaken using PCR primers designed by Primer3 software.

Amplified PCR products were purified and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3100 and 3130xl genetic analyzers (Applied Biosystems). Sequences were aligned and compared with consensus sequences obtained from the human genome databases (NCBI, UCSC) using the Applied Biosystems software package SeqScape v2.5. Patient and control samples were sequenced using the same methodology.

Five computer programs (PMut, SHIFT, PolyPhen, SNP3Ds, and HOPE) were used to investigate the potential effect of the amino acid changes on the protein structure and/or function.

TaqMan assays

The NPHP4 c.3131G>A and c.3706G>A variants in exons 22 and 27 (p.Arg1044His and p.Val1236Met) were screened by an allelic discrimination method (Custom TaqMan SNP genotyping assay, Applied Biosystems). Heterozygote samples were confirmed by direct sequence analysis.

Zebrafish

Zebrafish (Danio rerio) embryos were collected and cultured as previously described.

Identification of zebrafish nphp4

A zebrafish nphp4 ortholog was assembled by combining two overlapping transcripts, ENSDART00000100063 and ENSDART00000111181. The first 10 exons of ENSDART00000100063 were sequenced using wildtype zebrafish cDNA and gDNA to corroborate the reference sequence spanning the morpholino oligonucleotides (MO) design regions. To assess whether zebrafish nphp4 has two transcripts we used quantitative real-time-PCR (qPCR). Primers were designed to target both transcripts and embryos were injected with splice-blocking SB-MO2, targeting the ENSDART00000100063 transcript only. Knockdown efficiency of both nphp4 transcripts was between 44-58% (data not shown). Therefore, we refer to the two database transcripts as a single nphp4 ortholog.

Morpholino Injections and rescue experiments

Antisense morpholino oligonucleotides (MO) were purchased from Gene Tools, LLC. To knockdown nphp4, we designed a MO to bind to the start codon and block translation: nphp4 TB-MO 5′-GCTTCCACACTCAGACATCGGT-3′ and two MOs to interfere with RNA splicing: nphp4 SB-MO1 5′-CGGTCAAGTGCATCCTACGTCA-3′ and nphp4 SB-MO2 5′-TGTGTGTTGCCATCATTACCTGCT-3′. All MO sequences were aligned with the Danio rerio genome using UCSC Blast and NCBI Blast to confirm specificity to the intended nphp4 genomic region. Different amounts of each MO were injected to determine the optimum dose. Subsequent experiments were carried out using 4.5 ng nphp4 TB-MO, 10ng SB-MO1 and 0.8 ng SB-MO2 unless noted otherwise. A standard control MO (5′-CCTTACACTCAGTCAATTATA-3) obtained from Gene Tools, LLC, was used for TB-MO and SB-MO2 experiments.
For MO rescue experiments, wild type human \textit{NPHP4} cDNA was cloned into pcDNA 3.1-V5-His vector (Invitrogen) and the point mutations c.3131G>A (p.Arg1044His) and c.3706G>A (p.Val1236Met) were generated by using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Single nucleotide changes were confirmed by sequencing. Wild-type and mutant mRNAs were synthesized with mMESSAGE mACHINE® T7 ULTRA kit (Ambion Inc). 100pg mRNA was co-injected with 4.5ng \textit{nphp4} TB-MO in 1-cell stage embryos. Heart looping and body curvature phenotypes were evaluated at 48 hour-post-fertilization.

**RNA Isolation and qPCR**

Pooled embryos were quickly frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated from 80 embryos, at 48-53hpf, using an RNA-Bee (Tri-Test, Inc.) protocol. To remove any remaining genomic DNA, the RNA was treated with RNase-free DNase for 30 minutes at 37°C. To synthesize cDNA, 5µg of RNA was reverse-transcribed with oligo-dT primers and Superscript III reverse transcriptase (Invitrogen, California, USA). To measure mRNA levels, qPCR on cDNA samples was carried out using the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Inc., MA, USA). Samples were analyzed on the Bio-Rad CFX96 qPCR detection system.

All the primers used for qPCR, including the reference gene \textit{SDHA}, were designed using Primer Express software (version 2.0.0). Primer pairs used to measure knockdown efficiency of \textit{nphp4} with the two splice-blocking morpholinos SB-MO1 and SB-MO2 were 5′-GACCCTCTGCGATGATGC-3′ (forward) and 5′-GAGCTGGATGTGGCTGTATGG-3′ (reverse); and 5′-GATGAGGGACGTGGATTTCAG-3′ (forward) and 5′-GCGCACACACTGCATAACG-3′ (reverse), respectively.

**In situ hybridization and Immunohistochemistry**

Two fragments (<900 bp) of zebrafish \textit{nphp4} cDNA were amplified by RT-PCR and ligated into the pCRII-TOPO vector (Invitrogen). Positive clones confirmed by DNA sequencing were used to generate antisense and sense probes to detect \textit{nphp4} mRNA. Whole-mount \textit{in situ} hybridization was carried out as previously described 4. A DIG RNA labeling kit (Roche) was used to generate digoxigenin-labeled riboprobes against \textit{nphp4}, cmlc2 5, foxa3 6, spaw 7, ntl 8 and shh 9. Stained embryos were mounted in 70% glycerol and imaged with either a Leica DFC300 FX digital colour camera mounted on a Leica MZ16 FA Fluorescence Stereomicroscope or a Nikon DS-Fi1 camera on a Nikon SMZ800 microscope. Images were edited using Photoshop (Adobe) software.

Whole-mount fluorescent immunostaining of cilia was performed using anti-acetylated Tubulin primary antibodies (Sigma) and goat anti-mouse Alexa 488 secondary antibodies (Molecular Probes), as previously described 10. Embryos were mounted in Slow Fade Reagent (Molecular Probes) and analyzed with a Zeiss Axioam HSm digital camera on a Zeiss AxioImager M1 AX10 microscope. Images were captured with Zeiss AxioVision software and assembled using ImageJ (NIH) and Photoshop (Adobe) software. Cilia length was measured using ImageJ software.

**Visualisation of fluid flow in Kupffer’s vesicle**

Live embryos were immobilized in 1% low melting point agarose at 6-10 somite stage (SS) and fluorescent beads (Polysciences, Inc.) were injected into Kupffer’s vesicle to analyze fluid flow as described 10. Beads were visualized using a 63X water dipping objective on a Zeiss AxioImager M1 AX10 microscope. Movies were captured with a Zeiss Axioam HSm digital camera and Zeiss Axiovision software and edited using Quicktime (Apple) software. Fluid flow in each embryo KV was classified as strong, reduced or absent by blind scoring of the movies.
Online Figure Ia. Haplotypes with SNP and microsatellite genotypes corresponding to chromosome 1p36 locus.

Summarized genealogical tree of the index family. Persons II-1 and II-3 are brothers; II-2 and II-4 are sisters. Open symbols indicate normal individuals, solid black symbols indicate patients with cardiac laterality defects and grey symbols indicate asymptomatic (clinically unexamined) individuals. Note that the patients share homozygous haplotypes extending from rs2722782 (5.26 Mb) to rs1203696 (14.21 Mb). The borders of the homozygous region are shown (boxed haplotypes) in person IV-8.
Person IV-14 (unaffected, normal clinical examination) has homozygous haplotypes overlapping the same region. Genotypes from generation II were inferred. Physical position (Mb) of each marker is shown according to NCBI build 37.1.

Online Figure Ib. Haplotypes with SNP and microsatellite genotypes corresponding to chromosome 11p15 locus.
Symbols are the same as described in Online Figure Ia. The patients show homozygous haplotypes (depicted in black) in a region delimited by markers D11S4188 (9.07 Mb, telomeric) and rs2896598 (12.05 Mb, centromeric). The borders of the homozygous region are shown (boxed haplotypes) in person IV-1. Note that individuals IV-3 and IV-4 (asymptomatic, clinically unexamined) share the same homozygous region. Genotypes from generation II were inferred. Physical position (Mb) of each marker is shown according to NCBI build 37.1.
Online Figure II. Comparison of human and zebrafish NPHP4 proteins.

ClustalW protein alignment of human NPHP4 (ENSP00000367398) with zebrafish (Danio) (ENSDARP00000090835 and ENSDARP00000101221 combined) orthologs. The number of amino acids is indicated at the end of each line. Amino acids indicated with a star (*) are identical among human and zebrafish. The amino acids marked in grey indicate the exon-exon boundaries.

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Online Figure III. Temporal and spatial nphp4 expression patterns in the zebrafish embryo.

(a) The expression of nphp4 mRNA at multiple developmental stages between the 4-cell stage and 100 hours post-fertilization (hpf). Amplification of β-actin was used as an internal control. Negative (-ve) controls lacked template. (b-g) in situ hybridization of nphp4 mRNA expression. Maternal nphp4 expression was detected at the 4-cell stage using an antisense nphp4 probe, whereas a sense probe showed reduced or absent staining (b). nphp4 expression was ubiquitous during epiboly stages (c, d) and somite stages (SS) (e-f) and at 24 hpf (g). Enriched staining was detected in the tailbud at 4 SS (arrow in f) where Kupffer’s vesicle develops and in anterior regions at 24 hpf (g). A second probe for the nphp4 gene showed identical results (data not shown).
Online Figure IV. Zebrafish nphp4 MO knockdown.

(a) Schematic of the zebrafish nphp4 gene showing the location of nphp4 SB-MO1 targeting the exon4/intron4 splice donor site and nphp4 SB-MO2 targeting the exon9/intron9 splice donor site. The pink bar represents the exons and exon/intron boundaries we confirmed by re-sequencing cDNA and gDNA from wildtype zebrafish. The mis-splicing outcomes represent splicing defects in nphp4 SB-MO1 or SB-MO2 injected embryos. (b) PCR was performed on cDNA from wild-type embryos and embryos injected with nphp4 SB-MO1 or SB-MO2. Injecting SB-MO1 or SB-MO2 resulted in the excision of exon 4 or exon 9, respectively. Mis-splicing was confirmed by direct sequencing. Negative (-ve) control lacked template. (c) Quantitative PCR analysis of nphp4 in 50 hpf wildtype and injected embryos confirmed gene knockdown in response to SB-MO1 and SB-MO2. All sample expressions were normalized to the control gene sdha. Relative expression was calculated by setting the wildtype expression level at 1. Error bars represent standard error of the mean. (d) Relative to control MO injected embryos, nphp4 TB-MO and SB-MO2 injected embryos showed a curved body axis phenotype at 48 hpf, whereas nphp4 SB-MO1 embryos had normal axial development.
Online Figure V. *nphp4* knockdown leads to otoliths defects and (low penetrance) pronephric cysts.

(a) Uninjected and control MO injected embryos show normal otoliths (arrows) while a proportion of *nphp4* TB-MO and *nphp4* SB-MO2 injected embryos displayed abnormal number (1 or 3) of otoliths.

(b) The distribution of number of otoliths observed in uninjected (n=147), control MO (n=130), *nphp4* TB-MO (n=88) and *nphp4* SB-MO2 (n=96) injected embryos. (c) Kidney cysts (arrows) were observed in a small proportion of the *nphp4* TB-MO and SB-MO2-injected embryos. No cysts were observed in the SB-MO1 injected embryos. (d) Graph shows the distribution of kidney cysts observed in uninjected (n=124), control MO (n=109), *nphp4* TB-MO (n=89) and *nphp4* SB-MO2 (n=87) embryos at 5 dpf.
Online Figure VI. *nphp4* MO knockdown does not disrupt embryonic midline development. (a, b) Whole-mount *in situ* hybridization analysis of *no tail* (*ntl*) expression in the notochord (a) and *sonic hedgehog* (*shh*) expression in the notochord and floorplate of the neural tube (b). Embryos were injected with control MO, *nphp4* TB-MO or *nphp4* SB-MO2.

**Online Table I.** All novel and rare genetic variants found in the chromosome 1 and chromosome 11 loci

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<th>Locus</th>
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<th>Controls</th>
<th>Type of variant</th>
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**Movie I.** Counter clockwise fluid flow in control Kupffer's vesicle.
**Movie II.** Injection of TB-MO stops Kupffer's vesicle fluid flow.
Movie III. Injection of SB-MO2 reduces Kupffer's vesicle fluid flow.

REFERENCES


10. Essner JJ, Amack JD, Nyholm MK, Harris EB, Yost HJ. Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart and gut. *Development*. 2005;132:1247-1260.