Cardiac Homeobox Gene NKX2-5 Mutations and Congenital Heart Disease

Associations With Atrial Septal Defect and Hypoplastic Left Heart Syndrome

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OBJECTIVES
We sought to examine the importance of mutations in the cardiac transcription factor gene NKX2-5 in patients with an atrial septal defect (ASD), patent foramen ovale (PFO), or hypoplastic left heart syndrome (HLHS).

BACKGROUND
Mutations in NKX2-5 have been found in families showing secundum ASD and atrioventricular (AV) conduction block and in some individuals with tetralogy of Fallot. The prevalence of NKX2-5 mutations in sporadic cases of ASD/PFO and other forms of congenital heart disease is unknown.

METHODS
A cohort of 146 individuals with secundum ASD, PFO complicated by paradoxical embolism, or HLHS were evaluated. Patients with ASD or PFO were ascertained irrespective of family history or associated cardiac abnormalities. The coding region of the NKX2-5 locus was amplified by polymerase chain reaction and sequenced.

RESULTS
Among 102 ASD and 25 PFO patients screened, 13 patients (10%) had a positive family history and 5 patients (4%) had AV conduction block. We found one previously documented NKX2-5 missense mutation, T178M, in members of a family with ASD without AV conduction block. One NKX2-5 mutation-positive child from this family had HLHS, although no mutations were subsequently found in 18 patients with sporadic or familial HLHS. In a second ASD family without AV conduction block, we found a missense change, E21Q, previously reported as pathogenic. Because this change did not segregate with disease status, we propose that it is a non-disease-causing polymorphism.

CONCLUSIONS
Our findings suggest that NKX2-5 mutations are a relatively infrequent cause of sporadic ASD and HLHS. Screening for NKX2-5 mutations may be warranted in individuals with ASD and a positive family history, irrespective of the presence or absence of AV conduction block. (J Am Coll Cardiol 2003;41:2072–6) © 2003 by the American College of Cardiology Foundation

The secundum atrial septal defect (ASD) accounts for ~10% of congenital cardiac malformations (1). Large ASDs can lead to cardiac failure in childhood, but the morbidity associated with untreated ASD is largely due to the development of pulmonary hypertension with right heart dilation and atrial arrhythmia in later life. In adults, percutaneous closure techniques are being increasingly used as an alternative to surgery to prevent ASD complications.

The ASD is causally heterogeneous. Population-based studies suggest that ASD is multifactorial in origin, but with a strong genetic component, and estimates of heritability are in the order of 0.6 to 0.7, similar to figures for congenital heart disease (CHD) in general (1). It can occur in mono- or polygenic familial conditions, sometimes in association with other cardiac malformations, or as one feature of syndromes, although most cases appear sporadic. The ASD has also been associated with in utero exposure to teratogens such as alcohol.

Recently, mutations in the cardiac homeobox transcription factor gene NKX2-5 (or CSX1) have been found in families with inherited autosomal-dominant ASD and atrioventricular (AV) conduction block (2–4). Eleven disease-associated mutations have been documented to date. Other congenital heart abnormalities have been observed at low penetrance in these families, including the ventricular septal defect, Ebstein’s anomaly, tetralogy of Fallot (TOF), subvalvular aortic stenosis, and tricuspid valve abnormality (2,3). A survey of patients with nonsyndromic TOF also identified NKX2-5 mutations in ~4% of individuals, including three new mutations (5). In mice, heterozygous loss-of-function NKX2-5 mutations lead to a mild conduc-
tion delay and atrial septal dysmorphogenesis manifest as an increased frequency of patent foramen ovale (PFO), atrial septal aneurysm, and ASD (6). The higher penetrance of ASD and conduction disease in humans, as compared with mice, may reflect genetic background (6) or the dominant-negative nature of human mutations (7).

It is currently unknown to what extent dominant or modifying NKX2-5 mutations contribute to sporadic or familial CHD. Here, we examined whether NKX2-5 mutations play an important role in the causation of ASD and PFO in individuals unselected for a family history or AV conduction abnormalities. Accordingly, we sequenced the coding region of NKX2-5 in a cohort of 102 ASD and 25 PFO patients ascertained only by their requirement for septal repair. We also evaluated patients with sporadic or familial hypoplastic left heart syndrome (HLHS) after discovering an individual with HLHS within one NKX2-5 mutant family.

**METHODS**

**Patient recruitment.** The study population comprised consecutive individuals with secundum ASD or PFO who underwent percutaneous closure at St. Vincent’s Hospital (from 2000) and individuals identified retrospectively from hospital records (St. Vincent’s and Sydney Children’s Hospitals) in whom ASD closure had been performed (1982 to 1999). After identification of HLHS in one individual in family 1024, a group of 18 HLHS individuals identified prospectively through the Children’s Hospital-San Diego and U.S. HLHS support groups was included. These HLHS patients were karyotypically normal. Sequencing of the *connexin-43* gene, mutated in a subgroup of HLHS patients (8), was not undertaken. Subjects were evaluated clinically by taking a medical history and performing 12-lead electrocardiography and transthoracic or transesophageal echocardiography. A verbal family history was available for all but 15 individuals. Informed, written consent was obtained from all participants. The study protocol was approved by institutional research ethics committees.

**Molecular methods.** The *NKX2-5* locus was amplified from 50 to 100 ng of blood genomic deoxyribonucleic acid (DNA) using Expand Polymerase (according to the manufacturer’s instructions, Roche Applied Sciences, Basel, Switzerland) and cycling conditions: 95°C, 2 min; 30 cycles of 94°C, 30 s; 68°C, 2 min. The polymerase chain reaction (PCR) products were sequenced using the BigDye nucleotide mix (according to the manufacturer’s instructions, Applied Biosystems, California), ABI 3700 sequencer, and Lasergene DNASTAR (Wisconsin) software.

**RESULTS**

**Clinical details.** The *NKX2-5* gene was sequenced in 102 ASD patients ascertained only because of their need for ASD closure. Thirty-two patients were children (age range birth to 14 years; mean 3.9 years) and 70 were adults (age range 18 to 80 years; mean 44.7 years). Within the cohort, four individuals (3.9%) also had first-degree AV conduction block and 3 (2.9%) had some other form of CHD (Table 1). Thirteen patients (10%) had a positive family history of ASD, although no family members had evidence of AV conduction block. Twenty-five PFO patients were also assessed, because our previous studies in mice suggested that ASD and PFO exist within a pathologic continuum (6). The PFO cohort was drawn from patients with suspected paradoxical embolism in whom PFO was subsequently found. Thus, there is a likely selection bias toward more severe PFO in this group (9).

**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th></th>
<th>ASD (n = 102)</th>
<th>PFO (n = 25)</th>
<th>HLHS (n = 19)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>37/65</td>
<td>12/13</td>
<td>11/8</td>
</tr>
<tr>
<td>Mean age in yrs (range)†</td>
<td>31.9 (birth to 80)</td>
<td>48.7 (12–73)</td>
<td>0.9 (birth to 4)</td>
</tr>
<tr>
<td>Positive family history‡</td>
<td>12</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>AV conduction block†</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Other cardiac conditions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anomalous pulmonary venous drainage</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tricuspid annular dilation</td>
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<tr>
<td>Ebstein’s anomaly</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Double-outlet right ventricle</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Complete heterotaxy</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>NKX2-5 mutation</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*Includes one unaffected individual who was an obligate carrier of a familial mutation. †At the time blood was drawn. ‡First- or second-degree relative with congenital heart disease; §§both members of family 1024.

ASD = atrial septal defect; AV = atrioventricular; HLHS = hypoplastic left heart syndrome; PFO = patent foramen ovale.
Molecular findings. Within the ASD/PFO cohort, only one ASD patient with an NKX2-5 mutation was identified. This individual had a normal electrocardiogram and a positive family history (family1024; Fig. 1A). The proband's father (individual I-2) had an ASD complicated by episodes of atrial and ventricular fibrillation, although it is unclear whether these were primary or induced, because they occurred postoperatively after surgical treatment for constrictive pericarditis and mitral valve ring implantation. He had no evidence of AV conduction block. Individual II-1 had ASD in association with HLHS. Sequencing of NKX2-5 revealed a heterozygous C-T transition at nucleotide 642 (GenBank AB021133), resulting in a T178M amino acid change (Figs. 1A and 1C). This mutation introduces a diagnostic NlaIII restriction enzyme cleavage site, which was used to screen PCR-derived NKX2-5 DNA of other family members (Fig. 1A). Cleavage was evident in all affected members, including the individual with HLHS, as well as in individual II-4, who was phenotypically normal. As far as we are aware, this is the first documented case of an NKX2-5 mutation correlating with HLHS. Hypoplastic left heart syndrome is genetically heterogeneous and can be associated with distal deletions of chromosome 11 as part of Jacobsen syndrome (10), as well as in a subset of sporadic cases with mutations in the connexin–43 gene (8). We examined the NKX2-5 sequence from 18 additional patients with sporadic or familial HLHS who had a normal karyotype, but no mutations were found. The T178M change identified in this study was previously found in two ASD/conduction disease families (3,5) and occurs within the NKX2-5 homeodomain, thus severely diminishing its ability to bind DNA (7).

The other 12 individuals in our ASD/PFO cohort with a positive family history did not carry NKX2-5 mutations. This is consistent with findings that familial ASD is genetically heterogeneous (11). The NKX2-5 family described here differs from other mutant families reported in the literature, all showing a combination of ASD/conduction disease families (3,5) and occurs within the NKX2-5 homeodomain, thus severely diminishing its ability to bind DNA (7).

A second NKX2-5 amino acid change (E21Q) was identified within a family with ASD but no conduction disease (family AF1) (Fig. 1). However, evaluation of other family members showed that that mutation did not segregate with disease. This change has previously been reported as pathogenic in one individual with TOF, although two
unaffected family members were also carriers (5). These findings suggest that the amino acid change represents a non-disease-causing polymorphism.

DISCUSSION

The T178M mutation and CHD. We found a single individual within our cohort of 127 ASD/PFO patients who carried a mutation in NKX2-5. This individual had a family history of ASD without AV conduction block. The T178M amino acid change in family 1024 is clearly pathogenic. The change diminishes the ability of NKX2-5 to bind DNA (7) and has been found in two other families showing highly penetrant ASD and high-grade AV conduction disease (3). The presence of an NKX2-5 mutation in a member of family 1024 with HLHS raised the possibility that the mutation may also cause this disorder. However, no mutations were subsequently found in 18 individuals with HLHS and a normal karyotype. Hypoplastic left heart syndrome is believed to result from decreased flow to the left side of the heart during ontogeny (10), and this could occur as a consequence of haplo-insufficiency for NKX2-5 through the effects on the conduction system (3,12) or left ventricular development (13). Analysis of larger populations will be required to confirm the suggested role for NKX2-5 mutations in HLHS.

The penetrance of CHD in T178M families is high but incomplete, as evidenced by the existence in family 1024 of a child who was genotype-positive but phenotype-negative, as assessed by transthoracic echocardiography with Doppler...
analysis. However, a small ASD or one that had closed spontaneously earlier in life cannot be excluded. Furthermore, because the AV conduction block found in other Nkx2-5 mutant families is progressive, it is possible that individuals from family 1024 are at risk of developing late-onset conduction disease.

Implications for genetic causation in CHD. The principal conclusion of this study is that exonic Nkx2-5 mutations are a relatively infrequent cause of sporadic secundum ASD. The single mutation detected in our ASD cohort occurred in an individual with a family history of ASD; none were found in 90 sporadic cases. Previous work has associated Nkx2-5 mutations with familial ASD and AV conduction disease at a high penetrance, but also with a spectrum of other abnormalities in families at a lower penetrance. Hypoplastic left heart syndrome might now be included in the growing list of defects attributable to Nkx2-5 mutation. Our studies also show that Nkx2-5 mutations can occur in families in the absence of conduction disease. These observations support a critical role for Nkx2-5 in diverse cardiac developmental processes.

The status of the E21Q change in family AF1 is unclear. Although this polymorphism alters a conserved amino acid within a known functional domain of Nkx2-5 (TN domain) (Fig. 1C), it leads to a conservative substitution (albeit one with an altered charge) and inserts glutamine into an already glutamine-rich microenvironment. The change has also been found in one TOF patient and multiple unaffected members of the same family (5). In family AF1, there was one ASD patient who carried the change, but at least two members with ASD who did not, clearly indicating that this change was not the disease-causing mutation. We postulate that this polymorphism is, in itself, unlikely to cause ASD, conduction disease, or TOF, although a possible contribution to polygenic CHD cannot be excluded.

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REFERENCES