NKX2.5 Mutations in Patients With Tetralogy of Fallot
Elizabeth Goldmuntz, MD; Elizabeth Geiger, MS; D. Woodrow Benson, MD, PhD

Background—Recent reports have implicated mutations in the transcription factor NKX2.5 as a cause of tetralogy of Fallot (TOF). To estimate the frequency of NKX2.5 mutations in TOF patients and to further investigate the genotype-phenotype correlation of NKX2.5 mutations, we genotyped 114 TOF patients.

Methods and Results—Patients were recruited prospectively (November 1992 through June 1999) and tested for a 22q11 deletion; those with 22q11 deletion or recognized chromosomal alteration were excluded from the present study. Patients were screened for NKX2.5 alterations by conformation-sensitive gel electrophoresis and sequencing of fragments with aberrant mobility. Four heterozygous mutations were identified in 6 unrelated patients with cases of TOF, including 3 with pulmonary atresia and 5 with right aortic arch; none had ECG evidence of PR interval prolongation. Three of 4 mutations (Glu21Gln, Arg216Cys, and Ala219Val) altered highly conserved amino acids, of which 2 mapped in the conserved NK2 domain. The fourth mutation (Arg25Cys) was identified in 3 unrelated probands in the present study and has been previously reported. No homeodomain mutations were identified.

Conclusions—NKX2.5 mutations are the first gene defects identified in nonsyndromic TOF patients. NKX2.5 mutation is present in ≥4% of TOF patients. Mutations identified in the present study mapped outside of the homeodomain, were not associated with atrioventricular conduction disturbances, and were not fully penetrant, in contrast to mutations previously reported that impair homeodomain function. (Circulation. 2001;104:2565-2568.)

Key Words: genetics ■ heart defects, congenital ■ tetralogy of Fallot

Tetralogy of Fallot (TOF) is the most common type of cyanotic congenital heart disease; it is estimated to occur in 3.3 per 10,000 live births and accounts for 6.8% of all congenital heart disease.1 Approximately 15% of patients with TOF have a deletion of chromosome 22q11,2–6 and nearly 7% of TOF patients have trisomy 21 (Down syndrome).7 TOF patients with Alagille syndrome have mutations in JAG1.8,9 Recent reports have implicated mutations in the transcription factor NKX2.5/CSX as a nonsyndromic cause of TOF.10,11 To estimate the frequency of NKX2.5 mutations in TOF patients and to further investigate the genotype-phenotype correlation of NKX2.5 mutations, we genotyped 114 TOF patients.

Methods

Patient Recruitment
Patients were recruited prospectively from November 1992 through June 1999 by the Clinical Core of the Special Center of Research on the Genetic Basis of Conotruncal Malformations at The Children’s Hospital of Philadelphia into a protocol approved by the Institutional Review Board. Any patient with TOF, regardless of sex or race, was approached to participate in the protocol. All subjects were tested for 22q11 deletion as previously described.5 Some patients underwent other cytogenetic testing if such was clinically indicated. Patients with a 22q11 deletion, trisomy 21, or other chromosomal anomalies were excluded from the present study. After informed consent was obtained from each patient, whole blood was collected, a lymphoblastoid cell line established, and DNA extracted by use of standard techniques. Complete family medical histories were obtained by a genetic counselor. Parental blood samples were obtained when possible. Previously reported subjects were not included.11

Patient Characterization
A total of 114 subjects were studied (58% male, 42% female). The cohort was racially diverse and included patients from the following categories: whites (71%), blacks (11%), Asians (3%), Hispanics (2%), and other/unknown (13%).

Diagnosis of TOF
All subjects were clinically evaluated for TOF at The Children’s Hospital of Philadelphia. Diagnosis of TOF was confirmed for study by reviewing ≥1 of the following reports: echocardiography (80%), cardiac catheterization (70%), and operative note (77%); all subjects had ≥1 ECG. MR imaging and autopsy each were performed on 1 subject. Original echocardiograms or cine films were reviewed to resolve lack of specificity or ambiguity in the reports. In addition to an anterior malalignment-type ventricular septal defect, 4 patients had concomitant conal septal hypoplasia and 4 had ≥1 additional muscular ventricular septal defect. In addition, 4 patients had a secundum atrial septal defect. Pulmonary valve anatomy reflected previously reported trends, with possible overrepresentation of the absent-valve syndrome, and included: stenosis/hypoplasia (69%), atresia (24%), and absent-valve syndrome (7%). Aortopulmonary collaterals were reported in 17% of the patients. Aortic arch anatomy was specifically defined for 97% of the patient cohort: 32% (36 of 114)
111) of the cohort had right aortic arch, of which 86% (31 of 36) had mirror-image branching pattern with left-sided innominate artery and 8% (3 of 36) had aberrant left subclavian artery. Of those with left-sided aortic arch, 3% (2 of 74) had aberrant right subclavian artery and 4% (3 of 74) had a common brachiocephalic trunk.

Polymerase Chain Reaction Amplification of NKX2.5 Coding Region
The coding region, including exon/intron boundaries, was amplified from genomic DNA by 4 polymerase chain reactions (PCRs, Table 1). All reactions started with 2 minutes at 95°C followed by 35 cycles of 45 seconds at 95°C, 30 seconds at 60°C or 61°C, and 45 seconds at 72°C and finished with a 10-minute extension period at 72°C. DMSO (0.2 mL/20-µL reaction) was added to standard reagents for reaction 2A. Enhancer solution (PCR enhancer system, Gibco BRL) was added to standard reagents at 1X concentration for reaction 2B.

Conformation-Sensitive Gel Electrophoresis
PCR products were screened for sequence alterations by conformation-sensitive gel electrophoresis (CSGE), a modification of heteroduplex analysis.12 PCR products were heated for 5 minutes at 98°C and incubated for 30 minutes at 68°C. PCR reaction mixture (2 µL) was mixed with loading dye and loaded onto a 0.8-mm-thick 10% polyacrylamide gel. Gel was run at 400 V for 22 hours at room temperature, stained in ethidium bromide, inspected, and photographed.

Sequencing
If an aberrant band was detected by CSGE, the corresponding PCR reaction was repeated and the product isolated (Qiaquick PCR purification kit, Qiagen) for direct sequencing. Bidirectional sequencing was performed by an automated cycle sequencer (ABI Prism 373 or 377 sequencer, ABI BigDye terminator cycle-sequencing kit). Sequence alterations were examined in context of the open reading frame to determine whether the alteration would change the corresponding amino acid. When available and applicable, parental samples were sequenced with the appropriate primer pair as listed in Table 1 and sequence data were compared with those of their offspring.

CSGE False-Negative Rate
To investigate the false-negative rate of CSGE, we sequenced directly the coding region, including intron/exon boundaries, in 14 subjects with no apparent alterations by CSGE. Sequence data identified 1 sequence alteration that was not initially detected by CSGE. In retrospect, the variant caused a subtle change in the CSGE gel that initially had not been appreciated. Direct sequencing also identified a previously described, common polymorphism (A63G) in 7 of 14 patients. This polymorphism is particularly difficult to detect by CSGE because the base-pair change results in slight thickening of the corresponding band rather than a distinct shift on the gel. In retrospect, those subjects heterozygous for the polymorphism demonstrated subtle thickening that had not been appreciated initially, whereas those homozygous for the polymorphism could not be distinguished from those without the polymorphism.

Screening Control Population for Sequence Alterations
A total of 100 normal chromosomes from a random control population were sequenced completely for the NKX2.5 open reading frame from genomic DNA.11 A total of 43 black control subjects were screened for the Arg25Cys mutation by restriction-enzyme digest analysis. The mutation introduces a HhaI site in the first exon. The first exon was PCR amplified and digested with HhaI (Promega) by use of manufacturer’s buffer C and by following manufacturer’s directions. PCR and restriction-enzyme digest fragments were resolved on 1.5% agarose gel and stained with ethidium bromide for visualization.

Results
NKX2.5 Mutations in Patients With TOF
Four heterozygous sequence variants predicted to alter the encoded protein were identified in 6 unrelated cases of TOF. Three of 4 (Glu21Gln, Arg216Cys, and Ala219Val) variants resulted in substitution of highly conserved amino acids, 2 of which occurred in the NK2 domain (Figure 1). The fourth alteration (Arg25Cys) was identified in 3 unrelated probands in the present study and has been reported previously.11

Characterization of First-Degree Relatives
Family history was available for all 6 patients with a sequence variant (patients 122, 229, 324, 328, 393, and 518). The father of patient 393 had a history of a ventricular septal defect (details unavailable). First-degree relatives of the other 5 patients were reported to be clinically normal. Blood samples from family members were available for 3 of 6 patients (Figure 2). Patient 393 and his affected father both had the Arg25Cys alteration. In the remaining 2 families, Glu21Gln and Ala219Val variants were carried by some family members. Thus, in the 3 families in which other members were available for genotyping, sequence alterations were identified.

![Figure 1](https://example.com/Figure1.png)

Figure 1. Location of new and previously reported NKX2.5 mutations. Mutations identified in the present article are noted above diagram of the gene. Number of patients with each mutation is noted in parentheses. Short arrows below diagram note mutations reported previously. Mutations reported in patients with TOF are depicted as black arrows. Mutations reported in other patients are depicted as arrows above asterisks. UTR indicates untranslated region; TN, conserved domain; HD, homeodomain; and NK, NK-2 box conserved domain. Hatched boxes represent the coding region outside of conserved domains. Broken line represents the single intron.
in 7 subjects (3 probands, 4 family members); among these 7 individuals, 4 were reported to have a cardiac defect. These findings are consistent with decreased penetrance.

**Evidence That Sequence Alterations Are Mutations**

Sequence alterations identified in the TOF cohort were not present in 100 normal chromosomes from a random control population completely sequenced for the NKX2.5 open reading frame from genomic DNA. The 3 unique variants (Glu21Gln, Arg216Cys, and Ala219Val) also change highly conserved amino acids. Given that these 3 variants change highly conserved amino acids and were not identified in normal controls, they were considered to be significant missense mutations.

The Arg25Cys variant has been previously reported and was previously reported and demonstrated that 3 probands had TOF with pulmonary stenosis and 3 probands had TOF with pulmonary atresia (Table 2). Two of 3 patients with pulmonary atresia had aortopulmonary collaterals. Although the sample size is too small to reach statistical significance, the proportion of patients with pulmonary atresia in the subgroup with NKX2.5 mutation exceeds the reported frequency of pulmonary atresia in the general TOF population and the present study cohort (50% versus 20% and 24%, respectively). Pulmonary valve anatomy did not correlate with mutation site; however, as noted previously, the number of mutation-positive patients is small.

Right aortic arch was diagnosed in 5 of 6 patients. Frequency of right aortic arch in the NKX2.5 mutation-positive subgroup appeared to be higher than reported frequency of right aortic arch in the general TOF population and the present study cohort (83% versus 25% and 32%, respectively), although subgroup frequency did not reach statistical significance. All mutation-positive patients had normal or mirror-image branching patterns. All mutation-positive patients had right bundle-branch block after cardiac surgery, but none had PR prolongation either before or after operation on review of surface ECG. Noncardiac malformations were not reported in the medical records or study pedigrees of any mutation-positive patient.

**TABLE 2. Clinical Characteristics of Mutation-Positive Patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>PV Anatomy</th>
<th>APC, Yes/No</th>
<th>Arch Side, Right/Left</th>
<th>Arch Branching</th>
<th>AV Block, Yes/No</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>229</td>
<td>G61C</td>
<td>Stenosis</td>
<td>No</td>
<td>Right</td>
<td>Mirror image</td>
<td>No</td>
<td>Retroaortic innominate vein</td>
</tr>
<tr>
<td>122</td>
<td>C73T</td>
<td>Stenosis</td>
<td>No</td>
<td>Left</td>
<td>Normal</td>
<td>No</td>
<td>PFO or secundum ASD</td>
</tr>
<tr>
<td>324</td>
<td>C73T</td>
<td>Atresia</td>
<td>Yes</td>
<td>Right</td>
<td>Mirror image</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>393</td>
<td>C73T</td>
<td>Atresia</td>
<td>Yes</td>
<td>Right</td>
<td>Mirror image</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>518</td>
<td>C646T</td>
<td>Stenosis</td>
<td>No</td>
<td>Right</td>
<td>Unknown</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>328</td>
<td>C656T</td>
<td>Atresia</td>
<td>No</td>
<td>Right</td>
<td>Mirror image</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

PV indicates pulmonary valve; APC, aortopulmonary collaterals; AV, atrioventricular; PFO, patent foramen ovale; and ASD, atrial septal defect.
gene for nonsyndromic cases of TOF. The small number of mutation-positive patients in this report does not permit detailed genotype/phenotype analyses, but pulmonary atresia or right-sided aortic arch was found more frequently in the mutation-positive subgroup than in the general TOF population.

Mutations identified in the present study did not disrupt the homeodomain, were not associated with atrioventricular conduction disturbances, and were not fully penetrant, as evidenced by the mutation-positive relatives with no apparent phenotype. In contrast, previously reported mutations predicted to impair homeodomain function were associated with atrioventricular conduction disturbances, pleiotropic cardiovascular malformations, and full penetrance. These findings suggest a genotype/phenotype correlation and may help to elucidate functional regions of the encoded protein.

Kasahara and colleagues have evaluated nuclear localization, DNA binding and transcriptional activation, and dimerization of mutant human NKKX2.5 in vitro. Homeodomain mutations demonstrated severely reduced DNA binding and minimal to absent transcriptional activation. In contrast, protein with an intact homeodomain that carried the Arg25Cys mutation had normal transcriptional activity and normal DNA binding to a monomeric site but 3-fold to 9-fold reduction in DNA binding to dimeric sites. Additional studies are required to identify the mechanism by which the novel mutations identified in the present study affect transcription factor function and thereby affect cardiac development.

The present study and other recent investigations increasingly illustrate the importance of single-gene defects in the origin of congenital heart disease. The variable expressivity associated with heritable monogenic mutations suggests an influence of environmental or other genetic factors. However, identification of specific genetic causes for congenital cardiac malformations will allow for improved family counseling and provide insight into the developmental mechanisms that result in normal and abnormal cardiac development.

Acknowledgments
The present study was supported by NIH/NHLBI grant HL-51533, The Ethel Brown Foerdener Fund for Excellence, The University of Pennsylvania Research Foundation (Dr Goldmuntz), and NIH/NHLBI grant P50-HL-61006 (Dr Benson).

References
NKX2.5 Mutations in Patients With Tetralogy of Fallot
Elizabeth Goldmuntz, Elizabeth Geiger and D. Woodrow Benson

Circulation. 2001;104:2565-2568
doi: 10.1161/hc4601.098427
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/104/21/2565

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/