Combined Mutation Screening of NKX2-5, GATA4, and TBX5 in Congenital Heart Disease: Multiple Heterozygosity and Novel Mutations

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ABSTRACT

Background. Variants of several genes encoding transcription modulators, signal transduction, and structural proteins are known to cause Mendelian congenital heart disease (CHD). NKX2-5 and GATA4 were the first CHD-causing genes identified by linkage analysis in large affected families. Mutations of TBX5 cause Holt–Oram syndrome, which includes CHD as a clinical feature. All three genes have a well-established role in cardiac development.

Design. In order to investigate the possible role of multiple mutations in CHD, a combined mutation screening was performed in NKX2-5, GATA4, and TBX5 in the same patient cohort. Samples from a cohort of 331 CHD patients were analyzed by polymerase chain reaction, double high-performance liquid chromatography and sequencing in order to identify changes in the NKX2-5, GATA4, and TBX5 genes.

Results. Two cases of multiple heterozygosity of putative disease-causing mutations were identified. One patient was found with a novel L122P NKX2-5 mutation in combination with the private A1443D mutation of MYH6. A patient heterozygote for a D425N GATA4 mutation carries also a private mutation of the MYH6 gene (V700M).

Conclusions. In addition to reporting two novel mutations of NKX2-5 in CHD, we describe families where multiple individual mutations seem to have an additive effect over the pathogenesis of CHD. Our findings highlight the usefulness of multiple gene mutational analysis of large CHD cohorts.

Key Words. Congenital Heart Disease; Mutations; Multiple Heterozygosity

Introduction

Congenital heart disease (CHD) is a complex trait, as both environmental and genetic factors have been implicated in its pathogenesis. The etiology of CHD has a strong genetic component, as shown by extensive epidemiological studies in large series of consecutive births.1,2 In approximately one in four cases, CHD occurs associated with other congenital anomalies within a single-gene disorder (e.g., Holt–Oram syndrome), sporadic malformative complex (e.g., VACTERL association), or as a consequence of a chromosomal abnormality (e.g., trisomy 21).3 Cases of isolated CHD appear mainly as sporadic events. However, a small fraction present as familial cases, often showing Mendelian segregation with widely variable penetrance.4 The first genes involved in Mendelian isolated CHD, NKX2-5 and GATA4, which encode transcription factors, were identified...
by genetic linkage studies in large affected families.5,6

The mammalian NKX2-5 gene was discovered during a screening for mouse homologues of tinman,7,8 a Drosophila gene essential for cardiac development,9,10 the product of which has been categorized as a Class I NK-2 homeodomain protein.11 This group of transcription factors bind to the 5'-CAAGTG-3' motif in target promoters.12 Murine NKX2-5 is expressed from day 7 in the cardiac primordia8 and is an early marker of both embryonic heart fields.13 Although mutations of NKX2-5 are associated with a wide spectrum of CHDs and thyroid dysgenesis,14 most manifest as atrial septal defects (ASDs) and atrioventricular block.15

The product of GATA4 belongs to the zinc-finger family of transcription factors and, as the other GATA proteins, it binds to the 5'-(A/C/T)GATA(A/G)-3' motif within its target sequences.16 The mammalian GATA4 gene was identified in a screen of zinc-finger encoding cDNAs in a mouse library,17 and it was first suspected to be implicated in CHD when a deletion18 and a duplication19 of the chromosomal segment in which it is contained were discovered in patients with cardiac malformation. Most mutations of GATA4 occur in the segments of the gene encoding the zinc-finger motifs16 and have been related to diverse types of CHDs.20

Mutations of TBX5 cause Holt–Oram syndrome, the most common of the heart–hand syndromes.21,22 If strict criteria are used in Holt–Oram syndrome diagnosis (i.e., at least one family member with radial ray defect and cardiac septal defects), mutation of TBX5 is found in more than 70% of cases.23 The vertebrate TBX5 gene was discovered in a screen of mouse cDNA clones using a probe complementary to the TBX2 T-box region.24 It encodes a transcription factor and alternative splicing regulator25 expressed in the developing heart, among other tissues.21 Except for somatic mutations,26 no variants of TBX5 have been found to cause nonsyndromic CHD.

Physical and functional interaction to modulate transcription of cardiac genes has been documented between the NKX2-5, GATA4, and TBX5 proteins.5,27,28 In order to evaluate the contribution to multiple mutations in these genes in the pathogenesis of CHD, we conducted a mutational scan of the NKX2-5, GATA4, and TBX5 genes in a large cohort of sporadic nonsyndromic CHD cases that complements the findings of our previous analysis of MYH6 in the same cohort.29 We found two novel mutations of the NKX2-5 gene and two instances in which the distribution of changes in families is consistent with additive effects of variants in different genes in the pathogenesis of CHD.

Methods

Patients and Samples

The patient cohort comprised 331 patients with a wide variety of CHDs. Peripheral blood samples from all participants were taken after informed consent and approval of the project by the local ethics committees. Genomic DNA was purified from blood using the QIAamp DNA blood Maxi kit (Qiagen, Hilden, Germany) following the manufacturer directions. Anonymous human control DNA panels were obtained from the European Collection of Cell Culture (Salisbury, UK).

Denaturing High Performance Liquid Chromatography (dHPLC)

Mutational analysis by dHPLC was performed as described previously.30 Briefly, to analyze the combined 18 exons of the NKX2-5, GATA4, and TBX5 genes, 35 polymerase chain reaction (PCR) amplicons were designed. Most of the amplicons spanned individual exons and short segments of flanking introns to each side to detect mutations of splicing regulatory elements. Large exons were spanned by two overlapping amplicons. A pair of PCR primers was designed for each amplicon (see Table 1). PCR reactions were performed using patient and control DNA samples following standard protocols. A final hybridization step for heteroduplex formation was carried out heating the PCR products to 95°C and cooling down 1.5°C per minute until a temperature of 25°C was reached. Sequences of individual amplicons were processed by the Navigator software (Transgenomic, Omaha, NE, USA) in order to plot the melting profile of each DNA segment to determine the optimal dHPLC temperatures. PCR products were analyzed on the dHPLC WAVE System (Transgenomic). PCR products displaying a trace indicative of heterozygosity were sequenced by dHPLC in samples from 384 ethnically matched control subjects.

Results

We identified two novel nonsynonymous mutations in NKX2-5 compared with sequence accession number NM_004387.2 for the cDNA and
NP_004378.1 for the protein. In exon 2, a 541T>C transition (L122P) was discovered in a patient with secundum ASD (Figure 1A). The mutation was transmitted by the unaffected father.

The same patient also harbors a private mutation we reported previously (a 4395C>A transversion resulting in A1443D) in exon 30 of the MYH6 gene, which was transmitted by the mother.29 A

Table 1. Summary of the Primers, Size, Annealing Temperature (Ta) of the Amplicons Used for the PCR Amplification of the NKX2.5, GATA4, and TBX5 Genes and Temperatures Used in dHPLC

| Amplicon Forward Primer | Reverse Primer | Size (bp) | Ta | dHPLC Temperatures |
|-------------------------|----------------|-----------|----|--------------------|
| NKX2.5-E1P1              | tgacagaaactgctcattg | 416       | 56.6 | 63.3, 65.3, 67.3   |
| NKX2.5-E1P2              | cgtgctgtggaacctg    | 422       | 56  | 62, 63.6, 66.2, 67.4 |
| NKX2.5-E2P1              | ccagcgctctctcacaagc | 467       | 59.6 | 62.4, 65.6, 66.4   |
| NKX2.5-E2P2              | ccatctctctctctctctc | 530       | 60.9 | 62.2, 63.9         |
| NKX2.5-E2P3              | acatctcatctcattcattc | 461       | 54  | 59.3, 62.8, 64, 66.3 |
| GATA4-E1                | ctcagacatctcattcattc | 389       | 58.4 | 62.1, 64.3, 67.7   |
| GATA4-E2P1              | gttggttcgaactgctcattc | 497       | 58.4 | 57.4, 59.6, 62.3, 65.1 |
| GATA4-E2P2              | cactctcatctcattcattc | 267       | 54.7 | 64.3, 66.1, 68.8   |
| GATA4-E2P3              | cgcagagtaactgctcattc | 586       | 60.9 | 66.2, 68.2, 68.8   |
| GATA4-E3                | aaagaagatctcattcattc | 344       | 54.1 | 58.6, 61.5, 64.1, 64.7 |
| GATA4-E4                | gcttctctctctctctctc | 336       | 60.3 | 63.2, 65.1         |
| GATA4-E5                | cgcagacatctcattcattc | 229       | 57.7 | 58.5, 60.5         |
| GATA4-E6                | ccgtctctctctctctctc | 269       | 59.7 | 62.6, 65.1         |
| GATA4-E7P1              | cactctcatctcattcattc | 499       | 59.1 | 60.8, 61.4, 62.2   |
| GATA4-E7P2              | gcaacatctcattcattcattc | 470     | 58.4 | 59.4, 61.5, 63.3   |
| GATA4-E7P3              | gccgctctctctctctctc | 482       | 59.8 | 58.2, 61.2         |
| GATA4-E7P4              | aagcagtaactgctcattc | 484       | 59.4 | 61.2, 62.2         |
| GATA4-E7P5              | ccagtctctctctctctctc | 384       | 58.3 | 56.5, 58.1, 60.6   |
| TBX5-E1P1               | ggcagacatctcattcattc | 478       | 57.9 | 53.6, 59.2, 63.5, 64.9 |
| TBX5-E1P2               | gcaacatctcattcattcattc | 390       | 57.8 | 57.8, 60.4, 62.8   |
| TBX5-E2                 | ttctctctctctctctctctc | 297      | 60.2 | 62.8, 68.2         |
| TBX5-E3                 | gtcagctctctctctctctc | 243       | 57  | 58.9, 60.4         |
| TBX5-E4                 | gcgctctctctctctctctc | 248       | 56.7 | 57.7, 60.2         |
| TBX5-E5                 | ccagctctctctctctctctc | 282       | 60.3 | 62.7, 65.1         |
| TBX5-E6                 | gctctctctctctctctctc | 296       | 54.3 | 54.6, 57.8, 61.1   |
| TBX5-E7                 | tgtctctctctctctctctc | 294       | 53.4 | 56.5, 58.9         |
| TBX5-E8                 | atctcctctctctctctctc | 390       | 60.3 | 58.7, 61.3         |
| TBX5-E9P1               | gctgagctctctctctctc | 465       | 54.1 | 56.3, 60.5, 63.8   |
| TBX5-E9P2               | acctctctctctctctctc | 474       | 50.3 | 55.6, 59, 61.3, 63.5 |
| TBX5-E9P3               | gctgagctctctctctctc | 496       | 53.4 | 52.8, 56.4, 58, 59.3 |
| TBX5-E9P4               | gcccagctctctctctctc | 481       | 49.9 | 56.9, 58, 59.2     |
| TBX5-E9P5               | gcctctctctctctctctc | 482       | 60.3 | 53.1, 54.5, 56.7, 58.4 |
| TBX5-E9P6               | gtcagactctctctctctc | 361       | 56.9 | 52.1, 54.2, 57     |
| TBX5-E9P7               | aagcgctctctctctctctc | 276       | 56.2 | 56.5, 58.7         |

PCR, polymerase chain reaction; dHPLC, denaturing high performance liquid chromatography.

Figure 1. Sequence traces showing the nonsynonymous nucleotide changes causing amino acid replacements in the NKX2.5, GATA4, and TBX5 proteins. The changes are indicated with arrows.

The same patient also harbors a private mutation we reported previously (a 4395C>A transversion resulting in A1443D) in exon 30 of the MYH6 gene, which was transmitted by the mother.29 A
870G>A (G232R) mutation (Figure 1B) was also identified in exon 2 of a patient with pulmonary valve stenosis that was transmitted by the apparently unaffected mother. The G232R replaces an uncharged glycine residue with a positively charged arginine residue. Neither NKX2-5 mutation was found in 384 ethnically matched control subjects (768 chromosomes).

Three rare and one common nonsynonymous variants of GATA4 were found in our CHD cohort. They are identified according to their positions in sequence accession number NM_002052.3 (cDNA) and NP_002043.2 (protein). A 1591C>T transition (A346V) (Figure 1C) was discovered within exon 6 in a patient with transposition of the great arteries. A346V has been previously described in a patient with endocardial cushion defect.20 Also within exon 6, a transition 1692G>A (V380M) was identified in a patient with a large ventricular septal defect (VSD) (Figure 1D). V380M has been reported as a pathogenic mutation in CHD in one study,11 but another study reports the same variant in 3 out of 318 control subjects.12 V380M was not found in our control cohort of 384 ethnically matched subjects. In exon 7, an 1827G>A (D425N) transition was found in a patient with a large patent foramen ovale (Figure 1E). This mutation has been reported previously in three CHD patients.34,35 The patient also carries a private mutation in exon 18 of MYH6, a 2165G>A transition (V700M).29 Two clinically normal sons are both heterozygous for the GATA4 mutation but do not carry the MYH6 mutation.

Analysis of the TBX5 gene revealed one synonymous variant in our patient cohort. In exon 4, a transversion 998G>T (cDNA sequence accession NM_000192.3) (Figure 1F) resulting in D111Y (protein sequence accession NP_000183.2) was discovered in a patient with double outlet right ventricle, large ventricular septal defect, large atrial septal defect, and patent ductus arteriosus. We also found this variant in 3 out of 384 ethnically matched control samples.

Discussion

We have conducted a mutational scan of the NKX2-5, GATA4, and TBX5 genes in a large cohort of patients with a wide variety of CHDs. We have identified two novel changes of NKX2-5 (L122P, G232R), one for TBX5 (D111Y), and three previously known variants of GATA4 (A346V, V380M, and D425N).

The L122P change lies N-terminal to the homeodomain of NKX2-5. An analysis of the NKX2-5 wild-type and L122P mutant-derived protein sequences using the nnpredict program indicates that the variant occurs within a segment of the molecule that normally adopts an α-helical secondary structure that is disrupted in the mutant (Figure 2). This can be explained by the observation that, when a proline residue is located within an α-helical structure, the helix is kinked by approximately 30 degrees to prevent a steric clash between the pyrrolidine ring and the preceding carbonyl oxygens.37 This suggests that the private L122P mutation could affect the function of the NKX2-5 protein by modifying its three-dimensional conformation. This mutation was inherited from the father. In the same individual, there is another private mutation in a different gene, MYH6, (A1443D) which was transmitted by the mother (Figure 3A).29 Both

Figure 2. nnpredict output showing the prediction of the structural consequence of the L122P mutation in NKX2.5. The panels show the sequence of the wild-type (A) and mutant (B) NKX2.5 proteins. The relevant residues at position 122 are underlined. The secondary structure prediction is shown for each sequence. The fourth helix predicted in the wild-type sequence is 18 residues long (in bold). An eight-residue stretch within that segment is predicted to adopt a different configuration in the mutant peptide (H, helix, E, strand, -, no prediction).
parents are of white descent. The A1443D mutation introduces a negatively charged residue in a position where only noncharged amino acids exist in sarcomeric myosins from mammals, chick, zebrafish, Xenopus, and Caenorhabditis elegans and is predicted to interfere with the interaction between the two molecules of the myosin dimer or with other sarcomeric proteins. The frequency of the alleles encoding either L122P (NKX2-5) or A1443D (MYH6) in the CHD and control cohorts is 0.000619. The probability of both private alleles occurring in the same individual by chance, assuming the variants do not have causal relation to the phenotype is estimated to be $4.82 \times 10^{-7}$. The most common phenotype resulting from mutations in either NKX2-5 or MYH6 is secundum ASD. As this patient has the same diagnosis, double heterozygosity could at least partially explain the development of the defect in the proband and the incomplete penetrance of the L122P and A1443D mutations in the parents. Incomplete penetrance is a common finding in documented cases of mutations causing Mendelian CHD. In the case of NKX2-5, recent data highlight the profound influence of modifier loci in the pathogenesis of single-gene cardiac malformation.

The novel NKX2-5 G232R mutation occurs in the C-terminal end of the NK2-specific domain (NK2-SD) and introduces a positively charged residue in a position where only noncharged residues exist in every vertebrate NKX2-5 ortholog sequence available (Figure 4A). The NK2-SD interaction with bone morphogenetic protein (BMP)-dependent mothers against decapentaplegic (SMAD) proteins is required for the binding of the homeodomain with the histone deacetylase (HDAC)/Sin3A complex. This domain is also thought to stabilize the interaction of the tinman domain to the corepressor Groucho proteins and to modulate, in an intramolecular fashion, the activity of the C-terminal activation domain. These data suggest that the G232R mutation could compromise the transcriptional-repressor activity of NKX2-5 and its responsiveness to BMP signaling.

The novel D111Y TBX5 variant occurs within the conserved T-box element of the molecule. A three-dimensional structure model of the human TBX5 protein (PDB: 2X6U) has been made available recently. This model allows more precise predictions of the consequences of the variant D111Y (Figure 5A). The structural model predicts that the salt bridge present between the K126 and D111 is disrupted when the aspartic acid residue (D) is replaced, as in the case of the D111Y variant, by an uncharged tyrosine (Y) residue (Figure 5B). A model of the human TBX3 bound to its target sequence (PDB: 1H6F) reveals that the side-chains of both K126 and D111 point to opposite directions during interaction with DNA, canceling the salt bridge (Figure 5C) that suggests that the D111Y variant could have an impact on the conformational change of TBX5 upon binding to its target promoters. Both residues involved in the salt bridge (D111 and K126) are universally conserved in every TBX5 vertebrate sequence available (Figure 4B). We suggest that these data warrant the inclusion of the TBX5 D111Y variant in future case-control studies for sporadic CHD, as this variant could confer susceptibility to the disease.

The D425N mutation of GATA4 has been reported previously in three patients with secundum ASD, VSD, and tetralogy of Fallot. This variation replaces a negatively charged aspartic acid (D) residue for an uncharged asparagine (N) residue in a position where only negatively charged amino acids are found in GATA4.

**Figure 3.** Pedigrees of the families CHD where multiple heterozygosity for the NKX2.5, GATA4, and MYH6 genes was detected. ASD, atrial septal defect; PFO, patent foramen ovale.
orthologs in wide range of vertebrate species (Figure 4C). The patient heterozygous for D425N also carries a private mutation in exon 18 of MYH6, a 2165G>A transition (V700M) (Figure 3B). This mutation is located within the segment of the MYH6 gene encoding a part of the SH1 helix that is conserved in every myosin sequence available and is predicted to hinder the movements of the helix at different stages of the myosin cycle. Two clinically normal sons of the proposita are both heterozygous for the D425N GATA4 mutation but do not carry the V700M MYH6 mutation. The patient suffered a stillbirth but no sample from the product was available for analysis. The structure of this pedigree is consistent with complementation of both variants to contribute to the phenotype (Figure 3B).

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Conclusions

Mendelian nonsyndromic CHD is known to be caused by mutations of genes encoding transcription factors and, notably, muscle proteins expressed in the developing heart or great vessels like ACTC1, MYH6, MYH7, and MYH11. Here, we describe two novel mutations of NKX2-5 associated with CHD. Also, we present cases where multiple heterozygosity of variants could contribute, by additive effects, to the development of individual cases of cardiac malformation. Our findings highlight the usefulness of multiple gene analysis in large patient CHD cohorts in order to identify variants whose combined effects may cause the phenotype.

Although array-based genome-wide association studies (GWAS) have been successful in identifying chromosomal segments and variants associated with complex phenotypes, in general, they only explain a small fraction of their heritability. Recent findings suggest that a great proportion of the “missing heritability” in GWAS studies can be masked by incomplete linkage disequilibrium between the causal variants and the single-nucleotide polymorphisms (SNPs) typed by the arrays, as causal variants would tend to have lower minor allele frequencies than the typed SNPs. Thus, we suggest there is a need for further detailed analysis of common, rare, and private variants of genes implicated in Mendelian CHD in association studies aimed at identifying the genetic causes of the more common, sporadic forms of the disease.

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