Identification of Candidate Genes for Congenital Ventricular Septal Defects With HSA22q11 Loss of Heterozygosity

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ORIGINAL ARTICLES

Introduction and objectives. Ventricular septal defect (VSD) is one of the major forms of congenital heart disease (CHD) in individuals with Homo sapiens chromosome 22q11 (HSA22q11) deletion syndrome. The objective was to identify candidate genes associated with VSD located within HSA22q11 by analyzing loss of heterozygosity (LOH) using microsatellite genotyping and by gene dosage analysis in 7 candidate genes.

Methods. The study involved 82 families with CHD, which included 261 individuals (85 patients and 176 siblings and parents). All were screened for LOH in the HSA22q11 region by microsatellite (n=10) genotyping. Bioinformatic strategies were used to characterize 7 candidate genes located within this region in greater detail. Quantitative polymerase chain reaction analysis was used to determine the dosages of the 7 candidate genes in 16 patients with LOH of HSA22q11.

Results. Overall, 42 out of 85 patients (49.4%) with CHD had at least 1 LOH in the HSA22q11 region. Moreover, LOH of HSA22q11 was found in 17 out of 29 patients with a VSD and in 3 out of 4 families with 2 offspring affected by CHD. Dosage analysis of the 7 candidate genes showed recurrent heterozygous deletion of HIRA, GNB1L, and TUBA8 genes in 16 VSD patients with a LOH of HSA22q11.

Conclusions. Microsatellite genotyping identified LOH of HSA22q11 in several types of CHD. Heterozygous deletion of HIRA, GNB1L, or TUBA8 genes might play an important role in ventricular septum development. Since CHD can be a familial disease, screening the siblings of a proband for LOH of HSA22q11 could be valuable for early diagnosis and treatment.

Key words: Congenital heart disease. Gene dosage analysis. Genotyping 22q11del.
INTRODUCTION

Congenital heart disease (CHD) is one of the most common types of birth defects, occurring in 1% of live births and 10% of spontaneously aborted fetuses. Genetic aberrant expressions of certain genes may lead to defects of heart development. In the past decade, molecular genetic studies have exploited CHD families with multiple affected individuals and have provided further insight into the genetic bases of several forms of CHD, such as atrial septal defect or patent ductus arteriosus. Fifteen representative chromosomal disorders associated with CHDs and 28 genes associated with CHDs in the young were summarized in Pierpont et al (2007). The CHDs are present in 75% to 80% of patients with a Homo sapiens autosomal 22q11 (HSA22q11) deletion and most cases were regional hemizygosities or monosomics, and resulting in haploinsufficiencies, including syndromic cases (CHD associated with at least 1 extra-cardiac anomaly) and non-syndromic familial cases. Septation of heart chambers starts at early stages in embryogenesis and is completed only at birth with the closure of the foramen ovale. Defects in heart separation, including atrial septal defect, atroventricular canal septal defects, conotruncal septal defects, and ventricular septal defect (VSD), represent a major cause of congenital heart disease.

A substantial proportion of patients with double outlet right ventricle, interrupted aortic arch, posterior malalignment type VSD, pulmonary atresia with VSD, subpulmonary VSD, truncus arteriosus, transposition of great arteries, tetralogy of Fallot, and VSD associated with other conotruncal heart defects have an HSA22q11 deletion. Fluorescent in situ hybridization (FISH) with DNA probes from the DiGeorge Syndrome chromosome region is the clinical diagnostic strategy that has been used for molecular genetic testing of HSA22q11 deletion. However, microsatellite genotyping provides a higher resolution (locus-based) than karyotyping (Mb-based resolution) to diagnose HSA22q11 microdeletions in karyotypically normal patients. Therefore we selected microsatellite genotyping technology to perform the current study. Also, in our previous genetic analysis of HSA22q11 markers in CHDs, a significant deviation from the expected level of heterozygosity at a microsatellite D22S1648 locus implied that the HSA22q11 region might be hemizygous among syndromic CHD patients. The HSA22q11 deletion in syndromic CHD patients was further confirmed by haplotype analysis, dual-labeling FISH and quantitative polymerase chain reactions (PCR).

To improve our understanding of whether specific CHD types are relevant to HSA22q11 deletion and to further narrow down causative candidate genes, microsatellite genotyping and gene dosage analysis were performed in this study.

METHODS

Blood Samples and DNA Extraction

A total of 82 CHD families that had been referred to Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan), including 261 individuals (85 patients and 176 siblings and parents) participated in this study. Informed consent was obtained from each participant. The age of the 85 patients ranged from 1 to 14 years of age (mean [SD], 3.6 [2.9]). They were randomly selected from our pediatric cardiology outpatient department. Genomic DNA was prepared from peripheral blood leukocytes using the salting-out DNA extraction method.

Microsatellite Genotyping

Microsatellites are polymorphic loci present in genomes that consist of repeating units of 1-6 bp in length, typically neutral and co-dominant. Because their copy numbers could be highly polymeric within and between individuals, these markers have been used to identify loss of heterozygosity (LOH), ie, loss of 1 allele or more alleles in an individual, compared to the parents. A total of 9 polymorphic microsatellite markers covering the HSA22q11 region and 1 distal marker, D22S315, located at HSA22q12.1, were used for genotyping. Nine highly polymorphic markers in Asia population spanning a total of 8.1 Mb were selected based on previous studies and the distal marker, D22S315, was selected from Map Viewer (http://www.ncbi.nlm.nih.gov/mapview; NCBI, USA). Primer sequences for microsatellite genotyping (not shown) were directly retrieved from Electronic PCR (http://www.ncbi.nlm.nih.gov/sutils/e-PCR; NCBI, USA). To multiplex PCR-based genotyping, one primer of each pair was either labeled with FAM (D22S264,
Spain) (Table 1). In addition to 7 candidate genes, an X chromosome-linked gene, 6-phosphofructo-2-kinase (PFKFB1),23 was analyzed as a dosage reference of single-copy gene. Each quantitative PCR experiment was performed in a 15 µL volume (7.5 µL of SYBR® Green Mix and 0.75 µL of forward and reverse primers and 6 µL [12.5 ng/µL] genomic DNA). The concentration of each primer was 4 µM and the annealing temperature was optimized to 55-60°C. Amplifications were carried out in 96-well plates for 55-60 cycles. Equal amounts from an aliquot of 12 genomic DNA samples were pooled to be a common reference genomic DNA for further calculation of the relative expression fold in each candidate transcript. To assure equivalent amplification efficiencies of all primer sets used in this study, validation assays were performed according to Senchenko et al24 (2003).

Quantitative PCR data were expressed as mean (SD). The starting copy number of unknown genomic DNA samples was determined relative to the known copy number of the calibrator sample (common reference genomic DNA) using the following formula: $D_{DCt} = D_{Ct}(\text{candidate gene, unknown sample}) - D_{Ct}(\text{common reference gDNA})$. The relative gene copy number was calculated by the expression $2^{-D_{DCt}}$. Prior to the dosage analysis on candidate genes, standard ranges of 1- and 2-copy gene dosages were established by quantifying the X chromosome-linked housekeeping gene, PFKFB1,23 in genomic DNA from 12 males and 12 females.

Bioinformatics Strategies to Identify Candidate Genes

Ensemble Genome Browser17 (http://www.ensembl.org/index.html), UCSC Genome Browser18 (http://genome.ucsc.edu/) and NCBI Map Viewer16 were used to identify CHD-related candidate genes in the HSA22q11 region. Tissue specificities of candidate genes were inspected with the Swiss-Prot database19 (http://www.expasy.ch/sprot/). The FatiGOplus data mining tool20 was further employed to search for genes with similar Gene Ontology terms21 to one well-known CHD candidate gene, TBX1.

Gene Dosage Analysis by Quantitative Polymerase Chain Reaction

Dosage analyses of candidate genes were examined by quantitative PCR (iCycler thermal cycler, BioRad, CA, USA) along with comparative Ct method (DDCt method).22 Primer pairs were designed with the Beacon Designer 2.1 (Biomedal, LCR22-1 LCR22-2 LCR22-3 LCR22-4 LCR22-5 LCR22-6 LCR22-7 LCR22-8

Figure 1. Genomic organization of the Homo sapiens (HSA) autosomal 22q11 (HSA22q11) and markers used in this study. D22S- are polymorphic microsatellites. A total of 8 low copy repeats, termed LCR22-1 to -8, have been mapped to this region. The common ~3-Mb typically deleted region, which is present in more than 85% of velocardiofacial syndrome (VCFS) and DiGeorge syndrome (DGS) patients and the ~1.5-Mb deletion (~8%) are shown. Atypical deletions are not shown. Deletion in VCFS/DGS: common ~3-Mb deletion (>85%); ~1.5-Mb deletion (~8%).
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RESULTS

The Phenotypes of CHD patients

No CHD phenotypes were reported in parents of any recruited families. The VSD was identified in 29 patients (34.1%), which is the most prevalent CHD in this cohort. The tetralogy of Fallot and the atrial septal defect were identified in 10 patients (11.7%) and 7 patients (8.2%), respectively. The VSD cases were further subtyped as follows: (1) membranous (n=18); (2) subpulmonary (n=6); (3) muscular (n=4); and (4) atrioventricular canal (n=1). The presence more than 3 anatomical defects in 1 patient was defined as a complex CHD (n=4; 4.7%) (Table 2). Four families, ie, numbers 102, 106, 260, and 352, had two children affected. Two children in family 352 had a membranous VSD. The VSD was also diagnosed in two children in family 102, but the subtypes were apical muscular and membranous. Two CHD patients in families 106 and 260 were diagnosed as membranous VSD (106-1) and pulmonary stenosis (106-2), and membranous VSD (260-1) and atrial septal defect (260-2), respectively (Table 3).

The Loss of Heterozygosity in HSA22q11 Was Identified in Several Types of CHDs

In addition to 85 patients and 164 unaffected parents, 12 unaffected siblings were genotyped for 10 microsatellites as well (partial data are shown in Table 3). While both parents share one allele (eg, D22S1623 in family 260) or both alleles (eg, D22S941 in family 297), the LOH statuses in their offspring might not be identified (uninformative, denoted as “UI” in Table 3 and Figure 2), indicating that no information was provided. Namely, UI situation does not exclude the possibility that the region is involved. Alternatively, failure to inherit any allele from the parents with homozygotes (eg, D22S1648 in family 100, child 1 (100-1) and 3 (100-3)) or heterozygotes (eg, D22S420 in family 106, child 2 (106-2), pulmonary stenosis case; Table 3) might result in LOH. Number and percentage of patients with HSA22q11 deletions by microsatellite genotyping and LOH analysis for different types of CHD are shown in Table 2. Forty-two (49.4%) of 85 CHD patients were identified to have at least 1 LOH in the HSA22q11 region. Although the children of family 352 were both affected by membranous VSD, microsatellite genotyping was not able to identify any LOH within the HSA22q11 region. Distinct HSA22q11 LOHs were identified in family 102 with 2 different subtypic VSD patients and in family 106 with 2 different pathologic CHDs (Table 3). The LOH of D22S944 was identified in both VSD and atrial septal defect patients of family 260. LOH HSA22q11 was not found in 2 of 4 families with more than 1 child, none of whom was affected. However, in the other 2 families (157 and 201), HSA22q11 LOHs were also

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**TABLE 1. Primers Used for Quantitative Polymerase Chain Reaction**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers Sequence (5' to 3')</th>
<th>Length, mers</th>
<th>Amplicon Size, bp</th>
<th>Tm, °C</th>
<th>Slope a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>F: CCCAG CCATG TAGT TGCT</td>
<td>19</td>
<td>86</td>
<td>59.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>R: CGTCA CGGGA GTCGA TCAC</td>
<td></td>
<td></td>
<td>60.1</td>
<td></td>
</tr>
<tr>
<td>CLTCL1</td>
<td>F: CCCTT GTTGAC GAGGA GGGAA TG</td>
<td>22</td>
<td>120</td>
<td>60.4</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td>R: TTGTC CACTG GTGTC TGATA CTC</td>
<td></td>
<td></td>
<td>60.1</td>
<td></td>
</tr>
<tr>
<td>DGCR2</td>
<td>F: CCAGA GCTAG AGGTT GATGC</td>
<td>20</td>
<td>121</td>
<td>59.9</td>
<td>–0.074</td>
</tr>
<tr>
<td></td>
<td>R: GGCGA CAGGG AACCT GATAA</td>
<td></td>
<td></td>
<td>59.9</td>
<td></td>
</tr>
<tr>
<td>DGCR14</td>
<td>F: ACTTG AGAAA GGGGC TGTT</td>
<td>19</td>
<td>106</td>
<td>60.2</td>
<td>–0.089</td>
</tr>
<tr>
<td></td>
<td>R: TCAAG GGGCC ATTTA AACAG</td>
<td></td>
<td></td>
<td>59.9</td>
<td></td>
</tr>
<tr>
<td>GNB1L</td>
<td>F: CCGTG GCTTT GGGAG GTGGT G</td>
<td>21</td>
<td>107</td>
<td>60.7</td>
<td>–0.081</td>
</tr>
<tr>
<td></td>
<td>R: AATCT GAACT GCTGC GTGC C</td>
<td></td>
<td></td>
<td>60.3</td>
<td></td>
</tr>
<tr>
<td>HIRA</td>
<td>F: AGGTT GAGAA TGAG TGAGA GTGG</td>
<td>24</td>
<td>116</td>
<td>60.2</td>
<td>–0.064</td>
</tr>
<tr>
<td></td>
<td>R: GCCCG CAGCA GTGAG GATC</td>
<td></td>
<td></td>
<td>60.1</td>
<td></td>
</tr>
<tr>
<td>PFKFB1</td>
<td>F: AGGCC ACTAC TGACC CCTTT C</td>
<td>21</td>
<td>146</td>
<td>56</td>
<td>0.097</td>
</tr>
<tr>
<td></td>
<td>R: GTTGG GGAGG ACTTC TCCAC TG</td>
<td></td>
<td></td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>TBX1</td>
<td>F: TGGGA GAAAT GGGG CCTTG GC</td>
<td>22</td>
<td>142</td>
<td>60.3</td>
<td>–0.070</td>
</tr>
<tr>
<td></td>
<td>R: TTGCG TGAGAT CAGGA AGGTG AG</td>
<td></td>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>TUBA8</td>
<td>F: GACAA CGAG CCATG ATAGG CATC</td>
<td>24</td>
<td>119</td>
<td>57.7</td>
<td>–0.054</td>
</tr>
<tr>
<td></td>
<td>R: AAGCG GAGAG CATC GGTG TGATG TG</td>
<td>22</td>
<td></td>
<td>58.4</td>
<td></td>
</tr>
</tbody>
</table>

*Validation assays for the efficiency of each primer pair were performed. Serial dilutions (from 75 ng to 7.5 pg in 15 µL reactions) were assayed for each target gene and the internal control gene, ACTB, and the result plotted with the log input concentration for each dilution on the x-axis, and the difference in Ct (∆Ct= Ct target gene-CtACTB) for each dilution on the y-axis. If the absolute value of the slope of the line is <0.1, the comparative Ct method may be used.24.*
identified in non-affected individuals (Table 3). Of 3 children in family #157 only 1 was affected by VSD, both siblings showed HSA22q11 LOHs. Similarly, in family 201, HSA22q11 LOH was detected in the other non-affected child (Table 3).

Identification of Candidate Genes

A total of 75 genes were first identified to be intersected from 3 genome browsers in the HSA22q11 region. NCBI gene annotations indicated

<table>
<thead>
<tr>
<th>Type of Disease</th>
<th>No. of Patient</th>
<th>HSA22q11 LOH</th>
<th>Percentage With LOH, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital heart disease</td>
<td>85</td>
<td>42</td>
<td>49.4</td>
</tr>
<tr>
<td>Ventricular septal defect (VSD)</td>
<td>29</td>
<td>17</td>
<td>58.6</td>
</tr>
<tr>
<td>Membranous</td>
<td>18</td>
<td>12</td>
<td>66</td>
</tr>
<tr>
<td>Muscular</td>
<td>4</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Subpulmonary</td>
<td>6</td>
<td>4</td>
<td>66</td>
</tr>
<tr>
<td>Atrioventricular canal</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetralogy of Fallot (TOF)</td>
<td>10</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Atrial septal defect</td>
<td>7</td>
<td>3</td>
<td>42.9</td>
</tr>
<tr>
<td>Complex congenital heart disease</td>
<td>4</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>Pulmonary stenosis</td>
<td>4</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>TOF with pulmonary atresia</td>
<td>4</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Aortic stenosis</td>
<td>2</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>4</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Left ventricular non-compaction</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Mitral valve regurgitation</td>
<td>4</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>3</td>
<td>1</td>
<td>33.3</td>
</tr>
<tr>
<td>Transposition of great arteries</td>
<td>2</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Vascular ring</td>
<td>2</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Aortic valve regurgitation plus mitral valve regurgitation</td>
<td>4</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Coarctation plus mitral valve stenosis</td>
<td>2</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Coarctation plus VSD</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Pulmonary atresia with intact ventricular septum</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Pulmonary stenosis plus VSD</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 2. Combination of loss of heterozygosity (by microsatellite genotyping) and gene dosage (by quantitative polymerase chain reaction) data in 16 ventricular septal defect patients. For microsatellite markers (D22S-). H indicates retention of heterozygosity; L, loss of heterozygosity; UI, uninformative; for candidate genes: 1 indicates 1 copy; 2, 2 copies; UI, uninformative.

Table 2. Number and Percentage of Patients With HSA22q11 Loss of Heterozygosity (LOH) by Microsatellite Genotyping in Patients With Congenital Heart Diseases

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TABLE 3. Ten Microsatellite Genotypes (D22S-)a in Patient Families With More Than 1 Child and More Than 1 Affected Children

<table>
<thead>
<tr>
<th>Family</th>
<th>ID°</th>
<th>CHD Subtype</th>
<th>D22S420</th>
<th>D22S427</th>
<th>D22S1638</th>
<th>D22S1648</th>
<th>D22S594</th>
<th>D22S941</th>
<th>D22S944</th>
<th>D22S1623</th>
<th>D22S264</th>
<th>D22S303</th>
<th>D22S315</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>F</td>
<td>VSD</td>
<td>136</td>
<td>134</td>
<td>136</td>
<td>134</td>
<td>136</td>
<td>134</td>
<td>136</td>
<td>134</td>
<td>136</td>
<td>134</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>VSD</td>
<td>138</td>
<td>134</td>
<td>136</td>
<td>134</td>
<td>136</td>
<td>134</td>
<td>136</td>
<td>134</td>
<td>136</td>
<td>134</td>
<td>134</td>
</tr>
</tbody>
</table>

that only 20 of them were related to embryogenesis, mesoderm differentiation or heart development (data not shown). By using the information of tissue specificities from the Swiss-Prot database, 5 genes, ie, CLTCL1, DGCR2, DGCR14, GNB1L, and TUBA8, were verified to be expressed in heart specifically. Another gene, HIRA, was found to be identical to TBX1 at level 5 of gene ontology terms in molecular function, cellular component, and biological process. The TBX1 is a gene encoding the T-box transcription factor, which is hemizygously deleted on HSA22q11, and was found to be a strong candidate for DiGeorge syndrome. As a consequence, including TBX1, 7 candidate genes within HSA22q11 LOH region were identified.

Gene Dosage Analysis

The results of the PCR efficiency assay for every candidate gene was plotted with the log input concentration for each dilution of DNA templates on the x-axis, and the difference in Ct (candidate gene-ACTB) for each dilution was plotted on the y-axis (data not shown). When the absolute value...
of the slope of the line was less than 0.1, the
comparative Ct method would be appropriate. As
shown in Table 1, all primer sets in this study had
slopes <0.1.

The genomic dosage profiles of PFKFB1 gene in
males (n=12) and females (n=12) were subsequently
established. Because PFKFB1 is an X chromosome-
linked gene, average relative fold changes (2-ΔΔCt)
in the male and female genomes, represent the relative
genomic organization of low-copy repeats. The
most common rearrangement is the 3.0- and 1.5-Mb
recombination mechanisms mediate these deletions.27

DISCUSSION

The HSA22q11 region is prone to rearrangements
that lead to congenital anomaly disorders due to
its genomic organization of low-copy repeats. The
most common rearrangement is the 3.0- and 1.5-Mb
hemizygous deletions detected in velo-cardio-facial
syndrome/DiGeorge syndrome patients.26 (Figure 1).
Both intra- and interchromosomal homologous
recombination mechanisms mediate these deletions.27
However, atypical deletions are more common than
has been reported.28 Accordingly, we hypothesized
that the HSA22q11 region encompasses genes that
associate with cardiac and craniofacial anomalies.
Earlier, HSA22q11 deletions were found in 12
(10%) of 125 VSD patients by FISH examination,
using the Oncor cosmid probe N25(D22S75).10

With Vysis probe set targeting TUPLE1 (ie, HIRA),
Beauchesne et al12 (2005) identified 6 patients (5.8%)
to have HSA22q11 deletions (3 tetralogy of Fallot, 2
pulmonary atresia/VSD, and 1 truncus arteriosus).
Combining several probes (100c10, 48F8, KI-506,
and Sc11.1), 50 (48%) of 104 newborns with
conotruncal defects and HSA22q11 deletions were
detected.29 Most of these cases were syndromic
CHD. Few studies have used polymorphic
microsatellites that span the HSA22q11 region to
detect microdeletion.3,13 Microsatellite genotyping
have detected 10 (48%) of 21 patients with tetralogy
of Fallot and 10 (100%) of 10 patients with either
velo-cardio-facial syndrome or DiGeorge syndrome
showing HSA22q11 deletions in our previous
report.13 In the present study, we as well achieved
higher identification efficiency than previous FISH
results.2,10 The possible explanations were (1) higher
resolution of microsatellite genotyping compared
to FISH analysis and (2) some of our patients were
syndromic CHD cases. It was difficult to evaluate
whether patients had syndromic CHD because
subtle noncardiac features might not be apparent
in infancy. Some of these features are age-dependent.
In addition, the diagnosis of facial dysmorphia is
more subjective.30 Syndromic CHDs might exist
in our patients. However, we speculated that the
probable case number was small because they had
been evaluated initially for facial dysmorphia by
at least one cardiologist. We therefore suggest that
the microsatellite genotyping technology provides a
higher resolution than traditional FISH analysis in
such diagnoses.

After genotyping, no LOH in HSA22q11 was
detected from 43 CHDs patients (including 1 family
with 2 VSD patients), indicating other chromosomal
regions might be responsible for their abnormalities.
On the other hand, the HSA22q11 LOHs were
identified in both patients in 3 of 4 families with
2 CHD-affected individuals, suggesting that genes
within this region might be responsible for more than
1 CHD phenotype and that these CHDs might not be
sporadic but fundamentally familial. To our surprise,
LOHs in HSA22q11 were also identified in 3 non-
sporadic but fundamentally familial. To our surprise,
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LOH region. To narrow down specific genes that cause VSDs, genomic dosages of 7 candidate genes in 16 probands were examined by quantitative PCR. Results exhibited recurrent heterozygous deletion of GNBIL, HIRA, and/or TUBA8 genes in membranous and subpulmonary VSDs. In HSA22q11 region, TBXI was thought to be a major candidate gene to the cardiac phenotype or its severity in patients carrying the HSA22q11 microdeletion. Although Tbx1+/Tbx1 heterozygous mutant mice survived at normal Mendelian ratios and had only mild cardiovascular defects, Tbx1−/Tbx1 homozygous mice died at birth with cleft palate, thymus gland aplasia, a single outflow tract and VSD. Unexpectedly, in this study, no hemizygous or homozygous deletion of TBXI gene was identified in 16 VSD probands with HSA22q11 LOH. In contrast, recurrent heterozygous deletion of GNBIL, HIRA and/or TUBA8 genes was found. These are genes that are frequently deleted in the DiGeorge syndrome critical region, thus suggesting that heterozygous deletion might play a crucial role in the development of VSD. Alternatively, mutations of TBXI gene in patients with HSA22q11 deletion syndrome but without 22q11 microdeletion or any other detectable rearrangements in this region were identified. In our dosage screening, heterozygous deletion of any candidate gene was not observed in 6 of 16 VSD probands with H22q11 LOH, implying that potential mutations of TBXI or other genes in these patients might exist; however, further experiments will be required to validate these assumptions.

Of 3 recurrently deleted genes, HIRA is a homolog of yeast transcriptional corepressors Hir1p and Hir2p, possessing 7 WD dipeptide motifs and 1 LXXLL motif in its N-terminal and C-terminal regions, respectively. Whole mount in situ hybridization indicated HIRA mRNA is expressed in a temporal and spatial pattern during early embryogenesis, with high levels of expression in the neuroepithelium during neurulation. By exposing a portion of the premigratory chick neural crest to phosphorothioate end-protected antisense oligonucleotides, ex ovo, following by orthotopic backtransplantation to the untreated embryos, Farrell et al. (1999) showed that the functional attenuation of chicken HIRA in the chick cardiac neural crest results in a significantly increased incidence of persistent truncus arteriosus, a phenotypic change characteristic of DiGeorge syndrome. But this does not affect the repatterning of the aortic arch arteries, the ventricular function, or the alignment of the outflow tract. High levels of mouse HIRA transcripts were detected in the cranial neural folds, frontonasal mass, the first 2 pharyngeal arches, circumpharyngeal neural crest, and limb buds. The TUBA8 is a tissue-specific isoform of alpha-tubulin that is highly conserved in human and mouse. Reverse transcription-PCR assay performed on a panel of 16 different human tissue cDNAs using primers specific for TUBA8 isoform found preferential expression in heart, skeletal muscle, and testis. Moreover, sequence comparison of TUBA8 with other known alpha-tubulins shows that TUBA8 is the most divergent member of the mammalian alpha-tubulin family. The sequence peculiarity of the TUBA8 in the human and mouse suggests that it might have functional significance and, according to the multi-tubulin hypothesis, that it might play specific functional roles in the cell cytoskeleton. The GNBIL encodes a G-protein β-subunit-like polypeptide. Northern blot analysis by Gong et al. (2000) revealed its high expression level in the heart, but low expression levels in brain, placenta, lung and pancreas, suggesting it might be a strong candidate for heart development. A chicken model using vector-based RNA interference to knock down TUBA8 and GNBIL genes in early embryogenesis underway in our laboratory will perhaps provide functional interpretations of these VSD candidate genes in the near future.

Limitations

Minor noncardiac features might not be diagnosed at an earlier stage in CHD patients. Hence karyotyping or detection for HSA22q11 deletion could be neglected, resulting in limitations to the power of genetic counseling. In patients without the HSA22q11 deletion, CHD might be a result of other chromosome/gene abnormality/deletion/mutation.

CONCLUSIONS

By applying microsatellite genotyping within HSA22q11 region to CHD families along with the LOH analysis, we identified the HSA22q11 LOH in several CHD types. Recurrent heterozygous deletion of 3 candidate genes, HIRA, TUBA8, and GNBIL, is potentially responsible for some VSD cases. More than 1 affected individual within 2 families with HSA22q11 LOH strongly suggests that CHD is a familial disease. To screen HSA22q11 LOHs in siblings of the proband will be beneficial for early diagnosis and the design of effective treatment strategies.

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