

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

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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 HSA22q could be valuable for early diagnosis and treatment.

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

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Identificación de genes candidatos en las comunicaciones interventriculares congénitas con pérdida de heterocigosis de HSA22q11

Introducción y objetivos. La comunicación interventricular (CIV) es una de las principales formas de cardiopatía congénita (CPC) en los individuos con el síndrome de delección del cromosoma 22q11 del *Homo sapiens* (HSA22q11). Con objeto de identificar los genes candidatos situados en el HSA22q11 asociados a la CIV, se analizó la pérdida de heterocigosis (LOH) mediante determinación del genotipo de microsatélites y análisis de dosis de 7 genes candidatos.

Métodos. Se investigó a un total de 82 familias con CPC, en las que había 261 individuos (85 pacientes, 176 hermanos y progenitores). Se efectuó un examen de detección de la LOH en la región HSA22q11 mediante determinación del genotipo de microsatélites (n = 10). Las estrategias de bioinformática permitieron caracterizar más detalladamente 7 genes candidatos situados en esa región. Se aplicaron reacciones en cadena de polimerasa cuantitativas para el análisis de dosis a siete genes candidatos de 16 pacientes con LOH de HSA22q11.

Resultados. En 42 (49,4%) de los 85 pacientes con CPC se identificó la presencia de al menos una LOH en la región HSA22q11. Se observaron LOH de HSA22q11 en 17 de 29 pacientes con CIV y en 3 de 4 familias con ambos hijos afectados por CPC. El análisis de dosis realizado para siete genes candidatos indicó una delección heterocigota recurrente de los genes *HIRA*, *GNB1L* y *TUBA8* en 16 pacientes con CIV que presentaban LOH de HSA22q11.

Conclusiones. La determinación del genotipo de microsatélites identificó la LOH de HSA22q11 en varios tipos de CPC. La delección heterocigota de los genes *HIRA*, *GNB1L* o *TUBA8* podría desempeñar un papel importante en el desarrollo del tabique ventricular. Dado que la CPC puede ser una enfermedad familiar, el examen de detección de LOH de HSA22q en los hermanos del caso índice será útil para un diagnóstico y un tratamiento precoces.

Palabras clave: Cardiopatías congénitas. Análisis de dosis génica. Genotipo 22q11del.

ABBREVIATIONS

CHD: congenital heart disease
 FISH: fluorescent in situ hybridization
 HSA22q11: *Homo sapiens* chromosome 22q11
 LOH: loss of heterozygosity
 PCR: polymerase chain reactions
 VSD: ventricular septal defect

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.¹ 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).² The CHDs are present in 75% to 80% of patients with a *Homo sapiens* autosomal 22q11 (HSA22q11) deletion and most cases were regional hemizygosity or monosomies, and resulting in haploinsufficiencies,^{3,4} including syndromic cases (CHD associated with at least 1 extracardiac anomaly) and non-syndromic familial cases.^{2,5,6} 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, atrioventricular canal septal defects, conotruncal septal defects, and ventricular septal defect (VSD), represent a major cause of congenital heart disease.⁷

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.^{3,8-11} 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.^{9,11,12} However, microsatellite genotyping provides a higher resolution (locus-based) than karyotyping (Mb-based resolution) to diagnose HSA22q11 microdeletions in karyotypically normal

patients.^{5,8,13} 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.¹³ The HSA22q11 deletion in syndromic CHD patients was further confirmed by haplotype analysis, dual-labeling FISH and quantitative polymerase chain reactions (PCR).^{13,14} 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.¹⁵ 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^{13,14} and the distal marker, D22S315, was selected from *Map Viewer* (<http://www.ncbi.nlm.nih.gov/mapview/>; NCBI, USA)¹⁶ (Figure 1). Primer sequences for microsatellite genotyping (not shown) were directly retrieved from *Electronic PCR* (<http://www.ncbi.nlm.nih.gov/sutils/e-pcr/>; NCBI, USA).¹⁶ To multiplex PCR-based genotyping, one primer of each pair was either labeled with FAM (D22S264,

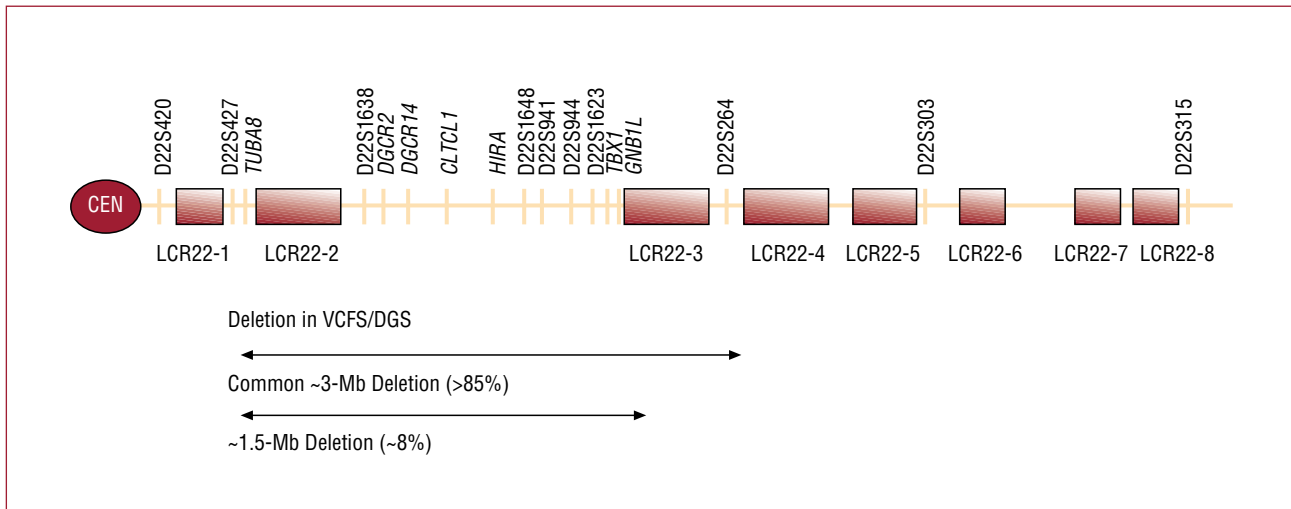


Figure 1. Genomic organization of the *Homo sapiens* (HSA) autosomal 22q11 (HSA22q11) and markers used in this study. D22S- are polymorphic microsatellites. A total of 8 low copy repeats, termed LCR22-1 to -8, have been mapped to this region. The common ~3-Mb typically deleted region, which is present in more than 85% of velo-cardio-facial 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%).

D22S303, D22S420, D22S941, D22S1638, and D22S1648), HEX (D22S427, D22S944, D22S1623), or TET (D22S315). Multiplex PCRs were performed in primers with different fluorescent labels or with the same label but a different range (in size) of amplicons. The PCR conditions and microsatellite genotyping methods have been described in our earlier studies.^{5,13} For each sample, all markers were analyzed at least 3 times to confirm the genotypes.

Bioinformatics Strategies to Identify Candidate Genes

*Ensemble Genome Browser*¹⁷ (<http://www.ensembl.org/index.html>), *UCSC Genome Browser*¹⁸ (<http://genome.ucsc.edu/>) and *NCBI Map Viewer*¹⁶ were used to identify CHD-related candidate genes in the HSA22q11 region. Tissue specificities of candidate genes were inspected with the *Swiss-Prot database*¹⁹ (<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,

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: $DDCt = DcT(\text{candidate gene, unknown sample}) - DcT(\text{candidate gene, common reference gDNA})$. The relative gene copy number was calculated by the expression 2^{-DDCt} . 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.

TABLE 1. Primers Used for Quantitative Polymerase Chain Reaction

Gene	Primers Sequence (5' to 3')	Length, mers	Amplicon Size, bp	Tm, °C	Slope ^a
<i>ACTB</i>	F: CCCAG CCATG TACGT TGCT	19	86	59.8	–
	R: CGTCA CCGGA GTCCA TCAC	19		60.1	
<i>CLTCL1</i>	F: CCGCT GTTAC GAGGA GGGAA TG	22	120	60.4	0.093
	R: TTGTC CACTG CTGCC TGATA CTC	23		60.1	
<i>DGCR2</i>	F: CCAGA GCTAG AGGGT GATGC	20	121	59.9	–0.074
	R: GGGCA CAGGG AACTT GATAA	20		59.9	
<i>DGCR14</i>	F: ACTGG AGAAA GGGGC TGTG	19	106	60.2	–0.089
	R: TCAAG GGGCC AATTA AACAG	20		59.9	
<i>GNB1L</i>	F: CCGTG TGCTT GGAGA GTGTG G	21	107	60.7	–0.081
	R: AATCT GAACC TCGTC GCTGC C	21		60.3	
<i>HIRA</i>	F: AGGTG GAGAA TGAAG TGACA GTGG	24	116	60.2	–0.064
	R: GCCCG CAGCA GTGAG GATC	19		60.1	
<i>PFKFB1</i>	F: AGCCC ACTAC TGAGC CCTTT C	21	146	56	0.097
	R: GTTGC GGAGG ACTTC TTCAC TG	22		57	
<i>TBX1</i>	F: TGGGA GAAAT GGGCG TCTTG TC	22	142	60.3	–0.070
	R: TTGCG TGGAT CAGGA AGGTG AG	22		60	
<i>TUBA8</i>	F: GACAA CGAAG CCATC TATGA CATC	24	119	57.7	–0.054
	R: AAGCG GAGAG AAGCA GTGAT TG	22		58.4	

^aValidation 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 ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{ACTB}}$) 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

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

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

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

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

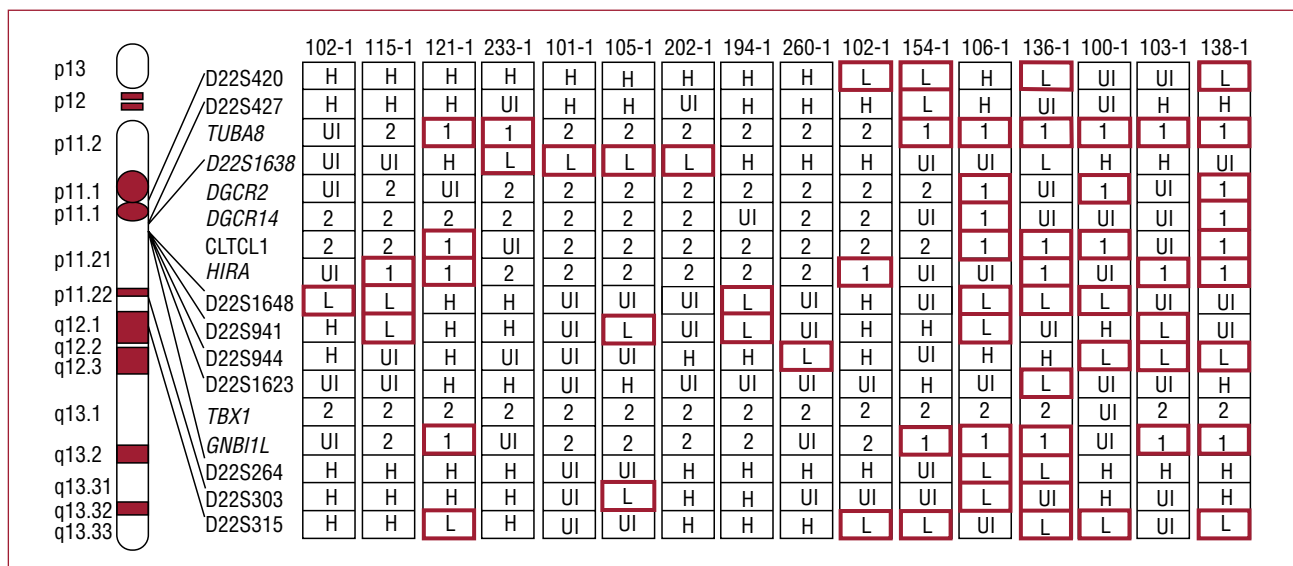


Figure 2. Combination of loss of heterozygosity (by microsatellite genotyping) and gene dosage (by quantitative polymerase chain reaction) data in 16 ventricular septal defect patients. For microsatellite markers (D22S-). H indicates retention of heterozygosity; L, loss of heterozygosity; UI, uninformative;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

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

Family	ID ^b	CHD	Subtype	D22S420	D22S427	D22S1638	D22S1648	D22S941	D22S944	D22S1623	D22S264	D22S303	D22S315
102	F			140	98/102	116	178	230/256	158	152	198/206	228/230	194
	M			136	98/102	114/116	176	244	156/158	152	200	228	194/196
	1	VSD	Apical muscular	136/140	98/102	116 (UI)	178 (LOH)	244/256	158/158	152 (UI)	198/200	228/230	194/194
	2	VSD	Membranous	136 (LOH)	102/102	114/116	176/178	230/244	156/158	152 (UI)	200/206	228 (UI)	196 (LOH)
352	F			140/148	110	114/118	172/176	244	164/166	152/154	198/200	218/230	192/200
	M			136/142	110	110/114	176	230	164/166	148/154	198/200	230	194/200
	1	VSD	Membranous	142/148	110 (UI)	110/114	172/176	230/244	164/166	148/154	198/200	230 (UI)	192/200
	2	VSD	Membranous	136/140	110 (UI)	114/118	176 (UI)	230/244	164/166	148/154	198/200	218/230	194/200
260	F			132/140	96/104	102/108	172/176	230/244	162	152	198/208	218	194/198
	M			134/144	98/102	110/116	176	244	164	152/168	202	218	184/194
	1	VSD	Membranous	140/144	98/104	102/116	176 (UI)	244 (UI)	164 (LOH)	152 (UI)	198/202	218 (UI)	184/198
	2	ASD		132/144	98/104	102/116	176 (UI)	244 (UI)	164 (LOH)	152 (UI)	198/202	218 (UI)	184/198
106	F			136/140	96/98	114/122	178	244	150/162	152	190/206	218	194/198
	M			136/142	96/98	112/122	176	230/256	146/162	152	200	232	194/198
	1	VSD	Membranous	140/142	98/98	122 (UI)	178 (LOH)	244 (LOH)	146/162	152 (UI)	200 (LOH)	218 (LOH)	198 (UI)
	2	PS		140 (LOH)	96 (UI)	122 (UI)	178 (LOH)	244 (LOH)	162 (UI)	152 (UI)	190/200	218/232	194 (UI)
157	F			140	96/98	108/114	174/176	260	158	150/154	206	220/234	180/200
	M			126/142	94/98	106/112	174/176	244/256	158	154	202/206	220/234	188
	1	VSD	Atrioventricular canal	140 (LOH)	96/98	108/112	174/176	256/260	158 (UI)	154 (UI)	206 (UI)	220 (UI)	188/200
	2			140/142	96/98	106/114	174/176	256/260	158 (UI)	150 (LOH)	206 (UI)	220 (UI)	200 (LOH)
100	F			142 (LOH)	96/98	114 (LOH)	176 (UI)	244/260	158 (UI)	150/154	206 (UI)	220/234	200 (LOH)
	M			174/184	98	114/120	178	244	156/158	152	200/204	218/230	194
	1	VSD	Subpulmonary	184	98	112/114	174	240/244	158	152	204/208	218/232	196
	2			184 (UI)	98 (UI)	112/120	178 (LOH)	240/244	156 (LOH)	152 (UI)	200/204	218/232	196 (LOH)
297	F			174/184	98 (UI)	114 (UI)	174/178	244 (UI)	158 (UI)	152 (UI)	204 (UI)	218/232	194 (LOH)
	M			174/184	98 (UI)	114 (UI)	178 (LOH)	244 (UI)	158 (UI)	152 (UI)	204 (UI)	218/232	194 (LOH)
	1	VSD	Membranous	134/136	94/98	106/114	172/176	230/244	164/168	148/152	190/206	218/230	186/196
	2			132/140	98/102	112	172/176	230/244	162/164	148/152	190/204	228/230	186/196
201	F			132/136	98/102	106/112	176 (UI)	244 (UI)	162/168	152 (UI)	204/206	218/230	186/196
	M			134/140	98/102	112/114	176 (UI)	244 (UI)	162/164	152 (UI)	204/206	228/230	186/196
	1	PDA		130/138	98/102	110	172	244/256	162	150/154	200/202	218/234	194/198
	2			132/138	96/98	114/120	172/176	N/A	160/164	148/152	200/204	218/230	186/188
204	F			132/138	96/102	110 (LOH)	172/176	256/262	162/164	148/150	200/202	218/234	186/194
	M			138	96/102	112/118	172/176	N/A	164	152	198/204	218	186/192
	1	TOF		134/142	94/98	108/116	172/176	230	162	152	198/200	218/230	182/186
	2			138/142	98/102	108/118	172/176	230 (UI)	162/164	152 (UI)	198 (UI)	218/230	182/186
				134/138	94/102	112/116	176 (UI)	244 (UI)	162/164	152 (UI)	198/204	218/230	182/186

ASD indicates atrial septal defect; CHD, congenital heart disease; PDA, patent ductus arteriosus; PS, pulmonary stenosis; TOF, tetralogy of Fallot; VSD, ventricular septal defect.

^aMicrosatellite genotypes: number, allele size (allele); UI, uninformative; LOH, loss of heterozygosity when compared to parents' genotypes; N/A, DNA sample non-available.^bID: F, father; M, mother, numbers are the order of children within the family.

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*, *GNBIL*, 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 hemizygotously deleted on HSA22q11, and was found to be a

strong candidate for DiGeorge syndrome.²⁵ 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 DCt (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.²⁴ 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 ($2^{-\Delta\Delta C_t}$) in the male and female genomes, represent the relative gene dosages of 1 copy and 2 copies. By quantitative PCR, average gene dosages (ie, the relative fold changes, $2^{-\Delta\Delta C_t}$) of 1 copy (*PFKFB1* in males) and 2 copies (*PFKFB1* in females) were detected as 0.52 (0.15) (mean [SD]) and 1.0 (0.17), respectively. Thus, ranges of 95% coverage (mean [2SD] in 2-tail distribution) of gene dosages with 1 and 2 copies were 0.22 (0.82) and 0.66 (1.34). "Uninformative" (UI) was designated when gene dosages fell below 0.22, between 0.66 and 0.82, or above 1.34 (data not shown).

Genomic dosages of 7 candidate genes, ie, *TUBA8*, *DGCR2*, *DGCR14*, *CLTCL1*, *HIRA*, *TBX1*, and *GNB1L* (from centromere to telomere), in HSA22q11 region as identified by bioinformatic strategies were further analyzed by quantitative PCR. Due to inadequate DNA samples in patient 119-1, a total of 16 VSDs encompassing at least 1 LOH in HSA22q11 region were examined. Genomic dosages of candidate genes were compared with ranges of the standard fold change ($2^{-\Delta\Delta C_t}$) as established above. No gene deletion was detected in six VSD patients (102-1 apical muscular; 101-1, 105-1, 194-1, 202-1, and 260-1 membranous type; data not shown). Patients 138-1 (subpulmonary) and 106-1 (membranous) were identified to be heterozygous deletion of 6 and 5 candidate genes, respectively. Sixteen examined VSD patients preserved both copies of *TBX1* gene. The *HIRA* or *TUBA8* were examined as the only heterozygous deleted genes in 2 (115-1 subpulmonary; 102-2 membranous) and 1 (233-1 subpulmonary) patients, respectively (Figure 2). Among 7 candidate genes, heterozygous deletion of *TUBA8*, which was found in 8 probands, was predominant. Heterozygous deletions of *HIRA* and *GNB1L* genes were found to be the second most prevalent (6 cases in each gene) (Figure 2).

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 patients²⁶ (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.^{5,8,13} 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 dysmorphism 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 dysmorphism 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 identified 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-affected children from 2 patients' families, prompting us to monitor their CHD incidence carefully. These results indicate the clinical significance of screening all siblings of each proband for early diagnoses and of the design of treatment strategies.

Bioinformatics strategies additionally identified 7 candidate genes located within the HSA22q11

LOH region. To narrow down specific gene(s) 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, *TBX1* was thought to be a major candidate gene to the cardiac phenotype or its severity in patients carrying the HSA22q11 microdeletion.³¹ Although *Tbx1*^{+/−}/*Tbx1*^{−/−} heterozygous mutant mice survived at normal Mendelian ratios and had only mild cardiovascular defects, *Tbx1*^{−/−} homozygous mice died at birth with cleft palate, thymus gland aplasia, a single outflow tract and VSD.²⁵ Unexpectedly, in this study, no hemizygous or homozygous deletion of *TBX1* 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 *TBX1* gene in patients with HSA22q11 deletion syndrome but without 22q11 microdeletion or any other detectable rearrangements in this region were identified.^{32,33} 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 *TBX1* 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.³⁴ 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.³⁵ 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 al³⁶ (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 aortic arch arteries, the ventricular function, or the alignment of the outflow tract.³⁶ 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.³⁷ 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.³⁸ The *GNBIL* encodes a G-protein β -subunit-like polypeptide. Northern blot analysis by Gong et al³⁹ (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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