Genetic Analysis of Chromosome 22q11.2 Markers in Congenital Heart Disease

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Congenital heart disease (CHD) is a common cardiac defect found in infants and children. Despite advances in diagnosis and treatment, our understanding of the causative mechanism and etiology of CHD is limited. To determine the genetic etiology of CHD, we selected 11 consecutive short tandem-repeat polymorphic (STRP) markers located in the interval of the 22q11.2 region to perform genotype analysis on a large number of CHD patients (>120) and their normal relatives (>220). The results show that as regards the distribution of allelic size and frequency of these STRP markers, there were no significant differences between the CHD patients and the normal volunteers. This indicates that there is no linkage disequilibrium with these markers in CHD. In the level of

heterozygosity for each marker in non-syndromic CHD and conotruncal heart defect (CTD), there were no significant differences between the two populations. In syndromic CHD, the level of heterozygosity for D22S1648 was significantly lower than that observed in the unaffected population ($\chi^2 = 11.25$; $P = 0.001$). This suggests that there may be a deletion at the D22S1648 locus, and the low heterozygosity of D22S1648 indicates that this marker can be used as a genetic marker for detecting microdeletions in 22q11.2. With the use of fluorescence in situ hybridization (FISH) and real-time quantitative polymerase chain reaction (PCR) performed on syndromic patients, we confirmed the molecular results. *J. Clin. Lab. Anal.* 17:28–35, 2003. © 2003 Wiley-Liss, Inc.

Key words: CHD; STRP marker; chromosome 22q11.2; microdeletion

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 fetus (1). CHD can be caused by polygenic inheritance or multiple factors (2). Molecular analysis has indicated that 8% of CHD cases are due to chromosomal abnormality, 2% to environmental teratogens, and 90% to multiple factors (3).

The genetic cause of CHD, which manifests as cardiac defects, abnormal face, thymic hypoplasia, cleft palate, and hypocalcemia, is deletions in chromosome 22 (3). The cardiac defects involve abnormalities of conotruncal development (such as persistent truncus arteriosus, tetralogy of fallot (TOF), and interrupted aortic arch), and three other syndromes: DiGeorge syndrome (DGS), velocardiofacial syndrome (VCFS), and conotruncal

anomaly face syndrome (CTAFS) (4–8). Cytogenetic and molecular studies have revealed that microdeletions in the same specific region of chromosome 22q11.2 occur in 80–95% of these patients (9,10).

Since chromosome 22q11.2 plays an important role in cardiac development, this region may contain the genetic factors involved in the pathogenesis of CHD.

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In an attempt to determine the molecular basis of CHD, we chose 11 consecutive highly polymorphic short tandem-repeat polymorphic (STRP) markers located in chromosome 22q11.2, and examined variant phenotypic CHD patients with different types of cardiac abnormalities (both syndromic and nonsyndromic) and conotruncal heart defect (CTD), and their normal relatives. By genescan analysis in a large number of CHD patients (> 120) and normal subjects (> 220), the heterozygosity status and allele assessment of the 11 STRP markers in 22q11.2 were defined. According to the present results, linkage disequilibrium for these markers among CHD patients was detected, and integral genotyping data for chromosome 22q11.2 in CHD patients and the general population in Taiwan were established.

MATERIALS AND METHODS

DNA Extraction

Genomic DNA was prepared from peripheral blood leukocytes using a genomic DNA isolation kit (Blossom, Taipei, Taiwan).

Genotyping

Based on the physical map constructed by Morrow et al. (11), we selected 11 short tandem-repeat polymorphic markers (D22S420, D22S427, D22S1638,

D22S941, D22S1648, D22S944N, D22S1623, D22S264, D22S311, D22S303, and D22S257) for genotyping analysis. Primers for these markers were divided into three groups and labeled with different fluorescent dyes. Group 1, including D22S1648, D22S264, D22S311, and D22S257, was labeled with FAM-blue (6-carboxyfluorescein.). Group 2, including D22S1638, D22S941, D22S944N, and D22S420, was labeled with TET-green (tetrachloro-6-carboxyfluorescein). Group 3, including D22S427, D22S1623, and D22S303, was labeled with HEX-yellow (hexachloro-6-carboxyfluorescein).

Multiplex polymerase chain reaction (PCR) was carried out with the primers in each group separately. The total volume of PCR amplification is 25 μ L containing 100 ng DNA, 1X Taq polymerase buffer (1.5 Mm $MgCl_2$), 200 μ M dNTPs, and 10 pmole of each primer labeled with three different fluorescent dyes. The cycling conditions consisted of 1 cycle at 95°C, followed by 25 cycles of touch-down PCR at 95°C for 20 sec, 58°C for 20 sec, and 72°C for 30 sec. The annealing temperature was decreased 0.5°C per cycle during the amplification and followed by one cycle at 72°C for 30 min, and held at 4°C.

The PCR products were denatured by adding 2.5 μ L of deionized formamide, 0.5 μ L of blue dextran, and 0.5 μ L DNA ladder, which were used to determine the size of the allele. The mixture were heated for 3 min at

TABLE 1. The Clinical Features and Numbers of CHD

Diagnosis		Developmental abnormality and physical findings	Numbers of patients
Conotruncal defect (CTD)	TOF, DORV, Truncarterosus	A	25
	TOF, ECD, ASD, LV hypoplasia		1
Non-syndromic CHD	Isolated valvular stenosis	B	8
	ASD	B	8
	Heterotray		10
	Coronary AV fistula		1
	Ebstein anomaly	C	2
	ECD	D	1
	Hypertrophic cardiomyopathy		1
	MR		1
	PDA	A, B	13
	VSD	A, B, C	45
	CoA,VSD	B	5
Syndromic CHD	CATCH 22		6
	Di-George		11
	VCFS		8
	VCFS, Pirre-Robin syndrome		1

A. Abnormal tissue migration—tetralogy of fallot (TOF), double outlet right ventricle (DORV), pulmonary atresia with ventricular septal defect, truncus arteriosus, interruption of the aortic arch, type B, complete transposition of the great vessels.

B. Abnormal blood flow patterns—ventricular septal defect-membranous (VSD), atrial septal defect-secundum (ASD), coarctation of the aorta (CoA), aorta valve stenosis, hypoplastic left heart, pulmonary valve stenosis, pulmonary atresia with intact septum, patent ductus arteriosus (PDA).

C. Abnormal cellular death—ventricular septal defect-muscular, Ebstein's anomaly.

D. Abnormal extracellular matrix—atrial septal defect-primus, atrioventricular septal (atrioventricular canal) defect.

95°C, followed by electrophoresis of 1.5 µL of each sample, through 4% denaturing polyacrylamide gel. ABI Prism 377 DNA sequencer and Genescan 3.1 software (PE Applied Biosystems, Foster City, CA) were used to analyze the data.

RESULTS

The clinical diagnoses of the CHD patients examined in this study, and the patients' numbers are listed in Table 1. Based on anatomic pathology, the 157 CHD patients were classified into three groups: CTD ($n = 26$), nonsyndromic ($n = 105$), and syndromic ($n = 26$). All of the CHD patients were sporadic. The unaffected relatives from 124 families of the 157 CHD patients were examined in this study as a normal population. Eleven consecutive STRP markers, which exist in 22q11.2~qter and extend about 5 Mb in length, were selected for genetic analysis. On the genetic map, D22S420 is the most proximal and D22S257 is distal (Fig. 1). These STRP markers were dinucleotide repeat polymorphic markers. The status of the allelic numbers and the length for each marker were defined by genescan and genotyping analyses. The details for each of the STRP markers in 22q11.2, analyzed in CHD patients and their unaffected relatives, are summarized in Table 2. By χ^2 test, each marker was tested formally for evidence of linkage disequilibrium occurring in CHD patients, and compared with the results obtained from the unaffected population. However, the results showed that none of these markers yield a significant P -value. Although no significant allelic association was found in these markers with CHD, we calculated the heterozygosity of each marker and compared the results with the data from the normal population.

The heterozygosity of these STRP markers in the normal population was high. The values were >0.7 , except for D22S1648, D22S1623, D22S303, and D22S257, which were 0.47, 0.44, 0.42, and 0.48 (Table 3). The standard χ^2 test was performed as with the markers/disease calculations, and examined separately in the normal group and three groups of CHD patients: syndromic and nonsyndromic, and CTD. The heterozygosity value for each marker in the CTD and nonsyndromic groups was not significantly different from the normal population. However, in the syndromic groups, including VCFS, DGS, and CATCH22 (cardiac defects, abnormal faces, thymic hypoplasia, cleft palate, hypocalcemia, and 22q11 deletion), the level of heterozygosity for D22S1648 was 0.12, which is significantly lower than that observed in the unaffected population ($0.47; \chi^2 = 11.25; P = 0.001$).

Fig. 1. Genetic map of the STRP marker (D22S420–D22S257) intervals in chromosome 22q11.2.

DISCUSSION

In this study, we obtained integral genetic data for 11 STRP markers in chromosome 22q11.2. The results indicate that the allelic numbers and distribution of these markers are not significantly different between CHD patients and their normal relatives. This suggests that there is no linkage disequilibrium occurring in CHD.

Previous studies have suggested that the molecular etiology of VCFS and DGS may be related to a chromosomal 22q11.2 deletion (9,10). According to the clinical findings, we divided the patients into three groups: syndromic and nonsyndromic CHD, and CTD. In the nonsyndromic CHD group, the levels of heterozygosity did not differ from those in the normal population. The heterozygosity values of D22S427, D22S1648, and D22S303 obtained from the CTD group were lower than those obtained from the normal population, but they were not significantly different. However, the heterozygosity of D22S1648 in the syndromic patients was significantly different from that in unaffected individuals. As calculated by a standard χ^2 test, the P -value was significantly less than 0.05.

These results indicate that nonsyndromic CHD is not associated with the 22q11 deletion. In CTD patients, the

TABLE 2. Details of Polymorphic Markers Used in This Study.

Allele	Allele length	Number of of chromosome		χ^2	df	P
Normal	Affected					
D22S420 (n=8)		440 ^a	258 ^b	1.11	7	0.99
1	163	16 (3.6)	9 (3.5)			
2	161	16 (3.5)	10 (3.9)			
3	159	53 (12)	35 (13.6)			
4	157	131 (29.8)	72 (27.9)			
5	155	184 (41.8)	110 (42.6)			
6	153	27 (6.1)	16 (6.2)			
7	151	12 (2.7)	5 (1.9)			
8	149	1 (0.2)	1 (0.4)			
D22S427 (n=9)		422 ^a	254 ^b	6.81	8	0.55
1	109	9 (2.1)	3 (1.2)			
2	107	2 (0.5)	2 (0.8)			
3	105	9 (2.1)	5 (2.0)			
4	103	112 (26.5)	51 (20.1)			
5	101	19 (4.5)	11 (4.3)			
6	99	32 (7.6)	28 (11.0)			
7	97	231 (54.7)	147 (57.9)			
8	95	5 (1.2)	4 (1.6)			
9	93	3 (0.7)	3 (1.2)			
D22S1638 (n=11)		448 ^a	258 ^b	6.78	10	0.74
1	123	9 (2.0)	4 (1.6)			
2	121	10 (2.2)	3 (1.2)			
3	119	21 (4.7)	14 (5.4)			
4	117	120 (26.8)	73 (28.3)			
5	115	74 (16.5)	39 (15.1)			
6	113	50 (11.2)	32 (12.4)			
7	111	96 (21.4)	53 (20.5)			
8	109	18 (4.0)	9 (3.5)			
9	107	45 (10.0)	26 (10.1)			
10	105	1 (0.2)	4 (1.6)			
11	103	4 (0.9)	1 (0.4)			
D22S941 (n=14)		434 ^a	256 ^b	10.27	13	0.67
1	264	1 (0.2)	0			
2	262	2 (0.5)	2 (0.8)			
3	260	2 (0.5)	3 (1.2)			
4	258	9 (2.1)	10 (3.9)			
5	256	8 (1.8)	4 (1.6)			
6	254	44 (10.1)	26 (10.2)			
7	252	122 (28.1)	75 (29.3)			
8	250	9 (2.1)	12 (4.7)			
9	248	2 (0.5)	2 (0.8)			
10	244	1 (0.2)	0			
11	242	23 (5.3)	10 (3.9)			
12	240	170 (39.2)	90 (35.2)			
13	236	1 (0.2)	1 (0.4)			
14	226	40 (9.2)	21 (8.2)			
D22S1648 (n=5)		438 ^a	248 ^b	0.74	4	0.94
1	177	34 (7.8)	18 (7.3)			
2	175	308 (70.3)	172 (69.4)			
3	173	24 (5.5)	17 (6.9)			
4	171	71 (16.2)	40 (16.1)			
8	169	1 (0.2)	1 (0.4)			
D22S944N (n=11)		422 ^a	256 ^b	5.06	10	0.79
1	208	3 (0.7)	1 (0.4)			
2	204	8 (1.9)	5 (2.0)			
3	202	5 (1.2)	2 (0.8)			
4	200	19 (4.5)	11 (4.3)			
5	198	50 (11.8)	28 (10.9)			
6	196	187 (44.3)	127 (49.6)			
7	194	120 (28.4)	62 (24.2)			

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TABLE 2. Continued

Allele	Allele length	Number of of chromosome		χ^2	df	P
		Normal	Affected			
8	192	12 (2.8)	4 (1.6)			
9	190	11 (2.6)	10 (3.9)			
10	188	1 (0.2)	1 (0.4)			
11	182	6 (1.4)	5 (2.0)			
D22S1623 (n = 5)		410 ^a	256 ^b	5.09	4	0.27
1	155	1 (0.2)	0			
2	153	91 (22.2)	71 (27.7)			
3	151	293 (71.5)	176 (68.8)			
4	149	14 (3.4)	6 (2.3)			
5	147	11 (2.7)	3 (1.2)			
D22S264 (n = 10)		432 ^a	258 ^b	2.149	9	0.98
1	213	4 (0.9)	2 (0.8)			
2	208	16 (3.7)	11 (4.3)			
3	206	65 (15.0)	45 (17.4)			
4	204	137 (31.7)	83 (32.2)			
5	202	41 (9.5)	21 (8.1)			
6	200	114 (26.4)	69 (26.7)			
7	198	36 (8.3)	17 (6.6)			
8	196	5 (1.2)	3 (1.2)			
9	194	1 (0.2)	1 (0.4)			
10	190	13 (3.0)	6 (2.3)			
D22S311 (n = 12)		434 ^a	258 ^b	8.20	11	0.69
1	269	6 (1.4)	1 (0.4)			
2	267	21 (4.8)	12 (4.7)			
3	265	59 (13.6)	25 (9.7)			
4	263	109 (25.1)	69 (26.7)			
5	261	32 (7.4)	14 (5.4)			
6	259	25 (5.8)	18 (7.0)			
7	257	165 (38.0)	103 (39.9)			
8	255	4 (0.9)	6 (2.3)			
9	253	4 (0.9)	4 (1.6)			
10	251	2 (0.5)	1 (0.4)			
11	245	6 (1.4)	4 (1.6)			
12	243	1 (0.2)	1 (0.4)			
D22S303 (n = 7)		414 ^a	258 ^b	3.41	6	0.75
1	230	1 (0.2)	0			
2	228	76 (18.4)	52 (20.2)			
3	226	25 (6.0)	21 (8.1)			
4	220	1 (0.2)	0			
5	218	6 (1.4)	4 (1.6)			
6	216	304 (73.4)	181 (70.2)			
7	212	1 (0.2)	0			
D22S257 (n = 7)		444 ^a	258 ^b	2.06	6	0.91
1	135	1 (0.2)	0			
2	133	119 (26.8)	69 (26.7)			
3	131	1 (0.2)	1 (0.4)			
4	129	1 (0.2)	1 (0.4)			
5	127	25 (5.6)	14 (5.4)			
6	125	295 (66.4)	170 (65.9)			
7	121	2 (0.5)	3 (1.2)			

^aNumber of normal unaffected chromosomes were analyzed.

^bNumber of affected chromosomes were analyzed.

low heterozygosity of D22S427, D22S1648, and D22S303 showed that there may be a deletion in these regions. Previous studies (8,12) of CTD found that TOF with pulmonary atresia and conotruncal anomaly face syndrome (CTAFS), double outlet right ventricle

(DORV), and truncus arteriosus were associated with a deletion within 22q11 (8,12). In the present study, we did not investigate the microdeletion in CTD because the parents of the CTD patients were unavailable for examination.

TABLE 3. Results of Heterozygosity of STRP Markers on Chromosome 22q11.2

	D22S420	D22S427	D22S1638	D22S941	D22S1648	D22S944N	D22S1623	D22S264	D22S311	D22S303	D22S257
Heterozygosity ^a in unaffected relatives (n = 220)	0.71	0.62	0.83	0.75	0.47	0.70	0.44	0.79	0.76	0.42	0.48
Heterozygosity in CTD patients (n = 25)	0.69	0.53	0.82	0.73	0.42	0.64	0.49	0.72	0.72	0.33	0.63
Heterozygosity in non-syndromic patients (n = 92)	0.71	0.62	0.81	0.76	0.51	0.68	0.43	0.77	0.75	0.45	0.50
Heterozygosity in syndromic patients (26)	0.76	0.71	0.86	0.67	0.12	0.66	0.35	0.78	0.68	0.49	0.51
(χ^2) ^b					11.25						
<i>p</i>					0.001						

^aHeterozygosity was calculated as $1 - \sum P_i^2$, where P_i is the individual allele frequency.

^b χ^2 test and *P* value were calculated in unaffected relatives and in syndromic patients.

Heterozygosity of D22S1648 in the syndromic group was significantly lower than that observed in the unaffected population. The significant deviation from the expected level of heterozygosity at D22S1648

loci implied that the region might be hemizygous among the syndromic CHD patients. By haplotype analysis, comparing the data of syndromic patients with those of their unaffected parents and relatives, we

Fig. 2. FISH of DGS/VCFS region probe (TUPLE1-Texas Red) and a control probe (N85A3-FITC) hybridized to metaphase peripheral blood cells (VCSF 3). Absence of the red signal on one chromosome 22 (arrow del (22)) indicates deletion of the TUPLE1 locus at 22q11.2.

Fig. 3. The amplification plots of the target gene (D22S1648) for triplication reactions of two probands (VCFS 3 and VCFS 5) and their unaffected parents (VCFS 3.2 and VCFS 5.1). The graphs show the fluorescence intensity above baseline (ΔRn) of each PCR vs. the cycle number, and the y-axis (ΔRn) is displayed in linear scale. Using β -actin as an endogenous control, the two probands showed lower ΔRn than the unaffected parents. This means that the amplification of D22S1648 in the two probands was significantly lower than in their unaffected parents, and that both of the probands have lost one allele at this locus.

found that 17 out of 26 syndromic patients had detected deletions. The deletion region was estimated to be 1.5–3 Mb in length and boundaries spanned seven interstitial markers (from D22S1638 to D22S311) (13). This result suggests that D22S1648 can provide a clue as to the identity of the region that is commonly deleted in syndromic CHD. Moreover, we also conducted dual-labeling fluorescence in situ hybridization (FISH) and real-time quantitative PCR analyses on the syndromic patients to confirm the molecular results. The results of the FISH analysis are shown in Fig. 2, and the real-time quantitative PCR for VCFS results are shown in Fig. 3 and Table 4.

These data identified the deletion status in VCFS and confirmed that D22S1648 can be used as a genetic marker for detecting microdeletions in 22q11.2 region.

In this study we selected STRP markers that span 5 Mb in the 22q11.2 region (resolution = ~300 kb) (13). By haplotype analysis, the heterozygous, homozygous, or hemizygous status of each loci was accurately determined. This method is more useful than FISH or quantitative PCR for the rapid detection of microdeletions in chromosomes (13). Because of the low level of heterozygosity of D22S1648, this marker can be applied as a genetic probe to detect the chromosomal

TABLE 4. Comparison of Contents of Target Gene (D22S1648) to Control Gene (β -actin)

	Gene	C_T average	$\Delta C_T(\beta\text{-actin-D22S1648})^a$	$2^{\Delta C_T^b}$
VCFS 3	β -actin	19.5167 ± 0.1901		
	D22S1648	20.7467 ± 0.0503	-1.23	0.4263
VCFS 3.1	β -actin	20.750 ± 0.2758		
	D22S1648	20.320 ± 0.2170	0.43	1.3472
VCFS 3.2	β -actin	20.015 ± 0.0212		
	D22S1648	19.695 ± 0.1677	0.32	1.2483
VCFS 5	β -actin	19.8700 ± 0.2546		
	D22S1648	20.9600 ± 0.0872	-1.09	0.4698
VCFS 5.1	β -actin	19.1250 ± 0.3323		
	D22S1648	18.6650 ± 0.5445	0.46	1.3755
VCFS 5.2	β -actin	19.4067 ± 0.1242		
	D22S1648	18.8267 ± 0.1955	0.58	1.4948

^a ΔC_T : D22S1648 normalized to β -actin.^b $2^{\Delta C_T}$: the content of D22S1648 relative to β -actin.

deletion in syndromic CHD patients. Understanding the molecular genetics of CHD will reduce the cost of diagnosis and treatment, and help prevent the morbid and sometimes lethal anomalies associated with this disease.

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