

Spectrum of *CHD7* Mutations in 110 Individuals with CHARGE Syndrome and Genotype-Phenotype Correlation

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CHARGE syndrome is a well-established multiple-malformation syndrome with distinctive consensus diagnostic criteria. Characteristic associated anomalies include ocular coloboma, choanal atresia, cranial nerve defects, distinctive external and inner ear abnormalities, hearing loss, cardiovascular malformations, urogenital anomalies, and growth retardation. Recently, mutations of the chromodomain helicase DNA-binding protein gene *CHD7* were reported to be a major cause of CHARGE syndrome. We sequenced the *CHD7* gene in 110 individuals who had received the clinical diagnosis of CHARGE syndrome, and we detected mutations in 64 (58%). Mutations were distributed throughout the coding exons and conserved splice sites of *CHD7*. Of the 64 mutations, 47 (73%) predicted premature truncation of the protein. These included nonsense and frameshift mutations, which most likely lead to haploinsufficiency. Phenotypically, the mutation-positive group was more likely to exhibit cardiovascular malformations (54 of 59 in the mutation-positive group vs. 30 of 42 in the mutation-negative group; $P = .014$), coloboma of the eye (55 of 62 in the mutation-positive group vs. 30 of 43 in the mutation-negative group; $P = .022$), and facial asymmetry, often caused by seventh cranial nerve abnormalities (36 of 56 in the mutation-positive group vs. 13 of 39 in the mutation-negative group; $P = .004$). Mouse embryo whole-mount and section in situ hybridization showed the expression of *Cbd7* in the outflow tract of the heart, optic vesicle, facio-acoustic preganglion complex, brain, olfactory pit, and mandibular component of the first branchial arch. Microarray gene-expression analysis showed a signature pattern of gene-expression differences that distinguished the individuals with CHARGE syndrome with *CHD7* mutation from the controls. We conclude that cardiovascular malformations, coloboma, and facial asymmetry are common findings in CHARGE syndrome caused by *CHD7* mutation.

CHARGE syndrome (MIM 214800) is a distinctive syndrome with a complex constellation of multiple congenital anomalies. The incidence of CHARGE syndrome may be as high as 1 in 8,500 births (Issekutz et al. 2005). It is characterized by variable occurrence of coloboma, choanal atresia or cleft lip and/or palate, cranial nerve dysfunction, characteristic external ear malformations with distinct temporal bone anomalies, cardiovascular malformations, and hypogonadotropic hypogonadism with genitourinary anomalies (Hall 1979; Hittner et al. 1979; Pagon et al. 1981; Blake et al. 1998). Inner ear defects, including Mondini malformation and/or semi-circular canal hypoplasia/aplasia are common and often cause hearing loss and vestibular abnormalities in the affected individuals (Amiel et al. 2001). Many types of cardiovascular malformations have been reported, but

conotruncal and aortic arch malformations appear to be more common than the others (Blake et al. 1998). Growth delay, distinctive facial features, DiGeorge sequence, and tracheoesophageal fistula are some of the additional clinical features of this condition. In the 25 years since its initial characterization, the diagnostic criteria for the original CHARGE acronym were revised and standardized (Blake et al. 1998), with a further proposal to update them (Verloes 2005). What was considered a prototypic “association” is now viewed as a syndrome in many well-defined cases (Lubinsky 1994), for which the name “Hall-Hittner syndrome” may also apply (Graham 2001). Vissers et al. (2004) reported mutations in the *CHD7* gene, which encodes chromodomain helicase DNA-binding protein, in ~60% of individuals with CHARGE syndrome. Chromatin remod-

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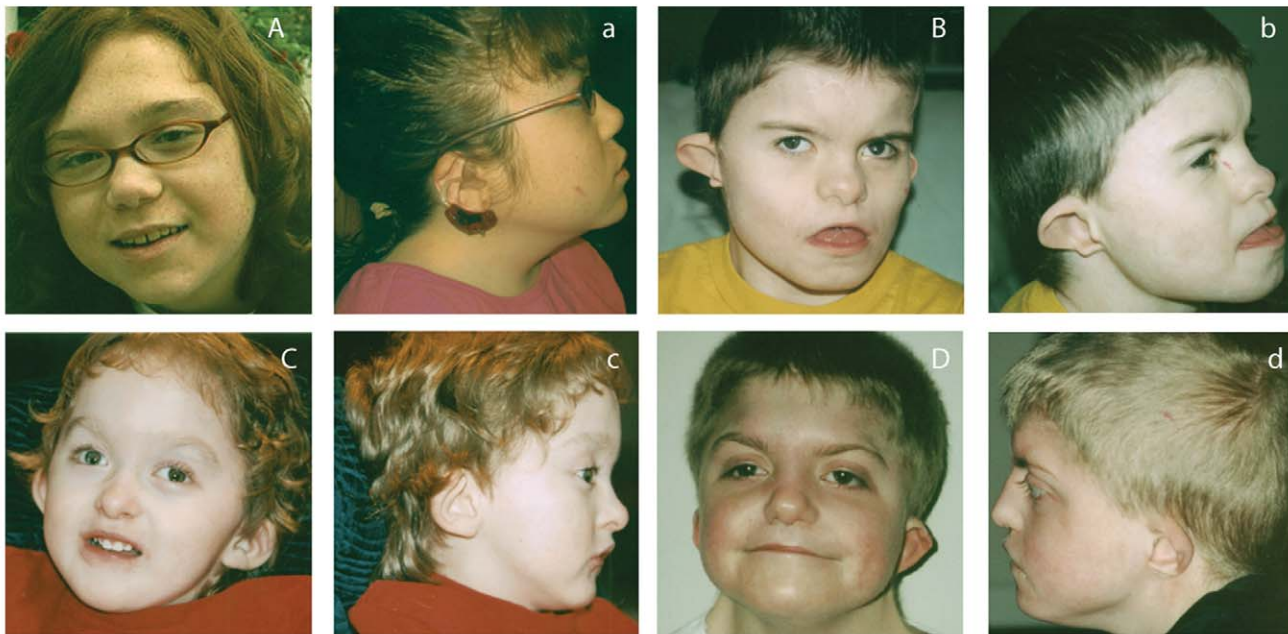


Figure 1 Individuals with CHARGE syndrome who have *CHD7* mutations. *A* and *a*, A 17-year-old girl with retinal coloboma, characteristic ear malformation, hearing loss, partial atrioventricular canal defect, growth retardation, and delayed puberty who has a frameshift mutation in exon 37. *B* and *b*, An 11-year-old boy with colobomas of the iris and the macula, choanal stenosis, characteristic ear malformation, bilateral Mondini malformation, profound hearing loss, submucous cleft palate, bilateral facial palsy, undescended testes, and pulmonary atresia with ventricular septal defect who has a de novo frameshift mutation in exon 18. *C* and *c*, A 3-year-old boy with bilateral iris coloboma, choanal stenosis, ear malformation, hearing loss, facial palsy, and abnormal cochlea but without cardiovascular malformation who has a nonsense mutation in exon 13. *D* and *d*, A 12-year-old boy with coloboma involving the optic nerve, choanal atresia, characteristic ear malformation, profound hearing loss, abnormal inner ear, patent ductus arteriosus, and genital abnormalities who has a frameshift mutation in exon 2. Note that the individuals in panels *B* and *D* illustrate the characteristic facial appearance of CHARGE syndrome.

eling is a recognized mechanism of gene-expression regulation, and the *CHD7* gene is likely to play a significant role in embryonic development and cell-cycle regulation (Woodage et al. 1997). The prevalence of *CHD7* mutations in a large cohort of individuals with CHARGE syndrome and the possible correlation of specific mutations with clinical characteristics have not been formally addressed. We report the systematic molecular and phenotypic evaluation of 110 individuals with clinical CHARGE syndrome. We identified mutations in the *CHD7* gene in 58% (64/110) of the affected individuals and analyzed the phenotypic data of the study cohort, using multivariate analysis conditional on the mutation status. Our results indicate significant phenotypic differences between *CHD7* mutation carriers and noncarriers and are consistent with locus heterogeneity as the likely explanation for the group of individuals with CHARGE syndrome whose sequence analysis of *CHD7* was normal. Nucleotide sequence data reported herein are available in the DDBJ/EMBL/GenBank databases; for details, see the Web Resources section.

Material and Methods

Clinical Evaluation

The study sample included 110 individuals with CHARGE syndrome who either had four major criteria for the diagnosis of CHARGE syndrome (coloboma, choanal atresia, characteristic ear abnormalities, and cranial nerve dysfunction), had three major and three minor criteria (genital hypoplasia, developmental delay, cardiovascular malformation, growth deficiency, orofacial cleft, tracheoesophageal fistula, and characteristic face), or had one or two major criteria with several minor criteria (Blake et al. 1998) (fig. 1). According to Blake et al. (1998), individuals with all four major criteria or with three major and three minor criteria definitely have CHARGE syndrome, and individuals with one or two major criteria and several minor criteria possibly have the syndrome. The children with CHARGE syndrome and their parents were enrolled through the CHARGE Syndrome Foundation meetings in 1999 and 2001 and through the Baylor College of Medicine Cardiovascular Genetics Web site. Informed consent was obtained from participant families. Clinical histories were obtained for all probands, as well as for any other possibly affected family members. The medical records of the individuals with

CHARGE syndrome were analyzed. The probands were ascertained by one of the dysmorphologists (S.R.L., J.W.B., C.A.B., J.M.G., or S.L.H.D.). Several individuals with CHARGE syndrome were also enrolled through evaluation of medical records. Photographs were reviewed when the individual with CHARGE syndrome was not available for clinical assessment.

The research protocol was reviewed and approved by the Baylor College of Medicine Institutional Review Board.

DNA Sequencing

Primers were designed using the program Primer 3, on the basis of the annotated genomic sequence across each exon, including regions of at least 40–50 bp of flanking intervening sequence (table A1). Larger exons were subdivided to allow for optimal product lengths. All PCR reactions were performed in a 25- μ l reaction volume containing 40 ng of genomic DNA, 10 pmol of each primer, and 12.5 μ l of Qiagen Multiplex PCR Master Mix. Conditions for PCR were 15 min of initial activation step at 95°C, followed by 40 cycles of denaturation at 94°C for 30 s and annealing at 60°C for 90 s, and a final extension at 72°C for 60 s. An annealing temperature of 55°C was used for PCR amplification of exons 30 and 38b. Sequencing of the *CHD7* coding region (exons 2–38) for mutations was performed using Big Dye Terminator Cycle Sequencing Reactions on an ABI 3700 (PE Applied Biosystems) by use of the manufacturer’s standard protocol and reagents. Sequence electropherograms were compared with gene sequences from GenBank accession number NM_017780. Sequence analysis of both the sense and antisense strands of the PCR-amplified fragments was performed with the aid of Sequencher 4.5 software.

FISH

FISH was performed on DNA from 23 individuals with CHARGE syndrome whose sequence analysis of *CHD7* was normal, by use of two clones, RP11-33111 (GenBank accession number AC113143) and RP11-174G1 (GenBank accession number AC022182), encompassing the *CHD7* gene. The BAC clones were obtained from the Children’s Hospital Oakland Research Institute BACPAC Resources Center. FISH analysis was performed as described elsewhere (Lalani et al. 2003). After hybridization and washing, biotinylated probes were detected by avidin-fluorescein, and digoxigenin-labeled probes were detected using anti-digoxigenin antibody coupled with rhodamine. The chromosome preparations were counter stained with 4',6-diamidino-2-phenylindole and were analyzed with a Zeiss Axioskop fluorescence microscope.

Mouse Embryo In Situ Hybridization

Whole-mount mouse embryo in situ hybridization was performed at 10.5 d post coitum (dpc), with modification of the

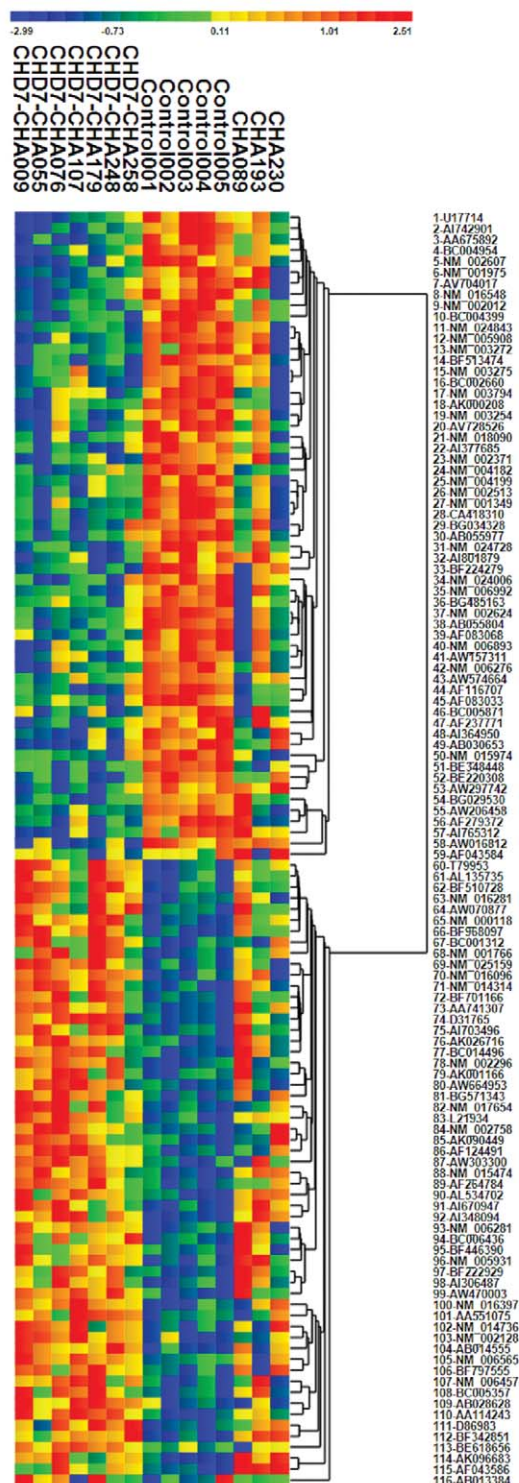


Figure 2 Microarray gene-expression profile and the resulting cluster analysis. Patterns of gene expression were assessed on Affymetrix U133Plus2.0 arrays (see “Material and Methods” section for details). After identification of a subset of transcripts with apparently significant differences in expression between the individuals with CHARGE syndrome with *CHD7* mutations and the controls, the combined data were used to produce a hierarchical cluster analysis. Expression values shown were standardized using the gene-specific mean and SD of \log_2 expression. Note that the profile of CHA230 (who had no detectable *CHD7* mutation) is highly similar to those of individuals with *CHD7* mutations.

protocol described elsewhere (Wilkinson 1992) and with the use of blocking reagent (Boehringer Mannheim). Probes were labeled using a DIG RNA Labeling kit (Boehringer Mannheim). *Chd7* riboprobes were prepared from a cDNA fragment (1587–2034) of KIAA1416 (GenBank accession number BC034239). The probe spanning 447 bp in the 3' region of the gene was generated using PCR. The primers used for PCR were forward primer 5'-GGAAACGACAATGAAGACGAGAAC-3' and reverse primer 5'-CGTTTCCGGTGTTCATCTCAT-3'. In situ hybridization on sagittal sections of 10.5-dpc embryos was performed using the same probe.

Microarray Analysis of Gene Expression

RNA was isolated from the lymphoblastoid cell lines of seven mutation-positive, three mutation-negative, and five control samples by use of the Qiagen RNeasy Mini kit. The quality and purity of total RNA was assayed in a 2% agarose gel, and the recovery was calculated after absorbance was measured with a spectrophotometer at 260 nm. Samples were labeled using the standard Affymetrix T7 oligo(dT) primer protocol and reagents supplied in the Affymetrix One-Cycle Target Labeling and Hybridization kit (catalog number 900493). Total RNA was reverse transcribed to produce double-stranded cDNA. The cDNA product was used as a template for the in vitro transcription reaction, producing biotin-labeled cRNA. The labeled cRNA was quantified by spectrophotometry (NanoDrop ND-1000). Fifteen μ g of the labeled cRNA was fragmented and rechecked for concentration. A hybridization cocktail containing Affymetrix spike-in controls and fragmented labeled cRNA was loaded onto GeneChip U133 2.0 Plus arrays. The array was hybridized overnight, for 16 h at 45°C with rotation at 60 rpm. Arrays were then washed and stained on Affymetrix Fluidics Station 400 equipment with a streptavidin, R-phycoerythrin conjugate stain. Signal amplification was done using biotinylated anti-streptavidin. The stained array was scanned on the Affymetrix GeneChip Scanner 3000. The images were analyzed, and quality-control metrics were recorded using Affymetrix GCOS software, version 1.1.2. By use of reference standards, data were normalized from different experiments and were compared with multiple experiments on a quantitative level. The expression profiles of

both groups were compared and further evaluated with control samples.

Affymetrix Data Analysis

Microarray data were analyzed with ChipST2C 1.14 (see ChipST2C Web site). Normalization of the probe-level hybridization data was performed using the Robust Multiarray Average measure (Affy, R-package) (Irizarry et al. 2003a, 2003b). Pairwise *T* tests were used to compare expression values from individuals with CHARGE syndrome and *CHD7* mutations ($n = 7$) with those from matched controls ($n = 5$) by use of ChipST2C. With a threshold of $P < .001$, 116 transcripts were identified. The analysis was repeated for these transcripts but with the addition of the individuals with CHARGE syndrome without detectable *CHD7* mutation ($n = 3$), by use of an *F* test (analysis of variance) and within-gene permutation to evaluate the empirical *P* values ($B = 1,000$ iterations). Storey *q* values were calculated to estimate the positive false-discovery rate (Storey and Tibshirani 2003). This resulted in 85 transcripts, with $q < 0.01$ for all transcripts (positive false-discovery rate 1%). The results are displayed after hierarchical cluster analysis for genes (fig. 2).

Phenotypic Data Analysis

Association of *CHD7* mutations with clinical phenotypes was computed using SPSS 12.0 (SPSS). The analysis was performed for each clinical variable, and the analysis was compared between mutation-positive and mutation-negative individuals. Types of mutations—including nonsense, frameshift, missense, and splice-site mutations—and the position of the mutations within the protein were analyzed and compared with the phenotypic data of the individuals with CHARGE syndrome. The Fisher's exact test with 1 df was used for all comparisons. The significance threshold was set at $P < .05$. All *P* values were two sided.

Results

Mutation Analysis

Among 110 individuals with clinical CHARGE syndrome in the study group, there were 105 sporadic cases;



Figure 3 Genomic and protein map of *CHD7*, indicating the spectrum of mutations in CHARGE syndrome. On the protein map, blackened circles signify nonsense mutations, blackened diamonds signify frameshift mutations, unblackened squares signify missense mutations, and the unblackened circle specifies a mutation with an in-frame deletion of 3 aa. On the genomic sequence, triangles represent splice-site mutations.

Table 1

CHD7 Mutations Observed in 64 Individuals with CHARGE Syndrome

Mutation Number	Exon	Individual ^a	Mutation	Origin ^b	Domain
1	2	95	Q112X	NA	
2	2	330	W145X	De novo	
3	2	3	Q180X	De novo	
4	2	315	T262fsX304	De novo	
5	2	125	V424fsX466	NA	
6	2	107	P366fsX377	De novo	
7	2	134	R494X	De novo	
8	2	284	H556fsX564	De novo	
9	3	28	G623fsX626	De novo	
10	4	308	c.2097-1G→A	De novo	
11	4	297	c.2238+1G→A	De novo	
12	8	34	Q839fsX843	De novo	Chromo
13	11	22	c.2836-15C→G	NA	
14	12	116	L1007C	NA	SNF2
15	12	71	T1027_P1029del	De novo	SNF2
16	12	233	W1031G	De novo	SNF2
17	13	66	R1069X	De novo	SNF2
18	13	281	R1069X	De novo	SNF2
19	13	119	V1070fsX1071	NA	SNF2
20	14	307	E1152X	NA	
21	15	206	Q1214R	De novo	
22	15	158	c.3778+1G→A	NA	
23	16	172	E1271X	NA	
24	16	182	E1271X	NA	
25	16	215	L1294P	De novo	HELICc
26	17	151	R1339X	De novo	HELICc
27	17	184	R1339X	De novo	HELICc
28	18	236	V1409fsX1420	De novo	
29	18	248	M1442fsX1443	De novo	
30	19	221	R1465X	NA	
31	19	55	R1493fsX1501	De novo	
32	20	146	W1534X	De novo	
33	20	171	L1545X	De novo	
34	21	9	R1571fsX1572	De novo	
35	21	25	S1577X	De novo	
36	21	113	c.4850+1G→A	De novo	
37	22	58	c.5050+3A→T	De novo	
38	24	49	E1751fsX1752	NA	
39	26	16	R1810X	De novo	
40	26	85	R1810X	De novo	
41	26	98	L1815P	De novo	
42	26	154	G1835fsX1843	De novo	
43	26	143	c.5534+1G→T	De novo	
44	29	300	A1896fsX1898	NA	
45	29	42	W1903X	De novo	
46	29	290	R1945X	NA	
47	30	287	C2017X	De novo	
48	30	29	R2024X	De novo	
49	30	339	c.6103+8C→T	De novo	
50	31	258	R2053X	De novo	
51	31	292	H2096R	De novo	
52	31	92	E2202fsX2208	De novo	
53	33	166	R2319S	Inherited	
54	33	76	W2332X	De novo	
55	33	176	W2332X	De novo	
56	33	179	P2333fsX2334	De novo	
57	34	239	E2450GfsX2468	De novo	
58	34	203	K2510X	De novo	
59	35	224	c.7830+11A→G	De novo	
60	36	131	R2653X	NA	BRK
61	36	255	R2653X	NA	BRK
62	36	304	R2653X	De novo	BRK
63	37	37	Q2691fsX2709	NA	
64	38	77	L2916fsX2940	NA	

^a For individual IDs, add "CHA" before the number shown.

^b NA = not available.

four familial cases and one pair of MZ twins were also included in the study. The individuals with CHARGE syndrome comprised 61 (55%) females and 49 (45%) males. Apparently pathogenic mutations in *CHD7* were found in 64 (58%) of the individuals with CHARGE syndrome, distributed throughout the gene (fig. 3 and table 1). Truncating mutations were seen in 47 (73%) of patients, including 28 stop-codon mutations and 19 frameshift mutations. Seven (11%) were missense mutations, and 9 (14%) were intron-exon boundary mutations (table 2). Either one or both parental samples were unavailable for 17 (26%) of the mutation-positive patients. All truncating mutations for which parental samples were available were found to be de novo. Of the seven missense mutations, parental samples were unavailable for one, five were found to be de novo, and one was inherited from an affected mother. Of the nine intron-exon boundary mutations, seven were found to be de novo; parental samples were unavailable for the other two. Three familial cases in this study group were found to have mutations in the *CHD7* gene (fig. 4). The missense mutation R2319S, seen in CHA166, was also identified in the mother, who was mildly affected. In another familial case, the E1271X mutation was identified in both MZ twins. The nonsense mutation W2332X was identified in a mildly affected girl whose brother died of complications of CHARGE syndrome in infancy. Although most mutations were unique, R1339X in exon 17, involving the HELICc domain, was present in both CHA151 and CHA184; R1810X in exon 26 was observed in both CHA16 and CHA85; W2332X in exon 33 was present in both CHA76 and CHA176; and R2653X in exon 36, involving the BRK domain, was present in three individuals: CHA131, CHA255, and CHA304. One mutation was detected in the chromo-domain, six in the SNF2 domain, three in the HELICc domain, and three in the BRK domain.

Of 64 individuals with the *CHD7* mutation, there were 40 (63%) females and 24 (37%) males. No sex difference was noted when they were compared with the group without *CHD7* mutations. Three of the clinical criteria showed statistically significant positive correlations with the *CHD7* mutation: cardiovascular malformations (54 of 59 in the mutation-positive group vs. 30 of 42 in the mutation-negative group; $P = .014$), coloboma of the iris, retinal, or optic nerve (55 of 62 in the mutation-positive group vs. 30 of 43 in the mutation-negative group; $P = .022$), and facial asymmetry (36 of 56 in the mutation-positive group vs. 13 of 39 in the mutation-negative group; $P = .004$). There were no significant differences between the two groups with respect to choanal atresia, external or inner ear malformations, deafness, cleft lip and palate, tracheoesophageal fistula, esophageal atresia, and urogenital abnormalities (table 3). The major and minor clinical diagnostic criteria

Table 2**Summary of Mutations Observed in the *CHD7* Gene in Individuals with CHARGE Syndrome**

Type of Mutation	Frequency			Parental Samples Unavailable
	(<i>n</i>)	De novo	Inherited	
Nonsense	28	20	0	8
Frameshift	19	13	0	6
In-frame deletion (3 aa)	1	1	0	0
Missense	7	5	1	1
Splice site	9	7	0	2

scores for each individual, as suggested by the criteria from Blake et al. (1998) and Verloes (2005), were also compared with mutation status. All 10 individuals with the combined three major signs of Verloes (coloboma, atresia of choanae, and hypoplastic semicircular canals) were found to have mutations in the *CHD7* gene ($P = .005$).

The phenotype-genotype analysis of the *CHD7* mutation-positive group revealed no clear correlation between the severity of the clinical findings and the type of mutation. Individuals with a milder phenotype were found to have mutations involving both the middle third and the C-terminal part of the protein. Ten SNPs with minor-allele frequency >0.01 in the white subjects ($n = 86$) were detected within the *CHD7* gene (table 4). There were no significant differences in allele frequencies between mutation carriers and noncarriers.

Mouse Embryo *In Situ* Hybridization

Previous research has shown that *CHD7* expression is widespread in human tissues, but the method used did not allow estimation of relative expression levels or correlation of expression pattern with the defects in organogenesis observed in individuals with CHARGE. Therefore, we examined the expression of the mouse homolog, *Chd7*, at a stage in which the relevant anlage might be visualized. Mouse embryo whole-mount and section *in situ* hybridization at 10.5 dpc showed expression of *Chd7* in the cardiac outflow tract, truncus arteriosus, facio-acoustic (VII–VIII) preganglion complex, hindbrain, forebrain, mandibular component of the first branchial arch, otic vesicle, optic stalk/optic vesicle, and olfactory pit (fig. 5) (Kaufman 1992). Although *Chd7* is widely expressed, these tissues in the early embryo exhibit differentially increased expression. The tissues with high levels of expression appear to correspond to the mature structures that are most severely affected in CHARGE syndrome.

Gene-Expression Analysis

Because *CHD7* is broadly expressed and because its protein motifs suggest that it may play a role in global regulation of chromatin structure, and hence gene expression, we wished to determine whether a characteristic pattern of expression abnormality is associated with mutation. Microarray analysis of gene-expression patterns by use of lymphoblastoid cell lines from seven *CHD7* mutation-positive individuals showed significant gene expression differences when compared with those from five control individuals and three affected individuals with no detected mutation of *CHD7* (fig. 2). The gene-expression signature created by the 116 transcripts with the best statistical support shows that the cases and controls cluster into two distinct groups. Interestingly, individual CHA230 appears to cluster with the case patients bearing a *CHD7* mutation, although we have not

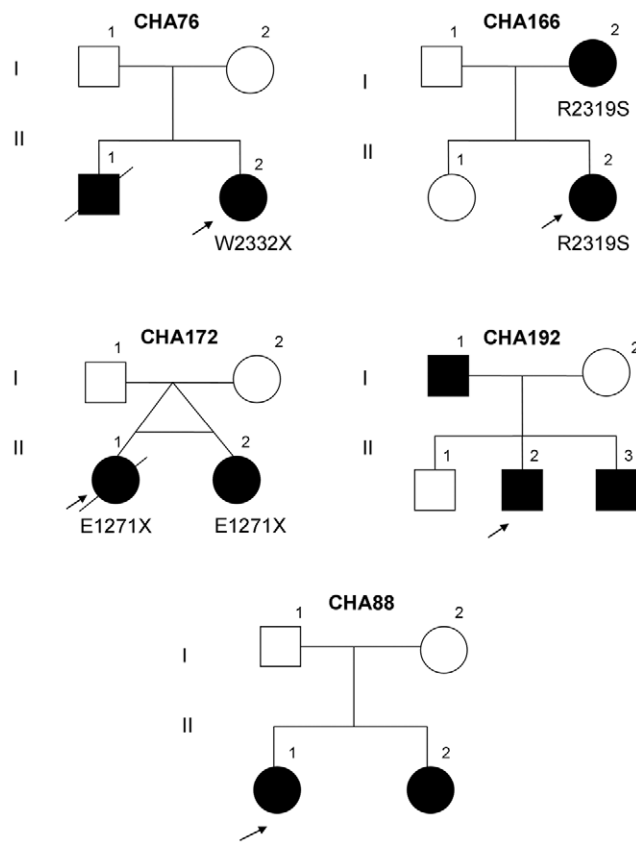


Figure 4 Five pedigrees with CHARGE syndrome illustrating four familial cases and an affected pair of MZ twins. *CHD7* mutations were identified in pedigrees CHA76, CHA166, and CHA172. Germ-line mosaicism is suggested in CHA76, whereas CHA166 indicates autosomal dominant transmission of the missense mutation in this mildly affected family. CHA172 shows a nonsense mutation in MZ twins. Mutations in *CHD7* were not identified in families CHA192 and CHA88. Probands are indicated by arrows.

Table 3**Distribution of CHARGE Syndrome Features in *CHD7* Mutation-Positive and Mutation-Negative Individuals**

SYNDROME FEATURE	NO. OF INDIVIDUALS WITH FEATURE/NO. EVALUATED (%)		P ^a
	With	Without	
	<i>CHD7</i> Mutation	<i>CHD7</i> Mutation	
Cardiovascular malformation	54/59 (92%)	30/42 (71%)	.014
Coloboma	55/62 (89%)	30/43 (70%)	.022
Facial asymmetry	36/56 (64%)	13/39 (33%)	.004
Choanal atresia	34/57 (60%)	23/39 (59%)	1.000
Hearing loss	54/59 (92%)	36/38 (95%)	.701
Inner ear abnormality	21/22 (95%)	9/10 (90%)	.490
External ear malformation	59/62 (95%)	39/42 (93%)	.683
Urogenital abnormality	29/53 (55%)	26/39 (67%)	.287
Cleft lip/palate	18/60 (30%)	9/41 (22%)	.493
Tracheoesophageal fistula	10/55 (18%)	3/40 (8%)	.226

^a All *P* values are two sided. Significant *P* values are in bold italics.

been able to find an alteration of the coding sequence in that individual.

Discussion

CHARGE syndrome is a recognizable multiple-malformation syndrome, the etiology of which was unknown until recently (Vissers et al. 2004). Autosomal dominant inheritance in rare families (Mitchell et al. 1985), concordance in MZ twins (Blake et al. 1989), advanced paternal age (Tellier et al. 1996), and variable chromosomal abnormalities in rare individuals with CHARGE syndrome (Clementi et al. 1991; Lee et al. 1995; North et al. 1995; Devriendt et al. 1998; De Krijger et al. 1999; Lev et al. 2000) supported a genetic basis for the condition. Genomewide deletion screening by use of comparative genomic hybridization (CGH) (Sanlaville et al. 2002), STR markers (Lalani et al. 2003), and SNPs (Lalani et al. 2005) in affected families did not identify a common microdeletion in this syndrome. Vissers et al. (2004) identified mutations in the gene for a chro-

modomain helicase DNA-binding protein, *CHD7*, in 10 of 17 individuals with CHARGE syndrome, after BAC CGH analysis of two unrelated individuals with CHARGE syndrome, one with an apparently balanced translocation involving chromosomes 6 and 8. These two subjects had overlapping microdeletions in chromosome 8q12, both of which spanned the *CHD7* locus. Subsequent mutation analysis demonstrated that de novo point mutations in this gene are associated with CHARGE syndrome. Chromatin remodeling is an important mechanism for regulation of gene expression. Like the other CHD genes, *CHD7* is likely to play a significant role in development and cell-cycle regulation. The gene encodes a protein with chromatin remodeling, an SNF2-like ATPase/helicase domain, and DNA-binding motifs. *CHD7* is 188 kb in length and is composed of 38 exons, of which 37 are coding. In our study, pathogenic mutations were identified throughout the gene, although many mutations were identified in exons that encode the middle third of the protein. Most of the mutations are truncating mutations that are likely to result in haploinsufficiency. Most of the affected individuals have de novo mutations in the *CHD7* gene. However, rare familial cases with recurrence were observed. In at least one family in our study cohort, autosomal dominant inheritance from the mother was documented. In CHA166, the R2319S mutation seen in the proband was also found in the affected mother who had iris coloboma, facial palsy, hearing loss, and delayed puberty. Neither the mother nor the child had a cardiovascular malformation. CHA76, a mildly affected girl, was found to have truncating mutation W2332X, not present in either of her parents. Interestingly, this child had a sibling who died at age 6 mo due to complications related to the diagnosis of CHARGE syndrome. The phenotype of the deceased infant included coloboma, choanal atresia,

Table 4**Common *CHD7* Polymorphisms**

EXON	POLYMORPHISM	FREQUENCY	
		Allele 1	Allele 2
2	c.1665+34G→A	.793	.207
4	c.2238+39G→A	.802	.198
6	c.2442+38A→T	.976	.024
6	c.2442+93A→G	.810	.190
9	c.2614-45G→A	.779	.221
10	c.2698-54A→G	.540	.460
15	c.3523-39C→G	.989	.011
19	c.4533+45G→A	.731	.269
31	c.6276G→A	.971	.029
34	c.7356A→G	.951	.049

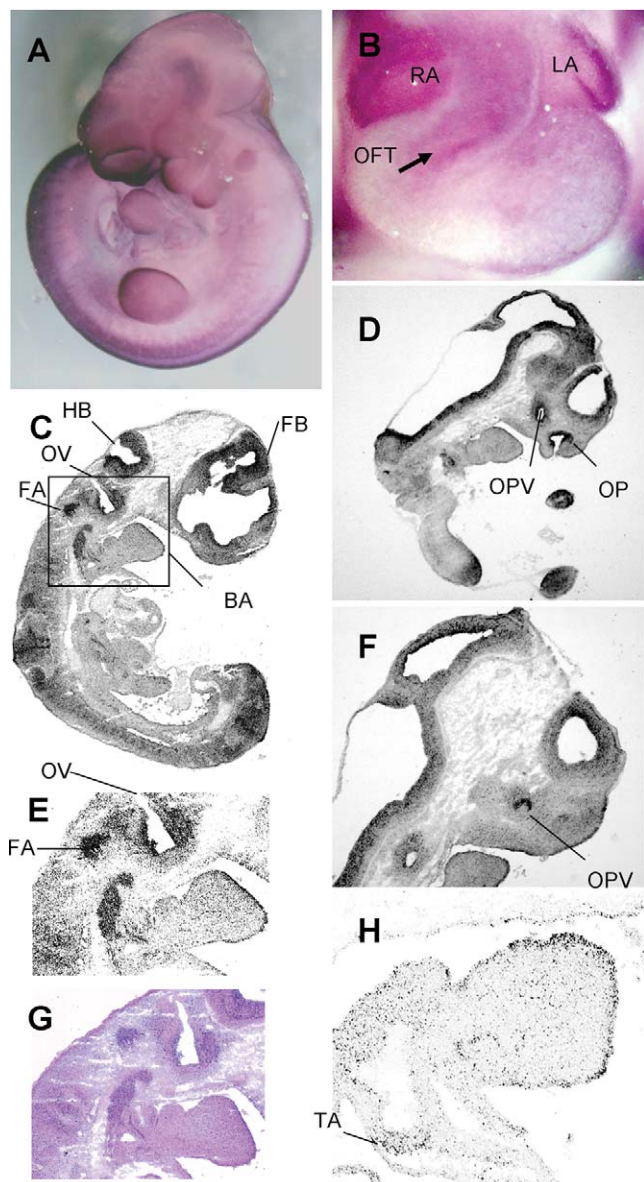


Figure 5 Mouse embryo whole-mount and section in situ hybridization showing the expression of *Chd7*. *A*, Whole-mount in situ hybridization of the embryo at 10.5 dpc, demonstrating expression of *Chd7* in the craniofacial region. *B*, Expression of *Chd7* observed in cardiac outflow tract (OFT). LA = left atrium; RA = right atrium. *C*, Sagittal section of embryo at 10.5 dpc, showing expression in the facio-acoustic (VII–VIII) preganglion complex (FA), hindbrain (HB), forebrain (FB), mandibular component of the first branchial arch (BA), and otic vesicle (OV). *D* and *E*, Sagittal section of embryo with expression in optic stalk/optic vesicle (OPV) and olfactory pit (OP). *F* and *G*, Magnified images of panel *C*, indicating expression of *Chd7* in facio-acoustic preganglion complex and otic vesicle, with hematoxylin and eosin stain. *H*, Sagittal section of the embryo, demonstrating expression in truncus arteriosus (TA) at 10.5 dpc.

hearing loss, pulmonary atresia with aortic stenosis and ventricular septal defect, kidney abnormality, duodenal atresia, and swallowing difficulties that necessitated G-tube and Nissen fundoplication. A tissue sample was not available for analysis from this sibling. Nonpaternity was excluded for this study subject. These findings suggest germline mosaicism in this family.

Proband CHA151 was found to have a de novo heterozygous mutation, R1339X, in the *CHD7* gene. His features included coloboma, choanal atresia, deafness, ear malformation, undescended testes, and partial atrioventricular canal. His sister was born with coarctation of the aorta, and their mother had unilateral deafness and mild facial palsy. The parents and sister were negative for the *CHD7* R1339X mutation detected in the proband. We conclude that, in this apparently familial case, the clinical features in other family members are unrelated to the R1339X mutation seen in the proband.

This study group also included a pair of female MZ twins. Both had the nonsense mutation E1271X in exon 16. They were born prematurely at 31 wk gestation. Twin A died at age 4 mo due to sepsis, and a complete autopsy was performed. The phenotype included bilateral optic-nerve coloboma, complete atrioventricular canal defect, mild-to-moderate hypoplasia of the ascending aorta, anomalous aortic arch branches including common brachiocephalic trunk, unilateral choanal atresia, olfactory bulb hypoplasia, growth retardation, unilateral cleft lip and palate, bilateral simplified ears, and renal glomerular cysts. Twin B survived and had bilateral coloboma, choanal stenosis, external ear malformation, profound bilateral hearing loss, bilateral cleft lip and palate, ventricular septal defect and patent ductus arteriosus, growth retardation, and normal renal sonogram (table 5). The causes of phenotypic variation between MZ twins remain poorly understood. However, the variation of phenotype in these twins could be explained by differential epigenetic regulation, such as variable methylation, resulting in altered gene expression and cellular phenotypes.

Cardiovascular malformations were more common in the *CHD7* mutation-positive group than in the mutation-negative group. On the basis of its function in nucleosome remodeling and transcription regulation, this chromodomain helicase DNA-binding protein may affect the functions of multiple genes involved in the patterning of the cardiogenic developmental fields. Mouse in situ hybridization studies demonstrate expression of *Chd7* in embryonic heart at 10.5 dpc. Assorted cardiovascular malformations are seen in the individuals with CHARGE syndrome with *CHD7* mutations, including tetralogy of Fallot, septal defects, patent ductus arteriosus, coarctation of the aorta, and complete and partial atrioventricular canal. Patent ductus arteriosus is more common in the *CHD7* mutation-positive group than

Table 5

Clinical Features of CHARGE Syndrome in Familial Cases

CLINICAL FEATURES	CHA76		CHA166		CHA172		CHA192			CHA88	
	II1	II2	I2	II2	II1	II2	I1	II2	II3	II1	II2
Coloboma	+	+	+	+	+	+	-	-	-	+	+
Choanal atresia	+	-	-	-	+	+	-	+	-	+	-
Cranial nerve anomalies	+	+	+	+	+	+	+	+	+	+	+
Hearing loss	+	+	+	+	U	+	+	+	+	+	+
Inner ear abnormality	U	U	U	U	U	U	U	+	+	U	U
External ear malformation	+	+	-	+	+	+	+	+	+	+	+
Cardiovascular malformation	+	+	-	-	+	+	-	-	-	+	-
Urogenital abnormality	+	-	+	-	+	-	-	+	-	+	-
Cleft lip/palate	-	-	-	-	+	+	+	-	+	-	-
Tracheoesophageal fistula	-	-	-	-	-	-	-	-	-	-	-
Growth retardation	-	+	-	-	+	+	+	+	-	+	+
Developmental delay	+	+	-	-	+	+	U	+	+	+	+

NOTE.—U = unknown; + = presence; - = absence.

in the mutation-negative group (31 of 54 vs. 7 of 38; $P < .001$). There was no clear difference between the two groups with respect to other cardiovascular malformations, including septal defects, left ventricular outflow-tract obstructive lesions, tetralogy of Fallot, and other outflow-tract anomalies. All five individuals with CHARGE syndrome with atrioventricular canal defects had *CHD7* mutation; however, the number of individuals with CHARGE syndrome with this cardiovascular malformation was too small for meaningful comparison.

Postnatal growth retardation is known to be a distinct phenotype in CHARGE syndrome. Various studies indicate presence of growth retardation in ~75% of individuals with CHARGE syndrome (Tellier et al. 1998). The etiology of short stature in these individuals is not understood in detail. Some children are known to have growth-hormone deficiency, although systematic studies of endocrine problems in these children are lacking. In our study, growth retardation was obvious in both *CHD7* mutation carriers and noncarriers; however, the Z scores of stature (normalized for age and sex) were slightly lower for the mutation-positive group (mean $Z = -2.5329$) than for the mutation-negative group

(mean $Z = -1.7594$). Detailed endocrine evaluation of these individuals with CHARGE syndrome may identify specific endocrine defects caused by aberration of *CHD7* function.

Cranial nerve dysfunction, including facial and auditory/vestibular nerves, is a major characteristic of CHARGE syndrome. In our study, facial asymmetry was observed more often in the *CHD7* mutation-positive group than in the mutation-negative group (36 of 56 vs. 13 of 39; $P = .004$) (fig. 1). Neuronal migration in vertebrate brain development is an important phenomenon, and the migration of embryonic facial motoneurons in mice and chicks has been studied extensively (Studer 2001). Despite detailed analyses, the cellular and molecular mechanisms that direct the migration of distinctive neuronal populations remain largely unknown. *CHD7* may play a role in neuronal migration, either directly or through its interaction with various partners, thus causing the multiple cranial nerve dysfunction commonly observed in CHARGE syndrome.

We have detected *CHD7* mutations in ~60% of individuals with clinical CHARGE syndrome. Because CHARGE syndrome has considerable phenotypic heterogeneity, clinical criteria were developed to aid reliable diagnosis. Although individuals with four major criteria or with three major and three minor criteria were designated as having definite CHARGE syndrome, the clinical guidelines called for consideration of the diagnosis of CHARGE syndrome in children with either one or two major characteristics and multiple minor characteristics (Blake et al. 1998). In our study, we included these individuals given a possible diagnosis of CHARGE syndrome and found pathogenic mutations in three of them. Inner ear defects, including semicircular canal hypoplasia with or without Mondini malformation, is a distinct feature of CHARGE syndrome. In our study cohort, temporal bone CT scans were available for re-

Table 6

Characterization of Verloes's Major Signs in 32 Individuals with CHARGE Syndrome

INDIVIDUALS	NO. OF VERLOES'S MAJOR SIGNS ^a			TOTAL	P
	1	2	3		
<i>CHD7</i> mutation negative	4	5	0	9	
<i>CHD7</i> mutation positive	1	12	10	23	.005
All	5	17	10	32	

NOTE.—Significant results are in bold italics.

^a Major signs include coloboma (iris or choroid, with or without microphthalmia), atresia of choanae, and hypoplastic semicircular canals (Verloes 2005).

Table 7**Characterization of Blake's Major Criteria in 98 Individuals with CHARGE Syndrome**

INDIVIDUALS	NO. OF BLAKE'S MAJOR CRITERIA ^a				TOTAL	P
	1	2	3	4		
<i>CHD7</i> mutation negative	1	10	16	12	39	
<i>CHD7</i> mutation positive	0	3	29	27	59	.014
All	1	13	45	39	98	

^a Major criteria include ocular coloboma (iris, retina, choroid, or disc; microphthalmia), choanal atresia (unilateral/bilateral; stenosis/atresia), cranial nerve anomalies (facial palsy-unilateral/bilateral, sensorineural deafness, and/or swallowing problems), and characteristic ear anomalies (external ear-lop or cup-shaped ear, middle ear ossicular malformations, mixed deafness, and cochlear defects) (Blake et al. 1998).

view for 32 individuals with CHARGE syndrome. Of these, 30 (94%) had abnormal findings, with hypoplastic or absent semicircular canals with or without Mondini malformation. There was no statistically significant difference in the carriers and noncarriers of *CHD7* mutation when this clinical entity was separately analyzed (8 of 9 in the mutation-negative group vs. 22 of 23 in the mutation-positive group; $P = .490$). However, within this group of 32 individuals, for which temporal bone imaging scans were available, Verloes's major signs—that is, iris or choroid coloboma, atresia of choanae, and hypoplastic semicircular canals—were analyzed. Of the 32 individuals, 10 displayed all three of Verloes's major signs, and all were found to have a *CHD7* mutation ($P = .005$) (table 6). Interestingly, these 10 individuals also displayed Blake's four major criteria and had associated cranial nerve dysfunction and characteristic ear abnormalities. Eight of these individuals also had a cardiovascular malformation. We suggest that individuals with combined coloboma, choanal atresia, and hypoplastic semicircular canals are likely to have mutations in the *CHD7* gene. Many individuals with all four of Blake's major criteria also have mutations in *CHD7* (table 7). The minor criteria of Verloes were difficult to score accurately for each individual, because growth hormone and gonadotropin deficiencies were not documented for most of the individuals in the study group. Also, formal assessment of mental retardation, another minor sign, was not performed for these individuals. Similarly, distinctive CHARGE facies, one of Blake's minor criteria, was difficult to score for many individuals enrolled through the Baylor College of Medicine Cardiovascular Genetics Web site by various referring physicians.

In the initial study, *CHD7* mutations and/or deletions were seen in ~63% (12/19) of individuals with CHARGE syndrome (Vissers et al. 2004). These data are similar to those of our present study and suggest that

CHARGE syndrome is likely a genetically heterogeneous condition. This is also implied by various chromosomal aberrations described in rare individuals with CHARGE. Additionally, the microarray analysis of gene expression with the use of lymphoblastoid cell lines from *CHD7* mutation-positive and mutation-negative individuals with CHARGE syndrome shows significant gene expression differences between these two groups. The differential gene-expression pattern in the *CHD7* mutation-negative group may further assist in the understanding of the molecular basis of the disorder in this group of affected children. Previously, we reported a de novo mutation in *SEMA3E*, a gene encoding a semaphorin protein involved in axon guidance and cell migration, in an individual with CHARGE syndrome (CHA6). *SEMA3E* became a candidate gene after a chromosomal breakpoint was narrowed in an unrelated affected individual, CHA193, with an apparently balanced chromosomal translocation, 46,XY(2;7)(p14;q21.11), within 200 kb of *SEMA3E* on chromosome 7 (Lalani et al. 2004). Both CHA6 and CHA193 were studied for mutations in *CHD7*. No mutation in the coding region or the exon-intron boundaries of *CHD7* was detected in these individuals. Moreover, the lymphoblast cell line from CHA193 was also analyzed for gene-expression pattern and was consistent with a signature pattern seen in *CHD7* mutation-negative individuals with CHARGE (fig. 2). It will be important to study the association between *CHD7* and this semaphorin protein gene in a mutant animal model, to help further delineate the pathogenesis, particularly as it relates to the cranial nerve dysfunction seen so frequently in individuals with *CHD7* mutation.

No significant distinct phenotype-genotype correlations were identified for different types of *CHD7* mutations (missense, splice site, and truncating) in the mutation-positive cohort. Similarly, no correlation was observed between the clinical severity and the inferred length of the mutant *CHD7* protein. Three unrelated individuals with CHARGE syndrome with the same mutation, R2653X, in the BRK domain of the *CHD7* gene have different clinical phenotypes. We have also observed variable expression of a *CHD7* mutation within individuals in the same family. The MZ twins with the same mutation, E1271X, have differences in their phenotype; twin A had atrioventricular canal defect, and twin B had a ventricular septal defect and patent ductus arteriosus. CHA166, with the R2319S mutation, has iris coloboma, ear malformation, mixed hearing loss, and facial palsy. Her mother, who has the same mutation, has iris coloboma, hearing loss, facial palsy, and a history of delayed puberty.

There was no correlation of severity of phenotype with mutation in specific domains of the protein. However, we have observed that five individuals with CHARGE

syndrome with mutations in the SNF2 domain have variable cardiovascular malformations.

In this study, the *CHD7* mutations in the coding region and exon-intron boundaries were analyzed, and these data were used for the statistical analyses. One limitation of the study is the lack of sequence information in the regulatory and promoter elements of the *CHD7* gene. Heterozygous deletions within the *CHD7* gene may not have been detected by sequencing. We addressed this concern by performing FISH analyses on 23 *CHD7* mutation-negative individuals with CHARGE syndrome, using RP11-33111 and RP11-174G clones encompassing *CHD7*, and excluded any larger heterozygous deletion in these individuals. However, microdeletions of ≤ 100 kb could potentially be undetected by the FISH methodology used.

Previously, the expression profile by semiquantitative RT-PCR analysis of *CHD7* showed ubiquitous expression (Vissers et al. 2004). Mouse embryo in situ hybridization, however, shows patterned expression of *Chd7* in tissues that are frequently affected in CHARGE syndrome, such as optic stalk/optic vesicle, facio-acoustic preganglion complex, olfactory pit, forebrain, hindbrain, mandibular component of the first branchial arch, and outflow tract of the heart. Remodeling of eukaryotic chromatin is known to play a significant role in regulation of gene transcription. Tong et al. (1998) have shown that the ATP-dependent nucleosome-remodeling activity of the human histone deacetylase complex derives from the helicase/ATPase domains of CHD3 (Mi-2 α) and CHD4 (Mi-2 β) proteins. This functionally coupled complex is referred to as the “nucleosome remodeling and deacetylating (NuRD) complex.” The NuRD complex from HeLa cells contains seven major polypeptides, including Mi2, MTA2, MBD3, and the histone deacetylase core, HDAC1/2 and RbAp46/48. Current models suggest that NuRD complexes are involved in producing a repressing chromatin environment (Feng and Zhang 2001; Brehm et al. 2004). Through its interaction with MBD3, a protein related to the methylcytosine-binding protein, NuRD is involved in remodeling and deacetylating nucleosomes containing methylated DNA. It is also known that MeCP1 complex, which is composed of 10 major polypeptides, including MBD2, CHD3, and CHD4, and all of the other known NuRD components, preferentially binds, remodels, and deacetylates methylated nucleosomes to cause gene silencing (Zhang et al. 1999). Another chromatin-remodeling protein, Chd1, is known to be a component of yeast SAGA and SILK, two highly homologous and conserved multisubunit histone acetyltransferase complexes (Pray-Grant et al. 2005). Chd1 was shown to specifically interact with the methylated lysine 4 mark on histone H3, which is associated with transcriptional activity. The association of CHD7 with histone deacetylases has not

been studied yet. It will be important to study the role of this chromodomain helicase DNA-binding protein in gene transcription and to understand its possible role in gene repression.

In conclusion, *CHD7* mutations account for ~60% of cases of CHARGE syndrome in our study. Cardiovascular malformations, coloboma, and facial asymmetry were seen more often in those with mutations in the *CHD7* gene. We also suggest that most individuals with CHARGE syndrome with combined coloboma, choanal atresia, and hypoplastic semicircular canals will have mutations in the *CHD7* gene. Functional studies are needed to address the question of how *CHD7* mutation alters gene expression, as expected on the basis of its similarity to other chromodomain helicase DNA-binding proteins.

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Appendix A

Table A1

CHD7 Oligonucleotide Sequences

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table A2

Annotated Transcript List from Microarray Cluster Analysis

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Web Resources

Accession numbers and URLs for data presented herein are as follows:

Baylor College of Medicine Cardiovascular Genetics, <http://www.cardiogene.org/>
 ChipST2C, <http://chipst2c.org/ChipST2C.html>
 GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for gene sequences [accession number NM_017780], clones RP11-33I11 [accession number AC113143] and RP11-174G1 [accession number AC022182], and cDNA fragment of KIAA1416 [accession number BC034239])
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for CHARGE syndrome)

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