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Exclusion of *PITX2* Mutations as a Major Cause of CHARGE Association

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CHARGE is a nonrandom association of ocular coloboma, congenital heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia, and ear anomalies including deafness. The cause of CHARGE remains unknown; however, there is considerable evidence of an underlying genetic basis, as discussed by Tellier et al. [1996: Clin Genet 50:548–550; 1998: Am J Med Genet 76:402–409] and by Martin et al. [2001: Am J Med Genet 99:115–119]. Based on the ocular, cardiac, and craniofacial expression pattern of *Pitx2*, a homeodomain transcription factor, and the pleiotropic effects of loss of *PITX2* function in both mouse and human, we hypothesized that *PITX2* mutations may contribute to the multiple phenotypic anomalies present in CHARGE individuals. By direct sequencing of DNA from 29 individuals with CHARGE, we did not identify any mutations in *PITX2*. We did, however, identify two *PITX2* sequence polymorphisms. Large deletions of *PITX2* were excluded in most patients by heterozygosity in at least one of several polymorphic markers near the *PITX2* locus. Together, these data indicate that *PITX2* mutations are unlikely to be a major contributing cause of the multiple anomalies present in individuals with CHARGE. © 2002 Wiley-Liss, Inc.

KEY WORDS: CHARGE; PITX2; mutation; sequence polymorphism

INTRODUCTION

CHARGE association was first described in 1979 [Hall, 1979], and the CHARGE acronym was proposed in 1981 [Pagon et al., 1981]. CHARGE is a collection of congenital anomalies including ocular coloboma (anterior or posterior), cardiac defects, choanal atresia (bony and/or membranous), delayed growth and development, external and inner ear anomalies, deafness, and cranial nerve dysfunction. Major diagnostic features of CHARGE syndrome, are present-a potential subset of individuals affected by CHARGE, and include coloboma, characteristic ear and cranial nerve abnormalities, and choanal atresia [Blake et al., 1998]. CHARGE affects the craniofacial region, teeth, and cardiac outflow tract, leading to the suggestion that CHARGE is a disorder of neural crest migration and development [Bolande, 1997].

Most cases of CHARGE are sporadic with an unidentifiable cause. However, there is considerable evidence that CHARGE may have a genetic basis, including an association with increased paternal age [Tellier et al., 1996], rare familial cases, concordance among monozygotic twins, and discordance among dizygotic twins [Tellier et al., 1998]. Several chromosomal rearrangements have been reported among CHARGE individuals, including duplications of 14q and 1q [Dev et al., 1985; North et al., 1995] and a balanced translocation involving 2p14 and 7q21 [Martin et al., 2001]. To date, no single chromosomal region appears to be preferentially involved in the CHARGE phenotype. CHARGE might result from mutations in a dosage-sensitive

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developmental pathway required by all the involved tissues. Alternatively, pleiotropic activity of a single factor in multiple developmental fields could explain the complex phenotype. The rarity of familial CHARGE cases, combined with high early mortality among CHARGE individuals, renders positional cloning and linkage analysis difficult. Instead, candidate gene analysis is a potentially useful approach to understanding the etiology of multiple congenital anomaly conditions, and has been used successfully to exclude *PAX2* mutations as a major cause of CHARGE [Tellier et al., 2000].

Pitx2, a transcription factor essential for proper formation of the eyes, heart, and craniofacial region, is expressed in several areas of the developing murine neural crest [Hjalt et al., 2000]. In humans, *PITX2* mutations cause ocular anomalies, including Axenfeld-Rieger syndrome (ARS), a haploinsufficiency condition affecting the eyes, teeth and umbilicus with associated abnormalities of the craniofacial region (midfacial hypoplasia, prognathism) [Semina et al., 1996; Alward et al., 1998; Doward et al., 1999; Kozlowski and Walter, 2000; Perveen et al., 2000; Priston et al., 2001]. Some patients with ARS and unknown mutation also exhibit congenital heart disease (atrial septal defect, conotruncal anomalies), choanal atresia, and growth hormone deficiency, indicating some clinical overlap with CHARGE association [Feingold et al., 1969; Sadeghi-Nejad and Senior, 1974; Brooks et al., 1989]. In mice, complete loss of *Pitx2* results in embryonic lethality, with severe ocular, cardiac, and abdominal abnormalities, and disruptions in tooth, pituitary and craniofacial development [Gage et al., 1999; Lin et al., 1999]. The *PITX2* locus produces three isoforms designated PITX2a, b, and c that arise through differential splicing and alternative promoter usage (Fig. 1) [Arakawa et al., 1998]. In mice, the Pitx2c isoform plays a particular role in early mesoderm differentiation, establishment of embryonic left–right axis, and cardiac development [Liu et al., 2001]. The striking overlap between the phenotypic anomalies in *Pitx2*-deficient mice and the clinical features of CHARGE led us to hypothesize that *PITX2* mutations may contribute to the multiple anomalies present in individuals with CHARGE.

We found, by direct sequencing of genomic DNA from 29 individuals with CHARGE, no identifiable mutations in the coding region or intron–exon borders of *PITX2*. Two polymorphic variants in the *PITX2* coding sequence were identified based on comparative sequencing of unaffected parent DNA samples. Large deletions near the *PITX2* locus were also excluded in 28 of 29 CHARGE individuals by heterozygosity at highly polymorphic markers near *PITX2*. Taken together, these results indicate that *PITX2* mutations are unlikely to be a major cause of the multiple congenital anomalies seen in CHARGE individuals.

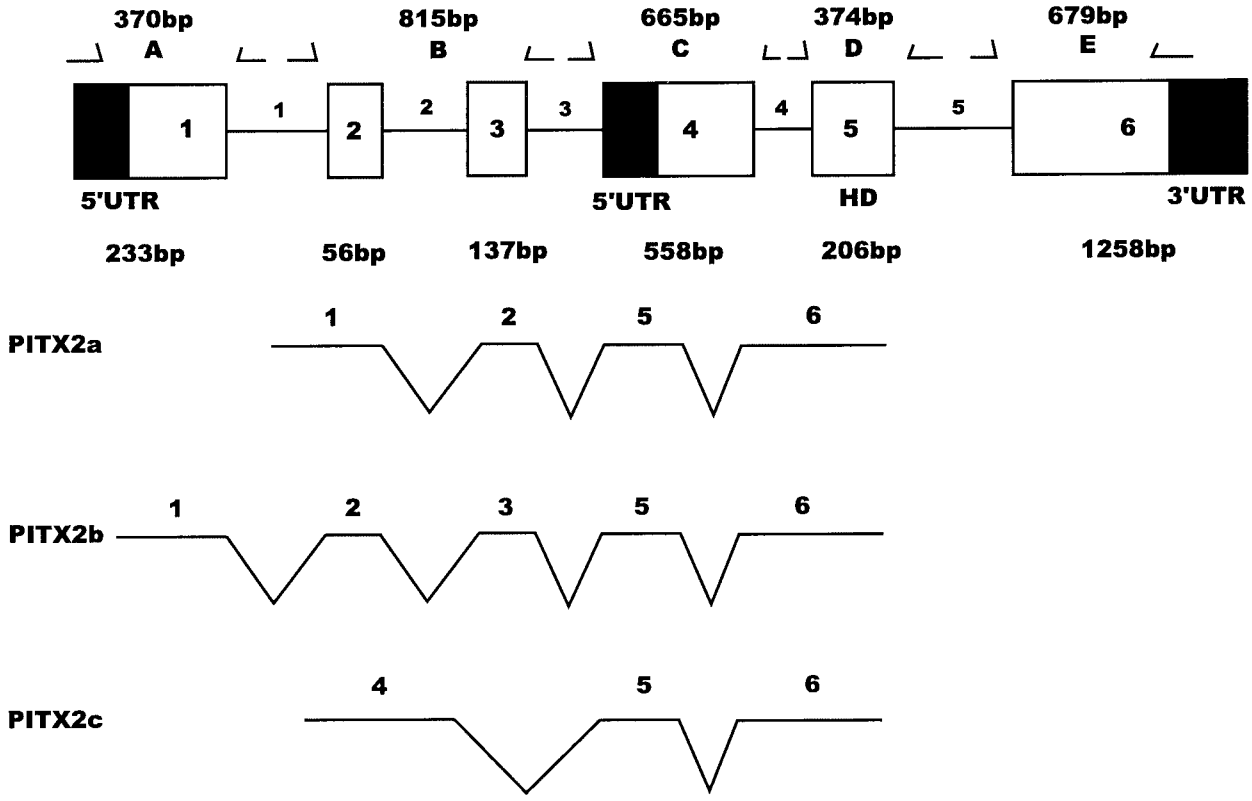

Fig. 1. Diagram of human *PITX2* gene and three different mRNA isoforms (PITX2a, PITX2b, PITX2c), with exons numbered in boxes and introns numbered above intervening segments. Sizes of exons in base pairs are given below each box; filled areas are untranslated regions. Introns are not drawn to scale. Primers used for sequencing are indicated as arrows, with five polymerase chain reaction products (A–E) and sizes in base pairs indicated. Adapted from Amendt et al., 2000.

METHODS

Patient Information

Patient and parent samples (10 mL peripheral blood) were obtained with IRB consent from each of three participating institutions (University of Michigan, Baylor College of Medicine, University of California, Los Angeles). Twenty-seven of 29 patients were from the CHARGE Syndrome Foundation Research Resource. Inclusion criteria were a clinical diagnosis of CHARGE on the basis of at least four of the originally described six major features (coloboma, heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia, and ear anomalies including deafness). Clinical features of the patients are outlined in Table I. All but two patients had normal peripheral blood chromosomes and no 22q11 deletion by fluorescence in situ hybridization testing or STR genotyping; similar testing information was not available for the other two patients. Prior approval for this study was obtained through the Internal Review Board of the University of Michigan.

DNA Isolation and Sequencing

Peripheral blood samples were obtained from affected patients and their parents. Lymphoblastoid cell lines were prepared from blood samples and DNA isolated with QIAGEN columns [Qiagen, Valencia, CA]. Primer pairs were as shown in Figure 1: region A, AATCTCTGCTGACGTCACGT and CCAGACTCGCATTATCTCAC; region B, TAGTCTCATCTGAGCCCTGC and TTCTTGCGCTTTCGCCCGA; region C, CTTGACACTTCTCTGTCAGG and AAGCGGGAATGTCTGCAGG; region D, CAGCTCTTCCACGGCTTCT and TTCTCTCCTGGTCTACTTGG; region E, GTAATCTGCACTGTGGCATC and AGTCTTTCAAGGGCGGAGTT. PCR was at 55°C annealing temperature. Amplified products were separated on agarose gels, reamplified, separated on agarose gels, purified with QIAEX II or QIAQUICK kits (Qiagen) and sequenced directly, using Applied Biosystems DNA Sequencers with the manufacturer's protocols (BigDye Version1 terminator chemistry, DNA Sequencing core, University of Michigan). Chromatograms were visualized with Sequencer software (Gene Codes Corporation, Ann Arbor, Michigan) and compared to the published genomic *PITX2* sequence [Genbank accession number AF238048, or chr4:118538103-118558100 in the April 1, 2001 freeze of the Golden Path sequence assembly (http://genome.ucsc.edu/)].

TABLE I. Clinical Features of CHARGE Subjects (N = 29)

Feature	No. of individuals	Ratio or percentage
Male: female	14:15	1:1.07
Ocular coloboma	26	90%
Heart defect	25	86%
Undescended testes or small penis	12	86%[a]
Ear malformation	24	83%
Hearing loss	24	83%
Development delay	20	72%
Swallowing problems	19	65%
Choanal atresia or stenosis	17	59%
Respiratory disease	17	59%
Recurrent otitis media	17	59%
Growth retardation	15	52%
Facial nerve palsy	14	48%
Increased secretions	12	41%
Renal or other urinary tract anomaly	11	38%
Hernia (unspecified)	7	24%
Cleft lip or palate	6	21%
Tracheoesophageal fistula	6	21%
CNS defect	5	17%
Growth hormone deficiency	3	10%

[a]Percentage given is that of the total number of males. Other data are presented as percentage of the total (N = 29). Single cases with microcephaly, seizures, absence of thymus, hypoparathyroidism were also reported. Average age at evaluation was 5.7 years. Ocular coloboma is defined as involving the retina and/or iris. Hearing loss includes sensorineural or conductive types, and respiratory disease includes multiple pneumonias or breathing problems.

RESULTS AND DISCUSSION

In this study, we demonstrate absence of *PITX2* mutations in a series of 29 individuals with CHARGE association. We sequenced the *PITX2* coding region and intron–exon boundaries, and identified two sequence changes in introns, both near splice junctions but not within consensus splice sites (Table II). These sequence changes were also present in unaffected parents, and likely represent polymorphic variants. Heterozygosity was detected in 28 of 29 affected patients for at least one of three polymorphic markers (D4S406, D4S2989, D4S2945 with heterozygosity values of 0.8843, 0.8423 and 0.783, respectively) near the *PITX2* locus. Data presented in this study do not exclude mutations in the distal 3′ UTR or in complete intronic regions of *PITX2*, nor have we excluded mutations in the *PITX2* promoter region that may affect transcript levels. Nevertheless, our observations indicate that mutations in *PITX2* are unlikely to contribute to the phenotypic abnormalities present in CHARGE association.

PITX2 mutations have been identified in association with a variety of clinical features, including Peters' anomaly, iridogoniodysgenesis, iris hypoplasia, and ARS; many of these patients also have maxillary hypoplasia and prognathism [Semina et al., 1996; Alward et al., 1998; Amendt et al., 1998; Kulak et al., 1998; Doward et al., 1999; Amendt et al., 2000; Kozlowski and Walter, 2000; Perveen et al., 2000; Priston et al., 2001; Saadi et al., 2001]. In addition to the phenotypic variability, there is considerable genetic heterogeneity among patients with ARS, and in a recent study of 76 individuals, eight were found to have *PITX2* mutations [Perveen et al., 2000]. Several different *PITX2* mutations have been reported, including

TABLE II. *PITX2* Sequence Variants

Sequence location	Sequence
Intron 2	CCCTCTTTCT (A/C) CTCCGGCCT
Intron 3	GCGTGGGGGGGG (G) CGGGCAG

nonsense mutations resulting in prematurely truncated PITX2 protein, missense mutations with decreased PITX2 homeodomain DNA binding, and a recently identified dominant negative mutation that leads to overexpression of a novel PITX2 protein [Priston et al., 2001].

The ocular abnormalities in patients with ARS and *PITX2* mutations typically involve the anterior segment of the eye, including the iris and lens. Similar anterior segment abnormalities (corectopia) are present in mice with complete loss of *Pitx2*, consistent with *Pitx2* mRNA and protein expression in the lens and anterior ocular mesenchyme during development [Gage et al., 1999; Hjalt et al., 2000]. Surprisingly, however, loss of *Pitx2* function in mice also leads to absence of the extraocular muscles and atresia of the optic nerve, even though only the extraocular muscles (and not the optic nerve) express *Pitx2* [Gage et al., 1999]. Thus, loss of *Pitx2* in the ocular mesenchyme may negatively influence development of adjacent posterior structures like the optic nerve and extraocular muscles. Data presented here indicate the *PITX2* mutations are not a major contributing cause of the ocular, cardiac, and craniofacial anomalies in CHARGE association; however, further studies will determine whether genes that are regulated by *PITX2* are involved in the CHARGE phenotype.

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