Mutation Screening of the *GJA7* (Cx45) Gene in a Large International Series of Probands with Nonsyndromic Hearing Impairment

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Direct evidence of the critical physiological role of connexins (Cxs) has come through the associations of several human diseases with pathogenic mutations in specific Cx genes. Currently, mutations in genes coding for five Cx proteins (Cx26, Cx30, Cx31, Cx32, and Cx43) have been shown to cause sensorineural hearing loss. Cx45 is another gap junction protein, coded by the *GJA7* gene. To investigate the possible contribution of *GJA7* mutations to deafness, we sequenced the *GJA7* gene in 341 unrelated probands with nonsyndromic hearing loss from Turkey, South Africa, United Kingdom, United States, and China. Three nucleotide variants not affecting the amino acid sequence, c.213C>T, c.906C>T, and c.912G>T, and one missense change, c.889C>A (p.D297N), were found. None of the identified changes appeared to be pathogenic. Our data suggest that *GJA7* alterations have no or low genetic relevance in nonsyndromic hearing loss in these populations.

Introduction

ESTIMATES SUGGEST THAT >270 million people worldwide have hearing loss that affects normal communication. Incidence of congenital severe hearing impairment is at least 1 in 1000 births, half of which can be attributed to genetic factors (Marazita *et al.*, 1993; Fortnum and Davis, 1997). Mutations in the *GJB2* (Cx26) gene are the most common cause of sporadic and autosomal recessive nonsyndromic sensorineural hearing loss (NSSHL) in many countries, which account for up 50% of inherited and 40% of sporadic cases with NSSHL (Estivill *et al.*, 1998).

Connexins (Cxs) are transmembrane proteins that form channels allowing rapid transport of ions or small molecules between cells. At least five Cx proteins have been reported to be involved in deafness (syndromic and nonsyndromic): Cx26 (*GJB2*), Cx30 (*GJB6*), Cx31 (*GJB3*), Cx32 (*GJB1*), and Cx43 (*GJA1*) (Kelsell *et al.*, 1997; Richard *et al.*, 2003). Autosomal-recessive and autosomal dominant forms of hearing impairment have been associated with >150 mutations in the coding region of *GJB2* (Cx26), *GJB3* (Cx31), *GJB6* (Cx30), *GJB1* (Cx32), and *GJA1* (Cx43) (Kelsell *et al.*, 1997; White 2000) (<http://davinci.crg.es/deafness/>). The *GJB1* (Cx32) gene is also responsible for X-linked Charcot-Marie-Tooth disease type I (Bergoffen *et al.*, 1993a, 1993b). *GJB3* (Cx31) is involved in both deafness or a skin disease, erythrokeratoderma variabilis, depending on the location of the mutations (Richard *et al.*,

1998, 2003). Mutations in *GJB6* encoding Cx30 have been shown to underlie hearing loss and hidrotic ectodermal dysplasia (Clouston's syndrome) (Grifa *et al.*, 1999; Lamartine *et al.*, 2000), and *GJA1* (Cx43) has been shown to be involved in oculodentodigital syndrome (Pazkenas *et al.*, 2003). On the basis of the important role different Cx-coding genes plays in NSSHL, any gene having a product present in the inner ear is a prime candidate for deafness.

Although it has been demonstrated that Cx45 is expressed in the inner ear (Cohen-Salmon *et al.*, 2004), mutation screening in *GJA7* as a cause of deafness has not been reported. This study was performed to clarify the relevance of mutations in *GJA7* through a large international collaboration.

Subjects and Methods

Subjects

Altogether, 341 unrelated families were investigated. Ninety-seven families were from Turkey, 60 from South Africa, 84 from South Florida, 40 from United Kingdom (UK), and the remaining 60 from China. All 97 families from Turkey included at least two affected siblings born to consanguineous parents, suggesting autosomal recessive inheritance. Probands were ascertained through the Genetics Clinic at the Department of Pediatrics at Ankara University and at the Department of Otolaryngology, University of Miami, as well as at the University of the Witwatersrand Medical School,

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TABLE 1. PRIMERS USED FOR SEQUENCING AND SINGLE-STRAND CONFORMATIONAL POLYMORPHISM SCREENING OF THE *GJA7* GENE

Primer	Primer sequences	PCR product (bp)
Cx45-1F	5'-TTG TTC CAC TGT GAA CCT TGA-3'	253
Cx45-1R	5'-ACG ATC CGG AAG ACA ATC AG-3'	
Cx45-2F	5'-AGT TGG AGC TTC CTG ACT CG-3'	
Cx45-2R	5'-CAT GGG AGA GAG GTG CAA AC-3'	217
Cx45-3F	5'-GTG TGC AAC ACA GAA CAG CC-3'	
Cx45-3R	5'-TTG TCC TCC TCC GTT TCT TC-3'	
Cx45-4F	5'-AGC AGA CAA GAA GGC AGC TC-3'	248
Cx45-4R	5'-CTG CCC TAT CAG AAA ACC CA-3'	
Cx45-5F	5'-CTA TGT GCT GCA GTT GCT GG-3'	
Cx45-5R	5'-GAG TCT CGA ATG GTC CCA AA-3'	255
Cx45-6F	5'-ATG GTG TTA CAG GCC TTT GC-3'	
Cx45-6R	5'-CAT ACT GCT GTT CCT GGG CT-3'	
Cx45-7F	5'-CAC CGA ACT GTC CAA TGC TA-3'	267
Cx45-7R	5'-GTC TTC CCA TCC CCT GAT TT-3'	
Cx45-8F	5'-AAA GTG GGG TCC AAA GCT G-3'	
Cx45-8R	5'-GAT CAT GAG CCA ACA GCA TC-3'	273
		205

PCR, polymerase chain reaction.

South Africa; Center for Audiology, University of Manchester, UK; and at the Department of Otolaryngology, Head and Neck Surgery, Chinese PLA General Hospital, Beijing, China. Informed consent was obtained from all individuals or the parents of minors. A clinical evaluation and family history was obtained on each proband. All participants were carefully evaluated for the presence of environmental factors and syndromic findings for their hearing loss using a standard questionnaire. Clinical evaluation included a thorough examination for the syndromic forms of deafness. Individuals with syndromic findings or with unequivocal evidence for environmental factors were excluded. The hearing of all affected individuals in the present series was examined using pure tone audiometry. Air conduction thresholds were measured at 250 and 500 Hz and 1, 2, 4, 6, and 8 kHz. Otoimmittance measurements were undertaken on all individuals and all were otoscopically examined to ascertain function of the middle ear. Genomic DNA was extracted from whole blood, prescreened for mutations in the coding and noncoding exons of the *GJB2* gene, and only negative samples were included in this study.

Mutation screening

We established the exon-intron structure of the gene by comparing the human cDNA sequences (GenBank accession number NM_005497) with the draft human genome sequence (<http://genome.ucsc.edu>, UCSC, Human, July 2003). A total

of eight pairs of primers were designed for the amplification and sequencing of one coding exon and its flanking splice sites (Table 1) using the Primer 3 program (www-genome.wi.mit.edu/genome-software/other/primer3.html). The complete coding sequence of *GJA7* was amplified from genomic DNA by polymerase chain reaction (PCR). Approximately 40 ng of genomic DNA was amplified with *GJA7*-specific primer pairs in a total volume of 12.5 μ L containing 10 $\times$ PCR Buffer (pH 8.5), 0.4 mM, dNTP mix (Promega Corporation), 0.4 pmol/ μ L of each primer, and 0.0625 unit of *Taq* DNA polymerase (Eppendorf AG). DNA templates were amplified using the following program: 95°C for 3 min; 30 cycles of 94°C for 50 s, 60°C for 50 s, 72°C for 60 s; and final extension of 72°C for 5 min. The PCR products were initially run on a 1-mm-thick 8% nondenaturing polyacrylamide gel (acrylamide: *N,N'*-methylene bisacrylamide 49:1) at 4°C. Single-strand conformational polymorphisms (SSCPs) were detected using silver staining as previously described (Liu *et al.*, 1997). Direct sequencing of PCR products from patients with SSCP variants was subsequently performed on both strands using the ABI Prism BigDye Terminator reaction kit and ABI 3100 DNA sequencer or with Beckman Coulter 2000 XL instrument and appropriate kits.

Since Turkish families included at least two affected siblings born to consanguineous parents, an autozygous mutation inherited from a common ancestor was the most likely explanation for hearing loss. Thus, an autozygous genomic segment flanking *GJA7* was sought as initial screen. All af-

TABLE 2. NUCLEOTIDE CHANGES IN THE Cx45 GENE

Nucleotide substitute	Location	Predicted codon change	Allele frequency in NSHL	Origin
213C>T	Exon 2	L74L	2/168	United States
889G>A	Exon 2	D297N	4/194 ^a	Turkey
906C>T	Exon 2	T302T	13/120	United States
912G>T	Exon 2	L304L	1/80	United Kingdom

^aSee the Subjects and Methods section for mutation screening.
NSHL, nonsyndromic hearing loss.

affected individuals were genotyped using D17S1861, D17S930, D17S934, and D17S965 microsatellite markers (66 families) or Affymetrix GeneChip 10K 2.0 Xba Arrays (51 families). Twenty-three families were genotyped with both methods). When a homozygous haplotype flanking the *GJA7* gene was obtained in all affected members of a family, direct DNA sequencing was performed.

Results

We have studied 341 unrelated probands with non-syndromic hearing loss from Turkey, South Africa, South Florida, UK, and China. Several different SSCP migration patterns in the entire coding region of the *GJA7* gene were observed in these patients. Sequencing of the DNA fragment displaying an abnormal SSCP pattern showed three single-nucleotide substitutions, which do not affect the Cx45 amino acid sequence. None of these changes have been described previously (Table 2). We found 2 individuals with the c.213C>T substitution in a heterozygous status, 13 subjects with the c.906C>T substitution (2 individuals in homozygous status and 9 individuals in heterozygous status), and 1 individual with the c.912G>T substitution (heterozygous status). Homozygous haplotypes flanking the *GJA7* gene were found in 4 out of 97 Turkish families. Unexpectedly, one previously unreported heterozygous missense change, c.889C>A (p.D297N), was identified in two unrelated families. Aspartic acid at position 297 is not a conserved residue and this change does not cosegregate with deafness in the two families. Our initial observation of homozygous haplotypes was likely spurious.

Discussion

Cx45 is a gap junction consisting of a cluster of closely packed pairs of transmembrane channels, the Cxs, through which materials of low molecular weight diffuse from one cell to a neighboring cell. The *GJA7* gene (GenBank accession number NM_005497) is comprised of two exons, part of exon 2 is coding. The 1191 bp open reading frame encodes a 396 amino acid protein.

Using an assay based on lacZ reporter gene activity, expression of Cx45 in the inner ear in mouse was detected from embryonic day E17.5. Cx45 is weakly expressed in epithelial and connective tissues during embryonic development. From birth onward, it is expressed in the inner ear capillaries, and this expression remains stable in the mature inner ear. Cx45 is involved in the inner ear vascular development. These findings suggest that cell-cell communication mediated by Cx45 plays different roles during early inner development and in mature ear (Cohen-Salmon *et al.*, 2004). However, by reverse transcription-PCR, we were not able to confirm expression of Cx45 in mouse cochlea (data not shown). This discrepancy may be attributed to difference in methods used. In this study, we carried out a mutation screening of the *GJA7* gene in 341 unrelated probands. Only four nonpathogenic nucleotide variants were found. We speculate that (1) due to differential selective constraints at the molecular level in the genome and/or because of the base composition, there is low level of nonsynonymous nucleotide variation in the *GJA7* gene, and (2) the gene is not expressed in the inner ear and therefore is unlikely to be required for hearing.

Cxs are transmembrane proteins involved in formation of intercellular channels. These gap junction channels permit the rapid exchange of ions and are thought to play an important role in maintaining hearing function by circulation of potassium ions between the fluids of the inner ear (Bruzzone *et al.*, 1996; Goodenough *et al.*, 1996). Blockage of K⁺ circulation causes hearing impairment. Direct evidence of the critical physiological role of Cxs has come through the linkage of several human diseases with pathogenic mutations in five different Cxs: Cx26 (GJB2), Cx31 (GJB3), Cx30 (GJB6), Cx32 (GJB1), and Cx43 (GJA1).

Although *GJA7* was a prime candidate for deafness because it codes for a gap junction protein shown to play a role in the inner ear cells, we could not demonstrate a germline DNA change that is associated with nonsyndromic hearing loss in a large cohort. None of the identified sequence changes appeared to be pathogenic. The incidence of nonpathogenic Cx45 variants suggests that Cx45 polymorphisms are common in UK and U.S. populations. We conclude that the *GJA7* mutations have low relevance in NSSHL.

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Disclosure Statement

No competing financial interests exist.

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