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Report

Familial Deafness, Congenital Heart Defects, and Posterior Embryotoxon Caused by Cysteine Substitution in the First Epidermal-Growth-Factor–Like Domain of *Jagged 1*

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In the present study, we report a kindred with hearing loss, congenital heart defects, and posterior embryotoxon, segregating as autosomal dominant traits. Six of seven available affected patients manifested mild-to-severe combined hearing loss, predominantly affecting middle frequencies. Two patients were diagnosed with vestibular pathology. All patients had congenital heart defects, including tetralogy of Fallot, ventricular septal defect, or isolated peripheral pulmonic stenosis. No individual in this family met diagnostic criteria for any previously described clinical syndrome. A candidate-gene approach was undertaken and culminated in the identification of a novel *Jagged 1* (*JAG1*) missense mutation (C234Y) in the first cysteine of the first epidermal-growth-factor–like repeat domain of the protein. JAG1 is a cell-surface ligand in the Notch signaling pathway. Mutations in *JAG1* have been identified in patients with Alagille syndrome. Our findings revealed a unique phenotype with highly penetrant deafness, posterior embryotoxon, and congenital heart defects but with variable expressivity in a large kindred, which demonstrates that mutation in *JAG1* can cause hearing loss.

Congenital heart disease is the most common birth defect, occurring in ~1% of live births. Although multifactorial inheritance has been postulated for the majority of cases, single-gene transmission is demonstrated by the observation of mutations in *NKX2.5* or *Jagged 1* (*JAG1* [MIM 601920]; GenBank accession number XM_056118), within families segregating congenital heart defects (Schott et al. 1998; Eldadah et al. 2001). *JAG1* encodes a highly conserved ligand within the Notch family, which consists of components of an intercellular signaling pathway shown to be crucial for cell-fate decisions in organisms spanning the phylogenetic spectrum (Lindsell et al. 1996; Lissemore and Starmer 1999). *JAG1* mRNA is abundantly expressed during development, and targeted disruption of *Jag1* in mice results in vascular defects and embryonic lethality (Loomes et al. 1999; Xue et al. 1999). The JAG1 protein has several domains that show high interspecies conservation, including an N-terminal "DSL" motif found in the Delta, Serrate, and Lag-2 ligands in the Notch family; 16 tandemly repeated epidermal-growth-factor (EGF)–like domains; and a transmembrane region (Lindsell et al. 1995; Oda et al. 1997). Among the 16 EGF-like domains, the first and second ones have been shown to be important for the formation of a high-affinity complex with Notch (Shimizu et al. 1999). Mutations in *JAG1* have been identified in ~70% of patients with Alagille syndrome (AGS [MIM 118450]), a rare autosomal dominant condition (Krantz et al. 1998; Yuan et al. 1998; Crosnier et al. 1999, 2001; Onouchi et al. 1999; Pilia et al. 1999; Heritage et al. 2000; Colliton et al. 2001; Giannakudis et al. 2001; Spinner et al. 2001) that comprises three of these five main features: paucity of intrahepatic bile ducts, congenital heart defects (predominantly peripheral pulmonic stenosis), skeletal de-

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fects, ocular anomalies, and characteristic facies (Alagille et al. 1975; Krantz et al. 1997). The absence of phenotypic differences between total gene deletions and protein-truncating mutations suggests that haploinsufficiency is a pathogenic mechanism causing AGS (Krantz et al. 1998; Crosnier et al. 1999). To date, 26 unique missense mutations have been identified in *JAG1,* most of them leading to AGS. A missense mutation (G274D) in JAG1 has also been identified, in a kindred with right-heart obstructive disease and characteristic facies, that is distinct from that seen in AGS (Eldadah et al. 2001). In addition, two mouse mutants, *Slalom* and *Headturner,* showed that missense mutations (P269S and G289D, respectively) in the second EGF-like domain of the *Jag1* gene lead to disturbances of the patterning of the organ of Corti in the inner ear (Kiernan et al. 2001; Tsai et al. 2001).

We studied a kindred (eight affected patients and seven unaffected individuals) with mild-to-severe combined deafness and congenital heart defects segregating as autosomal dominant traits (fig. 1). Auditory thresholds were determined by standard pure-tone audiometry with air and bone conductions at 250–8,000 Hz. In addition, previous audiological tests were collected. The phenotype of affected patients in this family was variable (table 1). Six of seven available affected individuals manifested mild-to-severe combined hearing loss, predominantly affecting middle frequencies. Two patients were diagnosed with vestibular pathology consistent with unstable equilibrium most prevalent during walking. To characterize vestibular dysfunction, a computed tomography (CT) scan was performed on patient III-6 and revealed bilateral aplasia of the anterior semicircular canal and hypoplasia of the posterior semicircular canal (fig. 2). All patients, who were evaluated in the Service de Cardiologie at CHU, exhibited congenital heart disease, including tetralogy of Fallot (ToF [MIM 187500]), ventricular septal defect (VSD), and/or peripheral pulmonic stenosis (PPS). Patient II-7 had ToF that did not allow surgical correction, because of severe PPS. He also had severe combined hearing loss that required a hearing aid (fig. 3), as well as vestibular dysfunction. One of his daughters (III-8) had very severe ToF, defined as the presence of pulmonary atresia and ventricular septal defect, which had been successfully surgically corrected in early childhood. Her audiogram was normal. His second daughter (III-6) had a VSD that spontaneously closed, as well as moderate combined hearing loss that required surgical correction and a hearing aid, associated with vestibular dysfunction. Patient II-1 had VSD, PPS, and moderate combined hearing loss. Patients II-2, II-3, and III-1 had isolated PPS and mild-to-moderate combined hearing loss. Patient III-2 had acute lymphoblastic leukemia and died at age 2.5 years; autopsy showed isolated PPS. All individuals had normal growth and cognition. No history of hepatic dysfunction was noted for any of the patients.

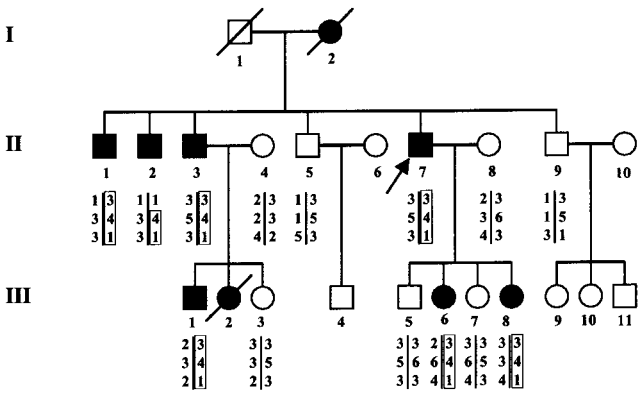

Figure 1 Pedigree of the family with deafness and congenital heart defects, showing three-locus genotypes and inferred haplotypes. Unblackened symbols denote unaffected individuals; blackened symbols denote affected individuals. Genotypes are shown in the following order, from top to bottom: *D20S115*, *D20S186*, and *D20S112*. The arrow indicates the proband.

Our protocol was approved by the appropriate institutional review boards. After informed consent was obtained, blood samples were collected from 14 family members. FISH analysis excluded microdeletion at 22q11, a common cause of syndromic and isolated conotruncal heart defects. Genomic DNA was prepared from peripheral lymphocytes. Linkage analysis was performed by standard methods, through use of fluorescence-labeled polymorphic STRs (ABI Prism Linkage Mapping Set version 2). Genotypes were analyzed with an ABI 377 automated sequencer. Alleles were assigned using the Genotyper version 2.0 program. Two-point linkage analysis was calculated using the MLINK program version 5.1, assuming a disease penetrance of 0.95. Because congenital heart defects are linked to mutations in *JAG1* or *NKX2.5* in humans and in *TBX1* or *NTF3* in mice, we considered these genes as suitable candidates (Donovan et al. 1996; Schott et al. 1998; Eldadah et al. 2001; Jerome and Papaioannou 2001; Lindsay et al. 2001; Merscher et al. 2001). Linkage analysis excluded the *NKX2.5, TBX1,* and *NTF3* loci but was conclusive for marker *D20S186,* in the vicinity of the *JAG1* gene (maximum LOD score [Z_{max}] 3.01 at recombination fraction 0.00) (Lathrop and Lalouel 1984). Mutation analysis was conducted by direct sequencing of the *JAG1* gene through use of an ABI 377 automated sequencer. All exons were amplified using previously described primers (Krantz et al. 1998). Direct sequencing of *JAG1* was performed on an affected family member (II-7). An amplicon spanning *JAG1* exon 5 revealed heterozygosity for a G→A transition at nucleotide 701 of the coding sequence, predicting substitution of cysteine (C) for tyrosine (Y) at position 234 (C234Y) of

Table 1

Comparison of the Clinical Features of Affected Individuals in the Family, with the Frequencies of Clinical Criteria in Patients with AGS

	Clinical Feature[a]									
Patient or Sample	Cardiac Disease	Deafness	Vestibular Pathology	Chronic Cholestasis	Ocular Abnormalities	Caracteristic Facies	Vertebral Anomalies	Growth Retardation	Renal Dysfunction	Mental Retardation
II-7	ToF	Severe	+	−	+	−	−	−	−	−
III-6	VSD	Moderate	+	−	+	−	−	−	−	−
III-8	ToF	−	−	−	+	−	−	−	−	−
II-1	VSD, PPS	Moderate	−	−	+	−	−	−	−	−
II-2	PPS	Mild	−	−	+	−	−	−	−	−
II-3	PPS	Mild	−	−	+	−	−	−	−	−
III-1	PPS	Mild	−	−	+	−	−	−	−	−
III-2	PPS	NA	NA	NA	NA	−	NA	−	−	NA
Patients with AGS[b]	92%	Rare	Rare	95%	78%	91%	70%	50%–90%	23%–74%	0%–16%

[a] + = Present; − = absent; NA = not available.
[b] Data are from Krantz et al. (1997) and are expressed as percentages of patients showing each clinical feature.

the JAG1 protein (fig. 4). The mutation occurred within the first of the 16 tandemly repeated EGF-like domains of JAG1. This sequence change was confirmed in all affected individuals with available genomic DNA ($n = 7$) in this family but was absent in unaffected family members. No DNA was available for the deceased sibling with PPS (III-2). C234Y was not found in 120 chromosomes from unrelated and unaffected individuals. Since the *JAG1* gene has been shown to cause AGS, a clinical geneticist (C.L.C.) reexamined all affected patients for subclinical manifestations of this syndrome. In all gene carriers, no characteristic facies or vertebral defects were identified, but all had asymptomatic posterior embryotoxon. Blood examination showed normal electrolytes, calcium, urea nitrogen, creatinine, total and direct bilirubin, transaminases, gamma-glutamyl transferase, and alkaline phosphatase consistent with normal renal and hepatic functions.

Deafness has been described in a few patients with AGS and cytogenetically visible deletions on 20p (Anad et al. 1990) but in none of the studies of large samples of patients with AGS. Therefore, hearing loss has been reported in patients with AGS who have chromosomal abnormalities on 20p (Krantz et al. 1997), suggesting a contiguous gene syndrome. First, however, the *JAG1* gene is expressed in the sensory regions of the ear (Crosnier et al. 2000). Second, the mouse mutant *Headturner* showed dominant head-shaking behavior indicative of a vestibular defect. It also demonstrates disturbances of the patterning of the organ of Corti in the inner ear, characterized by reduced numbers of outer hair cells, as well as loss of sensory structures in the vestibular system, including missing posterior and sometimes anterior ampulae and a truncation of the respective semicircular canals (Kiernan et al. 2001). Despite these patterning anomalies in the organ of Corti, *Headturner* mutants were not deaf but had slightly raised thresholds for compound action potentials, which is an indication of cochlear nerve responses, although these differences were not significant. A similar phenotype has also been seen in the mouse mutant *Slalom*, which also carries a mutation in *Jag1* (Tsai et al. 2001). Third, severe anomalies have been observed in temporal bones of four individuals with AGS. These patients exhibited partially or totally absent posterior and anterior semicircular canals. The cochlea was observed to be shortened in one case. An audiogram of one patient showed a bilateral mod-

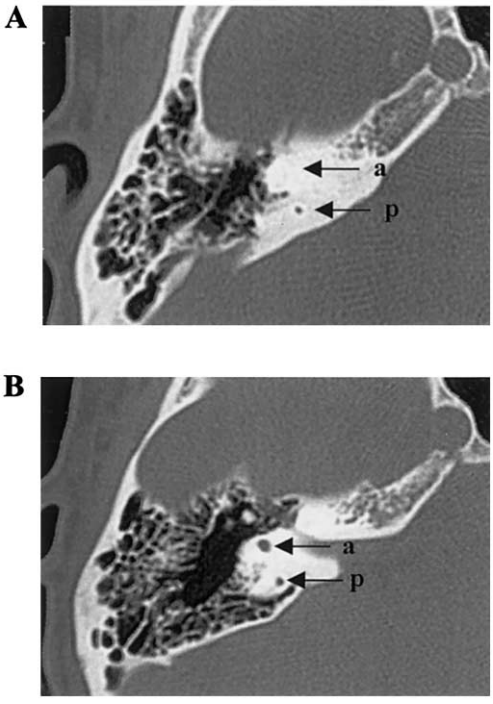

Figure 2 *A*, Axial CT scan of patient III-6, showing aplasia of the anterior semicircular canal (a) and hypoplasia of the posterior semicircular canal (p). *B*, Comparison with an unaffected individual.

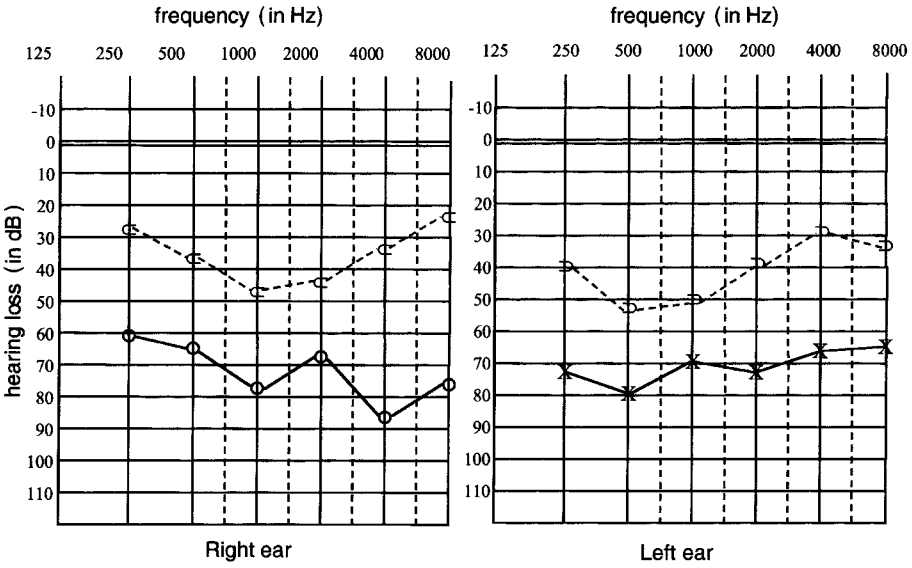

Figure 3 Representative audiogram for the family (individual II-7)

erate combined hearing loss (Okuno et al. 1990). Finally, hearing loss was described in a large kindred with classical AGS (LaBrecque et al. 1982). Patients were found to have normal karyotypes. The father and two children demonstrated a mild conductive hearing loss. At least two other family members were known to have hearing difficulties. The present study describes six of seven C234Y carriers with mild-to-severe combined hearing loss. JAG1 C234Y is the first mutation described in a large kindred with hearing loss.

The vast majority of *JAG1* mutations that cause AGS create premature termination codons, which predict an unstable message or the expression of truncated polypeptides that lack essential domains (Krantz et al. 1998; Crosnier et al. 1999). AGS is also caused by hemizygosity for *JAG1,* establishing haploinsufficiency as the relevant pathogenetic mechanism. However, the mechanism by which missense mutations in AGS lead to disease is unknown, and a dominant negative mechanism cannot be ruled out. In *Drosophila,* mutant Delta and Serrate (Notch ligands that are highly homologous to JAG1) act in a dominant negative fashion (fig. 5*A*) (Rebay et al. 1991; Sun and Artavanis-Tsakonas 1996). Among the 26 unique missense mutations identified in *JAG1,* 12 occurred in the EGF repeats (fig. 5*A*). Eight result in loss or gain of a conserved cysteine residue that forms intramolecular disulfide linkages, an event known to perturb folding and to target proteolytic degradation in other proteins (Schrijver et al. 1999). Each EGF-like domain contains six cysteine residues that form stable disulfide bridges creating stable three-dimensional conformations. Cysteine substitutions disrupt one of three disulfide bridges that covalently connect three pairs of cysteine residues that are highly conserved in EGF-like domains (Aoyama et al. 1993; Downing et al. 1996). This has a predictable detrimental effect on the domain itself, as shown in structural studies of similar domains in other proteins, but it also interferes with calcium binding even when the required consensus sequence is intact

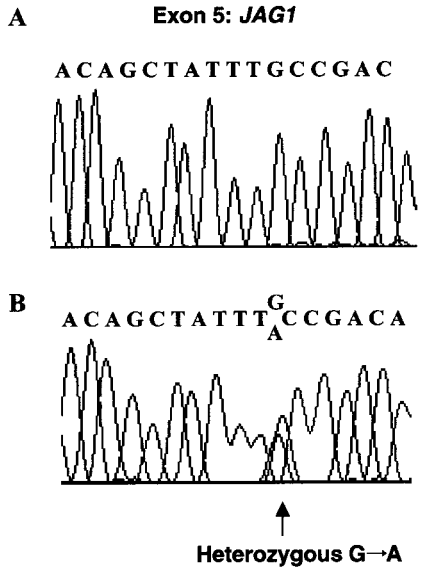

Figure 4 Sequence chromatograms showing the mutation found in the kindred. *A,* Sequencing data from individual II-8, representing the normal *JAG1* allele. *B,* Sequencing data from an affected patient (II-7), representing the normal and the mutant *JAG1* alleles. A G→A transition changes the codon for cysteine (TGC) in position 234 of the JAG1 protein to the codon for tyrosine (TAC).

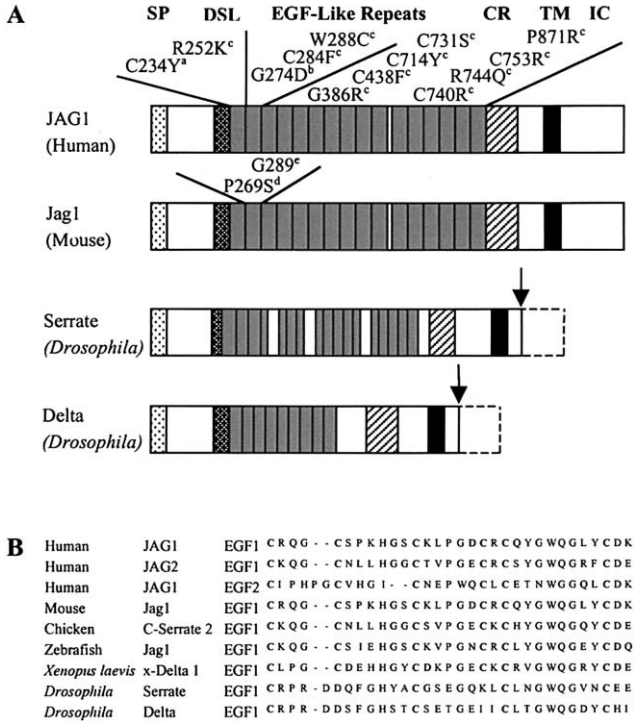

Figure 5 *A,* Schematic diagram of human JAG1, mouse Jag1, and *Drosophila* Delta and Serrate protein structures, and the locations of missense mutations identified in EGF-like domains of human JAG1 and in the second EGF-like domain of mouse Jag1. The arrows indicate C-terminal deletions of Delta and Serrate. Superscripts associated with mutations are as follows: a = missense mutation described in the present study; b = mutation described in a kindred segregating ToF and characteristic facies; c = mutations described in patients with AGS; d = mutation described in mouse *Slalom* mutant; e = mutation described in mouse *Headturner* mutant. SP = signal peptide; DSL = conserved domain shared by Delta, Serrate, and Lag2; CR = cysteine-rich region; TM = transmembrane domain; IC = intracellular domain. *B,* Alignment of the amino acid sequence of the first EGF-like domain of human JAG1 with the corresponding sequences of the first EGF-like domain of human JAG2, the second EGF-like domain of human JAG1, and those from other species. Note the first cysteine is conserved in all of the wild-type sequences.

(Reinhardt et al. 1997). The novel missense mutation we describe implicates the first cysteine in the first EGF-like domain that disrupts the C1-C3 disulfide bridge and probably affects calcium binding. The mutation alters a residue that is conserved through evolution within this and the other EGF-like domains of JAG1, as well as other proteins, including Jagged 2, Delta, and Serrate (fig. *5B*).

In the present study, we describe a kindred segregating a unique phenotype with highly penetrant deafness, posterior embryotoxon, and congenital heart defects. We have identified a novel missense mutation (C234Y) in the extracellular domain of JAG1 in the first of the 16 EGF-like domains, which demonstrates that mutations in *JAG1* can cause hearing loss. In the presence of a congenital heart defect suggestive of AGS-associated cardiopathies, a audiological test in infancy should be proposed. Further studies of the missense mutations in the EGF-like repeats of *JAG1* may provide new insights in JAG1-related diseases.

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Electronic-Database Information

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

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for the *JAG1* gene [accession number XM_056118])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for AGS [MIM 118450], JAG1 [MIM 601920], and ToF [MIM 187500])

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