

Connexin 43 (GJA1) Mutations Cause the Pleiotropic Phenotype of Oculodentodigital Dysplasia

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Gap junctions are assemblies of intercellular channels that regulate a variety of physiologic and developmental processes through the exchange of small ions and signaling molecules. These channels consist of connexin family proteins that allow for diversity of channel composition and conductance properties. The human connexin 43 gene, or *GJA1*, is located at human chromosome 6q22–q23 within the candidate region for the oculodentodigital dysplasia locus. This autosomal dominant syndrome presents with craniofacial (ocular, nasal, and dental) and limb dysmorphisms, spastic paraplegia, and neurodegeneration. Syndactyly type III and conductive deafness can occur in some cases, and cardiac abnormalities are observed in rare instances. We found mutations in the *GJA1* gene in all 17 families with oculodentodigital dysplasia that we screened. Sixteen different missense mutations and one codon duplication were detected. These mutations may cause misassembly of channels or alter channel conduction properties. Expression patterns and phenotypic features of *gja1* animal mutants, reported elsewhere, are compatible with the pleiotropic clinical presentation of oculodentodigital dysplasia.

Introduction

Oculodentodigital dysplasia (ODDD [MIM *164200]), also known as “oculodentoosseous dysplasia,” is an autosomal dominant disorder with high penetrance, intra- and interfamilial phenotypic variability, and advanced paternal age in sporadic cases. To date, >70 reports in the literature describe the clinical features of ODDD in >240 patients, the majority of whom were white (Gorlin et al. 2001; Loddenkemper et al. 2002). The typical craniofacial anomalies include a thin nose with hypoplastic alae nasi, small anteverted nares, prominent columnella, and microcephaly (fig. 1). Brittle nails and hair abnormalities of hypotrichosis and slow growth are present. Some cases have dysplastic ears and conductive hearing loss. Ophthalmic findings include microphthalmia, microcornea, fine porous spongy iris abnormalities, cataracts, glaucoma, and optic atrophy. Anomalies observed in the oral region are mandibular overgrowth and cleft

palate. The majority of cases have abnormal primary and permanent dentition with microdontia, partial anodontia, enamel hypoplasia, multiple caries, and early tooth loss. Hand and foot abnormalities in ODDD include syndactyly involving the third, fourth, and fifth fingers and second to fourth toes, camptodactyly, and clinodactyly due to hypoplasia or aplasia of the middle phalanges. Other skeletal abnormalities are cranial hyperostosis, mandible with wide alveolar ridge, and broad tubular bones.

Neurologic symptoms are frequent in ODDD and include dysarthria, neurogenic bladder disturbances, spastic paraparesis, ataxia, anterior tibial muscle weakness, and seizures (Loddenkemper et al. 2002). Symptoms of spastic bladder or gait disturbances are the usual presenting neurologic manifestations evident by the 2nd decade of life. Mild mental retardation occurs infrequently. Brain magnetic resonance imaging studies of patients with ODDD have shown diffuse bilateral abnormalities in the subcortical cerebral white matter, which can define a slowly progressive leukodystrophy.

The ODDD locus was mapped to chromosome 6q22–q24 by linkage analysis (Gladwin et al. 1997), and the location of the disease gene was further refined between markers D6S266 and D6S1639 (Boyadjiev et al. 1999). A detailed physical map with a YAC contig of the region

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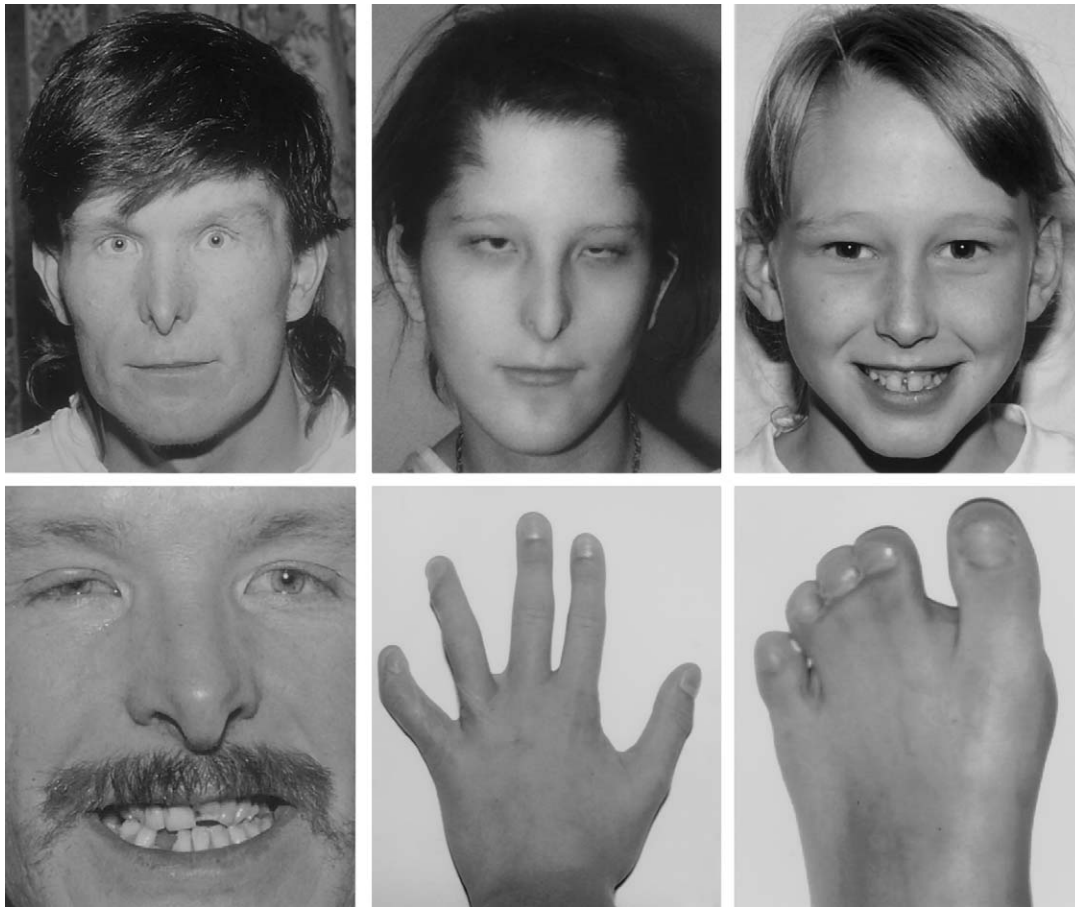


Figure 1 ODDD phenotype. Across the top are an adult, a teenager, and a child with microcornea, narrow nose, and/or hypoplastic alae nasi. Across the bottom are additional features: partial ano- and microdontia and enamel hypoplasia, syndactyly, and clinodactyly. The child's hand had surgical correction of the fourth and fifth finger syndactyly. Note that the child has a mild facial phenotype, but significant digital involvement, as shown in the bottom two right panels. The individuals in the top and bottom left panels are brothers with the Y17S mutation. The teenager has a G22E mutation, and the child has an Y98C mutation.

was created and fourteen candidate genes were identified and excluded by mutation analysis (Boyadjiev et al., in press; unpublished data). Another gene in the region is *GJA1*, which encodes gap junction protein alpha 1, a member of a large family of homologous connexin proteins.

GJA1 or connexin 43, like other connexin proteins, consists of an intracellular N-terminus, four transmembrane domains, two extracellular loops, one cytoplasmic loop, and an intracellular C-terminus (Kelsell et al. 2001). Six connexins can form a connexon, a specialized intracellular structure surrounding a pore. Two connexons in apposing cell membranes can align to form an intercellular gap junction. These channels provide a direct low-resistance intercellular pathway for the passage of ions and small molecules that confer distinct physiological properties. Gap junctions have been found in the majority of mammalian tissues. Most tissues ex-

press more than one type of connexin, and multiple types of connexins can assemble to form gap junctions between cells, with the diversity of combinations influencing the nature of the cell-to-cell communication. These properties are important for many physiological and developmental processes.

In mouse embryogenesis, *Gja1* is expressed, beginning at the blastocyst stage, in discrete spatially restricted domains in the developing brain, neural tube, prevertebrae, and limb, and in various aspects of organogenesis (Ruangvoravat and Lo 1992). Its expression is associated with developmental processes mediated by inductive interactions, involving the branchial arches, eye, and otic vesicle, as well as migratory cells of the neural crest and sclerotomes. In postnatal tooth development, rat connexin 43 is demonstrated between the odontoblasts that actively secrete dentin matrix (Murakami et al. 2001), and its expression is seen in the epithelial-derived ame-

loblasts synthesizing and secreting enamel matrix proteins (Fried et al. 1996). A BLASTn search of the NCBI human est database, using the *GJA1* mRNA sequence NM_000165, revealed *GJA1* expression in libraries derived from placenta, brain, nerve, thyroid, lung epithelial cells, bone, bone marrow, testis, uterus, skin, and duodenal adenocarcinoma.

Despite the near-ubiquitous expression of the connexins, previous disorders associated with mutations in various connexins have a restricted range of phenotypic abnormalities, isolated deafness, skin abnormalities with or without deafness, or peripheral neuropathy. The human *GJA1* gene is a candidate gene for the pleiotropic condition ODDD because of its map location and wide expression pattern. In this study, we screened the candidate gene *GJA1* for mutations in families affected with ODDD.

Patients and Methods

We recruited seventeen unrelated white families affected with ODDD, with a total of 60 family members ranging in age from 2 wk to adulthood. All affected individuals had the typical ODDD craniofacial appearance and limb involvement. Members of nine of these families had neurologic manifestations. We obtained informed consent (protocol approved by the institutional review board of Johns Hopkins University) from all participants who underwent physical examination, photo documentation, and sample collection. DNA was isolated from patients' blood samples or Epstein Barr virus-transformed lymphoblastoid cell lines. Centre d'Etude du Polymorphisme Humain (CEPH) DNAs were used as controls.

The organization of the *GJA1* gene was determined from the human chromosome 6 sequence (map element NT_033944.2 in NCBI Sequence Viewer). The gene is composed of two exons with an intervening intron 11 kb in size. The mRNA sequence (NM_000165 or XM_027459) contains 191 bp of the first exon, which is untranslated. The second exon consists of 16 bp of additional 5' untranslated sequence, 1,149 bp of coding sequence, and ~1,732 bp of 3' untranslated sequence. There are two potential poly (A) signal sites in the 3' UTR at nucleotides 1759–1764 and at 3062–3067 of NM_000165 with the latter being utilized. There is a *GJA1* pseudogene, *GJA1P1*, located within chromosome 5 at 5q21–5q22 (map element NT_006654.9).

PCR primers were designed with mismatches to the pseudogene sequence to specifically amplify *GJA1* and avoid amplification of *GJA1P1*. A 925-bp product, which contains the coding sequence for the first 238 amino acids of the GJA1 protein, was amplified with forward primer 5'-GATCTTTTCTTCGTTGGC-3' (within intron 1) and reverse primer 5'-CTCTTCCCTTAACCCG-3'. A 1,079-bp product, which contains the coding

region for the latter 178 amino acids of the GJA1 protein and extends into the 3' UTR, was amplified with forward primer 5'-TTCCTCTCTCGCCCCAC-3' and reverse primer 5'-GGCCTAGAAAGCTTACCTT-3'. Conditions of amplification were determined on a Bio-Rad Laboratories iCycler. All amplifications were performed using Platinum *Taq* High Fidelity DNA polymerase (Invitrogen). PCR products were sequenced in the forward and reverse directions by the dideoxy chain termination method on an ABI Prism 3700 automated fluorescent DNA analyzer (Applied Biosystems).

Six rare polymorphisms were observed within the 100 control alleles examined: IVS1-65G/A (1% incidence), IVS1-12T/A (1%), 717G/A (2%), 758C/T (1%), 11152_1153insA (2%), and 1322G/A (3%). Two of these were within the coding region, one resulting in a synonymous change, R239R (CGG→CGA), and the other resulting in A253V (GCG→GTG). The polymorphic amino acid change within the C-terminal domain, A253V, seen in one control allele, occurred in an amino acid that is not conserved among the GJA1 proteins of various species or among the human connexins. It should be noted that most of the variations listed in the *GJA1* mRNA entry NM_000165 between nucleotides 160–1384 can be accounted for by nucleotide differences between *GJA1* and its pseudogene and are not polymorphisms within *GJA1*.

Results and Discussion

GJA1 Mutations

We found mutations in 100% of the individuals studied who were affected with ODDD (table 1; fig. 2). A different missense mutation was found for each family, with the exception of a codon duplication in one family. These mutations segregated with the disease and were not found in any unaffected family members or in 100 control alleles from white subjects. The presence of different mutations in each family provides evidence that ODDD did not arise from a founder effect in the white population.

These amino acid changes are distributed throughout the protein, with the majority occurring in the amino terminal half (table 1; fig. 3). Mutations that produced pronounced changes—on the basis of differences in amino acid size and charge and lack of conservation among the connexins—are present in the cytoplasmic (Y17S, Y98C, I130T, and G138R), transmembrane (G21R and G22E), and extracellular (R76S) domains. Each mutation affects an amino acid highly conserved among the connexin 43 protein sequences for various species (fig. 4). Additionally, many of these same amino acids are conserved among the human connexin protein family, which are expressed from at least 20 different

Table 1**GJA1 Mutations in ODDD**

Family/Case	Inheritance ^a	Nucleotide Change ^b	Amino Acid Change ^b	Reference(s) ^c
FOD1118-4881	F*	50A>C	Y17S	Rajic and deVeber 1966; Boyadjiev et al. 1999
FOD1120-1902	F*	52T>C	S18P	Zellweger and Ionasescu 1974; Zach 1975; Judisch et al. 1979
FODMS2630	S*	61G>A	G21R	This report
FODHN2441	S	65G>A	G22E	Traboulsi and Parks 1990
FOD1083-4795	S	68A>C	K23T	Gorlin et al. 1963
FODCL2603	S*	119C>T	A40V	This report
FODJC1858	F*	145C>A	Q49K	Boyadjiev et al. 1999
FODLM1899	F	154_156dupTTT	F52dup	Gellis and Feingold 1974; Weintraub et al. 1975
FOD1113-4855	F*	226C>A	R76S	Stanislaw et al. 1998; Boyadjiev et al. 1999
FOD1140-4922	F*	268C>G	L90V	Mohr 1939; Opjordsmoen and Nyberg-Hansen 1980; Boyadjiev et al. 1999
FOD1119-4896	F*	293A>G	Y98C	Wooldridge et al. 1977; Wooldridge 1993; Boyadjiev et al. 1999
FODBC36590	F*	306G>C	K102N	This report
FODRB2514	F	389T>C	I130T	This report
FODOK2770	S	400A>G	K134E	This report
FOD975-4595	F*	412G>C	G138R	Shapiro et al. 1997; Boyadjiev et al. 1999
FODHB00-153-0388	F*	605G>A	R202H	This report
FODJS2375	F	646G>T	V216L	Norton et al. 1995; Boyadjiev et al. 1999

^a F = familial; S = sporadic. An asterisk (*) denotes that more than one sample was available to document, in multiplex families, segregation of the mutation or, in sporadic cases, the absence of the mutation in both parents.

^b Mutation nomenclature is according to den Dunnen and Antonarakis (2000).

^c References are for families previously reported with clinical description or who have participated in linkage studies.

genes. Several of the amino acid alterations occur at the same residues that are mutated in other connexin (26, 31, and 32) disorders (table 2). The phenotypes do not correlate between those generated by connexin 43 mutations and by analogous amino acid mutations in other connexins. Multiple connexin 26 and 32 mutations in amino acids corresponding to connexin 43 R76 and R202 result in more than one phenotype.

Major Phenotypic Features (Syndactyly and Neurodegeneration) and GJA1 Genotype Analysis

Our mutation analysis supports clinical observations that ODDD is fully penetrant, since all carriers of mutations exhibited craniofacial and limb dysmorphisms. Intrafamilial variability of the major phenotypic characteristics was observed for all mutations segregating in multiplex families. For example, all of our probands have syndactyly involving at least the fourth and fifth fingers. Four individuals with sporadic disease have syndactyly type III (involvement of only the fourth and fifth fingers) and different missense mutations in the first transmembrane domain (G21R, G22E, K23T, and A40V). Two of our multiplex families have a proband with syndactyly type III but have other affected family members with additional digital involvement and mutations in domains beyond the first transmembrane (F52dup and R202H). These data suggest, as already stated in the literature, that ODDD and “isolated” syndactyly type III (MIM 186100) may represent a disease spectrum rather than separate

genetic conditions (Schrandt-Stumpel et al. 1993). The association of GJA1 mutations with abnormal limb development can be inferred by the strong expression of connexin 43 in the distal part of early limb buds and its restricted pattern in the developing digits and regions of precartilaginous condensation, as shown in *Xenopus laevis*, mouse, and chick (Ruangvoravat and Lo 1992; Meyers et al. 1997; van der Heyden et al. 2001).

It should also be noted from our data that specific phenotypic characteristics of ODDD did not appear to segregate with mutation(s) in specific protein domain(s). Thus, no obvious phenotype-genotype correlations were noted. As an example, nine of our families with neurologic symptoms, including five with documented white-matter degeneration, have mutations distributed in the N-terminal, membrane and intracellular domains (Y17S, G22E, K23T, L90V, K102N, I130T, K134E, G138R, and V216L). Epilepsy, which has been observed previously in ODDD, is present in two of our case subjects with the mutations R76S in the first extracellular loop and L90V in the second transmembrane domain. Two individuals with sporadic cases of ODDD and with G21R and A40V mutations do not manifest neurologic symptoms but are <2 years of age. It is of note that no neurologic symptoms were present among all affected adults in five of our affected families (S18P, Q49K, F52dup, Y98C, and R202H). Mutations in *GJB1* (connexin 32), the major connexin of myelin-forming Schwann cells result in X-linked Charcot-Marie-Tooth

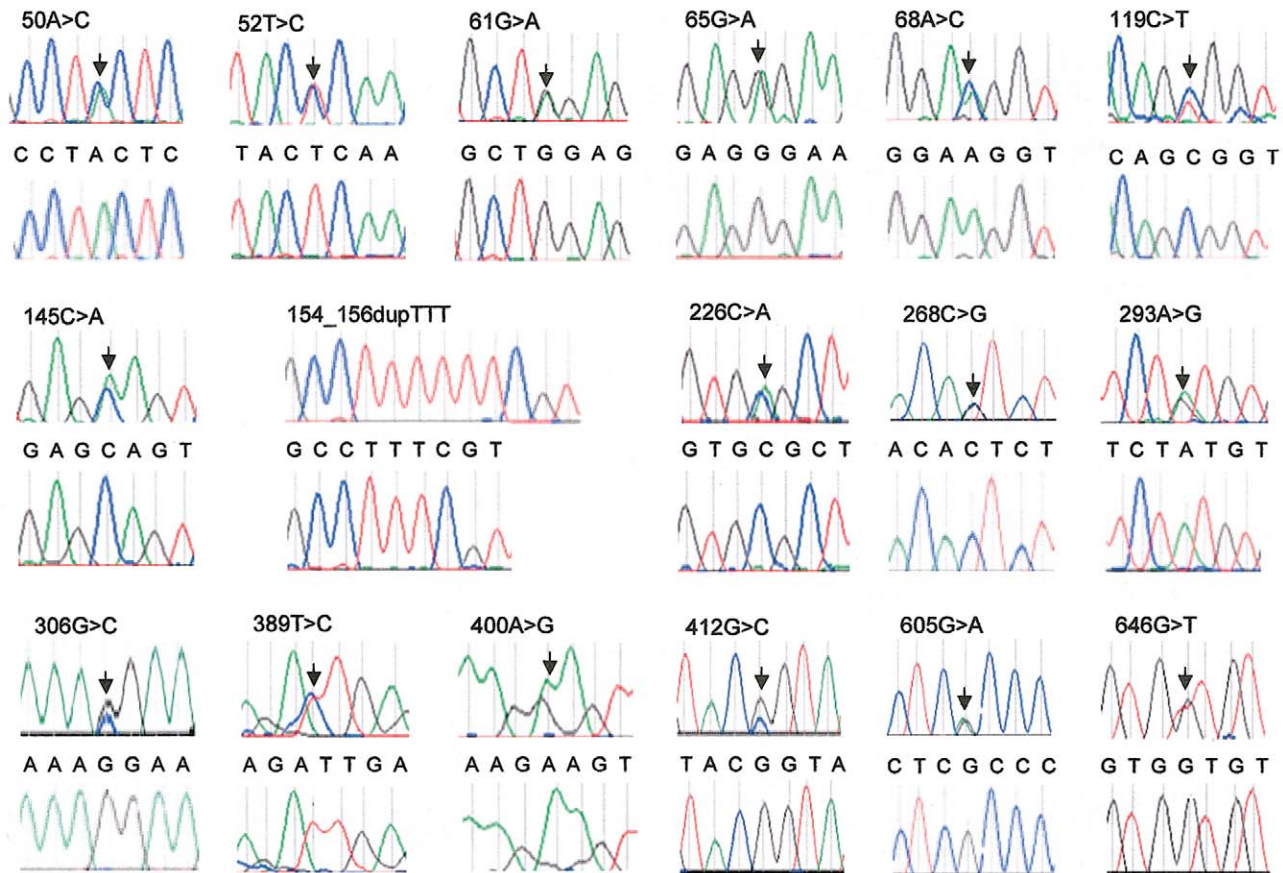


Figure 2 Sequences of 17 *GJA1* mutations found in families affected with ODDD. The top panels show the heterozygous mutations, with the exception of the sequence of the cloned duplication, 154_156dupTTT, and the bottom panels show the control sequences.

disease (MIM 302800), a congenital demyelinating disorder of peripheral nerve (Bergoffen et al. 1993; Ressot and Bruzzone 2000). The type of degeneration in this disease differs from ODDD, since it involves myelin of peripheral nerves rather than myelin of the CNS.

In the CNS, connexin 43 is the major connexin of astrocytes and ependymocytes but is not expressed in oligodendrocytes or neurons. The role of connexin 43 in astrocyte gap junctions is not well understood, but they have been implicated in the propagation of cortical “calcium waves” and “spreading depression” (Martins-Ferreira et al. 2000), and epilepsy is associated with cortical astrocytes with increased gap junction coupling (Lee et al. 1995). Astrocytes may also express limited quantities of other connexins, such as connexins 26, 30, and 45 (Dermietzel et al. 2000). Myelin-forming oligodendrocytes predominantly express connexin 32 but may also express connexin 45 (Dermietzel et al. 1997). Although astrocytes or oligodendrocytes form gap junctions with themselves, astrocytic-oligodendroglial gap junctions are also observed. The latter junctions may result from several possible heterotypic pairings, includ-

ing connexin 43 with connexin 45 (Rash et al. 2001). Aberrant heterotypic pairings may be a molecular explanation of how mutations in connexin 43, which is normally expressed in astrocytes but not in oligodendroglial cells, can contribute to the dysfunction of CNS myelin observed in ODDD leukodystrophy.

Additional Phenotypic Features (Conductive Hearing Loss, Cataracts, Glaucoma, Keratoderma, and Cardiac Defects) and GJA1 Genotype Analysis

Eight of our families affected with ODDD have conductive hearing loss and mutations located in the N-terminal, transmembrane, and cytoplasmic loop regions (Y17S, S18P, G22E, K23T, L90V, Y98C, G138R, and V216L). *GJA1* and other connexins are expressed in the cochlea (Rabionet et al. 2000), and mutations in other connexin proteins (connexin 32, 26, 31, and 30) have been associated with both autosomal dominant and autosomal recessive nonsyndromic hearing impairment, usually sensorineural. The type of hearing impairment in ODDD differs from the typical hearing loss associated

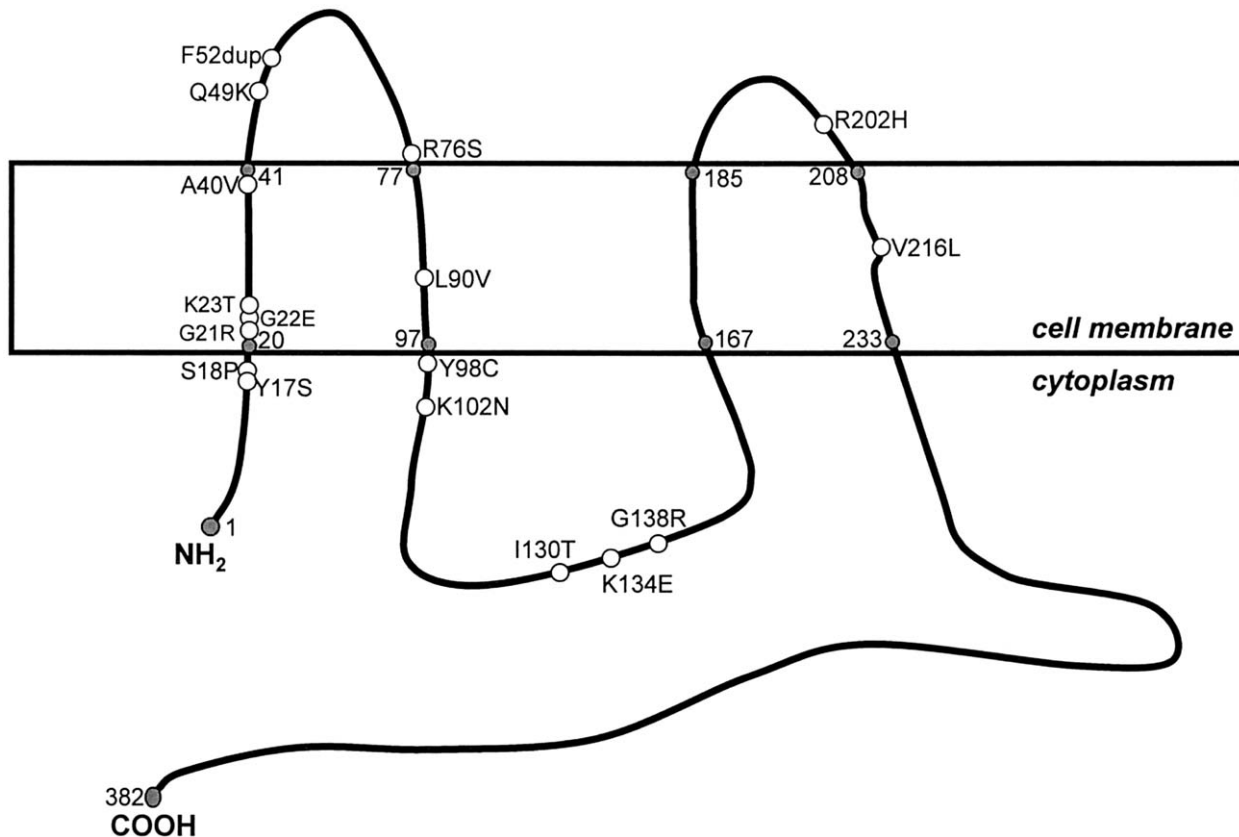


Figure 3 Diagram of the GJA1 protein. Locations of the amino acid changes are shown. The first and last amino acids of the protein and of each transmembrane domain are indicated as predicted by the program TMpred (see the TMpred Web site).

with other connexin mutations, because it is conductive rather than sensorineural. Lui and his colleagues (2001) reported L11F and V24A mutations in connexin 43 in association with sensorineural recessive deafness. However, these mutations have subsequently been shown to involve the pseudogene of connexin 43 on chromosome 5 (W. E. Nance, personal communication).

One familial case had cataracts and the Y17S mutation. In the literature, there are mutations in GJA3 and GJA8 (connexins 46 and 50) that are found in patients with dominant zonular pulverulent cataracts (Shiels et al. 1998; Mackay et al. 1999). Glaucoma presented in this same individual with the Y17S mutation and in two other case individuals with G22E and L90V mutations. Connexin 43 plays an important role in eye development. It is expressed in the lens placode and during fetal stages in the anterior and lateral lens epithelium in the mouse, sheep, and chick (White 2002). Other connexins including connexin 46 and 50 are also expressed in the developing lens. In connexin 50 knockout mice, White et al. (1998) demonstrated that there was little if any prenatal effect, but postnatally two ocular abnormalities were present—microphthalmia and pulverulent cata-

racts. Similar features have been observed in ODDD patients. Connexin 43 is also localized in the outer pigmented cell layer of the ciliary body and pairs to the nonpigmented cell layer to form heterotypic junctions (Vaney et al. 2000). Glaucoma can result from an imbalance in intraocular fluid production by the ciliary body and outflow through the trabecular meshwork. Since connexin 43 deficient mice fail to thrive past birth, it can only be postulated that connexin 43 deletion or another type of mutation may unbalance connexin diversity and result postnatally in cataracts and glaucoma.

Some of our patients with ODDD exhibited phenotypic features rarely associated or not previously described for this condition. Two members of the family with the F52dup mutation had cleft palate. A familial case with the K102N mutation had plagiocephaly with no synostosis. One individual with sporadic ODDD had hyperkeratosis of the palms and soles and the K134E mutation. It is of interest that missense mutations in *GJB2* (connexin 26) have been implicated in other skin conditions: palmar and plantar keratoderma with sensorineural deafness (Richard et al. 1998a), Vohwinkel syndrome (keratoderma, constriction of the digits, and

		Amino Acid at Connexin 43 Number																
		17	18	21	22	23	40	49	52	76	90	98	102	130	134	138	202	216
Accession no.	Protein (connexin) species																	
gi:4504001	GJA1 (Cx43) human	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:6753992	Gja1 (Cx43) mouse	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:117708	Gja1 (Cx43) rat	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:117704	gja1 (Cx43) bovine	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:482508	gja1 (Cx43) chicken	Y	S	G	G	K	A	H	F	R	L	Y	K	I	K	G	R	V
gi:17939336	gja1 (Cx43) Gallus gallus	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:117709	gja1 (Cx43) Xenopus laevis	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:16024889	gja1 (Cx43) Silurana tropicalis	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:10717217	gja1 (Cx43) Cyprinus carpio	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:2921388	gja1 (Cx43) Danio rerio	Y	S	G	G	K	A	Q	F	R	L	Y	K	I	K	G	R	V
gi:3192831	gja1 (Cx43) Danio aequipinnatus	Y	S	G	G	K	A	Q	F	R	L	Y	K	N	E	G	R	V
gi:4504003	GJA4 (Cx37) human	H	S	V	G	K	A	Q	F	R	L	Y	R	I	M	S	R	V
gi:22779877	GJA3 (Cx46) human	H	S	I	G	K	A	Q	F	R	L	H	M	-	D	S	R	V
gi:6631083	GJA5 (Cx40) human	H	S	V	G	K	A	Q	F	R	L	H	M	A	E	W	R	V
gi:4885275	GJA8 (Cx50) human	H	S	I	G	R	A	Q	F	R	L	H	M	Q	K	G	R	V
gi:6980948	GJB2 (Cx26) human	H	S	I	G	K	A	Q	F	R	L	Y	-	-	-	-	R	V
gi:6014760	GJB6 (Cx30) human	H	S	I	G	K	A	Q	F	R	L	Y	-	-	-	-	R	A
gi:4504005	GJB1 (Cx32) human	H	S	I	G	R	A	K	F	R	L	H	-	-	-	-	R	A
gi:12229761	GJB4 (Cx30.3) human	Y	S	L	S	R	A	Q	F	R	L	Y	-	-	-	-	R	T
gi:10835079	GJB5 (Cx31.1) human	Y	S	F	G	R	T	H	F	R	L	Y	-	-	-	-	K	T
gi:13128960	GJB3 (Cx31) human	Y	S	F	G	R	A	Q	F	R	L	Y	-	-	-	-	R	A
gi:14211941	Cx62 human	H	S	V	G	K	A	Q	F	R	L	Y	A	D	R	E	R	I
gi:13540537	GJA10 (Cx59) human	H	S	I	G	K	A	Q	F	R	L	Y	V	L	L	E	R	I
gi:4885169	GJA7 (Cx45) human	H	S	V	G	K	G	Q	F	R	V	H	K	E	P	-	R	V
gi:9966911	GJA12 (Cx46.6) human	H	S	V	G	K	G	Q	F	R	V	H	R	A	P	G	R	V
gi:22550106	GJC1 (Cx31.9) human	Q	S	V	G	R	V	Q	F	R	V	H	K	-	P	R	R	V
gi:10190698	Cx36 human	H	S	I	G	R	V	Q	F	R	L	H	K	L	P	R	R	V
ODDD Mutations		S	P	R	E	T	V	K	Fdup	S	V	C	N	T	E	R	H	L

Figure 4 Amino acid conservation in connexins. The amino acids of GJA1 that are affected in ODDD are aligned to the corresponding amino acids in gja1 in other species and in other human connexins. Accession numbers given (from NCBI's Entrez-Protein Web site) are for the versions used in the alignment. Connexin amino acid alignments were performed using the ClustalW program (see ClustalW Web site). Proteins are ordered according to homology. Gray shading indicates amino acid differences from the human GJA1 protein. Darker shading indicate amino acids observed only once in the connexins.

sensorineural deafness) (Maestrini et al. 1999), and KIDS (keratitis, ichthyosis, and sensorineural deafness) (Richard et al. 2002). Mutations in *GJB3* and *GJB4* (connexin 31 and 30.3) can cause erythrokeratoderma variabilis (Richard et al. 1998b; Macari et al. 2000), and mutations in *GJB6* (connexin 30) are present in patients with hidrotic ectodermal dysplasia (Lamartine et al. 2000).

Two of our families with ODDD had cardiac abnormalities. One individual with sporadic ODDD who had the G21R mutation had an atrioseptal defect. In addition, a familial case had recurrent ventricular tachycardia and an atrioventricular block with the I130T mutation. The latter proband's paternal grandmother and father both died at 43 years of age, from cardiac arrhythmia and sudden cardiac death, respectively. Two paternal aunts have ventricular tachycardia and sick sinus syndrome. The proband and father have craniofacial features of ODDD. Although some of the family members with cardiac findings do not present with the craniofacial features of ODDD, they have not been screened for the mutation. The presence of congenital heart dis-

ease in ODDD has been noted previously. Schneider et al. (1977) reported a patient with an endocardial cushion defect, ostium primum atrial septal defect with a cleft mitral valve, which may result from a conotruncal heart defect. In addition, a heart murmur attributed to a ventricular septal defect was reported in an ODDD patient (Judisch et al. 1979).

The results of molecular studies are not clear as to the role that GJA1 mutations, rare or mosaic, may play in human cardiac malformations such as visceratrial heterotaxia (Britz-Cunningham et al. 1995) and hypoplastic left heart (Dasgupta et al. 2001). However, it should be noted that a major site of expression of connexin 43 in mammals is cardiac muscle, where other connexins (37, 40, and 45) are also expressed (Kanter et al. 1993). Connexin 43 expression is not detected in the heart of day 8.5 mouse embryos, but it is quite prominent in the ventricle by day 10.5. Transgenic mice containing a cytomegalovirus (CMV) promoter driven connexin 43 construct caused *Gja1* overexpression and conotruncal heart, right ventricle, pulmonary outflow tract, coronary vasculature, and neural tube defects (Ewart et al. 1997).

Table 2**Connexin Mutations in Conserved Amino Acids**

Protein	Mutations	Phenotype	Reference
GJA1 (Cx43)	S18P G22E A40V R76S L90V K134E R202H	ODDD	This report
GJB2 (Cx26)	S17F	KIDS	Richard et al. 2002
GJB2 (Cx26)	R75W	ADN-SHI and PPK	Richard et al. 1998a
GJB2 (Cx26)	R75Q	ADN-SHI	Connexin-Deafness Home Page
GJB2 (Cx26)	K122I	ARN-SSNHI	Connexin-Deafness Home Page
GJB2 (Cx26)	R184Q	ADN-SSNHI	Connexin-Deafness Home Page
GJB2 (Cx26)	R184P	ARN-SHI, compound heterozygote with delE138	Connexin-Deafness Home Page
GJB2 (Cx26)	R184W	ARN-SHI, compound heterozygote with M34T	Connexin-Deafness Home Page
GJB3 (Cx31)	R180X	ADN-SHI	Connexin-Deafness Home Page
GJB1 (Cx32)	L89V	ADHI	Connexin-Deafness Home Page
GJB1 (Cx32)	G21D A39P R75P L89P	R183C	CMT1X
GJB1 (Cx32)	R75Q	R183H	CMT1X
GJB1 (Cx32)	R75W	R183S	CMT1X
GJB1 (Cx32)	A39V	CMT1X and 48,XXYY	CMT Mutations by Gene Web site
GJB1 (Cx32)	A39V	CMT1X and CNS involvement	CMT Mutations by Gene Web site

NOTE.—AD = autosomal dominant; AR = autosomal recessive; N-S = nonsyndromic; SN = sensorineural; HI = hearing impairment; PPK = palmoplantar keratoderma; CMTX1 = X-linked Charcot-Marie-Tooth syndrome.

The developmental abnormalities in the coronary vasculature are consistent with neural crest involvement, since deployment of the coronary vessels is dependent on neural crest–derived parasympathetic innervation in the heart. The Purkinje conduction fibers codistribute with the formation of coronary vasculature in avian embryos (Gourdie et al. 1995). Connexin 43 heterozygous knockout mice bred with connexin 40 null mice to yield haploinsufficiency of both genes show additive effects on ventricular (not atrial) conduction and cardiac morphogenesis of hypertrophy, in conjunction with atrioventricular junction or ventricular septal defects (Kirchhoff et al. 2000). The detection of the conduction system abnormalities in one of our families with ODDD may be consistent with altered expression of connexin 43. It is less clear whether the septal defect found in the other ODDD case mentioned above is directly related to connexin 43 dysfunction.

It was hypothesized that ODDD displayed the phenomenon of genetic anticipation (Shapiro et al. 1997). At least four ODDD families in the literature showed increasing severity of neurologic symptoms—such as white matter changes of the brain, spastic bladder, spastic paraplegia, and ataxia—in subsequent generations. Three families have shown more involvement of dysmorphic features, particularly syndactyly, in successive generations. However, mutation analysis of four of these families revealed changes (S18P, L90V, Y98C, and G138R) that cannot account for the genetic anticipation. Although unlikely, additional changes might be observed in the noncoding regions, which we did not screen, where many of the repeated trinucleotide sequences have been found for other disorders showing genetic anticipation. It is more likely that there are genetic and environmental modifiers that can account for the “observed” anticipation.

Relevance of Gja1 Null Mouse

Features observed in connexin 43 knockout mice are relevant but do not mimic the craniofacial, limb, or cardiac findings associated with ODDD. The lack of phenotypic correlation between the knockout mouse and ODDD suggests that loss of function of connexin 43 is not the mechanism by which missense mutations cause the human phenotype. For example, connexin 43 is the major gap junction protein present in osteoblasts (Civitelli et al. 1993). Lecanda et al. (2000) determined that, during embryonic development of connexin 43 null mutant mice, generalized intramembraneous and endochondral bone development was delayed as a result of osteoblast dysfunction. At birth, these mice exhibited ossification defects of the skull, originating from hypoplastic, migratory neural crest cells, and mineralization defects in non–neural crest–derived bone. The mandible was small, with a rounded alveolar ridge and flattened arch that resulted in the development of small and pointed snouts and less prominent incisors. These features suggest that connexin 43 affects craniofacial development and normal osteogenesis, which is compatible with the ODDD phenotype. However, the craniofacial features are not consistent with those of ODDD, and limb abnormalities are lacking in the null mouse (Reaume et al. 1995; Lecanda et al. 2000). Furthermore, cardiac malformations involving blockage of the right ventricular outflow tract, present in the null mice (Reaume et al. 1995; Guerrero et al. 1997), are not obvious in the ODDD phenotype.

Potential Functional Consequences of Mutant GJA1

Mutations in the connexins can have variable functional consequences, which explains how mutations in different connexins or different mutations in the same

connexin can cause an isolated or pleiotropic phenotype. In general, the deletion of one copy of a connexin gene does not appear to be of major consequence, as can be seen in animal-model knockouts as well as in human conditions that are recessive in nature. Deletion of both copies will be of consequence in cells and tissues where this connexin is vital and/or there is a lack of other connexins of compensatory function. In the case of missense mutations, cellular function of gap junctions may be affected in a number of ways. One possibility is that the protein alteration may cause mislocation to the cytoplasm and, for recessive mutations, failure to form connexons (either homotypic or of the appropriate heterotypic composition). Other protein alterations may allow mutant connexon formation, yet may disallow the formation of gap junctions with connexons of other cells. Lastly, some alterations may allow complete gap junctions to form across cells, but their properties of conductance, molecular permeability, phosphorylation, and voltage gating are significantly altered, and cellular function is affected either as a dominant negative or gain of function. In this regard, it is notable that five ODDD missense mutations (Y17S, S18P, G21R, G22E, and K23T) are clustered within a region homologous to a calmodulin-binding motif of connexin 32 (Peracchia et al. 2000). Gating of gap junctions by calmodulin is believed to occur in response to changes in intracellular calcium.

Pal et al. (1999) demonstrated that a connexin 50 mutation, P88S, failed to form functional gap junctions, as measured by conductance in *Xenopus* oocytes, when paired homotypically, and was able to inhibit the functional form of wild type connexin 50 in a dose-dependent fashion; Pal et al. concluded that only one mutant subunit per gap junction was necessary to abolish channel function. Richard et al. (1998a) had previously shown that the connexin 26 autosomal dominant mutation R75W (analogous to connexin 43 residue R76, which is mutated to a serine in ODDD) (table 2) also acted in a dominant-negative fashion against wild-type connexin 26; however, the autosomal recessive mutant W77R, though failing to form functional gap junctions itself, did not greatly interfere with wild-type connexin as measured by conductance in *Xenopus* oocytes. Rouan et al. (2001) further demonstrated *trans*-dominant inhibition of connexin 43 by mutant connexin 26 in *Xenopus* oocytes. Richard et al. (2002) showed lack of coupling and dye transfer in HeLa cells expressing mutant connexin 26 S17F (analogous to connexin 43 residue S18, which is mutated to a proline in ODDD) (table 2) and proposed a *trans*-dominant inhibition of connexin 30.

One can, then, speculate that the GJA1 missense mutations found in ODDD have a dominant negative effect. A single hit that alters the function of the mutant allele

causes the pleiotropic phenotype of ODDD because of the wide expression pattern of GJA1, and this allele may then cause aberrant connexons with the wild-type allele and other connexins. However, we cannot exclude the possibility that these missense mutations result in gain of function. Additionally, there must be modifiers, perhaps in different racial groups that enhance the degree of pleiotropy and variability of expression observed in ODDD. The complex combinatorial interactions among the connexins suggest that additional mutational analysis of connexins other than connexin 43 in ODDD patients may give us insights into which specific connexins may modify the functional consequences of the GJA1 missense mutations. Also, future studies using *GJA1* site-directed mutagenesis in combination with other normal or mutant connexins in cellular and animal model systems will shed light on how the observed missense mutations contribute to the pathogenesis of ODDD.

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Electronic-Database Information

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

ClustalW, <http://www.ebi.ac.uk/clustalw/> (for ClustalW program)
 CMT Mutations by Gene, <http://molgen-www.uia.ac.be/CMTMutations/DataSource/MutByGene.cfm>
 Connexin-Deafness Home Page, <http://www.crg.es/deafness>
 Entrez-Protein, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=protein> (for accession numbers in fig. 4)
 NCBI Sequence Viewer, <http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=22056350> (for human chromosome 6 sequence)
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for ODDD [MIM *164200], “isolated” syndactyly type III [MIM 186100], and X-linked Charcot-Marie-Tooth disease [MIM 302800])
 TMpred Server, http://www.ch.embnet.org/software/TMPRED_form.html (for TMpred program)

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