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Familial Tetralogy of Fallot caused by mutation in the jagged1 gene

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Tetralogy of Fallot (ToF) is the most common form of complex congenital heart disease, occurring in ~1 in 3000 live births. Evaluation of candidate loci in a large kindred segregating autosomal dominant ToF with reduced penetrance culminated in identification of a missense mutation (G274D) in *JAG1*, the gene encoding jagged1, a Notch ligand expressed in the developing right heart. Nine of eleven mutation carriers manifested cardiac disease, including classic ToF, ventricular septal defect with aortic dextroposition and isolated peripheral pulmonic stenosis (PPS). All forms of ToF were represented, including variants with pulmonic stenosis, pulmonic atresia and absent pulmonary valve. No individual within this family met diagnostic criteria for any previously described clinical syndrome, including Alagille syndrome (AGS), caused by haploinsufficiency for jagged1. All mutation carriers had characteristic but variable facial features, including long, narrow and upslanting palpebral fissures, prominent nasal bridge, square dental arch and broad, prominent chin. This appearance was distinct from that of unaffected family members and typical AGS patients. The glycine corresponding to position 274 is highly conserved in other epidermal growth factor-like domains of jagged1 and in those of other proteins. Its substitution in other proteins has been associated with mild or atypical variants of disease. These data support either a relative loss-of-function or a gain-of-function pathogenetic mechanism in this family and suggest that *JAG1* mutations may contribute significantly to common variants of right heart obstructive disease.

INTRODUCTION

Tetralogy of Fallot (ToF; MIM 187500) is the most common form of complex congenital heart disease, occurring in ~1 in 3000 live births. Its cardinal features are ventricular septal defect, obstruction to right ventricular outflow, aortic dextroposition and right ventricular hypertrophy. Although multifactorial inheritance has been postulated for the majority of cases, single gene transmission is suggested by the observation of disease recurrence within families.

Classic ToF and its variants have been observed as components of a number of heritable syndromes: most notably, the branchial arch disorders associated with microdeletions at chromosome 22q11, including the DiGeorge, velocardiofacial and conotruncal anomaly face syndromes (1,2). ToF is also among the variable cardiac defects occurring in Alagille syndrome (AGS), a rare autosomal dominant condition characterized by biliary atresia, growth retardation, vertebral and ocular anomalies, cognitive deficits, characteristic facies and right-sided heart defects, predominantly peripheral pulmonic stenosis (PPS) (3). Mutations in the jagged1 gene (*JAG1*) have been implicated in AGS (4,5).

JAG1 encodes a highly conserved ligand within the Notch family of proteins, components of an intercellular signaling pathway shown to be crucial for cell fate decisions in organisms spanning the phylogenetic spectrum (6,7). *JAG1* mRNA is abundantly expressed in the developing mammalian heart, and targeted disruption of *JAG1* in mice results in abnormal cardiovascular development and embryonic demise (8,9). The gene is comprised of 26 exons spanning >36 kb of genomic DNA at chromosome 20p12. It encodes a 1218 amino acid protein with several characteristic features, 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 (10,11).

We studied a large kindred segregating ToF as an autosomal dominant trait with reduced penetrance (Fig. 1A). A candidate gene approach was taken and culminated in the identification of a *JAG1* missense mutation (G274D) in 13 members of this kindred whose DNA was available. No individual in this family met diagnostic criteria for any previously described clinical syndrome, including AGS. The unique character of the phenotype and mutation in this family provides compelling evidence for a true phenotype–genotype correlation.

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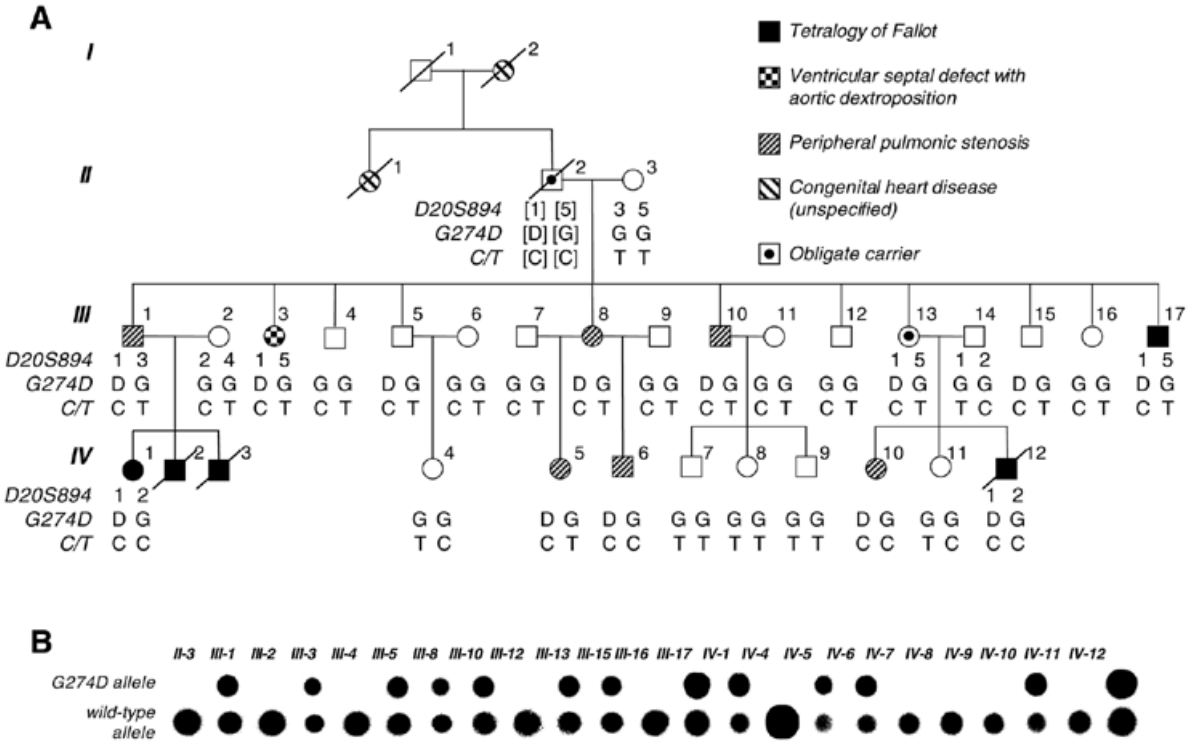

Figure 1. Study kindred. (**A**) Four generations of the kindred under study are shown. Haplotypes are listed including marker alleles of *D20S894* (1, 2, 3, 4 or 5), glycine (G) or aspartic acid (D) at the site of the mutation (G274D), and cytosine (C) or thymidine (T) at nucleotide 765 of *JAG1*, a silent dimorphism 56 bp upstream of the mutation site. Information in brackets was inferred from pedigree analysis. Genotyping for *D20S894* was performed only in individuals with ToF and obligate carriers. All patients with a clinical phenotype (cardiac or facial) inherited the D274 allele. The C/T silent dimorphism is shown to segregate independently from the severity of cardiac manifestations. For example, individuals IV-10 and IV-12 inherited the same wild-type allele marked by C. The former had only mild peripheral pulmonic stenosis whereas the latter had classic and severe ToF with early childhood death. (**B**) Genomic DNA from each individual indicated was hybridized with radiolabeled oligonucleotides specific for the mutant (G274D) and wild-type coding sequences.

RESULTS

A candidate gene approach was applied to a large kindred that segregated ToF as an autosomal dominant trait with reduced penetrance (Fig. 1A). Fluorescence *in situ* hybridization analysis excluded microdeletion at 22q11, a common cause of syndromic and isolated conotruncal heart defects (2). Linkage analysis, restricted to individuals with ToF and obligate carriers, further excluded genes at this map position as well as *NTF3*, which is located on chromosome 12p13 and also associated with cardiac defects, including ToF in homozygous targeted mice (data not shown) (12). The data were consistent with (but not conclusive for) linkage to *JAG1* at 20p12 (LOD 1.5 at $\theta = 0.00$). *JAG1* mutations conferring haploinsufficiency cause AGS, a rare, autosomal dominant condition characterized by biliary atresia, growth retardation, vertebral and ocular anomalies, cognitive deficits, characteristic facies and right-sided heart defects, predominantly PPS (3,13).

Single-strand conformation polymorphism (SSCP) analysis was used to screen affected individuals for mutations in *JAG1*. An amplicon spanning *JAG1* exon 6 from an affected family member was found to migrate aberrantly (data not shown). Direct sequencing of this PCR product revealed heterozygosity for a G→A transition at nucleotide 821, predicting substitution of aspartic acid (D) for glycine (G) at position 274 (G274D) of the jagged1 protein (Fig. 2A). G274D was not found on analysis of 100 chromosomes from unrelated and unaffected individuals (data not shown). The mutation occurs within the second of 16 tandemly repeated EGF-like domains in jagged1 and alters a residue that is evolutionarily conserved within this and other EGF-like domains of jagged1 (Fig. 2B), as well as other proteins, including jagged2, delta and serrate.

Screening of available genomic DNA identified 13 individuals who were heterozygous for G274D, including one deceased and two living children with ToF and all obligate carriers (Fig. 1B). No DNA was available for two deceased siblings with ToF born to a G274D carrier (individuals IV-2 and IV-3). These brothers died in early childhood despite corrective surgery. Eleven of the 13 individuals were available for comprehensive clinical and echocardiographic evaluation by examiners blinded to genotype. As summarized in Fig. 1A, two had classic ToF which was successfully surgically corrected in early childhood. One had ventricular septal defect with aortic dextroposition. Six others had isolated PPS—defined as acceleration of blood flow in the branch pulmonary arteries—that did not manifest with an audible murmur in five individuals. Two G274D carriers did not have overt cardiac abnormalities on physical or echocardiographic examination; one gave birth to two G274D-carrying children with cardiac phenotypes, one with classic ToF and another with PPS. All forms of ToF were

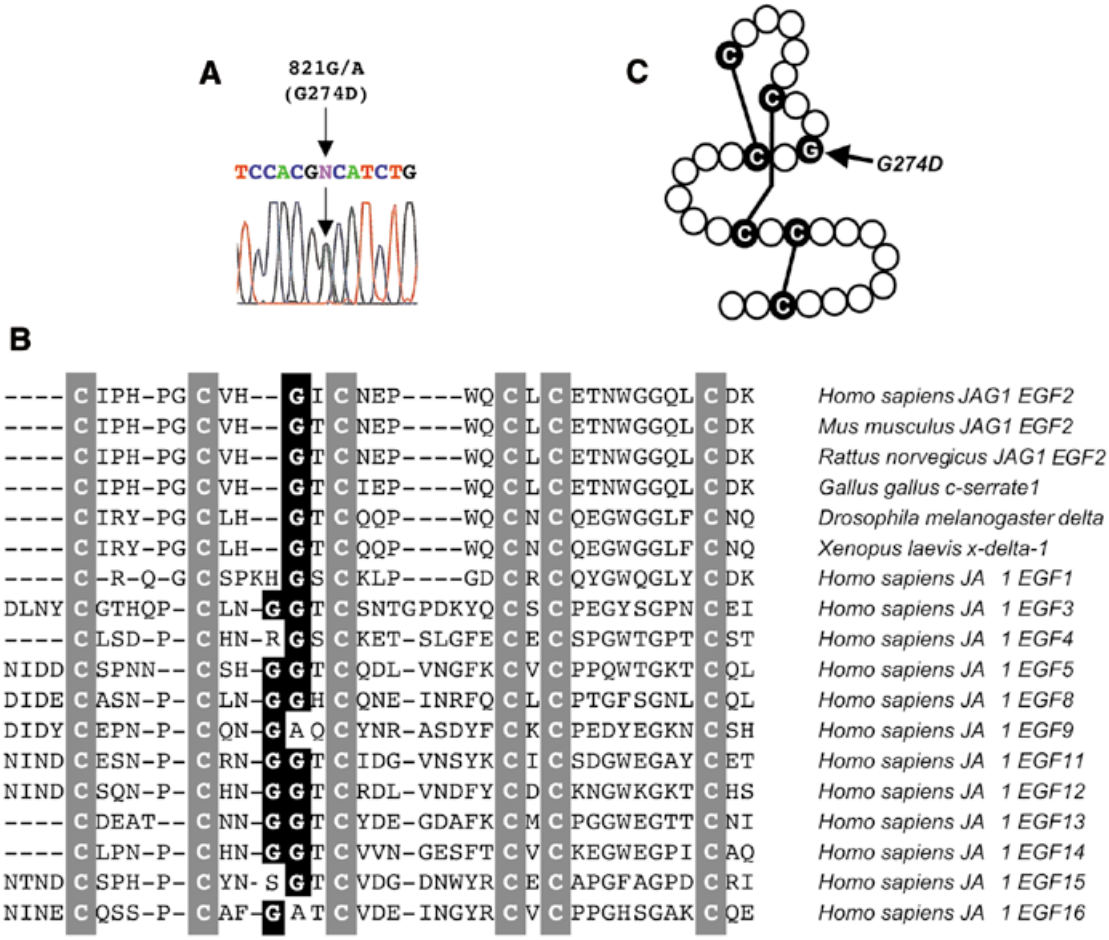

Figure 2. *JAG1* mutation 821G→A. Genomic DNA from IV-1 was used as template for PCR amplification of *JAG1* exon 6. (**A**) Automated sequencing revealed heterozygosity of G/A at nucleotide 821 of the coding sequence, which predicts a glycine-to-aspartic acid change at position 274 of the protein. (**B**) Alignment of the amino acid sequence of the second EGF-like domain of human *JAG1* with the corresponding sequence from other species and with other EGF-like domains within human *JAG1*. Shading indicates the six invariant cysteines (C) and the evolutionarily conserved glycine (G) residues in the variable loop region between the second and third cysteines, most often at the exact position corresponding to G274 in jagged1 (31). (**B**) Schematic of an EGF-like domain. Shown are the relative positions of the six invariant cysteine residues, intramolecular disulfide linkages and the site of G274D.

represented in this kindred, including variants with pulmonic stenosis, pulmonic atresia and absent pulmonary valve.

Detailed physical examination revealed a characteristic though often subtle facial appearance in all G274D carriers: long, narrow and upslanting palpebral fissures; wide nasal bridge; prominent and high zygomatic arches; prominent, broad chin; and square dental arch (Fig. 3). These features were not evident in family members who lacked the mutation. Eye examinations were documented for 11 G274D carriers, and one was noted to have asymptomatic posterior embryotoxon, a common finding in AGS that has been reported in up to 25% of a general ophthalmologic clinic population (14,15). Mutation carriers had normal growth and cognition, and no abnormalities in the pulmonary, gastrointestinal or skeletal systems were documented. Autopsy of the two brothers presumed to carry G274D who died after unsuccessful correction of ToF revealed no evidence of bile duct paucity or other non-cardiac organ defects. Analysis of serum from 11 carriers revealed normal electrolytes, calcium, urea nitrogen, creatinine, total and direct bilirubin, transaminases, γ-glutamyl transferase (GGT) and alkaline phosphatase, consistent with normal renal and hepatic function. No individual in this kindred met diagnostic criteria for AGS. Clinical features of G274D carriers and AGS patients are summarized in Table 1. The LOD score calculated for linkage of this mutation to the clinical phenotype, defined by facial or cardiac involvement, was 6.02 ($\theta = 0.00$). Affected status was assigned by a clinical geneticist who was blinded to genotype.

DISCUSSION

We present an extended kindred that segregates a *JAG1* mutation with right heart obstructive disease and characteristic facies that is distinct from that seen in AGS, as is the complete absence of other organ system involvement (Table 1). These data appear to demonstrate a true correlation between phenotype and genotype. The vast majority of *JAG1* mutations that cause AGS create premature termination codons that predict an

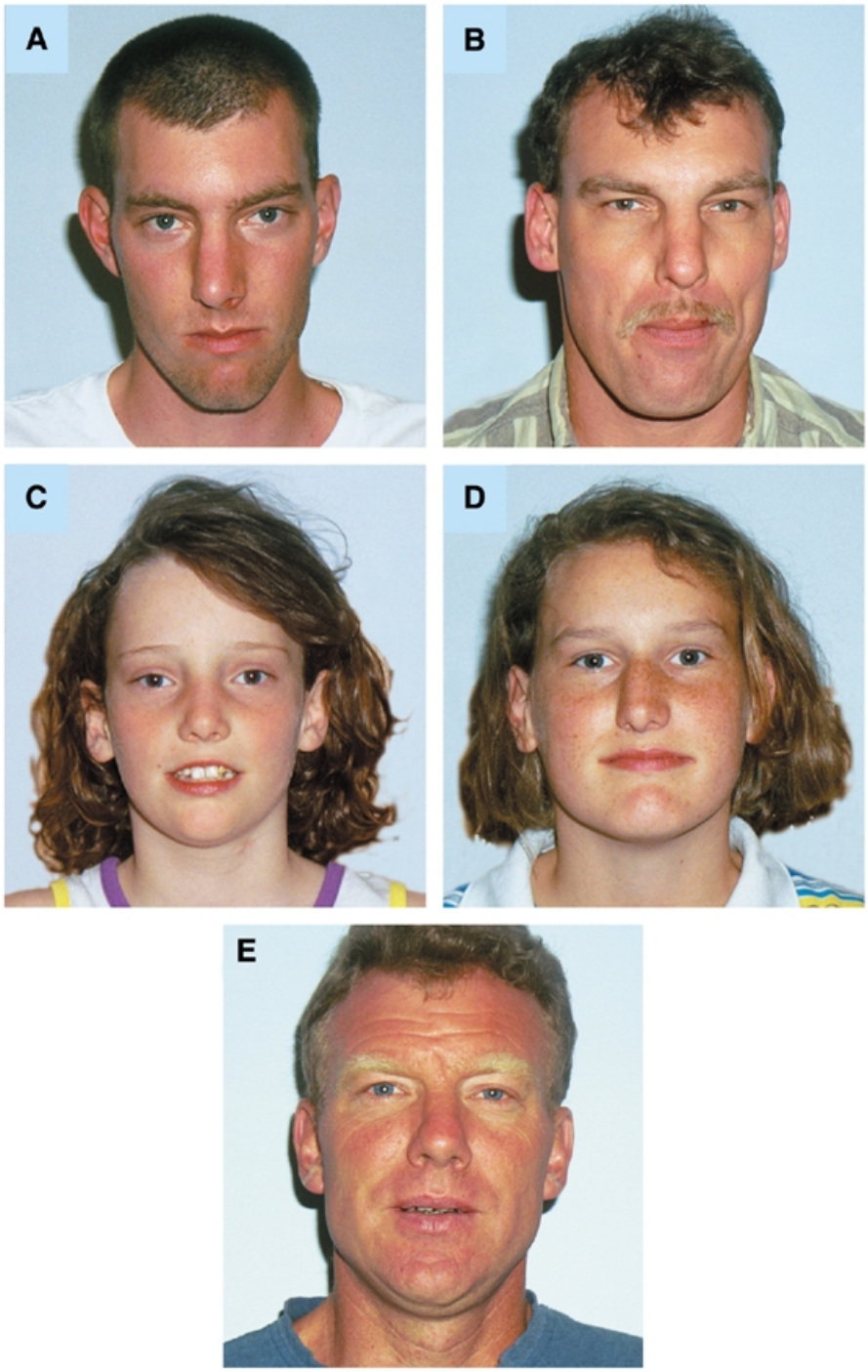

Figure 3. Facial features associated with G274D. Four representative members of the kindred are shown (A–D). All carry the G274D mutation and exhibit varying degrees of long and upslanting palpebral fissures, broad nasal bridge and chin and prominent and high zygomatic arches. (**A**) Individual III-17 with classic ToF (absent pulmonary valve); (**B**) individual III-10 with peripheral pulmonic stenosis; (**C**) individual IV-1 with classic ToF (pulmonic atresia); (**D**) individual IV-5 with peripheral pulmonic stenosis. These features were not present in unaffected family members, an example of whom is shown in (**E**).

unstable message or the expression of truncated polypeptides that lack essential domains (13,16,17). AGS is also caused by hemizygosity for *JAG1*, establishing haploinsufficiency as the relevant pathogenetic mechanism. A subset of reported missense mutations involves substitution of one of the six invariant cysteines in jagged1 EGF-like domains that form intramolecular disulfide linkages, an event known to perturb folding and to target proteolytic degradation in other proteins (18). Other missense mutations create cysteine residues, predicted to similarly derange protein structure and stability.

Table 1. Prevalence of clinical features in patients with G274D versus Alagille syndrome (AGS)

Clinical feature	AGS patients (%)[a]	G274D carriers (%)
Bile duct paucity	81–100	0 (0/2)[b]
Chronic cholestasis	93–96	0 (0/13)
Cardiac disease		
All forms	85–97	85 (11/13)
ToF	14	23 (3/13)[c]
Characteristic facies	70–96	100 (13/13)[d]
Vertebral anomalies	33–87	0 (0/9)[e]
Ocular abnormalities	56–88	8 (1/13)
Renal dysfunction	40–73	0 (0/13)
Growth retardation	50–87	0 (0/13)
Mental retardation	0–16	0 (0/13)
Developmental delay	16–52	0 (0/13)

[a]Refs 9,27,28.
[b]The two brothers with ToF (IV-2 and IV-3) born to a G274D carrier are included here assuming that they inherited the mutation. Neither had histopathologic evidence of bile duct paucity or any other non-cardiac organ malformation on autopsy.
[c]Inclusion of individuals IV-2 and IV-3 increases this figure to 33% (5/15).
[d]The characteristic facies in this family was subtle and variable yet distinct from that described in AGS. The classic AGS appearance includes prominent forehead, hypertelorism with deep-set eyes, pointed chin and straight nose with bulbous tip. Features of patients with G274D are summarized in Figure 3.
[e]No radiographic evidence of skeletal abnormalities, including butterfly vertebrae, was observed.

Most remaining reported missense mutations cluster at the extreme N-terminus, presumably affecting protein processing or trafficking.

Two *JAG1* mutations, 684insG and del(20)(p11.23p12), previously have been associated with subdiagnostic variants of AGS (19). One patient had hepatic, ocular and facial features of AGS with pulmonic stenosis; the other had facial, skeletal and developmental defects and ToF. The considerable intrafamilial variability in AGS and the lack of extended pedigrees precluded determination of whether these alleles specifically predisposed to atypical disease or were simply modified in their clinical expression. The observation that these mutations (which cause a premature stop codon and allele deletion, respectively) were characteristic of the types of gene defect causing AGS supported the latter hypothesis.

Although G274D substitutes an amino acid that is conserved in EGF-like domains, the function of this residue and the consequence of its alteration are not obvious. Remarkably, mutation of the corresponding glycine in EGF-like domains of factor IX and fibrillin-1 has been associated with atypically mild variants of hemophilia B and Marfan syndrome, respectively (20,21). Substitution of the corresponding glycine in synthetic domains causes focal and incomplete abnormalities of peptide folding (22). Unlike G274D, these mutations substituted serine for glycine in EGF-like domains that satisfy the consensus for calcium binding, perhaps limiting generalization of these results. As predicted, analysis of lymphoblasts cultured from

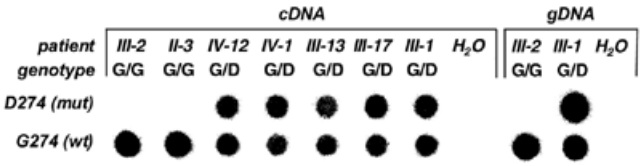

Figure 4. Assessment of mutant transcript level. Semi-quantitative allele-specific oligonucleotide hybridization analysis of PCR-amplified lymphoblast cDNA indicated no significant difference in levels of wild-type and mutant transcript in G274D heterozygotes.

members of our study kindred revealed no reduction in the level of transcript derived from the D274 allele (Fig. 4).

The extreme intrafamilial clinical variability seen in this large family suggests the existence of alleles that modify right heart obstructive disease and may offer an opportunity for their identification. A C/T silent dimorphism 56 nucleotides upstream from the site of G274D segregated independently of the severity of cardiac manifestations, suggesting that the character of the wild-type *JAG1* allele in G274D heterozygotes was not a major contributor to the observed clinical variation (Fig. 1A). The absence of AGS or a more severe variant of disease in this kindred makes a dominant negative mechanism of pathogenesis less likely. Loss of function that is tissue or developmental stage specific may underlie the phenotype: the absence of multi-system involvement may reflect residual function of the mutant gene product. Alternatively, the unique facial features may indicate a dominant gain of function. Indeed, these mechanisms are not mutually exclusive. The identification of a *JAG1* mutation in association with only mild and variable facial dysmorphisms suggests that this gene may contribute significantly to common and apparently sporadic forms of right heart obstructive disease.

MATERIALS AND METHODS

Patients

Written informed consent was obtained from all adult members of the kindred and from parents of minors. Available individuals underwent a complete medical history and physical examination. Echocardiography was performed on G274D carriers with an Acuson Cypress unit. Whole blood was collected and used to establish lymphoblastoid cell lines at the Johns Hopkins University School of Medicine Core facility. Genomic DNA was extracted from lymphocytes using a QIAamp DNA Midi kit (Qiagen). Blood was also submitted to the clinical chemistry laboratory at the Johns Hopkins Hospital, where measurements of electrolytes, blood urea nitrogen, creatinine, total and direct bilirubin, transaminases, GGT and alkaline phosphatase were performed. Available medical records for all family members were reviewed. Individuals with classic ToF underwent karyotyping at the Cytogenetics Laboratory of the University of Oklahoma Medical Center. Fluorescence *in situ* hybridization was performed at the Cytogenetics Laboratory of the Johns Hopkins Hospital.

Linkage analysis

Polymorphic markers (*D22S264*, *D20S894*, *D20S1154*) were selected based on prior associations with congenital heart disease (23,24). Human *NTF3* was screened using previously reported intragenic markers (25). Genotyping was performed as previously described (26). Automated sequencing of the entire *NTF3* gene was also performed for an affected proband and revealed no mutation (see below). LOD scores were calculated assuming autosomal dominant inheritance with complete penetrance in an affecteds-only analysis using LINKAGE software (27).

Mutation identification

Genomic DNA was used as the template for PCR amplification of the *JAG1* coding sequence using previously reported primer sequences and conditions (5). SSCP analysis was performed as previously described (28). Aberrant migration was observed for the product of PCR-amplified exon 6 in affected individuals. This exon was amplified in reactions containing 1 µl of genomic DNA template (50 ng), 10 µl of 10× PCR buffer (Perkin Elmer), 10 µl of 2 mM dNTP mix, 4 µl of sense primer (5′-AAGGCTAACCTGGAGGTGTGCTG-3′), 4 µl of the antisense primer (5′-TCCCACCCTGGGTCTCATCC-3′), 1 µl of AmpliTaq DNA polymerase (Perkin Elmer) and 70 µl of deionized dH_2O. After initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30s, 60°C for 30s and 72°C for 30 s were performed, followed by a final extension at 72°C for 10 min. The entire reaction volume was purified using a QIAquick PCR purification kit (Qiagen) and resuspended in 30 µl. The product was visualized by agarose gel electrophoresis, and 1–5 µl was used as template for automated sequencing using an Applied Biosystems 377 sequencer.

Mutation screening

Exon 6 was amplified as above from genomic DNA of all members of this kindred as well as 50 unaffected, unrelated individuals. Oligonucleotide probes specific for the mutant (5′-GCGTCCACG**A**CATCTGTA-3′) and wild-type (5′-GCGTC-CACG**G**CATCTGTA-3′) alleles were used in a dot-blot assay, performed as previously described (29). Final wash temperatures for the mutant and wild-type probes were 57 and 59°C, respectively.

Assessment of mutant transcript levels

mRNA was extracted from whole blood (QIAamp RNA Blood Mini kit; Qiagen), treated with RNase-free DNase (Life Technologies) and carried through an RT–PCR-based allele-specific oligonucleotide hybridization assay previously shown to be quantitative (30). PCR amplification of genomic DNA from a heterozygote served as an internal control for varying autoradiographic exposure times and differential affinities of the probes to their target sequence.

Inheritance of the 765C/T silent dimorphism

Fifteen microliters of each *JAG1* exon 6 amplification reaction (see above) was digested with 15 U of *Rsa*I (New England Biolabs). The presence of thymidine at nucleotide 765 resulted in enzymatic cleavage. Visualization of fragments after gel electrophoresis enabled assignment of genotype to each individual.

Nucleotide numbering

Numbering followed the published *JAG1* sequence (GenBank accession no. 4557678).

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