NOTCH2 Mutations Cause Alagille Syndrome, a Heterogeneous Disorder of the Notch Signaling Pathway

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Alagille syndrome (AGS) is caused by mutations in the gene for the Notch signaling pathway ligand Jagged1 (*JAG1*), which are found in 94% of patients. To identify the cause of disease in patients without *JAG1* mutations, we screened 11 *JAG1* mutation-negative probands with AGS for alterations in the gene for the Notch2 receptor (*NOTCH2*). We found *NOTCH2* mutations segregating in two families and identified five affected individuals. Renal manifestations, a minor feature in AGS, were present in all the affected individuals. This demonstrates that AGS is a heterogeneous disorder and implicates *NOTCH2* mutations in human disease.

Alagille syndrome (AGS [MIM 118450]) is a dominant, multisystem disorder defined clinically by hepatic bile duct paucity and cholestasis in association with cardiac, skeletal, and ophthalmologic manifestations. There are characteristic facial features and less-frequent clinical involvement of the renal and vascular systems.[1,2] Expressivity is known to be highly variable. AGS is caused by mutations in the gene encoding Jagged1 (*JAG1*), a ligand in the Notch signaling pathway.[3,4] Notch signaling is involved in cell fate determination and is essential for normal embryonic development. At least five ligands and four Notch receptors are expressed in humans, and many genes have been identified that function downstream of Notch.[5] Mutations in *JAG1* were identified in 94% of individuals with a clinically confirmed diagnosis of AGS.[6] Failure to identify mutations in the remaining patients could have been because the mutations were located in noncoding regions not screened by present techniques or were in another gene. Data from the mouse have implicated the *Notch2* gene in the etiology of clinical features associated with AGS. Although the Jagged1 knockout heterozygote mouse did not mimic the AGS phenotype,[7] a Jagged1/Notch2 double heterozygote was found to have liver, cardiac, ocular, and renal manifestations similar to those seen in patients with AGS.[8] Additionally, the spatial and temporal expression pattern of Notch2 in tissues involved in AGS makes it an excellent candidate to be the receptor interacting with Jagged1.[9,10] This led us to screen a cohort of *JAG1* mutation-negative patients with AGS for alterations in *NOTCH2*.

Eleven probands were screened for the coding region (34 exons) of *NOTCH2*. All individuals were enrolled in an institutional review board–approved protocol at The Children's Hospital of Philadelphia, after informed consent was obtained. Screening was accomplished by direct sequencing of purified genomic DNA after PCR amplification. The first four exons of *NOTCH2* (located within chromosome band 1p12) have a high degree of homology with a distinct but related gene, *N2N,* which is located at 1q21.[11] We therefore designed primers for the first four exons of *NOTCH2* so that the 3′ end sat on a nucleotide unique to *NOTCH2,* to avoid amplification of *N2N*. Primers are listed in table 1.

Mutations were identified in two probands. Proband 1 had cholestatic liver disease, cardiac disease (peripheral pulmonic stenosis and a small atrial septal defect), characteristic facial features, and severe infantile renal disease (small kidneys with cysts bilaterally, renal tubular acidosis, and renal insufficiency) (fig. 1*a*). He died of cardiopulmonary arrest at age 2 years. His mother had valvular and peripheral pulmonic stenosis, characteristic facial features, and dysplastic kidneys and proteinuria that resulted in renal failure and a kidney transplant. A *NOTCH2* mutation of the splice acceptor of exon 33 (c.5930−1G→A) was identified in the proband and his mother (fig. 1*b*). Maternal grandparents and three of the mother's siblings were also tested, and they did not carry the mutation, indicating it was a de novo change in the proband's mother.

To determine the effect of this mutation on splicing, we analyzed cDNA prepared from a lymphoblastoid cell line from the proband. Gel electrophoresis of an amplified portion of the proband's *NOTCH2* cDNA encompassing exons 32–34 confirmed the presence of an abnormal band, which, by sequencing, was shown to have the 98-bp exon 33 spliced out (fig. 1*c*). The transcript resulting from this mutation is predicted to have a premature termination codon within exon 34, and, since this is the last exon, the transcript is not predicted to undergo nonsense-mediated mRNA decay.[12] The resulting protein is predicted to lack three of the seven ankyrin repeats and the ensuing 3′ se-

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Table 1. ***NOTCH2*** **Mutation-Screening Primers**

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

quence. (fig. 1*d*). The ankyrin repeat is found in a number of proteins and is responsible for mediating protein-protein interactions.[13] These repeats in the intracellular domain of the Notch proteins interact with nuclear cofactors that serve to modulate Notch signaling, and the ankyrin repeats have been shown to be crucial for Notch activity.[14]

Proband 2 had cholestatic liver disease, which led to a liver transplant. She had cardiac disease (tetralogy of Fallot) and ocular findings (posterior embryotoxon). She demonstrated renal disease (tubular acidosis and dysplastic kidneys), and currently, at age 8 years, she is awaiting a renal transplant. Her mother has a history of asymptomatic hematuria and proteinuria. She came to medical attention at age 26 years with a mildly elevated urine protein level (155 mg/24 hr), which increased steadily over the next 10 years (584 mg/24 hr at age 36 years). Hypertension was diagnosed when she was age 36 years. Abdominal ultrasound indicated normal renal sizes. No cardiovascular or gastrointestinal abnormalities were present. The diagnosis was subnephrotic-range proteinuria with microscopic hematuria and no evidence of renal insufficiency. Examination by a dysmorphologist revealed the presence of facial features characteristic of AGS (fig. 2*a*). The proband's maternal grandmother has advanced chronic renal insufficiency of undetermined etiology, which was first noted at age 59 years. Her renal insufficiency worsened until age 65 years, when she began peritoneal dialysis. An ultrasound of the kidneys showed a right atrophic kidney, which was thought to be congenital. Cardiac evaluation was negative for a murmur, and there was no history of liver disease. Adult-onset diabetes was diagnosed just before dialysis was begun, but this condition is well controlled with diet alone.

Screening of the *NOTCH2* gene in the proband, her mother, and her grandmother identified a G→A change at position 1331 in the cDNA sequence (c.1331G→A) in exon 8, which causes a substitution of a tyrosine for a cysteine residue in the 11th epidermal growth factor (EGF)–like repeat (C444Y) (fig. 2*b* and 2*c*). The EGF-like repeat motif consists of six cysteine residues, forming three intramolecular disulfide bridges, which are crucial for conformational stability of the protein.[15] Loss or gain of cysteine residues in the EGF-like repeats of other genes has been

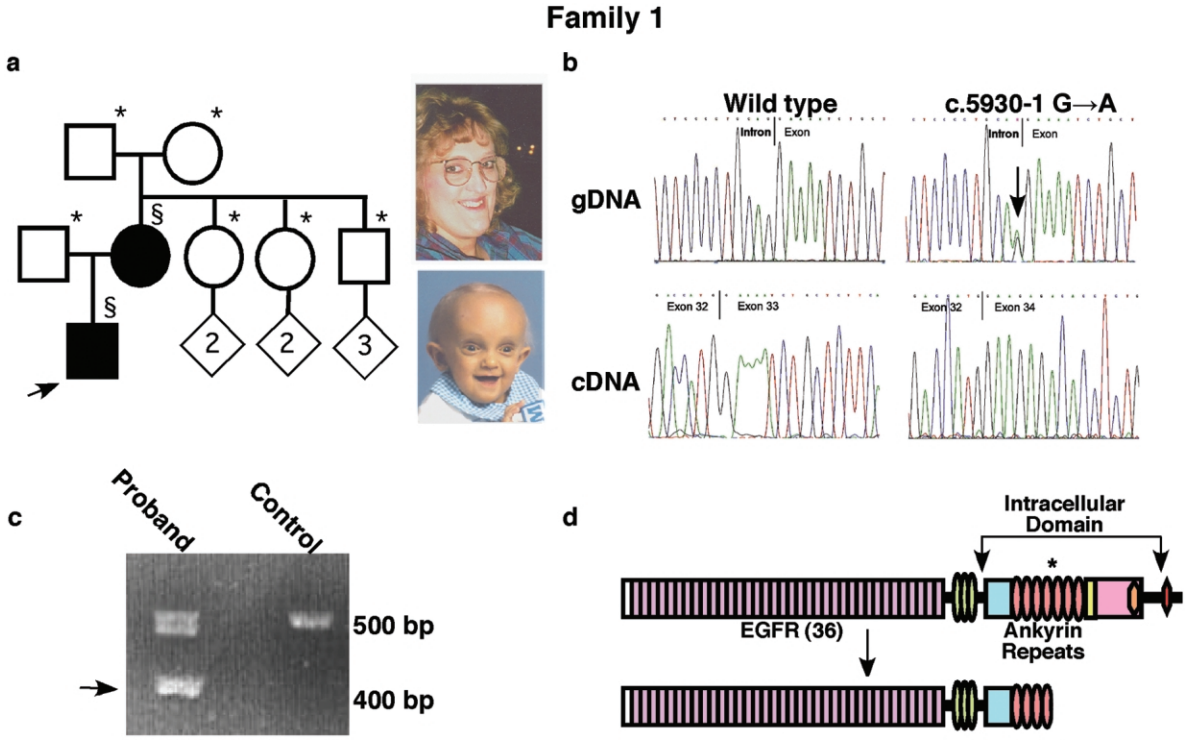

Figure 1. *NOTCH2* mutation in family 1. *a,* Proband and his mother demonstrate features of AGS. Family members marked with an asterisk (*) were tested for the presence of the mutation but had only wild-type sequence. Individuals marked with a section symbol (§) had mutant sequences. *b,* Genomic DNA (gDNA) and cDNA sequencing demonstrates the presence and consequences of a mutation in the splice site of exon 33 in the proband and his mother. *c,* cDNA amplification and electrophoresis demonstrate the abnormally spliced product (*arrow*). *d,* Predicted protein product is represented below the diagram of the wild-type Notch2 protein (mutation marked by an asterisk). EGFR = EGF-like repeats.

shown to cause disease. Examples include loss or gain of EGF-like cysteine residues in fibrillin, which results in Marfan syndrome,[16] and Notch3, which results in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).[17] The *Drosophila* and mammalian Notch genes are highly conserved, and both contain 36 EGF-like repeats, with EGF repeats 11 and 12 required for ligand binding of the *Drosophila* Notch, providing further support for the significance of the observed mutation.[18]

Neither of the mutations we identified was found in 110 ethnically matched control individuals (220 chromosomes). These studies identified a number of polymorphisms in *NOTCH2,* which are listed in table 2.

Data presented here indicate that AGS is a genetically heterogeneous disorder caused by mutations in the Notch signaling pathway ligand gene, *JAG1,* or the gene for its receptor, *NOTCH2*. However, at this time, the vast majority of patients with AGS have mutations in the *JAG1* gene, with only a small subset showing *NOTCH2* mutations. This may be a matter of ascertainment bias, in that more patients with *NOTCH2* mutations may be found who do not meet the full criteria we used to select patients with AGS. It is also possible that a single *NOTCH2* mutation does not, by itself, cause AGS, but perhaps there are polymorphisms in *JAG1* or other Notch signaling pathway genes that are necessary to cause the phenotype. We analyzed the complete *JAG1* gene in the two probands with *NOTCH2* mutations, and both had multiple *JAG1* polymorphisms, although all of these were common and did not result in an amino acid alteration. Further work is needed to understand why *NOTCH2* mutations are relatively rare in the AGS population.

To our knowledge, this is the first report of mutations in the *NOTCH2* gene causing human disease. Germline mutations in two other Notch receptor genes have been

Table 2. Polymorphisms Identified in *NOTCH2*

Nucleotide Change[a]	Exon or Intron (IVS)	Amino Acid	Frequency[b]
c.−214C→G	Exon 1		7/138
c.15C→T	Exon 1	Arg5	16/138
c.874+10G→A	IVS 6		3/138
c.939C→T	Exon 6	Gly507	2/138
c.1396C→A	Exon 8	Glu466Lys	1/358
c.1681+142A→C	IVS 11		1/138
c.1915+89C→T	IVS 12		5/138
c.1915+201G→C	IVS 12		6/138
c.2365+39T→G	IVS 15		17/138
c.2365+47T→A	IVS 15		18/138
c.2479+20G→C	IVS 16		46/138
c.2753−44C→T	IVS 18		20/138
c.3034T→C	Exon 19	Leu1012	1/138
c.3184−76G→A	IVS 20		19/138
c.3523−58G→A	IVS 22		25/138
c.3655+96G→T	IVS 23		26/138
c.3656−22G→A	IVS 23		20/138
c.3980A→G	Exon 24	Asp1327Gly	3/358
c.4005+45A→G	IVS 25		16/138
c.4005+232C→G	IVS 25		1/138
c.4014C→T	Exon 25	Ser1338	2/138
c.4305G→A	Exon 25	Arg1435	3/138
c.4859+54G→C	IVS 27		1/138
c.4859+152C→T	IVS 27		20/138
c.4859+191G→A	IVS 27		3/138
c.5103A→G	Exon 28	Lys1701	1/138
c.5310+171G→A	IVS 30		24/138
c.5310+195T→G	IVS 30		3/138
c.6028−116C→T	IVS 34		12/138
c.6028−84G→T	IVS 34		2/138
c.6224G→A	Exon 34	Val2075Met	2/358
c.6421C→T	Exon 34	Leu2141	2/138
c.7341T→A	Exon 34	Gly2447	20/138

[a] Numbering is based on *NOTCH2* cDNA sequence (GenBank accession number NM_024408.2).

[b] Frequency is presented as number of occurrences per number of chromosomes sequenced and refers to both patients and controls.

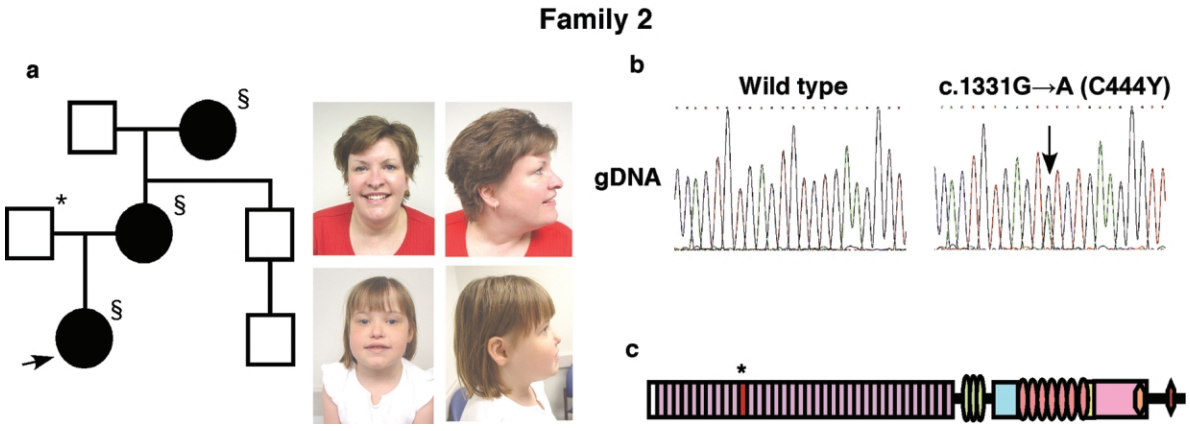

Figure 2. *NOTCH2* mutation in family 2. *a,* Proband, her mother, and her grandmother had clinical features associated with AGS. The family member marked with an asterisk (*) was tested for the presence of the mutation but had only wild-type sequence. Individuals marked with a section symbol (§) had mutant sequences. *b,* Sequence analysis shows a mutation in exon 8 in all three affected individuals. *c,* The mutation (*asterisk*) is predicted to cause substitution of a tyrosine for a cysteine residue in EGF-like repeat 11 of Notch2.

associated with human disease: congenital cardiac disease, in patients with *NOTCH1* mutations,[19] and CADASIL, in patients with *NOTCH3* mutations.[17] Both probands we identified with *NOTCH2* mutations clearly meet the diagnostic criteria for AGS, which require the presence of three of five clinical features (cholestasis, cardiac disease, ocular abnormality, skeletal abnormality, and characteristic facial features). It is of interest that the renal disease in both probands was severe. In addition, renal disease was present in all three mildly affected relatives who also carried the *NOTCH2* mutation. Renal disease has been reported in 40%–70% of patients with a clinical diagnosis of AGS.[1,2] Renal tubular acidosis and small kidneys are the most common findings, although severe renal phenotypes, including renal failure, have been described.[20] Microscopic examination of the kidneys of 26 children with AGS revealed glomerular lesions of varying severity in 18 children and mild changes in the remaining 8 children.[21] In our cohort, there were 59 *JAG1* mutation-positive parents. Of the 59, 3 had renal anomalies. These included two parents with renal failure (one complicated by the presence of diabetes) and one parent with a deformity of the left ureter. There is evidence from mouse studies that functional Notch2 is required for normal kidney development, because mice homozygous for a hypomorphic *Notch2* mutation died perinatally secondary to defects in glomerular development.[22] This work raises the possibility that AGS caused by mutations in *NOTCH2* will be found to have a phenotypic profile different from that of AGS caused by mutations in *JAG1*.

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Web Resources

The accession number and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *NOTCH2* cDNA [accession number NM_024408.2])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for AGS)

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