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# *NOTCH2* mutations in Alagille syndrome

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## Abstract

**Background—**Alagille syndrome (ALGS) is a dominant, multisystem disorder caused by mutations in the Jagged1 (JAG1) ligand in 94% of patients, and in the NOTCH2 receptor in <1%. There are only two NOTCH2 families reported to date. This study hypothesised that additional *NOTCH2* mutations would be present in patients with clinical features of ALGS without a *JAG1* mutation.

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**Methods—**The study screened a cohort of *JAG1*-negative individuals with clinical features suggestive or diagnostic of ALGS for *NOTCH2* mutations.

**Results—**Eight individuals with novel *NOTCH2* mutations (six missense, one splicing, and one non-sense mutation) were identified. Three of these patients met classic criteria for ALGS and five patients only had a subset of features. The mutations were distributed across the extracellular (N=5) and intracellular domains (N=3) of the protein. Functional analysis of four missense, one nonsense, and one splicing mutation demonstrated decreased Notch signalling of these proteins. Subjects with *NOTCH2* mutations demonstrated highly variable expressivity of the affected systems, as with *JAG1* individuals. Liver involvement was universal in *NOTCH2* probands and they had a similar prevalence of ophthalmologic and renal anomalies to *JAG1* patients. There was a trend towards less cardiac involvement in the *NOTCH2* group (60% vs 100% in *JAG1*). *NOTCH2* (+) probands exhibited a significantly decreased penetrance of vertebral abnormalities (10%) and facial features (20%) when compared to the *JAG1* (+) cohort.

**Conclusions—**This work confirms the importance of *NOTCH2* as a second disease gene in ALGS and expands the repertoire of the *NOTCH2* related disease phenotype.

## INTRODUCTION

Alagille syndrome (ALGS, OMIM 118450) is a multisystem autosomal dominant disorder which is classically defined by the presence of three of five major clinical criteria: cholestatic liver disease, cardiac disease, ocular abnormalities (typically posterior embryotoxon), skeletal abnormalities (most commonly butterfly vertebrae), and characteristic facial features.[1] The facial features in childhood are typically a broad forehead, deep-set eyes and a pointed chin, giving the face an overall triangular appearance. Additionally, renal and vascular defects are seen in a significant percentage of patients.[2–6] ALGS exhibits highly variable expressivity for each of the affected systems, ranging from no apparent clinical involvement to severe disease leading to transplantation or death.[78] Risk of mortality depends on severity of organ involvement, and is most commonly related to congenital heart disease or intracranial bleeding.[49]

ALGS is caused by mutations in one of two genes; the Notch signalling Pathway (NSP) ligand Jagged1 (*JAG1*) or the Notch receptor, *NOTCH2*. The NSP is an evolutionarily conserved intracellular signalling mechanism that regulates cell fate determination.[10] The pathway consists of four human Notch receptors (Notch 1, 2, 3, 4) that can be activated by binding of any of the five Notch signalling ligands (JAG1, 2, Delta-like 1, 3 and 4). Mutations in the NSP ligand, *JAG1* are found in 95% of patients with clinically defined ALGS.[1112] *JAG1* mutations include whole and partial gene deletions, frameshift, nonsense, and missense mutations. Haploinsufficiency is hypothesised to be the mechanism of disease causation in ALGS,[1314] although a dominant negative mechanism has been suggested in some cases.[15] Mutations in the NOTCH2 receptor have also been found in a small number of patients who met diagnostic criteria for ALGS. These two multi-generation families with *NOTCH2* mutations were described by our group, and in that small cohort of two probands and three mutation-positive relatives, there appeared to be a prominent renal phenotype associated with *NOTCH2* related ALGS.[16] In this study we report data on eight additional *NOTCH2* mutations identified in patients with clinical features associated with ALGS.

## PATIENTS AND METHODS

### Patient cohort

Patients with complete or partial clinical features associated with *ALGS*, who were found to be negative for a *JAG1* mutation, were screened for mutations in *NOTCH2*, either under an

institutional review board approved protocol at the Children's Hospital of Philadelphia, or by clinical testing at Centogene, in Rostock, Germany. The clinical and genetic characteristics of the patients in whom *NOTCH2* mutations were identified are presented in table 1. Eleven additional family members were screened after a genetic change was identified in the proband.

## Mutation analysis

Genomic DNA was isolated from peripheral blood using the ArchivePure DNA Blood Kit (5 Prime, Gaithersburg, Maryland, USA). In all patients a *JAG1* mutation was ruled out by sequencing the coding region and using a commercially available multiplex ligation dependent probe amplification (MLPA) kit (MRC-Holland, Amsterdam, the Netherlands).[17] We screened the coding region of *NOTCH2* as well as intron/exon boundaries as previously described.[16]

To analyse the effect of a nonsense mutation identified in exon 33 in patient 2, RNA was isolated from lymphoblastoid cell lines using an RNeasy Mini Kit (Qiagen, Valencia, California, USA). Superscript First Strand Synthesis System (Invitrogen, Carlsbad, California, USA) was used to reverse transcribe RNA using random hexamer primers. A PCR product was then amplified using primers from exons 32–34.[16]

## Creation of mutant NOTCH2 expression constructs

Mutant *NOTCH2* cDNAs were created using a human *NOTCH2* cDNA clone (courtesy of Dr Spyros Artavanis-Tsakonas at Harvard University Medical School). We subcloned the *NOTCH2* cDNA into the pCDNA3.1 mammalian expression vector (Invitrogen). Six mutant *NOTCH2* constructs were created in total. We introduced the three missense mutations identified in the present study (p.C373R, p.P383S, p.R1953C), the previously reported p.C444Y missense mutant, and the newly identified p.R2003X mutation into the construct via site directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene/Agilent, Santa Clara, California, USA). The primers used in these reactions are as follows (note only one primer sequence is provided because the `reverse' primer in a site directed mutagenesis reaction is simply the complement of the `forward' primer). The mismatched base pair is shown in bold:

p.Cys373Arg – GAAGGCAGGTCTCCTG**C**GTCATCTGGATGATGC;

p.Pro383Ser – GCATGCATCAGCAAT**T**CTTGCCACAA;

p.Cys444Tyr – CGCCTTCCACTGTGAGT**A**TCTGAAGGGT TATGCAGG;

p.Arg1953Cys – CCTGATCCTGGCTGCC**T**GCCTGGCTGT GGAGGG;

p.Arg2003X – GAAAAATGGGGCCAAC**T**GAGACATGCAGGAC.

The previously reported c.5930-1G→A *NOTCH2* splicing mutant was created via PCR of the *NOTCH2* cDNA with pFu Turbo polymerase (Stratagene) using primers flanking the gene up to the end of exon 32. The 3′ reverse PCR primer included a non-priming adapter region containing the first 44 bases of exon 34 (the splice site mutation leads to skipping of exon 33), which includes the newly recognised termination codon. Thus, the final construct contained the cDNA sequence of *NOTCH2* up to the end of exon 32 followed by the first 44 bases of exon 34. The primers also contained restriction sites for subcloning, resulting in an EcoRV site at the 5′ end and a NotI site at the 3′ end. Primer sequences for this reaction were:

F – TATATAGATATCATGCCCGCCCTGCGCCCCGC,

R –
GCAGTGGATGACCATGGAAGAGACACCTCTGTTTCTTGCTGCCCGGGAGGG
GAGCTATGAGCGGCCGCTATATA.

The constructs were all verified after mutagenesis using Sanger sequencing with custom cDNA primers.

### Reporter assays with *NOTCH2* mutants

The functional consequences of the *NOTCH2* mutations were analysed using a luciferase reporter system. We created human *NOTCH2* cDNA constructs containing the two previously reported mutations p.C444Y[16] and four of the eight mutations reported in this study, as described above. These mutant constructs or wild-type NOTCH2 or vector (pCDNA3.1) alone were transiently expressed in C3H/10T1/2 mouse embryonic cells maintained in DMEM/10%FBS/1%PennStrep in 24-well plate tissue culture wells that were coated with soluble JAG1 ligand or TRAIL (TNF related apoptosis inducing ligand) as a non-specific ligand control, as previously described.[18] Briefly, we obtained fusion protein expression constructs from the Kadesch Laboratory at the University of Pennsylvania containing the Fc region of human IgG attached to the extracellular region of JAG1 or the death receptor ligand TRAIL as a negative control. The soluble proteins were produced via transient transfection of the fusion constructs into HEK293T cells in 10 cm tissue culture plates using Fugene 6 transfection reagent (Stratagene). Twenty-four hours after transfection, the cells were washed and re-fed with 5 ml of DMEM/10%FBS. The following day the media was harvested, filtered with a 0.45 μm syringe, and either used immediately or stored at −80°C. Tissue culture wells in a 24-well plate were coated with 10 mg of mouse-anti-human IgG antibody, which then immobilised the Fc-JAG1 or Fc-TRAIL proteins to the bottom of the plate when applied to the well. Each well was coated with 1 ml of conditioned media. We then seeded $2\times10^4$ cells into each coated well of a 24-well plate, and 24 h later transfected 400 ng of total DNA comprised of the *NOTCH2* wild-type or mutant construct or vector alone, the previously described 4×CSL Notch dependent luciferase reporter construct[19] and the pRL-SV40 renilla internal control construct (Promega, Madison, Wisconsin, USA). Forty-eight hours post-transfection, whole cell lysates were made and luciferase values measured using the Dual-Luciferase Reporter Assay (Promega) following the manufacturer's protocol. Luciferase values are reported as fold-change over vector (pCDNA3.1) alone (figure 3). Functional consequences of the mutations were also analysed using four online tools for predicting the effect of mutations on NOTCH2 protein function: MutPred, SNPs&Go, Polyphen2, and SIFT. All four have different methods for predicting protein effect, but the results were classified as `damaging' or `benign' (table 2).

## RESULTS

### Mutation screening

Twenty-two individuals with suspected ALGS and no mutations in *JAG1* were screened for mutations in *NOTCH2*. We identified eight novel *NOTCH2* mutations in this cohort of *JAG1*-negative probands. One individual had a second change in *NOTCH2*, which is likely to be a polymorphism (p.Ile681Asn) (table 2). The mutations were all unique and were located in both the extracellular and intracellular domains (figure 1). There were five mutations in the extracellular portion of the protein, including four missense mutations in the epidermal growth factor (EGF)-like repeats (p.Cys373Arg, p.Pro383Ser, p.Pro394Ser, and p.Cys480Arg) (table 2) and one frameshift mutation (p.Ser856fs). The three intracellular mutations included two missense mutations (p.Arg1953Cys and p.Arg1953His) and one nonsense mutation (p.Arg2003X), all of which were localised within the ankyrin repeats. Parental samples were available in four cases, and the mutation was de novo in family 2 and

inherited in families 1, 4, and 5 (table 2 and discussed below). Table 2 displays the cDNA and protein coordinates for the mutations discovered in this study. Studies to gauge functional consequences of the mutations are discussed below.

## Clinical findings

*NOTCH2* mutations were identified in eight probands suspected of having ALGS, who had previously tested negative for a *JAG1* mutation (table 1). Three of these individuals met classic criteria for ALGS (CC in table 1) and the nature of the clinical involvement was similar to other *JAG1* mutation positive individuals (patients 1, 2, and 3). These children had cholestatic liver disease, a congenital cardiac defect, and either posterior embryotoxon, butterfly vertebrae or facial features to fulfil diagnostic criteria. The other five *NOTCH2* mutation-positive (+) individuals had only one or two typical ALGS diagnostic features and thus did not meet full criteria (table 1). In particular, patient 5 had bile duct paucity with no other syndromic features (non-syndromic paucity). None of the eight individuals had additional clinical features that are not usually associated with ALGS (table 1).

The frequency of clinical features in the *NOTCH2* (+) probands is represented in table 3. In these individuals, liver disease was a universal finding. Cardiac, renal, and ophthalmologic involvement was also prevalent. There was less frequent heart involvement in the *NOTCH2* (+) individuals, with 60% affected in comparison to 100% in the *JAG1* (+) cohort. The universal cardiac involvement in the *JAG1* (+) individuals included murmurs, peripheral pulmonary stenosis, and intracardiac disease. The frequency of structural intracardiac disease was similar between both cohorts (30% in *NOTCH2* (+) and 47% in *JAG1* (+)). Also of note, skeletal anomalies and facial features were infrequently found in *NOTCH2* (+) individuals with a frequency of only 10% and 20%, respectively (figure 2).

Parental samples were available for testing in four of the eight cases identified, and the mutation was de novo in family 2 (p.Arg2003X) and inherited in families 1, 4, and 5 (p.Cys373R, p.Pro383Ser, and p.Ser856fs). The mutation found in patient 1 (p.Cys373Arg) was paternally inherited and was found in multiple clinically affected family members including three of four siblings, father, paternal grandfather, and paternal great aunt. Clinical features seen in the proband's father include elevated liver enzymes, high cholesterol, and a heart murmur with a normal echocardiogram. He had no known renal, eye, and skeletal anomalies and he did not have the facial features associated with ALGS. Sibling 1 in this family had elevated serum transaminases, a murmur (with a normal echocardiogram), and vesico-ureteric reflux requiring surgery. Sibling 2 also had elevated liver enzymes and a murmur but no other diagnostic features. Mutation-positive sibling 4 had elevated liver enzymes and tetralogy of Fallot. The mutation found in patient 4 (p. Pro383Ser) was also inherited maternally, and was also seen in a sibling. The mother and sibling both had normal liver enzymes but no other formal evaluation, though they did not report any overt symptoms in other organ systems.

## Functional analysis of *NOTCH2* mutants

We studied the effect of six of the mutations (p.Cys373Arg, p.Pro383Ser, p.Cys444Tyr, p.Arg1953Cys, p.Arg2003X, c.5930-1G->A) on protein function using a luciferase assay, and the results are presented in figure 3. These six included the original two reported mutations. Wild-type NOTCH2 was able to activate the luciferase reporter ~3.5-fold higher than the vector (pCDNA3.1) alone, while no signalling was seen in the presence of the TRAIL ligand (control). In contrast, none of the mutants tested was able to induce Notch signalling. Lack of Notch2 signalling by these mutants suggests that these mutations are disease-causing mutations and disrupt the NSP. These data support the computer prediction models for the missense mutations studied (table 2).

The nonsense mutation R2003X was further studied by analysis of cDNA, as the location of the nonsense mutation is 21 nucleotides from the last exon junction and therefore is predicted to escape nonsense mediated decay. Sequencing of the NOTCH2 cDNA of patient 2 in both directions (forward and reverse) revealed the presence of the mutant sequence, without any evidence for degradation, of the mRNA.

## DISCUSSION

We report the identification of eight probands with clinical features of ALGS and mutations in *NOTCH2*. Three of these individuals met classic criteria for ALGS and five had only one or two typical ALGS clinical features. We identified six missense mutations, one frameshift, and one nonsense mutation, and we provide strong evidence for the pathogenicity of five of the six missense mutations (p.Pro394Ser is the exception and is discussed below). ALGS is caused by mutations in the Notch ligand JAG1 in 94% of patients, and before this report two patients had been identified with mutations in the Notch receptor, *NOTCH2*.[1,11,12,16] Therefore, a total of nine or possibly 10 individuals (if the case with the p.Pro394Ser mutation is included) with ALGS features who do not have a *JAG1* mutation, but have a disease-causing mutation in *NOTCH2*, have now been identified.

The frequency of clinical findings in the *NOTCH2* (+) cohort was different compared to a cohort of *JAG1* (+) probands (table 2). Liver disease was an almost universal finding in both groups, though this may reflect some referral bias as most of these children were ascertained through a hepatology clinic. In the four families in which the mutation was inherited, mutation-positive relatives had mild or no liver involvement and none had overt cholestasis that was clinically significant, demonstrating that *NOTCH2* mutations can be associated with minimal or no liver involvement. The frequency of renal involvement was also similar, which is notable as we had previously suggested that there may be a prevalent renal phenotype in *NOTCH2* associated ALGS, in the first two reported families.[16] There did appear to be a trend towards less frequent heart involvement in the *NOTCH2* (+) individuals, with 60% affected in comparison to 100% in the *JAG1* (+) cohort. However, the universal cardiac involvement in the *JAG1* (+) individuals included murmurs, peripheral pulmonary stenosis, and intracardiac disease. The frequency of structural intracardiac disease is more similar between both cohorts (30% in *NOTCH2* (+) and 47% in *JAG1* (+)). Of particular note, there was a much lower incidence of the characteristic facial features associated with ALGS in the *NOTCH2* cohort (20%) than in the *JAG1* cohort (97%), suggesting a weaker role for Notch2 in the formation of craniofacial features (figure 2). It should be noted that although we do not see typical Alagille facies in the patient from Vietnam in this study, we have recently shown that even with *JAG1* related ALGS, the facial features are less frequent in a Vietnamese cohort (Lin *et al*, in press). There was also a lower frequency of butterfly vertebrae in the *NOTCH2* individuals (10% vs 64%), further supporting the concept that NOTCH2 may be less important in the integrity of vertebral segmentation. However, it should be noted that since the individuals in the study cohort did not undergo a full skeletal survey, it is only possible to comment on the involvement of the spine and not other bony abnormalities that may exist in *NOTCH2* patients.

The eight unique mutations identified were found in both the extracellular and intracellular domains of the NOTCH2 receptor, with five in the extracellular domain, four missense mutations in the EGF-like repeats (p.Cys373Arg, p.Pro383Ser, p.Pro394Ser, and p.Cys480Arg), and one frameshift mutation (p.Ser856fs) in exon 16. There were three mutations in the intracellular domain (p.Arg1953Cys, p.Arg1953His, and p.Arg2003X), specifically in the ankyrin repeats, a region that is involved in the NOTCH intracellular domain's binding to DNA to effect gene transcription.[20] All of the missense mutations identified are predicted to lead to non-functional proteins (table 2). The evidence for the

pathogenicity of p.Pro394Ser is the least strong. The mutation was predicted to be benign by one of the computer predictions models (table 2); its inheritance is unknown and functional analyses were not performed. Another patient (patient 7) had a second change in *NOTCH2*, which is predicted to be a likely polymorphism, though parental studies and functional analyses are also lacking. Functional analysis with a Notch sensitive luciferase assay confirmed that six of 10 total mutations described to date are associated with a lack of Notch signalling, with complete loss of downstream Notch signalling (figure 3). The mechanism by which *NOTCH2* causes ALGS is not completely understood at this time. It is interesting that the overwhelming majority of *JAG1* mutations in ALGS patients are protein truncating (>70%), and 5–7% of patients have a complete gene deletion, consistent with haploinsufficiency for *JAG1* causing the disease. Among the 10 *NOTCH2* mutations identified, seven are missense mutations. Of the three that are not missense, two have been shown to result in mRNAs that escape nonsense mediated decay, suggesting that a mechanism other than haploinsufficiency may be in effect.

Recently, NOTCH2 has been implicated in the pathogenesis of the Hajdu-Cheney and serpentine fibula polycystic kidney syndromes.[21–25] Hajdu-Cheney syndrome is a rare condition characterised by focal bone destruction with other features including craniofacial abnormalities and renal cysts. In these studies the mutations are all localised in the last exon (exon 34) of *NOTCH2* and the mutations are predicted to disrupt the intracellular PEST (proline-glutamate-serine-threonine-rich) domain, which is hypothesised to result in increased NOTCH2 signalling.[26] Therefore, these mutations have an opposing effect on *NOTCH2* as compared to the Alagille associated mutations.

Mutations in other members of the NSP have also been associated with human disorders. Mutations in the Notch ligand delta like-3 (*DLL3*) cause the autosomal recessive skeletal disorder spondylocostal dysostosis.[27] This congenital syndrome has also been associated with mutations in other Notch pathway related genes including: the transcription factor mesoderm posterior 2 homologue (MESP2); lunatic fringe (LFNG), a glycosyltransferase that modifies the NOTCH proteins, altering signalling specificities; and HES7, a transcription factor that is regulated by Notch signalling. Mutations in *NOTCH1* are associated with left sided cardiac defects, including bicuspid aortic valve, coarctation of the aorta, and hypoplastic left heart syndrome.[28] *NOTCH3* mutations have been identified in patients with CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy), an autosomal dominant disorder characterised by stroke and vascular dementia.[29] The wide array of Notch pathway associated human diseases demonstrates the importance of Notch signalling during the normal development and functioning of various organ systems.

In summary, a total of 10 individuals with ALGS features and *NOTCH2* mutations have now been identified, with eight new patients described in this report. All 10 mutations are unique and are localised across the gene. The spectrum of clinical features associated with *NOTCH2* mutations differs from *JAG1* in this small cohort, with a notably lower prevalence of butterfly vertebrae and facial features. The low frequency of characteristic ALGS facies in the *NOTCH2* cohort significantly reduces the utility of this feature as a diagnostic tool. The distribution of mutation types (70% missense) is different from that seen in *JAG1* mutations in ALGS, suggesting a different mechanism may be operating. These data confirm the role of *NOTCH2* as a second disease gene in ALGS and further support the concept that this disorder is genetically and phenotypically heterogeneous. Screening for *NOTCH2* mutations in *JAG1*-negative patients is warranted, even in those who fulfil partial classic criteria. Clearly, these clinical diagnostic criteria no longer capture the heterogeneity of this disorder, and a molecular and clinical redefinition of ALGS is needed.

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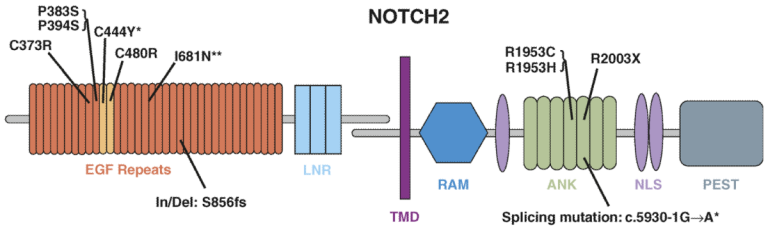


**Figure 1.**

NOTCH2 domain structure and Alagille syndrome (ALGS) mutation locations. The protein domain structure of the human NOTCH2 protein is depicted. NOTCH2 is a transmembrane protein with intracellular and extracellular components. The extracellular part of the protein contains 36 epidermal growth factor (EGF) repeats involved in ligand binding. The 11th and 12th of the EGF repeats (depicted in a different colour from the rest) are known to be required for efficient ligand binding. The intracellular portion of the protein is comprised of multiple domains, including seven ankyrin repeats (ANK) which are required for binding to the CSL (for CBF1 (mammalian C promoter-binding factor 1), suppressor of hairless (fly) Lag2 (worm)) transcription factor in the nucleus and activation of downstream transcription. All of the *NOTCH2* mutations identified in ALGS patients are listed in the corresponding region of the protein. Mutations previously reported in McDaniell *et al*[16] are marked with an asterick. The likely polymorphism in patient 7 is marked with a double asterix. ANK, ankyrin repeats; EGF Repeats, epidermal growth factor-like repeats; LNR, Lin/Notch repeats; NLS, nuclear localisation signal; PEST, proline/glutamic acid/serine/threonine rich domain; RAM, RBP-Jκ-associated module; TMD, Transmembrane domain.

**Figure 2.**
Facial features of *NOTCH2* (+) probands. Front and side profiles of two available *NOTCH2* (+) probands. The typical Alagille syndrome facial features associated with *JAG1* mutations (broad forehead, deep-set eyes, pointed chin) are not present in these individuals.

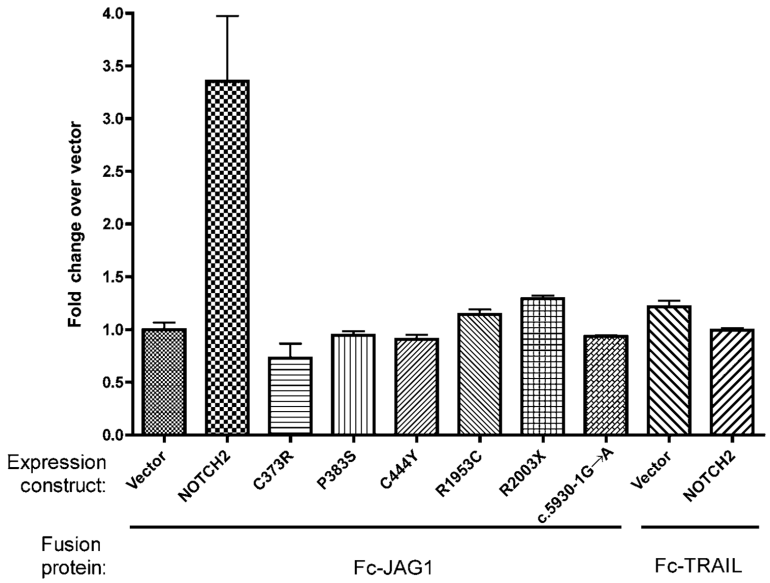


**Figure 3.**

Notch signalling ability of mutant NOTCH2 proteins. The ability of the mutant NOTCH2 proteins to activate Notch signalling in the presence of JAG1 ligand was examined. Cells transfected with wild-type or mutant NOTCH2 or vector alone, along with a CBF-responsive luciferase reporter, were plated in culture wells coated with soluble JAG1 ligand, or soluble TRAIL (TNF related apoptosis inducing ligand) as a ligand specific control. Forty-eight hours post-transfection lysates were collected and luciferase readings taken. Transfection reactions also included a *renilla* internal control for transfection efficiency. Luciferase readings were normalised to renilla readings, and values are expressed as fold change over vector alone. No mutant proteins were able to activate Notch signalling to levels significantly different from vector alone or TRAIL control wells, as assayed by Student t test.

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**Table 1**

Clinical and genetic features of *NOTCH2* probands

| Proband | Liver | Cardiac | Renal | Eye | Skeletal | Facies | Mutation |
|---|---|---|---|---|---|---|---|
| 1 CC | Cholestasis, bile duct paucity | Pulmonary artery stenosis | Vesico-ureteric reflux | Posterior embryotoxon | Normal spine | Present | p.Cys373Arg |
| 2 CC | Cholestasis | Atrial septal defect | Echogenicity of kidneys | NE | Butterfly vertebrae | Absent | p.Arg2003X |
| 3 CC | Cholestasis, bile duct proliferation, hepatic mass | Atrial septal defect, mild pulmonary stenosis | Normal | Posterior embryotoxon | Normal spine | Absent | p.Pro394Ser |
| 4 NC | Elevated LFTs, bile duct paucity | Normal | NE | Posterior embryotoxon | Normal spine | Absent | p.Pro383Ser |
| 5 NC | Cholestasis, bile duct paucity | Normal | Normal | Normal | Normal spine | Absent | p.Ser856fs16x |
| 6 NC | Cholestasis, portal hypertension | Mild pulmonary stenosis | Normal | Normal | Normal spine | Absent | p.Arg1953Cys |
| 7 NC | Cholestasis, bile duct paucity | Normal | Normal | Cataracts | Normal spine | Absent | p.Arg1953His (p.Ile681Asn) |
| 8 NC | Bile duct paucity | Normal | Normal | NE | Normal spine | Absent | p.Cys480Arg |
| 9*CC | Cholestasis | Pulmonary stenosis | Neonatal renal failure | Normal | Normal spine | Present | c.5930-1G→A |
| 10*CC | Cholestasis | Tetralogy of Fallot | Renal failure | Posterior embryotoxon | Normal spine | Absent | p.Cys444Tyr |

ALGS, Alagille syndrome; CC, consistent with ALGS per classic diagnostic criteria; NC, not consistent with classic ALGS, partial diagnostic criteria only; NE, not evaluated.

*Patients reported in original paper, McDaniell *et al*, 2006.

**Table 2**

*NOTCH2* mutations: genomic and protein coordinates, protein predictions, and results of functional analysis

| Mutation | | | | | | Predicted effect of mutation | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Genomic** | **Protein** | **Exon** | **Protein domain** | **Type of mutation** | **Origin** | **MutPred** | **SNPs&Go** | **Polyphen2** | **SIFT** | **Functional analysis** |
| c.1117T→C | p.Cys373Arg | 7 | EGF 9 | Missense | Paternal | 0.873 | Damaging | Damaging | Damaging | Damaging |
| c.1147C→T | p.Pro383Ser | 7 | EGF 10 | Missense | Maternal | 0.694 | Damaging | Damaging | Damaging | Damaging |
| c.1180C→T | p.Pro394Ser | 7 | EGF 10 | Missense | Unknown | 0.611 | Damaging | Damaging | Benign | Not tested |
| c.1331G→A* | p.Cys444Tyr | 8 | EGF 11 | Missense | Maternal | 0.999 | Damaging | Damaging | Damaging | Damaging |
| c.1438T→C | p.Cys480Arg | 9 | EGF 10 | Missense | Unknown | 0.99 | Damaging | Damaging | Damaging | Not tested |
| c.2043T→A† | p.Ile681Asn | 13 | EGF 17/18 | Missense | Unknown | 0.519 | Damaging | Damaging | Benign | Not tested |
| c.2566_2567delAG | p.Ser855fs | 16 | EGF 22 | Frameshift | Maternal | N/A | N/A | N/A | N/A | Not tested |
| c.5857C→T | p.Arg1953Cys | 32 | ANK 4 | Missense | Unknown | 0.588 | Damaging | Damaging | Damaging | Damaging |
| c.5858G→A† | p.Arg1953His | 32 | ANK 4 | Missense | Unknown | 0.591 | Benign | Damaging | Damaging | Not tested |
| c.6007C→T | p.Arg2003X | 33 | ANK 5 | Non-sense | De novo | N/A | N/A | N/A | N/A | Damaging |
| c.5930-1G→A* | N/A | 33 | ANK 5 | Splice site alteration | Maternal | N/A | N/A | N/A | N/A | Damaging |

MutPred score, probability of deleterious or disease associated mutation. MutPred: Li B, Krishnan VG, Mort ME, Xin F, Kamati KK, Cooper DN, Mooney SD, Radivojac P. Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics* 2009;**25**:2744–50. http://mutpred.mutdb.org/

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Polyphen: Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. *Nat Methods* 2010;**7**:248–9. http://genetics.bwh.harvard.edu/pph2/

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*Previously reported mutations.

†Both changes from patient 7.

**Table 3**

Phenotypic comparison between *JAG1*(+) and *NOTCH2*(+) individuals

| Frequency of clinical findings (%) | Liver | Cardiac | Renal | Eye | Skeletal | Facies |
|---|---|---|---|---|---|---|
| NOTCH2 probands (N=10) | 100 | 60 | 44 | 63 | 10 | 20 |
| JAG1 probands[7] N=34) | 100 | 100 | 40[9] | 75 | 64 | 97 |