Haploinsufficiency of the NOTCH1 Receptor as a Cause of Adams–Oliver Syndrome With Variable Cardiac Anomalies

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Background—Adams–Oliver syndrome (AOS) is a rare disorder characterized by congenital limb defects and scalp cutis aplasia. In a proportion of cases, notable cardiac involvement is also apparent. Despite recent advances in the understanding of the genetic basis of AOS, for the majority of affected subjects, the underlying molecular defect remains unresolved. This study aimed to identify novel genetic determinants of AOS.

Methods and Results—Whole-exome sequencing was performed for 12 probands, each with a clinical diagnosis of AOS. Analyses led to the identification of novel heterozygous truncating NOTCH1 mutations (c.1649dupA and c.6049_6050delTC) in 2 kindreds in which AOS was segregating as an autosomal dominant trait. Screening a cohort of 52 unrelated AOS subjects, we detected 8 additional unique NOTCH1 mutations, including 3 de novo amino acid substitutions, all within the ligand-binding domain. Congenital heart anomalies were noted in 47% (8/17) of NOTCH1-positive probands and affected family members. In leukocyte-derived RNA from subjects harboring NOTCH1 extracellular domain mutations, we observed significant reduction of NOTCH1 expression, suggesting instability and degradation of mutant mRNA transcripts by the cellular machinery. Transient transfection of mutagenized NOTCH1 missense constructs also revealed significant reduction in gene expression. Mutant NOTCH1 expression was associated with downregulation of the Notch target genes HEY1 and HES1, indicating that NOTCH1-related AOS arises through dysregulation of the Notch signaling pathway.

Conclusions—These findings highlight a key role for NOTCH1 across a range of developmental anomalies that include cardiac defects and implicate NOTCH1 haploinsufficiency as a likely molecular mechanism for this group of disorders.

Key Words: Adams–Oliver syndrome  ■ genetics  ■ haploinsufficiency  ■ heart defects, congenital  ■ receptor, NOTCH1

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Clinical Perspective on p 581

The molecular genetic basis of AOS appears heterogeneous and, to date, defects within 5 genes have been reported, providing limited insight as to the molecular mechanisms underlying these important aspects of early development. Mutations of the ARHGAP31 and DOCK6 genes underlie a proportion of AOS cases displaying autosomal dominant and recessive inheritance, respectively.6,7 Both ARHGAP31 and DOCK6 regulate the activity of the Rho GTPases Cdc42 and Rac1, which cycle between active, GTP-bound and inactive, GDP-bound states through the opposing modes of action of guanine nucleotide exchange factors and GTPase-activating proteins (GAPs). We have previously demonstrated that ARHGAP31 mutations cause AOS through a gain-of-function mechanism, which leads to an accumulation of inactive GTPase, disrupting...
actin cytoskeletal dynamics. Mutations in DOCK6 result in a more severe, multisystemic phenotype because of a homozgyous loss of guanine nucleotide exchange factor function.

More recently, the Notch signaling pathway has been implicated in AOS pathogenesis by the discovery of heterozygous alterations in the RBPJ gene, encoding the major transcription factor for Notch. Missense mutations within the DNA-binding domain of RBPJ result in impaired binding ability of the transcription factor to the HES1 promoter, likely disrupting the regulation of Notch target genes downstream. Moreover, 2 independent studies have identified homozygous mutations of EO GT, encoding an epidermal growth factor (EGF) domain-specific enzyme demonstrated as critical in the glycosylation of Notch1 in mammalian cells. Most recently, a report by Stüttrich et al identified mutations of the NOTCH1 gene in a proportion of an AOS cohort.

These studies have provided some important insights into the molecular processes key to the development of AOS. However, despite congenital heart anomalies affecting >1 in 5 subjects with AOS, the ARHGAP31- and RBPJ-positive pedigrees reported in the literature have a notable lack of cardiovascular involvement. Although the majority of these mutation carriers may not have been assessed by cardiac imaging, these data implicate distinct regulatory systems in the pathogenesis of autosomal dominant AOS with CHDs. We designed an exome-wide based study to further define the genetic mechanisms relevant to the pathogenesis of AOS. Through this work, we identified novel heterozygous mutations of NOTCH1, providing independent verification of a critical role for this gene as a common cause of AOS in both autosomal dominant and sporadic cases. We have further used gene expression studies to examine the effect of NOTCH1 mutation on downstream signaling and demonstrated a pathogenic effect in RNA extracted from AOS subjects harboring NOTCH1 defects. These cases display a striking genotype-phenotype correlation with a high prevalence of cardiac and vascular anomalies, highlighting the importance of Notch signaling in cardiovascular development and demonstrating a novel role for NOTCH1 in multiple developmental processes that include scalp and limb formation.

Methods

Patient Cohorts

Exome sequencing was performed for 12 unrelated probands, diagnosed with autosomal dominant AOS and negative for ARHGAP31 and RBPJ mutations. Criteria for diagnosis were according to the guidelines by Snape et al. Subsequent mutation screening of the NOTCH1 coding regions comprised a cohort of 52 additional individuals with a diagnosis of AOS (n=11 autosomal dominant cases; n=41 isolated cases with no known family history). Cardiac clinical evaluation and echocardiography of NOTCH1-positive patients and family members were conducted at specialist cardiology centers (United Kingdom, Germany, Italy, and France) after referral by the respective consultant clinical geneticist. The study complies with the Declaration of Helsinki, and informed written consent was obtained from all participants before taking part. The research protocol was approved by the local ethics committees (NRES Committee London [Bromley], United Kingdom and the Ethics Board of the Medical Faculty of the University of Erlangen, Germany). Patient samples were collected as either saliva (Origene DNA collection kit, DNA Genotek) or blood, and genomic DNA was extracted according to standard protocols.

Exome Sequencing and Mutation Detection

Exome libraries were generated with the SureSelect Human All Exon Target Enrichment kit (Agilent Technologies) using genomic DNA extracted from peripheral blood. Paired-end sequence reads were generated on an Illumina HiSeq 2000. Read alignment to the reference genome (hg19) and variant calling were performed as described previously, with variant annotation completed using the ANNOVAR software. Sequence variants were compared against publicly available databases (MapHap, 1000 Genomes Project, dbSNP, the National Heart, Lung, and Blood Institute Exome Sequencing Project, and an in-house repository of 400 exomes) to assess their novelty. Candidate genes were prioritized on the basis of novel truncating mutations (frameshift, nonsense, and splice-site) in ≥2 independent probands. Of these, NOTCH1 was taken forward for further study because of compelling biological relevance to AOS pathogenesis. Validation of variant segregation and mutation screening of all NOTCH1 coding regions and intron–exon boundaries was performed by direct DNA sequencing using BigDye Terminator version 3.1 chemistry on an ABI3730xl (Applied Biosystems).

Mutagenesis and Cell Culture

Wild-type NOTCH1 cDNA in pFN1A (Kazusa DNA Research Institute) was purchased from Promega. Identified AOS missense variants were introduced by site-directed mutagenesis using the QuikChange II XL kit (Agilent Technologies). Primer details are available on request. Cells were maintained at 37°C in a humidified incubator with 5% CO2. Human endometrioid cancer cells were cultured in Dulbecco modified Eagle medium with Glutamax (Gibco Life Technologies), supplemented with 10% fetal bovine serum. Human endometrioid cancer cells were seeded in 100-mm dishes and grown to 80% confluence. Transient transfection was performed using FuGene HD transfection reagent (Promega), and transfected cells were incubated for 48 hours before harvesting for RNA extraction.

Gene Expression Analysis

Total RNA was extracted from 2.5 mL of peripheral blood from NOTCH1-positive patients and a 26-year-old clinically unaffected female control using the PAXgene Blood RNA System (PreAnalytiX), following the manufacturer’s guidelines. For mutagenized constructs, RNA was extracted from transfected cells with the RNeasy Mini kit (Qiagen), according to the manufacturer’s instructions. RNA (500 ng) was used for first strand cDNA synthesis with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (PCR) was performed on a StepOnePlus real-time PCR machine (Applied Biosystems) using double-dye Taqman-style detection chemistry with the PrimerDesign 2x Precision Mastermix and custom-designed probe sets for NOTCH1, HEY1, and HES1 mRNAs (PrimerDesign). GAPDH and ACTB house-keeping genes were used for normalization in mRNA relative quantifications using SDS version 2.2 software. Gene of interest expression levels for patient and mutagenized samples were calculated by the 2^-ΔΔCt method relative to the wild-type baseline. Statistical analysis of real-time data was performed using a Mann–Whitney U test to generate 2-tailed P values (VassarStats software).

Results

Clinical Features of NOTCH1-Positive Families

The proband of family 1 (1-II-1) displayed cutis aplasia and marked terminal transverse limb defects at birth. The sibling 1-II-2 was also born with a severe cutaneous and bony scalp defect, with terminal transverse limb defects affecting both feet. On examination, the obligate carrier mother (1-I-3) exhibited no scalp or limb defects but was found to have an unexplained heart murmur. Subject 1-I-1 had died at 5 months because of a CHD, but no further details were available. Patient 1-II-4 presented with syndactyly of the left hand and both feet,
and cutis marmorata telangiectatica congenita on the abdomen and legs. Echocardiography revealed mild aortic stenosis and mild aortic regurgitation. Similar digit abnormalities and scalp ACC were displayed by subject 1-III:1. Although 1-III:2 appeared clinically normal on examination, sonography of the heart also revealed mild aortic regurgitation (Table).

Family 2 has been previously described by Dallapiccola et al. The proband (2-II:1) and his mother both exhibited ACC of the midline region of the scalp and cardiac investigation by ultrasonography identified coarctation of the aorta in both individuals. The mother (2-I:2) also had a vascular anomaly consisting of duplication of the right femoral artery. Surgical intervention to repair the aortic coarctation and femoral artery duplication were conducted at 14 and 17 years of age, respectively. The cardiac defect in subject 2-II:1 resembled the so-called Shone complex, an anatomic variant consisting of multiple levels of left-sided obstructive CHDs, including aortic coarctation, bicuspid aortic valve, and parachute non-stenotic mitral valve with mild regurgitation. Coarctectomy was performed at 5 months of age.

Patient 3-III:1 was born with a large area of scalp ACC with an underlying calvarial defect and shortened distal phalanges of the toes (Figure 1). A recent echocardiography detected no obvious abnormality. The obligate carrier father (3-II:2) was clinically normal, and cardiology examination was negative for cardiac defects. The relative 3-II:3 was considered to be affected with minor terminal hypoplasia of the phalanges of some toes, but no cardiac anomaly was detected on sonography.

The proband of family 4 (4-II:1) presented with scalp ACC of the posterior parietal region and brachydactyly of both hands. Cardiovascular abnormalities included coarctation of the aorta, valvular aortic stenosis, parachute mitral valve with valvular insufficiency, and a subaortic membranous ventricular septal defect, also indicative of Shone complex. Aortic coarctation was operated at 15 days of life; aortic valvulotomy and intervention for ventricular septal defect were performed at 9 years of age. Aortic valve substitution surgery was completed at 23 years. Echocardiography in the mother (4-I:2) revealed valvular aortic stenosis with thick fibrotic semilunar valves, moderate aortic valve insufficiency, and mild to moderate left ventricular hypertrophy (Table).

Specific clinical features of sporadic cases 5 to 11 are summarized in Table. Representative images of the limb and scalp defects observed across our AOS cohort are shown in Figure 1.

Identification of Novel NOTCH1 Variants

The analysis of exome profiles of affected male probands from families 1 and 2 identified novel heterozygous variants (c.1649dupA; p.Y550* and c.6049_6050delITC; p.S2017Tfs*9) in the NOTCH1 gene (NM_017617.3). Both mutations are predicted to result in premature stop codons of the mRNA transcript. Examination of available members of family 1 confirmed segregation of the c.1649dupA mutation with the phenotype (Figure 2). Unfortunately, DNA was not available from the affected mother of proband 2-II:1 for segregation analysis of the c.6049_6050delITC variant.

Subsequent mutation screening of the NOTCH1 coding regions was performed in an extended replication cohort of 52 individuals with a clear clinical diagnosis of AOS. Novel NOTCH1 heterozygous variants were identified in 9 additional subjects. Taken together with the exome data, we report a total of 10 distinct heterozygous mutations in the NOTCH1 gene, one of which (c.1343G>A; p.R448Q) is recurrent, in 4 autosomal dominant families and 7 apparently sporadic cases with no known family history (Figure 2; Table I in the Data Supplement). All variants were confirmed by independent Sanger sequencing and absent from public variant databases. In families, segregation of the observed variant was consistent with the disease phenotype where DNA from multiple family members was available (Figure 2). In AOS mutation carriers with no family history of AOS or isolated cardiovascular disease, the sequencing of available parental DNA demonstrated that 3 mutations occurred de novo, namely c.1343G>A (p.R448Q) in proband 5-II:1, c.1345T>C (p.C449R) in proband 6-II:1, and c.1367G>A (p.C456Y) in proband 10-II:1. In family 3, 2 unaffected paternal uncles and 2 clinically normal siblings of the proband 3-III:1 were negative for the c.4120T>C (p.C1374R) mutation (data not shown). However, the unaffected obligate carrier father (3-II:2) was confirmed to carry the mutation. Similarly, the unaffected mother of subject 7-II:1 was found to harbor the c.1220C>G (p.P407R) variant. Cardiovascular assessment of 3-II:2 and 7-II:2 by echocardiography detected no underlying cardiac abnormality, confirming that these mutation carriers are phenotypically normal and demonstrating incomplete penetrance for mutations in this gene.

NOTCH1 Missense Mutations Are Located Within Critical Functional Domains

Five of the 6 missense mutations identified in this study are predicted to be pathogenic by MutationTaster2,19 PolyPhen-2,20 or SIFT prediction software21 (Table II in the Data Supplement). The affected amino acids are located across the length of the receptor, in the main situated within the extracellular EGF-repeat domain. Specifically, 4 mutations (p.P407R, p.R448Q, p.C449R, and p.C456Y) occur in or adjacent to the ligand-binding domain, specified by EGF repeats 11 to 13 (Figures 3A and 4). The majority are strongly conserved across species and lie within highly conserved domains of the protein (Figure 3B). Furthermore, 3 amino acid substitutions (p.C449R, p.C456Y, and p.C1374R) affect cysteine residues, which are likely to disrupt disulfide bonds that are critical for the structure of EGF-like domains (Figure 4). By contrast, the p.A1740S mutation is located within the transmembrane domain and, while conserved across mammalian species, is not conserved in other vertebrate species and has a less clear effect on the structural integrity of the receptor, so remains a variant of unknown significance (Figure 3).

NOTCH1 Haploinsufficiency Is Implicated in AOS Pathogenesis

To assess the level of mutant mRNA transcripts, we conducted quantitative real-time PCR studies using RNA extracted from peripheral blood of 3 patients harboring NOTCH1 mutation (c.1343G>A and c.1649dupA [2 cases]).
NOTCH1 transcript levels were significantly reduced by comparison with an unaffected control individual, demonstrating >50% expression in all samples tested (Figure 5A). Although institutional ethical constraints precluded detailed cardiac evaluation of the control subject, it was made clear by personal testimony that there was no family history of

Table.  Clinical Characteristics of AOS Affected Subjects Harboring NOTCH1 Mutations

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Country of Origin</th>
<th>Current Age</th>
<th>Sex</th>
<th>ACC</th>
<th>TTLD</th>
<th>Cardiac or Vascular Features [Age at Diagnosis]</th>
<th>Echocardiographic Assessment [Age at Assessment]</th>
<th>Other Features [Age at Diagnosis]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-II:1*</td>
<td>United Kingdom</td>
<td>29 y</td>
<td>Male</td>
<td>++</td>
<td>+</td>
<td>Nd</td>
<td>Not assessed</td>
<td>Undefined heart murmur</td>
</tr>
<tr>
<td>1-II:2</td>
<td></td>
<td>28 y</td>
<td>Male</td>
<td>++</td>
<td>+</td>
<td>Nd</td>
<td>Not assessed</td>
<td>Undefined heart murmur</td>
</tr>
<tr>
<td>1-II:4</td>
<td></td>
<td>35 y</td>
<td>Female</td>
<td>–</td>
<td>++</td>
<td>[34 y]: AR; AS; CMT on abdomen and legs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-III:1</td>
<td></td>
<td>17 y</td>
<td>Male</td>
<td>+</td>
<td>++</td>
<td>Nd</td>
<td>Not assessed</td>
<td></td>
</tr>
<tr>
<td>1-III:2</td>
<td></td>
<td>10 y</td>
<td>Female</td>
<td>–</td>
<td>–</td>
<td>[9 yr]: AR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-II:1*</td>
<td>Italy</td>
<td>27 y</td>
<td>Male</td>
<td>+</td>
<td>–</td>
<td>[15 d]: BAV; CoA; PMV; [5 mo]: subclavian flap coarctectomy</td>
<td>[15 d]: nonstenotic PMV with mild regurgitation</td>
<td>Reference: Dallapiccola et al15 1992 (patient 2)</td>
</tr>
<tr>
<td>3-II:3</td>
<td>Germany</td>
<td>35 y</td>
<td>Male</td>
<td>–</td>
<td>+</td>
<td>None</td>
<td>[34 y]: normal</td>
<td></td>
</tr>
<tr>
<td>3-III:1*</td>
<td></td>
<td>8 y</td>
<td>Male</td>
<td>++</td>
<td>+</td>
<td>None</td>
<td>[7 y]: normal</td>
<td></td>
</tr>
<tr>
<td>4-I:2</td>
<td>Italy</td>
<td>47 y</td>
<td>Female</td>
<td>–</td>
<td>–</td>
<td>AR; AS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-II:1*</td>
<td></td>
<td>25 y</td>
<td>Female</td>
<td>+</td>
<td>++</td>
<td>[15 d]: AS; CoA; PMV; VSD</td>
<td>[15 d]: subaortic membranous VSD; severe AS (PG: 80 mm Hg, MG: 20 mm Hg); mild to moderate LWH narrowing of shunt, free pulmonary regurgitation</td>
<td>Long palpebral fissures</td>
</tr>
<tr>
<td>5-II:1*</td>
<td>United Kingdom</td>
<td>10 y</td>
<td>Male</td>
<td>++</td>
<td>–</td>
<td>[1 d]: PA-VSD; [6 d]: right MBTS; [2 yr]: Rastelli correction</td>
<td>[1 d]: PA, VSD Fallot type; [10 y]: some narrowing of shunt, free pulmonary regurgitation</td>
<td>[5 y]: portal vein thrombosis; portal hypertension; T-cell lymphopenia; complex learning disability; autism</td>
</tr>
<tr>
<td>6-II:1*</td>
<td>Russia</td>
<td>Deceased</td>
<td>Female</td>
<td>++</td>
<td>+</td>
<td>[In utero]: truncus arteriosus communis type I; [1 d]: VSD</td>
<td>[1 d]: membrane VSD, right-sided aortic bow descending on left-hand side</td>
<td>Bilateral cryptorchidism; bilateral abdominal wall hernia; hypertelorism; downslanting palpebral fissures</td>
</tr>
<tr>
<td>7-II:1*</td>
<td>Italy</td>
<td>8 y</td>
<td>Male</td>
<td>+</td>
<td>+</td>
<td>None</td>
<td>[7 y]: normal</td>
<td>Bilateral cryptorchidism; bilateral abdominal wall hernia; hypertelorism; downslanting palpebral fissures</td>
</tr>
<tr>
<td>8-II:1*</td>
<td>Italy</td>
<td>15 y</td>
<td>Male</td>
<td>+</td>
<td>+</td>
<td>[1 d]: CMTC</td>
<td>[15 y]: normal</td>
<td>Epilepsy; dyslexia</td>
</tr>
<tr>
<td>9-II:1*</td>
<td>Germany</td>
<td>33 y</td>
<td>Female</td>
<td>+</td>
<td>++</td>
<td>Nd</td>
<td>Not assessed</td>
<td></td>
</tr>
<tr>
<td>10-II:1*</td>
<td>France</td>
<td>16 y</td>
<td>Female</td>
<td>++</td>
<td>++</td>
<td>[3.5 y]: ASD; surgically closed at 4 y; arteriography; EHPVT, hepatopetal and hepatofugal collateral veins</td>
<td>[3.5 y]: ASD, suspected portopulmonary hypertension (mPAP: 30 mm Hg); [7 y]: mPAP: 45 mm Hg, PVR: 8.1 Wood U/m², PWP: &lt;15 mm Hg; [12 y]: mPAP: 27 mm Hg with Sildenafil treatment</td>
<td>[3.5 y]: HSM and portal hypertension with GI bleeding; OPV. Reference: Girard et al30 2005 (patient 1); Franchi-Abeilla et al31 2014</td>
</tr>
<tr>
<td>11-II:1*</td>
<td>Greece</td>
<td>19 y</td>
<td>Male</td>
<td>++</td>
<td>++</td>
<td>[6.5 y]: arteriography; EPVO, large hepatopetal coronary vein, tiny hepatopetal cavernoma</td>
<td>[6.5 y]: normal; [14 y]: normal</td>
<td>[6.5 y]: HSM and portal hypertension; OPV. Reference: Girard et al34 2005 (patient 2); Franchi-Abeilla et al35 2014</td>
</tr>
</tbody>
</table>

Subject identifiers refer to the pedigree structures in Figure 1. The proband for each family is marked with an asterisk. – indicates absent; +, present (for ACC: +, small defect [≤5 cm]; ++, large defect [>5 cm] with underlying osseous skull defect; for TTLD: +, feet or hands only; ++, both feet and hands affected); ACC, aplasia cutis congenita; AR, aortic regurgitation; AS, aortic valve stenosis; ASD, atrial septal defect; ACC, aposiopsis cutis congenita; AR, aortic regurgitation; AS, aortic valve stenosis; ASD, atrial septal defect; BAV, bicuspid aortic valve; CMTG, cutis marmorata telangiectatica congenita; CoA, coarctation of the aorta; EPVO, extrahepatic portal vein obstruction; EHPVT, extrahepatic portal vein thrombosis; GI, gastrointestinal; HSM, hepatosplenomegaly; LHV, left ventricular hypertrophy; MBTS, modified Blalock–Tausig shunt; MG: median gradient; mPAP, mean pulmonary arterial pressure (measured by right heart catheterization); Nd, not determined; OPV, obliterator portal venopathy; PA, pulmonary atresia; PG, peak gradient; PMV, parachute mitral valve; PV, peak velocity; PVR, pulmonary vascular resistance; PWP, pulmonary wedge pressure; TTLD, terminal transverse limb defects; and VSD, ventral septal defect.
developmental abnormalities relating to AOS–CHD. We additionally performed transient transfection of mutagenized NOTCH1 constructs to examine the functional effect of missense mutations for which patient RNA was not available. Real-time PCR of RNA extracted from transfected cells also showed a significant decrease of NOTCH1 expression when compared with cells transfected with a full-length wild-type construct and provided independent verification of NOTCH1 downregulation for the c.1343G>A mutation (Figure I in the Data Supplement).

To further interrogate the effect of NOTCH1 mutations on downstream signaling factors, we next performed gene expression studies to quantify the levels of HEY1 and HES1 transcript in patient-derived RNA samples. Subjects harboring the c.1649dupA frameshift mutation exhibited a particularly marked reduction of HEY1 transcript levels by comparison with wild-type control (P=0.0004). By contrast, downregulation of HEY1 expression was less profound for the c.1343G>A missense mutation (Figure 5B). In addition, HES1 mRNA levels were reduced in the c.1649dupA

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**Figure 1.** Clinical features of 3 representative Adams–Oliver syndrome patients with NOTCH1 mutation. **Left,** Patient 3-III:1 at age 2.8 years: residual skin defect after multiple operations on a large area of scalp aplasia cutis congenita; minor hypoplasia of terminal phalanges of the toes; normal fingers. **Middle,** Patient 6-II:1 as a newborn: large scalp defect involving the underlying bone; hypoplasia of terminal phalanges of both feet. **Right,** Patient 8-II:1 at age 14.7 years: small area of alopecia marking a healed scalp defect; hypoplasia of terminal phalanges and nails of the left foot; normal fingers.

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**Figure 2.** Pedigree structures and sequence chromatograms of identified NOTCH1 mutations. Asterisks denote the families included in the exome sequencing discovery cohort. Proband is marked by the black arrows, and asymptomatic carriers are indicated with black dots. For de novo mutations, paternity was confirmed by microsatellite analysis (data not shown). + indicates wild-type allele; and −, mutant allele.
Discussion

Molecular genetic studies of AOS have successfully provided vital insights into the pathways relevant to the pathogenesis of this serious disorder of morphogenesis, through the identification of multiple causative genes. Yet, there remains substantial unexplained locus heterogeneity with the underlying molecular genetic determinants still uncharacterized for the majority of cases. This degree of locus heterogeneity is uncommon for a rare disorder and suggests that AOS may represent a cluster of phenotypes with a related cause, analogous to the RASopathies or ciliopathies.23,24

Herein, we report 10 novel germline NOTCH1 mutations in a patient cohort with autosomal dominant and sporadic forms of AOS. By comparison with AOS cases reported in the literature (13%–20%),3–5,25,26 a significantly higher proportion of probands (5/11; 45%) presented with a congenital heart abnormality (Table). Similarly, cardiovascular anomalies were identified in 47% (8/17) of all affected variant carriers, thereby indicating that NOTCH1 variants may represent a distinct subtype of AOS associated with cardiac malformations. Many vascular complications, including cutis marmorata telangiectatica
congenita and portal vein abnormalities, were additionally observed in NOTCH1-positive cases. Importantly, 2 of the probands in this study and 2 related mutation carriers have not undergone echocardiographic assessment; therefore we are unable to define the exact proportion of NOTCH1-positive cases with cardiovascular defects. In contrast to other gene reports, these patients predominantly demonstrate ACC with mild terminal transverse limb defects, affecting only terminal phalanges with nail hypoplasia (Figure 1). This study is further corroborated by a recent report of distinct NOTCH1 mutations in 5 kindreds with AOS and cardiac spectrum defects.11

Emerging evidence is accumulating to implicate defects of the Notch signaling pathway in the pathogenesis of AOS. The Notch family of single-pass transmembrane receptors is well documented as playing a vital role in multiple cellular processes during embryogenesis, and Notch pathway members have an established role in development of the cardiovascular system.27 Mutations in a subset of Notch components have been shown to underlie CHDs in both mice and humans but in exclusion of limb and scalp developmental abnormalities.

For example, variants in the JAG1 gene, encoding a Notch ligand, underlie the majority of cases of Alagille syndrome,28 whereas endothelial-specific deletion of Jag1 in the mouse leads to embryonic lethality and cardiovascular defects.29 NOTCH2 mutations account for a proportion of Alagille syndrome cases30; however, distinct truncating variants in the terminal exon of NOTCH2 also lead to the osteolytic developmental disorder Hajdu–Cheney syndrome, indicating pleiotropic effects analogous to the NOTCH1 receptor.31,32 Of interest, mice homozygous for a targeted Jag2 deletion die perinatally because of craniofacial abnormalities and syndactyly of the fore- and hindlimbs, consistent features of the AOS spectrum.33 Furthermore, both Notch1 and Notch2 play key roles during mouse limb development in the regulation of apoptosis, a process mediated by Notch signaling through Jag2 in the apical ectodermal ridge,34,35 and the positive regulation of vascular growth through the promotion of angiogenesis and osteogenesis in bone.36 Despite numerous studies in lower organisms, the function of NOTCH1 during human fetal development remains to be fully elucidated.

In mammalian cells, canonical signaling through the Notch family (Notch1-4) is stimulated by ligand binding at the cell surface, which leads to proteolytic cleavage of the Notch intracellular domain, allowing for the formation of a transcriptional complex with RBPJ and coactivators (Figure 6).37 RBPJ is known to regulate the expression of the basic helix-loop-helix transcription factors HES1 and HEY1, both of which are related to the Drosophila hairy and enhancer of split 1 gene. The stimulation of HES1 and HEY1 gene expression is therefore a direct readout of Notch signaling activation.

In this study, we report predicted protein truncating mutations (4/10) most likely to be subject to nonsense-mediated decay. The N-terminal ligand-binding domain of NOTCH1 consists of a series of 36 EGF-like repeats. The majority (5/6) of the missense mutations identified in this study affect residues located within EGF domains of the receptor. These individual domains are characterized by a core β-pleated sheet, 3 disulfide bonds along with a series of variable loops.39 Within this region, EGF repeats 11 to 13 have been shown to be implicated with chelating Ca2+, which is essential for the maintenance of NOTCH1 function.38,39 Three of the identified amino acid substitutions resulting in AOS (p.R448Q, p.C449R and p.C456Y) lie within EGF11 and potentially perturb function by disrupting the tertiary structure and affecting Ca2+ binding and ligand interaction. The X-ray structure of this region of wild-type NOTCH1 indicates that the side chains of Arg448 and Glu424 interact electrostatically (Figure 4);22 thereby it is anticipated that the p.R448Q mutant is unstable. Similarly, the p.C449R and p.C456Y mutations will likely abolish the disulfide bonds between Cys440 and Cys449, and between Cys456 and Cys467, respectively, thereby disrupting the stability of the adjacent Ca2+ coordinated by the side chains Asp469, Glu455, and Asp452 (Figure 4). Although the 3-dimensional structure of the EGF35 region has not been experimentally resolved, it is anticipated that a similar effect would be seen with the Cys1374 mutation. Taken together, these observations imply that the majority of missense mutations have a substantial effect on the structural integrity vital to NOTCH1 activity. Determining the functional effect of the transmembrane
Mutations in EOGT and RBPJ have previously been identified in proteins (CoR) to repress transcription of downstream genes. Absence of Notch activation, RBPJ complexes with corepressor mind (MAML) and transcriptional coactivators (CoA). C to form an active transcriptional complex with RBPJ, master

**Figure 6.** Simplified schematic of the canonical Notch signaling pathway. A, Epidermal growth factor (EGF)–repeat domain of Notch is known to be glycosylated by EOGT in mammalian cells. Activation of the Notch signaling cascade is initiated by the binding of 1 of 5 ligands through direct contact of adjacent cells. B, Ligand activation leads to cleavage and release of Notch intracellular domain (NICD), which translocates to the nucleus to form an active transcriptional complex with RBPJ, mastermind (MAML) and transcriptional coactivators (CoA). C, In the absence of Notch activation, RBPJ complexes with corepressor proteins (CoR) to repress transcription of downstream genes. Mutations in EOGT and RBPJ have previously been identified in Adams–Oliver syndrome.

The 36 EGF-like repeats can be modified by the addition of an O-glucose sugar between the first and second conserved cysteines and an O-fucose between the second and third cysteines, which is essential for normal Notch function.40 Similarly, the target motif C5XXGXS/TGXXC6, located between the fifth and sixth conserved cysteines, has been shown to be recognized by EOGT in both Drosophila and mouse.41 EOGT functions as an O-linked N-acetylgalactosamine (GlcNAc) transferase, which catalyses the addition of an O-GlcNAc moiety.42 Although EOGT has not yet been formally demonstrated to target human Notch, it is notable that 4 of the 5 EGF-domain specific missense mutations identified here are located within this target motif, suggestive of a convergence of pathways previously implicated in the development of AOS (Figure 3B).

To examine the effect of identified mutations on Notch signaling, we have performed gene expression studies and demonstrated that NOTCH1 expression is downregulated in AOS subjects harboring NOTCH1 mutation in vivo, by comparison with a single healthy control female, aged 26 years. Although it is not uncommon to use a single control in real-time PCR studies, we acknowledge that these findings might have been strengthened by using additional controls as part of the experimental design. The data generated are corroborated by transient transfection studies of mutant constructs and, together, support the prediction of transcript loss by nonsense-mediated decay or, in the case of missense mutations, potential perturbation of mRNA stability.43 This observation is underpinned by a reduction of HEY1 and, to a lesser extent, HES1 transcript levels. Of interest, perturbation of both HEY1 and HES1 expression vary between the mutations tested, indicating allele-specific effects on downstream signaling. These data, although preliminary due in part to limited patient sample availability, suggest that downregulation of HEY1 is a common mechanism in AOS. HEY1 is known to have a prominent role in cardiovascular development, with Hey1/Hey2 double-knockout mice exhibiting defects of vasculogenesis and remodeling, particularly in the head region.44 Moreover, these results are compatible with the hypothesis that dysregulated Notch signaling caused by identified mutations is mediated via the transcription factor RBPI, a known causal factor in AOS pathogenesis. Taken together, these data offer support for loss-of-function or haploinsufficiency of NOTCH1 as an important factor in AOS pathogenesis and provide a compelling genotype–phenotype correlation between NOTCH1 mutation and AOS subjects with cardiac anomalies, which warrants further epidemiological investigation. As the overall study group herein has not been intensively examined for cardiac complications, these latter conclusions are at present indicative and will benefit from existing and future international collaboration.

This report establishes NOTCH1 mutation as the primary cause of AOS, accounting for 17% of cases in our cohort, and an important genetic factor in AOS with associated cardiovascular complications. Functional studies have indicated links to related genes associated with this condition, which together emphasize the central importance of the Notch signaling cascade in a series of key developmental systems in human embryogenesis.

**Acknowledgments**

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**Disclosures**

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**Appendix**

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References


**CLINICAL PERSPECTIVE**

Notch signaling provides a critical pathway for regulation of early development. However, the role of NOTCH1 during human embryogenesis remains unclear. Here, we show that deleterious mutations of the NOTCH1 gene lead to neonatal defects of the limb, scalp, and cardiovascular system. Mutation carriers demonstrate a notable genotype-phenotype correlation with an increased incidence of cardiac and vascular anomalies. Gene expression data implicate NOTCH1 haploinsufficiency as a likely molecular mechanism, leading to dysregulated Notch signaling. This study highlights the importance of NOTCH1 in limb, scalp, and heart formation and provides a diagnostic tool to aid clinical management of patients and families affected by this range of developmental disorders.
Haploinsufficiency of the NOTCH1 Receptor as a Cause of Adams–Oliver Syndrome With Variable Cardiac Anomalies


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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL TABLE I. Summary of identified *NOTCH1* variants

<table>
<thead>
<tr>
<th>Exon</th>
<th>Coding variant</th>
<th>Protein variant</th>
<th>Variant type</th>
<th>Protein domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>c.1220C&gt;G</td>
<td>p.P407R</td>
<td>Missense</td>
<td>EGF 10</td>
</tr>
<tr>
<td>8</td>
<td>c.1343G&gt;A</td>
<td>p.R448Q</td>
<td>Missense</td>
<td>EGF 11</td>
</tr>
<tr>
<td>8</td>
<td>c.1345T&gt;C</td>
<td>p.C449R</td>
<td>Missense</td>
<td>EGF 11</td>
</tr>
<tr>
<td>8</td>
<td>c.1367G&gt;A</td>
<td>p.C456Y</td>
<td>Missense</td>
<td>EGF 11</td>
</tr>
<tr>
<td>10</td>
<td>c.1649dupA</td>
<td>p.Y550*</td>
<td>Frameshift</td>
<td>EGF 14</td>
</tr>
<tr>
<td>25</td>
<td>c.4120T&gt;C</td>
<td>p.C1374R</td>
<td>Missense</td>
<td>EGF 35</td>
</tr>
<tr>
<td>26</td>
<td>c.4663G&gt;T</td>
<td>p.E1555*</td>
<td>Nonsense</td>
<td>LNR 3</td>
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<tr>
<td>26</td>
<td>c.4739dupT</td>
<td>p.M1580Ifs*30</td>
<td>Frameshift</td>
<td>-</td>
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<td>28</td>
<td>c.5218G&gt;T</td>
<td>p.A1740S</td>
<td>Missense</td>
<td>TM</td>
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<tr>
<td>32</td>
<td>c.6049_6050delTC</td>
<td>p.S2017Tfs*9</td>
<td>Frameshift</td>
<td>ANK 4</td>
</tr>
</tbody>
</table>

**Key to abbreviations:** EGF, epidermal growth factor-like repeat domain; LNR, Lin-12 NOTCH repeat domain; TM, transmembrane domain; ANK, ankyrin repeat domain.

SUPPLEMENTAL TABLE II. Predicted pathogenicity of identified *NOTCH1* variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>MutationTaster2</th>
<th>PolyPhen-2</th>
<th>SIFT Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.P407R</td>
<td>Disease causing (prob: 0.999)</td>
<td>Probably damaging (0.968)</td>
<td>Tolerated (0.51)</td>
</tr>
<tr>
<td>p.R448Q</td>
<td>Disease causing (prob: 0.999)</td>
<td>Probably damaging (0.984)</td>
<td>Tolerated (0.29)</td>
</tr>
<tr>
<td>p.C449R</td>
<td>Disease causing (prob: 0.999)</td>
<td>Probably damaging (1.000)</td>
<td>Damaging (0)</td>
</tr>
<tr>
<td>p.C456Y</td>
<td>Disease causing (prob: 0.999)</td>
<td>Probably damaging (1.000)</td>
<td>Damaging (0)</td>
</tr>
<tr>
<td>p.Y550*</td>
<td>Disease causing (prob: 1)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>p.C1374R</td>
<td>Disease causing (prob: 0.999)</td>
<td>Probably damaging (0.999)</td>
<td>Damaging (0)</td>
</tr>
<tr>
<td>p.E1555*</td>
<td>Disease causing (prob: 1)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>p.M1580Ifs*30</td>
<td>Disease causing (prob: 1)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>p.A1740S</td>
<td>Polymorphism (prob: 0.980)</td>
<td>Benign (0.134)</td>
<td>Tolerated (0.42)</td>
</tr>
<tr>
<td>p.S2017Tfs*9</td>
<td>Disease causing (prob: 1)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL FIGURE I. Real-time PCR of transient transfections

Real-time quantitative PCR of cells transiently transfected with mutagenized NOTCH1 cDNA constructs. All missense mutations tested demonstrate reduced NOTCH1 transcript level by comparison to cells transfected with a full-length wild-type construct (WT). Relative quantification of gene expression is calculated relative to the WT baseline value (set to 1) and normalized to GAPDH and ACTB. Graphs represent the mean of three independent experiments with error bars indicating SEM. **Key:** *p<0.01; †p<0.05