Congenital heart defects and pulmonary arterial hypertension
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A homozygous mutation in Smoothened, a member of the (SHH)-GLI pathway, is involved in human syndromic atrioventricular septal defect


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Submitted
ABSTRACT

Rationale: Studies in mice and zebrafish have strongly suggested that SHH/GLI signaling is involved in atrioventricular valve formation. Atrioventricular septal defect (AVSD) is a common feature of several syndromes associated with defects in the Sonic Hedgehog (SHH)/GLI pathway in humans, like Smith-Lemli-Opitz syndrome.

Objective: We aimed to find the genetic cause of a syndromic AVSD in a twin brother and sister with consanguineous parents, presenting with features suggesting Smith-Lemli-Opitz syndrome, which was excluded by DHCR7 mutation analysis. We focussed on SHH/GLI signaling.

Methods and Results: Homozygosity mapping in combination with exome sequencing was performed. A homozygous missense mutation c.1726C>T (p.R576W) was detected in SMO, a member of the SHH/GLI signaling pathway. Functional studies in fibroblasts of the patients showed normal expression but impaired translocation of the SMO protein to the cilia after stimulation with the SMO agonist purmorphamine. Moreover, a significant reduction of mRNA-expression of a SMO downstream gene, GLI1, was detected upon stimulation.

Conclusion: This is the first report of a SMO mutation in man, associated with human syndromic AVSD. Functional studies suggest that this mutation is causative and that SMO translocation to the cilia is a crucial step in SHH/GLI signalling.
INTRODUCTION
Atrioventricular septal defect (AVSD) is a congenital heart anomaly characterized by a defect in the lower part of the atrial septum, with AV valve defects and/or a ventricular septal defect. Complete AVSDs are characterized by a single AV valve, incomplete AVSDs are characterized by a left and a right AV valve. AVSDs can be surgically corrected, but patients often need life-long medical monitoring and may need several hospitalizations. The estimated birth prevalence of AVSD is 1.6-2.0/10,000. AVSDs can be isolated or syndromic, the latter meaning that the AVSD is accompanied by extra-cardiac anomalies. Most AVSDs (70%) are diagnosed in patients with Down's syndrome. However, AVSD is also a common feature of several syndromes associated with defects in the sonic hedgehog (SHH) pathway, like Smith-Lemli-Opitz syndrome (SLOS), Ellis van Creveld syndrome, and Mohr-Majewski syndrome. Overlapping features of these syndromes are heart defects, often being AVSD, polydactyly and/or syndactyly, a large fontanel, and several midline defects, like holoprosencephaly, pituitary abnormalities, neural tube defects, cleft palate, single incisor, and hypospadias.

We present the first report of a SMO mutation in man, in a brother and sister with an AVSD and with a phenotype resembling Smith-Lemli-Opitz syndrome.

SUBJECTS, MATERIAL AND METHODS
Patients
Dizygotic twins, a boy and girl, were referred to our hospital because of multiple congenital defects. The parents were first cousins, and had one healthy older child. The boy had a complete AVSD, a single atrioventricular (AV) valve, absent atrial septum (monoatrium) and coarctation of the aorta. The girl had a partial AVSD with two AV valves and insufficiency of the left AV valve. Both children had a very large fontanel, postaxial polydactyly of both hands and feet and almost complete syndactyly of the second and third toes of both feet; the boy also had a glandular hypospadias. Renal ultrasounds were normal. The boy died at the age of three months from severe pulmonary hypertension. The girl was operated on successfully for her AVSD and shows a normal psychomotor development at the age of 8 years. Karyotyping was normal and SLOS was excluded by DHCR7 gene analysis and 7-dehydrocholesterol analysis in fibroblasts. After signed consent, DNA was isolated from peripheral blood leukocytes and/or fibroblasts from the parents, the healthy sister, and the affected boy and girl using standard protocols.

Homozygosity mapping
Genome-wide genotyping with the Human Linkage-12 BeadChip® 6K SNP array (Illumina, San Diego, CA, USA) was performed in the affected twins, the parents, and unaffected sibling according to the manufacturer’s protocols. Data from the arrays were converted to genotypes using BeadStudio® data analysis software (Illumina). The genotype data were subjected to homozygosity mapping using Microsoft® Office Excel 2007 (Microsoft, Redmond, WA, USA) software by searching for homozygous
Chapter 5

stretches of five alleles or longer shared by the affected twins, and allowing 1% genotyping errors. The size of the homozygous stretches was calculated in cM, using Rutgers combined linkage-physical map of the Human Genome to locate the SNPs.  

Exome Sequencing and Mutation Detection

Three micrograms of DNA from each individual was used for exome sequencing. Exomes were enriched with the use of hybrid capture SureSelect Human All Exon V4 (Agilent Technologies, Amsterdam, the Netherlands), which targets most human exons (approximately 51 Mb). The captured fragments were sequenced on Illumina Hiseq2000 (Illumina). The sequence reads were aligned to the human genome build 19 (hg19) using the Burrows-Wheeler transform and NARWHAL pipeline. Subsequently, genetic variants were called using tools from the genome analysis toolkit (GATK). The resulting variant call format (VCF) files were processed with a custom variant annotation tool that determines the variant effects. Further filtering of known variants in the public databases, such as dbSNP134 and 1000 Genomes, was performed using the SeattleSeq (snp.gs.washington.edu/SeattleSeqAnnotation134/) exome variant server (evs.gs.washington.edu/EVS/) and the Genome of the Netherlands database (GoNL) (http://www.nlgenome.nl/). Variants with a minor allele frequency (MAF)>0.05 were excluded. As the pattern of inheritance suggested an autosomal recessive mode of inheritance, we focused on genes with homozygous or compound heterozygous variants. In silico prediction for the deleterious effect of candidate variants was performed by using Polyphen-2 and SIFT. Candidate variants were validated by Sanger sequencing in DNA from the twins, their unaffected sibling, their parents, and 144 healthy controls.

In situ hybridization

Digoxigenin-labeled probes for Smo were generated by subcloning mouse partial cDNA into a T-easy vector (Promega). Linearized plasmid DNA was transcribed into cRNA using T7 or SP6 (Promega) RNA polymerase in the presence of digoxigenin labeled dUTP (Roche) according to manufacturer’s instructions. In situ hybridization was essentially performed as previously described. Briefly, transverse 5 µm 4% paraformaldehyde-fixed paraffin-embedded sections through the thorax of an E13.5 mouse embryo were used. Sections were deparaffinized and rehydrated in H2O, incubated for 10 minutes in 1M HCl, digested with proteinase K for 20 minutes, refixed in 4% paraformaldehyde for 10 minutes and acetylated with acetic anhydride. Slides were then pre-hybridized for an hour in a mix of 2% Blocking Powder (Roche), 0.05% Chaps, 50% formamide, 5x SSC pH 4.5, 5 mM EDTA, 100µg ml-1 Heparin (Sigma) and 100µg ml-1 yeast RNA (Ambion). Subsequently slides were incubated for 72 hours at 68ºC with the digoxigenin labeled antisense cRNA probe. After incubation, slides were washed three times at 20 minutes at 65ºC in a stringency wash buffer containing 50% formamide and 2x SSC pH 4.5. Slides were then rinsed in TBS with 0.1% Tween-20, blocked with 0.5% Blocking Powder (Roche) in TBS-T and incubated overnight with sheep anti-digoxigenin alkaline phosphatase conjugated Fab fragments (Roche). Staining was developed with NBT/BCIP substrate.
(Sigma) over 48-72 hours.

**Fibroblast Cultures and SMO Expression (Western blotting)**

Fibroblasts from the affected twins and two healthy controls were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% of fetal calf serum (FCS) and penicillin (100 IU/ml)/streptomycin (100 µg/ml). Cells were incubated at 37°C and supplied with 5% of CO2. Cells were harvested and proteins were isolated using cell lysis buffer containing 150 mM NaCl, 20mM Tris-HCl pH 7.4, 1% Triton X-100, protease inhibitor, and Halt™ phosphatase cocktail inhibitor (Thermo Scientific). Forty micrograms of total proteins were separated in XT 412% Bis-Tris gradient gel (Bio-Rad) and transferred to nitrocellulose membrane, blocking was performed with 5% of skimmed milk in phosphate buffered saline (PBS) with 0.1% Tween-20 (PBST) at room temperature for one hour. The primary antibodies, rabbit polyclonal anti-SMO (Santa Cruz, 1:100) and mouse monoclonal anti-β-Actin (Sigma, 1:1000) were applied for overnight incubation at 4°C. After incubation with the respective secondary antibodies at room temperature for one hour, the membrane was scanned with the Odyssey™ infrared Imager (Li-COR Biosciences).

**Cilia, Basal Bodies and SMO Staining and Purmorphamine Treatment**

Fibroblasts (100,000) were seeded on a round 24 mm cover slip that was placed in one well of a 6-well plate in DMEM with 10% FCS. After 24 hours the medium was changed for DMEM containing 0.5% FCS. The next day, cells were fixed in ice cold methanol for 10 minutes at -20°C. Cells were rinsed three times in PBS before applying a blocking solution (50 mM Tris-HCl buffer pH 7.4, 0.9% NaCl, 0.25% gelatine, 0.5% Triton X-100) for 10 minutes at room temperature. To visualize the cillum, we performed double staining of anti-acetylated-tubulin (cilium: red) and anti-γ-tubulin (basal body: green) in the fibroblast cells. Primary antibodies, mouse monoclonal antibody anti-acetylated-tubulin (Sigma-Aldrich T7451, 1:8000) and rabbit monoclonal anti-γ-tubulin (Sigma-Aldrich T3320, 1:1000) were applied and incubated overnight at 4°C. Secondary antibodies antimouse CY™3-conjugated IgG (1:200) and anti-rabbit DyLight™488-conjugated IgG (1:300) were applied for one hour at room temperature. Cell nuclei were stained with 0.1 mg Hoechst/ml in PBS for 10 minutes at room temperature. Next, the cover slip was mounted with 8 µl Prolong Gold. We counted 100 basal bodies (y-tubulin positive cells) and the cilia (acetylated tubulin positive cells) per cover slip, in duplo, in the fibroblasts from the patients and from healthy controls. We grouped the cilia into normal-sized cilia (length: 3-6 µm), small cilia (length: <3 µm) and no cilia. To visualize the localization of SMO, we performed double staining of anti-SMO (red) and anti-γ-tubulin (basal body: green) in the fibroblast cells. Primary antibodies, mouse monoclonal antibody anti-SMO (Santa Cruz sc-166685, 1:400) and rabbit monoclonal anti-γ-tubulin (Sigma-Aldrich T3320, 1:1000) were applied and incubated overnight at 4°C. Secondary antibodies were applied as described above. For SMO activation, 24 hours before fixation, cells were treated with 2 or 20 µM of the SMO agonist purmorphamine (Calbiochem). This treatment mimicks activation by sonic hedgehog. We compared
the SMO localization between cells that were treated and untreated with purmorphamine. The images were captured with an AxioVision SE64 Rel. 4.8.3 fluorescence microscope, using AxioVision SE64 imaging software.

**Purmorphamine Treatment and Quantification of GLI1 Expression**

Fibroblasts (100,000) were seeded in a 6-well plate in DMEM with 10% FCS. The medium was changed the next day for DMEM containing 0.5% FCS and 20 μM purmorphamine (Calbiochem) was added. After 24 hours of incubation, cells were harvested and RNA was isolated using RNeasy Mini Kit (Qiagen). One microgram of RNA was used for cDNA synthesis using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. Seventy-eight nanograms of cDNA was used to quantify the GLI1 expression level by quantitative real-time (qRT) PCR Sybr Green, in triplo, on the 7300 Realtime PCR system (Applied Biostystems). The expression of the CLK2 housekeeping gene was used to normalize the GLI1 expression. The following primer sets were used for qRT-PCR of GLI1 and CLK2: GLI1-fw 5’-tccccatgactctgcccg-3’; GLI1-rv 5’-ccagcatgtccagctcaga-3’; CLK2-fw 5’-tcttagcaccttaggagagg-3’ and CLK2-rv 5’-tgatcttcagggcaactcg-3’. The normalized GLI1 expression of fibroblasts was compared to the normalized GLI1 expression of fibroblasts not treated with purmorphamine. Data were analyzed using the method described by Livak.18

**RESULTS**

**Homozygosity mapping**

Homozygosity mapping in the affected twins revealed a total of 66 shared homozygous stretches of at least five alleles, with an average size of 6.64 cM. Two of these stretches were substantially larger than the others: the largest region of homozygosity was located on chromosome 10, spanning 93 SNPs and 60.66 cM, from rs13005 (chr10: 13,685,766bp) to rs877783 (chr10: 13,685,766-chr10: 73,315,940bp), and containing 570 genes., and the second largest region of homozygosity was located on chromosome 7, spanning 79 SNPs and 50.25 cM, from rs758706 (chr7: 90,494,919bp) to rs10488598 (chr7: 90,494,919chr7: -136,587,843bp), and containing 531 genes.

**Exome sequencing in dizygotic twins with syndromic AVSD**

We performed exome sequencing on DNA samples from the affected twins, their parents and their unaffected sibling. A summary of the data is presented in the supplemental data (Table S1). After alignment to the human genome reference sequence (Hg19) and after variant calling using GATK software, we identified 88,148 – 100,878 SNPs and 6512 - 8076 small insertions and deletions (indels). The summary of the SNP- and indel-calling analysis is presented in the supplemental data (Tables S2 and S3).

An autosomal recessive pattern of inheritance was suspected because the parents were consanguineous. However, we analyzed compound heterozygous variants as well as homozygous variants. Several compound heterozygous variants were identified in the two affected sibs, but none
of these compound heterozygous variants were present in both. We identified 32 homozygous variants, present in both affected sibs. Only six variants were located either in coding sequences (CDS), 5'-utr, 3'-utr, or were affecting splice sites, or resulted in a premature stop (Table 1). Five of these homozygous variants were present in one of the two largest regions of homozygosity on chromosome 7 or 10. Four of these variants were not considered good candidates, because the homozygous mutation was also present in the unaffected sib (ZAN, ANKRD26, CXCL12, FRAT2). This left us with two variants: in SMO (NM_005631) and in JMJD1C (NM_032776), which were validated by Sanger sequencing. These variants were not present in the dbSNP134, 1000 Genomes, Exome Variant Server (EV5), or Genome of the Netherlands (GoNL) databases and were also not found in 144 healthy controls.

Table 1. Summary of homozygous variants, identical in the affected brother and sister

<table>
<thead>
<tr>
<th>Chr. Genomic Position</th>
<th>Gene</th>
<th>Ref. Alt.</th>
<th>II-2</th>
<th>II-3</th>
<th>I-1</th>
<th>I-2</th>
<th>Type</th>
<th>Effect</th>
<th>Exon</th>
<th>cDNA change</th>
<th>AA change</th>
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<tbody>
<tr>
<td>7 100348389</td>
<td>ZAN</td>
<td>T</td>
<td>A/A</td>
<td>A/A</td>
<td>A/A</td>
<td>T/A</td>
<td>CDS</td>
<td>missense</td>
<td>12</td>
<td>c.1391T&gt;A</td>
<td>M464K</td>
</tr>
<tr>
<td>7 128850879</td>
<td>SMO</td>
<td>C</td>
<td>T/T</td>
<td>T/T</td>
<td>C/T</td>
<td>C/T</td>
<td>CDS</td>
<td>missense</td>
<td>10</td>
<td>c.1726C&gt;T</td>
<td>R576W</td>
</tr>
<tr>
<td>10 27335297*</td>
<td>ANKRD26</td>
<td>T</td>
<td>A/A</td>
<td>A/A</td>
<td>A/A</td>
<td>T/A</td>
<td>CDS</td>
<td>missense</td>
<td>18</td>
<td>c.1970A&gt;T</td>
<td>D657V</td>
</tr>
<tr>
<td>10 44873197*</td>
<td>CXCL12</td>
<td>C</td>
<td>C/C</td>
<td>C/C</td>
<td>C/C</td>
<td>T/C</td>
<td>Stop codon</td>
<td>synonymous</td>
<td>4</td>
<td>c.423A&gt;G</td>
<td>=</td>
</tr>
<tr>
<td>10 64974069</td>
<td>JMJD1C</td>
<td>G</td>
<td>C/C</td>
<td>C/C</td>
<td>G/C</td>
<td>G/C</td>
<td>CDS</td>
<td>missense</td>
<td>8</td>
<td>c.1858C&gt;G</td>
<td>P620A</td>
</tr>
<tr>
<td>10 99094083*</td>
<td>FRAT2</td>
<td>C</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T</td>
<td>CDS</td>
<td>missense</td>
<td>1</td>
<td>c.247G&gt;A</td>
<td>A83T</td>
</tr>
</tbody>
</table>

Chr = chromosome; Ref = reference nucleotide; Alt = alternative nucleotide; II-2 = twin girl, II-3 = twin boy, I-1 = healthy sib, I-1 = father and I-2= mother; - AA = amino acid change
* These variants were introduced in dbSNP135 (http://www.ncbi.nlm.nih.gov/projects/SNP) with minor allele frequencies of 0.002 (ANKRD26 rs193178384), 0.002 (CXCL12 rs183966906), and 0.148 (FRAT2 rs181573119), respectively. & Not located in one of the two largest homozygous regions.

SMO transduces the SHH signal by forming a complex with EVC1 and EVC2. This complex is translocated into the cilium where it induces activation of the GLI family of transcription factors. Furthermore, the SMO variant is predicted to be probably damaging by the Polyphen-2 and deleterious by SIFT mutation prediction software programs. The JMJD1C variant, however, was predicted to be tolerated by SIFT and benign by Polyphen-2. JMJD1C is a gene that encodes for a nuclear protein and is thought to act as a histone demethylase. The protein is expressed ubiquitously, with high expression in the testis, but with a relatively low expression in the heart. The SMO variant was the most likely candidate, because of its function in the SHH/ GLI signaling pathway and the software prediction, so we focussed on this candidate. (Table 1).
**Smo expression in the embryonic (mouse) heart**

Embryonic day 13.5 wild type mouse embryos were analyzed for Smo expression in the developing heart using in situ hybridization (Figure 1). Lung tissue, trachea, and oesophagus were marked by high Smo expression. Endothelium and sub-endothelial mesenchyme of the developing tricuspid and mitral valves showed high expression of Smo.

![Figure 1. Smo expression in the embryonic (mouse) heart. Smo expression in the heart of E13.5 WT mouse embryo hybridized for Smo mRNA. Note high Smo expression in the developing tricuspid valve and mitral valve leaflets. L indicates lung tissue; B, bronchus; Oe, oesophagus; TV, tricuspid valve; MV, mitral valve; RV, right ventricle; LV, left ventricle. Scale bar represents 200 μm.](image)

**The fibroblasts of the affected twins have a comparable number of cilia to fibroblasts of healthy individuals**

To determine whether the SMO variant has an effect on the structure or formation of primary cilia, we stained and counted the numbers of cilia in fibroblasts of the patients (Figure 2, Table 2). We did not see a significant difference between the total numbers of cilia in fibroblasts of the affected twins and controls. The number of normal-sized cilia in fibroblasts of affected children were 40-60%, compared to 50-80% in the controls (Table 2). However, this difference is still within the normal range due to the large variance in the number of normal-sized cilia in healthy controls.20

**The SMO variant affects its translocation into the cilia needed for hedgehog signaling activation**

We hypothesized that the variant we had identified in this family results in a mutated SMO protein that might not bind to the EVC/EVC2 protein complex or to other proteins that are important for its translocation into the cilia, hence disturbing signal transduction. To validate this hypothesis we activated hedgehog signaling by adding purmorphamine to fibroblast cultures of both the patients
Figure 2. Cilia staining. Cilia staining using antibody anti-Acetylated-Tubulin (red: cilium) and γ-Tubulin (green: basal body). Graphic illustrating the visualisation of the basal bodies and the cilia in fibroblasts of healthy controls, used to count the number of normal and small-sized cilia in patients and controls (results in table 2).

Table 2. Cilia counts in patients and healthy controls. Cilia counts in the fibroblasts of the affected boy and girl compared to those in the healthy controls based on acetylated- and γ-tubulin double staining. Results of three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=20)</th>
<th>Affected girl</th>
<th>Affected boy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal-sized Cilia (3-6µm)</td>
<td>50 - 80%</td>
<td>44 - 58%</td>
<td>40 – 58%</td>
</tr>
<tr>
<td>Small-sized Cilia (&lt;3µm)</td>
<td>5 - 12%</td>
<td>11 - 15%</td>
<td>12.5 – 28%</td>
</tr>
</tbody>
</table>

and controls to determine whether the SMO protein trafficked properly into the cilium. In Figure 3A, the SMO translocation test is shown in a graph, illustrating that SMO antibodies stain cilia (axoneme) in fibroblasts only after purmorphamine or Shh stimulation. Figure 3B shows that in untreated control cells there was no cilium-like shape of SMO above the basal body (3B-I), while SMO was transported into the cilium when they were treated with 2 µM of purmorphamine, mimicking Shh stimulation (3B-II). In fibroblasts from the affected boy and girl there was no SMO signal (red) above the basal body (green) in cells treated with purmorphamine: in other words, there was no difference between the untreated (3B-III) and treated cells (3B-IV). This shows that the SMO protein failed to move into the cilium upon activation of hedgehog signaling.
Figure 3. SMO translocation test. A. Graphic illustrating the SMO translocation test: Smo antibodies stain cilia (axoneme) in fibroblasts only after purmorphamine or Shh stimulation. B. Anti-Smo (red) and anti-γ-Tubulin (green: basal body) double staining in fibroblast cells untreated with purmorphamine (panel I and III) and treated with 2 µM purmorphamine (panel II and IV): panels I-II healthy control fibroblasts, panels III and IV affected boy (results of the affected girl are similar, not shown). In control fibroblasts the cilium colors red after stimulation with purmorphamine, while in the affected children, no anti-SMO coloring of the cilium appeared after purmorphamine stimulation.
We also compared the SMO expression level in fibroblasts from the affected twins with healthy controls by a Western blot using an anti-SMO antibody. We did not see a significant difference in the SMO expression level between these fibroblasts (Figure 4). These data strengthen the idea that the SMO protein is expressed in the normal amount in the affected twins, but its mutation affects the transfer of the protein to the cilia and subsequently disturbs the GLI-mediated signaling pathway.

**Figure 4. Western blot showing SMO expression.** Western blot using antibody anti-SMO protein and β-actin on the protein lysate isolated from fibroblasts of: (1-2) Healthy controls, (3) affected boy and (4) affected girl. M : Marker. There is no difference in the level of SMO expression in the fibroblasts of the affected children and healthy controls.

**Activation of hedgehog signaling in fibroblasts from the affected twins did not increase the level of GLI1 mRNA**

To prove that the SHH/GLI signaling is affected, we also checked the expression level of a SHH/GLI signaling target gene. In this experiment, we performed qRT-PCR for GLI1 using RNA isolated from fibroblasts of the affected twins and controls. Cells were treated or not treated with 20 µM of purmorphamine to stimulate hedgehog signaling.

As shown in Figure 5, the level of GLI1 expression in control fibroblasts treated with purmorphamine is between 8 to 18-fold higher compared to that of untreated fibroblasts. However, using RNA from fibroblasts of the affected twins, no difference in GLI1 expression was detected between treated and untreated fibroblasts. These results show that the SHH/GLI signaling was hampered in both the affected children.

**DISCUSSION**

We report the first homozygous missense mutation in the SMO gene associated with syndromic AVSD in humans. We found this homozygous SMO mutation by homozygosity mapping in combination with exome sequencing of samples from a pair of dizygotic twins with syndromic (non-Down) AVSD, their healthy sibling, and their consanguineous parents.

SMO encodes for the transmembrane protein SMO, member of the G-coupled receptor family of proteins. It is active in cells with a primary cilium, located on the ciliary membrane, and is a key protein in the SHH/GLI signaling cascade. Activation of SHH signaling is triggered by binding of the extracellular SHH protein to PTCH1, relieving its inhibitory effect on SMO. SMO can then bind to the EVC1/EVC2 protein complex in the base of the cilium resulting in translocation of SMO to the tip of the cilium and recruitment of GLI. Subsequently, GLI translocates into the nucleus and induces
transcription of SHH target genes.\textsuperscript{21, 25-27} Numerous developmental defects are known to be caused by disruption of the SHH/GLI signaling cascade, including congenital heart defects.\textsuperscript{22, 23} In addition to the homozygous SMO mutation, we also detected a homozygous variant in JMJD1C, a gene coding for a histone demethylase and implicated in the reactivation of silenced genes in undifferentiated embryonic stem cells, pancreatic islet cells, and gastric cancer.\textsuperscript{28} The data in the literature and from the software prediction programs do not indicate that this variant plays a role in the pathogenesis of the congenital anomalies of these sibs.

**Why would this homozygous SMO mutation be pathogenic?**

The homozygous SMO mutation in our patients is a missense mutation (R576W). SIFT and Polyphen-2 predict the mutation to be damaging. Although this mutation results in normal protein levels of SMO, it hampers the translocation of the mutant protein to the primary cilium and inhibits SHH-GLI signal transduction. The most important domains of the SMO protein are the heptahelical domain, which is involved in ligand binding, and the C-terminal cytoplasmatic tail, which is involved in intracellular trafficking. Mutations in the first domain inactivate the protein\textsuperscript{29}, mutations in the second domain are thought to impair intracellular trafficking of SMO to the cilium by inhibiting phosphorylation of SMO.\textsuperscript{30} The mutation in exon 10 that we identified in our patients is localized in the latter domain consisting of an arginine cluster (between amino acid 500 and 693) which is thought to be involved.

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**Figure 5. GLI1 expression.** The fold change of the GLI1 expression level by qRT-PCR in the fibroblasts without (-) and with (+) stimulation of the Hedgehog pathway with 20µM of purmorphamine of three healthy individuals (positive controls 1-3), a patient with Meckel Syndrome (negative control), and the affected girl and boy. The patterns of GLI1 expression fold change with and without stimulation were similar from two independent experiments in triplo, the value of the fold change of untreated and treated control fibroblasts varied between 7-18 fold. No changes in GLI1 expression were observed after purmorphamine stimulation in the fibroblasts of the affected brother and sister with the homozygous SMO variant. * P ≤ 0.0005
in SMO trafficking. Our experiments confirm that the intracellular trafficking of SMO is hampered by this mutation.

Why would SMO-mutations cause a SLOS-like syndrome?
SLOS is a disorder of the endogenous cholesterol synthesis, resulting in a low total cholesterol level and accumulation of oxysterols, leading to altered hedgehog signaling. Hedgehog signaling has been shown to be compromised in a SLOS mouse model and in cholesterol-depleted cells. Patients with SLOS have multiple congenital anomalies, including mental retardation, postaxial polydactyly and syndactyly of the second and third toes, hypospadias, and congenital heart anomalies, almost half of which are AVSD. Overlapping features are reported in many hedgehog-associated syndromes. Humans with a SHH mutation either die in utero or are born with holoprosencephaly and often have an AVSD. Patients with a GLI2 mutation have holoprosencephaly, pituitary dysfunction, polydactyly, and orofacial clefting. We recently discovered that two unrelated patients with panhypopituitarism, polydactyly and AVSD had truncating GLI2 mutations (c.3502C>T; p.Gln1168X and c.1908dupC), suggesting again that the SHH/GLI pathway is crucial for human atrioventricular septation (data not published). Other syndromes known to be caused by disruption of the SHH/GLI pathway, also show considerable overlap with the clinical features of our patients. Ellis van Creveld and Mohr-Majewski syndromes are skeletal malformation syndromes with polydactyly, often accompanied by AVSD, and are also related to the sonic hedgehog pathway, with mutations reported in EVC and EVC2, and in TCTN3, respectively. Apart from the AVSD in these syndromes, the polydactyly, syndactyly, large fontanel and hypospadias seen in our patients, are common features of these syndromes, making it plausible that this one mutation is responsible for the entire phenotype. Noteworthy is that the surviving female sib has a normal development, while children with SLOS have variable intellectual deficits. This suggests that the SMO mutations found in these sibs do not affect cognition.

Is there evidence that a SMO mutation can cause AVSD?
Little is known on human SMO mutations and the only diseases reported in patients with somatic SMO mutations are those with medulloblastomas or basal cell carcinomas. The association with congenital heart defects in man has not been reported previously, although studies in mice and zebrafish already suggested such association. Smoothened knockout mice arrest at somite stage, have a linear heart tube, cyclopia and an open gut, and have a complete inactivation of all hedgehog signaling pathways. Deletion of Smo from the developing dorsal mesenchymal protrusion (DMP) in the heart resulted in AVSDs in mice. Furthermore, conditional Smo mutants exposed to the Smo inhibitor cyclopamine showed decreased dorsal mesocardium migration, also resulting in AVSD. Mice with a targeted deletion or mutation of the Pkd1, Ift88, cbs or Kif3a gene (genes all coding for proteins involved in cilia function and SHH/GLI signaling), display AVSD or hypoplasia of the endocardial cushions.
Smo in zebrafish resulted in a reduction in endocardial progenitors. 29

**How does impaired translocation of SMO cause AVSD?**

The SHH/GLI signaling pathway plays a major role in the functioning of primary cilia, which is a signaling organelle for many pathways and is present in various non-dividing cell types and tissues. 23, 47, 48 In the heart, primary cilia are present in cells in morphogenetically active areas, such as the endocardium, pericardium 49, the epicardium, parts of the sinus venosus myocardium, the outflow tract, and endocardial cushions. 33, 44, 50 SHH/GLI signaling is involved in AV cushion formation through several mechanisms. Firstly, SHH/GLI signaling plays a role in the survival of the neural crest cells, which contribute to the endocardial cushions. 23, 51 Secondly, SHH/GLI signaling is involved in a process called endocardial-to-mesenchymal transition (endoMT) and dorsal mesenchymal protrusion (DMP). 41, 42 EndoMT stimulates growth and maturation of the AV cushions that later form the AV valves, and relies on the dynamic regulation of endothelial primary cilia 52-54; DMP is involved in closure of the atrioventricular septum. 42, 55

Although we showed that the translocation of SMO to the primary cilia in fibroblasts was impaired, the exact mechanism by which downstream SHH signaling was impaired during atrioventricular cushion formation is still unclear. In addition to the role of the SMO mutation in a reduced transcriptional response of GLI, the mutation may also affect non-canonical SHH signaling. This SMO-mediated alternative SHH pathway, which does not involve activation of GLI transcription factors, induces cytoskeletal rearrangements and cell migration. 56, 57 Abnormal localization of SMO may enhance chemotactic responsiveness. 58 This pathway may also have contributed to the AVSDs in our patients. However, the GLI2 mutations that we found in two patients with AVSD favour a GLI-mediated pathway. Future studies could elucidate how impaired translocation of SMO in our patients led to developmental anomalies. An impaired binding of SMO to EVC2, a protein that is essential for trafficking of SMO to the cilium, is one of the hypotheses.

In conclusion, we report the first functional SMO mutation found in humans associated with disturbed SHH/GLI signaling and syndromic AVSD. Our data provide evidence that trafficking of the receptor SMO to the cilium is the crucial step for downstream SHH signaling.
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Homozygous SMO mutation in syndromic AVSD


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