Two Novel Mutations in SALL1 Cause Townes-Brocks-Like Syndrome: New Findings from an Old Case

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ABSTRACT

Background: All previous genetic testing has failed to identify the genetic cause of syndrome affecting this family for nearly 20 years. The advent of massively parallel next-generation sequencing technologies has provided an opportunity to affordably screen exomes to establish the genetic basis of disease. The utility of whole exome sequencing to identify causative variants of Mendelian disorders has been clearly demonstrated in the research arena. In this report, we describe a family with unique clinical features, including Hirschsprung disease, with a suspected genetic basis.

Methods: We employed whole exome sequencing to this case to identify causative mutations.

Results: We identified two novel compound heterozygous variants, following an autosomal recessive mode of inheritance, in the candidate gene SALL1, a gene known to cause Townes-Brocks syndrome (TBS) and central nervous system TBS (CNS-TBS). The pathogenicity of the two variants was supported by co-segregation, low frequency, location in mutation hotspot, pathogenicity program predictions, phenotype similarity, and immunohistochemical staining. As previously reported, we observed intrafamilial phenotypic variability among the affected individuals and they may represent an expansion of TBS, CNS-TBS or a new CNS-TBS-like syndrome by observing atypical features in affected individuals including mental retardation, developmental delay, tracheal anomalies, Hirschsprung disease and cleft lip and palate.

Conclusion: Solving this case brought a new view of the genetics of TBS and its relationship with SALL1. Another lesson learned is that advanced technologies have a profound impact on old, unsolved cases such as the one presented here. Thus, we further demonstrated the utility of exome sequencing in the research arena.

Keywords
Exome, Sequencing, Mutation, Syndrome, Townes, Brocks.

Introduction
There are a number of medical genetic cases with striking phenotype and of suspected genetic cause; however, their genetic etiology remained elusive due to the limitations of clinical diagnostic technology. Next generation sequencing (NGS) has demonstrated its power by screening the whole exome at a lower cost with sufficient coverage. The utility of whole exome sequencing...
SALL1 sonication, ligated to Illumina multiplexing paired-end adapters, Genomic DNA samples were fragmented with the use of Whole exome sequencing and (E) Sanger sequencing confirmation of the substitution and deletion shows heterozygous for the pathogenic variants in this small family. (A) Pedigree of family. II-2, II-3 and II-4 are compound Figure 1: A Caucasian/Hispanic family without consanguinity, consisting of unaffected parents and 3 of 4 children with a number of anomalies, namely, heart defects, tracheal anomalies, preaxial polydactyly, Hirschsprung disease, and development delay (language and motor), was examined over a decade ago [7]. This case was published as a new syndrome due to the unusual clinical characteristics, but the genetic etiology remained unknown even after a number of molecular mutation studies [7]. In this report, we employed WES in an effort to identify the genetic cause of this syndrome and we describe two novel heterozygous mutations in SALL1, a gene known to cause Townes-Brocks syndrome (TBS) and central nervous system TBS (CNS-TBS).

**Methods**

**Samples and approval**

Written informed consent was obtained from the parents/guardians of the under 16 participants for the publication of this case report, including consent to publish the identifiable images and this study was approved by the University of California Irvine Institutional Review Board (HS#: 2007-5805). DNA from the proband, siblings and parents were extracted from peripheral blood. In addition, a skin sample was collected from the proband (Figure 1, II-2).

![Figure 1: SALL1 pathogenic variants appear to segregate with disease in this small family. (A) Pedigree of family. II-2, II-3 and II-4 are compound heterozygous for the pathogenic variants in SALL1. (B) and (C) NGS data shows SALL1 substitution and deletion in the affected individuals. (D) and (E) Sanger sequencing confirmation of the substitution and deletion in affected patients.](Image)

**Whole exome sequencing**

Genomic DNA samples were fragmented with the use of sonication, ligated to Illumina multiplexing paired-end adapters, amplified by means of a polymerase-chain-reaction assay with the use of primers with sequencing barcodes (indexes), and hybridized to biotin-labeled V2, a solution-based exome capture reagent (NimbleGen). Hybridization was achieved at 47°C for 64 to 72 hours. After washing and reamplification, paired-end sequencing (100 bp) was performed on the Illumina HiSeq 2000 platform to provide a mean sequence coverage of more than 70×, with more than 98% of the target bases having at least 10× coverage.

**Data annotation and interpretation**

Before interpretation, the data were analyzed and annotated by means of a pipeline that was developed in-house. Briefly, the output data from the HiSeq 2000 were converted from a bcl file to a FastQ file by means of Illumina Consensus Assessment of Sequence and Variation software and mapped to the reference haploid human-genome sequence (hg19) with the use of the NexGENe 2.2.3 program. Variant calls, which differed from the reference sequence, were obtained with NexGENe 2.2.3. Variant prioritization was based on inheritance model, allele frequency, pathogenicity program predictions and mutation database searches. Specifically, to focus on Mendelian variants, inheritance modeling was performed in NexGENe. Alamut HT 1.1.8 and in-house scripts annotated the variants. Variants in this database with a minor allele frequency of less than 1% according to exome sequencing project database (http://evs.gs.washington.edu/EVS), an in-house unaffected control database of less than 5% and +/- 5 bp of the exon/intron boundary were retained. Furthermore, prioritization was based on the employment of phenomizer, a phenotype-genotype tool, which associated the proband’s clinical features to a gene list. In addition, damaging mutations were examined by focusing on frameshift, start loss and nonsense changes as well as missense changes with pathogenic scores as predicted by SIFT, POLYPHEN-2 and Grantham scores. Moreover, variants reported in the Human Gene Mutation Database were also prioritized.

**Sanger sequencing**

Post-filtering, promising candidate gene variants were confirmed by Sanger sequencing (Figure 1). Primers were designed to amplify each exon containing the variants in SALL1. Samples were prepared by fluorescence sequencing on the ABI 3730XL DNA analyzer with BigDye Terminator chemistry and the BigDye XTerminator purification kit (Applied Biosystems).

**Immunohistochemistry**

Using a BenchMark Ultra staining module, anti-SALL1 antibody (Abcam, ab31526) was applied to cryostat sections of skin from the patient and control unaffected human skin (age-matched) at a dilution of 1:200, incubated with horseradish peroxidase conjugated with anti-human IgG, and diaminobenzidine for 32 minutes at 37 oC, and counterstained with hematoxalin.

**Results and Discussion**

SALL1 encodes a protein that contains multiple distinct DNA-binding zinc finger domains and alanine- and glutamine-rich domains that are commonly found in transcription factors [8]. SALL1 is extensively involved in the development of heart, limbs,
and brain and other organ systems [9].

The \textit{SALL1} gene causes TBS, an autosomal dominant condition, and more recently shown to be inherited in an autosomal recessive manner as CNS-TBS, first described in 1972 by Townes and others [10-13]. The major clinical features of autosomal dominant TBS include gastrointestinal anomalies (anal stenosis or imperforate anus), dysplastic ears (overfolded superior helices, microtia), and thumb malformations (preaxial polydactyly, triphalangeal thumbs, hypoplastic thumbs) [17; OMIM #107480]. Minor features of TBS include sensorineural hearing loss and/or conductive hearing impairment, foot malformations (fusion of metatarsals, short metatarsals, absent/hypoplastic third toe, fifth toe clinodactyly or 3-4 toe syndactyly), renal anomalies with or without renal malformations (hypoplastic kidneys, multicystic kidneys or dysplastic kidneys), genitourinary malformations, congenital heart defects (tetralogy of fallot or ventricular septal defect) and mental retardation (rarely noted).

Atypical clinical features that have been observed were radius hypoplasia on clinical examination or radiographs and cleft lip/palate. Consistent with autosomal dominant TBS, the anomalies in noted in patient II-2 were telecanthus with an inner canthal distance of 3.25 cm (95th percentile) (Figure 2A), duplication of the great toes, postaxial polydactyly (Figure 2B), bilateral complete cleft lip and palate, developmental delay, particularly expressive language, and cardiac defects (Table 1, Figure 2C). Moreover, consistent with TBS patient II-4 (Figure 3A) had duplication of the great toes (Figure 3B), cardiac defects including atrial septal, cleft mitral valve and anomalous venous and developmental delay (expressive language and motor delay; Figure 3C, Table 1). Additional findings in patients II-2 and II-4 were tracheal anomalies and Hirschsprung disease, based on histological studies.

Consistently, gastrointestinal features have also been reported in TBS including chronic constipation and gastroesophageal reflux. Moreover, activation and loss of function in the same gene cause different phenotypic outcomes such as in the RET proto-oncogene in which loss of function mutations are associated with familial Hirschsprung disease [15,16]. There have been multiple articles with Hirschsprung disease with other congenital associations [17-22]. The clinical manifestations of TBS are highly variable and show strong inter- and intrafamilial variability [14]. A family with \textit{SALL1} nonsense mutation presented a phenotype lacking most of the typical malformations of TBS and also demonstrated intrafamilial variability [23]. Patient III-3 only had craniofacial and tracheal findings (Table 1).

Compared to autosomal dominant TBS, Vodopiutz et al. 2013 reported a homozygous \textit{SALL1} mutation, c.3160C > T (p.R1054*) in two female siblings with central nervous system Townes-Brocks syndrome (CNS-TBS). The common clinical features between CNS-TBS and patients II-2 and II-4 were cardiac defects, limb anomalies and mental retardation (Table 1). Interestingly, CNS-TBS and TBS had additional overlapping features, namely, renal anomalies, imperforate anus and hearing loss. Therefore, we demonstrated similarities in clinical features between our three patients, CNS-TBS, TBS and other reports with comparable phenotypes (Table 1). Whether our patients have TBS, TBS spectrum features, CNS-TBS or a new CNS-TBS-like syndrome requires further study. It is clear that our patients have a TBS-like disorder with biallelic mutations in \textit{SALL1}.

A study reported a heterozygous frameshift mutation in \textit{SALL1} (c.995delC) with a relatively severe form of TBS [24]. The level of mutant \textit{SALL1} cDNA from fibroblasts from a patient (c.995delC) with typical TBS was equal to wild-type even with cycloheximide, indicating nonsense-mediated decay (NMD) escape. Furniss et al. demonstrated that the phenotype of the c.995delC patient was caused by a truncated \textit{SALL1} protein acting in a dominant-negative manner. By contrast, another patient with c.3414_3415delAT (C1139WfsX14) presented with an isolated unilateral preaxial polydactyly and has not been reported previously, is milder than
Table 1: Phenotypic comparison of patients with TBS, CNS-TBS and other cases with Hirschsprung with congenital anomalies. Reprinted with permission from publisher [7], but was content modified.

<table>
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<th>TBS</th>
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<th>Other cases with Hirschsprung</th>
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<td>TOF, ASD, VSD</td>
<td>ASD, VSD, PDA, cleft MV, hypoplastic aorta, AVR</td>
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<td>Limb anomalies</td>
<td>broad thumb, bifid thumbs, preaxial polydactyly</td>
<td>bilateral tripalangeal thumbs, extra preaxial digit left hand, incomplete preaxial polydactyly, bilateral cutaneous 1,2 and 3,4 toe syndactyly</td>
<td>duplication of bilateral great toes and right 4th toe</td>
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<td>Tracheal anomalies</td>
<td>-</td>
<td>short epiglottis, short aryepiglottic fold</td>
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<tr>
<td>MR or DD</td>
<td>+</td>
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<td>(oligo-hydramnios)</td>
<td>+</td>
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<td>Hearing loss</td>
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<td>NA</td>
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<td>Renal anomalies</td>
<td>+</td>
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<td>CNS anomalies</td>
<td>+</td>
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<td>Addition features</td>
<td>radius hypoplasia, genitourinary malformations, dysplastic ears, imperforate anus or anal stenosis</td>
<td>pregnancy terminated</td>
<td>imperforate anus, cortical blindness, ear malformation, hyptonia</td>
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either patients with the same mutation reported by Botzenhart et al. or than the mild phenotype associated with heterozygous SALL1 deletions [24-26]. As expected for NMD, the c.3414_3415delAT mutant mRNA was present at only ~40% of the level of the wild-type allele, but this was fully restored after cycloheximide treatment. In SALL1, the c.995delC mutant allele causing TBS was unexpectedly resistant to NMD, whereas the c.3414_3415delAT mutation causing a much milder phenotype was susceptible to NMD. Another study applied quantitative real time PCR to detect and map SALL1 deletions in 240 patients with the clinical diagnosis of TBS, who were negative for SALL1 mutations [25]. Deletions were found in three families; two brothers (inherited, one family) and two sporadic cases. In all affected people, the TBS phenotype is rather mild as compared to the phenotype resulting from point mutations. These results confirm that SALL1 haploinsufficiency is sufficient to cause a mild TBS phenotype but not sufficient to cause the severe, classical form.

In contrast to heterozygous SALL1 mutations that have been associated with dominantly inherited features, Vodopiutz et al. 2013 reported a homozygous SALL1 mutation, c.3160C > T (p.R1054*) in two female siblings with CNS-TBS of a consanguineous pedigree after homoygosity mapping and candidate gene sequencing [20], (Table 1). The mutant SALL1 transcript was shown to undergo NMD and was present at 43% of the wildtype transcript level in the fibroblasts of a healthy carrier. Thus, Vodopiutz et al. 2013 demonstrated an allelic recessive SALL1-related CNS-TBS [27]. Consistent with this report, our findings
demonstrated an autosomal recessive mode of inheritance with two novel pathogenic variants supported by co-segregation, low frequency, location in mutation hotspot, pathogenicity program predictions, phenotype similarity, and immunohistochemical staining, in SALL1 (Figures 1, 4 and 5). Eleven and 1 genes were ruled out from the compound heterozygous and homozygous models, respectively, based on weak phenotypic overlap and variant classification of likely benign or benign (Supplementary Table 1). Variants in SALL1 appeared to co-segregate with the clinical phenotype in the family, even though it is a small family (Figure 1A). Both variants are very rare and are unlikely to co-occur/co-segregate by chance without phenotypic consequences. The missense variant was reported to have a low frequency of 0.002 (ExAC MAF: Total=0.00145, European (Non-Finnish) = 0.002188, Latino=0.0008639, South Asian=0.0009085, European (Finnish)= 0.0003024, African=9.61X10^-5, Other=0.002203, East Asian=0) and 0.00145 (ESP European American) in the exome sequencing project (ESP) and exome aggregation consortium (ExAC) databases, respectively. The amino acid at position 1265 is highly conserved across species (Figure 4C). Variant interpretation/classification uses the principle of conservation as one of the evidence points that supports the pathogenicity of variants. This principle has been adopted by the clinical molecular geneticists and the American College of Medical Genetics as stated in their interpretation guidelines [28].

In contrast, the deletion was not reported in the ESP database, but has a low frequency in ExAC (MAF: Total=0.003042, European (Non-Finnish) = 0.004399, Latino=0.004291, South Asian=0, European (Finnish)= 0.001694, African=0.001032, Other=0.004902, East Asian=0). Additionally, internal unaffected exome controls (n = 230) were examined and none had this in-frame deletion. Also, other deletions have been reported in the surrounding vicinity showing the potential importance of this region [26,29]. Based on these frequencies one can postulate that the true prevalence of TBS is much lower than reported. The mutations were inherited from unaffected parents, thus, supports the proposed autosomal recessive mode of inheritance.

Immunohistochemical staining with anti-SALL1 showed a strong reaction in our patient’s perivascular stromal cells; thus, demonstrating that mutations affect the localization of the SALL1 protein in vivo (Figure 5). This type of general deposition suggests the involvement of many organs in the affected family members. Similarly, manifestations of systemic light chain deposition have been reported in multiple individuals and include disease development in multiple organ systems such as hepatic, neurologic, gastrointestinal, cardiac and endocrine [30]. Clinical and pathologic correlations suggest that the retention and tissue deposition of light chains produced the organ dysfunction. In contrast to the reported loss of function mutations due to nonsense-mediated decay [27], the in-frame deletion and missense pathogenic variants may be activating or loss of function changes because the protein amount was not reduced and mislocalized (Figure 5).

Because of the clinical similarity to TBS, we examined the exome data for variants in GLI3 and other related genes but no mutations were detected. However, there are limitations of exome sequencing analysis. Even though the data strongly supports the pathogenicity of the aforementioned pathogenic variants, it is possible that other undetermined genetic changes undetected by exome sequencing, namely, non-coding variants, methylation and translocations, may contribute to this complex phenotype; however, further studies need to be performed in the future to assess their involvement.

**Conclusion**

In summary, clinical information and NGS data support a diagnosis of a TBS-like disorder for the affected individuals of this family. We report two novel SALL1 mutations in patients with clinical features that uniquely include tracheal and neurological findings. Whether these biallelic mutation was a clinical spectrum of TBS or a new CNS-TBS syndrome remains to be further studied. In this family, SALL1 mutations followed an autosomal recessive mode of inheritance. This study emphasizes the necessity to retest DNA samples from old cases with negative results with the latest genetic technology.

**Availability of data and Materials**

The dataset supporting the conclusions of this article is included within the article.
References
familial cases and refinement of the SALL1 hot spot region. Hum Mutat. 2007; 28: 204-205.


