

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

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Received: 04 January 2019; **Accepted:** 29 January 2019

Citation: Guoli Sun, Kevin E. Bove, Fanggeng Zou, et al. Two Novel Mutations in *SALL1* Cause Townes-Brocks-Like Syndrome: New Findings from an Old Case. *Genet Mol Med.* 2019; 1(1): 1-7.

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

(WES) to identify variants causative of Mendelian disorders has been clearly demonstrated in identifying novel candidate genes for Miller syndrome and other disorders. A combination of NGS and computational biology offers a great chance to pursue those fascinating, unsolved medical genetic cases [1-6].

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).

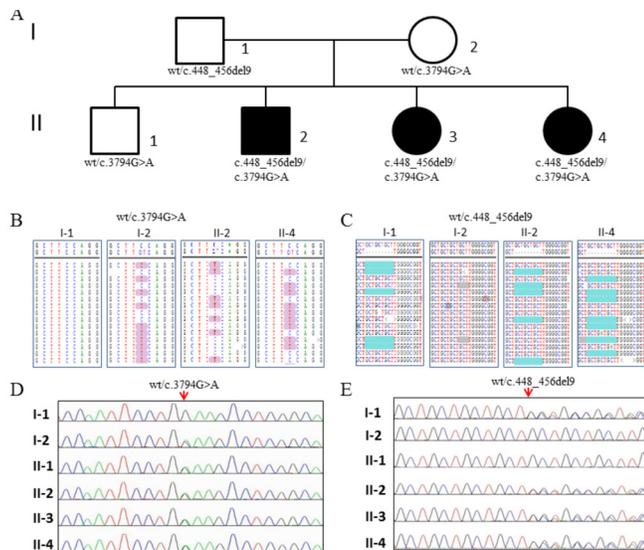


Figure 1: *SALL1* pathogenic variants appear to segregate with disease in this small family. (A) Pedigree of family. II-2, II-3 and II-4 are compound heterozygous for the pathogenic variants in *SALL1*. (B) and (C) NGS data shows *SALL1* substitution and deletion in the affected individuals. (D) and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

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 NextGENe 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 NextGENe. 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 hematoxylin.

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 *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.

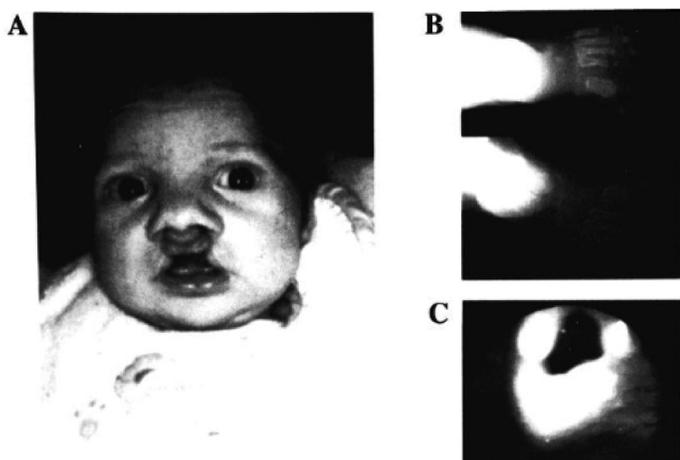


Figure 2: (A) Case 1 at age 2 ½ months, showing bilateral cleft lips and cleft palates. (B) A foot X-ray shows bilateral duplication of the halluces and duplication of the right 4th toe. (C) Direct laryngoscopy shows short aryepiglottic fold. Reprinted with permission from publisher (7).

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 *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).

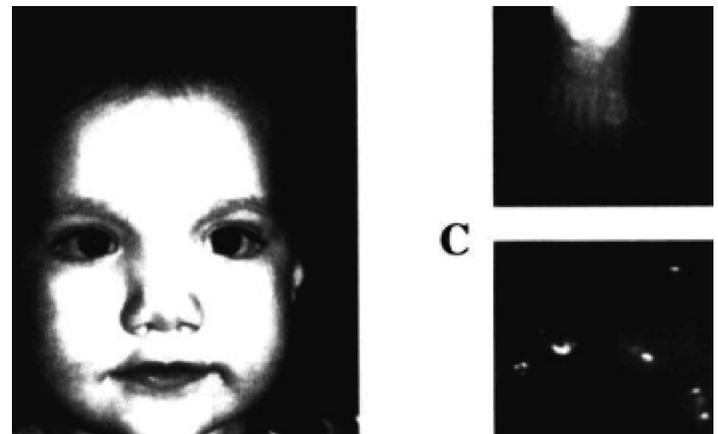


Figure 3: Case 2 at age of 2 ½ year. (B) A foot X-rays shows bilateral duplication of the great toes, but the 4th right toe is normal. (C) Direct laryngoscopy shows a bifid epiglottis and an unusual central mass of the epiglottis between separated epiglottis. Reprinted with permission from publisher (7).

Compared to autosomal dominant TBS, Vodopiutz et al. 2013 reported a homozygous *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 *SALL1*.

A study reported a heterozygous frameshift mutation in *SALL1* (c.995delC) with a relatively severe form of TBS [24]. The level of mutant *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 *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

	Townes-Brooks syndrome (AD)	central nervous system Townes-Brooks syndrome Vodopiutz et al.	central nervous system Townes-Brooks syndrome Vodopiutz et al. Patient 2	This study Patient II-2	This study Patient II-3	This study Patient II-4	Nowaczyk et al.	Lawrence et al.	McPherson et al.	Santos et al.	Pierpont et al.	Jespers et al.
Hirschsprung	-	-	-	+	-	+	+	+	malrotation	+	+	+
Cleft Palate	+	-	-	+	-	-	-	-	+	-	+	-
Cleft Lip	+	-	-	+	-	-	-	-	+	-	+	-
Cardiac defects	VSD, TOF	TOF	TOF, ASD, VSD	ASD, VSD, PDA cleft MV, hypoplastic aorta, AVR	-	ASD, VSD, AVR	ASD	VSD	hypoplastic left heart, DORV, PS	-	-	ASD, aneurysm, PDA
Limb anomalies	broad thumb, bifid thumbs, preaxial polydactyly	bilateral triphalangeal thumbs, extra preaxial digit left hand, incomplete preaxial polydactyly, bilateral cutaneous 1,2 and 3,4 toe syndactyly	bilateral triphalangeal thumbs, extra preaxial digit left hand, incomplete preaxial polydactyly	duplication of bilateral great toes and right 4th toe	-	duplication of bilateral great toes	postaxial polydactyly of hands	postaxial polydactyly of hands	bifid thumbs	preaxial polydactyly	-	-
Tracheal anomalies	-	-	-	short epiglottis, short aryepiglottic fold	bifid uvula	bifid epiglottis and an unusual epiglottic mass	-	-	-	-	-	laryngeal pharyngeal hypoplasia
MR or DD	+	+										
(oligo-hydramnios)	+	+	+	+					+	+		
Hearing loss	+	NA	+	-	-	-				+	-	-
Renal anomalies	+	+	+	-	-	-						
CNS anomalies	-	+	+	-	-	-				+		
Addition features	radius hypoplasia, genitourinary malformations, dysplastic ears, imperforate anus or anal stenosis	pregnancy terminated	imperforate anus, cortical blindness, ear malformation, hyptonia		Macrocephaly/midface hypoplasia							

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.

ASD: Atrial Septal Defect; VSD: Ventricular Septal Defect; MV: Mitral Valve; AVR: Anomalous Venous Return; MR: Mental Retardation; DD: Developmental Delay; CNS: Central Nervous System; DORV: Double Outlet of the Right Ventricle; PS: Pulmonic Stenosis; PDA: Patent Ductus Arteriosus; TOF: Tetralogy of Fallot; ?, Clinical features are not available.

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 homozygosity 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

Abbreviations

NGS: Next generation sequencing; WES: Whole Exome Sequencing; TBS: Townes-Brocks Syndrome; CCHMC: Cincinnati Children's Hospital Medical Center (CCHMC); ESP: Exome Sequencing Project; ExAC: Exome Aggregation Consortium; NMD: Nonsense-Mediated Decay; CNS-TBS: Central Nervous System Townes-Brocks Syndrome.

Acknowledgement

Research in the Huang Laboratory is supported by the Cincinnati Children's Hospital Research Foundation and by NEI 1R01EY018876.

Author Contributions

Conceived and designed the experiments: TH, KZ, CAV; Patient recruitment and clinical data collection: TH, JBM; Analyzed the data: GS, FZ, AM, AH, JD; Performed the experiments: GS; Contributed reagents/materials and analysis tools: TH, KZ, JBM, CAV; Contributed to writing: GS, TH, KZ, CAV. All authors read and approved the final manuscript.

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