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Original research

Molecular diagnosis, clinical evaluation and phenotypic spectrum of Townes-Brocks syndrome: insights from a large Chinese hearing loss cohort

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ABSTRACT

Background Townes-Brocks syndrome (TBS) is a rare genetic disorder characterised by multiple malformations. Due to its phenotypic heterogeneity and rarity, diagnosis and recognition of TBS can be challenging and there has been a lack of investigation of patients with atypical TBS in large cohorts and delineation of their phenotypic characteristics.

Methods We screened *SALL1* and *DACT1* variants using next-generation sequencing in the China Deafness Genetics Consortium (CDGC) cohort enrolling 20 666 unrelated hearing loss (HL) cases. Comprehensive clinical evaluations were conducted on seven members from a three-generation TBS family. Combining data from previously reported cases, we also provided a landscape of phenotypes and genotypes of patients with TBS.

Results We identified five novel and two reported pathogenic/likely pathogenic (P/LP) *SALL1* variants from seven families. Audiological features in patients differed in severity and binaural asymmetry. Moreover, previously undocumented malformations in the middle and inner ear were detected in one patient. By comprehensive clinical evaluations, we further provide evidence for the causal relationship between *SALL1* variation and certain endocrine abnormalities. Penetrance analysis within familial contexts revealed incomplete penetrance among first-generation patients with TBS and a higher disease burden among their affected offspring.

Conclusion This study presents the first insight of genetic screening for patients with TBS in a large HL cohort. We broadened the phenotypic-genotypic spectrum of TBS and our results supported an underestimated prevalence of TBS. Due to the rarity and phenotypic heterogeneity of rare diseases, broader spectrum molecular tests, especially whole genome sequencing, can improve the situation of underdiagnosis and provide effective recommendations for clinical management.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Townes-Brocks syndrome (TBS), characterized by the triad of imperforate anus, thumb malformations and dysplastic ears as well as other abnormalities, is caused by heterozygous variants in the *SALL1* and *DACT1* gene.
⇒ So far, all reported pathogenic/likely pathogenic (P/LP) *SALL1* variants are loss-of-function variants.

INTRODUCTION

Townes-Brocks syndrome (TBS, MIM #107480) is an autosomal dominant disorder with a prevalence estimated at 1 out of 250 000.[1] The syndrome was initially associated with mutations in the *SALL1* gene, which was identified as the primary causative factor for TBS. To date, more than 70 pathogenic/likely pathogenic (P/LP) variants of *SALL1* have been described, all characterised as loss-of-function (LoF) variants. In 2017, Webb *et al*[2] identified a second causal gene of TBS, *the dishevelled binding antagonist of beta catenin 1 (DACT1*), whose P/LP variants lead to a phenotype spectrum referred to as Townes-Brocks syndrome 2 (TBS2, MIM #617466). Currently, there have been five documented P/LP variants of *DACT1*. These discoveries have significantly broadened our comprehension of the genetic underpinnings of TBS.[2 3]

TBS is characterised by a triad of major features including anorectal, outer ear dysplasia and thumb malformations.[4] Additionally, five minor features, including hearing loss (HL), foot malformations, renal impairment, urinogenital malformations and congenital heart disease (CHD), commonly coexist in different combinations.[4] TBS shows prominent phenotypic heterogeneity, and its diagnosis is primarily clinical, rooted in recognising this specific symptom constellation. Up to now, most reported patients with TBS essentially have cases in their families who exhibited typical TBS phenotypic spectrum. The presence of atypical TBS cases underscores the importance of genetic diagnosis. Delving into these atypical cases can provide insight into potential diagnostic oversights or errors. Such missteps might arise from a physician's limited awareness of the syndrome or from an absence of comprehensive clinical assessments.

We undertook an extensive study of both patients with typical and atypical TBS within the China Deafness Genetics Consortium (CDGC) cohort. This cohort encompasses 20 666 unrelated HL cases spanning various nationalities across mainland China. Our goal was to achieve a holistic understanding of the phenotypic and genotypic characteristics of TBS. With this comprehensive study, we

WHAT THIS STUDY ADDS

⇒ This is the first study to targetedly screen TBS based on both genetic and phenotypic data in a large nationwide hearing loss cohort.
⇒ We reported five novel P/LP variants of *SALL1* and identified a series of novel otological phenotypes of patients with TBS, including asymmetrical hearing loss and malformations of the middle and inner ear.
⇒ We conducted comprehensive clinical evaluations on a three-generation TBS family and confirmed the association between TBS and certain endocrine abnormalities.
⇒ Combining our cases and literature review, we divided patients with TBS into first-generation patients and their affected offspring and summarised their clinical characteristics. Phenotypic heterogeneity between generations was observed.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our research highlights that TBS is often underdiagnosed, which is attributed to its rarity and the presentation of atypical phenotypes in certain patients. This suggests that there may still be a significant number of patients with unrecognised atypical TBS . This challenge is not unique to TBS but is also observed in other rare disorders that display phenotypic variability. In light of this, whole genome sequencing is suggested for undiagnosed cases suspected with rare diseases.
⇒ A comprehensive clinical evaluation is crucial for drawing links between uncommon phenotypes and their underlying genetic causes. Establishing these connections greatly enhances accurate diagnoses. Moreover, such detailed evaluations are essential for anticipating and managing potential symptoms that may emerge later in a patient's life.

aim to offer valuable insights for genetic counselling and guide more tailored clinical management for patients with TBS.

SUBJECTS AND METHODS

The CDGC cohort

The CDGC cohort was established in 2013 and has continuously enrolled individuals with diverse HL conditions, aiming to reveal the genetic basis of HL and related syndromes. Patients (n=20 666) affected with disabling HL (pure tone audiometry, >40 dB) were recruited from 101 special education schools, 95 rehabilitation centres for deaf children and 31 hospitals representing all 31 provincial administrative divisions across mainland China. Peripheral blood samples were collected, and available medical examination reports were reviewed. Additionally, pure tone tests and physical examinations were carried out. As a control group (n=7258), unrelated adults (≥18 years of age) without self-reported hearing impairment were recruited by the CDGC work group and the Fudan Huabiao project.[5] Signed informed consent was obtained from all caregivers prior to any procedure was initiated.

Genetic testing

Genomic DNA was extracted from whole blood samples using the QIAamp DNA Blood Kit (Qiagen, Limburg, the Netherlands) following the manufacturer's instructions. For genetic analysis, all cases were first screened by an SNP scan assay (Shanghai Genesky Biotech, Shanghai, China) which covered 96 single nucleotide variants (SNVs), 19 insertions/deletions (indels) and 3 CNV loci in *GJB2*, *SLC26A4* and *MT-RNR1*. Then, undiagnosed patients and all controls were sequenced for the exons and ±50 flanking bases of 785 HL-related genes including *SALL1* using Agilent technology (Agilent, Santa Clara, California, USA). Target genome sequencing was performed on Illumina sequencers. DNA variants were called following the Genome Analysis Toolkit software (GATK) best practices workflow (supplementary methods in the previous work[6]). Next, we proceeded with whole genome sequencing (WGS) for further analysis for undiagnosed patients (n=7258) using the DNBSEQ-T7 platform (BGI, Shenzhen, China) with paired-end 150 base reads (figure 1A).

For the individual F5-II:2, who exhibited typical TBS phenotypes without detectable candidate variants in *SALL1* or *DACT1*, we targeted employed HiFi long-read sequencing to explore potential disease-causing structural variants (SVs).[7] DNA sample of F5-II:2 was processed according to the manufacturer's instructions (PacBio, Menlo Park, California, USA).

Pathogenicity analysis of *SALL1* and *DACT1* variants

Variant annotation was completed with VEP (V.105).[8 9] Refseq used for variants of *SALL1* and *DACT1* were separately NM_002968.3 and NM_016651.5. The American College of Medical Genetics and Genomics (ACMG) guidelines that were outlined in 2018 by the Hearing Loss Variant Curation Expert Panel (HL-EP) were then used for the determination of pathogenicity.[10–12] An overview of variants filtering strategy and interpretation of pathogenicity of variants was presented in figure 1A. In reference to ACMG guidelines and the ACGS Best Practice Guidelines, after a multidisciplinary panel discussion, VUS variants were refined into three categories: Benign leaning VUS (VUS-B), Pathogenic leaning VUS (VUS-P), and VUS. VUS-B represents variants with at least one benign supporting evidence, without pathogenic evidence at any levels, and are not qualified to be assigned as B/LB variants. VUS-P represents variants with at least one pathogenic supporting evidence, without benign evidence at any levels, and are not qualified to be assigned as P/LP variants. VUS represents variants with neither pathogenic nor benign evidence or with both pathogenic and benign evidence (online supplemental tables 1; 2).

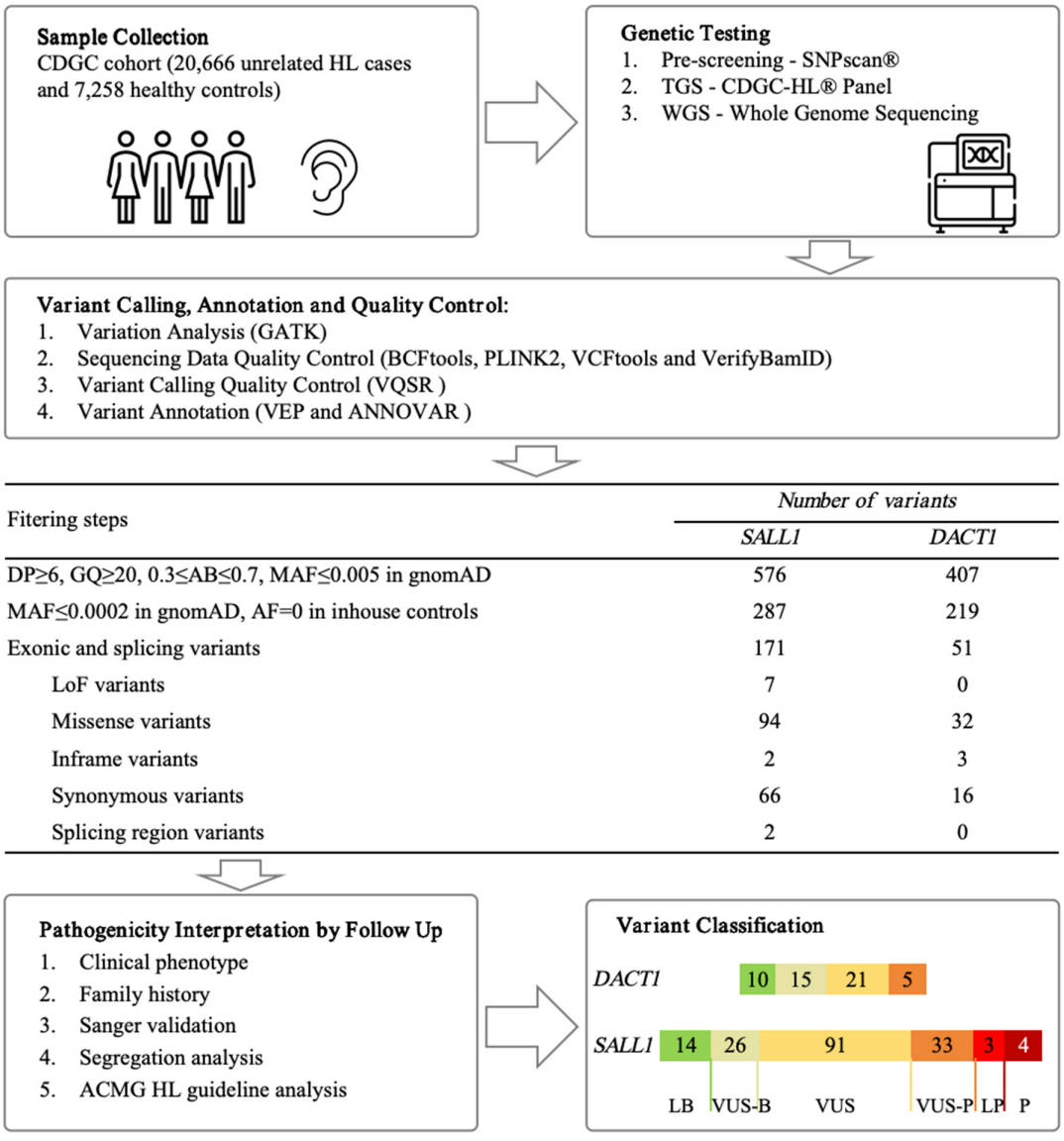
To identify CNVs, we employed cn.MOPS (V.1.36.0)[13] and CNVnator (V.0.4.1)[14] using WGS data. The CNVs were first detected using the R package cn.MOPS, then filtering of CNVnator was carried out considering zero mapping quality (q0) <0.5 and Pval1 <0.05. Manta (1.6.0)[15] was used for structural variation (SV) calling, SVs with GQ >20 and categorised as 'Pass' were enrolled as candidates.

HiFi reads were aligned to both GRCh38 and T2T-CHM13[7] assemblies using minimap2 (2.26-r1175) (https://github.com/lh3/minimap2) and SAMtools (1.10) (https://github.com/samtools/samtools/actions). Structural variants calling was conducted using Delly (1.1.6) (https://github.com/dellytools/delly) and Sniffles2 (2.0.7) (https://github.com/fritzsedlazeck/Sniffles).

Clinical evaluations and characterisation

To ensure accuracy, candidates underwent comprehensive follow-up assessments, including detailed evaluations of clinical presentations and family histories. Additionally, candidates were re-evaluated to confirm their clinical diagnoses. In cases whose further investigations were required, blood samples and phenotypic data were collected from accessible family members

A



Fitering steps	*Number of variants*	
	SALL1	*DACT1*
DP≥6, GQ≥20, 0.3≤AB≤0.7, MAF≤0.005 in gnomAD	576	407
MAF≤0.0002 in gnomAD, AF=0 in inhouse controls	287	219
Exonic and splicing variants	171	51
LoF variants	7	0
Missense variants	94	32
Inframe variants	2	3
Synonymous variants	66	16
Splicing region variants	2	0

B

Genomic position	HGVSc (NM_002968.3)	HGVSp (NC_002959.2)	ACMG evidence	Pathogenic classification	Study
chr16:51174786	c.1341_1347del	p.Phe447Leufs*44	PVS1+PM2+PP4	Pathogenic	This study
chr16:51174644	c.1489C>T	p.Gln497*	PVS1+PM2+PP4	Pathogenic	This study
chr16:51174634	c.1499_1500del	p.Lys500Argfs*15	PVS1+PM2+PP4	Pathogenic	This study
chr16:51171312	c.3686dup	p.Asp1229Glufs*48	PVS1+PM2	Likely pathogenic	This study
chr16:51172929	c.3207dup	p.Asn1070Glnfs*32	PVS1+PM2	Likely pathogenic	This study
chr16:51175307	c.826C>T	p.Arg276*	PVS1+PM2+PP4	Pathogenic	PMID: 14627694
chr16:51174740	c.1393C>T	p.Gln465*	PVS1+PM2	Likely pathogenic	VCV000373187.2*

Figure 1 Strategy of analysing the pathogenicity of *SALL1* and *DATC1* variants. (A) Process of variant filtering and pathogenic interpretation. (B) *SALL1* P/LP variants identified in the CDGC cohort. AB, allele balance at heterozygous sites; AF, allele frequency; DP, read depth; GQ, genotype quality; MAF, minor allele frequency. VUS-B, benign leaning VUS. VUS-P, pathogenic leaning VUS. Asterisk indicates Clinvar accession number.

as needed to support the diagnostic process. Clinical diagnosis of TBS was established based on the clinical diagnostic criteria[4]: (1) the presence of all three major features and the absence of cleft lip/palate or radius hypoplasia, or (2) the presence of two major features along with minor features and the absence of cleft lip/palate or radius hypoplasia. Patients satisfying clinical diagnostic criteria were referred as typical patients, otherwise were referred as atypical patients.

We performed comprehensive clinical evaluations on seven family members from family 1 (table 1), aiming to gain a deeper understanding of the TBS-related phenotypic spectrum within this family. This included two individuals with TBS (F1-II:3 and

Table 1 Genotype and clinical features of family 1

	F1-II:3*	F1-III:1*	F1-II:4	F1-I:1	F1-I:2
Gender	Male	Male	Female	Male	Female
Age range*	Adulthood	Early childhood	Adulthood	Adulthood	Adulthood
Relationship	Father	Proband	Mother	Grandfather	Grandmother
c-Notation	**c.1341_1347del**	**c.1341_1347del**	WT	WT	WT
p-Notation	**p.Phe447Leufs*44**	**p.Phe447Leufs*44**	WT	WT	WT
Major features					
Imperforate anus/anal stenosis	Y	Y	N^E	N^E	N^E
Dysplastic ears	Y	Y	N^E	N^E	N^E
Thumb malformations	N^E	N^E	N^E	N^E	N^E
Minor features					
HL	Y	Y	N^E	N^E	N^E
HL severity	Moderate	Severe	N^E	N^E	N^E
Foot malformations	N^E	N^E	N^E	N^E	N^E
Renal impairment	Y	N^E	N^E	N^E	N^E
Genitourinary malformations	N^E	N^E	N^E	N^E	N^E
CHDs	Y	Y	N^E	N^E	N^E
Rare features					
Thyroid change	Subclinical hypothyroidism; heterogeneous echogenicity	Subclinical hypothyroidism	N^E	N^E	N^E
Eye abnormalities	Myopia	Astigmatism; myopia	N^E	N^E	N^E
Hyperuricaemia	Y	Y	N^E	N^E	N^E
Temporal CT	N^E	Membranous atresia of EAM	N^E	N^E	N^E

Two patients with TBS (F1-II:3 and F1-III:1) and three unaffected family members from family 1 were evaluated for all TBS-related phenotypes including physical examination, hearing test (pure tone test for adults and ABR for the proband), abdomen ultrasound, echocardiography, radiological examinations for limbs and the temporal bone, and haematuria biochemistry. Phenotypes were classified into major features, minor features and rare clinical features.
Variants in bold were novel variants.
*Age range was defined according to suggested ranges for paediatrics and young adults from the BMJ ethics team: preterm neonatal—the period at birth when a newborn is born before the full gestation period; term neonatal—birth—27 days; infancy—28 days–12 months; toddler 13 months–2 years; early childhood—2–5 years; middle childhood—6–11 years; early adolescence—12–18 years; late adolescence—19–21 years. Adulthood was defined as ages over 21 years.
CHD, congenital heart disease; EAM, external acoustic meatus; HL, hearing loss; N^E, not present, confirmed by medical examinations; WT, wild type; Y, present.

F1-III:1) and five healthy members (F1-I:1, F1-I:2 and F1-II:4). Our evaluations encompassed a wide range of TBS-related phenotypes, incorporating the following assessments: (1) ultrasonic examinations of the abdomen, neck and breasts (restricted to female family members); (2) X-ray examinations of the limbs; (3) high-resolution computed tomography (HRCT) scans of the temporal bone; (4) auditory brainstem response (ABR) and auditory steady-state response (ASSR) tests conducted for F1-III:1; (5) pure tone tests performed for other adult family members; (6) haematuria biochemistry tests to assess renal function, liver function, immunological function, endocrinological function and other relevant parameters.

Literature review and statistical analysis

We enrolled all genetically diagnosed TBS cases reported since January 1998, when *SALL1* was identified as the causal gene.[16] Databases included in search of literatures were PubMed, HGMD,[17] ClinVar[18] and DVD.[19] Following the clinical diagnostic criteria, we collected and categorised the reported phenotypes of each case into major and minor features. Additionally, we identified rare features among patients that were present in over 10% of the cases.

To analyse the various penetrance of TBS-related phenotypes across generations, we selected all families that included at least two generations of patients with TBS and calculated the prevalence of each phenotype among the first-generation patients with TBS and their affected offspring. Statistically analysis was performed using SPSS software (V.27, IBM SPSS Statistics, USA).

RESULTS

Pathogenic interpretation of *SALL1* and *DACT1 variants*

To identify disease-causing variants, we conducted quality control and filtered for variants with minor allele frequency (MAF) <0.0002 and absent in in-house control population. We then selected coding and splicing region variants of *SALL1* (n=171) and *DACT1* (n=51) for following pathogenic interpretation (figure 1).

In the *SALL1* gene, seven of them were classified as P/LP variants. Except for previously reported c.826C>T (p.Arg276*)[20] and c.1393C>T (p.Gln465*),[21] five novel variants were identified and confirmed by Sanger sequencing, including c.1341_1347del (p.Phe447Leufs*44), c.1499_1500del (p.Lys500Argfs*15), c.3207dup (p.Asn1070Glnfs*32), c.3686dup (p.Asp1229Glufs*48) and c.1489C>T (p.Gln497*) (figure 1, tables 1–2, online supplemental figure 1). The rest were classified as variants of VUS (n=150) or likely benign variants (n=14) (online supplemental table 1). No candidate CNVs/SVs were detected.

In the *DACT1* gene, 51 variants were classified as variants of VUS (n=41), likely benign variants (n=10) and no P/LP variants were identified (online supplemental table 2).

Interpretation of WGS data for undiagnosed cases and the long-reads sequencing data for F5-II:2, who was clinically diagnosed with TBS, did not identify any candidate P/LP CNVs/SVs in the region 100K upstream and downstream of *SALL1* and *DACT1*.

Table 2 Genotypes and clinical features of Family 2–8

	Family 2		Family 3		Family 4	Family 5	Family 6	Family 7	Family 8
Patient	F2-I:2	F2-II:1	F3-I:2	F3-II:1	F4-II:1	F5-II:2	F6-II:1	F7-II:1	F8-II:1
Gender	Female	Male	Female	Male	Male	Male	Male	Male	Female
Age range*	Adulthood	Middle childhood	Adulthood	Early childhood	Toddler	Early childhood	Toddler	Adulthood	Infancy
c-Notation	**c.1489C>T**		**c.1499_1500del**		c.826C>T	Undiagnosed	c.1393C>T	**c.3686dup**	**c.3207dup**
p-Notation	**p.Gln497***		**p.Lys500Argfs*15**		p.Arg276*	Undiagnosed	p.Gln465*	**p.Asp1229Glufs*48**	**p.Asn1070Glnfs*32**
Major features									
Imperforate anus/anal stenosis	N^E	Y	N^E	N^E	N^E	Y	N^E	N^E	N^E
Dysplastic ears	Y	Y	Y	Y	Y	Y	Y	N^E	N^E
Thumb malformations	N^E	N^E	N	Y	N^E	N^E	N^E	N^E	N^E
Minor features									
HL	Y	Y	Y	Y	Y	Y	Y	Y	Y
HL severity	Severe	Severe	Severe	Severe	Severe	Severe	Severe	Moderate	Severe
Foot malformations	N^E	N^E	N^E	N^E	N^E	N^E	N^E	N^E	N^E
Renal impairment	N^Q	N^Q	N^Q	N^Q	N^Q	N^Q	N^Q	N^Q	N^Q
Genitourinary malformations	N^Q	N^Q	N^E	N^E	N^Q	N^Q	N^Q	N^Q	N^Q
CHD	N^Q	N^Q	N^E	N^E	N^Q	N^Q	N^Q	N^Q	N^E
Rare features									
	N^Q	N^Q	N^E	Corneal dermoid cyst; amblyopia; deformation of the right middle ear ; bilateral vestibular enlargement	N^Q	N^Q	N^Q	N^Q	N^Q

Phenotypes were classified into major features, minor features and rare clinical features.
Variants in bold were novel variants.
*Age range was defined according to suggested ranges for paediatrics and young adults from the BMJ ethics team: preterm neonatal—the period at birth when a newborn is born before the full gestation period; term neonatal—birth–27 days; infancy—28 days–12 months; toddler—13 months–2 years; early childhood—2–5 years; middle childhood—6–11 years; early adolescence—12–18 years; late adolescence—19–21 years. Adulthood was defined as age over 21 years.
CHD, congenital heart disease; HL, hearing loss; N^E, not present, confirmed by medical examinations; N^Q, not present, confirmed by questionnaires; Y, present.

Clinical diagnosis and novel phenotypic findings

By integrating phenotypic data, a total of 11 patients with TBS spanning seven families were identified. Among patients with established genetic diagnosis, half of them (5/10) were typical patients with TBS (F1-III:1, F1-II:3, F2-II:1, F3-II:1 and F4-II:1) and the other half were atypical (F2-I:2, F3-I:2, F6-II:1, F7-II:1 and F8-II:1) (tables 1–2).

For family 1, five members across three generations, including two patients with TBS (F1-III:1 and F1-II:3) and three healthy members (F1-II:4, F1-I:1 and F1-I:2), accepted comprehensive clinical evaluations overlapping with almost all reported TBS-related phenotypes (table 1). During infancy of the proband (F1-III:1), he exhibited bilateral microtia, anal atresia (surgically corrected), patent foramen ovale, astigmatism (175° for both eyes) and bilateral SNHL (left: 75 dB; right: 100 dB) revealed by ASSR (figure 2). Additionally, he was diagnosed with subclinical hypothyroidism (diagnosis of subclinical hypothyroidism is established when TSH level is elevated and free thyroxine level is normal). In the current evaluation, he was uncovered with hyperuricaemia (serum uric acid, 494 µmol/L, normal control: <390 µmol/L) and speech delay. Moreover, HRCT of temporal bone revealed membranous atresia of the right external acoustic

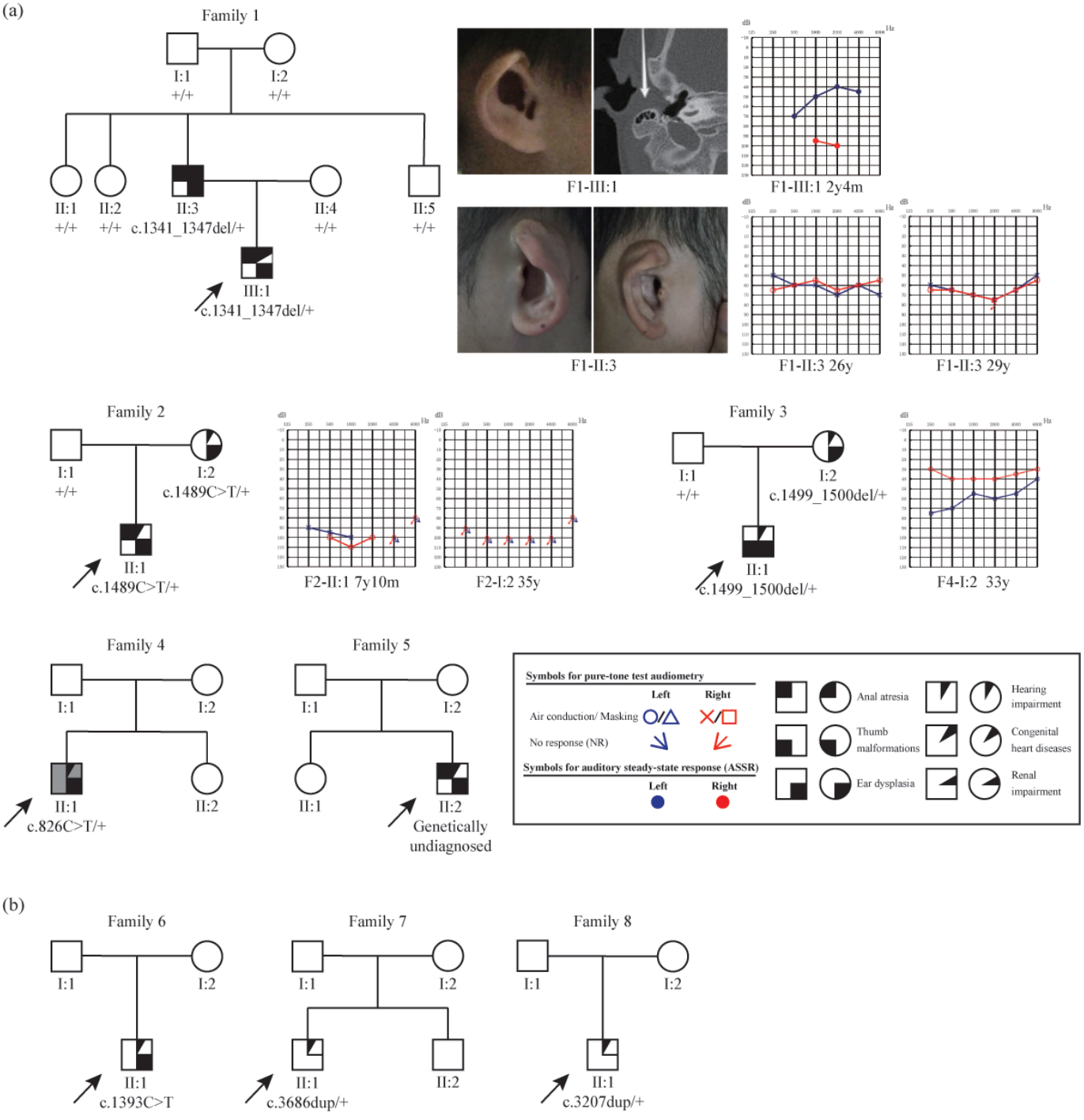


Figure 2 Pedigree, genetic data and clinical pictures of (A) typical and (B) atypical Townes-Brocks syndrome (TBS) families identified from China Deafness Genetics Consortium (CDGC) cohort. Black arrows, probands. Grey blocks, suspected presentation of phenotypes. White arrow, membranous atresia of external acoustic meatus of the right ear of F1-III:1.

meatus. Other TBS-related abnormalities, such as renal impairment, urogenital malformations or limb malformations, were not detected. F1-II:3 was the father of F1-III:1. He was born with CHD, anal atresia, bilateral hearing loss and myopia. No additional malformations were observed. In his mid-20s, he was diagnosed with gout and a pure tone test revealed moderate-to-severe SNHL (left, 63 dB; right, 60 dB). Three years later, the current evaluation revealed additional endocrine abnormalities, including heterogeneous echogenicity of thyroid and subclinical hypothyroidism (triiodothyronine, 1.94 nmol/L, normal control: 1.3–3.1 nmol/L; thyroxine, 104 nmol/L, normal control: 62–164 nmol/L; TSH, 9.84 mU/L, normal control: 0.27–4.2 mU/L). Mild-to-moderate renal impairment was also observed, characterised by proteinuria (0.3 g/L) and decreased estimated glomerular filtration rate (45.59 mL/min/1.73 m^2, normal control: >60 mL/min/1.73 m^2). His recent pure tone test identified hearing threshold of 69 dB on both ears. Other TBS-related abnormalities such as urogenital malformations and limb malformations were not detected. In summary, both patients exhibited typical TBS phenotypes (outer ear dysplasia, anal imperforation, HL, CHD). Additionally, they presented with rare features such as eye abnormalities. Unique to these two patients, and not observed in other family members, were endocrine abnormalities including hyperuricaemia and subclinical hypothyroidism. Notably, the presence of subclinical hypothyroidism in patients with TBS has not been documented in prior research.

F3-II:1 exhibited not only the typical TBS phenotypes but also a range of TBS-associated eye anomalies, including a corneal dermoid cyst and amblyopia. Notably, a temporal CT scan unveiled malformations in the right middle ear and bilateral enlargement of the vestibular system, otological observations that have not been documented in other patients with TBS before.

Among all our patients with TBS, severity of HL ranged from moderate to severe (figure 2A), with F7-II:1 and F1-II:3 demonstrated moderate HL while the rest had severe HL. Specifically, a 6–9 dB increase of average hearing threshold for F1-II:3 was observed. Additionally, asymmetrical HL was confirmed in both F1-III:1 and F3-I:2, which indicates a difference in loss >15 dB between ears at 0.5, 1 and 2 kHz or >20 dB at 3, 4 and 6 kHz on audiogram.[22]

Clinical review of TBS

We searched databases including PubMed, HGMD, ClinVar and DVD, and enrolled all TBS cases reported in the literature who were proved to carry *SALL1* pathogenic variants. Combining TBS cases identified in the CDGC cohort, a total of 166 patients with established genetic diagnosis were enrolled in our analysis (online supplemental tables 3–5). A total of 80 pathogenic *SALL1* variants were reported, with c.826C>T being the most frequently identified (identified in 19/103 probands) (figure 3A). We collected the phenotypes reported in each case and categorised them into major and minor features according to the clinical diagnostic criteria.[4] In addition, we included rare features found in more than 10% of cases.

We divided patients with TBS into two groups: first-generation patients with TBS and their affected offspring. We statistically compared penetrance of each TBS-related phenotype by χ^2 test (figure 3B,C). Results showed significantly higher rate of clinical diagnosis (p=0.000) and higher penetrance among offspring for dysplastic ears (p=0.002) and genitourinary malformations (p=0.003). In addition, among four rare features enrolled in our analysis, penetrance of craniofacial malformations (p=0.032) and psychomotor developmental delay (p=0.050) among affected offspring was significantly higher.

All three major features of TBS presented in more than 50% of patients. As one of the minor features, prevalence of HL in first-generation (62.5%) and affected offspring (69%) were both above 50%, and interestingly, in the first generation, more people suffered from HL than outer ear dysplasia (56.3%). Various types and severities of TBS-related HL was shown in figure 3D. Among the 108 patients, 11.1% (12/108) were diagnosed with conductive HL (CHL) or mixed HL, while over 60% (65/108) exhibited sensorineural HL (SNHL). Severe HL was observed in 19.4% (21/108) of patients, and an equal number of patients (13.9%, 15/108) demonstrated mild or moderate HL. Additionally, two patients were individually diagnosed with unilateral SNHL and mixed HL, with unknown severities.[23 24]

Renal impairment was the most common late-onset symptom of TBS, affecting 35 out of 166 patients, with varying onset times and severities (figure 3E). Eleven individuals were reported to get diagnosed between neonatal birth and middle childhood (0–11 years old), 3 of them had already advanced to end-stage renal disease (ESRD), necessitating dialysis or renal transplantation.[24–26] And for the 11 patients who got diagnosed during or after early adolescence (≥12 years old), 4 out of 11 suffered from ESRD at the time of evaluation[27–30] (online supplemental table 5).

DISCUSSION

Due to the significant phenotypic heterogeneity and low prevalence (1/250 000)[1] of TBS, recognition and diagnosis are often difficult for most primary care doctors or paediatricians. So far, only two TBS families with established genetic diagnosis had been reported in China.[28 31] In this study, we integrated the genotypic and phenotypic characteristics of TBS in the CDGC cohort in which HL was the predominant phenotype. A total of five patients were clinically diagnosed with TBS, and all but one received genetic diagnoses. Seven P/LP variants were also detected in the *SALL1* gene, five of which were novel variants. The comprehensive clinical evaluation on a three-generation TBS family (family 1) further identified associations between hyperuricaemia, subclinical hypothyroidism and *SALL1* variation. Our findings presented a highly variable phenotypic spectrum and an underestimated prevalence of TBS in China, underscoring the importance of WGS in patients with undiagnosed syndromic or non-syndromic HL.

In the preliminary evaluation of patients from the CDGC cohort, none was diagnosed with TBS. The condition was only recognised when we targetedly screened for P/LP variants of *SALL1* and *DACT1*. In addition, in 166 TBS cases enrolled in our review, 62.5% of first-generation patients and 14.9% of affected offspring exhibited atypical phenotypes (figure 3A). Due to its rarity, unfamiliarity to physicians and significant phenotypic variability, TBS is often underdiagnosed during initial consultations. Identifying patients with atypical TBS can be difficult, yet early diagnosis is vital. Patients with TBS often experience progressive dysfunction of specific organs/systems, especially in the kidneys. An early diagnosis enables timely renal assessments and interventions, encourages genetic screening for family members, aids in informed genetic counselling and helps in making family planning decisions while raising awareness of potential risks.

Combining with literature review, a total of 166 patients with TBS and 80 P/LP *SALL1* variants have been identified. We analysed all detected variants including frameshift, nonsense,

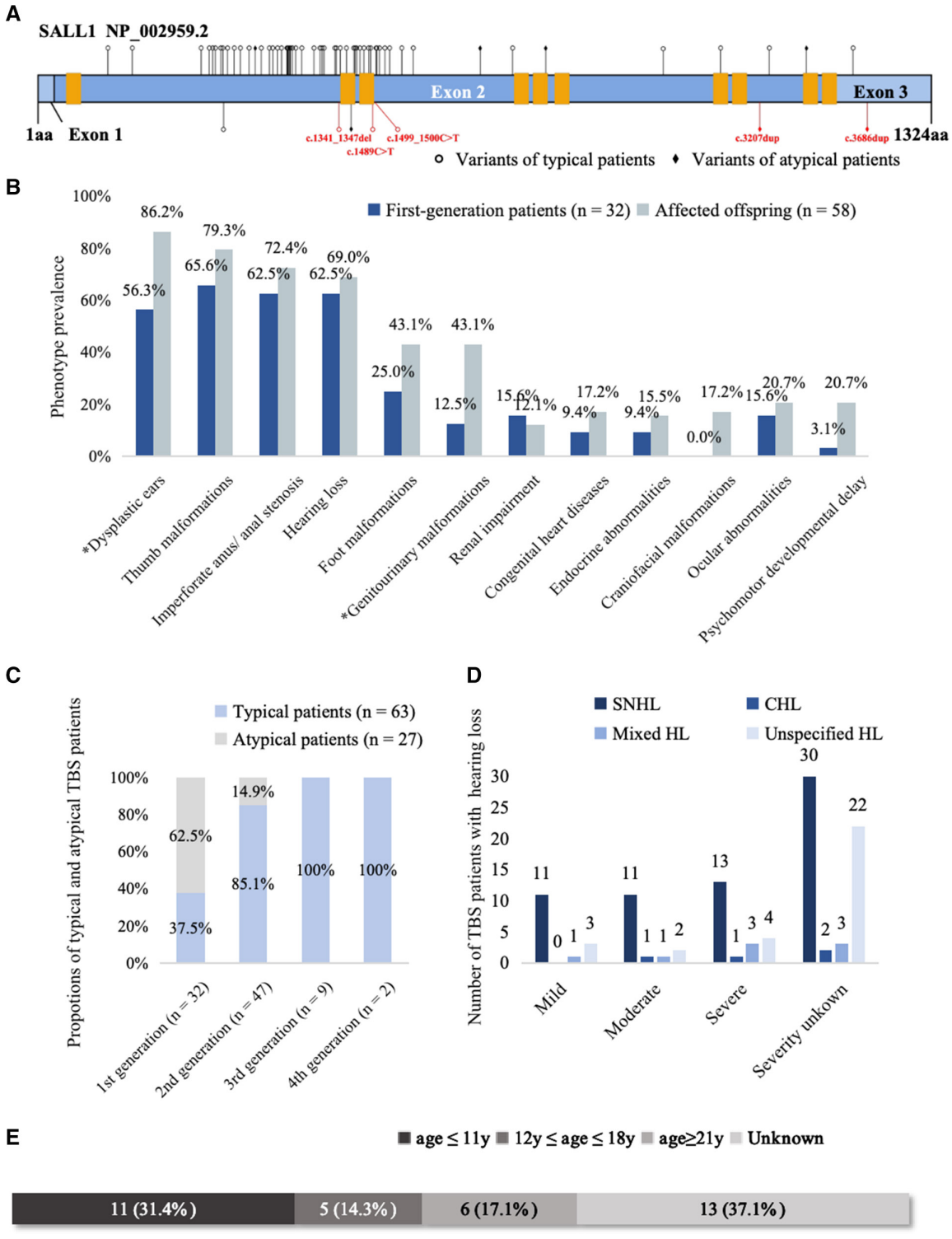


Figure 3 Overview of Townes-Brocks syndrome (TBS) genetic and phenotypic landscape. (A) Distribution of P/LP pathogenic *SALL1* variants (NM_002968.3). Upward strings, variants reported in other studies. Downward strings, variants identified in the China Deafness Genetics Consortium (CDGC) cohort. Red strings, novel variants identified in the CDGC cohort. Orange blocks, zinc-finger domains of SALL1 protein. (B) Prevalence of TBS-related phenotypes among first-generation patients and affected offspring. Asterisks indicate phenotypes with prevalence significantly different between first generation patients and affected offspring. (C) Proportions of patients with typical and atypical TBS among first generation patients and affected offspring. (D) Schematic showing HL across different severity levels and types in patients with TBS. (E) Distribution of age range at diagnosis of renal impairment among patients with TBS. *Age range was defined according to suggested ranges for paediatrics and young adults from the BMJ ethics team: preterm neonatal—the period at birth when a newborn is born before the full gestation period; term neonatal—birth–27 days; infancy—28 days–12 months; toddler—13 months–2 years; early childhood—2–5 years; middle childhood—6–11 years; early adolescence—12–18 years; late adolescence—19–21 years. Adulthood was defined as ages over 21 years. HL, hearing loss. CHL, conductive hearing loss. SNHL, sensorineural hearing loss.

splicing acceptor/donor, missense, in-frame indel, synonymous and splicing region variants in the *SALL1* and *DACT1* genes and performed follow-up phenotype and co-segregation analyses of carriers of variants of VUS and VUS-P, and there were no more candidate P/LP variants identified besides LoF variants. To date, all P/LP variants in *SALL1* gene are LoF variants or CNV/SVs. These variants are spreading throughout the exonic and splicing regions of *SALL1* but were more highly prevalent between 764 and 1565 bp in the cDNA, in agreement with the study by Botzenhart *et al*.[24] Of which, c.826C>T (p.Arg276*) showed the highest incidence (19/103) and was also detected in this study. Out of these 19 unrelated patients from around the globe, 10 were verified to have a de novo variant. For the remaining nine patients, no healthy parents were reported to have undergone testing. These findings highly suggested that the c.826C>T variant is a hotspot variant rather than a founder variant, which was consistent with previous studies.[32–34]

Since 2017, a total of five P/LP *DACT1* variants have been identified to cause TBS2,[2 3] which mainly includes outer ear, genitourinary and anal malformations, with no HL phenotype reported. Nevertheless, we also analysed the pathogenicity of *DACT1* variants detected in patients with HL in the CDGC cohort who had not yet been genetically diagnosed, but no P/LP variants were identified (online supplemental table 2).

In this study, F5-II:2 presented with typical TBS phenotypes but no candidate pathogenic variants (including SVs) in the *SALL1*, *DACT1* or other genes were identified by WGS or HiFi long-read sequencing. A similar case was reported by Liang *et al*,[35] who was a patient with typical TBS but no P/LP *SALL1* variants were detected. These observations aligned with study by Kohlhase,[4] stating that approximately 25% of patients with typical TBS do not harbour *SALL1* variants. For the remaining undiagnosed TBS cases, novel genes and non-coding variations should be considered. Therefore, more TBS cases need to be validated against each other to identify new causal genes, and we need communication platforms like the Matchmaker Exchange[36] to help with research on such rare diseases.

Combining reported TBS cases, HL accounted for 65.06% of 166 TBS cases as a minor feature, taking together the fact that there were 10 cases carrying *SALL1* variants in an HL cohort, all suggested the need to note the possibility of TBS-associated genetic diagnoses in patients presenting with minor features of TBS. We summarised characteristics of TBS-related HL: (1) mild (13.89%, 15/108) and moderate (13.89%, 15/108) HL among patients with TBS were unneglectable. Progressive HL was reported in at least two TBS families,[24 37] and in our cohort, F1-II:3 was also observed with elevated hearing threshold within 3 years (figure 2), which suggests the possibility of progressive exacerbation in patients with TBS with mild-to-moderate HL; (2) audiograms in two of our cases indicated the possibility of asymmetric HL in TBS.

To uncover more associations between *SALL1* variants and TBS phenotypes, we unbiasedly described the phenotypic spectrum of a three-generation TBS family by comprehensive clinical evaluations. Hyperuricaemia, previously reported in three patients with TBS, and subclinical hypothyroidism, newly identified in this family, were segregated with *SALL1* c.1341_1347del (p.Phe447Leufs*44) variant. It is widely known that both hyperuricemia and subclinical hypothyroidism are more prevalent than TBS itself. Therefore, their co-segregation with the *SALL1* variant in Family 1, where all family members lived with the same environmental factors, verified the causal relationship of the *SALL1* variant with these two endocrine abnormalities. Therefore, we recommend long-term monitoring on relevant physiological indicators in patients with TBS, especially in affected children, as hypothyroidism can significantly stunt growth and hyperuricaemia can lead to gout if left untreated.

Renal impairment emerged as the most prevalent late-onset disorder observed in patients with TBS. TBS-related renal impairment could present as ESRD during early childhood or progress insidiously from asymptomatic mild reduced clearance (figure 3E and online supplemental table 5). Notably, patient F1-II:3 was not revealed with renal impairment until the current comprehensive clinical evaluation. Previous studies had reported five cases diagnosed with renal impairment after adulthood, with three individuals already experiencing ESRD at the time of TBS diagnosis.[27 29 30] As one of the minor features, renal impairment may display an asymptomatic or delayed onset. Furthermore, due to the possibility of late-onset renal impairment, its prevalence in TBS is likely to exceed 21.1%. Continuous monitoring of renal function in patients with TBS and screening for *SALL1* variations in renal disease cohorts can assist in clarifying the penetrance and progress of TBS-related renal disease.

By analysing the proportion of clinical differential diagnoses of TBS in first-generation patients and their affected offspring (figure 3C), the results revealed that the proportion of typical patients is significantly greater in the affected offspring. Moreover, outer ear dysplasia and genitourinary malformations exhibited a significant higher penetrance among the offspring, indicating that these two traits are more pronounced in subsequent generations. It may be since most of the patients in the first generation are de novo variants, potentially chimeric and therefore have incomplete penetrance, as well as the possibility of other genetic early presentations. This needs to be studied in multigenerational family lines with high phenotypic heterogeneity.

Overall, our study provides valuable insights into the phenotypic and genotypic characteristics of TBS and underscores the importance of early diagnosis, appropriate management and genetic counselling for affected individuals and their families.

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