RESEARCH ARTICLE

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Mosaicism in tuberous sclerosis complex: Lowering the threshold for clinical reporting

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Abstract

Tuberous sclerosis complex (TSC) is a multi-system genetic disorder. Most patients have germline mutations in *TSC1* or *TSC2* but, 10%–15% patients do not have *TSC1/TSC2* mutations detected on routine clinical genetic testing. We investigated the contribution of low-level mosaic *TSC1/TSC2* mutations in unsolved sporadic patients and families with TSC. Thirty-one sporadic TSC patients negative on

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routine testing and eight families with suspected parental mosaicism were
sequenced using deep panel sequencing followed by droplet digital polymerase
chain reaction. Pathogenic variants were found in 22/31 (71%) unsolved sporadic
patients, 16 were mosaic (median variant allele fraction [VAF] 6.8% in blood) and 6
had missed germline mutations. Parental mosaicism was detected in 5/8 families
(median VAF 1% in blood). Clinical testing laboratories typically only report
pathogenic variants with allele fractions above 10%. Our findings highlight the
critical need to change laboratory practice by implementing higher sensitivity
assays to improve diagnostic yield, inform patient management and guide
reproductive counseling.

KEYWORDS

high-depth sequencing, mosaic mutations, parental mosaicism, tuberous sclerosis complex

1 | INTRODUCTION

Tuberous Sclerosis Complex (TSC) is a multisystem genetic disorder
characterized by benign hamartomas in multiple organs including
brain, skin, kidney, heart, eye, and lung. The estimated prevalence is
1 in 6,000 to 1 in 10,000 live births (Northrup et al., 2013). Frequent
neuropsychiatric features of TSC include epilepsy, intellectual
disability, and autism spectrum disorder (Curatolo, Moavero, de
Vries, 2015). Clinical manifestations and severity vary widely among
individuals, and within families (Caban et al., 2017; Northrup et al.,
2013). Overall, 85%–90% of patients have identified heterozygous
germline pathogenic variants in *TSC1* or *TSC2* (Tyburczy et al.,
2015). *TSC1* and *TSC2* encode negative regulators of the mammalian
target of rapamycin (mTOR) pathway. Loss-of-function variants in
TSC1 and *TSC2* lead to mTOR pathway hyperactivation and
abnormal cell growth, neuronal connectivity, and excitability (Baybis
et al., 2004; Curatolo et al., 2018; Hodges et al., 2001; Tavazoie
et al., 2005).

TSC is an autosomal dominant disorder with high penetrance
(Frost & Hulbert, 2015; Mayer et al., 2014). In about one-third of
patients, TSC is familial, following autosomal dominant inheritance,
with the remaining patients being sporadic due to de novo
pathogenic variants (Rose et al., 1999; Sampson et al., 1989). In
sporadic patients, germline *TSC2* variants are much more common
than germline *TSC1* variants (Au et al., 2007; Kingswood et al., 2017;
Martin et al., 2017), while for familial cases, the proportion of *TSC1*
and *TSC2* germline variants is almost equal (Au et al., 2004;
Au et al., 2007; Jones et al., 1999). Patients with *TSC2* germline
variants tend to have more severe phenotypes compared to those
with germline *TSC1* variants, characterized by higher number of
tubers, earlier age at seizure onset, and higher prevalence of
intellectual disability, affecting their reproductive fitness (Au et al.,
2007; Curatolo, Moavero, Roberto, et al., 2015; Dabora et al., 2001;
Giannikou et al., 2019; Ogórek et al., 2020).

While most patients have germline pathogenic variants detected,
10%–15% patients have "no mutation identified" (NMI) on clinical
genetic testing (Giannikou et al., 2019; Qin et al., 2010; Tyburczy et al.,
2015). Some NMI patients have germline pathogenic variants missed
on initial testing due to technical issues whereas, in others, mosaic
pathogenic variants may be present at allele fractions below the level of
detection of standard depth sequencing (Tyburczy et al., 2015). Mosaic
variants are DNA changes that occur postzygotically (after fertilization).
These variants may arise at any time a cell divides during development,
and are therefore found in only a fraction of the cells of an individual
(Ye et al., 2019). Variant allele fraction (VAF) of mosaic variants often
varies significantly in different organs of an individual, depending on the
timing of the mutational event and the embryonic lineage in which it
occurs (Giannikou et al., 2019; Treichel et al., 2019; Tyburczy et al.,
2015; Vadlamudi et al., 2010). Patients with TSC due to a mosaic
pathogenic variant usually have milder phenotypes compared to
patients with germline variants (Giannikou et al., 2019; Ogórek et al.,
2020; Treichel et al., 2019; Tyburczy et al., 2015; Verhoef et al., 1999).

Mosaicism in parents is a critical issue with serious reproductive
counseling consequences. Patients thought to have "de novo"
pathogenic variants based on routine genetic testing of parental
blood-derived DNA, may have a mosaic parent who has no or subtle
clinical features of TSC. Once a family with a mildly affected or
unaffected parent has more than one affected child, parental
mosaicism is highly likely. Conventional testing often misses low-
level mosaicism due to technological limitations.

Here, we performed a detailed study of mosaicism in TSC with
the following aims: (1) To study 31 mutation-negative sporadic
patients to identify missed pathogenic variants. (2) To test the VAF in
different tissues from mosaic patients and compare the clinical
features between mosaic patients and 213 patients with germline
pathogenic variants. (3) To study eight families with likely *TSC1/TSC2*
parental mosaicism and compare the phenotypes in mosaic parents
with their affected children.

2 | METHODS

2.1 | Editorial policies and ethical considerations

The Human Research Ethics Committee of Austin Health (Project No. H2007/02961) and the Research Ethics Committee of Shenzhen Children's Hospital approved this study. This study adhered to the Declaration of Helsinki. Written informed consent was obtained from all participants or their parents or legal guardians in the case of minors or those with intellectual disability. All patients' clinical and genetic data were deidentified.

2.2 | Participants and phenotyping

2.2.1 | Sporadic TSC patients

Thirty-one sporadic patients with a definite clinical diagnosis of TSC who had NMI on clinical genetic testing were recruited, including 8 adults and 23 children. TSC was diagnosed according to the updated international TSC diagnostic criteria (Northrup et al., 2021).

The adult patients were recruited between July 30, 2018 and February 20, 2020 through the Undiagnosed Diseases Program of Austin Health, Melbourne, Australia. The pediatric patients were recruited from a large TSC study of 425 patients between July 18, 2013 and January 17, 2020, at the Shenzhen Children's Hospital, Shenzhen, China. In this cohort, 216/242 (89%) patients had a known germline *TSC1* or *TSC2* pathogenic variant, and 26/242 (11%) had NMI on clinical testing; 23 of these NMI patients were available to be included in this study. Detailed clinical examination findings and investigation results for all TSC features of these patients are summarized in Table 1. Different tissues including blood, saliva, buccal cells, urine, brain and skin were collected, where possible. The clinical features of the pediatric mosaic sporadic patients were compared with the Chinese pediatric patients with germline pathogenic variants.

2.2.2 | Families with suspected parental mosaicism

To explore parental mosaicism, 35 members from 8 families were recruited from Shenzhen Children's Hospital based on meeting one of two criteria for suspected mosaicism: (i) multiple siblings with TSC sharing the same germline variant; or (ii) only one affected child with one parent negative on gene testing having clinical features of TSC. The eight probands were drawn from the 216 patients with known germline *TSC1* or *TSC2* pathogenic variants, and all of their parents had tested negative for the relevant pathogenic variant on Sanger sequencing. Five families were selected because there were multiple siblings with TSC sharing the same germline variants. None of the parents were diagnosed with TSC before molecular testing. The remaining three families were selected, because, although there was only one affected child, one parent in each family had clinical features

of TSC but was negative for the testing of their child's pathogenic variant. Two parents from these three families had been diagnosed with mild TSC before this study, whereas, one was diagnosed clinically through this study. Because germline mutations were not detected in the parents within these eight families via Sanger sequencing, it was possible they carried low-level mosaic variants below the limit of detection. Different tissues, including blood, saliva, buccal cells, and urine, were collected from all parents recruited, and semen samples were collected from three fathers.

2.3 | DNA and RNA extraction

For Melbourne participants, genomic DNA was extracted using the Qiagen QIAamp DNA Maxi Kit for blood, DNA GenotekPrepIT-L2P Kit for saliva, Isohelix BuccalPrep Plus DNA Isolation Kit for buccal cells, Qiagen DNA Micro Kit for urine, and Qiagen AllPrep DNA/RNA Kit for skin biopsy samples. RNA was extracted from whole blood collected in the BD PAXgene® Blood RNA Tube using Qiagen PAXgene Blood RNA Kit.

For Shenzhen participants, genomic DNA was extracted using the Magen MagPure Tissue&Blood DNA LQ Kit for blood, GENFINE FineQuick Saliva DNA Kit for saliva, GENFINE FineMag Swab DNA Kit for buccal cells, Qiagen DNA Micro Kit for urine, Magen SolPure Tissue DNA Kit for brain, Qiagen AllPrep DNA/RNA Kit for skin biopsy samples, and Magen HiPure Universal DNA Kit for semen. RNA was extracted from whole blood collected in the BD PAXgene® Blood RNA Tube using Magen HiPure Total RNA Plus Mini Kit.

2.4 | Deep massively parallel sequencing

The Melbourne sporadic patients were tested with a gene panel at Peter MacCallum Cancer Center, Melbourne. Sequencing was performed on DNA extracted from blood ($n = 7$) or facial angiofi-broma biopsy ($n = 1$). Libraries were prepared and enriched using SureSelect XT target enrichment (Agilent Design ID 0825941). The hybrid capture bait set covered all coding regions, splice sites, and 20bp up- and downstream of all exons of *TSC1* and *TSC2*. Indexed libraries were pooled and sequenced to a targeted coverage of ~1000 reads/base (Illumina NextSeq. 500, ×2 75 bp), achieving a mean coverage of 687-fold across the coding regions of *TSC1* and *TSC2*. Reads were aligned to the hg19 reference genome with BWA-MEM v0.7.17-r1188, then duplicate marking and base quality score recalibration were performed with the Genome Analysis Toolkit (GATK) version 3.7.0 (McKenna et al., 2010). Germline variant calling was performed with GATK HaplotypeCaller, and mosaic variant calling with GATK Mutect2. Variants were filtered using the following criteria: mutant reads ≥ 10, and variant frequency ≥ 2%. Variants were annotated using vcfanno (Pedersen et al., 2016) and ANNOtate VARiation (Wang et al., 2010).

The sporadic patients from Shenzhen were tested using a Tuberous Sclerosis 2-gene *(TSC1/TSC2)* gene panel using

TABLE 1 Phenotype of the 31 sporadic patients

Patient	Age (year)	Sex	AF (≥3) or forehead plaque	Hypomelanotic Macules (≥3)	Ungual Fibromas (≥2)	Shagreen patch	Multiple Retinal Hamartomas	Cortical dysplasia (no.)[a]	SEN	SEGA	Cardiac rhabdomyoma	LAM	AML (≥2)	Minor features	Seizures	ASD	ID
			Skin				Eye	Brain			Heart	Lungs	Kidney and liver		Neuropsychiatric features		
CHN-1	10	F	-	+	-	-	-	+ (2)	-	-	-	-	-	-	+	-	Moderate
CHN-2	11	F	-	+	-	-	-	+ (8)	+	-	+	-	-	-	+	-	Moderate
CHN-3	6	M	-	+	-	-	-	+ (16)	+	+	+	-	-	-	+	-	-
CHN-4	21	F	+	+	-	-	-	+ (2)	+	-	+	-	+	-	-	-	-
CHN-5	5	M	+	+	-	-	-	+ (3)	+	-	-	-	-	-	+	-	-
CHN-6	13	M	+	-	-	-	-	+ (8)	+	-	-	-	+	-	-	-	-
CHN-7	10	M	-	+	-	+	-	+ (16)	+	-	-	-	-	-	+	-	Borderline
CHN-8	13	F	+	+	-	+	-	+ (7)	+	-	+	-	+	-	-	-	-
CHN-9	16	M	-	-	-	+	-	+ (4)	-	-	+	-	+	-	+	+	Severe
CHN-10	7	F	+	+	-	-	-	+ (5)	+	-	+	-	+	-	+	-	-
CHN-11	8	F	-	+	-	-	-	+ (15)	+	-	+	-	+	Renal cysts	+	-	Mild
CHN-12	5	M	+	+	-	-	-	+ (17)	+	-	-	-	-	-	+	+	Moderate
CHN-13	3	M	-	+	-	+	-	+ (1)	+	-	+	-	-	-	+	-	Moderate
CHN-14	13	M	-	+	-	+	-	+ (4)	-	-	+	-	-	-	+	-	Moderate
CHN-15	7	F	+	+	-	-	NA	+ (28)	+	-	+	-	-	-	+	-	Mild
CHN-16	18	M	-	+	-	-	-	+ (21)	+	+	-	-	-	-	+	-	-
CHN-17	11	F	-	+	-	-	-	+ (4)	+	-	-	-	-	-	+	-	Moderate
CHN-18	14	F	+	+	-	-	-	+ (7)	+	-	-	-	+	-	+	-	Mild
CHN-19	3	F	-	+	-	-	NA	+ (11)	+	-	+	-	-	-	+	-	-
CHN-20	6	M	-	-	-	-	-	+ (5)	+	-	+	-	-	-	-	-	-
CHN-21	2	M	-	-	-	-	+	-	+	-	-	-	-	-	+	+	Moderate
CHN-22	7	F	+	+	-	-	-	+ (1)	-	-	-	-	-	Renal cysts	+	-	Mild
CHN-23	7	F	+	+	-	+	-	+ (17)	+	-	+	-	-	-	+	-	Mild

(Continues)

TABLE 1 (Continued)

| | | | Major features | | | | | | | | | | | | Neuropsychiatric features | | |
| | | | Skin | | | | Eye | Brain | | | Heart | Lungs | Kidney and liver | | | | |
Patient	Age (year)	Sex	AF (≥3) or forehead plaque	Hypomelanotic Macules (≥3)	Ungual Fibromas (≥2)	Shagreen patch	Multiple Retinal Hamartomas	Cortical dysplasia (no.)[a]	SEN	SEGA	Cardiac rhabdomyoma	LAM	AML (≥2)	Minor features	Seizures	ASD	ID
AUS-1	35	F	-	-	-	-	-	-	-	+	NA	-	+	-	-	-	-
AUS-2	18	F	+	+	-	-	-	-	-	-	NA	NA	+	Confetti lesion	-	-	-
AUS-3	29	F	-	+	-	-	-	+ (3)	-	-	NA	NA	-	-	+	NA	-
AUS-4	51	F	+	-	-	-	NA	+ (6)	+	-	-	NA	-	-	+	NA	-
AUS-5	43	M	+	-	-	-	NA	+ (4)	+	-	Suspected	NA	-	-	+	-	-
AUS-6	19	F	-	-	-	-	-	+ (1)	-	+	-	-	-	-	-	-	-
AUS-7	42	M	-	-	-	-	NA	+ (3)	+	-	-	NA	+	-	+	NA	-
AUS-8	32	F	-	-	-	-	-	+ (7)	-	-	-	+	+	-	-	-	-

Abreviations: AF, angiofibroma; AML, angiomyolipoma; ASD, autism spectrum disorder; ID, intellectual disability; LAM, lymphangioleiomyomatosis; MRI, magnetic resonance imaging; NA, not available; SEN, subependymal nodules; SEGA, Subependymal giant cell astrocytoma.

[a]Denotes number of tubers on MRI brain.

blood-derived DNA. The panel covers all the coding regions, splice sites, and introns of *TSC1* and *TSC2* (except six large introns: *TSC1* NM_000368.5 introns 1, 2, 8, and 23, and *TSC2* NM_000548.5 introns 9 and 16). *TSC1* and *TSC2* were amplified with long-segment polymerase chain reaction (PCR) (mean size ~8.5 kb). DNA libraries were prepared using a KAPA LTP Library Preparation Kit Illumina® platforms (KR0453) (KAPA BIO) according to the manufacturer's protocol, followed by paired-end sequencing to a targeted coverage of ~4000 reads/base on the NextSeq. 500 platform. Reads were aligned to the hg19 reference genome with NextGENe software v2.4.1.2 (SoftGenetics), then duplicate marking and base quality score recalibration were performed with the NextGENe software v2.4.1.2. The mean coverage achieved was 4019-fold. Germline variant calling and mosaic variant calling were also performed with NextGENe software v2.4.1.2. Variants were filtered using the following criteria: mutant reads ≥ 10, and variant frequency ≥ 2%. Variants were annotated using population and literature databases including 1000 Genomes, the single nucleotide polymorphism database, the genome aggregation database, the ClinVar database, the human gene mutation database, the online mendelian inheritance in man database and an internal database of 100,000 Han Chinese individuals from the AmCare Genomics Laboratory.

2.5 | Copy number variation (CNV) analysis

For Melbourne patients, clinical chromosomal microarrays from the Illumina Global Diversity array platform (GDAv1.0) were used to screen for genome-wide germline and mosaic (≥10% VAF) CNV and loss of heterozygosity for *TSC1* and *TSC2* at a reportable resolution of 200 kb or greater on blood-derived DNA. Gene specific microarray analysis for *TSC1* and *TSC2* excluded single or multi exon CNVs in these genes except for exon 1 of *TSC2* as there was a 1.4 kb probe gap over this region. The data was analyzed using NxClinicalv6.0 (BioDicovery) using genome reference sequence NCBI37/hg19. For Shenzhen patients, panel sequencing data was used to assess copy number of *TSC1* and *TSC2*. The normalized coverage depth of each exon of a test sample was compared with the mean coverage of the same exon in the reference file to detect potential CNVs with a VAF ≥ 10%. The data was analyzed using Amcarelab.ModelAnalysis.Tool software using genome reference sequence NCBI37/hg19 as previously reported (Feng et al., 2015). Breakpoints were verified by long-range PCR.

2.6 | Validation of candidate variants

Standard Sanger sequencing was performed to validate germline variants. Droplet digital PCR (ddPCR) was used to validate mosaic variants and determine their VAF in different tissues. Custom oligonucleotides and probes used for ddPCR assays are detailed in Supporting Information: Tables S1 and S2. For validation of mosaic

single nucleotide variants, ddPCR was performed as previously reported (Damiano et al., 2017). Positive results were defined as at least 3 positive droplets being detected using the BioRad Quantasoft software (Uchiyama et al., 2016). For validation of mosaic copy number variants, a 22 µl mixture was constructed for each reaction, containing 10 µl of ×2 ddPCR EvaGreen Supermix (Bio-Rad), 0.4 µl of each primer (5 µM) and 20 ng of DNA. Twenty microliterof the mixture was subjected to droplet generation using 70 µl of droplet generation oil for Evageen (Bio-Rad). Samples were manually transferred to a 96-well PCR plate, heat-sealed and amplified on a C1000 Touch thermal cycler using: 95°C for 5 min for one cycle, followed by 40 cycles at 94°C for 30 s and 60°C for 60 s, then one cycle at 4°C for 5 min and 90°C for 5 min, and finally infinite hold at 4°C. Post-PCR products were read on the QX200 droplet reader (Bio-Rad) and analyzed using QuantaSoft software.

2.7 | Complementary DNA (cDNA) synthesis and reverse-transcriptase PCR (RT-PCR)

To confirm aberrant splicing, reverse-transcriptase PCR was performed. cDNA was synthesized from 1 µg of RNA extracted from whole blood using the Invitrogen™ SuperScript™ III First-Strand Synthesis System Kit with the random hexamer protocol. RT-PCR was performed with 2 µl of cDNA using a standard protocol (Hildebrand et al., 2015).

2.8 | Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 27. Correlation analyses were performed using the Pearson correlation test. Comparison of the frequency of different TSC clinical features in mosaic and germline patients was analyzed using Fisher's exact test. Bonferroni correction was performed to adjust for multiple comparisons. A *p* value of less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Pathogenic variants in sporadic TSC patients

We identified a pathogenic variant in 22/31 (71%) patients in either *TSC1* or *TSC2*, including 16 with mosaic pathogenic variants and 6 with germline pathogenic variants that had been missed on clinical testing (Table 2 and Table 3). Overall, the VAF detected by ddPCR was consistent with that determined by gene panel sequencing in the same tissue. The only exception was patient (CHN-13) whose *TSC1* c.1888_1891del (p.Lys630Glnfs*22) variant was initially considered to be mosaic (VAF 29% in blood) on the basis of gene panel sequencing, but we determined it was germline by ddPCR (VAF ~50% in all tissues). This gene panel sequencing error was attributable to

TABLE 2 Variant Allele Fraction (VAF) in different tissues from sporadic patients with mosaic pathogenic variants

| Patient | Variant | ACMG Pathogenicity | Panel sequencing | | ddPCR | | | | | | Number of major clinical features | Number of minor clinical features |
			Tissue tested	VAF	Blood VAF	Saliva VAF	Buccal VAF	Urine VAF	Brain VAF	Skin VAF		
CHN-8	TSC2 c.598C > T (p.Gln200*)	Pathogenic	Blood	3.6%	3.7%	12%	12.8%	27.4%[a]	-	-	7	0
CHN-4	TSC2 c.4198delG (p.Asp1400Thrfs*11)	Pathogenic	Blood	5.8%	6.4%	5.3%	11.5%	12.5%	-	-	6	0
CHN-10	TSC2 c.4905C > A (p.Cys1635*)	Pathogenic	Blood	3.9%	3.0%	3.0%	9.2%	ND	-	-	6	0
CHN-11	TSC2 exon37-38 del	Pathogenic	Blood	30%	37%[a]	51.1%[a]	50.6%[a]	ND	-	-	5	1
CHN-3	TSC2 c.4881delC (p.Lys1628Argfs*44)	Pathogenic	Blood	11.3%	11.8%	18.7%	22.2%	19.2%	-	-	5	0
CHN-2	TSC2 c.4850-1G > C	Pathogenic	Blood	19.6%	19.8%	14.5%	11.5%	13.5%	18%	-	4	0
CHN-7	TSC2 c.729_730del (p. Cys244Serfs*93)	Pathogenic	Blood	7.2%	7.8%	8.6%	10.6%	19%[a]	-	-	4	0
CHN-6	TSC2 c.2015dupC (p.Ala673Glyfs*30)	Pathogenic	Blood	7.5%	7.3%	4%	2.5%	24.2%[a]	-	-	4	0
CHN-12	TSC2 c.838_839del (p.Met280Glyfs*57)	Pathogenic	Blood	6.0%	7.1%	10.2%	7.3%	12%	-	-	4	0
CHN-9	TSC2 c.5240T > A (p.Ile1747Asn)	Likely pathogenic	Blood	4.7%	4.6%	5.5%	2.7%	ND	-	-	4	0
CHN-5	TSC2 c.3685C > T (p.Gln1229*)	Pathogenic	Blood	8.9%	8.3%	9.7%	11%	12%	-	-	4	0
AUS-8	TSC2 c.4235dupC (p.Glu1413*)	Pathogenic	Blood	4.3%	4.8%	2.5%	-	9.1%	-	-	3	0
AUS-2	TSC2 c.1714_1716+4dup	Pathogenic	Angiofibroma	1.9%	0.5%	0.2%	0.07%	ND	-	Normal skin: 0.06% Lesion[b]: 2.5%	2	1
AUS-3	TSC1 c.1770_1777del (p.Pro591Serfs*12)	Pathogenic	Blood	21%	27%	-	-	-	-	-	2	0
CHN-1	TSC2 c.1912delG (p.Val638Cysfs*60)	Pathogenic	Blood	8.9%	5.2%	4.5%	1.4%	5.2%	1.6%	Lesion[c]:1.1%	2	0
AUS-1	TSC2 c.4537G > T (p.Glu1513*)	Pathogenic	Blood	2%	1.8%	2.2%	1.6%	1.3%	-	-	2	0

Abbreviations: ACMG, American College of Medical Genetics guideline; ddPCR, droplet digital polymerase chain reaction; ND, not detectable due to low DNA yield from urine samples; -, no sample.

[a] Mean VAF from 2 different samples of each tissue;

[b] Same angiofibroma DNA used for panel sequencing;

[c] Hypomelanotic macule skin lesion DNA.

TABLE 3 Sporadic patients with missed germline mutations

Patient	Variant	ACMG pathogenicity	Function	Number of major clinical features	Number of minor clinical features
CHN-13	*TSC1* c.1888_1891del (p.Lys630Glnfs*22)	Pathogenic	Known pathogenic variant[a]	5	0
CHN-15	*TSC2* c.976-15G > A	Pathogenic	Known pathogenic variant[b]	5	0
CHN-14	*TSC2* c.3598C > T (p.Pro1200Trp)	Pathogenic	Known pathogenic variant[c]	4	0
CHN-16	*TSC2* c.1443G > A (p.Glu481=)	Pathogenic	Exon 14 skipping	4	0
CHN-17	*TSC2* c.2639 + 4A > T	Likely pathogenic	Exon 23 skipping	3	0
AUS-4	*TSC1* c.362A > G (p.Lys121Arg)	Pathogenic	Exon 5 skipping	3	0

Abbreviation: ACMG, American College of Medical Genetics guideline.

[a]National Center for Biotechnology Information. ClinVar; [VCV000005097.6], https://www.ncbi.nlm.nih.gov/clinvar/variation/VCV000005097.6

[b]Tyburczy et al., 2015

[c]National Center for Biotechnology Information. ClinVar; [VCV000049770.12], https://www.ncbi.nlm.nih.gov/clinvar/variation/VCV000049770.12

strong strand bias, low coverage, and/or reduced alignment of reads containing the deletion.

Mosaic pathogenic variants were identified in *TSC2* in 15/16 patients, including 7 small insertions or deletions, 5 nonsense, 1 missense, 1 splicing and 1 CNV; whereas, only 1 mosaic pathogenic variant was in *TSC1*, an 8 base pair deletion (Table 2). Overall, VAF ranged from 0.5% to 37% in blood by ddPCR (median 6.8%), 0.2-51.1% in saliva (median 5.5%), and 0.07-50.6% in buccal cells (median 9.2%). Patient CHN-11 had a mosaic CNV with a VAF in blood of 30% on panel sequencing and 37% on ddPCR compared with 51.1% in saliva and 50.6% in buccal DNA. In view of the 50% VAF in saliva and buccal samples, we recollected all three tissues again and confirmed that this variant was indeed mosaic (Table 2).

Urine-derived DNA was less reliable for testing and generally of lower concentration than DNA from other tissues, resulting in variants detectable in the urine of only 11 patients, with VAF ranging from 1.3% to 27.4% (median 12.5%). In patients CHN-6, CHN-7, and CHN-8, their urine VAF was much higher than the VAF in their other tissues. We therefore collected a second urine sample from each child which confirmed the high urine VAF of 24.2%, 19% and 27.4%, respectively (Table 2). It is noteworthy that patients CHN-6 and CHN-8 both have multiple renal angiomyolipomata (AML) (Table 1), which may explain the higher VAF in urine compared to other tissues. The lower VAF of 19% was found in CHN-7 who did not have renal AML on ultrasound.

Three patients had lesion-derived DNA tested in addition to DNA from blood, saliva, buccal cells, and urine (Table 2). For patient AUS-2, VAF was highest in the facial angiofibroma biopsy (2.5%) compared to normal skin (0.06%) and other tissues (0.07-0.5%). For patient CHN-2, the VAF in cortical tuber (18%) was similar to that in blood (19.8%) and slightly higher than in other tissues (11.5% −14.5%). Conversely, for patient CHN-1, the VAF in cortical tuber (1.6%) and a hypomelanotic skin macule (1.1%) was much lower than in blood, saliva, and urine (4.5%–5.2%). Overall, we found that VAF across different tissues was relatively similar in some patients, but varied widely in others (Table 2).

Of the 6 germline pathogenic variants, 4/6 were in *TSC2* and 2/6 in *TSC1*. There were 4 splicing defects, including splice-site pathogenic variants in *TSC2* in CHN-15 and CHN-17, 1 synonymous variant in the last base pair of exon 14 of *TSC2* in CHN-16, and 1 missense variant in the second last base pair of exon 5 of *TSC1* in AUS-4 (Table 3). The recurrent heterozygous *TSC2* c.976-15G > A pathogenic variant found in CHN-15 has been shown to cause aberrant splicing (Tyburczy et al., 2015). Using RT-PCR and Sanger sequencing of patient RNA samples, we confirmed that the *TSC1* c.362A > G (p.Lys121Arg and AUS-4) variant leads to exon 5 skipping, *TSC2* c.1443G > A (p.Glu481= and CHN-16) variant causes exon 14 skipping, and *TSC2* c.2639 + 4A > T (CHN-17) variant induces exon 23 skipping (Table 3 and Supporting Informaton: Figure S1). The remaining two germline pathogenic variants were a missense variant in *TSC2* (CHN-14) and a four base pair deletion in *TSC1* (CHN-13), both recurrent pathogenic variants reported in ClinVar (VCV000005097.6 and VCV000049770.12) (Table 3).

3.2 | Genotype-phenotype correlation in mosaic patients

Overall, for patients with mosaic pathogenic variants, the number of major clinical features (Table 2) did not correlate with blood VAF ($R = 0.022$, $n = 16$, $p = 0.94$), saliva VAF ($R = 0.33$, $n = 15$, $p = 0.23$) or buccal VAF ($R = 0.44$, $n = 14$, $p = 0.11$). In contrast, the brain tuber load did correlate with saliva VAF ($R = 0.57$, $n = 15$, $p = 0.026$) and buccal VAF ($R = 0.56$, $n = 14$, $p = 0.037$), but not with blood VAF ($R = 0.37$, $n = 16$, $p = 0.16$).

Clinical data was available for 213 of the 216 Chinese patients from the Shenzhen cohort who had germline *TSC1* and *TSC2* pathogenic variants. We compared the frequency of each TSC feature in the 12 pediatric mosaic patients and the 213 pediatric germline patients. We found a trend that hypomelanotic macules, subependymal nodules (SEN), seizures and retinal hamartomas were

TABLEE 4 Comparison of frequency of clinical features between pediatric mosaic and germline patients

Features	Mosaic patients (n = 12)			Germline patients (n = 213)			Adjusted p value mosaic versus germline (Bonferroni test)
	Positive	Negative	Prevalence	Positive	Negative	Prevalence	
Cutaneous features							
Angiofibromas (≥3)	6	6	50%	97	101	49%	1
Forehead plaque	3	9	25%	51	146	26%	1
Hypomelanotic macules (≥3)	10	2	83%	203	2	99%	0.192
Ungual fibromas (≥2)	0	12	0%	9	200	4%	1
Shagreen patch	3	9	25%	106	102	51%	0.864
Neurological features							
Cortical dysplasias	12	0	100%	202	5	98%	1
Subependymal nodule	10	2	83%	197	9	96%	1
Subependymal giant cell astrocytoma	1	11	8%	83	124	40%	0.276
Seizure	9	3	75%	201	12	94%	0.444
Other organs							
Cardiac rhabdomyoma	7	5	58%	118	84	58%	1
Renal angiomyolipomata	6	6	50%	71	130	35%	1
Multiple retinal hamartomas	0	12	0	37	77	32%	0.144

less frequent in mosaic patients, however, none of them were statistically significant (Bonferroni adjusted p > 0.05, Table 4).

3.3 | Low-level parental mosaicism in families with TSC

Parental mosaicism for *TSC2* variants was detected in 5/8 families studied (Figure 1, Table 5). VAF in the mosaic parents ranged from 0.3% to 9.1%. Mosaic parents had milder phenotypes with fewer clinical features (range: 0–2) compared to their children with heterozygous pathogenic variants (range: 5–8) (Table 5). Although the parents in these families had low-level mosaicism, it is noteworthy that 6/11 of their children had TSC, while 5 did not and were negative for the familial pathogenic variant (Figure 1).

In Family 1, mosaicism was detected in the mother, with a VAF ranging from 2% to 3.9% in the tissues tested. After genetic testing was received, examination revealed multiple facial angiofibromas and multiple renal AML resulting in a clinical diagnosis of definite TSC. In Family 2, the father had multiple facial angiofibromas and multiple renal AML and was diagnosed with definite TSC before genetic testing, but he had been negative for his child's variant on routine clinical testing. Mosaicism was detected, with a VAF ranging from 7.3%–9.1%. In Family 3, mosaicism was detected in the mother (VAF 0.4%–2.2%). Following the molecular diagnosis, the mother had comprehensive physical examination and imaging and was found to only have an ungual fibroma without any other clinical features. In

Family 4, mosaicism was detected in the mother (VAF 0.3%–1.3%); subsequently she was diagnosed with multiple renal AML and possible TSC following the molecular testing, based on clinical criteria. In Family 5, both parents did not have any TSC features identified with comprehensive physical examination and imaging. Mosaicism was identified in the father's semen (VAF: 15.4%) and urine samples (VAF: 11.8%), but was not detectable in the other tissues tested.

4 | DISCUSSION

We sought to identify low-level mosaic pathogenic variants in *TSC1* or *TSC2* in individuals with unsolved TSC, and in families where an undetected low-level mosaic pathogenic variant could explain the observed TSC inheritance pattern. We solved 22/31 (71%) sporadic NMI patients with 16/31 having mosaic and 6/31 missed germline pathogenic variants in *TSC1* or *TSC2*, providing molecular diagnosis for most unsolved patients, consistent with an earlier report (Tyburczy et al., 2015). In the 16 patients with mosaic pathogenic variants, VAFs ranged from 0.5% to 37% in blood (median = 6.8%). Clinical genetic laboratories typically only report variants with an allele fraction greater than 10% (Batalini et al., 2019) and focus on blood or saliva-derived DNA. Therefore, it was not surprising that VAFs for 12/16 mosaic pathogenic variants in our cohort were below 10% in blood. Overall, our findings highlight the importance of applying sensitive assays in clinical laboratories to enable
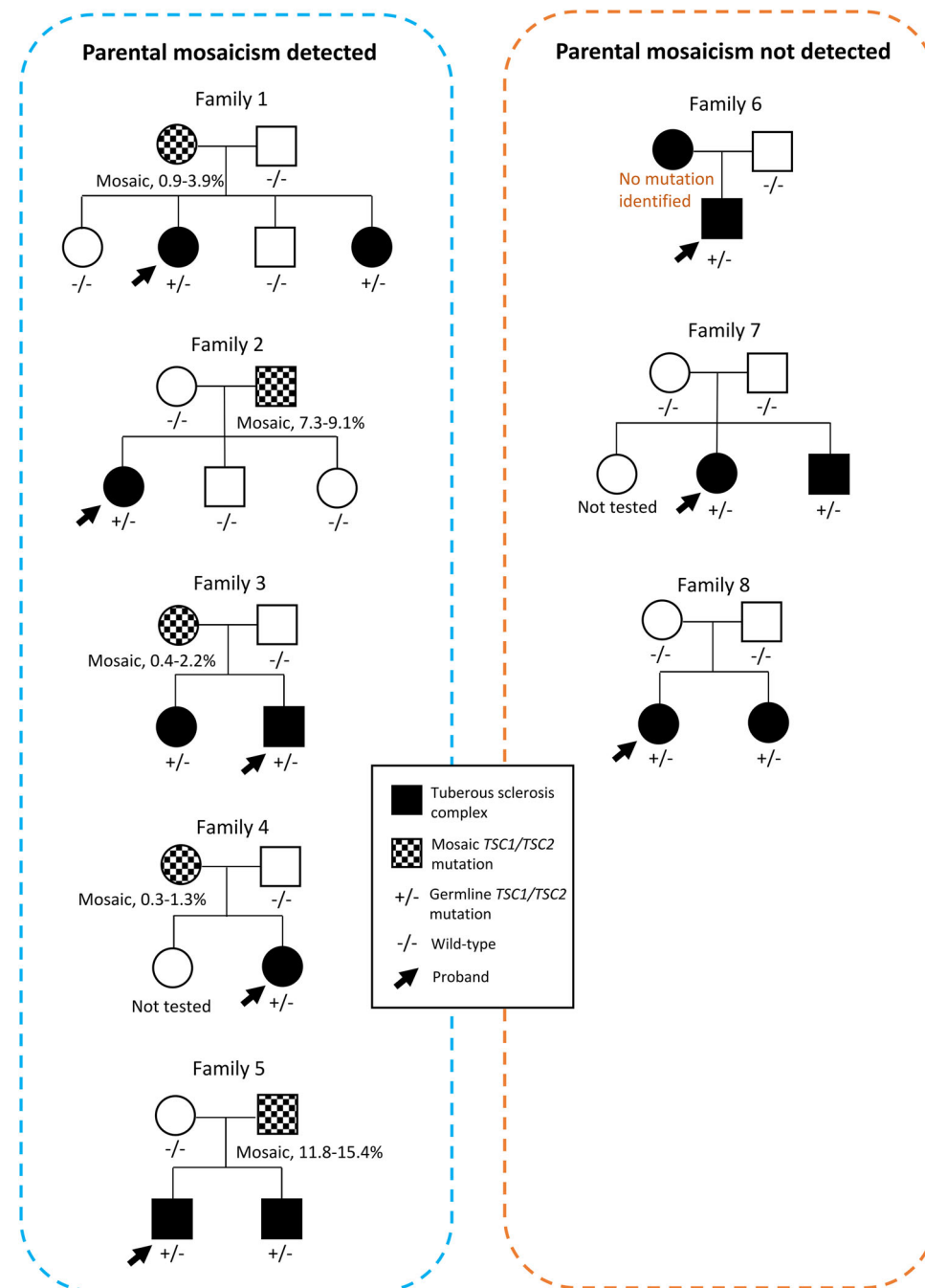
FIGURE 1 Family pedigrees of the eight families with potential parental mosaicism.
Phenotypes were classified according to the 2012 TSC diagnostic criteria guideline (Northrup et al., 2013). Black symbols represent patients with tuberous sclerosis complex; mosaic symbols represent individuals with parental mosaicism; arrows represent probands. Genotypes are provided for tested individuals as −/− for wild-type and +/− for heterozygous *TSC1/TSC2* pathogenic variants. For parents with mosaic pathogenic variants, variant allele frequency is provided. TSC, Tuberous sclerosis complex.

identification and reporting of disease-causing mosaic pathogenic variants down to at least 2% VAF. These findings have both clinical management implications for the mosaic individual, together with critical reproductive counseling implications.

Mosaicism explained half of the 11% of patients who were negative on prior clinical molecular testing in our large pediatric cohort from Shenzhen. Thus, we estimate that mosaic pathogenic variants explain 5.5% of all TSC patients. This is a large number of patients considering the prevalence of TSC, however, this estimate is based on a relatively small number of studies (Giannikou et al., 2019; Treichel et al., 2019; Tyburczy et al., 2015) with limited data on genotype-phenotype correlation. We found that specific cutaneous features (hypomelanotic macules), neurological features (SEN and seizures) and ophthalmological feature (multiple retinal hamartomas)

TABLE 5 Clinical and genetic diagnosis of parents and affected children in families with TSC

Family number	Family member	Clinical features for TSC	Variant	VAF				
				Blood	Saliva	Buccal	Urine	Semen
1	Mother	Definite TSC, 2 major features (multiple AF, multiple renal AML)	Mosaic *TSC2* c.439A > G	2%	3.1%	0.9%	3.9%	-
	Second child	Definite TSC, 7 major features	Germline *TSC2* c.439° > G	50%				
	Fourth child	Definite TSC, 6 major features		50%				
2	Father	Definite TSC, 2 major features (multiple AF, multiple renal AML)	Mosaic *TSC2* c.1832G > C	8.8%	9.1%	8.3%	7.3%	-
	First child	Definite TSC, 8 major features	Germline *TSC2* c.1832G > C	50%				
3	Mother	Does not meet TSC diagnosis criteria (only 1 ungual fibroma)	Mosaic *TSC2* c.668_676del	1%	1.6%	2.2%	0.4%	-
	First child	Definite TSC, 8 major features	Germline *TSC2* c.668_676del	50%				
	Second child	Definite TSC, 5 major features and 1 minor feature		50%				
4	Mother	Possible TSC, 1 major feature (multiple renal AML)	Mosaic *TSC2* c.3581G > A	0.3%	1.3%	0.7%	ND	-
	Second child	Definite TSC, 6 major features	Germline *TSC2* c.3581G > A	50%				
5	Mother	No TSC feature	ND	ND	ND	ND	ND	-
	Father	No TSC feature	ND	ND	ND	ND	11.8%	15.4%
	First child	Definite TSC, 5 major features	Germline *TSC2* c.4738_4741dup	50%				
	Second child	Definite TSC, 6 major features		50%				
6	Mother	Definite TSC, 2 major features (shagreen patch; multiple renal AML)	ND	ND	ND	ND	ND	-
	Only child	Definite TSC, 5 major features	Germline *TSC2* c.2015_2016insTT	50%				
7	Mother	No TSC feature	ND	ND	ND	ND	ND	-
	Father	No TSC feature	ND	ND	ND	ND	ND	ND
	Second child	Definite TSC, 6 major features	Germline *TSC2* c.1939dup	50%				
	Third child	Definite TSC, 7 major features		50%				
8	Mother	Does not meet TSC diagnosis criteria (1 suspected liver AML)	ND	ND	ND	ND	ND	-
	Father	No TSC feature	ND	ND	ND	ND	ND	ND
	First child	Definite TSC, 5 major features	Germline *TSC2* c.976-15G > A	50%				
	Second child	Definite TSC, 7 major features		50%				

Abbreviations: AF, facial angiofibroma; AML, angiomyolipoma; ND: not detected; -, not applicable.

were less common in pediatric mosaic patients compared to pediatric patients with germline pathogenic variants, but they did not achieve a statistical significance, most likely due to the small sample size, as we only have 12 pediatric mosaic patients for comparison. We were not able to compare the frequency of lymphangioleiomyomatosis, autism spectrum disorders and intellectual disability in the pediatric groups, and not able to compare adult mosaic patients with adult germline patients due to lack of available data.

Germline pathogenic variants were missed in six sporadic patients on prior testing, likely due to technical issues. These may arise due to poor coverage of certain genomic regions or because variants impacting splicing, such as synonymous changes, may be filtered out by standard analytic pipelines or may not have been considered potentially significant. RT-PCR should be considered to confirm the impact of putative splicing variants if tissue RNA is available. It is also worth mentioning that Patient CHN-17 was initially ascertained as sporadic because the patient was clinically diagnosed as definite TSC, while the parents did not have any clinical features of TSC on physical examination and declined further clinical investigation at that time. Following discovery of the germline splice

site mutation in Patient CHN-17, we Sanger sequenced both parents and found the mother also carried this variant. The mother subsequently received comprehensive clinical investigation and was found to have renal angiomyolipoma.

Nine sporadic patients remain unsolved, possibly due to the following limitations of our study: (1) deep intronic pathogenic variants not covered by gene testing panels; (2) mosaic pathogenic single nucleotide variants with a VAF < 2% and genomic deletions/rearrangements with a VAF < 10%, which were below the limit of detection using our methods with lower sequencing depth or microarray resolution than reported in some other studies (Giannikou et al., 2019; Tyburczy et al., 2015); or (3) mosaic somatic pathogenic variants confined to affected organs (brain, kidney) and not detectable in peripheral tissues available for testing. Novel technologies, including genome sequencing, may identify the remaining pathogenic variants, be they deep intronic or novel variation types. There has long been speculation as to whether there is a third TSC gene (Qin et al., 2010). Our findings reinforce that a third TSC gene is unlikely, consistent with previous reports of NMI patients (Giannikou et al., 2019; Tyburczy et al., 2015).

Parental mosaicism was found in 5 families with VAFs ranging from 0.3% to 9.1%. The affected offspring with germline pathogenic variants had more severe phenotypic manifestations and a greater number of clinical features on average. In the remaining three families, parental mosaicism was not detected. Family 6 has one affected child with the germline *TSC2* c.2105_2016insTT pathogenic variant. The mother has multiple bilateral renal AML and shagreen patch. However, mosaicism was not detected in any of her tissues, including urine. Her variant might be confined to certain organs which were not studied. Families 7 and 8 both have two affected children with the same germline pathogenic variant detected in both affected children. Both parents underwent comprehensive physical examinations, but no TSC features were identified, except for the mother in Family 8 who had a suspected liver AML. Different tissues from both parents were tested, including semen samples from the two fathers. Interestingly, mosaicism was not detected in any tissue, leaving a possible explanation of maternal gonadal mosaicism.

Renal AML was the most common major feature in parents with mosaicism, suggesting that renal ultrasounds should be routinely performed in parents of children with TSC. The mother in Family 4 had renal AML on re-examination following our detection of ultra-low *TSC2* mosaicism, with 0.3% in blood. Currently there is no consensus regarding variant threshold for diagnosis in the setting of mosaic pathogenic variants even in the updated diagnostic guidelines published in 2021 (Northrup et al., 2021).

Low-level *TSC1/TSC2* mosaicism may be under-recognized as such individuals may have no clinical TSC features or only subtle manifestations (Giannikou et al., 2019; Ogórek et al., 2020; Tyburczy et al., 2015). We observed this phenomenon; the sole feature in the mother in Family 3 was one ungual fibroma reflecting her ultra-low level of mosaic pathogenic variant (1% VAF in blood). This single subtle clinical feature would be insufficient for a TSC diagnosis based on clinical criteria alone; however, there is likely to be an increased

recurrence risk, acknowledging we could not determine the percentage mosaicism in gonadal tissue.

In conclusion, we found mosaic pathogenic variants in 52% of patients with TSC negative on conventional genetic testing. Most mosaic pathogenic variants had a VAF of less than 10%, highlighting the importance of using high-depth sequencing and ddPCR assays to increase sensitivity to detect and confirm low-level mosaic pathogenic variants. Mosaic patients have fewer cutaneous and neurological features compared to patients with germline variants. Higher VAFs in saliva and buccal cells positively correlated with tuber load in mosaic patients. Parents with low-level mosaic pathogenic variants and few or no clinical features of TSC can transmit their pathogenic variant to their offspring; careful clinical examination of parents is recommended. Genetic investigations, incorporating testing for low-level mosaicism, critically inform clinical management and reproductive counseling for patients and families with TSC.

CONFLICT OF INTEREST

I. E. S has served on scientific advisory boards for UCB, Eisai, GlaxoSmithKline, BioMarin, Nutricia, Rogcon, Chiesi, Encoded Therapeutics, Knopp Biosciences and Xenon Pharmaceuticals; has received speaker honoraria from GlaxoSmithKline, UCB, BioMarin, Biocodex, Chiesi, Liva Nova and Eisai; has received funding for travel from UCB, Biocodex, GlaxoSmithKline, Biomarin and Eisai; has served as an investigator for Zogenix, Zynerba, Ultragenyx, GW Pharma, UCB, Eisai, Xenon Pharmaceuticals, Anavex Life Sciences, Ovid Therapeutics, Epigenyx, Encoded Therapeutics and Marinus; and has consulted for Zynerba Pharmaceuticals, Atheneum Partners, Ovid Therapeutics, Care Beyond Diagnosis, Epilepsy Consortium and UCB; and is a Nonexecutive Director of Bellberry Ltd. She may accrue future revenue on pending patent WO61/010176 (filed: 2008): Therapeutic Compound; has a patent for SCN1A testing held by Bionomics Inc and licensed to various diagnostic companies; has a patent molecular diagnostic/theranostic target for benign familial infantile epilepsy (BFIE) [PRRT2] 2011904493 and 2012900190 and

PCT/AU2012/001321 (TECH ID:2012-009). The remaining authors declare no competing interests or conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available on reasonable request due to privacy/ethical restrictions. Variants identified in this study were submitted to the Leiden Open Variation Database (LOVD database): (https://databases.lovd. nl/whole_genome/variants/TSC1 and https://databases.lovd.nl/ whole_genome/variants/TSC2).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.