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Tuberous Sclerosis Complex in Chinese patients: Phenotypic analysis and mutational screening of *TSC1/TSC2* genes

Shan Lin[a,1], Jia-Bin Zeng[a,1], Gui-Xian Zhao[b], Zhen-Zhen Yang[a], Hui-Ping Huang[a], Min-Ting Lin[a], Zhi-Ying Wu[a,c], Ning Wang[a,d], Wan-Jin Chen[a,d], Ling Fang[a,d,*]

[a] Department of Neurology and Institute of Neurology, First Affiliated Hospital, Fujian Medical University, Fuzhou, China
[b] Department of Neurology, Huashan Hospital, Fudan University, Shanghai, China
[c] Department of Neurology and Research Center of Neurology in Second Affiliated Hospital, Zhejiang University School of Medicine, Key Laboratory of Medical Neurobiology of Zhejiang Province, Hangzhou, China
[d] Fujian Key Laboratory of Molecular Neurology, Fuzhou, China

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ABSTRACT

Purpose: Tuberous sclerosis complex (TSC) is characterized by the development of hamartomas in multiple organ systems. This study attempted to screen mutations and to investigate the mutation distribution and related phenotypes including epilepsy of Chinese TSC patients.

Methods: We performed the genotypic analysis of *TSC1* and *TSC2* genes in 77 unrelated Chinese TSC patients using direct Sanger sequencing and Multiplex ligation-dependent probe amplification (MLPA).

Results: Mutations were identified in a total of 63 (81.8%) cases, including 18 *TSC1* mutations (8 nonsense mutations, 6 frameshift, 1 in-frame shift, 1 missense and 2 splice-site) and 45 *TSC2* mutations (13 missense, 3 nonsense, 6 splicing, 6 in-frame shift,12 frameshift mutations and 5 large deletions). Large deletions were presented exclusively in *TSC2* gene, accounting for 7.9% of all mutations in this study. Fourteen novel mutations were identified in this study.

Conclusions: Epilepsy occurs in approximately 75.3% (58/77) of patients. Hypomelanotic macules occurred significantly more often in patients with *TSC2* mutations and cases with *TSC1/TSC2* mutations had a significantly higher frequency of cortical nodule than patients with no mutations identified. Overall, our data expands the spectrum of mutations associated with the TSC loci and will be of value to the genetic counseling in patients with the disease.

1. Introduction

Tuberous sclerosis complex (TSC) is a rare autosomal dominant inherited disorder characterized by the growths of benign hamartomatous tumors in multiple organ systems, primarily in the brain, eyes, heart, kidney, skin and lungs [1]. The prevalence of the disease was estimated to range from 1/6000 to 1/10 000 [2]. Inactivating mutations in either of two genes, *TSC1* on chromosome 9q34 (MIM 191100) and *TSC2* on chromosome 16p13 (MIM 191092), were identified as responsible for TSC [3,4]. The *TSC1* gene contains 23 exons, coding for a 130-kDa protein named Hamartin, while *TSC2* consists of 42 exons generating a transcript from which a 200-kDa protein called Tuberin is deriving. Both proteins form a complex that negatively regulates the activation of the mechanistic target of Rapamycin complex 1 (mTORC1), a master serine/threonine kinase that plays role in multiple processes, including translation, ribosome biogenesis, and autophagy [5].

It's said that patients with *TSC2* mutations tend to have more severe manifestation. The extensive clinical variability even for identical mutations means that establishing definite correlations between special mutation types and clinical feature is challenging. As the main affected organ, neurological symptoms presented in more than 90% of individuals with lesions in brain such as cortical tubers, subependymal nodules or subependymal giant cell astrocytomas (SEGA). These structural lesions may be related to neurological signs and symptoms, such as epilepsy, and neuropsychiatric disorders [6].

* Corresponding author at: Department of Neurology and Institute of Neurology, First Affiliated Hospital, Fujian Medical University, 20 Chazhong Road, Fuzhou, 350005, China.
E-mail address: paulinefz@126.com (L. Fang).
[1] These authors contributed equally to this work.

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Comprehensive mutation analysis has been reported previously in a series of cohorts with TSC till now. Among affected individuals, pathogenic mutations in either *TSC1* or *TSC2* account for 75%–94% of cases [7–25]. According to the Leiden Open Variation Database (LOVD, http://www.lovd.nl/3.0/home), all mutation types, including nonsense, missense, small deletions and insertions as well as large rearrangements involving whole gene deletion, have been described in TSC and the distribution of mutations is highly heterogeneous. However, 10%–15% of TSC patients still have no mutation identified (NMI) in *TSC1* and *TSC2* genes [21]. The clinical presentation and severity of TSC also significantly varies.

Genetic heterogeneity and phenotypic variability make the diagnosis of TSC more complicated and challenging [22]. As a result, the relationship between genotype and phenotype in TSC has been the focus of a number of studies which may have contributed to the development of TSC surveillance and management. To date, only a few studies regarding systematic analysis of the genotypic characteristics and clinical manifestations in TSC patients based on Chinese population have been reported [15,20,25]. In this paper we collected unrelated clinical TSC patients who were mainly from the southeast areas of China, detected both TSC1 and TSC2 gene mutations and analyzed the mutational distributions and possible genotype-phenotype correlations.

2. Materials and methods

2.1. Patients and DNA preparation

Seventy-seven unrelated patients (47 males; 30 females), including 13 index cases and 64 sporadic cases, were recruited in our study who were clinically diagnosed with TSC according to the criteria revised by the 2012 international Tuberous Sclerosis Complex Consensus Conference [19]. Clinical presentations, physical examination and imaging examination of collected cases with accessible clinical information were conducted. Clinical characteristics included central nervous features with epilepsy, cortical dysplasias, subependymal nodules and subependymal giant cell astrocytomas, dermatologic and dental features such as hypomelanotic macules, angiofibromas, ungual fibromas, shagreen patches and renal features. DNA samples were obtained from peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and TGuide Blood Genomic DNA kit (Tiangen, Beijing, China) according to the recommended protocol. Written consent was obtained from each patient or legal guardians of those who are under 18 years of age. This study was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University.

2.2. Molecular analysis of TSC1 and TSC2 genes

Direct sequencing was performed for all coding exons of the *TSC1* and *TSC2* genes using ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA). Primer sequences for polymerase chain reaction (PCR) amplification of all *TSC1* (GenBank, NM_000368.3) and *TSC2* (GenBank, NM_000548.2) coding exons and their flanking intronic regions were synthesized by Sangon Biotech Corporation (Shanghai, China) according to the previous studies 6. Patients with no mutation identified in both genes were screened for large copy number changes by Multiplex ligation-dependent probe amplification (MLPA) analysis. Commercial SALSA MLPA kits P124-C1 and P046-C1 (MRC-Holland, Amsterdam, Netherlands) were used for mutation scanning of *TSC1* and *TSC2* genes, respectively, according to the manufacturer's protocol, including denaturation, hybridization, ligation and amplification. Amplification products were subsequently separated through capillary electrophoresis on an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The raw data for copy numbers determination were analyzed using Coffalyser.Net (MRC-Holland) [22].

2.3. Prediction of variants pathogenicity

The pathogenicity of the variant was determined based on American College of Medical Genetics and Genomics Standards and Guidelines (ACMG). Mutations identified in *TSC1* or *TSC2* gene were compared with LOVD version 3.0 (http://chromium.lovd.nl/LOVD2/TSC/home) and the Clinvar database (https://www.ncbi.nlm.nih.gov/clinvar/). The possible influences caused by the identified mutations on proteins were evaluated through accessible websites such as SIFT (http://sift.jcvi.org/www/SIFT_seq_submit2.html)and Polyphen-2(http://genetics.bwh.harvard.edu/pph2/). A nucleotide change was classified as pathogenic variation when it was confirmed by previous researches or included by HGMD or LOVD database; resulting in abnormal transcription or protein truncation proved by functional assays; or when it was not observed in normal controls with co-segregation of mutation in families. A nucleotide change was considered as a variant of unknown significance when it was novel and parents were unavailable for study [16].

2.4. Statistical analysis

Statistical analysis was performed with SPSS version 23.0 software. The $\chi 2$ and Fisher exact tests were used to examine the differences in phenotypes and genotypes between patients in different groups.

3. Results

3.1. Clinical manifestation

A total of 77 unrelated patients with definite diagnosis of TSC based on the revised diagnostic criteria were enrolled in the mutation screening process. The ages of index patients at their first examination were 0.9–65 years old, with a median age of 10 years and an average age of 14.3 ± 13.8 years. Age of patients at their diagnosis mainly focuses under 10 years old, which can be obviously observed in the *TSC2* group (Fig. 1A). Clinical data of patients were collected and a summary of the manifestations was presented (Fig. 1B). As shown in the graph, neurological disorders such as subependymal nodules (83.1%), epileptic seizures (75.3%) and cutaneous lesions including facial angiofibroma (68.8%) and hypomelanotic macules (68.8%) were observed in most patients, all accounting for proportions over 50% (Fig. 1B). Other than that, male patients accounted for 61.0% of subjects (47/77) and no significant difference in distribution of clinical features between males and females was observed.

3.2. Mutational findings

Combination of Sanger sequencing and MLPA revealed variations in a total of 63 out of 77 patients (81.8%) (Fig. 2A). The proportions of sporadic and familial cases including those with no mutation identified were 83% and 17%, respectively. No significant differences were found between *TSC1* and *TSC2* genes with regard to the frequencies of familial and sporadic cases (P = 0.55).

3.3. Mutations at the TSC1 locus

Among the variations detected in *TSC1* gene, point mutations were identified in 18 patients, including 8 nonsense mutations (8/18, 44%), 6 frame shift(6/18, 33%), 1 in-frame shift (1/18, 6%), 1 missense (1/18, 6%) and 2 splice-site (2/18, 11%) mutations (Fig. 2B). Five out of the eighteen were not reported in some disease databases such as Clinvar, Leiden Open (source) Variation Database (LOVD), or the Exome Aggregation Consortium (ExAC). Among them, four variants were classified as pathogenic variants according to the ACMG Standards and Guidelines (Table 1). Exon 8 of *TSC1* gene present relatively higher mutation detecting rate (5/63; 7.9% of total mutations).

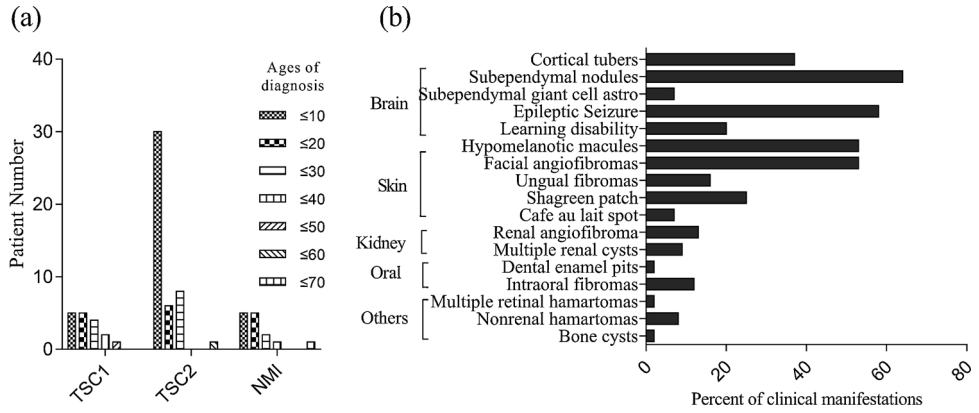

Fig. 1. Clinical characteristics of patients in our series. A. The age ranges for diagnosis among TSC patients; B. The distribution of diverse clinical features among 77 unrelated patients with tuberous sclerosis complex.

3.4. Mutations at the TSC2 locus

With regard to the *TSC2* locus, 40 small mutations including 13 missense mutations (13/40, 32%), 3 nonsense mutations (3/40, 8%), 6 splicing mutations (6/40, 15%), 6 in-frame shift mutations (6/40, 15%) and 12 frameshift mutations (12/40, 30%) were detected (Fig. 2C). 9 cases were novel, most of which were frameshift mutations. Exons 27 and 40 of the *TSC2* gene reveal relatively higher frequency of mutations in this study. Moreover, we observed a common in-frame deletion mutation (c.5238_5255del) in exon 40 of *TSC2* gene, which was seen in three independent patients in this study (Fig. 3A).Almost all the in-frame shift mutations identified in the *TSC2* gene were distributed in the GTPase activating-protein (GAP) and Rabaptin domains (spans exon 34–40). (Fig. 3A)

Large deletions of the *TSC2* gene were identified by MLPA in 5 out of 19 patients with no mutation identified using Sanger sequencing, accounting for 7.9% of all mutations. Of these, three were complete *TSC2* gene deletions combined with the last exon of *PKD1* gene adjacent to it. One was intragenic deletion from the exon 1 to the exon 37. Another one was identified heterozygous loss of the exon 16, which was also detected in her mother.

3.5. Genotype-phenotype correlation

Comparison of clinical features among patients carrying gene mutations or no mutation identified (NMI) in these genes presented some trends (Table 2). Cases with mutations identified had a significantly higher frequency of cortical nodule than patients with no mutations identified (NMI, 54.0% versus 21.4%, $P = 0.028$). Other clinical features such as learning disability, seizures, subependymal giant cell astrocytoma and intraoral fibroma occurred at a lower frequency in the group of NMI compared to the individuals with identified mutations, However, all these trends lacked statistical significance.

Furthermore, we compared affected individuals with a *TSC1* mutation to those with mutation in *TSC2* gene. The result indicated that hypomelanotic macules occurred significantly more frequently in patients with *TSC2* mutations than those with *TSC1* mutations (P = 0.03). (Table 2). In addition, shagreen patches occurred more often in the group of patients with a mutation in *TSC1* gene (44.4% versus 26.7%), yet the difference also lacks statistical significance. Other than that, the distribution of other clinical features between these two groups such as renal angiomyolipomas and learning disability were also not observed significantly different. The incidence of epilepsy of patients presenting cortical nodule and subependymal nodules were also respectively compared with cases without these lesions, but there were no statistical difference.

4. Discussion

According to the updated diagnostic criteria recommended, identification of a pathogenic *TSC1* or *TSC2* mutation is sufficient for diagnosis of TSC. The overall mutation detection rate was similar to the data of previous works in which the mean detection rate was 83% (range: 59%–96%) [1]. The mutation detection rate of reported cohorts from other countries such as Greek. Russia and Brazil were 87%˜89% by using the same methods as ours or next generation sequencing [25–27]. While the earlier reports analyzed about 117 TSC children and 84 TSC patients from China presented the rate to 100% and 76%, respectively [15,28]. Fourteen of these mutations were novel and variations were more often identified at the *TSC2* locus as compared to *TSC1,* with the

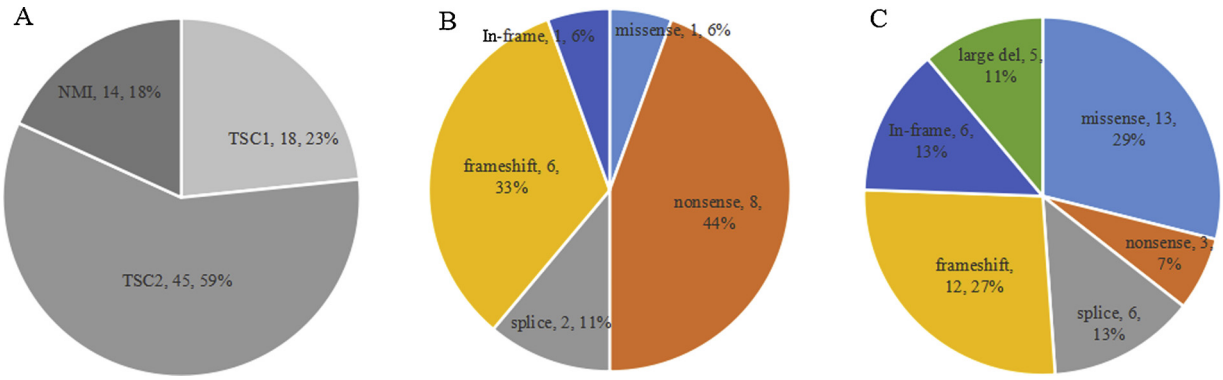

Fig. 2. Pie charts displaying the proportion of different mutation screening results and types in our cohort. A. The proportions of *TSC1 / TSC2* mutations and NMI (no mutation identified) in this series. B. Different types and ratios of identified mutations among *TSC1* patients. C. Different types and ratios of identified mutations among *TSC2* patients.

Table 1
Distribution of Novel Mutations and Large deletions identified in the TSC1/TSC2 gene.

Gene	Location	Nucleotide change	Protein change	LOVD	1000G	ExAC	Classification	Evidence of pathogenicity
Point Mutation of *TSC1*	Exon6	c.508 + 1G > T	–	N	N	N	P	1*PVS,2*PM,2*PP
	Exon8	c.737 + 2T > C	–	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon11	c.1065_1066del	p. Met355Ilefs*13	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon15	c.1665_1666ins†	p. Asp556Leufs*3	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon16	c.2031_2036del	p. Thr677delins3	N	N	N	VUS	2*PM, 1*PP
Point Mutation of *TSC2*	Exon6	c.629_630del	p. Ala210Valfs*24	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon9	c.932_936del	p. Ser311*	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon14	c.1537delG	p. Val513Trpfs*22	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon24	c.2771_2772del	p. Phe924*	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon26	c.3096_3097dup	p. Val1034Thrfs*20	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon26	c.3024insCA	p. Ala1009Glnfs*8	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon27	c.3253 T > C	p. Ser1085Pro	N	N	1	LP	2*PM, 2*PP
	Exon34	c.4541C > G	p. Ser1514*	N	N	N	P	1*PVS,2*PM, 2*PP
	Exon34	c.4569 + 1G > C	–	N	N	N	P	1*PVS,2*PM, 2*PP
CNV In Chr.band(16p13.3)	Exon 16	–	–	N	–	-–	P	1*PVS,2*PM, 1*PP
	Exons 1-37	–	–	N	–	–	P	1*PVS,2*PM, 1*PP
	Exons1-42(TSC2) +Exon46(PKD1)	–	–	14	–	–	P	1*PVS,2*PM, 1*PP
	Exons1-42(TSC2) +Exon46(PKD1)	–	–	14	–	–	P	1*PVS,2*PM, 1*PP
	Exons1-42(TSC2) +Exon46(PKD1)	–	–	14	–	–	P	1*PVS,2*PM, 1*PP

†: CTCCCATA.
N: Novel; CNV: copy number variant; P: pathogenic; LP: likely pathogenic; VUS: variants of uncertain significance; LOVD: Leiden Open Variation Database; 1000G: The 1000 Genomes Project Consortium; ExAC: Exome Aggregation Consortium.

ratio approaching to 2.5:1(45/18).

TSC is transmitted as an autosomal dominant inheritance pattern, but approximately 2/3 probands were sporadic. This can be explained by the poorer social adaptation of TSC patients that leads to lower marriage and fertility rate. In the present study, 39 of 77 patients (51.9%) carry de novo mutations in *TSC2* gene and 12 cases (15.5%) in *TSC1*, resulting in a *TSC1*:*TSC2* ratio of approximately 1:3. Among the familial cases, 6 (7.8%) and 4(5.2%) patients have mutations in *TSC1* and *TSC2* genes, respectively. It's reported that significantly more of the sporadic cases had mutations in *TSC2* than *TSC1* gene 15. However, the difference was not statistically significant (P = 0.051) due to the small sample size. All mutation types, including base substitution, insertions, and deletions, were described in TSC and the distribution of mutation spectrums within both genes is highly heterogeneous [7–9,12,15,16,29]. The results in our study demonstrated that nonsense mutations occurred significantly more frequently in *TSC1* gene (p = 0.001).

However, missense mutations were less often identified in *TSC1* gene (p = 0.049). The only missense mutation (c.1460C > G) in *TSC1* group is located in exon 15, the largest exon of *TSC1* with the highest percentage of identified mutations among Chinese TSC patients (Fig. 3B) [16]. In addition, large deletions or rearrangements are observed more frequently in *TSC2* [17]. Five patients in our cohort were detected multi-exon heterozygous deletion in *TSC2* gene using MLPA (Table 1), accounting for about 7.9% of mutations. Though no obvious mutation hot spots were observed, mutations were identified more often in exon 8, 15 of *TSC1* gene and exon 34, 36, 40 and 41 of *TSC2* gene (GAP-related and Rabaptin binding domains) among the cohorts

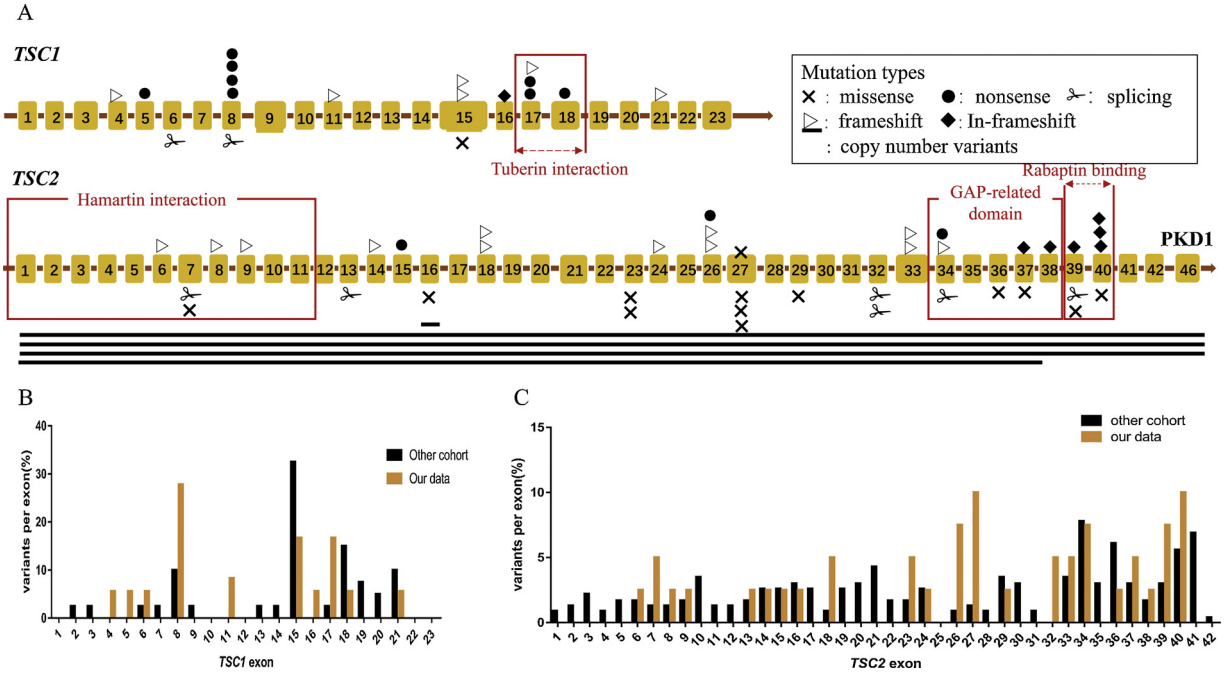

Fig. 3. Distribution of mutations along exons of both genes in our series and Chinese cohorts. A. Distribution of diverse mutation types along the whole exons in *TSC1* and *TSC2* genes. Numbered boxes denote individual exons. B&C. Distribution of variants along exons of *TSC1*(B9 and *TSC2* (C) gene.

Table 2
Clinical features in different groups of mutation types.

	Total (n = 77)	TSC1 (n = 18)	TSC2 (n = 45)	NMI (n = 14)	TSC1 vs TSC2	NMI vs Mut.
	No.	No.	No.	No.	P value	P value
Male	47	10	29	8	–	–
Female	30	8	16	6	–	–
Skin						
Hypomelanotic macules	53(69%)	9(50%)	35(78%)	9(64%)	0.030	ns
Facial angiofibromas	53(69%)	11(61%)	32(71%)	10(71%)	ns	ns
Ungual fibromas	16(21%)	2(11%)	11(24%)	3(21%)	ns	ns
Shagreen patch	25(33%)	8(44%)	12(27%)	5 (36%)	ns	ns
CNS						
Cortical dysplasia	37(48%)	10(56%)	24(53%)	3(21%)	ns	0.028
Subependymal nodules	64(83%)	15(83%)	37(82%)	12(86%)	ns	ns
SEGA	7(9%)	2(11%)	5 (11%)	0(0%)	ns	ns
Epileptic Seizure	58(75%)	15(83%)	34(76%)	9(64%)	ns	ns
Learning disability	20(26%)	4(22%)	15(33%)	1(7.1%)	ns	ns
Kidney						
Renal angiofibroma	13(17%)	0(0%)	9(20%)	4(29%)	0.05	ns
Multiple renal cysts	9(12%)	1(6%)	6(13%)	2(14%)	ns	ns
Renal cell carcinoma	1(1%)	0(0%)	1(2%)	0(0%)	ns	ns
Oral						
Dental pits	2(3%)	1(6%)	0(0%)	1(7%)	ns	ns
Intraoral fibroma	12(16%)	6(33%)	6(13%)	0(0%)	ns	ns
Other						
Non-renal hamartoma	8(10%)	0(0%)	7(16%)	1(7%)	ns	ns
Cafe au lait spot	7(9%)	2(11%)	3(7%)	2(14%)	ns	ns

Abbreviation: CNS: central nervous system; SEGA: Ependymal giant cell astrocytoma; NMI: No mutation identified; ns: no statistically significant difference.

in China (Fig. 3B & C) [30–34]. Nevertheless, using both direct sequencing and MLPA, there were still left with a set of 14 (18.2%) patients in whom no mutation was identified. It indicates that the sensitivity of any single currently available approach for mutation detection is limited and an efficient time-saving mutation detecting strategy is needed.

In our series, we also observed that most patients appear initial symptoms at an early age, especially in *TSC2* group whose age range of diagnosis among patients mainly focus under 10 years old, which may be due to their severer clinical situations.

The correlation between genotypes and phenotypes has been reported in numerous clinical and basic studies [6,7,9,16,35]. Cases with mutations identified had a significantly higher frequency of cortical nodule (Table 2). Epilepsy, which may give rise to learning disability in affected children especially during the course of cerebral development occur in approximately 75.3% (58/77) of patients. Studies of larger cohorts also demonstrated that patients with a *TSC2* mutation were more likely to appear learning disability [16]. However, this difference only indicated a trend. We observed that patients with learning disability were always accompanied by epilepsy. This finding is similar to reports elsewhere. However, the correlation between cortical or subcortical tuber and learning disability was relatively poor. And Associations between these two brain lesions and epilepsy were also not obvious. Interestingly, a rather striking correlation was noticed between renal AMLs and learning disability in other studies [9]. However, the relationship was not obvious in our cohort.

With regards to renal signs, more renal AML were associated with *TSC2* gene mutations., however, the difference was not statistically significant (P = 0.051) due to the small sample size and incomplete auxiliary examination of abdomen. It has been observed that most patients with TSC and polycystic kidney disease have contiguous deletions involving *TSC2* and the adjacent polycystic kidney disease 1 (*PKD1*) gene 16. However, we only find one case with deletions in both genes have multiple renal cysts (MRC). There also exist several cases with MRC identified *TSC2* mutations without involving *PKD1*. Other than that, Skin lesions such as hypopigmented patches also appeared significant higher frequency in patients with *TSC2 m*utations (35/45; 77.8%) compared to that of patients with *TSC1* mutations (9/18; 50%) (*P* = 0.03) (Table 2). Everolimus is the only FDA approved drug to treat renal angiomyolipoma or SEGA in TSC [36]. Previous researches has also indicated its efficacy in reducing seizures in patients with TSC and refractory epilepsy [37]. But the safety and efficacy in treatment of epilepsy still needs further evaluation among extensive patients from different regions and races or age groups.

5. Conclusion

In summary, this study provides a general perspective of the clinical features and gene mutations in TSC patients from southeast of China. MLPA and direct sequencing provide a feasible preliminary screening strategy with detection rate of 81.8% for *TSC1/TSC2* gene. Among them, fourteen variants have not been reported before, which expanded the spectrum of mutations. Because TSC remains a treatable disease by using rapamycin and its derivatives and most patients appear initial symptoms at an early age. Earlier genetic diagnosis is essential for promoting early intervention and management for TSC patients, which may decrease harmful effects of the disease.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Declaration of Competing Interest

All authors declare that they had no conflict of interest.

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