Identification of a novel *TSC2* pathogenic frameshift insertion causing tuberous sclerosis complex, an inherited tumor syndrome in a sporadic case

Punyaapa Srikirin[1], Thawornchai Limjindaporn[1], Chanin Limwongse[2,3,4], Manop Pithukpakorn[2,3,4], Ekkapong Roothumnong[3], Yupaporn Lalerd[2], Wanna Thongnoppakhun[2,4] and Chairat Turbpaiboon[1*]

[1]*Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.*
[2]*Division of Medical Genetics Research and Laboratory, Research Department, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.*
[3]*Division of Medical Genetics, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.*
[4]*Siriraj Center of Research Excellence in Precision Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.*

**Corresponding author: cturbpaiboon@hotmail.com*

ABSTRACT

Tuberous Sclerosis Complex (TSC) is a hereditary syndrome of multi-organ hamartomas or benign tumorigenesis with autosomal dominant mode of transmission. The tumorigenesis in this syndrome was discovered for its etiology as perturbed function of the mechanistic or mammalian target of rapamycin (mTOR) pathway. The hyperactivated function of mTOR pathway caused by the loss of upstream negative regulators or heterotrimeric tumor suppressor complex (hamartin-tuberin-TBC1D7) was found to be the molecular pathogenesis in this syndrome. Hamartin-coding *TSC1* and tuberin-coding *TSC2* loci are therefore the candidates for identification of disease-causing variant in TSC patients. In addition to the clinical manifestations, the presence of mutation in the causative genes of TSC with confirmed pathogenicity can be considered as one criterion for TSC definite diagnosis. As the prevalence of mutations found in *TSC2* is higher than that in *TSC1*, the molecular approach in this study was designed for detecting the variant in the coding sequence of *TSC2* by using the nuclease assay to determine the existence of mismatched base pairing of the amplicon in a 63-year-old Thai male patient with sporadic but definite TSC. After screening the whole coding sequence of *TSC2*, the sequence analysis of the suspected amplicon by Sanger sequencing revealed a novel frameshift, single-nucleotide insertion (NM_00548.5:c.1572dupC) in exon 15, leading to premature termination of coding region (p.Asn525GlnfsTer64). This pathogenic frameshift mutation causes the extremely truncated tuberin protein (587 compared with 1,807 amino acid residues of the wild-type) with mutated C-terminal domains (residues 525-587). This finding additionally provides better understanding of the TSC genotype-phenotype correlation when compared with previous reports of TSC patient. The approach for variant screening in the whole *TSC2* coding sequence used in this study is suggested to the routine laboratory service for molecular genetic analysis of TSC patients as another appropriate method of choice in practice.

Keywords: tuberous sclerosis complex; *TSC2*; tuberin; nuclease assay

INTRODUCTION

Tuberous Sclerosis Complex (TSC) is a hereditary syndrome of multi-organ hamartomas or benign tumorigenesis with autosomal dominant mode of transmission (Crino *et al.*, 2006). It was previously recognized as Bourneville disease after the report of Dr. D. M. Bourneville in 1880 about the brain appearance in the focal sclerotic areas. However, its organ involvement can be found outside the central nervous system, e.g. skin (or oral cavity), heart, lung, kidney or even eye (Northrup *et al.*, 2013). According to the diagnostic criteria

Received 7 April 2021, Revised 29 May 2021, Accepted 9 June 2021

established from The 2012 International Tuberous Sclerosis Complex Consensus Conference, the skin (or oral cavity) manifestations found in TSC patients are quite diverse when compared with other visceral organ/system involvements, for example, hypomelanotic macule, angiofibroma, ungual fibroma, shagreen patch, confetti skin lesion, dental enamel pit and intraoral fibroma. Nonetheless, the tumor growth at the visceral organs not only causes the disorder due to its mass effect but also gives rise to the physiological disturbance of that tissue/organ function. Only certain types of visceral manifestation are included in the diagnostic criteria, e.g. cortical dysplasia (brain), subependymal nodule (brain), subependymal giant cell astrocytoma or SEGA (brain), rhabdomyoma (heart), lymphangioleiomyomatosis (lung), angiomyolipoma (kidney) and renal cyst. In addition, the presence of mutations in the causative genes of TSC with confirmed pathogenicity can be considered as one criterion for TSC definite diagnosis. The neurological manifestation seems to cause significant morbidity in TSC patients, including developmental cortical dysplasia (tuber), seizure, delayed development, intellectual disabilities and autism (Hasbani and Crino, 2018). The incidence of TSC cases is about 1:6,000-10,000 without predominate gender or ethnicity (O'Callaghan *et al.*, 1998; Hong *et al.*, 2016). It also shows the variable penetrance (almost complete) and expressivity in phenotype (Uysal and Sahin, 2020).

The tumorigenesis of this syndrome was discovered for its etiology as perturbed function of the mechanistic or mammalian target of rapamycin (mTOR) pathway (Salussolia *et al.*, 2019). This pathway is normally responsible for controlling cellular metabolism, growth, and proliferation under both intracellular signals of cellular nutrients and energy and extracellular signals of growth factors. The hyperactivated function of mTOR pathway caused by the loss of upstream negative regulators (tumor suppressor) was found to be the molecular pathogenesis of this syndrome. This tumor suppressor protein complex is composed of 3 subunits which are hamartin (or TSC1, 1164 residues, 130 kDa), tuberin (or TSC2, 1807 residues, 200 kDa), and TBC1D7. The key domain for inhibiting downstream mTOR function is located at the C-terminal part of TSC2 called GTPase-activating protein (GAP) domain whereas TSC1 mainly functions in the TSC complex formation to maintain TSC2 stability (Huang *et al.*, 2008; Santiago Lima *et al.*, 2014; Gai *et al.*, 2016). *TSC1* and *TSC2* genes were identified as causative genes in TSC patients and mapped on chromosomal regions of 9q34.13 and 16p13.3, respectively (European Chromosome 16 Tuberous Sclerosis, 1993; van Slegtenhorst *et al.*, 1997). The *TSC1* gene spans 53,286 bp and comprises 23 exons with the coding sequence starts in exon 3 and ends in exon 23 (Ali *et al.*, 2003) while the *TSC2* gene spans 40,826 bp and being composed of 42 exons with only the first exon as a noncoding sequence (Ekong *et al.*, 2016). Although familial autosomal dominant inheritance is described in TSC, sporadic cases with *de novo* mutations in *TSC* genes are frequently observed. Haploinsufficiency of either *TSC* genes in the animal brain tissue was demonstrated for its tissue pathogenicity (Tavazoie *et al.*, 2005; Goorden *et al.*, 2007; Ehninger *et al.*, 2008). On the other hand, either germline or somatic mutation in one allele of either *TSC* genes causes the first hit according to the two-hit hypothesis which is followed by the second-hit mutation in the other allele of the affected somatic cell, leading to total loss of function and developing tumorigenesis (Henske *et al.*, 2016). Wide range of mutational spectrum identified in *TSC1* and *TSC2* genes has been reported on Leiden Open Variation Database from small variant as single nucleotide to large deletion extending to adjacent loci (Fokkema *et al.*, 2011).

The aim of this molecular genetic study is to search for the pathogenic variant in *TSC* loci in a sporadic case of clinically diagnosed patient with TSC visiting Siriraj Hospital, Bangkok, Thailand as the diagnosis based on only clinical manifestation cannot be definitely confirmed. Prevalence of mutations reported in TSC patients (both familial and sporadic cases) is greater in *TSC2* than in *TSC1*, therefore the *TSC2* locus of the patient in this study was focused for its mutational existence (Crino *et al.*, 2006).

MATERIALS AND METHODS

Patient characteristics

The studied case is a 63-year-old (year of birth: 1958) Thai male patient with epilepsy. His first visit at Siriraj Hospital was in 2013. His physical examination revealed the skin lesion of adenoma sebaceum (angiofibromas) at his nose and face, ash-leaf spots at his abdomen and hypopigmented lesions at his leg and back. His brain imaging showed a small focal lesion with hypodensity at left frontal periventricular white matter and multiple round calcification at bilateral periventricular white matter, bilateral caudate nuclei and right inner temporal lobe. A large heterogeneous liver

mass (12.2 x 9.1 cm) at hepatic dome was found. Both kidneys with small size and bilateral multiple renal cysts were demonstrated. There is no family history reported. Albeit sporadic, he was clinically diagnosed as a definite TSC case requiring a molecular diagnostic confirmation. His genomic DNA (gDNA) was extracted from collected venous blood for genetic analysis. This study was approved by Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University (COA no. *Si*343/2016). Informed consent for genetic studies was acquired from the participant.

DNA extraction and amplification

Peripheral leukocytes from the patient's venous blood sample was used for gDNA extraction by Puregene® Blood Core Kit, QIAGEN® according to a manufacturer's protocol (QIAGEN Sciences, USA). The concentration of purified gDNA sample was measured by spectrophotometric analysis using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., USA). and the sample was then diluted to the concentration of 25 ng/µL by TE buffer before being stored at 4°C.

Forty-one coding exons of the *TSC2* gene were amplified by polymerase chain reaction (PCR) in the total volume of 10 µL under the same condition together with at least 10-base-pair flanking noncoding sequence. PCR reagents contained 50 ng of gDNA template, 0.2 mM deoxynucleoside triphosphate (dNTP), 3.75 pmol of each primer (as shown in table 1), 0.2 unit of Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific Inc., USA) and Phusion GC Buffer (Thermo Fisher Scientific Inc., USA)**.** Touchdown approach was used as temperature profile of PCR amplification in the C1000 Touch, PCR Thermal Cycler (Bio-Rad Laboratories Inc., USA). The condition was started with initial denaturation step at 98°C for 1 minute, followed by 14 cycles of denaturation at 98°C for 10 seconds, annealing at 72°C with 0.5°C decrease per cycle for 10 seconds, and extension at 72°C for 15 seconds, then other 26 cycles with fixed annealing temperature at 65°C and the final extension step at 72°C for 10 seconds. Amplified PCR products were examined by 1.5% (w/v) agarose gel electrophoresis.

Table 1 Specific PCR primers for all coding exons of *TSC2* gene with their sizes of PCR amplicon.

Exon	Amplicon size (bp)	*Primer sequences (5' to 3')	
2	363	F-	ggaggtccgcagtggggaagg
		R-	ctggagacagcattcctgtgt
3	190	F-	aggagaccgtggcctgagcact
		R-	tctgaatagtctacgtgcctct
4	216	F-	cagggttcttggagagcaca
		R-	gacagtcagtgggcagcct
5	241	F-	gtgtgggcgacgctggcagg
		R-	ttgatcgtcaaggccagaga
6	294	F-	actgatgatggggtttctggc
		R-	cactgcggagctgaacttagg
7	142	F-	ggccatccaggcagtgctg
		R-	gaaaccagggtgaaatggg
8	326	F-	ctgggtgtcctctcctgtg
		R-	gacactggccgtccctcaa
9	156	F-	agggcttatgcctgccag
		R-	tggcctccactgccctgc
10	421	F-	tctgttccctgcccttccc
		R-	cccagctgcaaagcaactg
11	468	F-	ccccacctgctgtttctgc
		R-	cagacctgtctccggtgga

Table 1 Specific PCR primers for all coding exons of *TSC2* gene with their sizes of PCR amplicon. (cont.)

Exon	Amplicon size (bp)	*Primer sequences (5' to 3')	
12	523	F-	agctgccatcacagccact
		R-	ggcgcccacctggcccttg
13	238	F-	ctctcgaccagcagcccagt
		R-	cagacagagacagggccagg
14	203	F-	caggcagacgggctggtgtg
		R-	ccacggagaaatagccctga
15	380	F-	gctgtgctgaagtcccgagg
		R-	ccatccggtcactcgaagag
16	311	F-	gtgttctcacggctgctgac
		R-	ccgcaccctcagcaaatcca
17	344	F-	ggcccctgctccgggacaag
		R-	agtgggtgcagaggagggac
18	466	F-	gcctgtgtctgtgttgggat
		R-	gccgctggtcttcttctcag
19	517	F-	cacggcccatgaggctcag
		R-	cagcgtcaagggctatgga
20	360	F-	ccatagcccttgacgctgtg
		R-	gtgcagctgagtttgaggga
21	400	F-	aaggctccccagcccctttt
		R-	gcagccacccagggaagca
22	575	F-	ctgtgggatcgtgtcggaat
		R-	gccaggaaggagagcactca
23	262	F-	agccgtgttggccttcagag
		R-	ggcgctcaggccttggggac
24	292	F-	tcatgccctggggatgtttc
		R-	caggcacagccccaaacacc
25	324	F-	tgcccctagcctgcagcttg
		R-	tactccagggcacacaggac
26	283	F-	ggcttgttctccccttccc
		R-	gtcatgcaagcagccccat
27	312	F-	tgagctttggcccttggtg
		R-	ggagcgtgaaacccagctt
28	346	F-	tcacggctgtcccgaagag
		R-	agtccccaggctggtacga
29	255	F-	ggcccacgtggcaccctcgt
		R-	tcctgaacactgggaccagc
30	386	F-	cgggggggagcattcagctt
		R-	cccaagagggccaagtctg
31	428	F-	cagcgctggctccgacatc
		R-	aggagccacattgccgtca
32	257	F-	cacggggcctgtgctctctg
		R-	caatggaggcagacggaccat

Table 1 Specific PCR primers for all coding exons of *TSC2* gene with their sizes of PCR amplicon. (cont.)

Exon	Amplicon size (bp)	*Primer sequences (5' to 3')	
33	230	F-	cctggcccagccccacatcc
		R-	agccctgcctcccctaagga
34 (1st)	525	F-	gcctggtgctcgggctggt
		R-	ggccgagtcggagatggtg
34 (2nd)	474	F-	cccatcgagcgagtcgtct
		R-	aggttcccgcaggagaggt
35	185	F-	ggctctgtgttcctccctgt
		R-	cggatgcaggagagggaggc
36	206	F-	tggacgggcgtctggggctc
		R-	ctccctacccactgcaggct
37	405	F-	tgctggaatggatggtcttg
		R-	ccagggcgtggggtagcagg
38	365	F-	ccagagcccctggagtaatc
		R-	ccctgagcactgcggcctct
39	268	F-	cccatggagctgacaggtgt
		R-	cgccaggcccagggctcttg
40	263	F-	gccgggtggggccctgcagt
		R-	tgcacctgtgaggccatctg
41	329	F-	gccctgcacgcaaatgtgag
		R-	tcgcagatctgaaggcagag
42*	314	F-	gccacgcctcccagacttactg
		R-	gactgcaatctgtgcctctatgt

*The primers of exons 2-41 were newly designed in this study and primers of exon 42 were published elsewhere (Choi *et al.*, 2006).

Coding sequence screening by Surveyor® Nuclease Assay and direct sequencing

Mutation analysis in *TSC2* coding sequence based on Surveyor nuclease assay (CEL-1) was performed using the Surveyor® Mutation Detection Kit (Transgenomic, Omaha, NE, USA) to screen any mismatched site in the duplex strands of each amplified PCR product. To generate the DNA heteroduplex, the amplified PCR product was denatured at 95°C for 5 minutes and reannealed by decreasing 1°C for every 15 seconds to 85°C, followed by decreasing 0.5°C for every 15 seconds to 25°C and finally hold at 4°C. Reagents for the nuclease assay contained 4 µL of amplified PCR products, 0.2 µL of Surveyor® nuclease, 0.2 µL of Surveyor® enhancer, 0.3 µL of Surveyor® cofactor and 0.3 µL of 1X Surveyor® buffer. The assay was performed at 42°C for 60 minutes and then terminated by using 0.5 µL of stop solution. Samples stored at -20°C was detected for the reaction products by using the Microchip Electrophoresis System, MCE (MultiNA, Shimadzu, Kyoto, Japan). Any amplicon carrying putative variant demonstrated by MCE was subjected to Sanger sequencing for variant determination using Bigdye™ Terminator v.3.1 reaction mix with Applied Biosystems 3130XL Genetic Analyzer (Applied Biosystems, USA). Prior to direct sequencing, excess dNTPs and primers from the PCR amplification were enzymatic removed using ExoSAP-IT® (Affymetrix, Ohio, US). Sequencing primers of each amplicon were the same set as used in the PCR amplification. Variant existence in all amplicons was determined from the sequencing electropherogram by comparing with the reference sequence (RefSeq: NG_005895 and NM_000548.5).

RESULTS AND DISCUSSION

After screening 41 coding exons of the *TSC2* gene of the studied case by using Surveyor® Nuclease Assay, only an amplicon of exon 15 (380 bp) revealed a

positive result of heteroduplex existence, implying the mismatched base pairing. As shown in **Figure 1**, the first electropherogram (the studied case) demonstrated the mixture of cleaved (heteroduplex) and uncleaved (homoduplex) amplicons (approximately 127/252 and 363 bp, respectively), suggesting the heterozygosity of this amplicon. The other three electropherograms were the negative/uncleaved control of exon 15 amplicon of the wild-type *TSC2*, positive/cleaved control 1 (*RET10*) and the positive/cleaved control 2 (*RET11*). This suspected amplicon of *TSC2* was then analyzed for its variant existence by direct sequencing (**Figure 2**) which showed the heterozygous frameshift mutation caused by the cytosine insertion between the 1572nd and 1573rd coding nucleotides in exon 15 (NM_00548.5:c.1572dupC), leading to premature termination of coding region at the 63rd codon downstream of the mutated codon (p.Asn525GlnfsTer64). According to the classification of variant pathogenicity based on the 2015 ACMG-AMP standards and guidelines (Richards *et al.*, 2015) and the use of bioinformatics tools (Li *et al.*, 2017; Kopanos *et al.*, 2019), *TSC2* (NM_00548.5):c.1572dupC, p.Asn525GlnfsTer64 is interpreted to be a pathogenic variant. Several pieces of evidence supported this include (1) PVS1: the nature of the variant is null where loss of function (LOF) in the tumor suppressor gene, *TSC2* is a known mechanism of TSC *(TSC2* has 552 pathogenic LOF variants and LOF Z-Score = 8.37 is greater than 0.7); (2) PM2: the variant is absent from controls in all available databases (Exome Sequencing Project, 1000 Genomes Project, Exome Aggregation Consortium, gnomAD exomes, or gnomAD genomes); and (3) PM6: the variant is assumed *de novo*, but confirmation of paternity and maternity is not available. Additionally, one more supported evidence is the highly specific patient's phenotype for TSC (PP4).

This novel *TSC2* pathogenic frameshift mutation causes the extremely truncated tuberin protein (587 compared with 1,807 amino acid residues of the wild-type) with mutated C-terminal domains (residues 525-587). The altered C-terminal structure of this mutated protein, carrying the GAP domain could affect its physiologic function in the tumor suppressor protein complex, leading to the hyperactivated mTOR pathway and tumorigenesis. According to the clinical history of the studied case, one of the major pathologic findings is the neurological manifestation, e.g. epilepsy which can be in part explained by the structural lesions found in the brain. The dysregulated mTOR pathway has been studied for its macroscopic and microscopic effect on many processes of the nervous development from the early stem cell differentiation in nervous tissue till the late synaptic formation (Magri *et al.*, 2011; Costa *et al.*, 2016). In the presence of cortical tuber, seizure is assumed as its clinical consequence due to the upregulated mTORC1 and reduced mTORC2 activities (Fu *et al.*, 2012; Ruppe *et al.*, 2014).

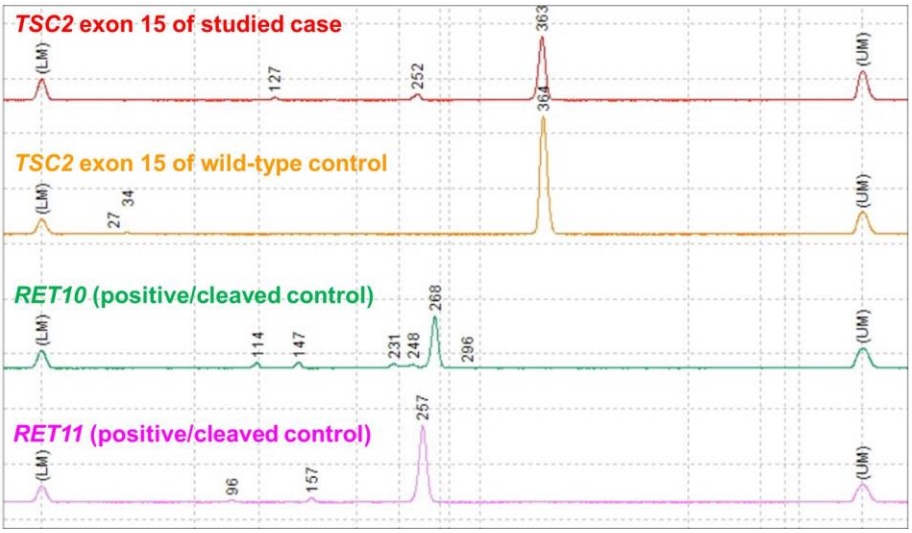

Figure 1 The electropherograms of amplicons from Surveyor® Nuclease Assay: the first as *TSC2* exon 15 amplicon of the studied case, the second as the negative/uncleaved control of exon 15 amplicon of the wild-type *TSC2*, the third as the positive/cleaved control 1 (*RET10*) and the fourth as the positive/cleaved control 2 (*RET11*).

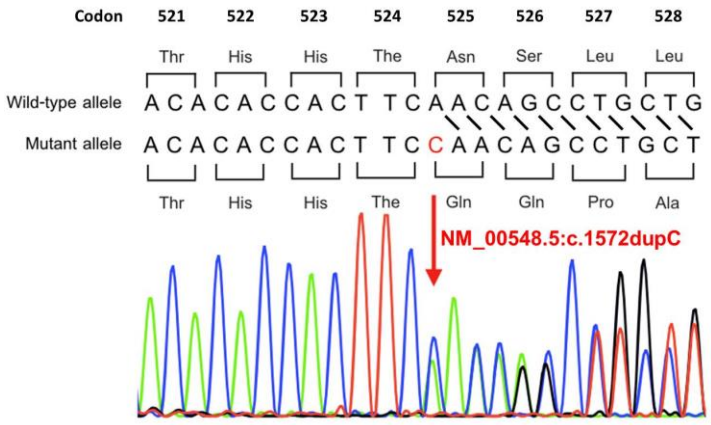

Figure 2 Sequence analysis of *TSC2* exon 15 amplicon by direct sequencing showing a heterozygous frameshift mutation caused by a cytosine insertion (red arrow) between the 1572nd and 1573rd coding nucleotides (NM_00548.5:c.1572dupC).

As this mutation was identified only in a sporadic case in the family, this study confirms the significant prevalence of *de novo TSC2* mutations. The ratio of sporadic to familial TSC cases (either with *TSC1* or *TSC2* mutations) is about 2:1 (Au *et al.*, 2007). This mutation can be recognized as the first hit in Knudson two-hit hypothesis as it is the germline pathogenic variant (Knudson, 1971). Some clinical manifestations follow this hypothesis in their pathogenesis, e.g. renal angiomyolipoma, facial angiofibroma, and SEGAs while certain types of brain pathology would rather be explained by haploinsufficiency (Chan *et al.*, 2004; Tyburczy *et al.*, 2014; Giannikou *et al.*, 2016; Martin *et al.*, 2017).

Spectrum of loss-of-function variants found in *TSC1*/*TSC2* ranges from small-sized mutations, e.g. single nucleotide, small insertion-deletion (indels) to large-sized deletions which may span the loci nearby (Kozlowski *et al.*, 2007; Fokkema *et al.*, 2011). Insertions and deletions are the most frequently reported mutation and account for about one-third of total reported mutations identified in *TSC2*. The distribution of mutations found in *TSC2* is scattered in all exons with the common areas found in exons 17, 24, 34 and 41, especially the downstream region coding the C-terminal GAP domain of tuberin protein (Salussolia *et al.*, 2019). However, lots of pathogenic variants other than the ones in hotspot areas have been identified only once in *TSC2*. Compared with *TSC1* mutations, *TSC2* mutations were observed to be more common in TSC patients with more severe manifestation (Curatolo *et al.*, 2015; Avgeris *et al.*, 2017). This can be explained by certain observations. Prevalence of mutations found in *TSC1* (one third) is lower than in *TSC2* (two thirds) and cellular consequence from single-allele loss of *TSC1* on the tumor suppressor protein complex is less severe than from the *TSC2* loss (Zeng *et al.*, 2011). However, they are not definitely associated in all TSC patient cases as the clinical phenotype in TSC patients is known for its diverse manifestation even observed among the same causative variant. Therefore, there is no significant correlation between genotype and phenotype in TSC patients.

For the studied case, the patient has the multi-organ/system involvement indicating the clinical diagnosis of definite TSC. Together with the mutation detected in the *TSC2* locus of this patient, this finding again confirms the observation of severe manifestation in TSC patient with *TSC2* mutation (Curatolo *et al.*, 2015; Avgeris *et al.*, 2017). In order to compare the genotype-phenotype correlation of the studied case with other reported TSC patients with similar mutations in *TSC2*, one of the common mutations causing truncated tuberin proteins with the approximate size of 500-600 residues is the nonsense mutation (NM_000548.5:c.1513C>T) at codon 505 (p.Arg505Ter) (Wilson *et al.*, 1996). Similar to the novel mutation detected in this study (p.Asn525GlnfsTer64), this truncated tuberin protein (p.Arg505Ter) still contains the N-terminal domain of hamartin interaction with the absence of C-terminal GAP domain. However, the reported clinical manifestation of the TSC patient carrying this nonsense mutation (NM_000548.5:c.1513C>T) shared some clinical signs and symptoms of the integumentary (adenoma sebaceum

and subungual fibroma) and nervous (positive finding in brain imaging and epilepsy) systems which are comparable to the patient case in this study. Certain phenotypic findings of the studied case were reported for their prevalence in the study of TSC patient cohort with tuberin protein truncation mutations (Au *et al.*, 2007): hypomelanotic macules, facial angiofibromas (adenoma sebaceum), epilepsy, and renal cysts at the prevalence of 96.2%, 73.3%, 84.3% and 34.2%, respectively. Therefore, the phenotypes caused by this novel mutation (NM_00548.5:c.1572dupC) can be considered to be consistent with other mutations sharing the same molecular pathogenesis.

For about 15% of TSC cases, there is no mutation identified (NMI) in either *TSC1* or *TSC2* locus by using conventional molecular methods. Some of this can be explained by the mosaicism phenomenon (Jones *et al.*, 1999; Dabora *et al.*, 2001), epigenetic regulation on the *TSC* locus expression (Patursky-Polischuk *et al.*, 2014; Dombkowski *et al.*, 2016) and disease-causing variants in non-*TSC* loci. Nonetheless, it is still important to establish a standard genetic testing because the presence of pathogenic variants in the *TSC* loci is considered as one criterion for making the diagnosis. This can much help in the cases of patient with inconclusive clinical findings.

Once the diagnosis has been made in this patient group, further proper clinical surveillance and management according to the prognosis can be achieved. Moreover, this can be beneficial for the prospective parents whose family planning requires appropriate genetic counseling. In case of the existence of mutation in their first affected child, such mutation needs to be verified in these parents. Recurrence risk in the second child is about 50% if this mutation is found in either of the parents but the risk should be reduced to 1-2% in case of no parental identification of this mutation. In addition, reliable approach of genetic analysis is necessary for either preimplantation or prenatal diagnosis in the suspected family. As conventional direct sequencing of all *TSC1*/*TSC2* exons including exon-intron boundaries and deletion-duplication study can provide about 75-90% yield of mutation detection, the current next generation sequencing (NGS) with targeted *TSC1*/*TSC2* panels is suggested for better yield of pathogenic variant detection, especially in certain conditions (mosaicism and splicing-affecting intronic variant) (Nellist *et al.*, 2015; Tyburczy *et al.*, 2015). By any approach, sufficient collected information from the genetic study will further help establish the genotype-phenotype correlation in the TSC patients even though this is not clearly observed at the moment.

In conclusion, this study reported a novel *TSC2* pathogenic frameshift insertion (NM_00548.5:c.1572dupC), leading to premature termination of coding region (p.Asn525GlnfsTer64) causing TSC in a Thai patient**.** Together with the current TSC genotype**-**phenotype information from previous studies, this finding provides better understanding of the TSC genotype-phenotype correlation. In addition, the screening method of the whole *TSC2* coding sequence using nuclease assay for detecting mismatched base pairing in this study is practically appropriate for batch analysis of TSC patients in routine laboratory service before direct Sanger sequencing of only the suspected exon for definite mutation identification. It requires only the simple heteroduplex generation by using conventional thermal cycler and then typical enzymatic incubation which products are detected by the automated system of MCE, thus providing a platform of batch screening of both multiple exons and multiple cases in the same time.

ACKNOWLEDGEMENTS

TL, CL, MP, WT and CT are supported by the Siriraj Chalermphrakiat Grant, Faculty of Medicine Siriraj Hospital, Mahidol University. CT is also supported by the Talent Management Project, Mahidol University. This research project is supported by Siriraj Research Fund, Faculty of Medicine Siriraj Hospital, Mahidol University.

REFERENCES

Ali M, Girimaji SC, Kumar A. Identification of a core promoter and a novel isoform of the human *TSC1* gene transcript and structural comparison with mouse homolog. Gene. 2003;320:145-154.

Au KS, William AT, Roach ES. Genotype/phenotype correlation in 325 individuals referred for a diagnosis of tuberous sclerosis complex in the United States. Genet Med. 2007;9(2):88–100.

Avgeris S, Fostira F, Vagena A, Ninios Y, Delimitsou A, Vodicka R, Vrtel R, Youroukos S, Stravopodis DJ, Vlassi M, *et al*. Mutational analysis of *TSC1* and *TSC2* genes in Tuberous Sclerosis Complex patients from Greece. Sci Rep 2017;7(1):16697.

Chan JA, Zhang H, Roberts PS, Jozwiak S, Wieslawa G, Lewin-Kowalik J, Kotulska K, Kwiatkowski DJ. Pathogenesis of tuberous sclerosis subependymal

giant cell astrocytomas: biallelic inactivation of *TSC1* or *TSC2* leads to mTOR activation. J Neuropathol Exp Neurol. 2004;63(12):1236-1242.

Choi JE, Chae JH, Hwang YS, Kim KJ. Mutational analysis of *TSC1* and *TSC2* in Korean patients with tuberous sclerosis complex. Brain Dev. 2006;28(7):440-446.

Costa V, Aigner S, Vukcevic M, Sauter E, Behr K, Ebeling M, Dunkley T, Friedlein A, Zoffmann S, Meyer CA, *et al*. mTORC1 inhibition corrects neurodevelopmental and synaptic alterations in a human stem cell model of tuberous sclerosis. Cell Rep. 2016;15(1):86-95.

Crino PB, Nathanson KL, Henske EP. The tuberous sclerosis complex. N Engl J Med. 2006;355(13):1345-1356.

Curatolo P, Moavero R, Roberto D, Graziola F. Genotype/phenotype correlations in tuberous sclerosis complex.Semin Pediatr Neurol. 2015;22(4):259-273.

Dabora SL, Jozwiak S, Franz DN, Roberts PS, Nieto A, Chung J, Choy YS, Reeve MP, Thiele E, Egelhoff JC, *et al*. Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1, disease in multiple organs. Am J Hum Genet. 2001;68(1):64-80.

Dombkowski AA, Batista CE, Cukovic D, Carruthers NJ, Ranganathan R, Shukla U, Stemmer PM, Chugani HT, Chugani DC. Cortical Tubers: Windows into Dysregulation of Epilepsy Risk and Synaptic Signaling Genes by MicroRNAs. Cereb Cortex. 2016;26(3):1059-1071.

Ehninger D, Han S, Shilyansky C, Zhou Y, Li W, Kwiatkowski DJ, Ramesh V, Silva AJ. Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. Nat Med. 2008;14(8):843-848.

Ekong R, Nellist M, Hoogeveen-Westerveld M, Wentink M, Panzer J, Sparagana S, Emmett W, Dawson NL, Malinge MC, Nabbout R, *et al*. Variants Within TSC2 Exons 25 and 31 Are Very Unlikely to Cause Clinically Diagnosable Tuberous Sclerosis. Hum Mutat. 2016;37(4):364-370.

European Chromosome 16 Tuberous Sclerosis Consortium. Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell. 1993;75(7):1305-1315.

Fokkema IF, Taschner PE, Schaafsma GC, Celli J, Laros JF, den Dunnen JT. LOVD v.2.0: the next generation in gene variant databases. Hum Mutat. 2011;32(5):557-563.

Fu C, Cawthon B, Clinkscales W, Bruce A, Winzenburger P, Ess KC. GABAergic interneuron development and function is modulated by the Tsc1 gene. Cereb Cortex. 2012;22(9):2111-2119.

Gai Z, Chu W, Deng W, Li W, Li H, He A, Nellist M, Wu G. Structure of the TBC1D7-TSC1 complex reveals that TBC1D7 stabilizes dimerization of the TSC1 C-terminal coiled coil region. J Mol Cell Biol. 2016;8(5):411-425.

Giannikou K, Malinowska IA, Pugh TJ, Yan R, Tseng YY, Oh C, Kim J, Tyburczy ME, Chekaluk Y, Liu Y, *et al*. Whole exome sequencing identifies TSC1/TSC2 biallelic loss as the primary and sufficient driver event for renal angiomyolipoma development. PLoS Genet. 2016;12(8):e1006242.

Goorden SM, van Woerden GM, van der Weerd L, Cheadle JP, Elgersma Y. Cognitive deficits in *Tsc1*+/- mice in the absence of cerebral lesions and seizures. Ann Neurol. 2007;62(6):648-655.

Hasbani DM, Crino PB. Tuberous sclerosis complex. Handb Clin Neurol. 2018;148:813-822.

Henske EP, Jozwiak S, Kingswood JC, Sampson JR, Thiele EA. Tuberous sclerosis complex. Nat Rev Dis Primers. 2016;2:16035.

Hong CH, Tu HP, Lin JR, Lee CH. An estimation of the incidence of tuberous sclerosis complex in a nationwide retrospective cohort study (1997-2010). Br J Dermatol. 2016;174(6):1282-1289.

Huang J, Dibble CC, Matsuzaki M, Manning BD. The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. Mol Cell Biol. 2008;28(12):4104-4115.

Jones AC, Shyamsundar MM, Thomas MW, Maynard J, Idziaszczyk S, Tomkins S, Sampson JR, Cheadle JP. Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis. Am J Hum Genet. 1999;64(5):1305-1315.

Knudson AG, Jr. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A. 1971;68(4):820-823.

Kopanos C, Tsiolkas V, Kouris A, Chapple CE, Aguilera MA, Meyer R, Massouras A. VarSome: the human genomic variant search engine. Bioinformatics. 2019;35(11):1978-1980.

Kozlowski P, Roberts P, Dabora S, Franz D, Bissler J, Northrup H, Au KS, Lazarus R, Domanska-Pakiela D, Kotulska K, *et al.* Identification of 54 large deletions/duplications in *TSC1* and *TSC2* using MLPA, and genotype-phenotype correlations. Hum Genet. 2007;121(3-4):389–400.

Li Q, Wang K. InterVar: clinical interpretation of genetic variants by the 2015 ACMG-AMP guidelines. Am J Hum Genet. 2017;100(2):267-280.

Magri L, Cambiaghi M, Cominelli M, Alfaro-Cervello C, Cursi M, Pala M, Bulfone A, Garcia-Verdugo JM, Leocani L, Minicucci F, *et al*. Sustained activation of mTOR pathway in embryonic neural stem cells leads to development of tuberous sclerosis complex-associated lesions. Cell Stem Cell. 2011;9(5):447-462.

Martin KR, Zhou W, Bowman MJ, Shih J, Au KS, Dittenhafer-Reed KE, Sisson KA, Koeman J, Weisenberger DJ, Cottingham SL, *et al*. The genomic landscape of tuberous sclerosis complex. Nat Commun. 2017;8:15816.

Nellist M, Brouwer RW, Kockx CE, van Veghel-Plandsoen M, Withagen-Hermans C, Prins-Bakker L, Hoogeveen-Westerveld M, Mrsic A, van den Berg MM, Koopmans AE, *et al*. Targeted Next Generation Sequencing reveals previously unidentified *TSC1* and *TSC2* mutations. BMC Med Genet. 2015;16:10.

Northrup H, Krueger DA, International Tuberous Sclerosis Complex Consensus Group. Tuberous sclerosis complex diagnostic criteria update: recommendations of the 2012 International Tuberous Sclerosis Complex Consensus Conference. Pediatr Neurol. 2013;49(4):243-254.

O'Callaghan FJ, Shiell AW, Osborne JP, Martyn CN. Prevalence of tuberous sclerosis estimated by capture-recapture analysis. Lancet. 1998;351(9114):1490.

Patursky-Polischuk I, Kasir J, Miloslavski R, Hayouka Z, Hausner-Hanochi M, Stolovich-Rain M, Tsukerman P, Biton M, Mudhasani R, Jones SN, *et al*. Reassessment of the role of TSC, mTORC1 and microRNAs in amino acids-meditated translational control of TOP mRNAs. PLoS One. 2014;9(10):e109410.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, *et al*. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405-424.

Ruppe V, Dilsiz P, Reiss CS, Carlson C, Devinsky O, Zagzag D, Weiner HL, Talos DM. Developmental brain abnormalities in tuberous sclerosis complex: a comparative tissue analysis of cortical tubers and perituberal cortex. Epilepsia. 2014;55(4):539-550.

Salussolia CL, Klonowska K, Kwiatkowski DJ, Sahin M. Genetic etiologies, diagnosis, and treatment of tuberous sclerosis complex. Annu Rev Genomics Hum Genet. 2019;20:217-240.

Santiago Lima AJ, Hoogeveen-Westerveld M, Nakashima A, Maat-Kievit A, van den Ouweland A, Halley D, Kikkawa U, Nellist M. Identification of regions critical for the integrity of the TSC1-TSC2-TBC1D7 complex. PLoS One. 2014;9(4):e93940.

Tavazoie SF, Alvarez VA, Ridenour DA, Kwiatkowski DJ, Sabatini BL. Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. Nat Neurosci. 2005;8(12):1727-1734.

Tyburczy ME, Wang JA, Li S, Thangapazham R, Chekaluk Y, Moss J, Kwiatkowski DJ, Darling TN. Sun exposure causes somatic second-hit mutations and angiofibroma development in tuberous sclerosis complex. Hum Mol Genet. 2014;23(8):2023-2029.

Tyburczy ME, Dies KA, Glass J, Camposano S, Chekaluk Y, Thorner AR, Lin L, Krueger D, Franz DN, Thiele EA, *et al*. Mosaic and intronic mutations in *TSC1/TSC2* explain the majority of TSC patients with no mutation identified by conventional testing. PLoS Genet. 2015;11(11):e1005637.

Uysal SP, Sahin M. Tuberous Sclerosis Complex: A review of the past, present and future. Turk J Med Sci. 2020;50(SI-2):1665-1676.

van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, Verhoef S, Lindhout D, van den Ouweland A, Halley D, Young J, *et al*. Identification of the tuberous sclerosis gene *TSC1* on chromosome 9q34. Science. 1997;277(5327):805-808.

Wilson PJ, Ramesh V, Kristiansen A, Bove C, Jozwiak S, Kwiatkowski DJ, Short MP, Haines JL. Novel mutations detected in the TSC2 gene from both sporadic and familial TSC patients. Hum Mol Genet. 1996;5(2):249-256.

Zeng LH, Rensing NR, Zhang B, Gutmann DH, Gambello MJ, Wong M. *Tsc2* gene inactivation causes a more severe epilepsy phenotype than *Tsc1* inactivation in a mouse model of tuberous sclerosis complex. Hum Mol Genet. 2011;20(3):445-454.