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ORIGINAL ARTICLE

Mutational analysis by targeted sequencing in 11 cases of tuberous sclerosis

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Abstract

Tuberous sclerosis (TSC) is an autosomal dominant genetic disorder mainly caused by mutations in the *TSC1* and *TSC2* genes. In this study, we performed sequencing analysis of target genes in 11 clinically diagnosed cases, including 10 definite cases and one possible case. Genetic mutations were detected in 7 of 11 patients (64%) (*TSC1* in one case and *TSC2* in six cases), of whom two patients had novel *TSC2* mutations. One case had a three-nucleotide insertion mutation in the GTPase-activating protein domain and the other had a mutation causing a 56-nucleotide deletion in the 19th exon transcript, which were considered "likely pathogenic" and "pathogenic", respectively. No mutations were found in genes related to the mTOR pathway, including *AKT1*, *AKT2*, *AKT3*, and *PTEN*. Genetic analysis is a useful adjunct diagnostic tool for TSC.

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Key words: *TSC1*; *TSC2*; tuberous sclerosis complex; genotype-phenotype correlations; splice-acceptor site.

Introduction

Tuberous sclerosis (TSC) is a genetic disorder that causes benign tumors, called hamartomas, in various organs throughout the body, resulting in damage to various organs, including the brain, lungs, heart, and kidneys[1-3)]. Central nervous system symptoms, such as mental retardation, epilepsy, and autism, are particularly serious clinical manifestations of the disease, with an incidence of 1 in 6,000 to 10,000[1-3)]. It is an autosomal dominant genetic disorder; however, 60–70% of cases are sporadic owing to de novo mutations[1-4)].

TSC1, located on chromosome 9p34, and *TSC2*, located on 16p13.3 have been identified as causative genes. Because hamartin and tuberin function as functional complexes inside the cell, mutations in either gene can result in similar clinical presentations. Mutations in both genes vary from point mutations to large deletions, and the distribution of mutations is diverse, with no obvious hot spots. Mutations are detected in only 70% of cases with clinically confirmed diagnosis[5-7)]. Regarding the relationship between genetic variants and clinical symptoms, it has been reported that patients with *TSC1* mutations tend to have milder symptoms than those with *TSC2* mutations; however, clinical symptoms differ even among patients with the same genetic mutation, suggesting that the severity of the disease is not determined by genetic variants alone and that other factors are involved in the development of symptoms[5-7)].

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Although genetic analysis has been considered an adjunctive diagnosis, it could be useful for appropriate treatment and management, especially in mild or atypical cases. In this study, we analyzed the causative genes of clinically diagnosed TSC using targeted next-generation sequencing (NGS).

Patients and Methods

Patients

We conducted a retrospective causal gene analysis of 11 patients clinically diagnosed with TSC based on the international diagnostic criteria (TSC Clinical Consensus Guideline for Diagnosis, 2012) [8] at five hospitals: Hirosaki University Hospital, Aomori Prefectural Central Hospital, Aomori National Hospital, Aomori City Hospital, and Odate Municipal General Hospital. Causative genes were analyzed retrospectively in 11 patients with clinically diagnosed TSC based on international diagnostic criteria. This study was approved by the Ethics Committee of the Hirosaki University Graduate School of Medicine and was conducted after the patients or their guardians received a full explanation and written consent was obtained.

Methods

Extraction of nucleic acids

Genomic DNA was extracted from the patient's peripheral blood using a QIAamp Blood Mini Kit (QIAGEN, Germantown, MD, USA), and RNA was extracted using a GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Genetic mutational analysis

The *TSC1* and *TSC2* gene regions were amplified by polymerase chain reaction (PCR) using the oligonucleotides listed in Table 1. The amplified DNA strands were converted into a library for NGS using Nextera-XT (Illumina, San Diego, CA, USA), according to the manufacturer's instructions, and then sequenced using MiSeq (Illumina). The obtained sequence data were subjected to mutation analysis using the CLC Genomic Workbench (QIAGEN) targeting the translational regions of *TSC1* and *TSC2*. For cases in which mutations could not be identified in *TSC1* and *TSC2*, further mutational analysis was performed using the TruSight One sequencing panel (Illumina). Genomic DNA or cDNA was amplified by PCR using the oligonucleotides listed in Table 1 as templates and labeled by direct sequencing using the BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific). SeqStudio (Thermo Fisher Scientific) was used for analysis. The variants identified in this study were classified according to the guidelines of American College of Medical Genetics and Genomic (ACMG).

Analysis of RNA splicing using *TSC2* mini-gene

The *TSC2* gene (NM_000548.5) from the middle of exon 17 to the middle of exon 21 of wild type and patient 10 was cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), and the 5' and 3' flanking the start and end codons were incorporated into the 5' and 3' sides of the pcDNA3.1 vector. The expression vector was transfected into Baby Hamster Kidney fibroblasts cells (BHK-21) using FuGene HD (Promega, Madison, WI, USA) according to the manufacturer's instructions and RNA was extracted 24 h later.

Genome editing of the *TSC2* gene

The target sequence of the *TSC2* gene was determined using the Custom Alt-R CRISPR-Cas9 guide RNA (gRNA) design tool (Integrated DNA Technologies, Inc. Coalville, IA, USA). Single-stranded oligonucleotide (ssODN) sequences were also designed to

Table 1. Primer sequences or oligos used in this study

Primers for Nexera-XT analysis of the *TSC1* gene

Forward primer		Reverse primer		Product size (bp)
TSC1_1f	TATCCTTCCTTTCGAGAAGGAGGGGAG	TSC1_1r	GGCAAGATAATTCCCTCCCATCTTCCT	9351
TSC1_2f	GTAGAAACCCTCTTCATAAACTCGCCA	TSC1_2r	CACATTTTCAATCTCTCGAAAGATTCT	7030
TSC1_3f	ACCATTTTAAACAGGGAAGGTATAGGA	TSC1_3r	GTACTTCACAATAAAATGGACCATTTAAC	6360
TSC1_4f	CCTGGAGTTTGAAACATTCTTATGCCA	TSC1_4r	AACACACTGCGAGGTAAATGAGAGG	8598

Primers for Nexera-XT analysis of the *TSC2* gene

Forward primer		Reverse primer		Product size (bp)
TSC2_1f	GCTCCCAAGGCGGCCCTCCGCGCAAT	TSC2_1r_a	AAGTGCAAACCAGATCATCGGCAGTCA	2666
TSC2_1f_a	AGGGTTCTTGGAGAGCACATCCT	TSC2_1r	AGAGGACAAGGACTCTCTAAGCCAGTG	3994
TSC2_2f	GCGGACCCTGGGACAGGGCCCTGCTCACAT	TSC2_2r_a	TCAGAAAGCTGCACTTCACCCAGCACC	3399
TSC2_2f_a	GAGAAGGAGAGCGCCGGAGGGGCAGA	TSC2_2r	AACAGACTCCAACACAACGCAGATGC	3278
TSC2_3f	GGACTGCGTTTTCACCTCCTGC	TSC2_3r_a	TTATGAGAAAAATGTGGGGCCTACCTG	2640
TSC2_3f_a	TGCCTCTGCTGCAAGCGGGTGG	TSC2_3r	AACCTGCTGAGGGGCTGAGGGGTGTTA	2612
TSC2_4f2	GATCTCTCCATCCTGACCCTGTGGCCT	TSC2_4r_a	GTTGCTGCTGGTCCCAGTGTTCAGGAA	2267
TSC2_4f_a	TGGTTTTGCATCAGGTAAGTGGTGGT	TSC2_4r	AATGGAGGCAGACGGACCATGGGGGT	2499
TSC2_5f	GCCAGGGCCTGGCCCAGCCCCACATC	TSC2_5r_a	TTTGGGACGCAAAGCCACCAGCCCCAT	2927
TSC2_5f_a	GCACAGAGGGCCTCAGCACTGG	TSC2_5r_b	AATCTGTGCCTCTATGTCTGTGCAC	2034

Primers for preparation of *TSC2* mini-gene constructs

Forward primer		Reverse primer	
TSC2_Ex17_ins_f	CCAAGCTGaagcttGCC**ATG**GCAAGCCACGC CACGCGTGT	TSC2_Ex21_ins_r	ATCAGCGGctcgag**CTA**CTGTTTGGTTTTGTC CAGGT

Bold text indicates artificial translation start or terminal codons. Lowercase letters indicate the sequence of restriction enzyme sites.

Primers for reverse transcription polymerase chain reaction (RT-PCR) analysis of patient #9

Forward primer	Reverse primer		
TSC2_09_f1	AGCTCCACTACAAGCACAGCTA	TSC2_09_r1	CAAGGGGAAGTAAAGATGAGCA

Primers for RT-PCR analysis of patient #10

Forward primer	Reverse primer		
TSC2_10_f2	CACGGGTCTCAGGAGAGATACT	TSC2_10_r2	CTGTCTTCACATCCTCCAAGG

Target sequences and ssODN sequence for genome editing

NAME	Sequence	Description
TSC2-1	AGTCTGTTCCGTTCCTGCTG	Target sequence for wild-type *TSC2*
TSC2-2	TTCTTCTCAGAGCCTCTCTC	Target sequence for wild-type *TSC2*
TSC2-3	AAGTCTGTTCCGTTCCTGAG	Target sequence for mutant *TSC2* (c.1947-41_1956del51)
TSC-009 ssODN	GATGTCCCAGGGTTGGGAAGAGCCAAGTC TGTTCCGTTCCTGCTGCGGGGACTTGGCCT CA**AGAAGCAA**GGAGCCAGAGAGAGGCTCT GAGAAGAAGACCAGCGGCCCCCTTTCTCC TCC	ssODN sequence for *TSC2* TSC-009
TSC2_1947-1G_A_L ssODN	AGGGTTGGGAAGAGCCAAGTCTGTTCCGT TCCTGCTGCGGGGACTTGGCCTCAGCTGCT TCTCTTGCTTCTGCA**A**GGAGCCAGAGAGA GGCTCTGAGAAGAAGACCAGCGGCCCCCT TTC	ssODN sequence for *TSC2* 1947-1G>A

TSC2 c.1947-41_1956del51 is a mutation carried by HEK293 TSC2 int18del51 cells. The bold letters in the ssODN sequences indicate the mutated sequences introduced. ssODN; single-stranded oligonucleotide.

introduce the case 9 mutation and *TSC2* c.1947-1G>A mutation via homology-directed repair (HDR). The target sequences for genome editing and ssODN sequences are listed in Table 1. A total of 104 pmol of Cas9 Nuclease V3 (IDT), 120 pmol of gRNA, and 100 pmol of ssODN were

transfected into 3.5 × 10^5 HEK293 cells using a 4D-Nucleofector (Lonza, Cologne, Germany). During this time, the cells were cultured according to a previous report[9] to increase the efficiency of HDR. In fact, no HDR was observed, although genome editing occurred; therefore, we used TSC2-1 gRNA and TCS2-2 gRNA to cleave and bind at two sites at once to create a new gene from 41 bases on the 3' side of the 18th intron of *TSC2* to 10 bases on the 5' side of the 19th exon of TCS2-2 gRNA. TSC2 int18del51 cells were generated in bulk, in which 51 bases from 41 bases on the 3' side of the 18th intron of *TSC2* to 10 bases on the 5' side of the 19th exon of TSC2 were deleted. Subsequent genome editing of HEK293 TSC2 int18del51 cells with TSC2-3 gRNA and the respective ssODNs resulted in a high rate of the desired HDRs. Six days after gene transfer, cells were cloned using a limiting dilution.

Results

The clinical characteristics of the patients examined in this study are presented in Table 2. Ten cases (91.6%) were definite, and one case (8.3%) was suspected. The male-to-female ratio was 6:5 (54.5%:45.5%), and the age at the time of the genetic diagnosis test ranged from 3 to 28 years (median, 11 years).

The results of the mutational analysis and clinical features are presented in Table 3. *TSC1* mutation was found in one case (case 2), and *TSC2* mutation was found in six cases (cases 1, 3, 8, 9, 10, and 11). Regarding TSC-associated neuropsychiatric disorders (TAND), 6 patients had autism spectrum disorder (ASD), and 1 patient had attention deficit hyperactivity disorder (ADHD). Of note, all patients with severe intellectual disabilities had ASD.

Among *TSC2* mutations, cases 8 and 9 were novel mutations, c.4829_4831dupGGC, p.Trp1610_His1611insArg and c.1947-22_1947-1delinsAGAAGCAA, respectively. The mutation observed in case 9 was located in the vicinity of the 19th exon, suggesting that it may cause splicing defects. Transcripts derived from the patient's peripheral blood were amplified by reverse transcription PCR (RT-PCR) (Figure 1A, lane 2) and directly sequenced. As a result, a deletion of 56 base pairs (c.1947_2002del, p.Glu650Alafs*34) was detected (Figure 1B (b)). To confirm that the mutation found in the genome caused the deletion of 56 base pairs in the transcript, we performed genome editing of the *TSC2* gene in HEK293 cells and performed in vitro analysis using the *TSC2* mini-gene. By genome editing, we obtained 13 clones homozygous for the *TSC2* mutation in case 9. RT-PCR and sequencing were performed on ten clones, all of which showed an identical deletion of 56 bp. In addition, nine clones homozygous for TSC2 c.1947-1G>A, a typical splice mutation, were obtained. All showed identical splice aberration patterns, as determined by RT-PCR. In four of these clones, sequencing of the RT-PCR products confirmed an identical deletion of 56 bp. In all cases, a deletion of 56 bp was reproduced (Figure 2). The mutation observed in case 9 causing this splicing abnormality was also confirmed by mini-gene assay. (Figure 3) The mutation (c.1443+1G>T) observed in case 10 was previously reported[10, 11], however, its effect on transcripts had not been explored,

Table 2. Patient characteristics

Characteristics		n (%)
Total patients		11
Sex	Male	6 (54.5%)
	Female	5 (45.5%)
Age (in years)		
	Median	11
	Range	3–28
Diagnosis		
	Definite	10 (91.6%)
	Possible	1 (8.3%)

Table 3. Mutations in the *TSC1* and *TSC2* genes and clinical types

Patient	Diagnosis	Gene	Variant	Codon change	Neurological findings			Renal findings		Dermatological findings		Other major criteria		Epilepsy		TAND			Number of criteria	
					CD	SEN	SEGA	AML	Renal cyst	HM	AF	CM	RH	Intractable seizure	Infantile Spasms	ID	ASD	ADHD	Major	Minor
1	Definite	*TSC2*	c.5073_5076 delGGAG	p.Glu1692fs	+	+	−	−	−	+	−	+	−	+	+	+	−	−	4	
2	Definite	*TSC1*	c.2293C>T	p.Gln765*	+	+	−	−	−	+	+	−	−	+	−	+	−	+	4	
3	Definite	*TSC2*	c.1099C>T	p.Arg367Trp	+	+	−	−	+	+	+	−	+	+	−	+	+	−	5	1
4	Definite	−	−	unidentified	+	+	−	−	−	+	+	−	−	+	−	++	+	−	4	
5	Definite	−	−	unidentified	+	+	−	−	−	+	+	+	−	+	+	++	+	−	5	
6	Possible	−	−	unidentified	+	−	−	−	−	−	−	−	−	+	−	−	−	−	1	
7	Definite	−	−	unidentified	+	+	−	+	−	+	+	+	+	+	−	++	+	−	7	
8	Definite	*TSC2*	c.4829_4831 dupGGC	p.Trp1610_His 1611insArg	−	−	−	−	−	+	−	+	−	−	−	+	−	−	2	
9	Definite	*TSC2*	c.1947-22_ 1947-1 delins AGAAGCAA	−	+	+	−	+	−	+	+	−	+	+	+	++	+	−	6	
10	Definite	*TSC2*	c.1443+1G>T	−	+	+	+	−	+	+	+	+	+	+	−	+	−	−	7	
11	Definite	*TSC2*	c.5045T>C	p.Leu1682Pro	+	+	+	+	−	+	+	−	+	+	−	++	+	−	7	

TAND: Tuberous sclerosis-associated neuropsychiatric disorders; CD: cortical dysplasias; SEN:subependymal nodule; SEGA: subependymal giant cell astrocytomas; AML: renal angiomyolipomas; HM: hypomelanotic macules; AF: angiofibromas; ID:intellectual disabilities; ASD: autism spectrum disorder; ADHD; attention deficit hyperactivity disorder; CM: cardiac rhabdomyomas; RH:retinal hamartomas, major and minor criteria are shown in dark gray and light gray, respectively.

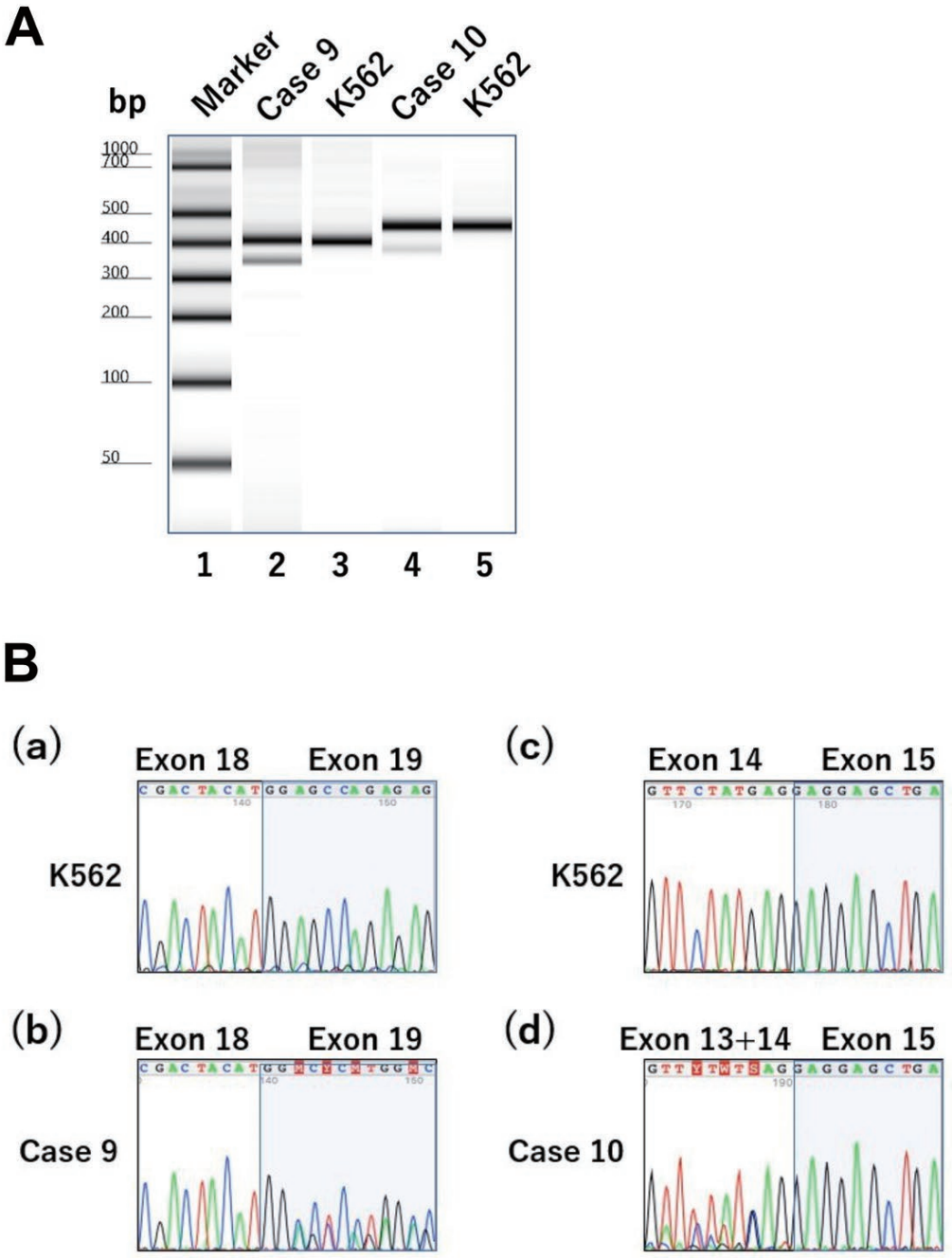

Figure 1 Analysis of TSC2 transcripts by RT-PCR in cases 9 and 10
A: The region containing the 18th and 19th or the 14th and 15th exons of *TSC2* was amplified by reverse transcription polymerase chain reaction (RT-PCR) using transcripts derived from the peripheral blood of patients 9, 10, or K562 as templates. The primers TSC2_09_f1, TSC2_ 09_r1, TSC2_10_f2, and TSC2_ 10_r2 (Table 1) were used for amplification. Short amplification products different from K562 were identified in the patient samples. B: Amplified products observed in 1a were analyzed by direct sequencing. Signals from the 15th and 19th exons are indicated by a blue background. Unlike K562 (a, c), duplicate signals were detected in the transcripts from patient samples. The deletions c.1947_2002del (b) and c.1362_1443del (d) were observed in cases 9 and 10, respectively.

therefore, we examined it by RT-PCR (Figure 1A, lane 4) and confirmed that the 14th exon was deleted (c.1362_1443del) (Figure 1B (d)).

Cases 4, 5, 6, and 7, in which no mutations were found in *TSC1* and *TSC2*, were analyzed by targeted sequencing using the TruSightOne sequencing panel. However, genes related to the

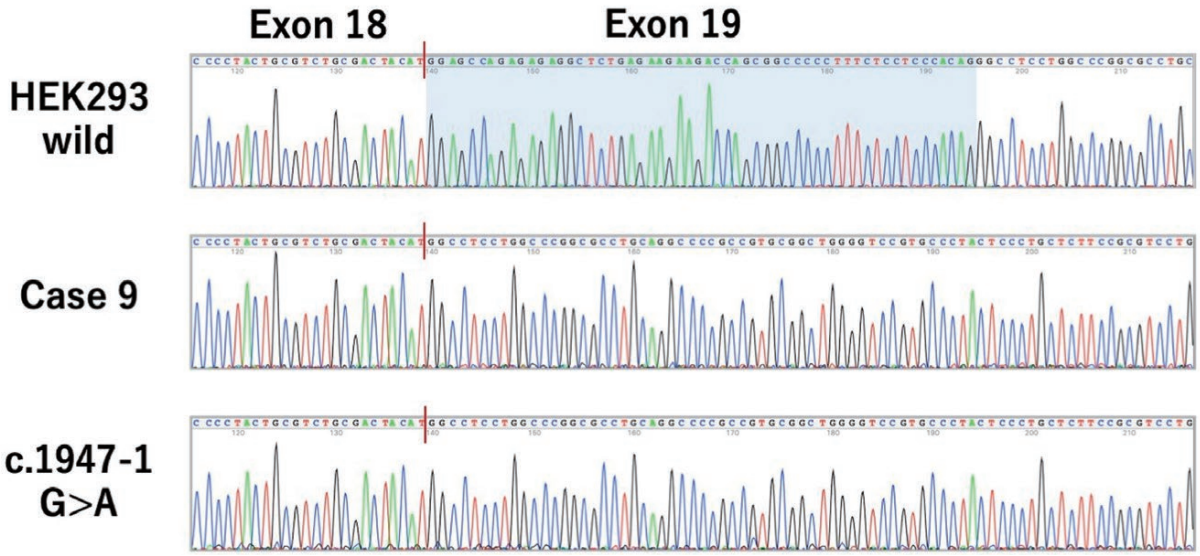

Figure 2 Verification of splicing mutations by genome editing of the *TSC2* gene
HEK293 cells were transfected with either the c.1947-22_1947-1delinsAGAAGCAA mutation observed in case 9 or the typical splicing mutation c.1947-1G>A using the CRISPR/Cas9 system. In clones harboring the mutation observed in case 9 or the c.1947-1G>A mutation, c.1947_2002del was detected, and the sequence found in the wild-type (blue background) was deleted. The red line indicates the linkage between exons.

mTOR system, including *AKT1*, *AKT2*, *AKT3*, and *PTEN*, were not mutated.

Discussion

In this study, we performed genetic analysis of 11 patients with clinically diagnosed TSC and detected causative gene mutations in 7 of 11 patients (64%), including two cases with novel gene mutations, one case with *TSC1* gene mutation, and six cases with *TSC2* gene mutation (Table 3).

The detection rate of the causative gene in the present study was similar to that reported previously. According to previous studies, the detection rate of NGS is approximately 54%[4]. Recently, the use of next-generation sequencers in genetic testing for TSC has been reported to be useful in terms of cost, labor, and time compared to conventional methods and to improve the detection rate when combined with other testing methods[5-7]. Regarding the ratio of *TSC1* to *TSC2* mutations detected, the ratio of *TSC2* was as high as 1:6. The ratio of *TSC1*:*TSC2* in Japan is 1:2.2, whereas it is reported to be 1:5.5 in Europe and the United States. It is known that the rate of intellectual disability is higher in patients with *TSC2* mutations than those with *TSC1*[4], which may explain why 10 out of 11 patients in this study had intellectual disabilities.

The novel *TSC2* mutation observed in case 8 was a one amino acid insertion in a non-repeat region, which has not been reported in genome projects, and is located in exon 37. This site is located in the GTPase-activating protein domain between exons 35 and 39, which is essential for tuberin function. *TSC2* mutations have been reported to were common in this region[12]. However, in this study, we did not conduct a functional analysis, we did not confirm that the mutations were loss-of-function mutations, and it was not clear whether the mutations were de novo. Based on these results, the mutation was determined to be likely pathogenic (PM1+PM2+PM4).

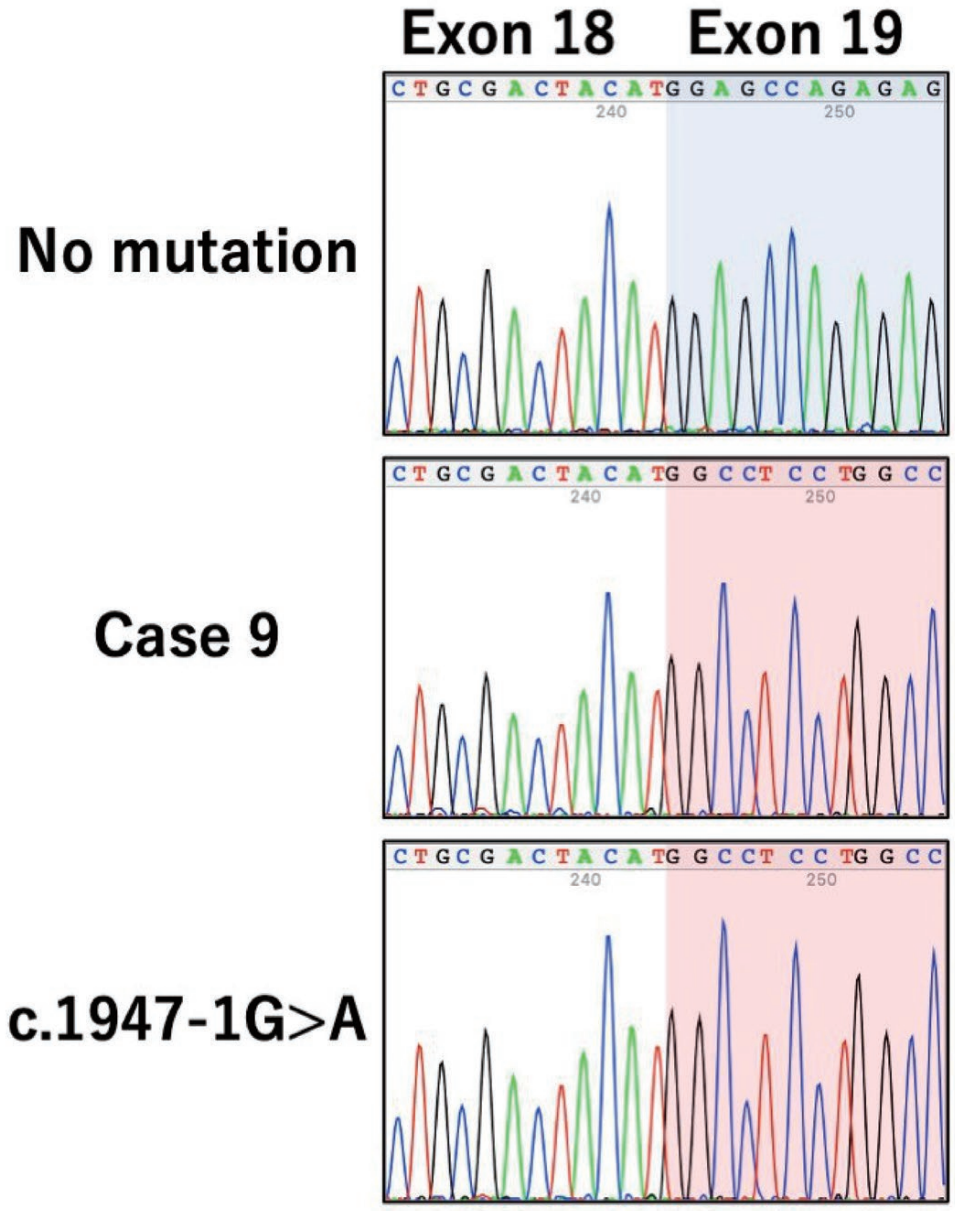

Figure 3 Confirmation of splicing mutation using the *TSC2* mini-gene experiment
The 17th to 21st exon region of *TSC2* was incorporated into the mammalian expression vector pcDNA3.1. This minigene was transfected into BHK-21 cells, and the transcripts were extracted and analyzed 24 h later. In wild-type *TSC2* cells, normal splicing was observed in the 18th to 19th exons (blue background). In the minigene carrying the mutation observed in case 9 or the c.1947-1G>A mutation, a deletion of 56 bp (c.1947_2002del) was found in the 19th exon (red background).

The mutation found in case 9 was considered to cause splicing errors because it is located in the proximal intron of the 19th exon and causes a deletion of 56 bases in the transcript (Figure 1b). To confirm that this mutation has a function to generate the splicing error, we used NetGene2[13, 14], a tool to predict splice sites. The results showed that the wild-type had a high score of 0.846 and 0.994 for the 1947-1 and 2002 sites, respectively. However, the mutation changed the score of the 1947-1 site to 0 (Table 4), suggesting that the 1947-1 site no longer functions as a splice site and promotes splicing at the 2002 site, 56 base pairs behind. This mutation was determined to be pathogenic (PVS1+PM2+PP5) because it produces the same

Table 4. Prediction of splice-acceptor sites by NetGene2

Genotype	Number of exons	Position in NM_000548.5	Nucleotide sequence	Donor site score	Acceptor site score	Confidence of donor site prediction	Confidence of acceptor site prediction
Wild		c.1947-5	T	0	0	0	0
		c.1947-4	G	0	0	0	0
		c.1947-3	C	0	0	0	0
		c.1947-2	A	0	0	0	0
		c.1947-1	G	0	0.846	0	0.528
	19	c.1947	G	0	0	0	0
	19	c.1948	G	0	0	0	0
	19	c.1949	A	0	0	0	0
	19	c.1950	G	0	0	0	0.054
	19	c.1951	C	0	0	0	0
Case 9		c.1947-5	A	0	0	0	0
		c.1947-4	G	0.007	0	0.022	0.015
		c.1947-3	C	0	0	0	0
		c.1947-2	A	0	0	0	0
		c.1947-1	A	0	0	0	0
	19	c.1947	G	0	0	0	0.015
	19	c.1948	G	0	0	0	0
	19	c.1949	A	0	0	0	0
	19	c.1950	G	0	0	0	0.015
	19	c.1951	C	0	0	0	0
Wild	19	c.2000	C	0	0	0	0
	19	c.2001	A	0	0	0	0
	19	c.2002	G	0	0.994	0	0.967
	19	c.2003	G	0	0	0	0
	19	c.2004	G	0	0	0	0
	19	c.2005	C	0	0	0	0

The blue background indicates sequences derived from the 19th exon. The orange background indicates the splice acceptor site scores for the c.1947-1 and c.2002 sites. Red letters indicate mutated bases.

transcript as TSC2 c.1947-1G>A, which has been previously reported to be pathogenic[15].

One of the limitations of this study is that it was a targeted sequencing analysis of the *TSC1* and *TSC2* genes and not an exhaustive analysis, and the number of cases was small. The reasons for the low detection rate of causative gene mutations may include the following: 1) the patient has somatic mosaicism with no mutations in blood cells or few mutated cells, 2) deep intronic mutations in the *TSC1* and *TSC2* genes, and 3) the presence of genes other than *TSC1* and *TSC2* genes that are responsible for TSC. With regard to the possibility of somatic mosaicism and deep intronic mutations, genetic analysis of samples derived from cells other than blood cells, such as tumors, and the addition of whole genome analysis and long-read sequence analysis may lead to an improvement in detection rates and the identification of new genetic abnormalities[16].

Conclusions

Genetic analysis of 11 patients with TSC was performed using NGS. Mutations were found in seven cases (*TSC1* mutation in one case and *TSC2* mutation in six cases), and the detection rate of mutations was similar to that reported previously. Among them, two cases were found to have novel mutations, and one case was found to have a characteristic splicing abnormality. Genetic diagnosis may be useful as an adjunctive diagnostic tool for atypical cases of TSC because

it allows for appropriate follow-up.

Statement of Ethics

This study was approved by the Ethics Committee of the Hirosaki University Graduate School of Medicine and was conducted after the patients or their guardians received a full explanation and written consent was obtained.

Conflicts of Interest

All authors have no conflicts of interest directly relevant to the content of this article.

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