Novel Mutations in 21 Patients with Tuberous Sclerosis Complex and Variation of Tandem Splice-acceptor Sites in *TSC1* exon 14

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Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by epilepsy, mental retardation, skin lesions, and tumors in various organs. However, TSC is sometimes difficult to diagnose because of its broad phenotypic spectrum. In such cases, it is essential to find a mutation in the disease-causing genes, *TSC1* and *TSC2*. In this study, we analyzed 21 TSC patients from 16 families using a combination method of DHPLC and nucleotide sequencing. We identified 16 novel mutations in the 16 families: nine mutations in *TSC1* (1 insertion, 7 deletion and 1 nonsense mutations) and seven mutations in *TSC2* (2 insertion, 2 deletion, 1 missense mutations and 2 splicing abnormalities). We also tested the possibility of very short alternative splicing due to a variation of the tandem splice-acceptor sites of *TSC1* exon 14 in a patient. RT-PCR and sequencing analysis indicated that no alternative splicing occurred in the patient. In conclusion, we confirmed the diagnosis of all patients using mutation analysis and clarified that variation of the tandem splice-acceptor sites in *TSC1* exon 14 does not cause a splicing abnormality.

Tuberous sclerosis complex (TSC; OMIM#191100) is an autosomal dominant disorder characterized by a broad phenotypic spectrum including epilepsy, mental retardation, skin lesions and tumors in various organs. The broad phenotypic spectrum reflected the development of hamartomas in multiple organs throughout the body (19). The incidence of TSC has been reported to be 1 in ~6000 (14). However, its true incidence is not known because of a number of undiagnosed cases consisting mostly of mildly affected or asymptomatic individuals (14).

Two disease-causing genes have been identified by positional cloning, *TSC1* (23), and *TSC2* (7). The *TSC1* gene is located on chromosome 9q34, and encodes the protein, hamartin (130 kDa, 1164 amino acids) (23). The *TSC2* gene is located on chromosome 16p13.3, and encodes another protein, tuberin (180 kDa, 1807 amino acids) (7). Recent investigation of somatic mutations in a variety of TSC hamartomas supports classification of the *TSC1* and *TSC2* as tumor suppressor genes (2). Interaction between hamartin and tuberin has a

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stoichiometry of 1:1 and is stable; a tight binding interaction between tuberin and hamartin forming a tumor suppressor heterodimer has been elucidated (12). This complex inhibits the mammalian target of rapamycin (mTOR) which is a key regulator in the signaling pathway of cell proliferation and organ size (12). Recently, it has been reported that hamartin-tuberin complex regulates mTOR via hydrolysis of Rheb-GTP into its inactive GDP bound state, Rheb-GDP (17,22).

The subtle or atypical cases lacking pathognomonic signs of TSC impose difficulties in diagnosis. In addition, diagnosis of TSC may be challenging early in life, particularly below 2 years of age. The value of the clinical criteria for early diagnosis and prompt management of TSC is limited by the fact that many symptoms become apparent only in late childhood or adulthood (19). In such cases, mutation identification is necessary for the diagnosis of TSC. However, both TSC-causing genes show huge structures: the *TSC1* gene consists of 23 exons (8617 nucleotides of cDNA sequence, GenBank NM_000368) and the *TSC2* gene of 41 exons (5675 nucleotides of cDNA sequence, GenBank NM_000548). Mutational hotspots are not observed in both genes. Moreover, more than half of the mutations in TSC patients are sporadic (11). The size of the genes, frequency of sporadic mutations and lack of mutational hotspots form great obstacles to mutation analysis. Thus, a rapid and accurate mutation screening method should be applied prior to final identification by DNA sequencing. Recently, denaturing high performance liquid chromatography (DHPLC) has been introduced for the purpose of *TSC1* or *TSC2* mutation screening (4,10,16).

In this study, we performed mutation analysis of 21 TSC patients using combination of screening by DHPLC and identification by nucleotide sequencing. We also analyzed the mRNA transcript from a *TSC1* allele with a nucleotide substitution within the tandem splice-acceptor sites of *TSC1* exon 14.

PATIENTS AND METHODS

Patients

A total of 21 Japanese patients from 16 unrelated families with TSC were enrolled in this study. All of these patients fulfilled the definitive tuberous sclerosis criteria of the 1998 Tuberous Sclerosis Consensus Conference (15). Molecular analyses were performed after obtaining informed consent from the patients or their parents.

Genomic DNA extraction, PCR, DHPLC, sequencing analysis

Genomic DNA was extracted from whole blood using a SepaGene Kit (Sanyo Junyaku, Tokyo, Japan). PCR amplification of all the exons in *TSC1* and *TSC2* genes were performed using the previously published primers (3). For *TSC2* exon 25, we designed a new primer set, 5'-GCA TGG CTC TTT TTG CTC A-3' and 5'-GAC GAT GAG GTC ATG CAA G-3'. We applied the PCR products into a DHPLC machine, WAVE® Nucleic Acid Fragment Analysis System equipped with a DNASep® cartridge (Transgenomic, Omaha, NE). The PCR products which showed heteroduplex peaks in the DHPLC chromatogram were then directly sequenced by an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). When a deletion or insertion mutation was suspected through direct sequencing analysis, we sequenced subcloned fragments in pGEM T-Easy Vector Systems (Promega, Madison, WI) to identify the mutation.

Total mRNA extraction, cDNA synthesis, and RT-PCR analysis

To see whether splicing anomalies exist within *TSC1* mRNA transcripts, we performed total mRNA extraction using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). First-strand cDNA synthesis with extracted mRNA was subsequently carried out using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim,

Germany). RT-PCR was performed using a primer on exon 12/13 junction, 5'-CAG GAA GGA AGA GAG AAT GG-3', and a primer on exon 15, 5'-AAA GGG AGA GTC AAA GCC TC-3'. The RT-PCR products were sequenced directly or after subcloning by an ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

RESULTS

1. Mutation status and clinical symptoms

We performed mutation analysis on the coding exons and the exon/intron junctions of both *TSC1* and *TSC2* in a total of 21 patients from 16 families with TSC. The determination of mutation vs. polymorphism was done by: 1) checking the SNP lists at the NCBI Entrez SNP database (http://www.ncbi.nlm.nih.gov/sites/entrez as of January 11 2008), 2) comparison of findings to those of 100 healthy Japanese controls. Figure 1 outlined our mutation analysis steps, representing a diagnostic procedure of *TSC1* gene mutation (found in Patient 018).
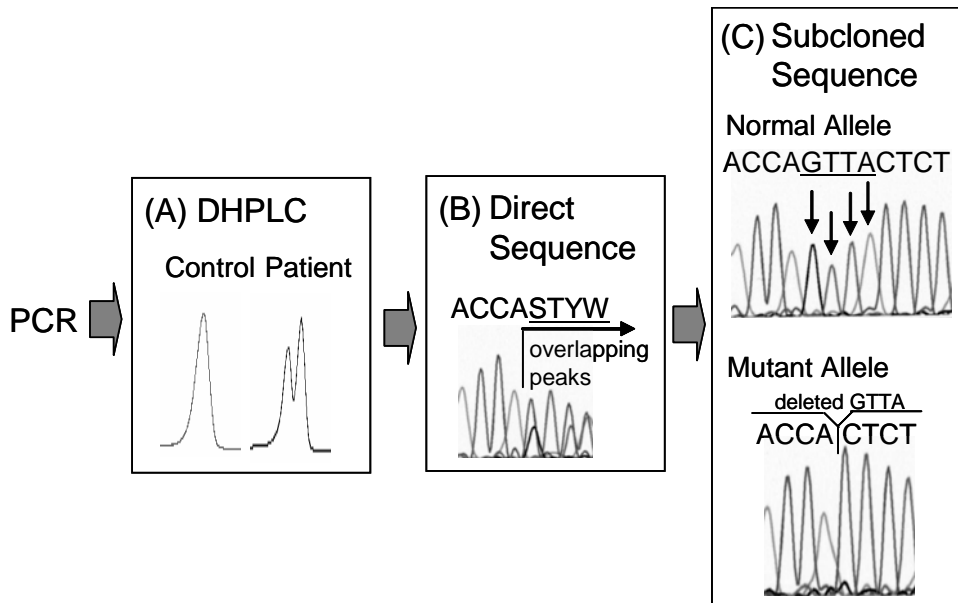

Figure 1. Mutation analysis of *TSC1* exon 17 (Patient 018). (A) DHPLC screening for mutations and SNPs. (B) Direct sequencing for the samples showing heteroduplex peaks. S: G/C; T: T/T; Y: T/C; W: A/T. (C) Subcloned sequencing for separating normal and mutant alleles.

Table 1 summarizes the mutations and single nucleotide polymorphisms (SNPs) found in the *TSC1* Mutations included one nonsense mutations with premature stop codon and eight insertion/deletion mutations which caused frameshifts and resulted in truncation of the protein. The *TSC2* mutations included one missense, four insertion/deletion mutations and two splice site mutations. All of these mutations identified in this study were novel.

Table 1. List of TSC1 and TSC2 mutations with the patients' clinical findings

No.	Patient	Sex	Age	Family Hx (2)	MR (3)	Epilepsy	Skin (4)	Brain	Tumors (5) Lung	Kidney	Heart	Mutations and SNPs (6)	Variation Types
1	032	f	~50y						O	O		*TSC1 exon 8 (c.953delT;p.P244PfsX249)	Deletion; mononucleotide
2	021	f	26y				O		O	O		*TSC1 exon 9 (c.971T>A; p.L250X);	Transversion; nonsense
												TSC1 exon 14 (c.1557A>G; p.E445E) (7)	Transition; SNP
3	012	m	29y			O	O	O				*TSC1 exon 9 (c.971delT; p.L250X)	Deletion; mononucleotide
4	022	f	36y	(+)			O	O	O	O		*TSC1 exon 9 (c.1053-1054delGT; p.V277VfsX298)	Deletion; dinucleotide
5	006	f	31y				O					*TSC1 exon 15 (c.1739-1740insTC; p.P506LfsX532)	Insertion; dinucleotide
6	016	m	62y	(+)		O	O					*TSC1 exon 15 (c.2010delT; p.F590LfsX628)	Deletion; mononucleotide
7	018	m	39y				O	O	O	O		*TSC1 exon 17 (c.2325-2328delGTTA; p.Q701HfsX735)	Deletion; tetranucleotide
8	023-1 (1)	m	57y	(+)			O		O			*TSC1 exon 18 (c.2579delG; p.R786QfsX806)	Deletion; mononucleotide
	023-2	f	27y	(+)		O	O		O			*TSC1 exon 18 (c.2579delG; p.R786QfsX806)	Deletion; mononucleotide
	023-3	f	26y	(+)			O	O	O			*TSC1 exon 18 (c.2579delG; p.R786QfsX806)	Deletion; mononucleotide
9	001-1 (1)	m	39y	(+)	O	O	O					*TSC1 exon 20 (c.2781-2784delGGTT; p.L853HfsX876)	Deletion; tetranucleotide
	001-2	m	5y	(+)	O	O	O				O	*TSC1 exon 20 (c.2781-2784delGGTT; p.L853HfsX876)	Deletion; tetranucleotide
10	004-1	f	21y	(+)				O				*TSC2 intron 1 (c.138+2T>C)	Transition; splice site
	004-2 (1)	f	47y	(+)			O		O	O		*TSC2 intron 1 (c.138+2T>C)	Transition; splice site
11	008-1 (1)	m	36y	(+)		O	O	O	O	O		*TSC2 exon 7 (c.736A>G; p.T246A)	Transition; missense
	008-2	m	1y	(+)		O	O	O	O			*TSC2 exon 7 (c.736A>G; p.T246A)	Transition; missense
12	020	f	39y			O	O	O	O	O		*TSC2 exon 16 (c.1737-1814del78bp; p.P579P inframe)	Deletion; 78 nucleotides
13	017	f	2y		O	O	O	O				*TSC2 intron 20 (c.2355+2T>C)	Transition; splice site
14	027	m	13y			O	O			O		*TSC2 exon 21 (c.2539delC; p.L847CfsX893)	Deletion; mononucleotide
15	003	f	12y			O	O	O		O		*TSC2 exon 23 (c.2710insC; p.F904LfsX914)	Insersion; mononucleotide
16	019	f	43y		O	O	O	O	O	O		*TSC2 exon 36 (c.4745insA; p.I1582NfsX1602)	Insersion; mononucleotide
												TSC1 exon 10 (c.1187T>C; p.M322T) (8)	Transition; SNP

(1) This patient has affected children with the same mutation.
(2) (+) denotes the presence of affected family member with apparent symptoms.
(3) The diagnosis of MR was based on the statement of doctors who referred these patients to us
(4) "Skin" lesions includes facial angiofibroma, shagreen patch, hypomelanotic macules (>3) and ungual/periungual fibroma.
(5) "Tumors" include hamartomas in brain, lung, kidney and heart. Brain tumors include subependymal nodule and calcification.
　　Lung tumors include lymphangioleiomyomatosis and multifocal mutinodular pneumocyte hyperplasia.
　　Kidney tumors include angiomyolipoma, renal cyst and renal cel carcinoma.
　　Heart tumor in Patient 001-2 is rhabdomyoma.
(6) The A of the initiator Met codon is denoted as nucleotide +222 for TSC1 cDNA, and nucleotide +1 for TSC2 cDNA, respectively.
(7) This SNP is registered in NCBI database (dbSNP ID: rs7862221).
(8) This SNP is registered in NCBI database (dbSNP ID: rs1073123).

O: presence of apparent symptoms *: TSC-causing mutation

Table 2. Comparison of mutation types with previously published reports (1)

Mutation	*TSC1*		*TSC2*	
	Cheadle et al. (2)	This study	HGMD (3)	This study
Large deletions/rearrangements	0 (0%)	0 (0%)	85 (18%)	0 (0%)
Insertions	26 (17%)	1 (11%)	46 (10%)	2 (29%)
Deletions	55 (36%)	7 (78%)	112 (24%)	2 (29%)
Nonsense	60 (39%)	1 (11%)	74 (15%)	0 (0%)
Missense	2 (1%)	0 (0%)	89 (19%)	1 (14%)
Splicing abnormality	11 (7%)	0 (0%)	65 (14%)	2 (28%)
Total	154 (100%)	9 (100%)	471(100%)	7 (100%)

(1) not the number of patients, but the number of mutation types

(2) reference 2

(3) reference 9

2. Missense mutation

Patients 008-1 and 008-2 in the same family exhibited no mutation except a nucleotide transition of A-to-G at position c.736 in *TSC2* exon 7 leading to an amino acid change of threonine-to-alanine at position p.246 (c.736A>G; p.T246A). This nucleotide transition is not listed in the NCBI Entrez SNP database. In addition, DHPLC screening showed that none of 100 healthy controls carried the nucleotide transition. Taken together, this nucleotide transition was regarded as a TSC-causing mutation, not simply a SNP.

3. Splicing abnormality

We identified two nucleotide transitions in a splice donor site. One is a nucleotide transition of T-to-C at position c.138+2 in *TSC2* intron 1 (c.138+2T>C) leading to activation of cryptic splice sites, and the other is a nucleotide transition of T-to-C at position c.2355+2 in *TSC2* intron 20 (c.2355+2T>C) leading to activation of cryptic splice sites and/or skipping of exons. Activation of cryptic splice sites and exon skippings may create a premature stop codon. Patients 004-1 and 004-2 in the same family exhibited no mutation except c.138+2T>C, and Patient 017 also exhibited no mutation except c.2355+2T>C. Thus, these nucleotide transitions at the splice donor sites were regarded as TSC-causing mutations, although the splicing abnormalities could not be shown because mRNA samples were not available.

4. Nucleotide substitution in tandem splice-acceptor sites with three AG-dinucleotides

One nucleotide transition was also found in the tandem splice-acceptor sites of *TSC1* exon 14 with a set of three AG-dinucleotides. Patient 021 exhibited a nucleotide transition of A-to-G at position c.1557 in *TSC1* exon 14 (c.1557A>G), creating a different set of three AG-dinucleotides. However, RT-PCR and sequencing analyses revealed that the second and third AG-dinucleotides were incorporated in the transcript sequences from normal and variant alleles, indicating that the first AG-dinucleotide was used as the splice-acceptor site in normal and variant alleles (Figure 2). This finding indicated that the nucleotide transition does not cause very short alternative splicing. In addition, the nucleotide transition does not cause an amino acid change (synonymous). DHPLC screening showed that 11 out of 100 normal controls carried this nucleotide transition. This nucleotide transition has been listed in the NCBI Entrez SNP database (dbSNP ID rs7862221). Patient 021 also exhibited a nonsense mutation, a nucleotide transversion of T-to-A at position c.971 in *TSC1* exon 9 creating a premature stop codon. Thus, we regarded this transversion, c. 971T>A, as a TSC-causing mutation.
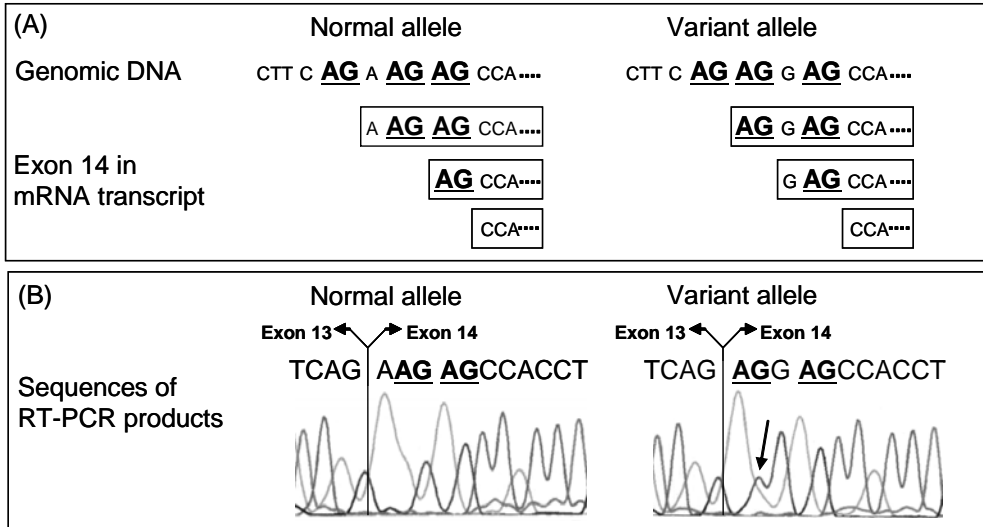
Figure 2. Three AG-dinucleotides within the splice-acceptor site of *TSC1* exon 14. (A) Selection model of "AG" as splice-acceptor site. Three alternative isoforms can be produced from each allele. (B) Sequences of RT-PCR product of normal and variant alleles. Regardless of normal or variant alleles, the second and third "AG" are incorporated into the transcripts, indicating that the first "AG" is used as the splice-acceptor site of exon 14.

DISCUSSION

In this study we analyzed 21 TSC patients from 16 families and confirmed 16 TSC-causing mutations in the *TSC1* or *TSC2* genes (including a missense mutation regarded as TSC-causing). We screened all patients for TSC-causing mutations using DHPLC and confirmed them by direct or subcloned sequencing. DHPLC is a rapid and sensitive method for detection of DNA sequence variation. The method requires only 10 minutes' run to complete the analysis of one sample. Detection of DNA sequence variation is based on differences in the retention of perfectly matched homoduplexes and heteroduplexes containing one mismatched base pair (20). The selected temperature and buffer gradient conditions during DHPLC can resolve mutations in various genes with detection rates of 90% or more (13). However, it should be noted that DHPLC does not always detect any mutation: DHPLC cannot detect any abnormality of genomic DNA structure, such as large deletions or rearrangements. According to the Human Gene Mutation Database (Table 2), large deletions or rearrangements have been frequently identified in patients with *TSC2* disease.

Comparisons between the types of mutations found in the previous reports and in this study are shown in Table 2. According to the *TSC1* mutation data of Cheadle et al. (2), the predominant type of mutation is a nonsense mutation (36%). However, insertion/deletion mutations in their data are taken together to be the most predominant (53%), which is similar to our finding that insertion/deletion mutations are the most predominant (89%). In the *TSC2* mutation data in the Human Gene Mutation Database (HGMD) (9), the mutations are almost evenly distributed among types, with deletion mutations as the most frequent (24%), followed by missense (19%). The *TSC2* mutation types found in this study are also distributed evenly: insertion mutations (29%), deletion mutations (29%) and splicing abnormality (28%).

As to the genotype-phenotype correlations in TSC patients, according to the large studies reported previously (6,11,18), *TSC1* disease is less severe than *TSC2* disease. Although the number of patients is limited in this study, we found a similar tendency: epilepsy appeared more frequently in patients with *TSC2* mutations compared to patients with *TSC1* mutations. We also showed that patients in the same family, with the same mutation, show a variety of symptoms. We experienced an extremely severe case of a boy with a *TSC1* mutation (Patient 001-2). Significant differences were observed between this boy and his father (Patient 001-1) with the same mutation. The boy suffered from cardiac rhabdomyoma, mental retardation and intractable convulsion, while his father had no symptoms except facial angiofibroma. It is known that even affected members of the same family often develop very different manifestations: the phenotype may not be determined by the specific gene mutation (21).

TSC1 exon 14 is known as an example of an exon with tandem splice-acceptor sites with NAGNAG sequence (8). Selection of tandem splice-acceptor sites, i.e. very short alternative splicing, may play a role in the structure-function diversity of proteins (24). Thus, the selection of tandem splice-acceptor sites with NAGNAG sequence might play an important role in the development of diseases (8). In fact, the splice-acceptor site of *TSC1* exon 14 has an additional AG-dinucleotide located downstream of NAGNAG sequence (CAG AAG AG) (Figure 2); there are three plausible selections in splice-acceptor site of *TSC1* exon 14. A nucleotide transition of Patient 021, c.1557A>G, makes different tandem splice-acceptor sites (CAG AGG AG), and the second AG-dinucleotide is shifted upstream in this variation. Three plausible selections of splice-acceptor site of *TSC1* exon 14 had also been hypothesized in this patient.

We also analyzed mRNA transcripts of Patient 021 in this study. Although the nucleotide transition, c.1557A>G, has been registered in NCBI Entrez SNP database, we tested whether it is associated with the development of TSC in Patient 021 or not, because the patient's tandem AG-dinucleotides may alter splicing patterns. The use of the second and third AG-dinucleotides as splice-acceptor site in the variant transcript may induce protein truncation. In this regard, the variation of tandem splice-acceptor sites in *TSC1* exon 14 can be a TSC-causing mutation. However, according to our analysis of the patient, only the first AG-dinucleotide was selected as a splice-acceptor site in both of normal and variant alleles: the second and third AG-dinucleotide are incorporated into the mRNA transcripts. This finding indicated that the nucleotide transition does not cause splicing abnormality.

These findings raise the question as to what factor determines the selection of tandem splice-acceptor sites. The tandem splice-acceptor sites of *TSC1* exon 14 suggest that the nucleotide upstream of an AG-dinucleotide has a great influence on splice-acceptor site selection: the C nucleotide upstream of the first AG-dinucleotide may determine that the first AG-dinucleotide was selected as the splice-acceptor site of the exon. Since the nucleotides upstream of the other AG-dinucleotides are A or G, then it may hamper their selection as a splice-acceptor site. This idea is supported by Clark F. and Thanaraj T.A. (5) who reported that the nucleotide upstream of any acceptor AG-dinucleotide is 95% C or T. However, it does not neglect other factors which may be involved in the selection of AG-dinucleotide as splice-acceptor sites (1).

In conclusion, we confirmed the diagnosis of all patients by identification of mutations in *TSC1* or *TSC2* genes and clarified that a nucleotide substitution in the tandem splice-acceptor sites of *TSC1* exon 14 does not cause a splicing abnormality.

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