SHORT COMMUNICATION

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Mutational analysis of *TSC1* and *TSC2* genes in Japanese patients with tuberous sclerosis complex

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Abstract We have surveyed the mutations of *TSC1* and *TSC2* from 38 (25 sporadic, 11 familial, and 2 unknown) Japanese patients with tuberous sclerosis complex. In 23 of 38 subjects, we detected 18 new mutations in addition to 4 mutations that had been previously reported. We also found 3 new polymorphisms. The mutations were not clustered on a particular exon in either of the genes. Seven *TSC1* mutations found in 3 familial and 4 sporadic cases were on the exons (3 missense, 2 nonsense point mutations, a 1-base insertion, and a 2-bp deletion). Fifteen *TSC2* mutations were found in 5 familial cases, 10 sporadic cases, and 1 unknown case. The 12 mutations were on the exons (8 missense, 1 nonsense point mutations, a 1-bp insertion, a 5-bp deletion, and a 4-bp replacement) and 3 point mutations were on the exon–intron junctions. Although the patients with *TSC2* mutations tend to exhibit relatively severe mental retardation in comparison to those with *TSC1* mutations, a genotype–phenotype correlation could not yet be established. The widespread distribution of *TSC1/TSC2* mutations hinders the development of a simple diagnostic test, and the identification of individual mutations does not provide the prediction of prognosis.

Key words Tuberous sclerosis complex · *TSC1* gene · *TSC2* gene · Hamartin · Tuberlin · Mutation

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Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disease with an estimated prevalence of 1 in 10,000 in the Caucasian population and 1 in 31,000 in the Japanese population (Ohno et al. 1981; Gomez 1988). Multiple organs are affected in TSC, including the central nervous system (mental retardation, epilepsy), skin (facial angiofibroma, shagreen patches, hypopigmented macules, periungual fibromas), heart (cardiac rhabdomyomas), and kidney (cysts, angiomyolipoma).

There are two causative genes of TSC. The *TSC1* (hamartin) is located on 9q34 and consists of 23 exons. The *TSC2* (tuberlin) is located on 16p13.3 and consists of 41 exons (European Chromosome 16 Tuberous Sclerosis Consortium 1993; Van Slegtenhorst et al. 1997). Loss of heterozygosity (LOH) of either *TSC1* or *TSC2* in the affected tissues indicates that both act as a tumor suppressor (Green et al. 1994; Henske et al. 1995, 1996; Sepp et al. 1996). Multiple isoforms of tuberlin are generated by alternative splicing at two different exons (exon 25 and 31) of *TSC2* (Xiao et al. 1997). Since the identification of the *TSC1/TSC2* genes, the mutations in Caucasian TSC patients have been extensively surveyed (Kumar et al. 1995 a,b; Vrtel et al. 1996; Wilson et al. 1996; Bajek et al. 1997; Jones et al. 1997; Maheshwar et al. 1997; Van Slegtenhorst et al. 1997; Au et al. 1997, 1998). The mutations in Japanese TSC patients, however, have remained unknown.

In the present study, we surveyed the mutations in Japanese patients and assessed the relationship between the mutations and clinical symptoms.

Materials and methods

Patients and sample collection

Thirty-eight (25 sporadic, 11 familial, and 2 unknown) Japanese TSC patients were clinically diagnosed according to the criteria (Gomez 1988). In some cases, ultrasound renogra-

phy, MRI, and CT brain scan gave supportive evidence for the diagnosis. Genomic DNAs were prepared from the blood samples as described (Nanba et al. 1995). Healthy Japanese volunteer DNA as normal controls were extracted by the Nucleic Acid Purification System (MagExtractor MFX-2000; Toyobo, Osaka, Japan) from 100 µl blood.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis

All coding exons of *TSC1* and *TSC2* were analyzed for every patient. Sets of PCR primers for the 41 exons of *TSC2* were designed based on the *TSC2* sequence, except for the primers for exon 13 and 25, which were kindly obtained by Dr. J.R. Sampson at the University of Wales, Cardiff, UK (Maheshwar et al. 1996) (Table 1). The primer information for the 21 coding exons of the *TSC1* gene was obtained from Dr. O Hino in the Department of Experimental Pathology,

Cancer Institute, Tokyo, Japan, and Dr. D.J. Kwiatkowski in Brigham and Womens' Hospital, Boston, USA, except for the primers for exon 9 (CACTGAGTTGACACTCTG-AAG and CTGAACTAAGTCTTACTCCAG), which were originally designed. PCR contained 25 ng of the genomic DNA as a template, 10 pmol of each primer, 0.25 µM of each dNTP, 0.1 unit of Ampli Taq Gold (Perkin-Elmer, Norwalk, CT, USA), and a buffer in a total volume of 5 µl. Cycling conditions were as follows: an initial denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min and elongation at 72°C for 1 min, with a final extension at 72°C for 5 min. All the PCR products were analyzed on 1.5% agarose gel. SSCP analysis was performed as described (Orita et al. 1989; Yuasa et al. 1997). Using a minigel (10 × 10 cm), the samples were analyzed under four different electrophoresis conditions, in a combination of two sets of gel mixtures (12% polyacrylamide gel with or without 5% glycerol) and two temperature conditions (4°C or 22°C).

Table 1. Primers for *TSC2* gene analysis

Exon		Product size (bp)
1	F: AAGGTTATGCCCACCAGAGA; R: AGACACAGGTAGCTCACTCA	296
2	F: CACTGGCCCTTTTCTTCT; R: CTCTAGTAGCTCAACTGGAT	150
3	F: CTTGGAGAGCACATCCTCAC; R: CAGCCTGACGTCACCCATCC	195
4	F: CTGATCCTGTGGCTTTTGTG; R: AGAAACCTCCAACCCAAGGT	199
5	F: TCGCAAACTGCGCGACTTCT; R: CAGAACAGGATCTCAATTCT	173
6	F: GACTGAGCTCGGTGCTCCCT; R: TGAAATGGGCCGGCCCTGGG	109
7	F: GAAGGAGGTGGGAAGGAAG; R: CATCCATGTGCTCTCAGGAA	278
8	F: CTTTGGGAGGAGATGGTGG; R: ATGTGTGCGTTGGTAAGGGA	286
9	F: CGTCTCTCTGGGGAACACTT; R: TCACTGCACACAGAAACCGC	176
10	F: TGCTGGCCGGGCTCGTGTC; R: TGCTCCCCGAGCTCCTGCC	198
11	F: GCCTGTGTATCGTGCCTGG; R: AAAGGCCCCACCCAGGAGGG	194
12	F: CAACACCGGCTCTTCTTTT; R: AGGCAAGGCTACAGAGGACC	153
13 ^a	F: GGGCTGTGGCGGGCACTC; R: AGAGGTCCGGCAGACCGAAGTC	245
14	F: TTGGCCTCCCTTGTGCCTGT; R: GCCCGGGTACAACGGCTCT	203
15	F: GACTCAGAACCATGAGCCTG; R: AACAGACTCCAACACAACGC	251
16	F: CGTGGTGAGCTGCGTCTCT; R: ACTGCGTGCGCAACCCACG	178
17	F: GGCTTTTACCATCCTCTTCC; R: TCATGGGCCGTGGGCAGGTC	160
18	F: GCCTCAGCTGCTTCTCTTGC; R: AACGTGGGACGGATGGTCCC	216
19	F: CCTGACGCCTCCTCTCCTCG; R: CCCAACCCAGGCCGGCATG	185
20	F: TCATGCCTGGATTGTGGTCAT; R: TCAAGCTCGCCTGCTCTGAC	187
21	F: GGGCCTGAGGTGTCCTGTCT; R: AGGTGCAGTGCTGCAGGGCG	246
22	F: GGCTCCCTGACCACCTCTCC; R: ACAGGGCCTGGGGCGACCAC	148
23	F: GTGCCAGCCCCCTTCTCATC; R: ACAGCCTTACCGCCCTGAG	152
24	F: TCACTGTCTGGGTGTGCTC; R: TGCCCTGCCTCACATACTC	260 ^b
25 ^a	F: CCCTCCACTGGCTTGTCTCC; R: CGGGCAAGACGATGAGGTCAT	—
26	F: CCTGGTCACGGCCTCTCCCT; R: CCAGCCCTGTAGTGCCGCCT	213
27	F: GGTTTCACGCTCCCTGTCTT; R: GGAGGAAAGGAAGGTGCAGT	200
28	F: ACCCTGTGCGTGGGATTCTC; R: AGGTGGCTGGGGCCAAGGCT	163
29	F: CCTCATGCCCCTCTTCTTC; R: AGATGGCGCTCAGGCCAC	267
30	F: AAGGTAGTCTGCCGCCTCCG; R: CCAAGGCCCGCCATGCCACT	251
31	F: CGTCGACACGGCCTCCCTT; R: ACCGCTTCCCTCTGAGAGGC	118
32	F: AGCAGCCCCGTCTGTGCTC; R: GTCCAGGCTCTGAGCCACAC	171
33	1F: CTCTTTGGGATGGTCCTTTC; 1R: CTGTGACCGGGCCTTAACCT	275
33	2F: CCTGGGGACAAGGCCGACGT; 2R: CCCGCCCCGAAGCAAGAGGC	317
34	F: CCACCATCCCCCTCCCTGT; R: TAGCCAGAGCCCTCTGCAA	206
35	F: CTCTGGCTAAGCTCCCTGT; R: TGAGCACTTCATGCTGTAGG	326 ^b
36	F: GGGAGTGATGCCACCCTG; R: ATGGGAGCCGTGCCCTGA	—
37	F: CATCCGGCCCTGCTCACCTC; R: GCCTCACTGACGGCCCTCAC	187
38	F: TGACCCCTTCTCTTGTCCGG; R: TCTGCACTGCAGCCCCACCC	131
39,40	F: TGGCGTGACCACCAAGTCTC; R: GCTGAGGAGCCCCATATTCC	325
41	F: CAGACTTACTGCCCCAAGCCGCT; R: CCCCACCAAGCAGACAAA	342

^aData were kindly provided by Dr. J.R. Sampson (University of Wales, UK)

^bThe exact sizes of the PCR products were not known

Sequencing

Variant bands were reamplified and subcloned into pGEM-T Vector (Promega, Madison, WI, USA). The DNA sequences were determined using the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham, Buckinghamshire, England) with Cy-5-labeled M13 universal or reversal primers and ALFred Automatic Sequencer (Pharmacia Biotech, Tokyo, Japan). At least ten independent clones of each PCR product were sequenced in both directions. The sequence data were analyzed by Genetyx computer software (Software Development, Tokyo, Japan).

Results

SSCP analysis

A total of 25 variants were detected by SSCP analysis (Fig. 1) from 23 patients (60% of the total 38 patients). Seven variants of *TSC1* gene were detected in 3 familial cases and 4 sporadic ones. Fifteen variants of *TSC2* gene were detected in 5 familial cases, 10 sporadic cases, and one unknown case. L574L of *TSC1* and I531I of *TSC2* genes were considered to be polymorphisms because the point mutations did not cause amino acid substitution. M322T of *TSC1* was detected in the normal controls (6 of 80) and was supposed to represent a polymorphism (Table 2A). All other missense mutations were not detected in 60 or 100 normal controls. Any DNA sample from the parents was not available except for P28.

Mutations in *TSC1* gene

Two nonsense, two frame shift, and three missense mutations were located in various exons of the gene. Two mutations (R509X, Q654E) in three patients were located in exon 15, and the R509X was found both in a sporadic patient (P26) and a familial patient (P3). Two mutations (W676X and T417I) were found in one patient (P31).

Mutations in *TSC2* gene

One nonsense, 3 frame shift, and 8 missense mutations were identified in 16 patients (Table 2B). The two single nucleotide substitutions (2760 + 1 G to A, 5179 - 1 G to T) of an exon-intron junction were predicted to cause a splicing error. Although a substitution (5178 + 5 G to A) in the intron was not in the consensus sequence of the splicing, the mutation was located 5bp distant from the exon and was not detected in 100 normal controls. The mutation might cause a splicing error. L717R in a patient with pulmonary tuberous sclerosis was reported previously (Zhan et al. 1999). Both the V769E mutation (P2 and P21) and R1459X mutation (P6 and P8) were detected in 2 unrelated sporadic patients. Two missense mutations (H137R and D647N)

Fig. 1. PCR-SSCP analysis of the *TSC1*/*TSC2* genes. Arrows indicate the aberrant bands found in the patients' samples. C, control samples from healthy subjects; P, patients' samples

Table 2A. Mutations in *TSC1*

Patient	Amino acid change	Location	Type of mutation	Nucleotide alteration	S/F*
46	216PheFS-216X(a)	exon 7	Frame shift	866delTT	F
11	311SerFS-340X	exon 10	Frame shift	1210insT	F
31	T417I	exon 12	Missense	1471 CtoT	S
26,3	R509X(b)	exon 15	Nonsense	1746 CtoT	F,S
23	Q654E(b)	exon 15	Missense	2181 CtoG	S
31	W676X	exon 16	Nonsense	2249 GtoA	S
12	T899S	exon 21	Missense	2918 CtoG	S
46,27,3	L574L	exon 15	Polymorphism	1947 TtoC	
17,34,36	M322T	exon 10	Polymorphism	1186 TtoC	

Table 2B. Mutations in *TSC2*

Patient	Mutation	Location	Type of mutation	Nucleotide alteration	S/F*
32	H137R	Exon 4	Missense	428 AtoG	S
48	238LeuFS-337X	Exon 7	Frame shift	725 insT	S
30	F320L	Exon 9	Missense	978 TtoG	
43	337GluFS-351X	Exon 10	Frame shift	1029GATCdel, CATGGCAT ins	F
22	R611Q(c)	Exon 16	Missense	1850 GtoA	F
47	597HisFS-614X	Exon 16	Frame shift	1808delACTAC	S
32	D647N	Exon 17	Missense	1957 AtoG	S
1	L717R	Exon 19	Missense	2168 TtoG	S
21,2	V769E	Exon 20	Missense	2324 TtoA	F,S
33	—	Intron 24	Splicing	2760 + 1 GtoA	S
27	V963M	Exon 25	Missense	2905 GtoA	S
6,8	R1459X	Exon 33	Nonsense	4393 CtoT	S,S
28	P1657L(d)	Exon 38	Missense	5042 CtoT	S
24	—	Intron 39	Splicing	5178 + 5 GtoA	F
41	—	Intron 39	Splicing	5179 – 1 GtoT	F
33	1531I	Exon 14	Polymorphism	1611 CtoT	

*S, sporadic; F, familial

The initiator ATG begins at base 222 of *TSC1* and at base 19 for *TSC2*

The original amino acid and its position in the protein (numbered from the initiator Met as 1) are followed by a new amino acid for missense mutations, and by X for nonsense mutation

^{a,b}The identical change was reported by Van Slegtenhorst et al. (1997)

^cThe identical change was reported by Wilson et al. (1996)

^dThe identical change was reported by Maheshwar et al. (1997)

were found in the same patient (P32). P1657L in P28 was confirmed to be a de novo mutation by examination of the parents' samples.

Clinical symptoms and mutation types

The clinical symptoms and the gene mutations are summarized in Table 3. The number of the patients with mental retardation (+ and ++) in *TSC2* was significantly larger than that in *TSC1* ($\chi^2 = 4.8$, $P = 0.0285$). The severity of mental retardation of the patients with *TSC1* mutations appeared to be milder than that of the patients with *TSC2* mutations. Complete information on renal and cardiac involvements was not available and could not be assessed. None of the mutation types (missense, nonsense, frame shift, and splicing) appeared to be correlated with a specific clinical symptom. In particular, the R509X mutation in *TSC1* was detected in two patients, and the severity of mental retardation in these patients were quite different.

Discussion

Mutations in a given gene may be screened by various methods such as Southern blot analysis, SSCP, heteroduplex analysis, protein truncation test, and RT-PCR followed by SSCP (Kumar et al. 1995a,b; Vrtel et al. 1996; Wilson et al. 1996; Bajek et al. 1997; Jones et al. 1997; Maheshwar et al. 1997; Au et al. 1997, 1998). In the present study, we employed SSCP to survey the mutations of *TSC1* and *TSC2* genes because the method is simple enough to cover the multiple numbers of exons. To increase the mutation detection rate of SSCP, we used four electrophoresis conditions by a combination of different sets of temperature and glycerol content of the gel. Our mutation detection rate was 60% (23 of 38), which is comparable with those in other reports (Jones et al. 1997; Van Slegtenhorst et al. 1997; Au et al. 1998).

There are several possible explanations for the failure to detect of *TSC1/TSC2* mutations in the 15 patients. First, it can be false negative. One report showed that the SSCP

Table 3. Clinical data of mutation detected patients

Patient number	Age	Sex	Mental retardation	AF	White macules	Subependymal nodules	Seizures	S/F	Mutations	Location of mutation
46	9	M	+	—	+	+	+	F	Frame shift	TSC1 exon 7
11	7	F	±	+	+	+	+	F	Frame shift	TSC1 exon 10
31	8	F	+	n.d.	+	+	+	S	Missense/nonsense	TSC1 exon 12; TSC1 exon 16
3	32	F	++	n.d.	n.d.	n.d.	n.d.	S	Nonsense	TSC1 exon 15
26	21	M	—	—	+	+	—	F	Nonsense	TSC1 exon 15
23	21	M	+	—	+	+	+	S	Missense	TSC1 exon 15
12	32	F	—	+	+	+	+	S	Missense	TSC1 exon 21
32	2	M	n.d.	n.d.	+	+	+	S	Missenses	TSC2 exon 4; TSC2 exon 17
48	24	M	++	+	+	+	+	S	Frame shift	TSC2 exon 7
30	nd	nd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Missense	TSC2 exon 9
43	8	M	++	+	+	+	+	F	Frame shift	TSC2 exon 10
22	6	F	—	—	+	+	+	F	Missense	TSC2 exon 16
47	6	F	+	+	+	+	+	S	Frame shift	TSC2 exon 16
1	17	F	—	+	+	+	+	S	Missense	TSC2 exon 19
21	44	F	++	+	+	n.d.	+	F	Missense	TSC2 exon 20
2	32	M	++	+	+	+	+	S	Missense	TSC2 exon 20
33	48	M	++	n.d.	n.d.	n.d.	+	S	Splicing	TSC2 intron 24
27	13	M	—	—	+	+	+	F	Missense	TSC2 exon 25
6	8	M	++	n.d.	n.d.	n.d.	n.d.	F	Nonsense	TSC2 exon 33
8	9	F	++	+	+	+	+	S	Nonsense	TSC2 exon 33
28	3	F	+	n.d.	+	+	+	S	Missense	TSC2 exon 38
24	12	M	—	—	+	+	+	F	Splicing	TSC2 intron 39
41	7	M	—	+	+	+	+	F	Splicing	TSC2 intron 39

n.d., information not available; AF, angiofibroma; S, sporadic; F, familial

method to detect a point mutation on a 250-bp DNA fragment was 63% effective (Sheffield et al. 1993). Second, it may be true negative in those cases in which the mutation lies outside of the coding sequence, either in the 5'-regulatory elements, within the intronic sequences, or in the 3'-UTR. In addition, a large-scale rearrangement of genomic DNA may yield true negative results in the SSCP of the coding exons. Although there is no report of a large-scale mutation in *TSC1*, there are indeed some in *TSC2* (Jones et al. 1997; The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Au et al. 1997; Van Slegtenhorst et al. 1997).

The mutation surveys of *TSC1/TSC2* genes in Caucasian TSC patients revealed no clustering of the mutation in either gene (Wilson et al. 1996; Jones et al. 1997; Van Slegtenhorst et al. 1997; Au et al. 1998). In accordance with these previous findings, the 22 mutations (18 new and 4 reported) found in this study were distributed on various exons and there was no clustering of the mutations. Again in accordance with the previous findings, there was no obvious relationship between the type and location of the mutation and the clinical symptoms. However, the patients with *TSC2* mutations appeared to have more severe mental retardation compared with the patients with *TSC1* mutations. The same tendency has been noted (Jones et al. 1997). It is therefore at least clear that the clinical phenotype cannot be predicted from the genotype. Because of the heterogeneity of the mutations, it also appears impossible at present to develop a simple diagnostic test for TSC.

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