ORIGINAL ARTICLE

Mutational analysis of *TSC1* and *TSC2* in Japanese patients with tuberous sclerosis complex revealed higher incidence of TSC1 patients than previously reported

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Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by multiple hamartias and hamartomas involving throughout the body. To date, many *TSC1* and *TSC2* mutations have been reported all over the world, however, few *TSC* mutation studies have been performed in the Japanese population, and genetic characteristics of Japanese TSC patients are not yet clear. In this study, we analyzed *TSC1* and *TSC2* in 57 Japanese patients with TSC (8 familial and 49 sporadic; 46 definite and 11 suspect TSC) and identified 31 mutations including 11 *TSC1* mutations (two familial and nine sporadic; all definite TSC) and 20 *TSC2* mutations (2 familial and 18 sporadic; 19 definite and 1 suspect TSC). We also reviewed all Japanese *TSC* mutations previously reported. Our study demonstrates significantly higher incidence ($P = 0.007$) of *TSC1* mutations among sporadic TSC patients in the Japanese population compared with US and European studies. No differences emerged in mutation distributions and types in precedent studies, excepting low frequency of the *TSC2* nonsense mutation. Comparing clinical manifestations, developmental delay and/or mental retardation were milder in TSC1 patients than TSC2 patients for its frequency ($P = 0.032$) and severity ($P = 0.015$); however, no other symptoms were clearly different.
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INTRODUCTION

Tuberous sclerosis complex (TSC) is a variably expressed multisystem disorder. It manifests lesions with misarranged undifferentiated dysplastic cells. These dysplastic cells do not grow more rapidly than normal cells in some lesions (called hamartias), but in other lesions, grow into benign tumors (called hamartomas) or rarely grow into malignant tumors such as renal cell carcinoma.[1] As is well known, characteristic hamartias of TSC are hypomelanotic skin macules and brain cortical tubers, whose disease name is derived from macroscopic pathological brain feature of cortical tubers described by Bourneville as 'tuberous sclerosis of the cerebral convolutions'.[2] Also characteristic hamartomas are subependymal nodules, facial angiofibromas, renal angiomyolipomas (AML) and cardiac rhabdomyomas. In practice, however, entire body organs are involved in patients with TSC, including retina, lung, liver, pancreas, intestines, endocrine system, bone and teeth,[1] and established clinical diagnostic criteria is widely used.[3] All hamartias are embryonic lesions, whereas hamartomas appear in an age-dependent fashion; that is, cardiac rhabdomyomas

grow in the fetus through early infanthood then regress, facial angiofibromas become apparent in puberty, renal AML emerge in adults and subungual fibromas present after middle age.[4] At least half of the patients with TSC have developmental delay or mental retardation, sometime with intractable epilepsy, though the spectrum of severity in individual patients varies widely between families or even within a family.[1]

TSC is an autosomal dominantly inherited disorder, and its estimated frequency in the population is about 1 in 6000 of whom approximately two-thirds are sporadic and the remaining segregating in families.[5] The disease is caused by the mutation of one of two tumor suppressor genes, *TSC1* (OMIM # 191100) and *TSC2* (OMIM #191092), which encode hamartin and tuberin, respectively.[6,7] These proteins form a functional heterodimer, and this intracellular complex (TSC complex) is a universal sensor of several intracellular and extracellular signals; for example, growth factor stimulation, hypoxia and energy levels.[8] The most remarkable function of the TSC complex is GTPase activating protein activity for Ras homolog enriched in

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brain (Rheb), which converts Rheb–GTP to Rheb–GDP. Since Rheb–GTP activates the mammalian target of rapamycin (mTOR), in cells lacking proper function of the TSC complex there are increased levels of Rheb–GTP, which leads to activation of mTOR, causing increased cell size and growth through phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1, ribosomal protein S6 kinase beta-1 and eukaryotic translation initiation factor 2 (eEF-2K).[8,9]

Concordant with tumor suppressor function, loss of heterozygosity (LOH) of both *TSC1* and *TSC2* loci have been demonstrated in TSC lesions; however, reported LOH frequencies differed depending on the genes and tumor types. LOH was demonstrated more frequently in *TSC2* (37%) than *TSC1* (6%), with the highest frequency observed in renal AMLs (56%), intermediate rates observed in subependymal giant cell astrocytoma (SEGA; 39%), skin lesions (33%) and cardiac rhabdomyoma (23%), and the lowest rates observed in cortical tubers (15%).[10] However, increased phosphorylated isoforms of S6 kinase beta-1 and 4E-binding protein 1, which exhibit hyperactive mTOR signaling, was shown in cortical tubers from patients with TSC.[11] Also, abnormal phosphorylation of tuberin in cortical tubers, which induced post-translational inactivation of the *TSC2* product without second hit on DNA level, was demonstrated.[12] Some investigators postulated that this modification occurs through activation of extracellular signal-regulated kinase (ERK).[8,13] More recently, hamartin and tuberin have been discovered to bind to at least 40 additional proteins, and thus there are numerous potentials and yet undefined effects of *TSC* gene mutations.[14]

To date, there are several large cohort studies for *TSC* mutations in US,[15–17] UK,[18] Denmark,[19] Netherland,[20] Australia,[21] Taiwan[22] and Korea.[23,24] Through these studies, several aspects of the molecular genetics of TSC are becoming apparent. Overall mutation distribution among patients with TSC shows higher frequency of *TSC2* mutations than *TSC1* mutations, with equally represented *TSC1* and *TSC2* mutations in familial TSC, but *TSC2* mutations are more common than *TSC1* mutations in sporadic cases.[15] Although all types of mutations including large deletions and point mutations have been identified in *TSC2*; the majority of *TSC1* mutations are frameshift, nonsense and splicing mutations, but missense mutations and large deletions are rare.[16] There are no mutational hot spots in either *TSC1* or *TSC2*. The type of *TSC1* or *TSC2* mutation (protein truncation vs missense) and the location of the mutation do not predict the severity of the phenotype. The exception is that some *TSC2* missense mutations (that is, p.R905Q and p.Q1503P) are associated with milder disease phenotypes,[25,26] and *TSC2/PKD1* contiguous gene deletion is correlated with polycystic kidney disease.[27] Detailed genotype/phenotype data indicated that *TSC2* mutations produce a more severe phenotype than *TSC1* mutations especially in intellectual disability.[16,17]

Although large amounts of data on *TSC* mutations and phenotypes have been reported worldwide, in Japan only three *TSC* mutation cohort studies, including our previous study,[28–30] and three *TSC* mutations collected reports have been presented.[31–33] The diversity of mutations, phenotype characteristics and genotype correlations in the Japanese TSC population are not yet clear. In this study, we report a mutational analysis of both *TSC1* and *TSC2* genes in 57 unbiased sequential Japanese patients with TSC. We screened whole-coding exons of both genes and performed long PCR and Southern blotting for detecting large gene deletions. Also, detailed clinical data were corrected by symptom check lists for each patient. In addition, we reviewed and verified all previously reported Japanese *TSC* mutations available in the literature and summarized them in a unified format and compared them to *TSC* mutations reported in other races.

MATERIALS AND METHODS
Patient collection and clinical evaluations
We analyzed 57 Japanese patients with TSC, 8 familial and 49 sporadic cases, collected between 2002 and 2011. These patients were clinically diagnosed according to established criteria,[3] and 46 patients diagnosed as definite TSC and 11 patients as suspect TSC. Of the 57 patients, 15 patients were referred to our department in Kanazawa University Hospital or Kanazawa Medical University Hospital. These patients were clinically evaluated by us with the clinical data check list, according to Dabora *et al.*,[16] and corrected DNA samples. The remaining 42 samples were collected from regions all over Japan in response to requests for genetic diagnosis of TSC with the same clinical data check list filled out by the primary doctors. The clinical data check list not only assesses TSC diagnosis, but also scores severity of the patients' phenotype in several aspects. In the original check list, mental retardation, facial angiofibroma, renal AML and liver AML are scored as grade 0–3, and renal cysts are scored as grade 0–4 depending on severity. Actual numbers of subependymal nodules and cortical tubers are counted on brain imaging (computed tomography or magnetic resonance).

In this study, we modified the original check list at two points for convenience. First, for the assessment of mental retardation, the original check list uses speech and reading abilities as applied to only patients 8 years of age or older. Our subjects included many infants and toddlers and we could not use this scale for these young patients. Thus, we defined new criteria for 'Developmental delay and/or mental retardation' for our patients as follows. Grade 0: intercultural quotient or developmental quotient evaluated in any established scale is 70 or more and no obvious psychomotor developmental delay was observed in infants and toddlers by a pediatric neurologist; or, grade 0 in the mental retardation item in the original check list for older children and adults, corresponding to reading ability above a third grade level (8 years of age). Grade 1: intercultural quotient or developmental quotient is between 50 and 69, and pediatric neurologist observed mild developmental delay, or grade 1 in the original check list, corresponding to reading at less than a third grade level, in older children and adults. Grade 2: intercultural quotient or developmental quotient is <50, and pediatric neurologist observed substantial developmental delay, or grade 2 in the original check list in older children and adults, corresponding to some speech ability, no reading ability or grade 3 in the original check list in older children and adults, no speech ability. We eliminated five patients <6 months of age who were too young to evaluate (family 12, 26, 31, 32 and 47).

Second, the number of subependymal nodules and cortical tubers are difficult to count precisely. It is dependent on performance of the imaging machine, the condition of the slice to the region and any body motion of the patients. So, we simply scored as grade 0: no lesion, grade 1: single lesion and grade 2: multiple lesions.

The study was approved by the institutional review board and all samples were obtained with informed consent.

Mutational screening
Genomic DNA was extracted from whole blood by rapid methods.[34] DNA samples obtained from 50 normal volunteer individuals (medical students of Kanazawa University) were used as a control panel.

We analyzed whole-coding exons of both *TSC1* and *TSC2* genes. For the first 10 patients (including nine definite and one suspect TSC, from two familial and eight sporadic pedigrees), we screened mutations by single-strand conformation polymorphism and heteroduplex analysis (SSCP/HA) followed by direct sequencing. Disease causative mutations were detected in four patients. For the remaining six patients, we performed whole-coding exon sequencing of both genes, and mutations were identified in two more patients. For the remaining four patients, large deletion analysis (see below) was performed; one *TSC1* and one *TSC2* large deletion were detected. Details of these mutations have been reported previously.[28]

For the remaining 47 patients (including 37 definite and 10 suspect TSC, from 6 familial and 41 sporadic pedigrees), we applied CEL nuclease-mediated heteroduplex incision with polyacrylamide gel electrophoresis and silver staining (CHIPS) for mutation screening.[35,36] CHIPS is the simple and convenient system we have recently developed, possessing very high

sensitivity for mutation screening as well or better than denaturing high-performance liquid chromatography (DHPLC). It is based on enzyme mismatch cleavage and optimizes each step of the system; that is, PCR protocol, heteroduplex formation, selection of endonuclease and DNA digesting condition and running gel matrix and developing methods. We reached 100% sensitivity and specificity by CHIPS in previous studies.[35,36] Hence, we did not perform full coding sequencing of *TSC1/TSC2* for the patients whose mutations were not detected by CHIPS. PCR primer sets using this study for SSCP/HA or CHIPS were the same as described previously, for exons 1–41 of *TSC2*[15,37] and exons 3–23 of *TSC1*.[15]

To detect large gene deletion or duplication of *TSC1* or *TSC2* in the all patients with no mutation detected by CHIPS screening, we performed long-range PCR as described by Dabora *et al.*,[16] and Southern blotting followed by densitometry analysis[28] for both genes.

Mutation nomenclature is in accordance with Dunnen *et al.*[38] For *TSC2*, complementary DNA (cDNA) sequence is according to GenBank accession number NM_000548.3, transcript variant 1. This recent version of the cDNA sequence includes a 5′ leader exon as exon 1 and following 41 coding exons from exon 2–42. Although codon number was not changed, exon number was shifted from previously reported *TSC2* mutations that do not count a leader exon. In this paper, we adhere to the previous exon numbering system of *TSC2* for convenience in comparison. For *TSC1*, cDNA sequence is according to Genbank accession number NM_000368.4, transcript variant 1, including two 5′ leader exons (exon 1 and 2) and 22 coding exons (exon 3 to 23) without any changes from previous reports.

We reviewed all *TSC* mutations reported in Japanese patients available in the literature and compared them to *TSC* mutations reported in other ethnic populations. We found inconsistent mutation nomenclature in each paper and several errors in mutation descriptions in the reviewing process. We standardized and corrected all reported *TSC* mutations in Japanese patients and summarized them in a unified nomenclature system.[38]

Statistical analysis

Differences in each clinical trait were evaluated between TSC1 patients and TSC2 patients, TSC1 patients and no mutation identified (NMI) definite TSC patients and TSC2 patients and NMI-definite TSC patients. Fisher's exact test was used for the analysis of categorical variables, and $P < 0.05$ (one-sided) was considered statistically significant. The quantitative variables—average of grade scales—were evaluated by F-test first and then equal variance data were analyzed by Student's unpaired *t*-test, and unequal variance data were analyzed by Welch's *t*-test. In both analyses $P < 0.05$ (double-tailed) was considered statistically significant. To compare the incidences of TSC1 and TSC2 patients between Japanese studies and studies of other ethnic groups, χ^2-test was used and $P < 0.05$ was considered statistically significant.

RESULTS

Identification and characterization of mutations

Among the total of 57 patients with TSC (46 definite and 11 suspect TSC), 11 *TSC1* mutations (2 familial and 9 sporadic cases, all of them were definite TSC) and 20 *TSC2* mutations (2 familial and 18 sporadic cases, including 19 definite and 1 suspect TSC) were detected. All *TSC* mutations detected by this study are summarized in Table 1. Overall mutation detection rate was 54.4% (31 among 57 patients) and 65.2% in definite TSC population (30 among 46 patients). Overall mutation detection rate by CHIPS was 48.9% (23 among 47 patients) and 62.2% in definite TSC (23 among 37 patients). Compared with the screening results of our first 10 patients (nine definite and one suspect TSC), this detection rate of CHIPS is reasonable and showing its high sensitivity. With the exclusion of two large gene deletions, six mutations were identified among the first 10 patients, of which, four mutations were positively screened by SSCP/HA analysis and remaining two were just detected by whole-coding exon sequencing of both *TSC1* and *TSC2*.

The 11 *TSC1* mutations included one missense, two splicing, two nonsense, five frameshift mutations and one large deletion. Eight of them are novel mutations detected by this study and two of them are previously reported only by us, whereas Q654X has been reported by the other US group.[39] In *TSC1*, several amino acid substituted nucleic acid changes are found in general population and these amino acid substitutions are recognized as polymorphisms not as missense mutations. So, we evaluated one novel missense substitution H181P detected in this study for its pathogenesis. In the *TSC1* mutation and polymorphism databases, H181P was not described in the human gene mutation database (http://www.hgmd.cf.ac.uk/ac/index.php), the Leiden open variation database (http://www.lovd.nl/2.0/index.php) or dbSNP (http://www.ncbi.nlm.nih.gov/snp/). So, four web-based algorithmic programs were applied to predict the effect of amino acid substitution on protein function. PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/),[40] PMut (http://mmb2.pcb.ub.es:8080/PMut/)[41] and Mutation taster (http://www.mutationtaster.org/index.html)[42] predicted that this substitution is pathological and SIFT (http://sift.jcvi.org/)[43] predicted that it is tolerated with relatively low credibility. H181P did not appear in other TSC patients we analyzed in this study nor in the DNA of the 50 normal Japanese volunteers. According to these results, we consider that H181P is a disease causative mutation.

Another *TSC1* missense substitution, T899S, appeared in family 15 who were diagnosed as suspect TSC with the only presenting TSC phenotype was hypomelanotic macules of the skin. The same substitution was previously reported as a causative mutation in a Japanese patient with TSC.[29] T899S was in agreement with the record of dbSNP rs76801599, in which clinical significance is not tested. Although T899S does not appear in the other TSC patients in this study, nor in the 50 Japanese normal volunteers; all of the four web-based programs mentioned above predicted that this substitution does not affect protein function. According to these results, we conclude that T899S is a rare polymorphism in the Japanese population. In family 10, *TSC1* large deletion was detected by long-range PCR, which spans exon 17–19 with somatic mosaicism of two different deletions. The details of this patient have been reported previously.[28]

The 20 *TSC2* mutations included four missense, four in-frame deletion, one stop codon, four splicing, six frameshift mutations and one large deletion were detected in our patients. However, no *TSC2* nonsense mutation was detected. Seven of them are novel mutations detected in this study and two of them are previously reported only by us, whereas the other 11 have been reported previously in other ethnic groups.[16,17,19,37,44–46] The stop codon mutation detected in family 31 is the same mutation as previously reported.[46] TG deletion of the stop codon (TGA) causes continuation of translation beyond the stop codon, without a new stop codon downstream. Goedbold *et al.*[46] demonstrated that this mutated tuberin could not bind to hamartin by the yeast two-hybrid experiment. The *TSC2* patient of family 1 had only a SEGA as a feature of TSC and was classified as suspect TSC by clinical diagnostic criteria. This patient was included in our first 10 patients and mutational screening was performed by SSCP/HA with SEGA-derived DNA at the first instance. After detected *TSC2* missense mutation R1743Q in the brain tumor, we screened whether the same mutation existed in other tissues of the patient including blood, hair, nail, buccal mucosa and urinary sediment, but R1743Q was not detected in these tissues. We concluded that R1743Q was a somatic mutation in SEGA and *TSC2* LOH was also confirmed in this tumor. Details of this TSC2 somatic mosaic patient have been described elsewhere.[47]

Table 1 TSC mutations

Family no.	F/S	Gene	Position	Mutation type	Mutation (DNA level)	Mutation (protein level)	Previous report
48	S	TSC1	Exon 7	Missense	c.542A>C	p.H181P	Novel
8	S	TSC1	Intron 16	Splicing	IVS16+1G>A		28[a]
30	F	TSC1	Intron 20	Splicing	IVS20-1G>A		Novel
14	S	TSC1	Exon 15	Nonsense	c.1759A>T	p.K587X	Novel
41	S	TSC1	Exon 15	Nonsense	c.1960C>T	p.Q654X	39
44	S	TSC1	Exon 7	Frameshift	c.648delT	p.F216LfsX223	Novel
36	S	TSC1	Exon 10	Frameshift	c.989delT	p.L330RfsX337	Novel
25	S	TSC1	Exon 12	Frameshift	c.1250delC	p.T417NfsX439	Novel
49	S	TSC1	Exon 18	Frameshift	c.2342_2343insAA	p.L782SfsX807	Novel
33	F	TSC1	Exon 20	Frameshift	c.2582delT	p.L861PfsX877	Novel
10	S	TSC1	Exon 17-19	Large deletion	Complex large deletion detected by long PCR		28[a]
53	S	TSC2	Exon 19	Missense	c.2198T>C	p.L733P	44
42	S	TSC2	Exon 21	Missense	c2423T>C	p.L808S	—[b]
27	S	TSC2	Exon 40	Missense	c.5228G>A	p.R1743Q	45
1	S	TSC2	Exon 40	Missense	c.5228G>A (tumor only)	p.R1743Q (tumor only)	17,19,28[a],47[a]
38	F	TSC2	Exon 17	In-frame deletion	c.1862_1864delTGC	p.L621del	Novel
24	S	TSC2	Exon 35	In-frame deletion	c.4654-4656delGAA	p.E1552del	16
5	S	TSC2	Exon 40	In-frame deletion	c.5238_5255delCATCAAGCGGCTCCGCCA	p.H1746_R1751del	16,28[a],37
43	S	TSC2	Exon 40	In-frame deletion	c.5238_5255delCATCAAGCGGCTCCGCCA	p.H1746_R1751del	16,37
31	S	TSC2	Exon 41	Stop codon mutation	c.5422_5423delTG	p.X1808RextX1841	46
12	S	TSC2	Intron 3	Splicing	IVS3-1G>A		Novel
6	S	TSC2	Intron 20	Splicing	IVS20-1G>A		28[a],44
32	S	TSC2	Intron 29	Splicing	IVS29+1G>A		44
22	S	TSC2	Intron 31	Splicing	IVS31-1G>C		Novel
19	S	TSC2	Exon 1	Frameshift	c.45_51delTAAGATT	p.K16CfsX43	Novel
7	F	TSC2	Exon 10	Frameshift	c.980-981insAT	p.M327IfsX363	28[a]
47	S	TSC2	Exon 15	Frameshift	c.1628_1629insC	p.E544GfsX588	Novel
11	S	TSC2	Exon 30	Frameshift	c.3620_3624delGCTGG	p.S1207TfsX1231	Novel
9	S	TSC2	Exon 33	Frameshift	c.4162_4178dupGAGCAAGTCCAGCTCCT	p.S1338X	28[a]
29	S	TSC2	Exon 39	Frameshift	c.5112-5113insT	p.D1705X	Novel
2	S	TSC2	5′ side	Large deletion	Large deletion detected by southern blot		16,28[a]

Abbreviations: F/S, familial/sporadic; TSC, tuberous sclerosis complex.
[a]Previously reported by our group.
[b]Tuberous sclerosis project (http://tsc-project.partners.org/index.htm).

Analysis of clinical manifestations and genotype–phenotype correlation

The clinical features associated with each TSC1 or TSC2 mutation-positive patients and patients with NMI are displayed in Table 2. A comparison of the incidence and grade score of these clinical manifestations between the TSC1, TSC2 and NMI-definite TSC patients are summarized in Table 3.

Among the clinical findings, subependymal nodule and cortical tuber are the most consistent features in all groups, and at least one of these features were found in all patients with TSC1 and TSC2 patients who were given brain imaging tests. However, two patients with NMI-definite TSC (family 34 and 57) had no brain lesions. Among the neurological findings, incidence of any developmental delay or mental retardation (grade 1 or 2) was significantly higher in TSC2 patients (12/15) in comparison with TSC1 patients (4/11; $P = 0.032$, Fisher's exact test). Also, the incidence of substantial developmental delay or mental retardation (grade 2) was significantly higher in TSC2 patients (9/15) in comparison with TSC1 patients (2/11; $P = 0.040$, Fisher's exact test). In addition, mean grade score was significantly higher in TSC2 patients (1.4) than TSC1 patients (0.5; $P = 0.015$, Student's unpaired t-test). This trend of worse psychomotor delay in TSC2 patients was also observed between TSC2 patients and NMI-definite TSC patients, in incidence of grade 1 or 2 (6/16 vs 12/15, $P = 0.020$, Fisher's exact test), grade 2 (3/16 vs 9/15, $P = 0.023$, Fisher's exact

test) and mean grade score (0.6 vs 1.4, $P = 0.005$, Student's unpaired t-test). However, no difference was observed between TSC1 patients and NMI-definite TSC patients. The incidence and severity of epilepsy, brain imaging and retinal hamartoma were not different in TSC1, TSC2 and NMI-definite TSC patients.

From the phenotypic skin lesions, only ungula fibroma was observed more in TSC1 patients than in TSC2 patients ($P = 0.034$, Fisher's exact test) but no difference was detected in facial angiofibroma, hypomelanotic macules and other skin lesions. Renal, hepatic, pulmonary and cardiac lesions were variable in all groups and the incidences and mean grade scores were not statistically different. We also examined the data for genotype/phenotype correlation and found no correlation between mutation type and phenotype in both TSC1 and TSC2 patients.

Mutation distributions of TSC1 and TSC2 genes in this study, as well as those reported in Japanese patients and previous studies of other races are summarized in Table 4. No obvious difference was indicated between the Japanese population and other ethnic groups, except for low frequencies of TSC2 nonsense mutation ($P = 0.015$, this study vs US and Europe studies; and $P = 0.000$, all reported Japanese mutations vs US and Europe studies, χ^2-test). Incidence of TSC1 and TSC2 mutation among patients with TSC are compared with either this study or the sum of Japanese cohort studies and other previous studies from different ethnic groups (Table 5). It is

Table 2 Clinical features of patients with TSC

Family no.	F/S	Clinical diagnosis	Gene	Age	Mutation type	Epilepsy	DD/MR	Brain SEN	SEGA	Cort. Tub.	HM	AF	SP	UF	FP	Renal cyst	Renal AML	Liver AML	Ret. Ham.	Card. Rhabd.	LAM
48	S	Definite TSC	TSC1	35	Missense	2	1	2	-	0	+	2	NS	NS	NS	2	2	0	NS	+	0
8	S	Definite TSC	TSC1	15	Splicing	2	0	2	-	2	+	2	-	+	-	0	0	0	-	-	0
30	F	Definite TSC	TSC1	35	Splicing	1	0	2	-	2	-	3	-	-	-	0	0	0	NS	NS	NS
14	S	Definite TSC	TSC1	48	Nonsense	0	0	2	-	2	-	2	-	+	-	0	3	0	-	-	0
41	S	Definite TSC	TSC1	7	Nonsense	2	2	0	-	2	+	0	-	-	-	0	0	0	NS	-	NS
44	S	Definite TSC	TSC1	22	Frameshift	2	2	2	-	1	+	2	+	+	-	0	0	0	-	-	0
36	S	Definite TSC	TSC1	50	Frameshift	0	0	NS	NS	NS	+	2	+	+	-	NS	NS	NS	-	NS	1
25	S	Definite TSC	TSC1	31	Frameshift	2	0	2	-	2	-	1	+	-	-	0	0	0	NS	-	NS
49	S	Definite TSC	TSC1	16	Frameshift	2	1	2	-	0	+	2	NS	NS	NS	2	0	0	NS	-|	0
33	F	Definite TSC	TSC1	31	Frameshift	2	0	2	-	2	-	3	-	-	-	3	3	0	-	NS	1
10	S	Definite TSC	TSC1	1	Large deletion	2	0	2	-	2	+	0	-	-	-	0	0	0	-	+	0
53	S	Definite TSC	TSC2	15	Missense	2	2	2	-	2	+	0	NS	NS	NS	0	0	0	NS	-	0
42	S	Definite TSC	TSC2	3	Missense	2	1	2	-	2	+	0	-	-	-	4	0	0	NS	+	0
27	S	Definite TSC	TSC2	26	Missense	2	2	2	-	NS	-	3	+	-	-	0	2	0	-	+	0
38	F	Definite TSC	TSC2	2	In-frame deletion	2	0	0	-	2	-	0	-	-	-	0	0	0	NS	-	NS
24	S	Definite TSC	TSC2	1	In-frame deletion	2	2	1	-	2	-	0	-	-	-	NS	NS	NS	-	-	NS
5	S	Definite TSC	TSC2	9	In-frame deletion	2	2	2	-	2	+	3	-	-	-	4	3	0	-	-	0
43	S	Definite TSC	TSC2	8	In-frame deletion	2	2	2	-	2	+	0	-	-	-	2	0	0	-	+	0
31	S	Definite TSC	TSC2	0	Stop codon mutation	0	NS	2	-	1	+	0	-	-	-	0	0	0	-	+	NS
12	S	Definite TSC	TSC2	0	Splicing	2	NS	2	-	2	+	0	-	-	-	NS	NS	NS	+	NS	0
6	S	Definite TSC	TSC2	28	Splicing	2	2	2	-	2	+	3	+	-	-	0	0	0	-	-	0
32	S	Definite TSC	TSC2	0	Splicing	2	NS	2	-	2	-	0	-	-	-	0	1	0	-	-	0
22	S	Definite TSC	TSC2	3	Splicing	2	2	2	-	2	+	0	+	-	-	0	0	0	-	+	NS
19	S	Definite TSC	TSC2	1	Frameshift	0	0	2	-	2	+	0	-	-	-	NS	NS	NS	-	-	NS
7	F	Definite TSC	TSC2	13	Frameshift	2	2	2	-	2	-	3	+	-	+	2	3	0	-	+	0
47	S	Definite TSC	TSC2	0	Frameshift	1	NS	2	+	2	+	0	-	+	-	NS	NS	NS	+	-	0
11	S	Definite TSC	TSC2	21	Frameshift	0	1	2	-	2	+	3	-	+	NS	0	3	2	-	+	0
9	S	Definite TSC	TSC2	17	Frameshift	2	1	2	-	2	+	2	NS	NS	+	0	3	0	+	+	0
29	S	Definite TSC	TSC2	36	Frameshift	1	0	NS	NS	NS	-	3	-	-	-	NS	0	NS	NS	NS	0
2	S	Definite TSC	TSC2	11	Large deletion	2	2	2	-	2	+	3	+	-	+	4	0	0	+	+	0
3	F	Definite TSC	NMI	9		2	1	2	-	2	+	0	+	-	-	0	2	0	+	+	2
4	S	Definite TSC	NMI	19		2	1	2	-	2	+	2	+	-	-	0	3	0	-	+	2
13	S	Definite TSC	NMI	64		0	0	2	+	2	-	0	-	-	-	1	0	0	-	-	0
17	S	Definite TSC	NMI	8		2	1	2	-	2	-	1	-	-	-	0	0	0	-	-	0
18	F	Definite TSC	NMI	30		2	0	2	-	0	+	3	+	+	-	3	3	2	-	-	2
34	S	Definite TSC	NMI	70		0	0	0	-	2	+	0	-	+	-	0	3	0	-	NS	NS
37	S	Definite TSC	NMI	1		0	0	2	-	2	+	0	-	-	-	0	0	0	-	-	NS
39	S	Definite TSC	NMI	2		2	2	0	-	2	-	0	-	-	-	0	0	0	NS	-	NS
40	S	Definite TSC	NMI	7		0	0	1	-	0	-	0	-	-	-	0	0	0	-	+	0
50	F	Definite TSC	NMI	27		2	2	2	+	2	+	2	NS	NS	NS	0	2	0	NS	+	0
51	F	Definite TSC	NMI	30		2	2	2	-	2	+	2	NS	NS	NS	2	0	0	NS	-	0
52	S	Definite TSC	NMI	1		2	0	2	-	2	+	0	NS	NS	NS	0	0	0	-	-	0
54	S	Definite TSC	NMI	8		0	0	2	-	2	+	2	NS	+	-	2	2	0	NS	-	0
55	S	Definite TSC	NMI	37		0	0	2	-	1	-	2	+	+	-	3	0	0	+	-	0
56	S	Definite TSC	NMI	43		1	0	2	-	NS	-	3	NS	NS	+	3	3	0	-	NS	2

Table 2 (Continued)

Family no.	F/S	Clinical diagnosis	Gene	Age	Mutation type	Epilepsy	DD/MR	Brain SEN	SEGA	Cort. Tub.	HM	AF	SP	UF	FP	Renal cyst	Renal AML	Liver AML	Ret. Ham.	Card. Rhabd.	LAM
57	S	Definite TSC	NMI	25		0	0	0	0	0	–	2	–	–	–	0	3	0	NS	–	0
1	S	Suspect TSC[a]	TSC2	20		0	0	0	+	0	–	0	–	–	–	0	0	0	–	–	0
15	S	Suspect TSC[b]	NMI	3		0	0	0	–	0	+	0	–	–	–	NS	NS	NS	–	–	NS
16	S	Suspect TSC	NMI	16		0	0	0	–	0	–	2	–	–	–	NS	0	1	–	–	0
20	S	Suspect TSC	NMI	1		1	0	0	–	NS	+	0	–	–	–	NS	NS	NS	NS	NS	NS
21	S	Suspect TSC	NMI	2		2	2	0	–	2	–	0	–	–	–	0	0	0	–	–	0
23	S	Suspect TSC	NMI	6		1	1	0	–	1	–	0	–	–	–	NS	NS	NS	NS	NS	NS
26	S	Suspect TSC	NMI	0		0	NS	0	–	0	–	0	–	–	–	0	0	0	–	+	0
28	S	Suspect TSC	NMI	10		2	1	1	–	0	–	0	–	–	–	0	0	0	NS	–	NS
35	S	Suspect TSC	NMI	3		1	0	2	–	0	–	0	–	–	–	0	0	0	–	–	NS
45	S	Suspect TSC	NMI	3		2	0	0	–	1	–	1	–	–	–	0	0	0	–	NS	0
46	S	Suspect TSC	NMI	5		2	0	0	–	1	–	0	–	–	–	0	0	0	–	–	0

Abbreviations: AF, facial angiofibroma; AML, angiomyolipomas; Card. Rhabd., cardiac rhabdomyoma; Cort. Tub., cortical tuber; DD/MR, developmental delay and/or mental retardation; FP, forehead plaque; F/S, familial/sporadic; HM, hypomelanotic macules; LAM, lymphangiomyomatosis; NMI, no mutation identified; Ret. Ham., retinal hamartoma; SEGA, subependymal giant cell astrocytoma; SEN, subependymal nodule; SP, shagreen patch; TSC, tuberous sclerosis complex; UF, ungual fibroma; –, screening negative; +, screening positive; NS, not screened.
Scoring grade: epilepsy, 0 = never, 1 = controlled, 2 = active seizure; DD/MR, 0 = normal range, 1 = moderate, 2 = significant (see text); brain SEN and Cort. Tub., 0 = no, 1 = single, 2 = multiple; AF, 0 = no, 1 = macular lesions only on cheek, 2 = papular lesions <3 mm, 3 = papular lesions >3 mm and or extending to below mouth; renal cyst, 0 = no, 1 = 1 or 2 small <2 cm, 2 = more than 2 small <2 cm, 3 = more than 2 and at least 1 >2 cm, 4 = polycystic kidney disease; AML, 0 = no, 1 = all <1 cm, 2 = all <4 cm, 3 = 1 or more >4 cm; LAM, 0 = no, 1 = multifocal micronodular pneumocyte hyperplasia, 2 = LAM.
[a]TSC2 missense mutation, R1743Q only in SEGA.[47]
[b]TSC1 T899S polymorphism (see text).

remarkable that incidence of TSC1 patients in Japan is significantly higher than in other studies. To compare US with Europe studies, overall *TSC2/TSC1* detected mutation ratio is lower in this study (1.7 vs 3.7, $P = 0.043$, χ^2-test) and in the Japanese cohort (1.9 vs 3.7, $P = 0.014$, χ^2-test). When comparing only sporadic cases, these variances are much more significant, both in this study (1.9 vs 5.5, $P = 0.009$, χ^2-test) and in the Japanese cohort (2.2 vs 5.5, $P = 0.007$, χ^2-test).

DISCUSSION

We screened both *TSC1* and *TSC2* for disease causative mutations in 57 Japanese families with TSC, including 46 definite and 11 suspect TSC, and identified 11 *TSC1* mutations and 20 *TSC2* mutations including one somatic mosaic mutation in the patient with suspect TSC (family 1). Mutation detection rate in definite TSC patients was 30/46 (65.2%). This rate is comparable to previous large cohort studies. In addition, no mutation was detected in suspect TSC patients other than the *TSC2* somatic mosaic case mentioned above.

First, this result proved the accuracy and utility of TSC clinical diagnostic criteria in general,[3] but a small fraction of suspect TSC could have *TSC* mutation. Au *et al*.[17] also showed similar results in large-scale cohort. They screened *TSC* mutations in 368 patient with TSC (including 325 definite TSC and 43 uncertain diagnosis or not-definite TSC patients), and disease causative mutations were detected in a total of 243 patients, of which, 234 were definite TSC, eight were patients with uncertain diagnosis because of poor medical records and one *TSC2* mutation was found in a not-definite TSC patient.

We could not identify *TSC1* or *TSC2* mutations in 16 definite TSC patients. Two of them have no brain lesions and no neurological symptoms (epilepsy, mental retardation, subependymal nodule, SEGA or cortical tuber). A 25-year-old male (family 57) has facial angiofibroma and renal AML, but no other TSC-related lesions. A 70-year-old female (family 34) has hypomelanotic macules, ungual fibroma, renal AML and pulmonary LAM, but no facial angiofibroma. As represented in these patients, several NMI-definite TSC patients seem to abortive type although fulfilled clinical diagnostic criteria.

It is reported that a significant population up to 10% of TSC patients have somatic mosaicism and it might be difficult to detect the mutations by peripheral blood DNA testing for these patients.[15,16] Actually, in family 1, we detected a *TSC2* somatic mosaic mutation only in the tumor DNA sample. If we could test tumor samples of these NMI-definite TSC families, it may increase the possibility to detect more disease causative *TSC* mutations. From another viewpoint, we might have missed *TSC1* or *TSC2* mutations in these patients due to technical problems. For point mutations, or small insertions or deletions, we detected a sufficient number of mutations; considering the high sensitivity of CHIPS technology and comparing it with the previous study using DHPLC,[22] it is less possible that we missed many small mutations in this population. For large deletions, we checked large intragenic rearrangements by long-range PCR and large gene deletions including the 5′ or 3′ end of the genes by Southern blot analysis. Although we did not perform multiplex ligation-dependent probe amplification (MLPA) for each exon, previous studies indicate that point exon deletion is a rare event for both *TSC1* and *TSC2*,[19] and the majority of *TSC2* large deletions could be detected by our methods. In addition, in our 16 NMI-definite TSC patients, nine patients have *TSC2* polymorphisms and seven patients have *TSC1* polymorphisms. Existence of polymorphisms proved two copies of each gene at these sites, and previous studies indicate that *TSC2* large deletions are detected in

Table 3 Observed frequencies and severities of clinical features and statistical analysis

Category	Features	TSC1 mutation positive (n = 11)	TSC2 mutation positive[a] (n = 19)	NMI- definite TSC (n = 16)	P[b] TSC1 vs TSC2	P[b] TSC1 vs NMI	P[b] TSC2 vs NMI
Family history		2/11 (18%)	2/19 (11%)	3/16 (19%)	0.874	0.684	0.415
Neurological	Epilepsy (grade 1 or 2)	9/11 (82%)	16/19 (84%)	9/16 (56%)	0.719	0.133	0.074
	Epilepsy (grade 2)	8/11 (73%)	14/19 (74%)	8/16 (50%)	0.796	0.311	0.137
	Average grade score	1.9	1.6	1.1	0.786	0.223	0.093
	DD/MR (grade 1 or 2)	4/11 (36%)	12/15 (80%)	6/16 (38%)	**0.032**	0.267	**0.020**
	DD/MR (grade 2)	2/11 (18%)	9/15 (60%)	3/16 (19%)	**0.040**	0.684	**0.023**
	Average grade score	0.5	1.4	0.6	**0.015**	0.958	**0.005**
	Brain SEN	9/10 (90%)	17/18 (94%)	13/16 (81%)	0.881	0.496	0.257
	Average grade score	2.0	1.8	1.6	0.943	0.384	0.264
	SEGA	0/10 (0%)	1/18 (6%)	1/16 (6%)	0.643	0.615	0.485
	Cort. Tub.	8/11 (80%)	17/17 (100%)	12/15 (80%)	0.128	0.699	0.092
	Average grade score	1.9	1.9	1.5	0.148	0.971	0.086
	Ret. Ham.	0/6 (0%)	3/15 (20%)	2/11 (18%)	0.342	0.404	0.654
Skin lesions	HM	7/11 (64%)	14/19 (74%)	9/16 (56%)	0.839	0.508	0.234
	AF	9/11 (82%)	9/19 (47%)	9/16 (56%)	0.069	0.167	0.427
	Average grade score	2.1	1.3	1.2	0.415	0.225	0.778
	SP	3/9 (33%)	3/17 (18%)	2/11 (18%)	0.916	0.396	0.710
	UF	4/9 (44%)	1/17 (6%)	2/11 (18%)	**0.034**	0.217	0.336
	FP	0/9 (0%)	2/17 (12%)	2/12 (17%)	0.418	0.314	0.822
Internal organs	Renal cyst	3/10 (30%)	5/14 (36%)	5/16 (31%)	0.561	0.648	0.741
	Average grade score	1.2	1.1	0.8	0.485	0.919	0.472
	Renal AML	3/10 (30%)	6/15 (40%)	8/16 (50%)	0.470	0.277	0.422
	Average grade score	0.8	1.0	1.3	0.718	0.363	0.534
	Liver AML	0/10 (0%)	1/14 (7%)	1/16 (6%)	0.583	0.615	0.791
	Average grade score	0.0	0.1	0.1	0.336	0.333	0.925
	LAM	2/8 (25%)	0/14 (0%)	3/14 (21%)	0.121	0.767	0.111
	Average grade score	0.3	0.0	0.4	0.171	0.592	0.082
	Card. Rhabd	2/8 (25%)	9/17 (53%)	4/14 (29%)	0.190	0.631	0.092

Abbreviations: AF, facial angiofibroma; AML, angiomyolipomas; Card. Rhabd., cardiac rhabdomyoma; Cort. Tub., cortical tuber; DD/MR, developmental delay and/or mental retardation; FP, forehead plaque; HM, hypomelanotic macules; LAM, lymphangiomyomatosis; NMI, no mutation identified; Ret. Ham., retinal hamartoma; SEGA, subependymal giant cell astrocytoma; SEN, subependymal nodule; SP, shagreen patch; TSC, tuberous sclerosis complex; UF, ungual fibroma.
Statistically significant values are represented in bold.
[a]Remove family 1, R1743Q somatic mosaic patient.
[b]Fisher's exact test was used for the categorical variables, and Student's unpaired *t*-test or Welch's *t*-test was used for average grade score. See text in details.

only 8% of *TSC2* patients at the maximum.[16] These data indicate it is less possible that we missed many large *TSC1* or *TSC2* deletions. Also, previous linkage analysis confirmed that there is no other TSC locus.

Another possibility could be hidden in the large 3′-UTR sequence in *TSC1* gene that has not yet been analyzed by us or any other researchers. *TSC1* cDNA consists of 234-bp 5′-UTR, 3045-bp coding and 5097-bp 3′-UTR sequence. Although many polymorphisms are reported in dbSNP, no pathological mutation has been detected in *TSC1* 3′-UTR, and the function of this long 3′-UTR sequence is still unknown. Put together with NMI-definite TSC patients that resemble TSC1 patients in that they manifest intellectually milder phenotype than TSC2 patients, precious analysis of this sequence is an issue in the future for determining the possibility that we missed *TSC1* 3′-UTR mutations in NMI-definite TSC population.

The spectrum and distribution of *TSC1* and *TSC2* mutations in this study were not different from previous reports, except for the low *TSC2* nonsense mutation finding. To date, including this study, 92 mutations (33 *TSC1*, 59 *TSC2*) of Japanese patients with TSC are reported in the literature, and no ethnically specific mutational hot spots are detected (Figure 1, Supplementary Table 1). The only

reported *TSC2* nonsense mutation in Japanese patients is R1459X in exon 33 found in two different families.[29] Why the *TSC2* nonsense mutation is rare in the Japanese TSC population is not clear at this time. We have to collect and analyze more *TSC* mutations in Japanese patients to elucidate the reason.

Comparing the phenotypes of TSC1, TSC2 and NMI-definite TSC patients, we conclude that TSC2 patients have much profound developmental delay or mental retardation than TSC1 patients and NMI-definite TSC patients. It is difficult to evaluate mental retardation of very young patients with the same scale because the patients were collected from different hospitals all over Japan. However, in clinical practice, evaluating developmental delay by physical examination and developmental scale is routine work for pediatric neurologists. So, our modified check list precisely evaluates developmental state even in young patients and also evaluates mental retardation of older patients per the original check list item.[16] The milder phenotypes of *TSC1* patients[16,17] and NMI patients[17] in comparison with *TSC2* patients have also been reported elsewhere. Our data indicate that this is consistent in the Japanese population. However, physicians must be cautious in two issues. First, a milder phenotype

Table 4 Distribution of TSC mutations

	This study	Japan[a]	US[15]	US[16]	US[17]	UK[18]	Denmark[19]	Netherland[20]	US + Europe[b]	Taiwan[22]	Korea[23]	Korea[24]
TSC1 mutations												
Missense	1 (9%)	3 (9%)	0 (0%)	0 (0%)	1 (2%)	1 (5%)	0 (0%)	0 (0%)	2 (1%)	0 (0%)	2 (100%)	1 (20%)
In-frame deletion	0 (0%)	0 (0%)	0 (0%)	0 (0%)	ND	0 (0%)	0 (0%)	1 (1%)	1 (0%)	1 (11%)	0 (0%)	0 (0%)
Splicing	2 (18%)	2 (6%)	2 (13%)	2 (7%)	4 (7%)	2 (9%)	1 (9%)	7 (9%)	18 (8%)	0 (0%)	0 (0%)	0 (0%)
Nonsense	2 (18%)	8 (24%)	7 (44%)	11 (39%)	21 (34%)	7 (32%)	4 (36%)	37 (45%)	87 (40%)	5 (56%)	0 (0%)	3 (60%)
Frameshift	5 (45%)	19 (58%)	7 (44%)	15 (54%)	35 (57%)	12 (55%)	6 (55%)	37 (45%)	112 (51%)	3 (33%)	0 (0%)	0 (0%)
Large del	1 (9%)	1 (3%)	NT	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	NT	NT	1 (20%)
TSC1 total	11	33	16	28	61	22	11	82	220	9	2	5
TSC2 mutations												
Missense	4[c] (20%)	22 (37%)	13 (22%)	31 (20%)	58 (32%)	22 (22%)	10 (25%)	56 (20%)	190 (23%)	12 (22%)	0 (0%)	2 (33%)
In-frame deletion	5[d] (25%)	8 (13%)	2 (3%)	13 (8%)	ND	5 (5%)	1 (3%)	15 (5%)	36 (4%)	2 (4%)	2 (20%)	0 (0%)
Splicing	4 (20%)	13 (22%)	8 (14%)	27 (17%)	25 (14%)	8 (8%)	6 (15%)	43 (15%)	117 (14%)	7 (13%)	1 (10%)	2 (33%)
Nonsense	0 (0%)[e]	2 (3%)[f]	14 (24%)	37 (23%)	39 (21%)	20 (20%)	9 (23%)	67 (24%)	186 (23%)	15 (27%)	4 (40%)	1 (17%)
Frameshift	6 (30%)	14 (23%)	21 (36%)	30 (19%)	57 (31%)	21 (21%)	10 (25%)	79 (28%)	218 (27%)	19 (35%)	3 (30%)	0 (0%)
Large del	1 (5%)	1 (2%)	NT	20 (13%)	3 (2%)	22 (22%)	4 (10%)	20 (7%)	69 (8%)	NT	NT	1 (17%)
TSC2 total	20	60	58	158	182	98	40	280	816	55	10	6
All	31	93	72	186	243	120	51	362	983	64	12	11

Abbreviations: ND, In-frame deletion was not distinguished from frameshift in the US[17] study; NT, not tested in the study; TSC, tuberous sclerosis complex .
[a]Add this study and other Japanese TSC mutation studies.[29–33]
[b]Add US and Europe studies.[15–20]
[c]Include family 1, R1743Q somatic mosaic mutation.
[d]Include family 31, TSC2 stop codon mutation.
[e]$P = 0.015$ to US + Europe (χ^2-test).
[f]$P = 0.000$ to US + Europe (χ^2-test).

Table 5 TSC2/TSC1 mutation ratio comparing between Japanese studies and previous studies

	Inheritance				TSC1 mutation				TSC2 mutation					TSC2/TSC1 ratio in all cases			TSC2/TSC1 ratio in sporadic cases		
Studies	F	S	U	Total	F	S	U	Total	F	S	U	Total	Mutation detected (%)		P[a]	P[b]		P[a]	P[b]
This study[c]	4	42		46	2	9		11	2	17		19	65.2	1.7			1.9		
Japan[29]	11	25	2	38	3	4		7	5	11	1	17	63.2	2.4			2.8		
Japan[30]	4	23		27			4	4			6	6	37	1.5			1.5		
Japan sum	19	90	2	111	5	13	4	22	7	28	7	42	57.7	1.9			2.2		
US[15]	40	86		126	11	5		16	16	42		58	58.7	3.6	0.113	0.094	8.4	**0.013**	**0.015**
US[16]	38	183		221	6	22		28	29	129		158	84.2	5.6	**0.004**	**0.001**	5.9	**0.013**	**0.012**
US[17]	71	297		368	25	36		61	28	154		182	66	3	0.175	0.137	4.3	0.065	0.07
UK[18]	19	130	1	150	9	13		22	9	88	1	98	80	4.5	**0.03**	**0.015**	6.8	**0.009**	**0.009**
Denmark[19]				65	2	2	7	11	6	17	17	40	84.6	3.6	0.14	0.131	8.5	**0.043**	0.078
Netherland[20]	63	137	290	490			82	82			280	280	85.1	3.4	0.083	**0.044**			
US + Europe				1420	53	78	89	220	88	430	298	816	72.9	3.7	**0.043**	**0.014**	5.5	**0.009**	**0.007**
Australia[21]			45	45			9	9			24	24	73.3	2.7	0.423	0.477			
Taiwan[22]			84	84	2	7		9	8	47		55	76.2	6.1	**0.013**	**0.007**	6.7	**0.023**	**0.026**
Korea[23]	9	35		44	1	1		2	3	8		11	29.5	5.5	0.163	0.178	8	0.179	0.213
Korea[24]			55	55			4	4			5	5	16.4	1.3	0.674	0.555			

Abbreviations: F, familial; S, sporadic; TSC, tuberous sclerosis complex; U, unknown.
Statistically significant values are represented in bold.
[a]P-value of each study vs this study (χ^2-test).
[b]P-value of each study vs Japan sum (χ^2-test).
[c]Indicate only definite TSC for comparison to other studies.

just means developmental delay or mental retardation, and there are no differences of incidence or severity in all other findings between TSC1 and TSC2 patients including skin, renal, cardiac, pulmonary manifestations and even epilepsy and brain images. Although previous studies have indicated a milder renal phenotype[16,17] and facial angiofibroma[17] in TSC1 patients, we could not detect these differences because of low patient numbers and actual phenotypic differences are slight. Second, even though overall incidence and severity of intellectual disability are milder in TSC1 than TSC2, some TSC1 patients have profound developmental delay or mental retardation as found in TSC2 patients. These points should be kept in mind, especially when providing genetic counseling.

We also identified a higher incidence of ungual fibroma in TSC1 patients than in TSC2 patients. However, ungual fibroma is a lesion
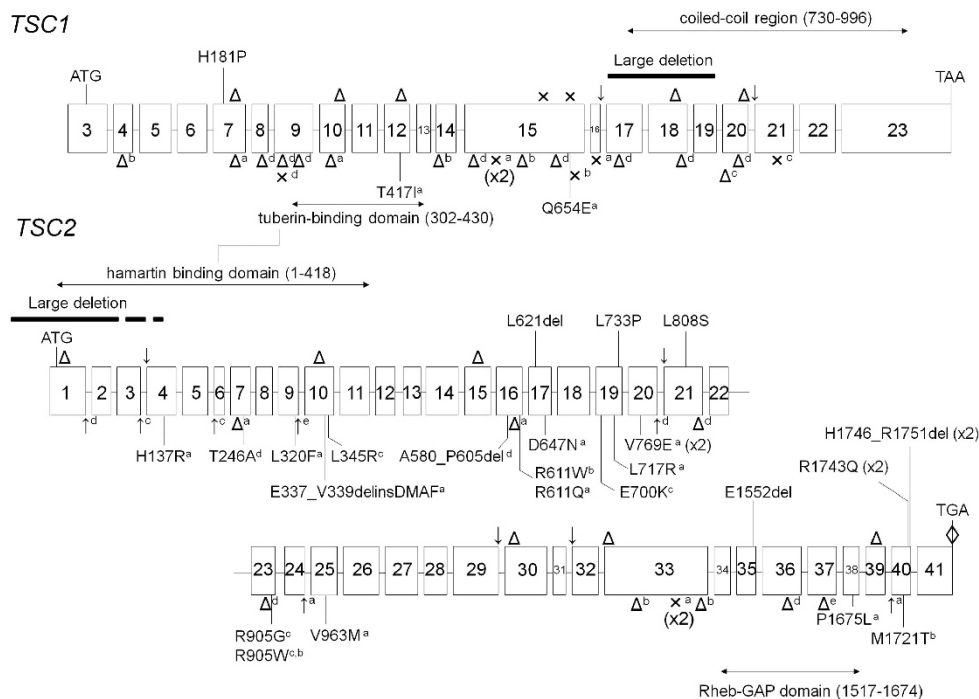
Figure1 Summary of *TSC* mutations reported in Japanese patients. Mutations reported in this study are indicated in the upper portion and mutations reported by others are indicated in the lower portion of each gene pattern diagram (a, Zhang *et al.*;[29] b, Yamashita *et al.*;[30] c, Yamamoto *et al.*;[31] d, Sasongoko *et al.*[32] and e, Sato *et al.*[33]). Missense mutations and in-frame deletions are described by amino acid changes. Δ, frameshift mutation; ↑, splicing mutation; ×, nonsense mutation; ◇, stop codon mutation; x2, reported two other non-related families.

that appears in adults, especially after middle age, the results was affected by younger TSC2 patients ages in our cohort (average 10.2 years old, one 21-year-old patient has ungula fibroma) than TSC1 patients (average 26.5 years old, four 50, 48, 22, 15-year-old patients have ungual fibroma).

One notable difference in the Japanese TSC population in comparison with previous studies in US and Europe is the higher TSC1 patient incidence among all mutation-positive TSC patients. The ratio of *TSC2/TSC1* mutation-positive cases is 1.7 in this study and is 1.9 after adding two other Japanese cohort studies, compared with 3.4–5.6 in US and Europe studies (3.7 as a whole). Though TSC1 patients are more detected in familial cases than in sporadic cases—because of higher reproductive fitness by its milder phenotype of intellectual faculties—higher incidence of TSC1 patients in Japan is not dependent on a high familial case ratio of mutation screened. Rather, in this study, familial cases comprise only 8 families among 46 definite TSC, and a higher TSC1 patient ratio is more evident when comparing only the sporadic cases: 1.9 in this study and 2.2 in Japanese cohort to 4.3–8.5 in US and Europe (5.5 as a whole). Other than US and Europe, *TSC2/TSC1* mutation ratio is 6.1 in Taiwan and 2.7 in Australia. In Korea, one *TSC* mutation study showed a high incidence of TSC1 patients (*TSC2/TSC1* is 1.3)[24] but another study did not (5.5).[23] We cannot conclude TSC1 incidence in Korea because overall mutation detection ratios in both of these studies are low. In any case, it seems that incidence of TSC1 patients among TSC differs across race and region.

In this study, eight *TSC1* mutations are novel and many other *TSC1* mutations detected in Japanese patients in other studies are also novel and unique. By contrast, many *TSC2* mutations detected in the Japanese population are also reported in US and Europe studies. It seems that the Japanese population has a specific *TSC1* mutational variation.

The reason why TSC1 patients are detected at a higher incidence in the Japanese TSC population is not clear. As mentioned above, it is not likely that we missed many *TSC2* mutations. *TSC1* mutations detected in the Japanese population are scattered throughout *TSC1* without hot spots. A majority of *TSC1* mutations in Japanese patients are detected in sporadic cases and TSC1 familial clusters are unlikely to exist. It is difficult to imagine that mutation ratio of the specific gene in the specific race or region would increase without the founder effect.

Another point of view is a social environmental factor. Differences of the medical service system or health disparity, including healthcare insurance and adoption rate of computed tomography and magnetic resonance units in the regions, may affect the results. TSC1 patients could be more frequently diagnosed in Japan owing to a generous healthcare insurance system and the convenient medical situation. Because TSC1 patients manifest a milder phenotype than TSC2 patients, it could be that less TSC1 patients visit a hospital in other countries, which does not have universal healthcare system like the United States. In addition, the adoption rate of computed tomography/magnetic resonance units in clinics and hospitals in Japan is world's best.[48] It seems that brain and abdominal imaging studies are performed more frequently in Japan compared with other countries including US and Europe, which consequently increases the chance for diagnosis of TSC among patients with milder phenotype. If this is true, many TSC1 patients are still undiagnosed in other countries. More research and analysis for *TSC1* mutations are vitally needed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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