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A Case of Solitary Subependymal Giant Cell Astrocytoma

Two Somatic Hits of TSC2 *in the Tumor, without Evidence of Somatic Mosaicism*

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Subependymal giant cell astrocytoma (SEGA) is a unique brain tumor arising in tuberous sclerosis complex (TSC), an autosomal dominant inherited phacomatosis. There are several case reports of solitary SEGA without any other manifestations of TSC. Usually these cases are thought to be forme fruste of TSC due to somatic mosaicism. However, no previous reports have used molecular methodology to fully investigate mutations in *TSC* genes or the possibility of somatic mosaicism. Here, we report a 20-year-old woman with a brain tumor. Pathological diagnosis was consistent with SEGA, but comprehensive clinical screening found no other lesions indicative of TSC. Molecular analysis of the tumor revealed loss of heterozygosity and allelic mutation (5228G>A, R1743Q) of *TSC2*. To detect the small fraction of mosaic mutation in somatic cells, we developed a highly sensitive new method: triple-nested polymerase chain reaction-restriction fragment length polymorphism. The identical *TSC2* missense mutation was not detected in any other tissues from the same patient, including peripheral blood, buccal mucosa, urinary sediment, nail, and hair. According to these results, this patient should be considered as having SEGA that developed from two somatic hit mutations in *TSC2*, rather than being a TSC2 patient with a very small fraction of somatic mosaicism. *(J Mol Diagn 2005, 7:544–549)*

Subependymal giant cell astrocytoma (SEGA) is a unique brain tumor that usually accompanies tuberous sclerosis complex (TSC), an autosomal dominant hereditary phacomatosis. Patients with TSC develop multiple hamartomas, mainly in the brain, heart, kidneys, eyes, and skin.[1,2] Two distinctive disease causative genes, *TSC1* and *TSC2,* have been cloned;[3,4] and germline and second hit mutations in hamartomas, mostly loss of heterozygosity (LOH), have been well studied.[5–8] Clinical expression of TSC is widely different in each case. Mosaicism is relatively common and well documented in TSC, because up to 10% of patients have somatic and/or gonadal mosaicism.[9–12]

Because SEGAs are characteristic lesions of TSC and were once even considered as pathognomonic, patients with isolated SEGA without any other TSC manifestations are usually considered as a forme fruste of TSC with somatic mosaicism. Although there are several case reports of isolated SEGA, no previous case was molecularly analyzed for *TSC* mutations or somatic mosaicism.[13–18] Here, we report a 20-year-old woman with isolated SEGA and no other clinical manifestations of TSC. We performed comprehensive molecular analysis of the surgically removed SEGA for both LOH and point mutations in the *TSC1* and *TSC2* genes. We also examined any available tissues throughout the body from the same patient, including peripheral leukocytes (two separate collections in a two year interval), urine, buccal mucosa, hair, and nail, to determine somatic mosaicism.

Materials and Methods

Case Report

A 20-year-old previously healthy woman presented with sudden onset of headache and vomiting. Brain MRI re-

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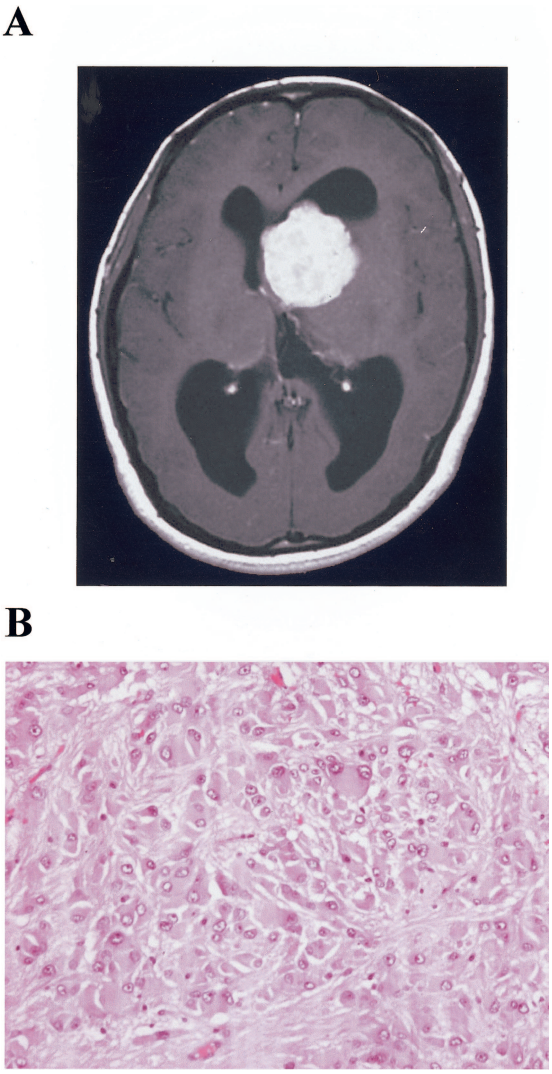

Figure 1. MRI and pathological findings of the patient. **A:** Preoperative MRI revealed a large, well-demarcated tumor protruding into the left lateral ventricle. The tumor homogeneously enhanced after Gd-DTPA administration. Neither SEN nor cortical tuber was observed. **B:** Photomicrograph of the tumor specimen; hematoxylin and eosin staining. The tumor was composed mainly of large polygonal cells resembling gemistocytic astrocytes. Mitotic figures were rare, and no endothelial proliferation or necrosis was seen. These findings were compatible with SEGA.

vealed a large, well-demarcated tumor in the left caudate head. The tumor was 4 cm in diameter and homogeneously enhanced after Gadolinium-diethyltriaminepentaacetic acid (Gd-DTPA) administration. The tumor was located near the foramen of Monro, which resulted in obstructive hydrocephalus (Figure 1A). No subependymal nodule (SEN) or cortical tuber was observed. Preoperative differential diagnosis included astrocytoma, oligodendroglioma, central neurocytoma, meningioma, germ cell tumor, and SEGA. Complete clinical screening for TSC, including dermatological and ophthalmologic exams, abdominal and cardiac ultra-sonography, and whole-body computed tomography found no lesions consistent with TSC. A left frontal craniotomy was performed, and the tumor was completely removed via a transcortical, transventricular approach. Macroscopically, the tumor arose from the caudate head and protruded into the anterior horn of the lateral ventricle. Pathological findings were compatible with SEGA (Figure 1B). Written informed consent for molecular analysis was obtained from the patient after explanation of the study, which was approved by the ethics committee of Kanazawa University Graduate School of Medical Science.

DNA and RNA Extraction

Peripheral blood leukocyte DNA and SEGA DNA were extracted by standard method. At the same time, peripheral leukocyte DNA from a healthy volunteer was extracted and used as a normal control. DNA was also extracted from a buccal mucosa swab, urinary sediments, hair, and nail by ISOHAIR (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. Total RNA of SEGA was extracted by Trizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized by standard method with Rivatra Ace reverse transcriptase (TOYOBO, Tokyo, Japan).

LOH Analysis

Both *TSC1* and *TSC2* loci of the SEGA and blood DNA were tested for LOH, as previously described.[19] Chromosomal markers D9S2126, D9S1830, D9S1199, and D9S1198 were used for detecting *TSC1* LOH; and D16S525, D16S3252, D16S665, D16S3403, D16S663, and D16S283 were used for *TSC2*. All polymerase chain reaction (PCR) products were run on 13.5% nondenatured polyacrylamide gel and developed with a silver stain kit (Bio-Rad, Hercules, CA). The sample was scored as positive for LOH only when the intensity of one allele of the SEGA was decreased more than 50% when compared with that of the blood control. Single-strand conformational polymorphism (SSCP) and sequence analysis of blood DNA revealed a polymorphism in *TSC2* intron 39, thus, we also added this polymorphism to *TSC2* LOH marker and determined LOH by direct sequencing.

TSC Mutation Screening

The coding exons of both *TSC1* and *TSC2* in blood and SEGA DNA were screened by SSCP as previously described.[19] All PCR products were separated on a SSCP gel (12% polyacrylamide, C = 2, with 8% glycerol) and developed with silver staining. Any samples with detected band shifts were confirmed with the BigDye Terminator v3.1 cycle sequencing kit and ABI PRISM 3100 Genetic analyzer (Applied Biosystems, Foster City, CA).

Investigation of Somatic Mosaicim by Single and Triple-Nested PCR-Restriction Fragment Length Polymorphism (RFLP)

With the above screening method, a missense mutation in *TSC2* was detected in SEGA (see Results). To explore the possibility of TSC somatic mosaicism, we tested ev-

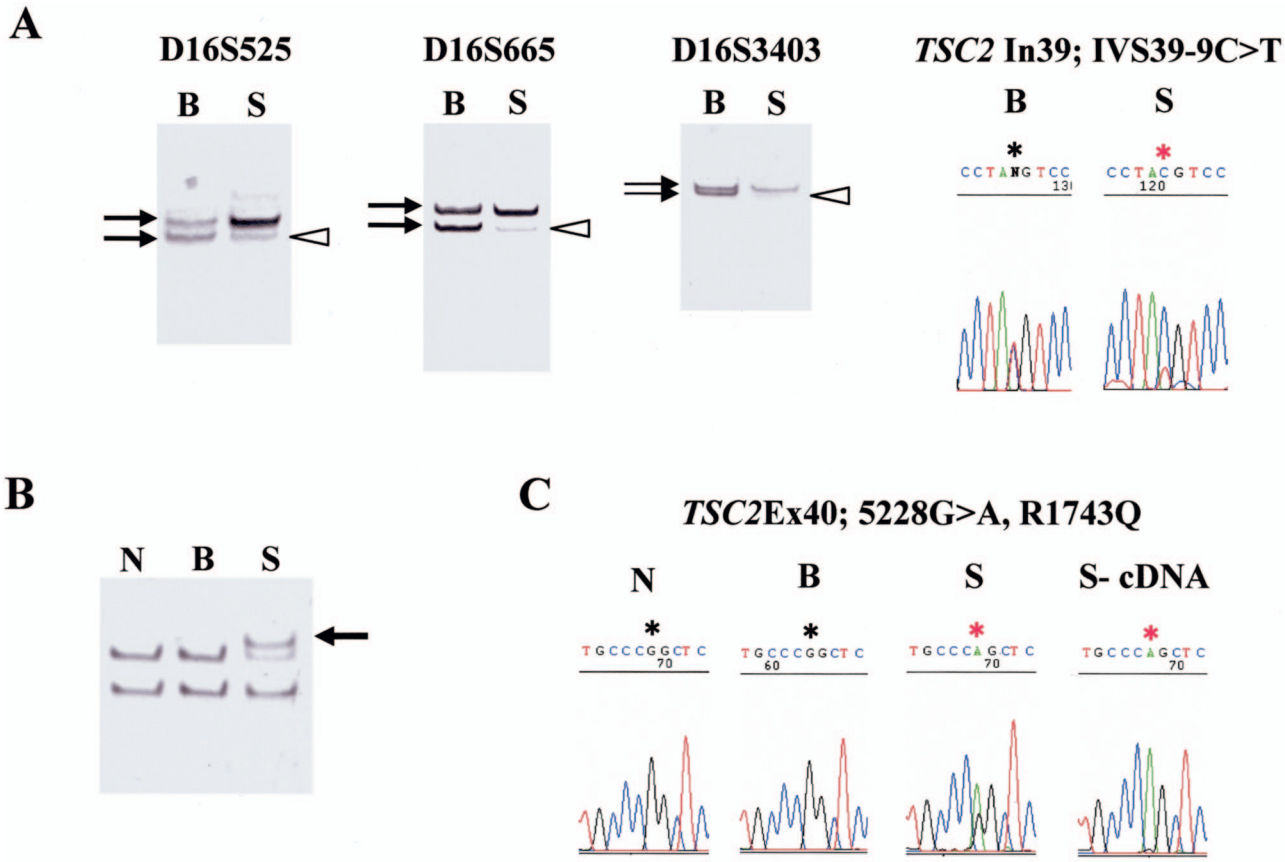

Figure 2. *TSC2* mutations in the SEGA. **A:** *TSC2* LOH was shown with three markers, D16S525, D16S665, and D16S3403, and the *TSC2* intron 39 polymorphism. **Black arrows** indicate the two alleles of each marker, and **white arrowheads** indicate the lost allele. **B:** SSCP analysis of *TSC2* exon 40 (primer set 66 and 66R) shows clear band shift (**arrow**) in the SEGA. **C:** Sequence analysis shows *TSC2* exon 40 missense mutation, 5228G>A, R1743Q, in the SEGA. B, blood DNA; S, SEGA DNA; N, normal control DNA; S-cDNA, SEGA cDNA.

ery available tissue from the patient, ie, peripheral blood leukocyte (two independent collections in a 2-year interval), buccal mucosa, urinary sediments, nail, and hair. Because the detected *TSC2* missense mutation destroys the *Msp*I site of the wild-type allele, we set up triple-nested PCR-RFLP (restriction fragment length polymorphism) to detect the small fraction of somatic mosaic mutation and compared its sensitivity with conventional single-step PCR-RFLP. Because only wild-type allele is digested by *Msp*I, a combination of nested PCR and *Msp*I digestion of PCR products can effectively concentrate the mutant allele at each step. A total of 0.1 μg of each DNA sample was completely digested by *Msp*I (TaKaRa, Tokyo, Japan) and then amplified for 10 cycles of 95°C for 10 seconds and 60°C for 5 seconds with the first PCR primer set 40 (5′-TGGCCAAGATCGTGTCTGAC-3′) and 67 (5′-TTCCGCTGGCCCACCTCATAGCCA-3′).

To eliminate the possibility of amplification of nucleotide change caused by polymerase error, we used a high-fidelity enzyme, KOD-Plus DNA polymerase (TOYOBO). The initial PCR products were digested with *Msp*I again and then amplified for 10 cycles with the second PCR primer set 40 and 66R (5′-AGGTGGCTTGGGCAGTAAGT-3′). The second PCR products were digested with *Msp*I again and a final PCR was performed with primer set 66 (5′-CCACCGATATCTACCCCTCCAAGT-3′) and 66R for 25 cycles. Final products were digested by *Msp*I and separated on 13.5% nondenatured polyacrylamide gel and developed by silver staining. Through these cycles, the wild-type allele was completely removed, and only the mutated allele was amplified. To compare the sensitivity of this analysis, we also performed conventional PCR-RFLP, ie, all samples were amplified by primer set 66 and 66R for 30 cycles and then digested by *Msp*I.

Results

LOH and Mutation Analysis

Through the LOH analysis of the SEGA, all *TSC1* markers were found to be informative and detected heterozygosity. On the other hand, *TSC2* LOH was detected with D16S525, D16S665, D16S3403, and *TSC2* intron 39 polymorphism (IVS39-9C>T) (Figure 2A). The centromere side-two *TSC2* markers D16S663 and D16S283 maintained heterozygosity. SSCP analysis revealed no band shift in the *TSC1* coding exons, whereas band shifts were detected at *TSC2* around exon 40 in SEGA DNA (Figure 2B). Direct sequencing confirmed exon 40 missense

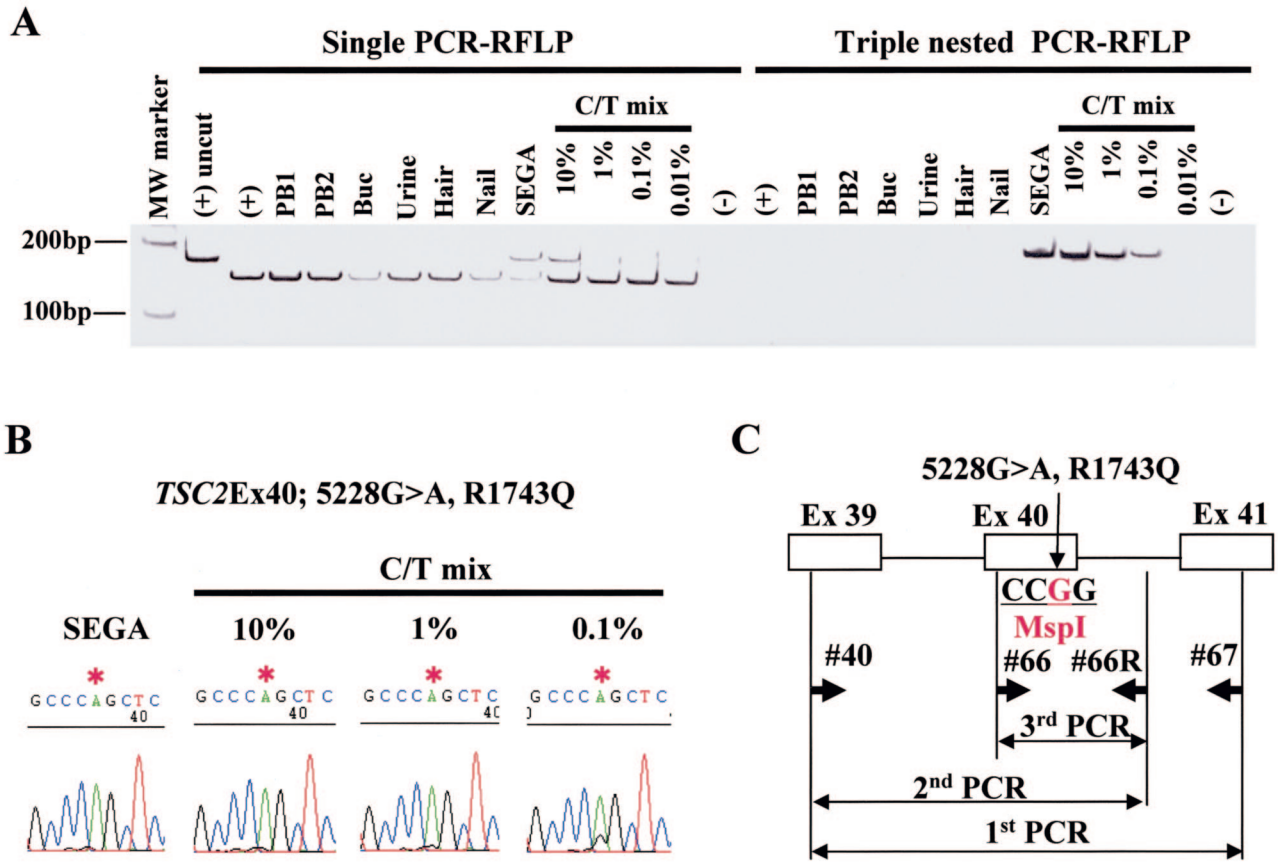

Figure 3. Analysis of somatic mosaicism. **A:** Comparison of single and triple-nested PCR-RFLP. Triple-nested PCR-RFLP can detect 0.1% of tumor DNA in the sample. **B:** Sequence analysis of the final PCR products of triple-nested PCR-RFLP demonstrated only mutated allele. **C:** Schematic representation of primer and mutation position for triple-nested PCR-RFLP. (+) uncut, normal control DNA PCR products not digested with *MspI*; (+), normal control DNA; PB1, peripheral blood collected in October 2001; PB2, peripheral blood collected in February 2003; Buc, buccal mucosa; Urine, urinary sediment; C/T mix, mixture of control and SEGA DNA with the percentage of SEGA DNA indicated; (−), H_2O (negative control).

change 5228G>A, R1743Q in the SEGA DNA (nucleotide number denotes A of the start codon ATG as nucleotide 1 and includes the sequence of exon 31; GenBank accession no. X75621) but not in blood DNA. Sequence analysis also revealed a polymorphism at intron 39 only in blood DNA, as mentioned above. However, SSCP could not detect this nucleotide substitution. The *TSC2* exon 40 missense change was previously reported,[20] and a different missense mutation in the same codon (R1743P) was also reported in patients with TSC.[21] The SEGA preferentially contained mutant allele both at the DNA and message level (Figure 2C). In conclusion, the SEGA contained both *TSC2* missense mutation and *TSC2* LOH, and the tumor lost wild-type allele by LOH.

Analysis of TSC2 Somatic Mosaicism

Somatic mosaicism of the *TSC2* mutation was examined by single and triple-nested PCR-RFLP. To determine the sensitivity of these methods, SEGA DNA was serially diluted with normal genomic DNA from a healthy volunteer to 10, 1, 0.1, and 0.01% SEGA DNA concentration. Using these sensitivity control DNA, conventional single PCR-RFLP detected as little as 10% of SEGA DNA in the sample. On the other hand, triple-nested PCR-RFLP could detect as little as 0.1% of SEGA DNA (Figure 3A). Amplified final products of triple-nested PCR were sequenced and confirmed to contain only mutant allele (Figure 3B). Mutant allele was only detected in the SEGA. These results showed that the patient does not have higher than 0.1% somatic mosaicism in these tested tissues.

Discussion

SEGA is a rare and unique astrocytoma that usually arises in patients with TSC and was once regarded as pathognomonic for TSC.[22] According to the diagnostic criteria of the National Tuberous Sclerosis Association in 1992, histologically confirmed SEGA alone fulfills the criteria of definite TSC.[1] There are several case reports of SEGA without any other clinical manifestation of TSC; these cases have been thought as forme fruste of TSC with somatic mosaicism.

The diagnostic criteria were revised at 1998 because no single major feature fulfills the diagnosis of definite TSC. According to these new criteria, our patient is diagnosed with possible TSC.[2] Molecular analysis of our patient revealed that the SEGA possessed both *TSC2* mis-

sense mutation and *TSC2* LOH according to Knudson's two-hit theory. To detect the small fraction of mosaic mutation in somatic cells, we have developed a new method, triple-nested PCR-RFLP. This method detects as little as 0.1% of mutated DNA in samples. Using this highly sensitive detection system, we demonstrated that all samples other than SEGA (peripheral blood, buccal mucosa, urinary sediment, nail, and hair) did not contain the same mutation detected in SEGA.

Because we could not check every cell of the human body, it is theoretically possible to miss a very small fraction of somatic mosaicism. Meanwhile, natural somatic mutation occurs constantly at some frequency. In fact, the somatic mutation rate *in vivo* has been experimentally determined for some genes. LOH ratio of adenine phosphoribosyltransferase gene in normal human T lymphocyte is estimated as 2 to 15 $\times$ 10^{-5};[23] somatic mutation frequencies of the hypoxanthine phosphoribosyltransferase gene in human kidney cortical epithelial cells are $\sim 5 \times 10^{-5}$ in the first decade of life and $\sim 2.5 \times 10^{-4}$ in the eighth and later decades of life.[24] Although the natural somatic mutation frequency of *TSC* genes has not been determined, every person has *TSC* somatic mosaicism at a certain small ratio. Furthermore, the new mutation ratio of TSC is estimated as 2.5 to 16 $\times$ 10^{-6} per gamete per generation, thus every person also possesses germline mosaicism of *TSC* with this ratio.[25]

On the other hand, we also have to consider technical limitations. Although we used KOD-Plus DNA polymerase, with fidelity 82 times higher than *Taq*DNA polymerase, misincorporation of nucleotide can occur during the nested PCR cycles. In addition, the amount of template DNA used in the first PCR cycle is 0.1 μg, which is equivalent to 2 $\times$ 10^4 cells on the assumption that each cell contains 5 pg of genomic DNA. According to these reasons, we could not improve the detection sensitivity of triple-nested PCR-RFLP infinitely. The actual sensitivity of this method, using the mixture of control and SEGA DNA, was 10^{-3}. So, the sensitivity is not only acceptably high enough to detect a small fraction of mosaicism, but also reliably low from the point of view of both the natural somatic mutation ratio and experimental artifact. In summary, our data strongly suggest that *TSC2* mutations of the patient are well localized to SEGA. Thus, it is reasonable to think that this patient has isolated SEGA without TSC, rather than a forme fruste of TSC with a very small fraction of somatic mosaicism. In the case of retinoblastoma, another autosomal dominant inherited tumor disease, it is well known that sporadic cases have developed their tumors through two successive somatic hit mutations of *RB1* gene.[26] Therefore, we can think similarly in cases of sporadic SEGA.

Among TSC lesions, LOHs were detected frequently in renal angiomyolipoma but less frequently in brain lesions, eg, SEGA and cortical tuber.[27] A recent study showed that some SEGAs do not have *TSC2* LOH but rather have phosphorylation of TSC2 protein tuberin.[28] These data suggest that *TSC2* LOH is not an absolutely necessary condition for development of TSC brain lesions. Our current data do not conflict with this previous data. Because there is some evidence that SEN and SEGA are consecutive lesions and SEN may grow to SEGA,[29] it is hypothesized that SEN is developed by a single-hit mutation of *TSC2* and tuberin phosphorylation, then *TSC2* LOH accelerates tumor growth and transforms SEN to SEGA.

Another rare TSC lesion, lymphangioleiomyomatosis (LAM), is a progressive interstitial lung disease characterized by diffuse proliferation of abnormal smooth muscle cells. LAM can occur as an isolated disorder (sporadic LAM) in a non-TSC patient. Recently, it has been proven that sporadic LAM contains two somatic *TSC2* mutations.[30–32] Therefore one could consider solitary SEGA to be similar to sporadic LAM. These are rare events; however, two successive somatic mutations of *TSC2* in those tissues create identical lesions to those of patients with TSC. Furthermore, we can consider inherited TSC, sporadic TSC, TSC with somatic and/or gonadal mosaicism, and isolated TSC lesions (SEGA or LAM) to be sequential spectrum phenomenon because these patients only differ as to when the first hit occurred.

Acknowledgments

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