Identification of a novel heterozygous TSC2 splicing variant in a patient with Tuberous sclerosis complex

A case report

Linli Liu, MD, Chunshui Yu, PhD, Gaowu Yan, MD

Abstract

Rationale: Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder characterized by facial angiofibromas, epilepsy, intellectual disability, and the development of hamartomas in several organs, including the heart, kidneys, brain, and lungs. Mutations in either TSC1 or TSC2 result in dysregulated mTOR activation, leading to the occurrence of TSC.

Patient concerns: A 44-year-old man was hospitalized for acute lumbago and hematuria.

Diagnosis: The patient presented with facial angiofibromas, epilepsy, fibrous plaques, periangual fibroma, renal angiomyolipomas (AML), pulmonary lymphangioleiomyomatosis (LAM), liver hamartomas, and osteosclerosis. A diagnosis of TSC was made based on clinical manifestations.

Interventions: Next-generation sequencing (NGS) was performed to screen for potential variants, which were verified using Sanger sequencing. The final variant was analyzed using a minigene assay.

Outcomes: A potentially pathogenic novel TSC2 variant (NM_000548.4, c.336_336 +15delGGTAAGGCCAGGGGC) was identified using NGS and confirmed using Sanger sequencing. The in vitro minigene assay showed that the variant c.336_336 +15delGGTAAGGCCAGGGGC caused erroneous integration of a 74 bp sequence into intron 4. This novel variant was not found in his unaffected parents or 100 unrelated healthy controls.

Lessons: We identified a novel heterozygous TSC2 variant, c.336_336 +15delGGTAAGGCCAGGGGC, in a patient with classical TSC and demonstrated that this variant leads to aberrant splicing using a minigene assay. Our results extend the understanding of the mutational spectrum of TSC2.

Abbreviations: CNS = central nervous system, CT = computed tomography, LAM = lymphangioleiomyomatosis, LOF = loss of function, mTOR = mechanistic target of rapamycin, NGS = next-generation sequencing, PKD = polycystic kidney disease, TSC = tuberous sclerosis complex.

Keywords: minigene, TSC2 variant, tuberous sclerosis complex

1. Introduction

Tuberous sclerosis complex (TSC, OMIM #191100 and #613254) is an autosomal dominant genetic disease caused by mutations in TSCI or TSC2. The morbidity of TSC is nearly 1:10,000 among newborns, and most patients are diagnosed during the first 15 months of life.[1] TSC is characterized by facial angiofibromas, epilepsy, intellectual disability, and development of hamartomas throughout the body, particularly in the brain,
skin, heart, and kidneys. Owing to the different locations of lesions, the spectrum of clinical manifestations is very wide among individuals. Some patients are severely affected at an early age and at multiple sites throughout the body. The major causes of mortality in patients with TSC are seizures and renal complications. TSC is believed to have a high spontaneous mutation rate, as suggested by the large number (approximately two-thirds of all cases) of aperiodic cases without a family history. TSC1 and TSC2, which act as tumor growth suppressors and encode the proteins hamartin and tuberin, have been found to be responsible for mTOR overactivation, which may be the underlying mechanism of pathogenesis.

The diagnosis of TSC depends mainly on clinical diagnostic criteria. Owing to genetic heterogeneity, the clinical phenotype of the disease presents high variability, thus making the prediction of the phenotype on an individual basis challenging. Even monozygotic twins with the same mutation can exhibit different clinical expressions. The atypical clinical presentation indicates the potential shortcomings of the current diagnostic criteria for TSC. A pathogenic variant of either TSC1 or TSC2 detected by genetic analysis represents a separate diagnostic criterion, regardless of clinical findings. With advances in genomic DNA sequencing technology, it is now easy to analyze the entire sequence of TSC genes. However, molecular diagnosis of TSC remains challenging because of the difficulty in correctly assessing gene variants of unknown significance. A functional analysis is required to determine the pathogenicity of these variants.

Here, we present the clinical manifestations of a patient with TSC and detected a novel TSC2 gene variant, c.336_336+15del16, by Next Generation sequencing (NGS) and Sanger sequencing. An in vitro minigene assay was performed to investigate the effect of this variant on the splicing process.

2. Ethics and methods

Written informed consent was obtained from the individual and approval of the Institutional Medical Ethics Committee of Suning Central Hospital. Genomic DNA was extracted from the peripheral blood samples of the patient. After quality control of the extracted DNA samples, a targeted NGS panel (Illumina HiSeq2000 platform, Inc., San Diego, CA, USA) was used to analyze the exons and flanking intronic regions of the TSC1 and TSC2 genes associated with TSC. The sequencing data were aligned to the human reference genome hg19 (University of California Santa Cruz (UCSC, http://genome.ucsc.edu). Sanger sequencing was performed to verify the NGS results. Primers were designed using PRIMER 5 software for PCR amplification, according to the potential pathogenic variants obtained by NGS. After purification, the PCR products were sequenced using an ABI PRISM 3730 Genetic Analyzer. Finally, a minigene assay was performed to study the effects of the variants at the transcript level. Mutant and wild-type pMINI-TSC2 vectors were transfected into 293T cells. After transfection for 48 hours, the total RNA was extracted and reverse-transcribed into cDNA. Following, the cDNA was amplified by PCR, analyzed by agarose gel electrophoresis, and sequenced.

3. Case presentation

A 44-year-old man from the Han dynasty presented with a chief complaint of “acute lumbago and hematuriesis” for three days and was admitted to the urology department of our hospital. He had a long history of seizures and was being treated with antiepileptic medication. Facial angiofibromas, fibrous plaques, and multiple periungual fibromas were discovered on physical examination (Fig. 1). Biochemical laboratory tests revealed renal insufficiency and moderate anemia. Non-contrast-enhanced computed tomography (CT) of the abdomen revealed large renal AMLs with prominent fatty components and prominent internal vessels bilaterally. Lung CT and consistent lymphangioleiomyomatosis (LAM) revealed multiple air-filled cysts of variable sizes bilaterally. Small hamartomas were noted on liver CT. Multiple calcified subependymal nodules were observed on the brain CT. Spinal CT revealed multiple patchy sclerotic lesions (Fig. 2). No treatment had been administered in the past. There was no family history of similar diseases. Ultimately, we concluded that the patient fulfilled the diagnostic criteria for TSC. Owing to the ruptured hemorrhage of his renal AMLs, the patient eventually underwent surgery. After year of follow-up, the patient remained stable.
Next-generation and Sanger sequencing revealed a novel heterozygous TSC2 splice variant (NM_000548.4, c.336_336+15del16). This variant was not found in his family members or in the 100 unrelated controls (Fig. 3). This was considered novel because it was not present in the ExAC, 1000G, or HGMD databases. It is noteworthy that the TSC2 variant c.336_336+15del16, resulting in the deletion of the last nucleotide G in exon 4 and the subsequent 15 bases in the intron region, is more likely to cause a complete loss of the splice donor site of intron 4. This variant was more likely to cause splice defects. Minigene assay electrophoresis showed that the c.336_336+15del16 variant led to a slightly larger TSC2 mRNA transcript than that in the wild-type clone. Sequencing showed that pMINI-TCS2-WT was considered to have normal splicing, whereas the mRNA sequence of pMINI-TCS2-MUT was changed, the original splicing site was lost, and erroneous insertion of a 74 bp sequence into intron 4 caused the splicing position moved back 90 bp (r.336delins336+16_336+90) (Fig. 4). This variant led to aberrant splicing, and this change was predicted to be p.Gly113Argfs*5. Based on classification standards and guidelines for ACMG genetic variation, the c.336_336+15del16 variant was classified as pathogenic (PVS1+PS2+PM2).

4. Discussion

TSC is an autosomal dominant neurocutaneous disorder affecting multiple organs. Two genes involved in TSC have been identified; the TSC1 gene coding for hamartin is located on chromosome 9q34, whereas the TSC2 gene coding for tuberin is located on chromosome 16p13.3.[1] Mutations in either TSC1 or TSC2 result in dysregulated mTOR activation, leading to the occurrence of TSC. In general, TSC2 variants cause more severe phenotypes than TSC1 variants.[9] TSC manifests in many organ systems including the brain, skin, heart, kidneys, and lungs.
Approximately 80% of TSC cases are associated with renal involvement, such as angiomyolipomas (AML) and polycystic kidney disease (PKD). In such patients, PKD is frequently associated with TSC2/PKD1 contiguous gene syndrome.[10] The central nervous system is almost invariably involved, with up to 85% of patients presenting with epilepsy, and at least half of the patients have intellectual disabilities or other neuropsychiatric disorders, including autism spectrum disorder.[11] In this study, the patient was characterized by classical multisystemic symptoms, including severe renal AMLs, pulmonary LAM, multiple calcified subependymal nodules, liver hamartomas, skin impairment, and osteosclerosis, indicating a more severe grade of illness. The patient eventually underwent surgery because of rupture hemorrhage of the renal AMLs.

In this study, using NGS and Sanger sequencing of TSC genes, a novel heterozygous TSC2 variant c.336_336+15del16 of exon 4 was identified. This variant leads to deletion of the last nucleotide G in exon 4 and the subsequent 15 bases in the intron region. Notably, this position may play an important role as a splicing modulator that may alter the splicing of the TSC2 transcript. The minigene splicing assay confirmed that the variant c.336_336+15del16 led to the loss of the original splicing site and erroneous insertion of a 74 bp sequence into intron 4, causing the splicing position moved back 90 bp (r.336delins336 +16_336 +90). This variant may disturb normal splicing alterations during splicing. Therefore, this variant might lead to a defective tuberin and severe impairment of the normal hamartin–tuberin interaction, thus resulting in upregulation of the mTOR pathway, which in turn might cause the disease. Notably, splice mutations may play a more important role in human hereditary diseases.[12]

Classical splicing variants that affect nucleotides at the splice acceptor and donor sites lead to aberrant RNA splicing. However, all types of variants (missense, nonsense, and small insertions or deletions) can lead to splicing defects either by disrupting or creating signals. Zhang et al.[13] reported a missense mutation, c.3610G > A, in which the last nucleotide of exon 29 in TSC2 was replaced. This leads to the substitution of a single amino acid from glycine to arginine at amino acid position 1204 (p. Gly1204Arg), affecting normal splicing. Qiu[14] reported a novel TSC1 frameshift mutation (TSC1 c.1550_1551del) that triggered aberrant splicing simultaneously, leading to TSC formation. Additionally, the in vitro minigene assay is an attractive alternative for assessing the impact of intronic variants on splicing.

The c.336_336+15del16 variant was considered a novel variant because it was not found in the ExAC, ESP, 1000G, and HGMD databases and was absent in his parents and 100 healthy controls. According to the classification standards and guidelines of ACMG genetic variation,[15] the c.336_336+15del16 variant was considered as the LOF variant (loss of function), which conforms to very strong disease-causing evidence (non-functional variant, splice site variants), strong pathogenicity evidence (the patient had a de novo mutation and no family history), and moderate pathogenicity evidence (the mutation was not found in ExAC, ESP, 1000G, HGMD database), namely PVS1+PS2+PM2, which was considered pathogenic.

In summary, we highlighted the clinical features of patients with classical TSC. DNA sequencing and minigene splicing assays showed that the novel splicing variant c.336_336+15del16 within exon 4 and intron 4 of the TSC2 gene is a pathogenic variant. This variant affects mRNA splicing, leading to loss of tuberin function, which may be the underlying cause of TSC. The identified variant extended the TSC2 mutational spectrum and, to some extent, increased our understanding of the molecular mechanism of TSC pathogenesis.

Acknowledgments

We thank the patient who agreed to use the images and clinical data.
Figure 4. Transcript analyses of pMINI-TSC2. Electrophoresis showed that the variant c.336_336 + 15del16 led to a subtly larger TSC2 mRNA transcript than the wild-type clone. Sequencing showed that the pMINI-TCS2-WT was considered as expected normal splicing, while the mRNA sequence of pMINI-TCS2-MUT was changed, the original splicing site was lost and erroneous insert of a 74 bp sequence into intron 4, making the splicing position moved back 90bp (c.336delins336 + 16_336 + 90) (the diagrams above and below represent the plasmid sequencing and mRNA sequencing, respectively).

Author contributions

Data curation: Linli Liu, Gaowu Yan.
Funding acquisition: Chunshui Yu.
Writing – original draft: Linli Liu.

References

[1] Portocarrero LKL, Quental KN, Samorano LP, et al. Tuberous sclerosis complex: review based on new diagnostic criteria. An Bras Dermatol 2018;93:323–31.
[2] Zöllner JP, Franz DN, Hertzberg C, et al. A systematic review on the burden of illness in individuals with tuberous sclerosis complex (TSC). Orphanet J Rare Dis 2020;15:23.

[3] Byers HM, Jensen DM, Glass IA, Bennett JT. Minimal mosaicism, maximal phenotype; discordance between clinical and molecular findings in two patients with tuberous sclerosis. Am J Med Genet C Semin Med Genet 2018;178:374–8.

[4] Uysal SP, Sahin M. Tuberous sclerosis: a review of the past, present and future [published online ahead of print, 2020 Mar 28]. Turk J Med Sci 2020;50:1665–76.

[5] Roach ES, Gomez MR, Northrup H. Tuberous sclerosis complex consensus conference: revised clinical diagnostic criteria. J Child Neurol 1998;13:624–8.

[6] Humphrey A, Higgins JN, Yates JR, Bolton PF. Monozygotic twins with tuberous sclerosis discordant for the severity of developmental deficits. Neurology 2004;62:795–8.

[7] Fox J, Ben-Shachar S, Uliel S, et al. Rare familial TSC2 gene mutation associated with atypical phenotype presentation of Tuberous sclerosis complex. Am J Med Genet A 2017;173:744–8.

[8] Northrup H, Krueger DA. International Tuberous Sclerosis Complex Consensus Group Tuberous sclerosis complex diagnostic criteria update: recommendations of the 2012 International Tuberous Sclerosis Complex Consensus Conference. Pediatr Neurol 2013;49:243–54.

[9] Ogórek B, Hamieh L, Hulshof HM, et al. TSC2 pathogenic variants are predictive of severe clinical manifestations in TSC infants: results of the EPISTOP study. Genet Med 2020;22:1489–97.

[10] Reyna-Fabián ME, Alcántara-Ortígoza MA, Hernández-Martínez NL, et al. TSC2/PKD1 contiguous gene syndrome, with emphasis on a case with an atypical mild polycystic kidney phenotype and a novel genetic variant. Nefrologia 2020;40:91–8.

[11] Curatolo P, Moavero R, Roberto D, Graziola F. Genotype/phenotype correlations in tuberous sclerosis complex. Semin Pediatr Neurol 2015;22:259–73.

[12] López-Bigas N, Audit B, Ouzounis C, Parra G, Guigó R. Are splicing mutations the most frequent cause of hereditary disease? FEBS Lett 2005;579:1900–3.

[13] Zhang R, Wang J, Wang Q, et al. Identification of a novel TSC2 c.3610G>A, p.G1204R mutation contribute to aberrant splicing in a patient with classical tuberous sclerosis complex: a case report. BMC Med Genet 2018;19:173.

[14] Qiu C, Li C, Tong X, et al. A novel TSC1 frameshift mutation c.1550_1551del causes tuberous sclerosis complex by aberrant splicing and nonsense-mediated mRNA degradation (NMD) simultaneously in a Chinese family [published online ahead of print, 2020 Jul 31]. Mol Genet Genomic Med 2020:e1410.

[15] Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405–24.