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TSC2 GAP Domain V1646Cfs*7 Variant Alters Protein Stability and Interaction Networks in Tuberous Sclerosis Complex

Kagistia Hana Utami,[1,*] Velda X. Han,[2,3] Nur Amirah Bte Mohammad Yusof,[4] Yazhini Ramaswamy,[2] Jia Feng,[5] Stacey Kiat Hong Tay,[2,3] Sarah R. Langley,[6] and Mahmoud A. Pouladi[5,*]

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Correspondence
Prof. Pouladi
map@pouladilab.org
or Dr. Utami
utami@pouladilab.org

Abstract

Objectives

Tuberous sclerosis complex (TSC) is autosomal dominant neurocutaneous disorder caused by TSC1/2 pathogenic variants. We report a child with refractory epilepsy and developmental delay who harbors a de novo *TSC2* p.V1646Cfs*7 variant. To elucidate its functional consequences, we assessed the variant's effects on proteostasis, providing a framework for developing therapeutic strategies addressing the underlying molecular disruptions.

Methods

The child was identified through genetic testing. Molecular characterization included bioinformatics prediction, protein stability assays, and mass spectrometry, which identified protein-protein interactions between control and TSC2 V1646Cfs*7. Pathway analysis and cross-referencing with autism and epilepsy databases were performed to assess functional significance.

Results

The TSC2 p.V1646Cfs*7 variant produced a truncated yet initially stable protein with distinct properties. The pathogenic variant retained subcellular localization and mTOR interactions but showed accelerated degradation. Proteomic analysis revealed enrichment in RNA metabolism and mitophagy pathways, with significant overlap with autism and epilepsy-related genes.

Discussion

This study expands our understanding of TSC2 variants, highlighting how altered protein stability and interaction networks contribute to disease pathology. Disruptions in RNA processing and mitochondrial homeostasis may underlie key aspects of the TSC2 phenotype. These findings highlight the potential for precision medicine in TSC and offer scalable framework for modeling other variants in syndromic disorders with uncharacterized pathogenicity.

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Supplementary Material

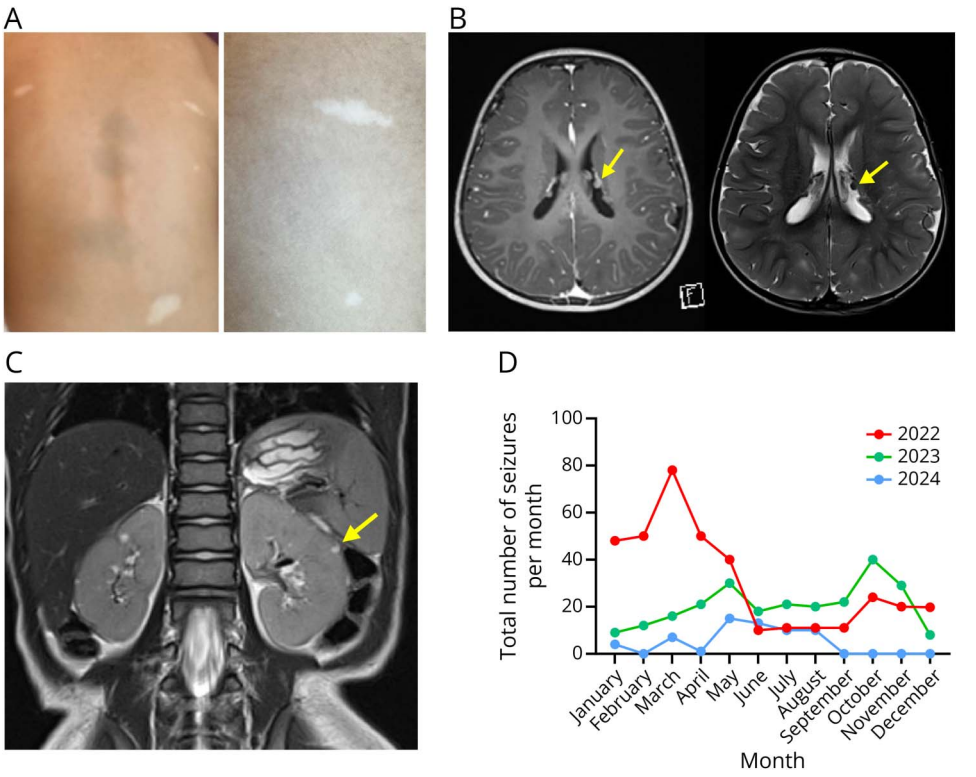
Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous disorder with multisystem involvement arising from *TSC1* or *TSC2* pathogenic variants. Individuals with TSC present with a wide range of neurologic manifestations, including drug-refractory epilepsy, autism spectrum disorder (ASD), and intellectual disability.[1] *TSC2* comprises 42 exons with a hamartin-binding domain and a GTPase-activating (GAP) domain.[2] Together with *TSC1* and *TBC1D7*, it forms a complex that negatively regulates mammalian target of rapamycin complex

*These authors contributed equally to this work as co-corresponding and co-senior authors.

[1]Keio University Regenerative Medicine Research Center, Kawasaki, Japan; [2]Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore; [3]Khoo-Teck Puat- National University Children's Medical Institute, National University Health System, Singapore; [4]Agency for Science, Research and Technology, Singapore; [5]Department of Medical Genetics, Centre for Molecular Medicine and Therapeutics, Djavad Mowafaghian Centre for Brain Health, Edwin S. H. Leong Centre for Healthy Aging, University of British Columbia, British Columbia Children's Hospital Research Institute, Vancouver, Canada; and [6]School of Biosciences, Cardiff University, United Kingdom.

Figure 1 Clinical Manifestations of the *TSC2* V1646Cfs*7 Variant



(A) Hypomelanotic macules, also known as ash-leaf spots, observed on the back (arrowhead). (B) MRI showing multiple subependymal nodules: Axial postcontrast T1-weighted image (left) reveals enhancing subependymal nodules along the lateral ventricular walls (arrow) with corresponding hypointense signals on axial T2-weighted images (arrowhead), consistent with subependymal hamartomas. (C) Abdominal MRI showing multiple kidney cysts: Coronal T2-weighted image of the abdomen highlights several cortical hyperintense foci in both kidneys (arrow, left interpolar region), representing renal cysts. (D) Total number of seizures tracked over 3 years: Year 1 (2022), year 2 (2023), and year 3 (2024). Lacosamide was introduced in the last quarter of year 2 and continued in year 3, improving seizure-free periods.

1 (mTORC1).[3] Pathogenic variants in *TSC1* or *TSC2* disrupt this regulation, leading to mTOR hyperactivation and abnormal cell growth.[4,5]

We examined a child with refractory epilepsy and global developmental delay carrying a single-nucleotide T duplication in exon 38 of *TSC2*, located within the GAP-domain. Although this variant appears in curated databases, it lacks functional characterization. Our study aims were to (1) define the functional consequences of the *TSC2* p.V1646Cfs*7 variant, (2) assess its effect on protein interactions and pathways, and (3) examine overlaps with ASD and epilepsy-related genes. By advancing these objectives, this study advances understanding of the p.V1646Cfs*7 variant by providing insights into genotype-phenotype correlations.

Methods

Ethics Approval

Institutional Review Board approval was not required for single case reports per institutional guidelines, as no patient-derived material was used. Parental consent was obtained.

Patient Consents

Because no patient samples were obtained, parental consent for use of clinical data and patient history was obtained in accordance with ethics guidelines.

Genetic Variant Analysis

Details on sequence analysis and molecular cloning are described in eMethod.

Protein Assays and Mass Spectrometry

Detailed protocols on immunoblot, immunofluorescence, protein stability, SunSET assays, immunoprecipitation, mass spectrometry, and downstream analyses are provided in eMethod.

Data Availability

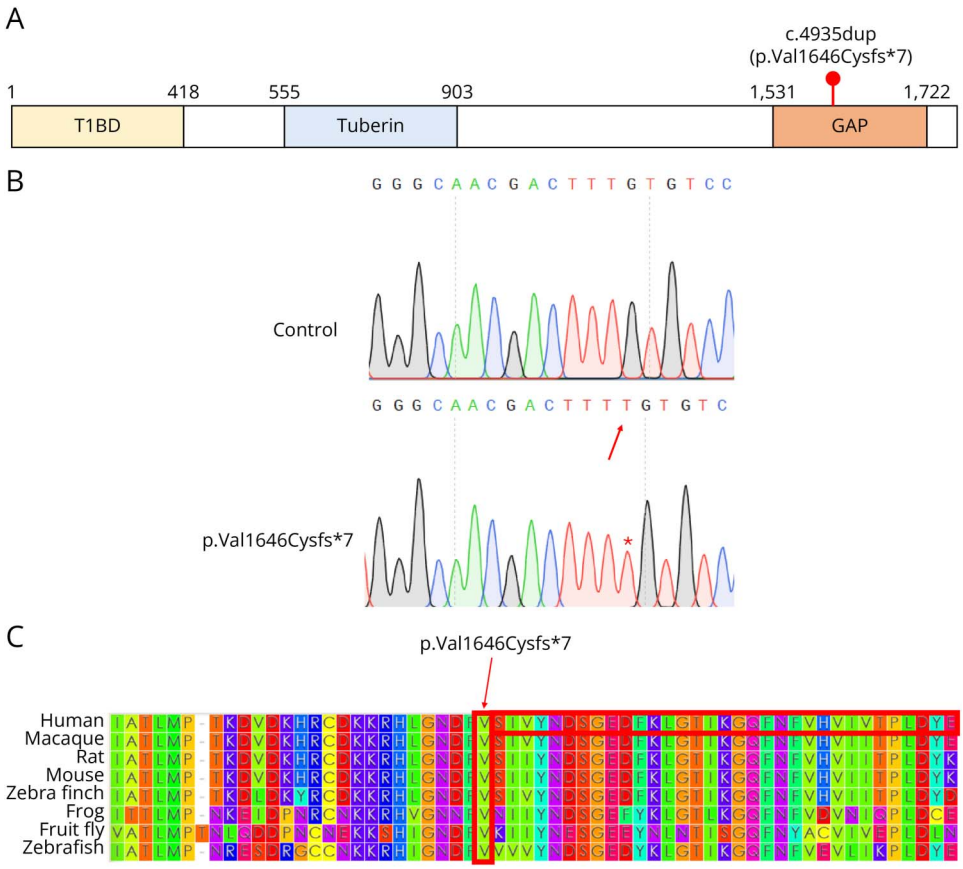
Affinity purification mass spectrometry raw data are available on request to the corresponding authors.

Results

Clinical Information

This child developed infantile spasms at 6 months, with EEG showing focal interictal discharges in the right frontotemporal region. Multiple ash-leaf spots prompted suspicion of TSC (Figure 1A), subsequently confirmed by identification of a pathogenic *TSC2* exon 38 variant; parental testing was negative. He met 7 major diagnostic criteria for TSC, including a shagreen patch, cortical tubers, subependymal nodules at the lateral ventricles (Figure 1B), retinal hamartoma, kidney cysts (Figure 1C), and cardiac rhabdomyomas.[6]

Figure 2 Pathogenic Variant Analysis of *TSC2* p.Val1646Cysfs*7 Variant



(A) Sanger sequencing identifies a single-base duplication (T) from site-directed mutagenesis to model similar variant identified in patient, marked by an arrow. (B) Schematic representation of the *TSC2* gene, showing key functional domains and the patient-derived variant (p.Val1646Cysfs*7). (C) ConSurf analysis demonstrating the conservation of *TSC2* protein sequences across multiple species.

Treatment with vigabatrin controlled seizures initially, but focal seizures recurred at 18 months. Despite treatment with levetiracetam and carbamazepine, the child's seizures remained refractory until the introduction of lacosamide which led to a marked reduction in seizures (Figure 1D). He also had global developmental and severe speech delay.

De Novo TSC2 Pathogenic Variant in GAP Domain

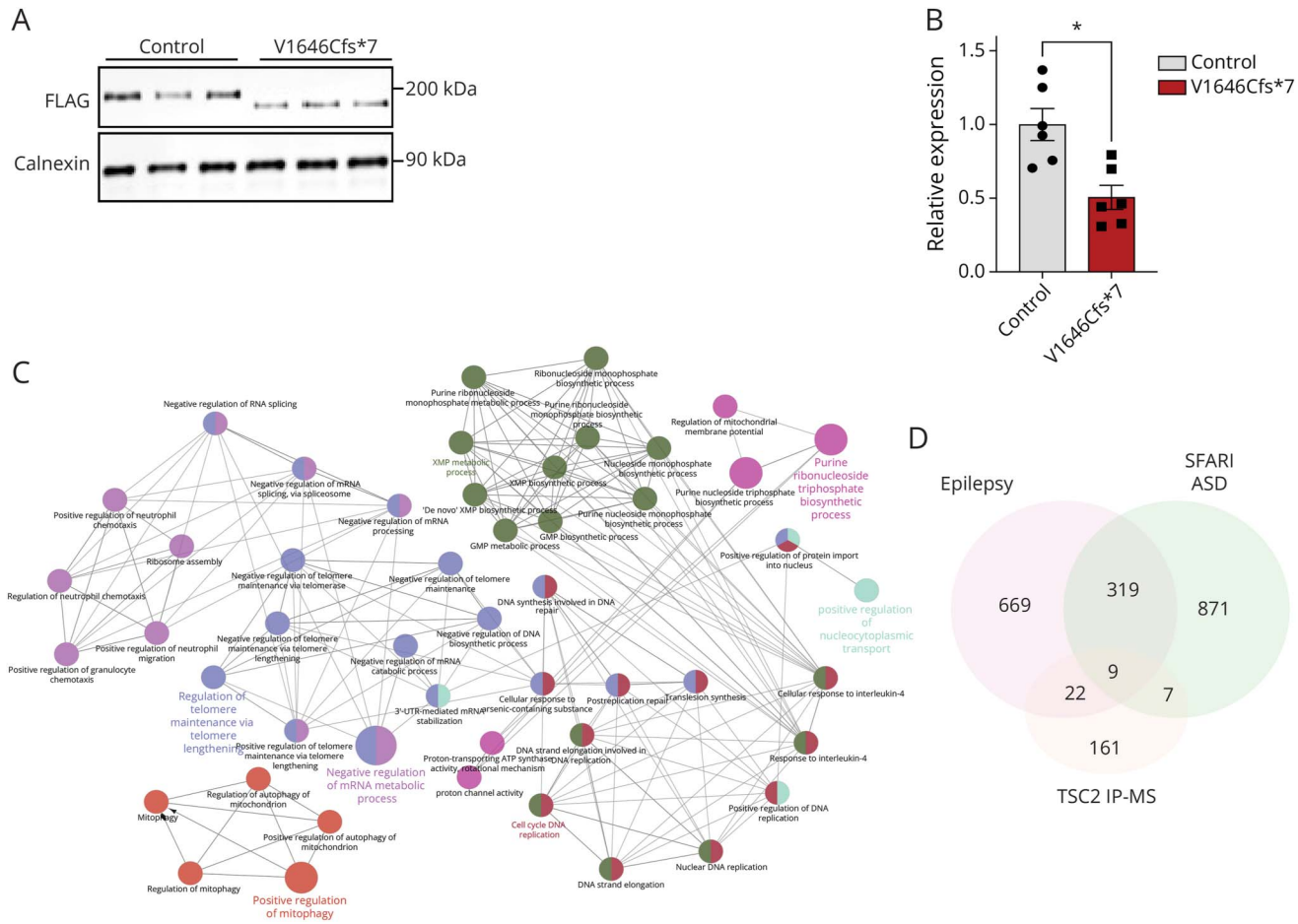
Genetic testing identified a single-base pair duplication at amino acid (AA) 1646, c.4935dupT (NM.000548.5), resulting in the p.V1646Cfs*7 frameshift within the GAP domain of *TSC2* (Figure 2A). We subsequently cloned this variant by performing site-directed mutagenesis from the *TSC2* full length cDNA (Figure 2B). Sequence analysis predicted a premature stop codon (eAppendix 1). Conservation analysis (ConSurf) showed the affected valine residue is highly conserved (Figure 2C). Seven patients with identical variants were identified through Leiden Open Variation Database (LOVD)[7] (eAppendix 2). However, limited or no clinical information was available for these cases, preventing a meaningful phenotypic comparison.

*TSC2 V1646Cfs*7*-Specific Protein Binding Interactions

We overexpressed FLAG-tagged control and TSC2 V1646Cfs*7 variant proteins in HEK293 cells, and performed anti-FLAG immunoprecipitation, followed by mass spectrometry (IP-MS). TSC2 V1646Cfs*7 produced a truncated protein with reduced expression (Figure 3A, Figure 3B, eFigure 1). After filtering out low-confidence and CRAPome-listed interactors,[8] we identified 59 control-specific, 50 TSC2 V1646Cfs*7-specific, and 90 shared proteins, including core mTOR components (TSC2, YWHAE, EEF2, and EIF4A1) (eFigure 2A, eTable1). Shared proteins suggest partial function retention, whereas variant-specific interactors imply gain-of-function effects or aberrant signaling.

Gene ontology (GO) enrichment analysis showed control interactors enriched in gene expression and ribosome biogenesis (eFigure 1B), while TSC2 V1646Cfs*7 interactors were linked to RNA metabolism, mRNA processing (HNRNPA3, HNRNPA2B1, HNRNPC, HNRNPA1), and mitophagy regulation (VDAC1, SLC25A5) (Figure 3C), suggesting disrupted protein stability and mitochondrial regulation.

Figure 3 TSC2 V1646Cfs*7 Variant Acquired Distinct Sets of Interactor Proteins Compared With Control *TSC2*



(A) Immunoblot showing FLAG-tagged TSC2 expression after immunoprecipitation (IP) with FLAG antibody. The full blot image is provided in eFigure 3. (B) The TSC2 V1646Cfs*7 protein exhibits significantly reduced levels compared with the control, quantified as pooled independent western blot experiments. Calnexin was used as loading control to normalize protein expression. (C) GO enrichment analysis of proteins specifically associated with the TSC2 V1646Cfs*7 variant. (D) Venn diagram showing the overlap between IP-MS and SFARI/epilepsy gene sets. GO = gene ontology; IP-MS = immunoprecipitation, followed by mass spectrometry.

TSC2 IP-MS Overlap With ASD and Epilepsy Risk Genes

It is important that the TSC2 V1646Cfs*7 interactome was significantly enriched for genes associated with ASD and epilepsy. Cross-referencing with the Simons Foundation Autism Research Initiative (SFARI)[9] and Genes4Epilepsy[10] databases identified 9 proteins overlapping with both disorders, including those related to RNA processing/translation (DHX9, EIF3F, HNRHNPK, or HNRNPU), cytoskeletal transport (DYNC1H1, YWHAE), and metabolism (PDHA1). In addition, 22 proteins were linked specifically to epilepsy and 7 to ASD (Figure 3D, eTable 2). This overlap highlights shared molecular pathways between TSC, ASD, and epilepsy, emphasizing the broader relevance of our findings.

Impaired Protein Stability of TSC2 V1646Cfs*7 via Akt-Independent Pathway

Protein stability emerged as a key GO term shared between control and TSC2 V1646Cfs*7 variants. Cycloheximide chase assays showed that while control TSC2 remained stable, TSC2 V1646Cfs*7 underwent progressive degradation (eFigure 3A, eFigure 4A). SunSET assay revealed no change in global protein synthesis (eFigure 3B), and immunoblotting showed unchanged Akt signaling (eFigures 3C and 4B), indicating that TSC2 V1646Cfs*7 degrades via Akt-independent mechanisms.

Subcellular Localization of TSC2 V1646Cfs*7 Variant

TSC2 normally localizes in the cytoplasm and regulates the actin cytoskeleton.[11] Using immunocytochemistry and phalloidin staining to label F-actin in HEK293 cells, we found that the TSC2 V1646Cfs*7 variant showed no changes in subcellular localization, actin morphology or cell size (eFigure 3D), indicating preserved cytoskeletal organization.

Discussion

TSC is characterized by significant phenotypic and genetic heterogeneity, posing challenges for establishing genotype-phenotype correlations and prognostic predictions.[12] In individuals with TSC, prediction of clinical severity is complicated by genetic

heterogeneity and incomplete penetrance, with even identical variants in monozygotic twins leading to divergent clinical outcomes.[e1]

We characterized the functional effect of *TSC2* V1646Cfs*7 frameshift variant within the GAP domain, essential for mTORC1 regulation via Rheb inactivation.[13] This pathogenic variant produces a truncated TSC2 protein with progressively reduced stability, while cytoplasmic localization is preserved. Proteomic pull-down analysis revealed altered protein interactions and identified mTOR pathway components shared between control and TSC2 V1646Cfs*7. Variant-specific enrichment analysis suggested that V1646Cfs*7 disrupts mitochondrial quality control. VDAC1, a key mitophagy regulator, was preferentially recruited by the TSC2 V1646Cfs*7 protein, consistent with its upregulation in *TSC2*-knockout Purkinje cells,[14] highlighting mitochondrial dysregulation as a key consequence. These findings warrant further validation via co-IP or proximity ligation to develop therapies to restore mitochondrial homeostasis.

Accelerated degradation of TSC2 V1646Cfs*7 variant occurs via an Akt-independent pathway, warranting investigation into lysosomal and proteasomal regulation.[2,15] Despite altered turnover of the variant protein, the variant does not affect cytoskeletal morphology, indicating preserved trafficking but disrupted downstream signaling. Enrichment of ASD and epilepsy-related genes in the TSC2 interactome,[9,10] particularly those involved in mRNA splicing and translation highlights shared molecular mechanisms and the association of TSC2 with neurodevelopmental comorbidities.

We acknowledge several limitations in this study. The translational effects of the frameshift variant were assessed using full-length cDNA in HEK293 cells; however, patient-derived samples are necessary to evaluate upstream splicing and transcriptional regulation. Future validation in iPSC-derived neurons and studies of protein degradation, autophagy, and mitochondrial activity are essential to define cell-type specific mechanisms underlying this pathogenic variant.

Our study highlights the complexity of *TSC2* variants, linking mTOR hyperactivity to disrupted protein stability, RNA metabolism, and mitochondrial regulation. *TSC2* V1646Cfs*7 variant retains partial function but affects mitophagy and RNA processing, revealing potential therapeutic targets. Shared molecular links to ASD and epilepsy suggest mTORC1 or autophagy-based therapies may provide broader benefits across neurodevelopmental comorbidities. This study provides the first *in vitro* validation of the *TSC2* V1646Cfs*7 variant, strengthening its pathogenic classification and enhancing its diagnostic relevance. By linking genotype to phenotype and offering a rapid, scalable approach to assess variant pathogenicity, our findings contribute to a valuable framework for precision medicine in TSC and related neurodevelopmental disorders.

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Author Contributions

K.H. Utami: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. V. Han: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. N.A.B. Mohammad Yusof: major role in the acquisition of data. Y. Ramaswamy: analysis or interpretation of data. J. Feng: major role in the acquisition of data. S.K.H. Tay: major role in the acquisition of data. S.R. Langley: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. M.A. Pouladi: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data.

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