Genetic and Functional Analysis of Glycosyltransferase 8 Domain–Containing Protein 1 in Taiwanese Patients With Amyotrophic Lateral Sclerosis

Pei-Chien Tsai, PhD, Kang-Yang Jih, MD, PhD, Ting-Yi Shen, BS, Yi-Hong Liu, MD, Kon-Ping Lin, MD, Yi-Chun Liao, MD, PhD, and Yi-Chung Lee, MD, PhD

Neurol Genet 2021;7:e627. doi:10.1212/NXG.0000000000000627

Abstract

Background and Objectives
To investigate the frequency, spectrum, and molecular functional effect of glycosyltransferase 8 domain-containing protein 1 (GLT8D1) variations in Taiwanese patients with amyotrophic lateral sclerosis (ALS).

Methods
We performed genetic analyses of GLT8D1 in 410 unrelated patients with ALS by Sanger sequencing. The 410 patients were selected from a cohort of 477 unrelated patients with ALS after excluding variations in common ALS disease genes. Functional effects of the GLT8D1 variation were investigated by in vitro functional analysis.

Results
We identified a novel heterozygous missense variation in GLT8D1, p.I290M (c.870C>G), in 1 single patient with familial ALS. The patient with the p.I290M variation had a spinal-onset ALS with disease onset at age 60 years and a survival of 6 years. Functional studies demonstrated that the variant I290M GLT8D1 protein was mislocalized to the endoplasmic reticulum (ER), provoked ER stress and unfolded protein response, compromised the glycosyltransferase activity, and led to an increased cytotoxicity.

Discussion
GLT8D1 variations account for 0.2% (1/477) of the patients with ALS in Taiwan. These findings expand the spectrum of GLT8D1 variation and support the pathogenic role of GLT8D1 variations in ALS.
Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by relentless loss of upper and lower motor neurons, leading to progressive muscle weakness and atrophy with a short survival of 3–5 years. Although the underlying causes of most patients with ALS are still unclear, approximately 10% of patients with ALS inherit their disease from their parents, suggesting that genetic factors have an important role in ALS pathogenesis. To date, more than 50 genes have been implicated in ALS pathogenesis, and variations in at least 10 of these genes have been clearly demonstrated to cause familial ALS. Moreover, only superoxide dismutase 1 (SOD1), fused in sarcoma (FUS), transactive response DNA binding protein (TARDBP), chromosome 9 open reading frame 72 (C9ORF72), valosin-containing protein (VCP), and TRAF family member-associated NF-kB activator binding kinase 1 (TBK1) variations account for a significant number of patients with ALS, indicating a high degree of genetic heterogeneity of ALS. However, the roles of newly identified causal genes of ALS, such as GLT8D1, are yet to be completely understood because the relevant studies are still sparse.

The GLT8D1 gene encodes glycosyltransferase 8 domain-containing protein 1 (GLT8D1), a single-pass transmembrane protein of 371 amino acids in length. GLT8D1 is a member of the glycosyltransferase family 8 and functions in transferring a glycosyl group from a donor to an acceptor molecule. Recently, a British study identified GLT8D1 variations in patients with familial or sporadic ALS. They first identified a GLT8D1 missense variation in exon 4, p.R92C, cosegregating with the disease in an autosomal dominant manner in the ALS family. Then, 4 additional rare deleterious variations in GLT8D1 exon 4 were further found in 4 patients with ALS. Statistical analysis showed that the rare deleterious variants affecting the conserved amino acids in exon 4 of GLT8D1 were significantly enriched in patients with ALS. Further functional studies revealed that R92C and G78W GLT8D1 variant proteins exhibited impaired glycosyltransferase activity, produced cytotoxicity, and led to motor deficits in zebrafish. Another Chinese study screened GLT8D1 variations in 977 patients with sporadic ALS and 47 patients with familial ALS and identified 1 likely pathogenic variant, p.G78A, in 2 patients within the same family. Another 2 Chinese studies and 1 Australian study screened 512, 539 and 699 patients with ALS for GLT8D1 variations, respectively, but failed to identify any pathogenic variation.

To further understand the role of GLT8D1 variations in ALS, we screened 410 unrelated Taiwanese patients with ALS for GLT8D1 variations. In addition, in vitro studies were conducted to assess the functional effects of the variant gene product.

**Methods**

**Standard Protocol Approvals, Registrations, and Patient Consents**

Informed consent was obtained from all patients in this study. The protocols for this study were approved by the Institutional Review Board of Taipei Veterans General Hospital.

**Patients**

Four hundred ten unrelated individuals (248 men and 162 women) with the diagnosis of probable or definite ALS based on the revised EL Escorial criteria were enrolled into this study. All participants were of Han Chinese descent and were recruited from the Neurology Service of Taipei Veterans General Hospital, which is a 2,974-bed tertiary medical center that serves both veterans and regular citizens in Taiwan. It accepts both self-referred patients and referrals of difficult cases from other hospitals. These 410 patients were selected from a consecutive series of 477 unrelated patients with ALS after excluding variations in SOD1 (20 patients), C9ORF72 (18), TARDBP (16), FUS (8), cyclin-F (2), Optineurin (1), Matrin 3 (1), and TBK1 (1). Among the 410 patients with ALS, the average age at onset was 55.8 years (range 15–89). Eleven patients (2.7%) had a positive family history of ALS (familial ALS), and 399 (97.3%) were apparently sporadic cases. Seventy-seven patients (18.8%) suffered from bulbar-onset ALS, and 211 (51.5%) had an upper limb-onset disease.

**Genetic Analyses**

Genomic DNA was extracted from peripheral blood cells. Genetic analysis of the coding exons and their flanking regions of GLT8D1 was performed by PCR amplification and Sanger sequencing with the intronic primers using the BigDye 3.1 dideoxy terminator methods with an ABI Prism 3700 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). Amplicon sequences were compared with the reference GLT8D1 coding sequence (NM_152,932.2). The sequence variations were validated by sequencing both sense and antisense strands of the amplicons. The putative pathogenic GLT8D1 variants were first discriminated by absence in the 2 population databases, the genome aggregation database (gnomAD) and Taiwan Biobank database (taiwanview.twbiobank.org.tw). In silico prediction of the pathogenicity of
the variant was conducted using 2 computational programs, MutationTaster\textsuperscript{14} and PolyPhen-2.\textsuperscript{15} Evolutionary conservation of the mutated amino acid residue was assessed by aligning the amino acid sequences of GLT8D1 orthologs of different species using the UniProt website (www.uniprot.org).\textsuperscript{16}

**Expression Plasmids, Cell Cultures, and Transfection**

A full-length coding region of GLT8D1 was cloned into pFLAG-CMV-5a (Sigma-Aldrich, St. Louis, MO) to generate the wild-type (WT) GLT8D1 expression construct. The 2 GLT8D1 variations, p.I290M (c.870C>G) and p.R92C (c.274 G>A), were separately introduced into the WT expression plasmids by using the QuickChange Site-Directed Mutagenesis method (Agilent, Santa Clara, CA). The endoplasmic reticulum (ER) marker pDsRed-ER and the Golgi marker pDsRed-Monomer-Golgi were purchased from Clontech (Mountain View, CA). HEK293T cells were maintained in Dulbecco modified eagle medium supplemented with 10% FBS. Transient transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific).

**Western Blot Analysis of the Steady-State Protein Levels**

GLT8D1-transfected HEK293T cells were lysed in RIPA buffer, fractionated on 10% SDS-PAGE, and analyzed by Western blotting with anti-FLAG (#8146; Cell Signaling, Danvers, MA) or anti-actin (Merck Millipore, Burlington, MA) antibodies. Detection was performed with a standard enhanced chemiluminescence method.

**Immunofluorescence Analyses**

GLT8D1-transfected HEK293T cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.2% tween-20. After blocking with 1% BSA, the cells were stained for GLT8D1 using the anti-FLAG antibody conjugated to Alexa 488 together with DAPI for counterstaining cell nuclei. The confocal images were acquired under a FluoView FV10i confocal microscope (Olympus, Tokyo, Japan).

**Assessing ER Stress by Real-Time Quantitative PCR**

To investigate the effect of the GLT8D1 variations on ER stress, the mRNA expression levels of the ER stress biomarkers, binding immunoglobulin protein (BiP), C/EBP homologous protein (CHOP), and spliced X-box–binding protein 1 (sXBP1), were examined in the GLT8D1-transfected HEK293T cells by using real-time quantitative PCR (RT-qPCR). Total RNA of transfected cells was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). The cDNA synthesis was performed with the SuperScript\textsuperscript{III} 1st strand synthesis kit (Thermo Fisher Scientific). The RT-qPCR reactions were performed with SYBR Green master mix using a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). The relative gene expression was normalized against glyceraldehyde 3-phosphate dehydrogenase expression.

**Glycosyltransferase Activity Assays**

GLT8D1-transfected HEK293T cells were lysed in IP lysis buffer (Thermo Fisher Scientific). The lysates were incubated with anti-FLAG antibody-conjugated Dynabeads (Thermo Fisher Scientific) at 4°C overnight. Then, beads were washed with lysis buffer, and the pulled-down GLT8D1 proteins were eluted with 0.2 M glycine (pH 2.6) then neutralized with 1 M Tris-HCl (pH 9.0). Glycosyltransferase activity was measured using a glycosyltransferase activity kit (R&D Systems, Minneapolis, MN). Briefly, reactions were initiated by adding 10 ng/μL of purified GLT8D1 proteins to a reaction mixture including 5 mM UDP-galactose, 5 mM GlcNAc, and 2 ng/μL coupling phosphatase. Reactions were incubated at 37°C for 1 hour and then terminated by addition of Malachite Green reagent and OD620 read.

**Cell Viability and Cytotoxicity Assays**

Cell counting kit-8 (CCK-8) assay (Enzo Life Sciences, Farmingdale, NY) was used to assess the cell viability. GLT8D1-transfected cells were grown in 96-well plates, and CCK-8 solution was added to growing cultures and incubated at 37°C for 2 hours. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices, San Jose, CA). Lactate dehydrogenase (LDH) activity was measured in the culture medium as an index of cytotoxicity, using the CyQUANT LDH cytotoxicity assay (Thermo Fisher Scientific). Culture medium of the transfected cells was transferred to 96-well plate and incubated with the reaction mixture from the kit for 30 minutes. Absorbance at 490 and 680 nm was measured to determine LDH activity.

**Data Availability**

The data that support the findings of this study are available from the corresponding author. Data will be shared on reasonable request and after ethics approval if requested by other investigators.

**Results**

**Identification of the Novel GLT8D1 Variation**

Genetic analyses of GLT8D1 in the 410 patients with ALS revealed 1 heterozygous missense variant, p.I290M (c.870C>G) (Figure 1A), in 1 single patient with familial ALS. GLT8D1 p.I290M was not found in the gnomAD as well as Taiwan Biobank database, which contains 1,517 Taiwanese healthy control exomes. The p.I290M variant alters an amino acid residue of GLT8D1 which is evolutionarily conserved from human to zebrafish (Figure 1B). PolyPhen2 and MutationTaster predicted the GLT8D1 p.I290M variant to be probably damaging (scores 1 for HumDiv and 0.999 for HumVar) and disease causing (probability 0.998), respectively.

**I290M GLT8D1 Variant Protein Is Aberrantly Retained in the ER**

GLT8D1 p.R92C was a well-confirmed pathogenic variation for ALS\textsuperscript{5} and was used as a positive control in this study. To
assess the functional effect of the \textit{GLT8D1} p.I290M variation, we first investigated whether this variation could affect \textit{GLT8D1} protein expression. Western blot analysis revealed that the steady-state expression of the I290M \textit{GLT8D1} was much increased than that of the WT and the R92C \textit{GLT8D1} (Figure 2A). We then performed immunofluorescence analyses to demonstrate the intracellular distribution of the \textit{GLT8D1} proteins. As shown in Figure 2B, cells expressing WT or R92C \textit{GLT8D1} displayed discrete punctate perinuclear localization. Combined staining of \textit{GLT8D1} with a Golgi marker revealed that WT or the R92C variant protein signals overlapped largely with the Golgi marker (Figure 2C), implicating that WT or R92C \textit{GLT8D1} was localized primarily in the Golgi apparatus. However, staining of I290M \textit{GLT8D1} displayed abnormal reticular pattern throughout the cytoplasm rather than in the Golgi apparatus (Figure 2B). Combined labeling of I290M \textit{GLT8D1} with the ER marker showed a significant overlap between an ER marker and the I290M protein signals (Figure 2D), suggesting that the I290M \textit{GLT8D1} was predominantly retained in the ER instead of being transported to the Golgi apparatus.

I290M \textit{GLT8D1} Induces ER Stress and Unfolded Protein Response

To examine whether the I290M \textit{GLT8D1} could induce ER stress and unfolded protein response (UPR), we measured the mRNA levels of several ER stress genes in HEK293T cells transfected with the WT or variant \textit{GLT8D1} constructs. Cells expressing R92C myelin protein zero (MPZ), a variant protein known to induce ER stress,\textsuperscript{17} were used as positive control. As shown in Figure 3A, cells expressing I290M \textit{GLT8D1}, as well as the positive control R92C MPZ, had significantly increased levels of ER stress markers, such as BiP, CHOP, and sXBP1, compared with cells expressing WT \textit{GLT8D1}, indicating that I290M \textit{GLT8D1} induced ER stress. Cells expressing R92C \textit{GLT8D1} did not trigger UPR as significantly as I290M did.

I290M \textit{GLT8D1} Compromises the Glycosyltransferase Activity

To assess the influence of the p.I290M variation on glycosyltransferase activity, we purified the FLAG-tagged \textit{GLT8D1} proteins from HEK293T cells expressing WT or variant \textit{GLT8D1} constructs using immunoprecipitation. The cell-free enzymatic activity assays demonstrated that the glycosyltransferase activities were significantly reduced in the I290M \textit{GLT8D1} and R92C \textit{GLT8D1} compared with the activity of the WT protein (Figure 3B). These findings indicated that the \textit{GLT8D1} p.I290M variation compromised the glycosyltransferase activity, which may subsequently perturb cellular lipid and protein synthesis.

I290M \textit{GLT8D1} Leads to an Increased Cytotoxicity

Moreover, to determine whether the I290M variant protein may cause cell toxicity, CCK-8 assays were used to assess the cell viability of the \textit{GLT8D1}-transfected cells while LDH assays were used to evaluate cell death. As shown in Figure 3C and D, compared with WT \textit{GLT8D1}, both R92C and I290M variants had a significantly increased cellular toxicity in HEK293T cells.

Clinical Information of the Patient Carrying the \textit{GLT8D1} Variation

The pathogenicity of \textit{GLT8D1} p.I290M has been supported by both genetic analyses and in vitro functional studies. The patient harboring the \textit{GLT8D1} p.I290M variation had an initial symptom of right hand atrophy and weakness at age 60 years. Then, the symptoms progressed to bilateral upper limbs and then lower limbs within 2 years. She developed dysarthria.
and dysphagia at age 64 years and received percutaneous endoscopic gastrostomy at age 65 years. Physical examination at age 65 years revealed tongue atrophy with fasciculation, severe dysarthria, and weakness and atrophy in 4 limbs (muscle strength of 1–2/5 according to the Medical Research Council scale), brisk deep tendon reflexes, spasticity over the lower limbs, and bilateral extensor plantar responses. She died of respiratory failure at age 66 years. Her younger brother was reported to die of ALS at age 30 years without additional clinical information (Figure 1C).

**Discussion**

*GLT8D1* was recently identified as an ALS disease gene, and all the disease-associated variations were found in the exon 4. Functional studies showed that the p.R92C and p.G78W variations could result in impaired *GLT8D1* glycosyltransferase function and contribute to in vitro cytotoxicity and motor deficits in zebrafish. To understand the contribution of *GLT8D1* variations to ALS in our population, we investigated *GLT8D1* in 410 unrelated Taiwanese patients with ALS and identified 1 novel heterozygous missense variation, p.I290M in 1 patient with familial ALS. This variation locates in exon 9 of *GLT8D1* and is predicted to alter a highly conserved amino acid residue within the glycosyltransferase domain of *GLT8D1* (Figure 1B). Several lines of evidence support the pathogenicity of the *GLT8D1* p.I290M variation. First, it was present in a patient with familial ALS and was absent in gnomAD and Taiwan biobank databases. Second, it has been predicted as a damaging or disease causing variant by PolyPhen2 and MutationTaster programs. Furthermore, in vitro functional studies revealed that the p.I290M variation caused mislocalization of the variant *GLT8D1* proteins to ER, provoked ER stress and UPR, compromised the glycosyltransferase activity, and led to an increased cytotoxicity. According to the American College
of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines, GLT8D1 p.I290M matches the PS3, PM2, PP3, and PP4 criteria and is classified as a likely pathogenic variant.

The GLT8D1 is a member of the glycosyltransferase family and catalyzes transglycosylation reactions, where the monosaccharide component of a high-energy nucleotide sugar donor is transferred to an acceptor. Aberrant glycosylation has been implicated in neurodegeneration through 2 main glycosyltransferase-related mechanisms: ganglioside synthesis and addition of O-linked β-N-acetylglucosamine to proteins (O-GlcNAcylation). Gangliosides can modulate cell signaling processes, and O-GlcNAcylation is vital for axonal and synaptic function.

Defective glycosyltransferase-related mechanisms have been shown in animal models and patients of neurodegenerative diseases, such as Parkinson disease, Huntington disease, Alzheimer disease, and ALS. In this study, we demonstrated impaired glycosyltransferase function of the I290M GLT8D1 variant protein, which is consistent with previous observations in other disease-associated GLT8D1 variants. Notably, we observed mislocalization of I290M GLT8D1 to ER as well as activation of ER stress and UPR. These phenomena were not shown in the cells expressing R92C GLT8D1. ER stress has been widely implicated in ALS. Our findings suggest that the GLT8D1 p.I290M variation has more than 1 mechanism disturbing cellular homeostasis, including impairment of glycosyltransferase activity and disruption of ER function, which contribute to both loss-of-function and toxic gain-of-function effects on the disease pathogenesis.

The clinical features of ALS associated with GLT8D1 variations are still not fully elucidated. GLT8D1 variations may be associated with a typical ALS phenotype with highly variable age at disease onset and survival. In our study, the patient with the p.I290M variation had a spinal-onset ALS with disease onset at age 60 years and a survival of 6 years. Clear information of her affected younger brother was unavailable except knowing he is having an earlier disease onset and dying at age 30 years. In the study by Cooper-Knock et al., all the patients with ALS with a GLT8D1 variation had a spinal or bulbar-onset disease with onset ages ranging from 33 to 66 years and disease survivals ranging from 6 to 101 months. In another study, the Chinese male patient harboring a heterozygous GLT8D1 p.G78A variation had a right upper limb-onset ALS since age 44 years. He was still alive in last evaluation at 10 months after disease onset. More studies are needed to conclude the phenotypic characteristics of GLT8D1-associated ALS.
The prevalence of GLT8D1 variations in ALS appears to be low. The British study identified GLT8D1 variations in 7 patients from 103 familial and young sporadic ALS cases, including 34 familial patients with ALS in whom a genetic cause had not been identified after screening for ALS-associated variations in SOD1, C9ORF72, TARDBP, and FUS.\(^6\) In our study, the GLT8D1 p.I290M variation was identified in 1 of the 477 unrelated patients with ALS (0.2%, 1/477). In another Chinese study, only 1 likely pathogenic variant of GLT8D1, p.G78A, was found in 1 single patient with familial ALS after screening 977 patients with sporadic ALS and 47 patients with familial ALS (0.1%, 1/1024).\(^8\) Another 2 Chinese studies and 1 Australian study failed to identify any pathogenic variation after screening 512, 539 and 699 patients with ALS, respectively.\(^9\) These findings suggest that GLT8D1 variations are not a common cause of ALS.

All the GLT8D1 pathogenic variations identified in patients with ALS previously are located within exon 4, including p.I70T, p.G78A, p.G78W, p.A82E, p.I87N, and p.R92C.\(^5,8\) However, the GLT8D1 p.I290M variation identified in this study resides in exon 9, demonstrating that variations in other exons of GLT8D1 could also contribute to ALS. Of interest, a GLT8D1 variant of unknown significance reported in a Chinese patient with familial ALS, p.V291I, is just located next to the p.I290M variation.\(^8\)

In conclusion, we identified a novel GLT8D1 variation, p.I290M, in 1 (0.2%) of 477 unrelated Taiwanese patients with ALS and demonstrated that this variation may lead to impaired GLT8D1 glycosyltransferase activity and aberrantly activation of ER stress. This study broadens the spectrum of GLT8D1 variations as well as highlights the role of GLT8D1 in ALS pathogenesis.

Acknowledgment
The authors would like to thank the patients who participated in this study. The authors also thank the High-throughput Genome Analysis Core Faculty of National Core Facility Program for Biotechnology of Taiwan for genetic analysis service.

Study Funding
This study was supported by Ministry of Science and Technology, Taiwan (109-2314-B-075-044-MY3, 109-2314-B-005-002-MY3); Taipei Veterans General Hospital (V110C-034); and Brain Research Center, National Yang Ming Chiao Tung University from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan.

Disclosure
The authors report no disclosure relevant to the article. Go to Neurology.org/NG for full disclosure.

Publication History
Received by Neurology: Genetics June 17, 2021. Accepted in final form August 5, 2021.

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Appendix Authors

| Name | Location | Contribution |
|------|----------|--------------|
| Pei-Chien Tsai, PhD | Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan | 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 |
| Kang-Yang Jhh, MD, PhD | Department of Neurology, Taipei Veterans General Hospital, Taipei, Taiwan | Major role in the acquisition of data; Analysis or interpretation of data |
| Ting-Yi Shen, BS | Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan | Major role in the acquisition of data |
| Yi-Hong Liu, MD | Department of Neurology, Taipei Veterans General Hospital, Taipei, Taiwan | Analysis or interpretation of data |
| Kon-Ping Lin, MD | Department of Neurology, Taipei Veterans General Hospital, Taipei, Taiwan; Department of Neurology, National Yang Ming Chiao Tung University School of Medicine, Taipei, Taiwan | Major role in the acquisition of data |
| Yi-Chung Liao, MD, PhD | Department of Neurology, Taipei Veterans General Hospital, Taipei, Taiwan; Department of Neurology, National Yang Ming Chiao Tung University School of Medicine, Taipei, Taiwan; Brain Research Center, National Yang Ming Chiao Tung University, Taipei, Taiwan | 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 |
| Pei-Chien Tsai, PhD | Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan | 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 |

Neurology.org/NG
Neurology: Genetics | Volume 7, Number 6 | December 2021
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