RESEARCH ARTICLE

A Novel *TFG* Mutation in a Korean Family with α-Synucleinopathy and Amyotrophic Lateral Sclerosis

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ABSTRACT: Background: *Tropomyosin-receptor kinase fused gene* (*TFG*) functions as a regulator of intracellular protein packaging and trafficking at the endoplasmic reticulum exit sites. TFG has recently been proposed as a cause of multisystem proteinopathy.
Objectives: Here, we describe a Korean family presenting with Parkinson's disease or amyotrophic lateral sclerosis caused by a novel variant of *TFG* (c.1148 G > A, p.Arg383His).
Methods: We collected clinical, genetic, dopamine transporter imaging, nerve conduction, and electromyography data from the seven subjects. To verify the pathogenicity of the R383H variant, we studied cell viability and the abnormal aggregation of α-synuclein and TAR DNA-binding protein 43 (TDP-43) in HeLa cells expressing R383H-TFG.
Results: The clinical phenotypes of the R383H-TFG mutation varied; of the five family members, one had Parkinson's disease, three had subclinical parkinsonism, and one (the proband) had amyotrophic lateral sclerosis. The individual with multiple system atrophy was the proband's paternal cousin, but the *TFG* genotype was not confirmed due to unavailability of samples. Our in vitro studies showed that R383H-TFG overexpression impaired cell viability. In cells co-expressing R383H-TFG and α-synuclein, insoluble α-synuclein aggregates increased in concentration and were secreted from the cells and co-localized with R383H-TFG. The levels of cytoplasmic insoluble aggregates of TDP-43 increased in HeLa cells expressing R383H-TFG and co-localized with R383H-TFG.
Conclusions: Clinical and in vitro studies have supported the pathogenic role of the novel *TFG* mutation in α-synucleinopathy and TDP-43 proteinopathy. These findings expand the phenotypic spectrum of *TFG* and suggest a pivotal role of endoplasmic reticulum dysfunction during neurodegeneration. © 2021 International Parkinson and Movement Disorder Society

Key Words: *tropomyosin-receptor kinase fused gene* (*TFG*); parkinsonism; amyotrophic lateral sclerosis; endoplasmic reticulum

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Glossary

^{18}F-FP-CIT	[18F] N-(3-fluoropropyl)-2β-carbonethoxy-3β-(4-iodophenyl) nortropane
ALS	amyotrophic lateral sclerosis
αSyn	α-synuclein
CMT2	Charcot–Marie–Tooth disease type 2
DM	diabetes mellitus
ER	endoplasmic reticulum
HMSN-P	hereditary motor and sensory neuropathy with proximal dominant involvement

MSA	multiple system atrophy
NCS/EMG	nerve conduction study/electromyography
NE	neurological examination
PD	Parkinson's disease
HSP	hereditary spastic paraplegia
TDP-43	TAR DNA-binding protein 43
TFG	tropomyosin-receptor kinase fused gene
UPS	ubiquitin-proteasome system
WES	whole-exome sequencing
WT-TFG	wild-type TFG

The human *tropomyosin-receptor kinase fused gene* (*TFG*) is located on chromosome 3q12.2 and was originally identified in oncogenic chromosomal translocations, an aberration which results in the formation of chimeric fusion proteins in various cancers.[1,2] *TFG* mRNA is ubiquitously expressed in human tissues, and rat studies have shown it to be predominantly expressed in nerve tissues, especially neurons in the dorsal motor nucleus of the vagus, dorsal raphe nuclei, and locus coeruleus in the brainstem, Purkinje cells in the cerebellum, and anterior horn cells in the spinal cord.[1,3] *TFG* encodes a 400-amino acid cytoplasmic protein which regulates endoplasmic reticulum (ER)-to-Golgi transport at ER exit sites and increases ER-resident proteins by inhibiting the ubiquitin-proteasome system (UPS).[4,5] Known pathogenic *TFG* mutations are associated with defects in protein secretion and aggregation, leading to ER stress, and abnormal axonal growth.[6,7] *TFG* has been implicated in three distinct neurological disorders through different mutations, including autosomal-dominant hereditary motor and sensory neuropathy with proximal dominant involvement (HMSN-P; p. Pro285Leu), the autosomal-dominant type of Charcot–Marie–Tooth disease type 2 (CMT2; p. Gly269Val), and an autosomal-recessive form of complicated hereditary spastic paraplegia (SPG57; p. Arg106Cys).[6,8-10] Although phenotypic heterozygosity of *TFG* mutations remains unclear, dysregulation of ER homeostasis might be linked to ER stress-associated neurodegeneration.

In this study, we report a novel heterozygous mutation in *TFG* (c.1148 G > A; p.R383H) in a Korean family. Two brothers were diagnosed with amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). Additionally, there was a female cousin on the paternal side who was diagnosed with multiple system atrophy (MSA). Further experimental analyses showed that this mutation is associated with abnormal protein aggregation.

Patients and Methods

Subjects

An index case (II-3) was diagnosed with ALS. We subsequently recruited five members across two generations of the family (Fig. 1A), collected blood samples for genetic analysis, and evaluated all participants by neurological examination (NE), nerve conduction study/electromyography, brain magnetic resonance imaging (MRI), and ^{18}F-fluorinated-N-3-fluoropropyl-2-b-carboxymethoxy-3-b-(4-iodophenyl) nortropane (^{18}F-FP-CIT) positron emission tomography (PET) studies. A cousin of the proband was further diagnosed with MSA (II-6); however, nerve conduction study/electromyography (NCS/EMG) and genetic analyses were not performed because she died before this study.

Standard Protocol Approvals, Registrations, and Patient Consents

All participants provided written informed consent, and the local institutional review board approved the study to conduct clinical investigations according to the principles of the Declaration of Helsinki.

Genetic Analysis

Whole-exome sequencing (WES) was performed in the proband (II-3, Fig. 1A), with Sanger sequencing confirming the *TFG* variant, which was subsequently also identified in the family members. SureSelect Human All Exon V5 (Agilent Technologies, Santa Clara, CA, USA) was used for library preparation, and sequencing was performed on the Illumina NextSeq500 platform (Illumina Inc., San Diego, CA, USA) generating 2 × 150 base pair (bp) paired-end reads. Alignment of sequence reads, indexing of the reference genome (hg19), and variant calling with a pipeline based on GATK Best Practice. [Alignment was done using the BWA-mem (version 0.7.12),[11] duplicated reads were marked with Picard (version 1.96, http://picard.sourceforge.net) and local alignment, base quality recalibration, and variant calling was performed using the Genome Analysis Tool kit (GATK, version 3.5)].[12] To reduce the false-positive variants, variants were filtered out to meet the following criteria: QUAL > 30.0, QD > 2.0, MQ > 40.0, strong strand bias (FS < 60.0), HaplotypeScore <13.0, MQRankSum > −12.5, ReadPosRankSum > −8.0, and low read depth (DP > 10). Variant annotation was performed using Variant Effect Predictor (VEP) 88,[13] dbNSFP v3.3.[14] A population frequency of 1000 Genome[15] and ExAC[16] of 1% was used to define variants as rare or common. Coverage was calculated using the coverageBed of bedtools (version 2.17.0). The mean depth of coverage is 102× and the percentage of >10× is 98.6%.

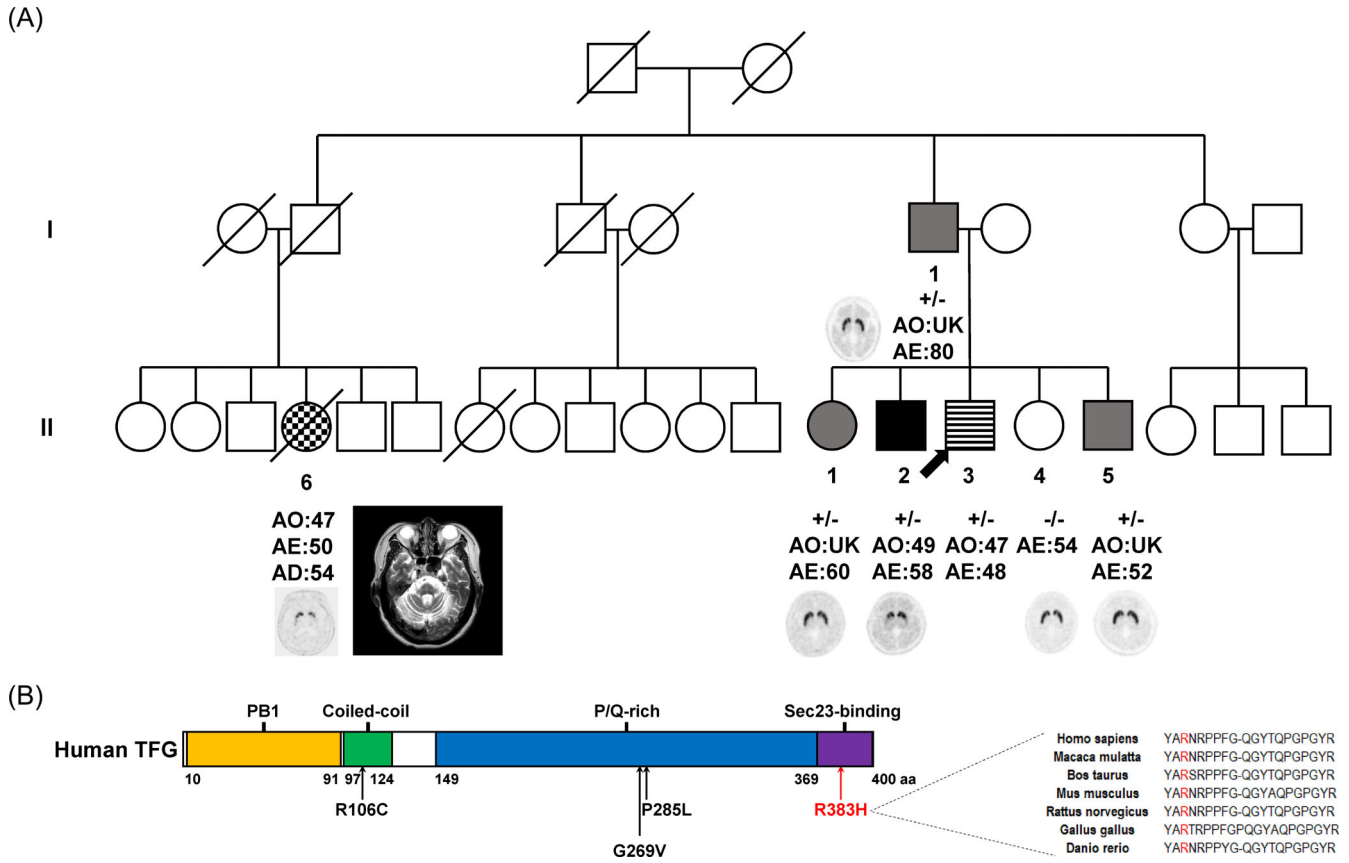

FIG. 1. A novel mutation of TFG (p.R383H) in a Korean family with heterozygous clinical phenotypes. (**A**) Pedigree of the family with clinical, genetic, and dopamine transporter imaging. Among patients with a heterozygous *TFG* mutation, the index case (II-3) has amyotrophic lateral sclerosis (horizontal stripes), II-2 has Parkinson's disease (black), and the others (I-1, II-1, and II-5) show subclinical parkinsonism (gray). II-6 (checkerboard) was diagnosed with multiple system atrophy showing a 'hot-cross bun' sign on her brain magnetic resonance imaging scan, with an unidentifiable *TFG* genotype. (**B**) Schematic illustration of the 400 amino acid (aa)-human TFG protein containing the Phox and Bem 1p (PB1) domain, a coiled-coil domain, proline and glutamate (P/Q)-rich domain, and Sec23-binding domain. The location of the known mutations (black arrows) and the novel mutation (c.1148 G > A; p.R383H; a red arrow) are indicated. Alignment of multiple *TFG* orthologues shows conservation of the Arg383 residue across species. AO, age at onset; AE, age at evaluation; AD, age at death; +/−, heterozygous mutation; −/−, wild-type. [Color figure can be viewed at wileyonlinelibrary.com]

The analysis of *C9orf72* repeat size was performed by fragment analysis. Fluorescently labeled polymerase chain reaction (PCR) primers were used as follows: F-5′-AGCCTGTAGCAAGCTCTGGA-3′ and R-5′-AG TCGCTAGAGGCGAAAGC-3′. Electrophoresis of amplicons was run on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed using GeneMapper v.3.7 (Applied Biosystems) software.

In Vitro Study: Pathogenic Role of the Mutant TFG in α-Synuclein (aSyn) and TAR DNA-Binding Protein 43 (TDP-43) Expressions

All experimental materials, preparation, cell viability test, immunoblotting, immunofluorescence staining, and statistical analysis are described in the Supplementary Material.

Results

Clinical Studies

In this family, the elder brother of the proband had PD (II-2), three of the family members showed subclinical parkinsonism (I-1, II-1, and II-5), and one sister was found to be normal upon neurological examination (NE) (II-4) (Fig. 1A). The cousin on the paternal side (predeceased) was previously diagnosed with MSA (II-6).

NCS/EMG was compatible with ALS in patient II-3. In one subject (I-1), who had a long history of diabetes mellitus (DM), NCS/EMG showed sensorimotor polyneuropathy and lumbosacral radiculopathy. The rest of the participants (II-1, II-2, II-4, and II-5) showed no abnormal findings on NCS/EMG.

The patient was an 80-year-old man with DM and hypertension. NE showed mild bradykinesia in the left arm and leg. ^{18}F-FP-CIT PET showed decreased uptake

in the right mid-to-posterior putamen and right caudate tail. MRI of the brain revealed diffuse cortical atrophy.

Individual II-1

The patient was a 60-year-old woman with no remarkable medical history. NE showed mild bradykinesia in the left limbs. Brain MRI was normal. ^{18}F-FP-CIT PET showed mildly decreased uptake in the right posterior and left posterior dorsal putamen.

Individual II-2

The patient was a 58-year-old man who had been treated for PD for 9 years. The initial symptom was the dragging of the left foot. Anti-PD drugs were effective. NE showed bilateral bradykinesia and rigidity, and the left side was more affected. The patient had mild torticollis on the right side. The Unified Parkinson's Disease Rating Scale motor subscales were 19, and Hoehn and Yahr staging was 2. He was taking rasagiline 1 mg, pramipexole extended-release 1.5–0.75 mg, trihexyphenidyl 2 mg, and amitriptyline 1 mg per day. Brain MRI findings were unremarkable. ^{18}F-FP-CIT PET revealed bilaterally decreased uptake, which was worse in the posterior and right sides.

Individual II-3

The patient was a 48-year-old man. He visited our hospital for progressive limb weakness over 1 year. He had frequent cramps, fasciculation, difficulty climbing stairs, running, and using chopsticks. NE showed decreased motor power (Medical Research Council (MRC) grade in upper extremities 4+/4+ and in lower extremities 4/4), muscle atrophy, and increased deep tendon reflexes in the four extremities. He further showed spastic gait and ankle clonus. EMG showed widespread denervation in multiple muscles innervated by the bulbar, cervical, thoracic, and lumbosacral segments. Brain MRI findings were unremarkable. At age 51 years, he underwent bedridden tracheostomy and

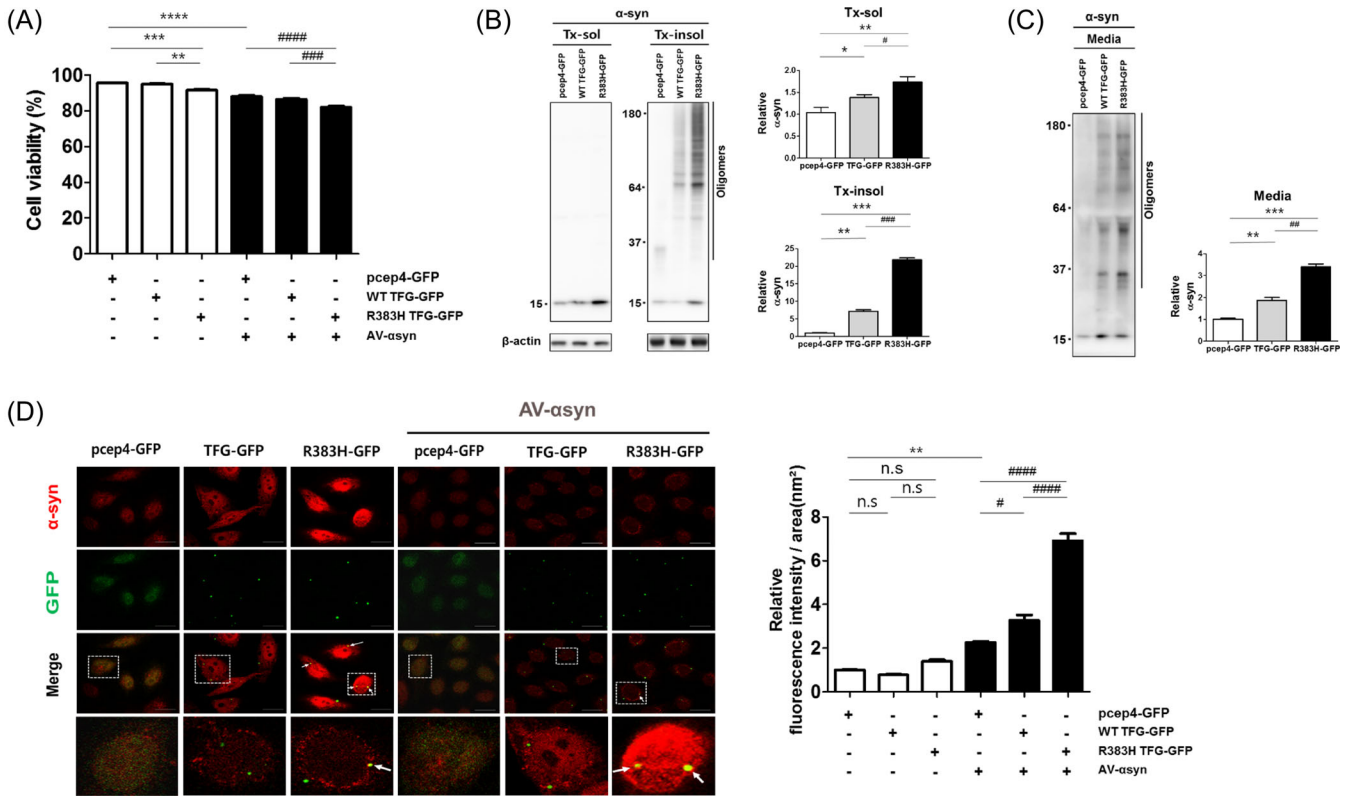

FIG. 2. R383H-TFG increases the intracellular expression and secretion of α-synuclein aggregates. (**A**) Cell viability test in HeLa cells stably expressing TFG-GFP. Human α-syn was found overexpressed using an adenoviral vector (AV). On day 3 post-infection, cell viability was measured ($n = 4$ per group; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$) by two-way ANOVA with Tukey's post-hoc test. One thousand cells were analyzed per experiment. (**B** and **C**) Western blot analysis of α-syn in cell lysates and culture media. Human α-syn was overexpressed in TFG-GFP-stable HeLa cells. On day 3 post-infection, the levels of α-syn were measured in Triton X-100-insoluble (Tx-insol) and soluble (Tx-sol) fractions. (**B**) Monomeric α-syn in the Tx-soluble fraction and accumulation of α-syn aggregates in the Tx-insoluble fraction. (**C**) Oligomeric α-syn released from cells was measured ($n = 4$ per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$) by one-way ANOVA with the Tukey post-hoc test. (**D**) TFG-GFP HeLa cells were immunolabeled with antibodies against human α-synuclein (LB509) and GFP (GF28R). α-syn fluorescence intensity was analyzed in randomly chosen areas (scale bars = 20 μm; $n = 3$ per group; n. s., non-significant, $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#\#\#}P < 0.0001$) using two-way ANOVA with Tukey post-hoc test. White arrows indicate co-localization of *TFG* and α-syn. One hundred cells were analyzed per experiment. The boxed areas in the upper images are magnified in the lower panels. [Color figure can be viewed at wileyonlinelibrary.com]

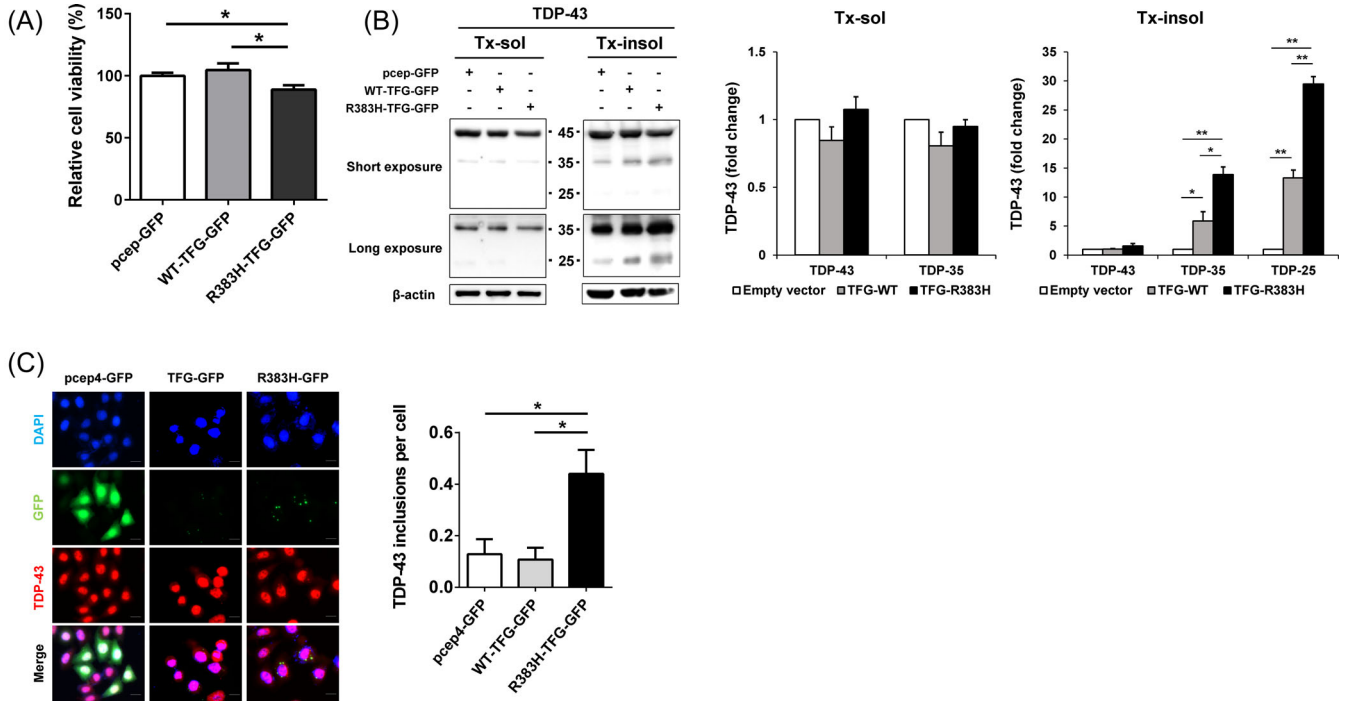

FIG. 3. R383H-TFG induces cytoplasmic TDP-43 inclusions. (**A**) Cell viability test was performed 2 days after transfection with WT-TFG or R383H-TFG ($n = 3$ per group; $^{**}P < 0.01$). (**B**) After dividing cell lysates from TFG-expressing HeLa cells into soluble (Tx-sol) and insoluble fractions (Tx-insol), immunoblotting was performed using an anti-TDP-43 antibody (c-terminal). We quantified band intensities corresponding to TDP-43 or its 35 and 25 kDa fragments, which were normalized with reference to β-actin. Data are presented as bar graphs with data from three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$. (**C**) We performed immunofluorescence staining with an antibody against the c-terminal of TDP-43 and DAPI to localize cytoplasmic TDP-43 inclusions and *TFG* proteins. The mutant R383H-TFG increased cytosolic TDP-43 inclusions compared to WT-TFG and was co-localized. (Scale bars = 10 μm; the number of cytoplasmic inclusions of TDP-43 was normalized with reference to the cell number and presented as a bar graph. $^{*}P < 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

percutaneous endoscopic gastrostomy. His medical and neurological conditions did not allow ^{18}F-FP-CIT PET imaging examination.

Individual II-5

The patient was a 52-year-old man with no remarkable medical history. The NEs showed mild limb bradykinesia on the left side. Brain MRI was normal. ^{18}F-FP-CIT PET showed decreased uptake in the left putamen.

Individual II-6

The patient was a 48-year-old woman who visited a neurology clinic complaining of urinary problems, such as urgency and frequency, orthostatic dizziness, and gait difficulty for 1 year. NEs showed bilateral bradykinesia and rigidity that were more affected on the right side, cerebellar speech, ataxia, limb dysmetria, and hyperreflexia. Systolic blood pressure dropped in the standing position up to 53 mmHg. Various medications, including levodopa, were ineffective. Brain MRI showed severe pontocerebellar atrophy with a 'hot-cross bun' sign. ^{18}F-FP-CIT PET showed decreased uptake on the left side. She finally became bedridden and died of pneumonia 7 years after onset.

Genetic Studies

The proband was examined using WES and a novel variant of *TFG* (c.1148 G > A; p.R383H) (Fig. 1B) was identified. Sanger sequencing confirmed this variant in the proband and all the living family members. Five family members (I-1, II-1, II-2, II-3, and II-5) harbored this variant. Alignment of multiple *TFG* orthologs showed that the Arg383 residue is highly conserved across species and is located in the Sec23 binding domain, through which the *TFG* protein directly interacts with the Sec23 protein, a component of coat protein complex II (COPII).[17] The *TFG* variant (c.1148 G > A; p.R383H) was segregated with neurologically affected individuals within the family and was predicted to be probably damaging in Polyphen-2, tolerated in SIFT, and scaled CADD scores of 26.2. The *TFG* variant is rarely found, with allele frequencies of 5.88×10^{-4} in the Korean Reference Genome Database ($n = 1722$) and 2.72×10^{-4} in East Asians. The gnomAD (125,748 exome sequences and 15,708 whole-genome sequences) also showed a variant at allele frequency of 2.39×10^{-5}. Functional studies

showed the pathologic effect of the variant. (PS3) The variant was co-segregated with disease in multiple family members: individual II-4 was normal without the variant and other members had disease with the variant. (PP1) Family history is the dominant trait for PD, and an ALS-phenotype with TDP-43 aggregation has previously been reported in those with *TFG* mutation.[6,18] (PP4) These findings suggest that the R383H variant is "*likely pathogenic*" according to the American College of Medical Genetics and Genomics (ACMG) guideline.[19]

A hexanucleotide repeat expansion in *C9ORF72* is the most common cause of ALS and frontotemporal dementia,[20] and is possibly associated with other phenotypes, including parkinsonism.[21] The lengths of the *C9ORF72* hexanucleotide repeat alleles were two and seven repeats in the proband, which were normal.

Laboratory Studies

R383H-TFG Induced Expression, Aggregation, and Secretion of aSyn

Overexpression of aSyn by an adenoviral vector (AV-α-syn) triggered cytotoxicity in HeLa cells, and the variant *TFG* (R383H-TFG) exacerbated cytotoxicity (Fig. 2A). When we examined the effects of R383H-TFG on aSyn expression and aggregation, higher levels of aSyn aggregates were detected in the Triton X-100-insoluble fractions from R383H-TFG cells than in the wild-type TFG (WT-TFG) cells and in the soluble fractions (Fig. 2B). When cells are exposed to stressful conditions, such as oxidative stress and nutrient depletion, the secretion of aSyn aggregates is increased.[22] We investigated changes in the secretion of aSyn aggregates by immunoblotting of the cell culture media at 3 days post-infection with AV-α-syn. Immunoblotting results revealed that the amount of secreted aSyn aggregates were higher from R383H-TFG cells than those from WT-TFG cells (Fig. 2C). Next, we confirmed the altered levels of aSyn aggregates by immunofluorescence staining. When aSyn was overexpressed, higher fluorescence intensities of aSyn were detected in HeLa cells expressing R383H-TFG-GFP than in the GFP vector or WT-TFG-GFP. Unlike WT-TFG-GFP, the R383H-TFG-GFP puncta co-localized with the aSyn aggregates (Fig. 2D).

R383H-TFG Increased Cytoplasmic TDP-43 Inclusions

We measured TDP-43 and its fragments (TDP-35 or TDP-25) by immunoblotting or immunofluorescence staining. In addition to increased cell death in R383H-TFG cells compared to WT-TFG cells (Fig. 3A), the levels of TDP-35 and TDP-25 in the Triton-X insoluble fraction were significantly increased, while those in the Triton-X soluble fraction were not (Fig. 3B). Immunocytochemical studies showed increased cytoplasmic inclusions of TDP-43 in R383H-TFG cells (Fig. 3C). Co-localization of TDP-43 and TFG was also observed in R383H cells.

Discussion

We identified a novel likely pathogenic variant of *TFG* (c.1148 G > A, p.Arg383His) with a heterozygous form in a family presenting with various clinical phenotypes, including ALS and PD. Since we could not obtain genetic information for the patient with MSA (II-6), we could not confirm whether her clinical presentation was caused by the same mutation.

In this study, functional studies showed a pathologic effect of the *TFG* variant on cell viability, with molecular evidence related to disease-specific pathomechanism. The variant was co-segregated with disease in multiple family members, in which individual II-4 was normal without the variant and other subjects had diseases with the variant. Family history is the dominant trait for PD, and an ALS-phenotype with TDP-43 aggregation has previously been reported in those with *TFG* mutation.[6,18] Since PD was not reported in patients with *TFG* mutations, assigning PD as a specific syndrome of *TFG* is limited at this stage. In summary, the R383H variant can be classified as "*likely pathogenic*" according to ACMG guidelines [1 strong (PS3) and 2 supporting (PP1, PP4) evidences of pathogenicity].

The clinical features of the PD, MSA, and ALS patients in this family showed little difference from those of sporadic PD, MSA, and ALS, except for mild cervical dystonia and younger onset age in patients with PD (II-2).[23]

Multisystem Proteinopathy (MSP) Due to *TFG* Mutations

It is difficult to understand the diversity of clinical phenotypes related to single gene abnormalities, such as those in this family. The umbrella term MSP was recently introduced, designating an inherited disorder characterized by a plethora of heterogeneous phenotypes with multiple organ involvement. Genes for RNA-binding proteins and proteins that mediate ubiquitin-dependent autophagy are associated with MSP, such as the valosin-containing protein (VCP) gene.[24] The spectrum of VCP-related disorders initially encompasses inclusion body myopathy, Paget's disease of the bone, frontotemporal dementia, and amyotrophic lateral sclerosis.[25,26] Other movement disorders such as PD and hereditary spastic paraplegia (HSP) have also been reported.[27,28]

Similar to VPS, the major biological role of TFG proteins is related to intracellular protein machinery, such as the ER and UPS. As expected, mutations in *TFG* have been associated with pleiotropic clinical presentations, such as HMSN-P, CMT2, and HSP.[6,8-10] Neuropathological studies have shown TFG-, TDP-43-, and

p62-positive aggregates in neuronal tissues, including the brain and spinal cord.[6,18] In this study, we suggest the addition of PD, ALS, and possibly MSA to the spectrum of *TFG* mutations, emphasizing *TFG*-related disorders as an MSP.[18,29]

Abnormal Aggregation of aSyn and TDP-43 by *TFG* Mutation as Pathomechanisms

The novel missense mutation in the *TFG* gene is located within the 20 C-terminal amino acids (Fig. 1B), which directly binds to the gelsolin-like domain of the Sec23 protein, enabling COPII-mediated trafficking from ER exit sites to ER−Golgi intermediate compartments.[30] ER homeostasis requires coordination of cargo deposition in the ER lumen, proper distribution to other cellular compartments, and turnover of unfolded or misfolded proteins. Dysregulation of these pathways could result in the accumulation of immature proteins in the ER lumen, which activates the unfolded protein response and promotes cell death by prolonged ER stress. Based on these observations, we performed in vitro experiments to investigate whether the novel *TFG* mutation (R383H) exerts cellular toxicity and induces protein aggregation.

R383H-TFG seems to facilitate α-synucleinopathy by increasing overexpression, aggregation, and secretion of aSyn proteins compared to WT-TFG, with co-localization of the proteins also observed. As aSyn is a major component of Lewy bodies, which is pathognomonic in PD, its inclusions support a potential role for the mutant *TFG* in α-syn pathology in in vitro cell culture models.[31] Studies of rare familial forms of PD recapitulated that ER stress in conjunction with dysfunction of the UPS could provide a potential link to PD pathogenesis.[32,33]

TDP-43 is the major component of ubiquitinated neuronal cytoplasmic inclusions in approximately 90% of ALS cases, and is predominantly localized to the nucleus, which regulates RNA transcription, splicing, transport, and other cellular processes.[34,35] Pathogenic TDP-43 proteins translocate from the nucleus to the cytoplasm, forming detergent-resistant inclusions that become phosphorylated, ubiquitinated, and truncated, to leave C-terminal fragments. In this study, overexpression of mutant R383H-TFG increased cytoplasmic inclusions. These inclusions contained more aggregation-prone C-terminal fragments of TDP-43, such as TDP-25 and TDP-35.[36,37] Only two pathologic studies showed co-localization of TDP-43 and TFG over multiple brain areas, suggesting a pathophysiological correlation between TDP-43 and TFG.[6,38] In this study, co-localization of TDP-43 and TFG was found only in R383H cells. These findings showed the pathological role of R383H in the cellular handling of TDP-43, supporting the detrimental effect of R383H variants on ALS.

Limitations and Conclusions

Our in vitro functional studies had several limitations. First, our findings provide functional evidence using a non-neuronal cellular model with overexpression of *TFG* variants. These studies showed a disturbance in cell viability and an increase in α-synuclein and TDP-43 aggregation. Although ectopic overexpression has been widely used to study the function of the protein of interest, this approach may not completely reflect the physiological functions of the protein. Therefore, further studies using more relevant model systems are needed to validate the conclusions of the present study. Second, the detailed pathomechanism that determines the fate of the mutation in specific disorders was left untouched, requiring further study. In general, the contribution of the *TFG* variant to α-synucleinopathy and TDP-43 proteinopathy requires further study.

In conclusion, clinical and experimental investigations demonstrate that the R3838H variant of *TFG* is a novel mutation responsible for MSP encompassing α-synucleinopathy (PD, MSA) and TDP-43 proteinopathy (ALS). Considering the function of *TFG* in the regulation of protein trafficking and degradation by an ER-centered mechanism, abnormal protein aggregation by *TFG* mutation in our study is in line with the protein-mishandling hypothesis of α-synucleinopathy and TDP-43 proteinopathy. ■

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Data Availability Statement

The corresponding author takes responsibility for the integrity of the data and the accuracy of the data analysis. Supporting data of the findings in this study are available upon reasonable request from the corresponding author.

References

1. Greco A, Mariani C, Miranda C, et al. The DNA rearrangement that generates the TRK-T3 oncogene involves a novel gene on chromosome 3 whose product has a potential coiled-coil domain. Mol Cell Biol 1995;15(11):6118–6127.
2. Hernández L, Pinyol M, Hernández S, et al. TRK-fused gene (TFG) is a new partner of ALK in anaplastic large cell lymphoma producing two structurally different TFG-ALK translocations. Blood 1999; 94(9):3265–3268.
3. Takeuchi S, Masuda C, Maebayashi H, Tooyama I. Immunohistochemical mapping of TRK-fused gene products in the rat brainstem. Acta Histochem Cytochem 2012;45(1):57–64.
4. Witte K, Schuh AL, Hegermann J, et al. TFG-1 function in protein secretion and oncogenesis. Nat Cell Biol 2011;13(5):550–558.
5. Yagi T, Ito D, Suzuki N. Evidence of TRK-fused gene (TFG1) function in the ubiquitin-proteasome system. Neurobiol Dis 2014;66:83–91.
6. Ishiura H, Sako W, Yoshida M, et al. The TRK-fused gene is mutated in hereditary motor and sensory neuropathy with proximal dominant involvement. Am J Hum Genet 2012;91(2):320–329.

7. Slosarek EL, Schuh AL, Pustova I, et al. Pathogenic TFG mutations underlying hereditary spastic paraplegia impair secretory protein trafficking and axon fasciculation. Cell Rep 2018;24(9):2248–2260.
8. Lee S-S, Lee HJ, Park J-M, et al. Proximal dominant hereditary motor and sensory neuropathy with proximal dominance association with mutation in the TRK-fused gene. JAMA Neurol 2013; 70(5):607–615.
9. Elsayed LEO, Mohammed IN, Hamed AAA, et al. Hereditary spastic paraplegias: identification of a novel SPG57 variant affecting TFG oligomerization and description of HSP subtypes in Sudan. Eur J Hum Genet 2016;25(1):100–110.
10. Tsai PC, Huang YH, Guo YC, et al. A novel TFG mutation causes Charcot-Marie-tooth disease type 2 and impairs TFG function. Neurology 2014;83(10):903–912.
11. Li H, Durbin R. Fast and accurate long-read alignment with Burrows–Wheeler transform. Bioinformatics 2010;26(5):589–595.
12. McKenna A, Hanna M, Banks E, et al. The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20(9):1297–1303.
13. McLaren W, Gil L, Hunt SE, et al. The Ensembl variant effect predictor. Genome Biol 2016;17(1):122
14. Liu X, Wu C, Li C, Boerwinkle E. dbNSFP v3.0: a one-stop database of functional predictions and annotations for human nonsynonymous and splice-site SNVs. Hum Mutat 2016;37(3): 235–241.
15. Auton A, Abecasis GR, Altshuler DM, et al. A global reference for human genetic variation. Nature 2015;526(7571):68–74.
16. Karczewski KJ, Weisburd B, Thomas B, et al. The ExAC browser: displaying reference data information from over 60 000 exomes. Nucleic Acids Res 2017;45(D1):D840–D845.
17. Hanna MG, Block S, Frankel EB, et al. TFG facilitates outer coat disassembly on COPII transport carriers to promote tethering and fusion with ER–Golgi intermediate compartments. Proc Natl Acad Sci 2017;114(37):E7707–E7716.
18. Yamashita S, Kimura E, Zhang Z, et al. Muscle pathology of hereditary motor and sensory neuropathy with proximal dominant involvement with TFG mutation. Muscle Nerve 2019;60(6):739–744.
19. 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(5):405–423.
20. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron 2011;72(2): 245–256.
21. Lesage S, Le Ber I, Condroyer C, et al. C9orf72 repeat expansions are a rare genetic cause of parkinsonism. Brain 2013;136(Pt 2): 385–391.
22. Jang A, Lee HJ, Suk JE, Jung JW, Kim KP, Lee SJ. Non-classical exocytosis of alpha-synuclein is sensitive to folding states and promoted under stress conditions. J Neurochem 2010;113(5):1263–1274.
23. Jankovic J, Rajput AH, McDermott MP, Perl DP. Group ftPS. The evolution of diagnosis in early Parkinson disease. Arch Neurol 2000;57(3):369–372.
24. Taylor JP. Multisystem proteinopathy: intersecting genetics in muscle, bone, and brain degeneration. Neurology 2015;85(8):658–660.
25. Watts GD, Wymer J, Kovach MJ, et al. Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. Nat Genet 2004; 36(4):377–381.
26. Johnson JO, Mandrioli J, Benatar M, et al. Exome sequencing reveals VCP mutations as a cause of familial ALS. Neuron 2010; 68(5):857–864.
27. Al-Obeidi E, Al-Tahan S, Surampalli A, et al. Genotype-phenotype study in patients with valosin-containing protein mutations associated with multisystem proteinopathy. Clin Genet 2018;93(1):119–125.
28. van de Warrenburg BP, Schouten MI, de Bot ST, et al. Clinical exome sequencing for cerebellar ataxia and spastic paraplegia uncovers novel gene–disease associations and unanticipated rare disorders. Eur J Hum Genet 2016;24(10):1460–1466.
29. Nicolau S, Liewluck T. TFG: at the crossroads of motor neuron disease and myopathy. Muscle Nerve 2019;60(6):645–647.
30. Peotter J, Kasberg W, Pustova I, Audhya A. COPII-mediated trafficking at the ER/ERGIC interface. Traffic 2019;20(7):491–503.
31. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. Alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc Natl Acad Sci U S A 1998;95(11):6469–6473.
32. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392(6676):605–608.
33. Leroy E, Boyer R, Auburger G, et al. The ubiquitin pathway in Parkinson's disease. Nature 1998;395(6701):451–452.
34. Neumann M, Sampathu DM, Kwong LK, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 2006;314(5796):130–133.
35. Mackenzie IRA, Rademakers R, Neumann M. TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. Lancet Neurol 2010;9(10):995–1007.
36. Cicardi ME, Cristofani R, Rusmini P, et al. Tdp-25 routing to autophagy and proteasome ameliorates its aggregation in amyotrophic lateral sclerosis target cells. Sci Rep 2018;8(1):12390
37. Che MX, Jiang LL, Li HY, Jiang YJ, Hu HY. TDP-35 sequesters TDP-43 into cytoplasmic inclusions through binding with RNA. FEBS Lett 2015;589(15):1920–1928.
38. Maeda K, Idehara R, Mukaisho KI. Presence of colocalised phosphorylated TDP-43 and TFG proteins in the frontotemporal lobes of HMSN-P. J Neurol Neurosurg Psychiatry 2020;91(11):1231–1232.

Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.