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Original Research

TGM6 variants in Parkinson's disease: clinical findings and functional evidence

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TGM6 encodes transglutaminase 6, which catalyzes the covalent crosslinking of proteins through transamination reactions. Variants in *TGM6* have been identified as the cause of spinocerebellar ataxia type 35. However, we found 12 *TGM6* variants of low frequency among 308 patients with Parkinson's disease using next-generation sequencing technologies and multiple ligation-dependent probe amplification, including two variants *TGM6* p.R111C and p.L517W, which have been reported to affect functions of transglutaminase 6 in spinocerebellar ataxia type 35 cases. The characteristics of these *TGM6* carriers were summarized. To clarify the role of *TGM6* variants in Parkinson's disease, we constructed the plasmids of wild-type *TGM6* and *TGM6* p.R111C, p.P359L, p.L517W to transfect A53T-SH-SY5Y cells and conducted transglutaminase assay, western blots, immunofluorescence, and cell viability assay. Results revealed that the distribution and expression levels of transglutaminase 6 were not affected by *TGM6* variants. However, the variants showed lower transglutaminase activity than wild-type transglutaminase 6. The overexpression of wild-type *TGM6* was proved to relieve the cell damage, down-regulate the level of α-synuclein and enhance autophagy. These effects were weakened in cells transfected with mutant *TGM6* plasmids. Our results suggested that there may be some relationship between *TGM6* and Parkinson's disease. *TGM6* carriers in Parkinson's disease patients presented with typical parkinsonism but progressed slower. The high expression level of wild-type transglutaminase 6 may protect cells by decreasing α-synuclein and enhancing autophagy.

Keywords

Parkinson's disease; genetic testing; transglutaminase 6; variant

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease (Poewe et al., 2017). α-synuclein is the main constituent of Lewy bodies, which is an essential pathological marker for PD (Sharon et al., 2003). These aggregates of misfolded α-synuclein may eventually become toxic and trigger neuronal death. Autophagy plays an essential role in the degradation of misfolded proteins. However, autophagy is impaired in PD, leading to further accumulation of misfolded α-synuclein (Anglade et al., 1997; Cai et al., 2016). As such, increasing autophagy flux for α-synuclein clearance represents a promising therapeutic strategy (Fowler and Moussa, 2018).

Over the past few decades, a growing body of genetic research has greatly enhanced our understanding of the genetic basis of PD. While approximately 5-10% of PD patients have a monogenic form of the disease with Mendelian inheritance, most cases are sporadic with unknown etiology (Kalinderi et al., 2016). So far, at least 17 genes have been identified as being associated with PD (http://www.genenames.org). Risk loci for PD continue to be discovered by way of genome-wide association studies and other sequencing technologies (Chang et al., 2017; Shi et al., 2018).

TGM6 (MIM *613900) encodes for transglutaminase 6 (TG6), a member of the transglutaminase (TG) family. TGs catalyze protein covalent cross-linking through transamination reactions, in which intra- or inter-molecular isopeptide bonds are formed between a glutamine residue and, usually, a lysine residue (Folk and Chung, 1973). TG6 is abundantly expressed in the brain, especially in the septal region, basal ganglia, and cerebellum (Liu et al., 2013; Schulze-Krebs et al., 2016). Some TGs have been related to neurodegenerative diseases (AbdAlla et al., 2009; Andringa et al., 2004; Zainelli et al., 2003). For example, TG2 is capable of catalyzing the formation of α-synuclein crosslinks, which is considered as an early step in PD pathogenesis (Andringa et al., 2004).

In recent years, variants in *TGM6* have been demonstrated to be the cause of the spinocerebellar ataxias (SCA) type 35 (MIM #613908) by whole-exome sequencing as well as some functional studies (Fasano et al., 2017; Guan et al., 2013; Guo et al., 2014; Li et al., 2013; Lin et al., 2019; Wang et al., 2010; Yang et al., 2018). Patients with SCA35 develop symptoms at 15-56 years of age, with most patients experiencing late-onset. Clinically, SCA35 presents as slowly progressing ataxia. Most patients encounter gait disturbance, dysarthria, and action tremor as initial symptoms. Mild pursuit aberrations, paresthesia, hyperreflexia and dystonia can

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also be found in some patients. There is usually evident cerebellar atrophy in MRI. However, the variants of *TGM6* that reportedly affect functioning in SCA were also found in our PD patients. These patients, who showed no symptoms of ataxia, had been diagnosed with PD according to the United Kingdom PD Society Brain Bank criteria and the Movement Disorder Society (MDS) clinical diagnostic criteria for PD. Given that α-synuclein has been demonstrated to be one of the substrates of TGs, we, therefore, wondered whether *TGM6* and PD are related and, if so, by what mechanism.

On these bases, we hypothesized that *TGM6* might play a role in PD pathogenesis through the regulation of α-synuclein and autophagy. In our research, *TGM6* variants of low frequency were screened in PD patients through next-generation sequencing. The clinical features of the *TGM6* carriers were analyzed. Further, we used a PD model of A53T-SH-SY5Y cells overexpressing wild-type (WT) or mutant-type (MT) TG6 to examine the impact of *TGM6* mutations on transglutaminase activity, α-synuclein, autophagy, and cell viability. Our data indicated that WT *TGM6* might protect cells by enhancing autophagy and decreasing α-synuclein. This protective effect was attenuated with MT *TGM6*, which may be related to PD pathogenesis. These findings might help to clarify the correlation between *TGM6* and PD and provide a potential target for the treatment of PD.

2. Materials and methods

2.1 Subjects and clinical evaluation

PD patients were recruited from the movement disorders clinic in Huashan Hospital between February 2014 and May 2017. Subjects who met the following criteria were selected for genetic tests: (1) a clinical diagnosis of PD according to the United Kingdom PD Society Brain Bank criteria (Gibb and Lees, 1988) (before December 2015) or the MDS clinical diagnostic criteria for Parkinson's disease (Postuma et al., 2015) (after November 2015) ; (2) age at onset < 50, or having a family history of parkinsonism. The ethics committee approved the research of Huashan Hospital. After the receipt of informed consent, blood samples and detailed information on each subject were collected. This information included demographic characteristics, clinical data, family records, neurologic examination, Hoehn and Yahr (H&Y) stage, Unified Parkinson's Disease Rating Scale part III (UPDRS-III) (Goetz et al., 2004), Mini-mental State Examination (MMSE) (Li et al., 2016), Beck Depression Inventory (BDI) (Huang et al., 2016), Sniffin' Sticks screening 12 test (SS-12) (Huang et al., 2016), Rapid-eye-movement Sleep Behavior Disorder Screen Questionnaire (RBDSQ) (Stiasny-Kolster et al., 2007) and Epworth Sleepiness Score (ESS) (Chen et al., 2002). Motor function was assessed during the wearing-off phase of levodopa, signified by a loss of drug effect.

2.2 Genetic test and variants analysis

Genomic DNA was extracted from peripheral blood leukocytes by blood genomic extraction kit (Qiagen) and subjected to target sequencing (HiSeq2500 sequencer, Illumina) for a panel of PD-related genes (Suppl. 1) and multiple ligation-dependent probe amplification (MLPA) by the SALSA MLPA P051-D1/P052-D2 Parkinson probemix kit (MRCHolland). Mean exon coverage was higher than 100 × , with 95% of target base positions having at least 10 × coverage. Variants different from the reference sequences were filtered to remove common polymorphisms (allele frequency > 1%) according to the 1000 Genomes Project (http://www.1000genomes.org/home), In-house database, ESP6500 (evs.gs.washington.edu/EVS/), and ExAC (Exome Aggregation Consortium, http://exac.broadinstitute.org/). The phenotypes of the filtered genes were compared with the clinical characteristics of the corresponding patient, and the inherited modes were considered to exclude irrelevant genes further. SIFT (http://sift.jcvi.org/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), and MutationTaster (http://www.mutationtaster.org/) were used to predict the impact of the variants on gene function. The possibly pathogenic variants were confirmed in the proband and other family members by Sanger sequencing.

2.3 Construction of recombinant plasmids

WT human *TGM6* cDNA (NCBI transcript NM_198994) was amplified by polymerase chain reaction with designed primers (forward nucleotide sequence 5′-CGCAAATGGGCGGTAGGCGTG-3′ and reverse nucleotide sequence 5′-TAGAAGGCACAGTCGAGG-3′). The MT *TGM6* constructs were amplified from the WT *TGM6*. The products were introduced into a pcDNA3.1-EGFP-3 × Flag vector via the 5′-EcoRI and 3′-BamHI restriction sites. Hence, the cells transfected by the recombinant plasmids expressed EDFP-TG6-3 × Flag fusion proteins. All constructs were verified by Sanger sequencing.

2.4 Cell culture and transfection

A53T-SH-SY5Y cells overexpressing α-synuclein have been constructed before by stably transfecting SH-SY5Y cells with human *SNCA* A53T cDNA in our laboratory. A53T-SH-SY5Y cells and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C in a humidified incubator of 5% CO_2. Mycoplasma testing was done for the cells. When greater than 80% confluent, cells were transiently transfected with the plasmids of empty vector, WT *TGM6,* or MT *TGM6* using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol [DNA (μg): Lipofectamine 2000 (μL) = 1 : 1.5].

2.5 Protein extraction and western blotting

A53T-SH-SY5Y cells in 6-well plates were harvested after transfection for 48 hours (h). The cells were washed with PBS and lysed with RIPA lysis buffer (Beyotime) containing 1% protease inhibitor cocktail (Thermo), 1% phosphatase inhibitor (Thermo) and 1% EDTA (Thermo) for each well. The cell lysates were incubated on ice for 5 minutes (min), sonicated, incubated on the ice again for 20 min and centrifuged at 14000 rpm for 15 min at 4 °C. Supernatants were collected to measure the protein concentration using the BCA protein assay kit (Beyotime) and diluted to keep the same concentration among different groups. Protein aliquots were added with SDS-PAGE loading buffer (Beyotime) and boiled at 100 °C for 10 min.

Aliquots of 20 μg protein were electrophoresed through a 12% SDS gel and transferred onto a PVDF membrane (Merck Millipore). The transferred membrane was blocked in TBST with 5% non-fat milk for 1.5 h at room temperature (RT) and incubated with primary antibodies overnight at 4 °C. The next day, after washing three times, the membrane was incubated for 1 h with

the secondary antibody at RT. After washing, the membrane was incubated with the ECL reagent (Merck Millipore). The signal was recorded and analyzed by the Bio-Rad imaging system. With GAPDH as control, the relative protein expression was calculated after normalization.

The primary antibodies used in western blotting included mouse monoclonal anti-flag antibody (Abmart), rabbit monoclonal anti-alpha-synuclein antibody (Abcam), mouse monoclonal anti-LAMP2 antibody (Abcam) and rabbit monoclonal anti-LC3B antibody (Abcam). The secondary antibodies were HRP-conjugated horse anti-mouse IgG (CST) and HRP-conjugated goat anti-rabbit IgG (CST). All of the antibodies were diluted in line with the manufacturer's instructions.

If the results of western blot reveal an increase in LC3B-II, it is essential to differentiate whether it was caused by an increased flux or a block in the pathway. As such, cells were treated with the autophagy inhibitor bafilomycin A1 (50 nM, MCE) for 6 h at the end of the transfection, with DMSO treated as control.

2.6 Transglutaminase assay

After transfection for 48 h, HEK293 cells overexpressing WT or MT TG6 were washed twice with PBS and added ddH_2O containing 10 mM DTT and 1 mM EDTA. Cells were harvested, lysed through repeated freezing and thawing six times, and centrifuged at 12000 g for 5 min at 4 °C. Supernatants were collected to estimate the protein concentration by the BCA protein assay kit (Beyotime) and diluted to keep the same concentration among different groups. Aliquots of 10 μg protein were used for TG assay using a TG assay kit (Sigma) according to the manufacturer's protocol. Absorbance at 450 nm was measured using a microplate reader (Thermo).

2.7 Immunofluorescence

Before transfection, A53T-SH-SY5Y cells were grown on coverslips pre-coated with poly-D-lysine in 24-well plates for 24 h. The transfection was performed as described above. After 48 h, the cells were fixed with prechilled 4% paraformaldehyde (500 μl/well) for 10 min at RT. Following three washes with PBS, the cells were incubated in PBS supplemented with 0.2% Triton X-100, 2% BSA and the primary antibody (rabbit monoclonal anti-alpha-synuclein filament antibody, 1: 2000, Abcam) overnight at 4 °C. After washing three times with PBS, the cells were incubated with the secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG, 1: 2000, Life Technologies) for 1 h at RT in darkness. Then, after being washed three times, the coverslips were mounted with DAPI Fluoromount-G (SouthernBiotech). Images were acquired with the epifluorescence microscope (Olympus BX60). Fluorescence levels were quantified from three randomly selected images for each group and analyzed through ImageJ software (Schneider et al., 2012). With an outline drawn around each cell, the integrated density of selected cells was acquired and adjusted for the background. The mean intensity of selected cells, equal to the ratio of integrated density adjusted for background and area of the selected cell, was calculated.

2.8 Cell viability assay and cytotoxicity assay

A53T-SH-SY5Y cells were plated in 96-well plates (1000 cells/well). 24 h later, cells were transfected with plasmids according to the protocol of Lipofectamine 2000. After 48 h of transfection, effects of WT and MT *TGM6* on cell proliferation and cytotoxicity were measured using Cell Counting Kit-8 (CCK-8, Dojindo) and LDH Assay Kit-WST (Dojindo), respectively, according to the manufacturers' instructions. Optical density (OD) values were measured at 450 nm in the CCK-8 test and 490 nm in the LDH assay. Relative cell viability was calculated according to the following equation: relative cell viability = (OD of the experimental group OD of the blank group)/(OD of the mock group OD of the blank group). Relative cytotoxicity was calculated in the same way.

2.9 Statistical analysis

Data were given as mean ± standard deviation (SD). Statistical analysis of clinical information was done using Chi-square test for categorical variables, unpaired Student's t-test for parametric continuous variables, and Mann-Whitney test for nonparametric continuous variables. All experiments were repeated at least three times. One-way ANOVA analyzed experimental data. Post-hoc tests included the Tukey test for the comparison among all groups or the Dunnett method for the comparison of specific groups. Statistical analysis was performed using Graphpad Prism. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Genetic findings in PD patients

Genetic tests were conducted in 308 PD patients. In 56 of these patients, pathogenic variants accounting for the disease were found. This included 43 patients with homozygous or compound heterozygous variants in *PARK2*, four patients with *PLA2G6* homozygotes or compound heterozygotes, three patients with homozygous variants in *PINK1*, two patients with *LRRK2* variants, two patients with *SNCA* variants, one patient with homozygous variants in *DJ-1,* and one patient with a *VPS35* mutation. Besides these pathogenic variants, risk factors comprising *GBA* variants and *PARK2* heterozygotes were found in 29 and 13 patients, respectively. The remaining 210 patients were defined as patients with negative genetic findings. In screening all patients, 12 *TGM6* variants of low frequency were found in 15 cases. Among these cases, 14 patients belonged to patients with negative findings, and 1 patient, who carried *TGM6* c.7 + 1G > T and compound heterozygous deletion of *PARK2*, belonged to patients with pathogenic variants. Since PARK2 may significantly influence the characteristic of this patient, this patient was not included in the analysis of *TGM6* carriers. Notably, two *TGM6* variants, c.331C > T (p.R111C) and c.1550T > G (p.L517W), were found in patients characterized by progressive ataxia and were found to affect the function of the protein TG6, such as the transglutaminase activity. The locations of the variants were labeled on the structure of *TGM6* (Fig. 1).

3.2 Clinical features of the probands carrying TGM6 variants

Among the 15 TGM6 carriers we found (14 probands and one brother of the proband in Family A), eight patients had a family history (Fig. 2 Family A-G), with the other seven patients being early-onset without a family history (numbered as EOPD 1-7). All of these TGM6 cases were summarized (Fig. 2 and Table 1). The redundancy of sequencing coverage for these patients is provided in Suppl. 2.

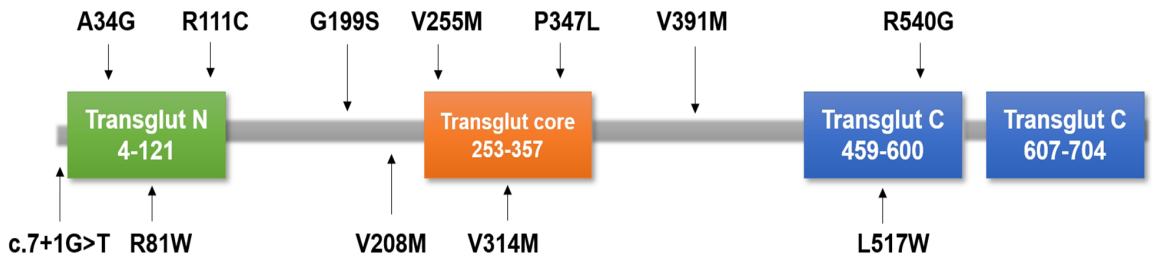

Figure 1. Schematic representation of the TGM6 protein, showing the position of the mutations found in PD patients.

3.2.1 *Family A and Family B*

The proband in Family A (AII: 6, Fig. 2) was a 64-year-old male who developed resting tremor, rigidity, and bradykinesia at the age of 56, responding well to dopaminergic therapies (pramipexole 0.375 mg/d). No signs of ataxia were found in this patient. His non-motor symptoms included olfactory dysfunction, constipation, and rapid-eye-movement sleep behavior disorder (RBD). His cognition was unimpaired, with an MMSE score of 28. The brother of the proband (AII: 5), a 66-year-old male, developed parkinsonism at 64 years with olfactory impairment, RBD, and depression, but good cognitive performance. The proband accepted the genetic test. The targeted sequencing achieved an average read depth of 329.22 × and a 96.89% breadth of coverage of the target region at a minimum depth of 10 × (Suppl. 2). No pathogenic mutations were detected other than *TGM6* c.331C > T (p.R111C). The variant was confirmed in the brother by Sanger sequencing.

The proband in Family B (BIII: 10, Fig. 2) was a 68-year-old male. At the age of 66, he presented with resting tremor, followed one year later by bradykinesia and instability, with a positive response to dopaminergic therapies (carbidopa-levodopa 50/200 mg/d, pramipexole 0.25 mg/d and trihexyphenidyl 2 mg/d), and some non-motor symptoms of constipation and depression. His cognition was normal in the course of the disease, with an MMSE score of 29. There were no signs of ataxia through physical examinations. Within his family, both his cousin (III-3) and niece (IV-2) had been diagnosed with PD according to the United Kingdom PD Society Brain Bank criteria. The genetic result of the proband showed a heterozygous mutation *TGM6* c.1550T > G (p.L517W). The mean depth of target sequencing was 278.71 x and the coverage of the target region was 99.74%. The percentage of the target region with mean depth > 10 x was 97.49%.

For both probands in Family A and Family B, dopamine transporter (DAT) imaging with [^{11}C]-2β-carbomethoxy-3β-(4-fluorophenyl) tropane (CFT) and [^{18}F]-fluorodeoxyglucos (FDG) positron emission tomography (PET) was performed. There was a marked reduction of DAT uptake in both putamen and caudate nuclei bilaterally for each proband. The uptake ratios for [^{11}C]-CFT are displayed in Table 2. The FDG PET of the proband in Family A displayed increased metabolism in the globus pallidus, thalamus, pontine, and cerebellum. The FDG PET of the proband in Family B showed increased metabolism in the globus pallidus, putamen, and thalamus.

3.2.2 *Other families*

TGM6 variants were also found in other families (Fig. 2 Family C-G). Because the probands refused to consent to genetic tests in family members, we report the genetic results of the probands only.

Among the 15 PD patients with *TGM6* variants, 10 cases (66.7%) were male. The mean ± SD of age at onset was 48.3 ± 13.9. The primary manifestation of disease in these patients was typical parkinsonism with slow progress. They exhibited a positive response to dopaminergic therapies (initial levodopa equivalent daily dosage ranging from 37.5 mg/d to 400 mg/d) and had several non-motor symptoms. Their PET results agreed with PD. Clinical information was compared between the 15 *TGM6* carriers and the 238 patients without pathogenic mutations (Table 3). No differences were found in gender, age, age at onset, disease duration, or non-motor symptoms between groups. However, UPDRS motor scores of *TGM6* carriers were significantly lower than those of patients without pathogenic variants, suggesting that patients with PD and *TGM6* variants may present with milder symptoms and progress slower.

3.3 *Expression, distribution, and transglutaminase activity of TGM6 WT and MT*

Among the *TGM6* variants of low frequency found in our PD patients, *TGM6* p.R111C and p.L517W were appraised as likely pathogenic, based on the standards and guidelines for the interpretation of sequence variants (Table 4) (Richards et al., 2015). Other *TGM6* variants were classified as of uncertain significance. *TGM6* p.P359L was previously reported in an early-onset PD pedigree by Westenberger et al. (2016). Therefore, the functional differences between *TGM6* WT, p.R111C, p.P359L, and p.L517W formed the focus of our further research. To investigate whether *TGM6* variants affect the amount of TG6 protein, plasmids of empty vector, *TGM6* WT, R111C, P359L, and L517W were respectively transfected into A53T-SH-SY5Y cells. Because the transfected cells expressing *TGM6*-3 × flag fusion protein, anti-flag antibodies were used in western blots to reflect the expression level of *TGM6*. After 48 h transfection, western blot analyses revealed that there was no significant difference among the level of the WT and three MT TG6 (Fig. 3A).

Since TG6 was EGFP-3 × flag-tagged, we could confirm that the transfection efficiency was similar among different groups through observation by fluorescence. In contrast to the mock group, whose EGFP distributed homogeneously in the nucleus and cytoplasm, cells expressing WT and three MT TG6 all displayed a predominantly cytoplasmic localization of TG6 (Fig. 3B).

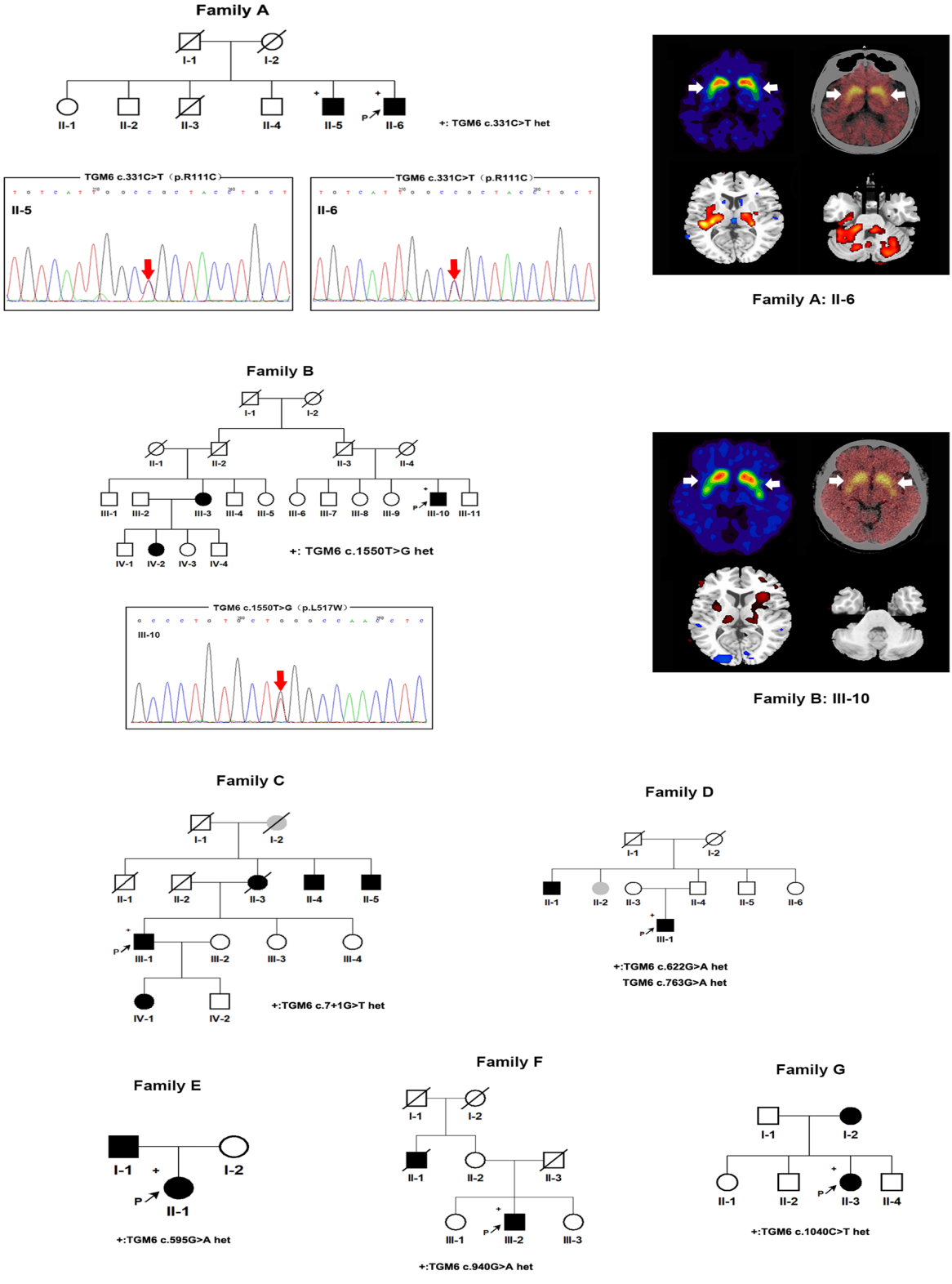

Figure 2. PD pedigrees with TGM6 mutations. Family A and B were displayed with the pedigree, the results of Sanger sequencing, and PET imaging. There was a marked reduction of DAT uptake in both bilateral putamen and caudate nuclei for each proband (white arrow). Their FDG PET displayed a PD-related pattern with increased metabolism in the putamen, globus pallidus, thalamus, pontine, and cerebellum (red: increased metabolism; blue: decreased metabolism). Family C-G was shown with the pedigree. Black-filled icons indicate PD patients. Grey-filled icons denote possible PD patients. P, proband; het, heterozygous.

Table 1. Summary of the manifestations of TGM6 carriers.

ID	Mutation		Het/ hom	Gender	Age	AAO	Initial symptoms	Main manifestations	Other symptoms	BDI	SS-12	MMSE	ESS	UPDRS-III	H&Y	PET
Family A II-5	c.331C > T	p.R111C	het	Male	66	64	Bradykinesia	Tremor + bradykinesia	Olfactory disorder + RBD + depression	12	3	29	14	20	2	NR
Family A II-6	c.331C > T	p.R111C	het	Male	64	56	Tremor + bradykinesia + rigidity	Tremor + bradykinesia + rigidity	Olfactory disorder + constipation + RBD + sexual impotence + sleep benefit	0	6	28	10	31	2	PD
Family B III-10	c.1550T > G	p.L517W	het	Male	67	66	tremor	Tremor + bradykinesia + Instability	Constipation + depression + sleep benefit	6	4	29	1	15	2	PD
Family C III-1	c.7+1G > T	splicing	het	Male	63	63	Tremor + bradykinesia + rigidity	Tremor + bradykinesia + rigidity	Constipation + RBD	0	6	28	10	31	2	NR
Family D III-1	c.622G > A	p.V208M	het	Male	32	32	Bradykinesia	Bradykinesia + rigidity	Sleep benefit	2	9	30	0	11	2	NR
	c.763G > A	p.V255M	het													
Family E II-1	c.595G > A	p.G199S	het	Female	66	59	Bradykinesia	Tremor + bradykinesia + rigidity + instability	Constipation + RBD + fall	23	8	25	11	45	4	PD
Family F III-2	c.940G > A	p.V314M	het	Male	65	56	Tremor	Tremor + bradykinesia + rigidity	Olfactory disorder + constipation + RBD + depression	16	3	NR	12	17	2	NR
Family G II-3	c.1040C > T	p.P347L	het	Female	59	54	Bradykinesia	Tremor + bradykinesia	Constipation + motor fluctuations	11	5	24	12	28	3	NR

Table 1. Continued.

ID	Mutation		Het/ hom	Gender	Age	AAO	Initial symptoms	Main manifestations	Other symptoms	BDI	SS-12	MMSE	ESS	UPDRS-III	H&Y	PET
EOPD 1	c.100A > G	p.A34G	het	Female	44	36	Tremor	Tremor + bradykinesia + rigidity + instability	Olfactory disorder + constipation + depression + fall + motor fluctuations + sleep benefit	16	6	28	5	20	3	NR
EOPD 2	c.241C > T	p.R81W	het	Male	49	47	Tremor	Tremor + bradykinesia + rigidity + instability	Olfactory disorder + constipation + depression + sexual impotence + orthostatic hypotension + sleep benefit	NR	NR	NR	NR	38 (on)	3	NR
EOPD 3	c.C1618G	p.R540G	het	Female	51	45	Tremor	Tremor + bradykinesia + rigidity	Fall + dementia + dyskinesia	4	NA	11	3	28 (on)	2	NR
EOPD 4	c.595G > A	p.G199S	het	Male	48	36	Tremor	Tremor + bradykinesia + rigidity + instability	Olfactory disorder + constipation + RBD	12	NA	30	8	24	2	NR
EOPD 5	c.1171G > A	p.V391M	het	Male	17	16	Bradykinesia	Tremor + bradykinesia	No	NR	NR	NR	NR	27	3	NR
EOPD 6	c.595G > A	p.G199S	hom	Female	53	50	Bradykinesia	Tremor + bradykinesia + rigidity	No	13	4	25	0	17	1	NR
EOPD 7	c.1171G > A	p.V391M	het	Male	47	44	Bradykinesia + rigidity	Tremor + bradykinesia + rigidity	Olfactory disorder + depression + Frozen gait + motor fluctuations + sleep benefit	NR	NR	NR	NR	39 (on)	2	NR

Table 2. Uptake ratios of DAT in [^{11}C]-CFT PET.

Subject	Uptake ratio					
	Caudate nucleus		Putamen (anterior)		Putamen (posterior)	
	Left	Right	Left	Right	Left	Right
Family A: II-6	1.442	1.507	1.502	1.509	0.878	0.868
Family B: III-10	1.63	1.608	1.693	1.613	0.965	0.992
Range of normal values	2.51-3.41		3.22-4.26		3.11-4.05	

To assess the enzymatic properties of *TGM6* variants, the TG assay was performed *in vitro*. Setting the result of the mock group as the background value, the OD values detected were used to represent the activity of TG6. Compared to WT, *TGM6* R111C, P359L, and L517W all displayed remarkably lower transglutaminase activity (Fig. 3C).

3.4 Effect of wild-type and mutant-type TG6 on cell viability

To explore the effect of WT and MT TG6 on cell viability, CCK8 and LDH assays were used to detect cell proliferation and cytotoxicity, respectively. Our results showed that, compared to *TGM6* WT, *TGM6* R111C and L517W reduced the cell proliferation in A53T-SH-SY5Y cells (Fig. 4A). Meanwhile, the LDH level in the *TGM6* WT group was significantly lower than that in the groups of *TGM6* R111C (Fig. 4B). Also, overexpressing WT TG6 tends to reduce cell damage in contrast to the mock group ($P = 0.076$).

3.5 Influence of TG6 on α-synuclein level

The α-synuclein, is an essential protein in the pathogenesis of PD, was reported to be one of the substrates of TGs. Therefore, we explored the influence of *TGM6* WT and MT on α-synuclein and its aggregates, using A53T-SH-SY5Y cells overexpressing α-synuclein. After the transfection of WT or MT *TGM6* plasmids for 48 h, western blot was conducted to detect the amount of α-synuclein, and immunofluorescence was performed to analyze the impact of *TGM6* on α-synuclein filaments. Results revealed that the cells overexpressing WT TG6 showed a decreased amount of α-synuclein compared to the mock group (Fig. 5A). However, this effect was attenuated when cells overexpressed MT TG6 rather than WT. Through immunofluorescence, we found that there was no significant difference in fluorescence intensity of α-synuclein filaments among WT and MT groups (Fig. 5B and 5C). But the expression of α-synuclein filaments within cell nuclei tended to be higher in the group of *TGM6* MT in contrast to *TGM6* WT.

3.6 Influence of TG6 on autophagy

We attempted to find a pathway to explain the change in α-synuclein and cell viability. Autophagy is a major protein degradation pathway to maintain cellular homeostasis. Furthermore, hyperactive autophagy may induce apoptosis. We investigated the key proteins in this pathway, including LC3B, whose conversion from LC3B-I to LC3B-II is essential for autophagosome formation; and P62, a receptor of autophagy; and LAMP2, a lysosome-associated membrane protein. Compared to the mock group, the WT group showed markedly higher levels of LC3B-II/I and LAMP2 and tended to have fewer P62 (Fig. 6). Cells were also treated with the autophagy inhibitor bafilomycin A1. The amount of LC3B-II was significantly higher in the presence of the inhibitor, signifying the occurrence of flux (Fig. 6A). There was no significant difference between the WT group and the variant groups in the LC3B-II/I ratio after bafilomycin A1 treatment. The results of western blots revealed elevated autophagy efficiency in the group of *TGM6* WT. In the MT *TGM6*, all of these trends were reduced.

4. Discussion

TGM6 is the causative gene of SCA35. Yet, we found *TGM6* variants of low frequency in PD patients, including two variants reported to affect protein function. Interestingly, all of these patients were characterized by typical parkinsonism and did not present with any signs of ataxia, which made us speculate if *TGM6* played a role in the pathogenesis of PD. We further investigated the function of WT *TGM6* and the MT *TGM6*, p.R111C, p.P359L, and p.L517W. The results showed that overexpression of WT *TGM6* could protect cells by enhancing autophagy and decreasing the α-synuclein level. But when *TGM6* was mutated, its TG activity decreased significantly and its protective effect was lost. As such, it is conceivable that in cells overexpressing MT *TGM6*, α-synuclein accumulation caused by the attenuated autophagy may partially account for PD pathogenesis.

In our research, PD patients with *TGM6* variants presented with typical parkinsonism without ataxia. Compared to PD patients with negative genetic findings, these patients exhibited slower and milder disease progression. PET imaging offered supportive evidence for the clinical diagnosis of PD (Matthews et al., 2018; Wu et al., 2013). Given these findings, we supposed that there might be genetic heterogeneity in *TGM6*, resulting in two different diseases. However, there are a few reports of a pure parkinsonian phenotype in molecularly confirmed SCA patients, especially in patients with SCA2 and SCA3. The previous study describes a parkinsonism-predominant SCA2 family with six affected individuals showing typical parkinsonism and two showing cerebellar ataxia (Sun et al., 2011).

Moreover, PET imaging conducted in an SCA2 family showed results similar to that found in idiopathic PD (Furtado et al., 2004). Some SCA3 patients have been found to initially present with a phenotype indistinguishable from PD, as well as responding positively to levodopa (Bettencourt et al., 2011; Buhmann et al., 2003). However, cerebellar signs have been reported to manifest in some of these patients after several years of disease progression (Lu et al., 2004). As yet, there are no reports of SCA35 patients with pure parkinsonism. The patients we reported will be followed up to confirm their diagnoses.

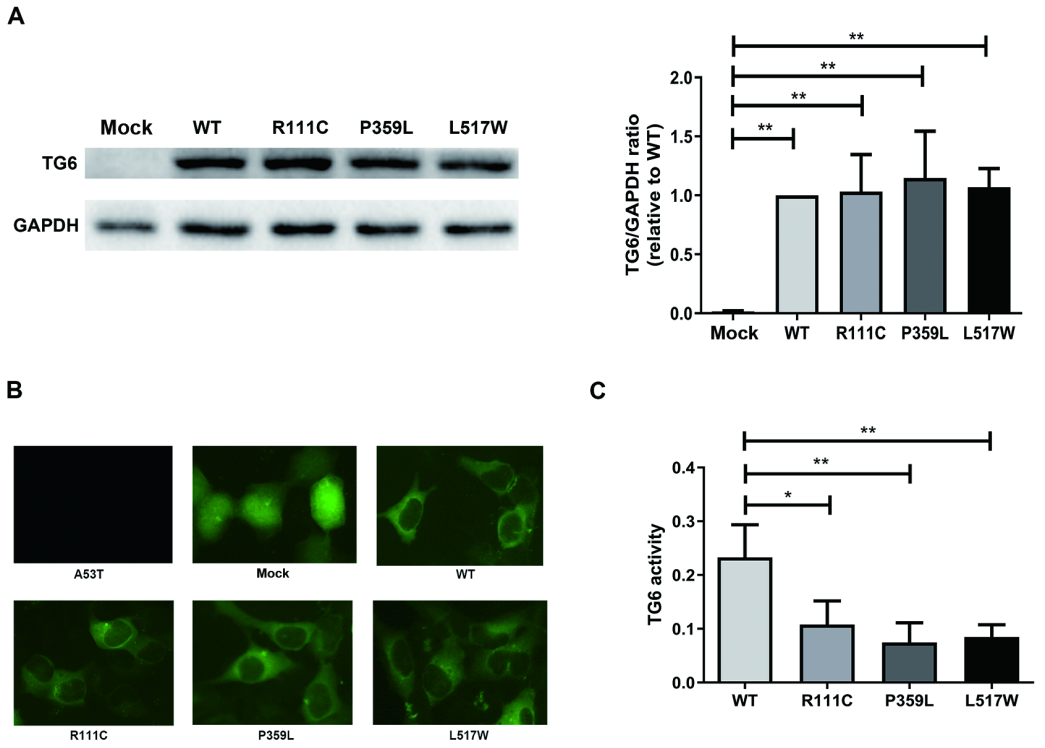

Figure 3. Expression, distribution, and enzymic activity of TGM6 WT and MT. (A) Western blots demonstrating no difference of TG6 levels among TGM6 WT, R111C, P359L and L517W by the Tukey method. (B) Immunofluorescence displaying a predominant cytoplasmic localization of WT and MT TG6. (C) TG assay showing decreased enzymic properties of TGM6 MT by the Dunnett method. Data were represented as mean ± SD (n = 3 per group). *, P <0.05; **, P <0.01.

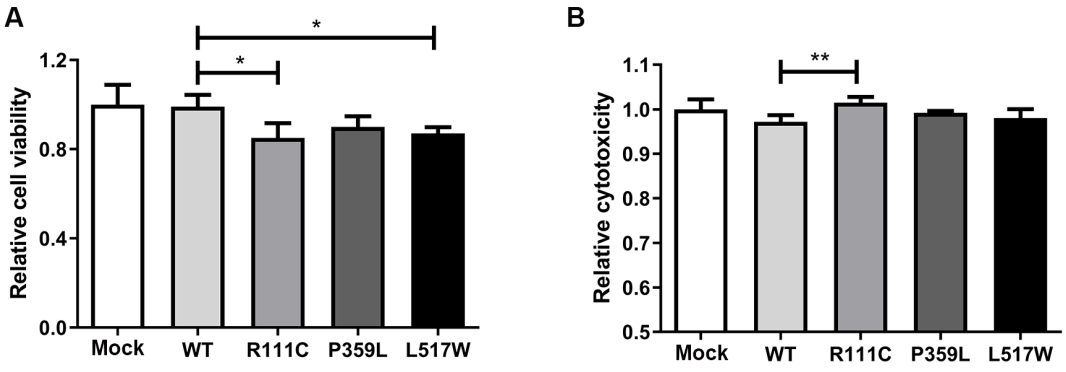

Figure 4. Effect of WT and MT TG6 on cell viability. (A) CCK8 assay to detect cell proliferation. (B) LDH assay to detect cell cytotoxicity. According to two assay results, overexpressing TGM6 R111C and L517W reduced cell viability in contrast with TGM6 WT. Data are represented as mean ± SD (n = 3 per group) and analyzed by the Dunnett method. *, P <0.05; **, P <0.01.

Table 3. Comparison of clinical features of *TGM6* carriers and PD patients without pathogenic mutations.

	TGM6 carriers	Patients without pathogenic mutations	*P*-value
N	15	238	
Gender (Male)[a]	10 (66.7%)	143 (60.1%)	0.787
Age[b]	52.7 ± 14.2	50.2 ± 11.6	0.389
Age at onset[b]	48.3 ± 13.9	45.6 ± 11.3	0.409
Disease duration[c]	5.5 ± 3.7	5.5 ± 4.0	0.984
Family history[a]	8 (53.3%)	115 (48.3%)	0.793
H&Y[c]	2.3 ± 0.7	2.1 ± 0.8	0.376
UPDRS-III[c]	23.3 ± 9.1	30.7 ± 13.9	0.042
BDI[c]	9.8 ± 7.0	13.1 ± 9.3	0.291
SS-12[c]	5.3 ± 2.0	5.3 ± 2.4	0.725
ESS[c]	6.6 ± 5.3	6.2 ± 4.4	0.744
MMSE[c]	26.0 ± 5.2	27.5 ± 2.9	0.342

a: Chi-square test. b: Student's t-test. c: Mann-Whitney test.

Table 4. *TGM6* Mutations of low frequency in PD patients.

Mutation	Carriers	SNP	1000 g	ExAC	SIFT	PolyPhen-2	MutationTaster	HGMD	ClinVar	ACMG
c.7+1G > T splicing	1	rs772567551	-	0.0002	NA	NA	Disease_causing	NR	NR	PVS1 + PM2 + PP3 + BP2 = VUS
c.100A > G p.A34G	1	rs566673079	-	-	Damaging	Probably_damaging	Disease_causing	NR	NR	PM2 + PP3 = VUS
c.241C > T p.R81W	1	NA	-	4.17E-05	Tolerated	Probably_Damaging	Polymorphism	NR	NR	PM2 = VUS
c.331C > T p.R111C	2	rs372250159	-	0.0000082	Damaging	Probably_damaging	Disease_causing	NR	SCA: pathogenic	PS3 + PM2 + PP3 = likely pathogenic
c.595G > A p.G199S	3	rs182249285	0.000199681	0.0000659	Tolerated	Benign	Polymorphism	NR	NR	PM2 + BP4 = VUS
c.622G > A p.V208M	1	rs61743614	0.0002	0.0001	Damaging	Probably_damaging	Polymorphism	NR	NR	PM2 = VUS
c.763G > A p.V255M	1	rs751618387	-	0	Damaging	Probably_damaging	Polymorphism	NR	NR	PM2 = VUS
c.940G > A p.V314M	1	rs202184911	0.000998403	0.0003	Damaging	Probably_damaging	Polymorphism	NR	SCA:likely benign	PM2 = VUS
c.1040C > T p.P347L	1	rs183670042	0.00239617	0.0004	Damaging	Possibly_damaging	Disease_causing	NR	SCA:likely benign	PM2 + PP3 = VUS
c.1171G > A p.V391M	2	rs116904482	0.00199681	0.0009	Damaging	Probably_damaging	Disease_causing	NR	SCA:likely benign	PM2 + PP3 = VUS
c.1550T > G p.L517W	1	rs387907097	0.0002	0.0001	Damaging	Probably_damaging	Disease_causing	SCA	SCA:pathogenic	PS3 + PM2 + PP3 = likely pathogenic
c.1618C > G p.R540G	1	NA	-	4.40E-05	Tolerated	Benign	Disease_causing	NR	NR	PM2 = VUS

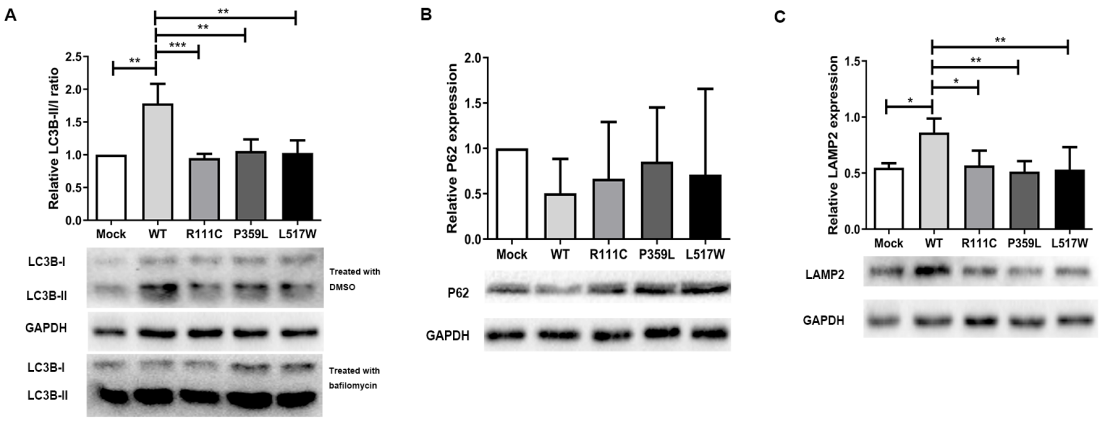

Figure 5. Impact of TGM6 on α-synuclein. (A) Impact of TGM6 on α-synuclein expression detected by immunoblotting. Cells overexpressing WT TG6 showed decreased α-synuclein compared to the mock group. This effect was attenuated in the groups of MT TG6. (B-C) Impact of TGM6 on α-synuclein filaments detected by immunofluorescence. TG6 was EGFP-3 × flag-tagged, shown in green. A53T-SH-SY5Y cells were stained for α-synuclein filament (red). Nuclei were stained with DAPI (blue). There was no difference in fluorescence intensity of α-synuclein filaments between the WT group and each other group. However, α-synuclein filament levels tended to be higher within nuclei in the TGM6 MT group compared to TGM6 WT. Data were analyzed using one-way ANOVA and post hoc Dunnett test (n = 3 per group).

Among the *TGM6* variants in our PD patients, *TGM6* p.R111C and *TGM6* p.L517W have been reported to affect function in SCA35. *TGM6* p.P359L has been published in an early-onset PD pedigree by Westenberger et al. (2016). Therefore, we chose *TGM6* WT, p.R111C, p.P359L, and p.L517W to explore the relationship between *TGM6* and PD.

We found that the variants of *TGM6* did not affect the distribution and expression level of TG6, but resulted in the reduction of transglutaminase activity. Several studies have revealed that the expression levels of TG6 MT were similar to that of TG6 WT (Guan et al., 2013; Guo et al., 2014), which was confirmed in our research. But reports vary concerning TG6 distribution. Guan et al. (2013) reported that TG6 WT was localized in the cytoplasm and that neither p.D327G nor p.L517W changed the subcellular localization of TG6 in HEK293 cells. In contrast, Tripathy et al. (2017) described TG6 WT localized mainly in the nucleus, but also in the cytoplasm, with different variants showing different patterns of distribution. For example, TG6 p.D327G was distributed similarly to TG6 WT, while TG6 p.R111C and TG6 p.L517W localized almost exclusively in the cytosolic space in COS-7 cells. Our results in A53T-SH-SY5Y cells were similar to those of Guan et al. (2013). *TGM6* WT and three MT were all distributed in the cytoplasm, and different variants did not alter the localization of TG6.

TGM6 variants have previously been reported to yield decreased TG activity (Guan et al., 2013; Guo et al., 2014). We further confirmed the enzymic activity of two reported variants p.R111C and p.L517W and explored whether the newly identified *TGM6* mutant, P359L, showed defects in transamidase activity. Using the transglutaminase assay kit, the enzymic properties of TG6 p.R111C, p.P359L, and p.L517W were remarkably reduced compared to TG6 WT.

The altered transamidase activity of TG6 may result in changes in its substrate α-synuclein, as well as in cell function. Our research revealed that, compared with the mock group, overexpressing WT TG6 can prominently reduce the level of α-synuclein. This suggests a protective effect of WT TG6 in PD. For MT TG6, this protection is weakened to varying degrees (Fig. 4). Nevertheless, visualized by immunofluorescence, the α-synuclein filament showed no difference among groups. However, we hypothesize that this may be due to the anti-α-synuclein filament antibody recognizing different kinds of aggregated α-synuclein, including both toxic and non-toxic forms, as such lacking the sensitivity to reflect changes in toxic components. It may also be pertinent that the expression of α-synuclein filaments tended to be higher in the nucleus within the *TGM6* MT group compared to *TGM6* WT. Previous studies have suggested that increased nuclear α-synuclein may reduce autophagy activity (Decressac et al., 2013), which can lead to further accumulation of α-synuclein and toxicity.

Macroautophagy (henceforth called autophagy) is crucial for the degradation of misfolded α-synuclein. In the process of autophagy, proLC3 should be cleaved to form LC3-I, followed by conjugation to phosphatidylethanolamine to generate LC3-II, to function at the stage of phagophore elongation. LAMP2 is one of the structural proteins of the lysosome. SQSTM1/P62 functions as receptors or autophagosome substrates (Feng et al., 2014). By western blot analysis, we found that in the group of *TGM6* WT, the expression level of LAMP2 and LC3B-II/I ratio was elevated significantly compared to the mock group. And the P62 level in the group of *TGM6* WT tended to decrease. This suggested increased autophagy efficiency in the *TGM6* WT group, which was confirmed by the treatment of the autophagy inhibitor. Therefore, WT TG6 may reduce α-synuclein through enhanced autophagy of abnormal α-synuclein. However, MT TG6 partially depletes this function, leading to increased apoptosis on account of the reduced autophagic activity and accumulated α-synuclein.

Although we found several rare variants of *TGM6* in PD patients and conducted functional research about the possible mechanism, our present research has several limitations. First, cosegre-

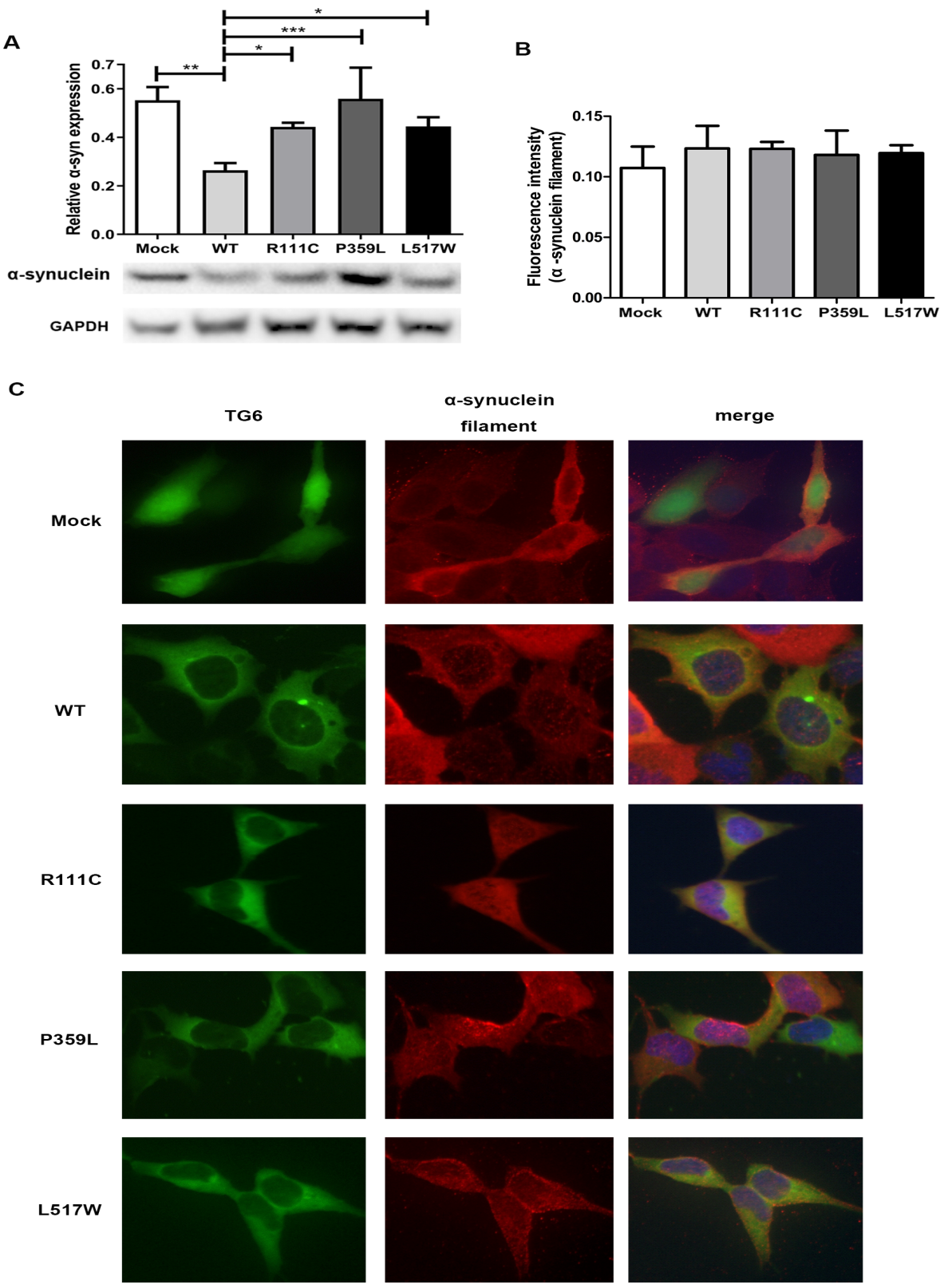

Figure 6. Expression levels of autophagy-related proteins. Compared to the mock group, the WT group showed markedly higher levels of LC3B-II/I and LAMP2, as well as tending to have less P62. Results from cells treated with bafilomycin A1 demonstrated that an enhanced autophagy flux caused the increase of LC3B-II/LC3B-I in the TGM6 WT group. Data are represented as mean ± SD (n = 3 per group) and analyzed by the Dunnett method. *, P <0.05; **, P <0.01; ***, P <0.001.

gation with the disease in most pedigrees was not confirmed because of the subjects' refusals to permit genetic testing of family members. Second, our experimental results were based on a gene overexpression system that differs from the physiological environment. Therefore, further research should be conducted through methods such as site-directed mutagenesis in vivo. Third, it remained unsolved as to why the same variant led to two different diseases, which is an aspect worth exploring. Fourth, it would have been instructive to monitor autophagy in the analysis of flux with the use of transmission electron microscopy (TEM) and fluorescence microscopy combined with GFP-LC3 reporter constructs.

5. Conclusions

In summary, we found *TGM6* variants of low frequency in PD patients, among which two have been reported to affect functioning in SCA cases. The clinical characteristics of *TGM6*-carrying PD patients presented with typical parkinsonism but progressed slower. Functional results suggested that overexpressing WT TG6 could protect cells by decreasing α-synuclein and enhancing autophagy and that this protective effect was attenuated in MT TG6. Moreover, with the decreased TG activity, *TGM6* variants seemed to be associated with α-synuclein accumulation, which helps to clarify the correlation between *TGM6* variants and PD.

Abbreviations

AAO, age at onset; BDI, Beck Depression Inventory; EOPD, Early-onset Parkinson's disease; ESS, Epworth Sleeping Scale; H&Y, Hoehn and Yahr Staging; LOPD, Late-onset Parkinson's disease; MMSE, Mini-Mental State Examination; NR, Not Recorded; RBD, Rapid-eye-movement Sleep Behavior Disorder; RBDSQ, Rapid-eye-movement Sleep Behavior Disorder Screen Questionaire; SS-12, Sniffin' Sticks Screening 12 Test; UPDRS-III, Unified Parkinson's Disease Rating Scale-Part III; NA, Not Available; PD, Parkinson's disease; SCA, spinocerebellar ataxia; VUS, variants of uncertain significance; DAT, dopamine transporter; [^{11}C]-CFT, [^{11}C]-2β-carbomethoxy-3β-(4-fluorophenyl) tropane; PET, positron emission computed tomography.

Authors' contributions

KC and YXZ designed the study. KC, FP and HLY collected the data, JYW and YT analyzed the data, KC and YL analyzed the results and drafted the manuscript.

Ethics approval and consent to participate

All authors hereby declare that this study involving human participants has been approved by the ethics committee of Huashan Hospital. Informed consent was obtained from all individual participants included in the study.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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Supplemental Materials

Suppl. 1. Gene list in the PD panel performed by target sequencing of the exons.

ADCY5	*CHMP2B*	*FUS*	*PARK2*	*SPR*
ADH1C	*CIZ1*	*FXN*	*PARK7*	*SPTBN2*
AFG3L2	*COASY*	*GBA*	*PDYN*	*STX1B*
ANO10	*COQ2*	*GCH1*	*PINK1*	*SYNJ1*
ANO3	*CP*	*GIGYF2*	*PLA2G6*	*TAF1*
APTX	*DCTN1*	*GNAL*	*PNKD*	*TARDBP*
ATM	*DDRGK1*	*GRID2*	*POLG*	*TBP*
ATP13A2	*DNAJC13*	*GRN*	*PPP2R2B*	*TENM4*
ATP1A3	*DNAJC6*	*HTRA2*	*PRKCG*	*TGM6*
ATP6AP2	*DRD2*	*HTT*	*PRKRA*	*TH*
ATP7B	*DRD3*	*IFRD1*	*PRRT2*	*THAP1*
ATXN1	*EEF2*	*IL1B*	*PSEN1*	*TMEM240*
ATXN10	*EIF4G1*	*INPP5F*	*RAB29*	*TOR1A*
ATXN2	*ELOVL4*	*ITPR1*	*RAB39B*	*TTBK2*
ATXN3	*ELOVL5*	*KCND3*	*SETX*	*TUBB4A*
ATXN7	*FA2H*	*LRRK2*	*SGCE*	*UCHL1*
C9orf72	*FBXO7*	*MAPT*	*SIPA1L2*	*VPS13A*
CACNA1A	*FGF14*	*MCCC1*	*SLC2A1*	*VPS13C*
CCDC88C	*FMR1*	*NOP56*	*SLC6A3*	*VPS35*
CHCHD2	*FTL*	*PANK2*	*SNCA*	*WDR45*

Suppl. 2. Redundancy of sequencing coverage for PD patients with *TGM6* variants.

Patients	Average sequencing depth on target	Coverage of target region	Fraction of target covered with at least 10 ×	Fraction of target covered with at least 20 ×
Family A II-6	329.22	99.50%	96.89%	94.36%
Family B III-10	278.71	99.74%	97.49%	94.89%
Family C III-1	378.66	99.21%	95.05%	90.46%
Family D III-1	196.34	99.49%	95.87%	92.12%
Family E II-1	361.06	99.54%	97.70%	95.80%
Family F III-2	448.29	99.42%	96.83%	94.08%
Family G II-3	414.68	99.59%	97.80%	95.80%
EOPD 1	399.95	99.59%	95.73%	91.59%
EOPD 2	475.85	99.67%	99.22%	98.23%
EOPD 3	236.37	99.73%	97.41%	94.62%
EOPD 4	395.55	99.93%	99.44%	98.72%
EOPD 5	415.15	99.96%	99.40%	98.45%
EOPD 6	260.78	99.77%	98.92%	97.68%
EOPD 7	555.22	99.74%	99.37%	98.65%