Deng *et al. Ageing Neur Dis* 2023;3:9
DOI: 10.20517/and.2023.06

Ageing and Neurodegenerative Diseases

Brief Communication

Open Access

Identification of *PLA2G6* variants in a Chinese patient with Parkinson's disease

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How to cite this article: Deng X, Zheng W, Yang Y, Yang Z, Li H, Song Z, Wang J, Deng H, Yuan L. Identification of *PLA2G6* variants in a Chinese patient with Parkinson's disease. *Ageing Neur Dis* 2023;3:9. https://dx.doi.org/10.20517/and.2023.06

Received: 5 Mar 2023 **First Decision:** 10 Apr 2023 **Revised:** 18 Apr 2023 **Accepted:** 24 Apr 2023 **Published:** 31 May 2023

Academic Editor: Weidong Le **Copy Editor:** Dong-Li Li **Production Editor:** Dong-Li Li

Abstract

Parkinson's disease (PD) is a clinical syndrome and a heterogeneous group of neurodegenerative conditions with variable pathologies and clinical sub-entities, characterized by motor symptoms and non-motor features. PD represents an outcome of the combination of genes and other risk or protective factors. Patients with variants in the phospholipase A2 group VI gene (*PLA2G6*) can present complex Parkinsonian phenotypes. This study reported a PD patient with typical motor symptoms of PD, including bradykinesia, gait disturbance, rigidity, and rest tremor, who also suffered from nocturia, constipation, and sleeping problems. Two *PLA2G6* variants, c.402C>T and c.2327_2328del, were identified in the patient by whole exome sequencing followed by Sanger sequencing. The transition c.402C>T was predicted to generate an alternative acceptor splice site, though the minigene splicing assay showed negative *in vitro* outcomes. The novel variant c.2327_2328del was predicted to result in a truncated protein. These two variants may be pathogenic in PD or increase the susceptibility to PD individually or collaboratively. This discovery may enrich the genetic landscape of *PLA2G6*-associated PD and confirm the notion of prioritizing whole exome sequencing analysis in patients with PD.

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Keywords: Parkinson's disease, genetics, *PLA2G6*, variants

INTRODUCTION

Parkinson's disease (PD) is a recognizable clinical syndrome and a heterogeneous group of neurodegenerative conditions with variable pathologies and distinct clinical sub-entities in the disease spectrum[1,2]. The diagnosis of PD is based on clinical manifestations characterized by three primary motor features: bradykinesia with either rest tremor or rigidity, or both[3]. The motor symptoms of PD include bradykinesia, changes in posture and gait, dysphagia, and dysarthria[2,3]. Non-motor features of PD usually precede motor symptoms, including hyposmia, orthostatic hypotension, constipation, urinary dysfunction, cognitive/psychiatric problems, pain, and sleep disturbances[1,4]. The pathological characteristic of PD is the accumulation of α-synuclein with dopaminergic neuronal loss in the substantia nigra as well as other brain areas[2,4]. PD represents an outcome of the combination of genes and other risk or protective factors such as aging, environmental toxins (pesticide and heavy metal exposure), and behavioral factors (coffee intake, chocolate consumption, or cigarette smoking)[5,6]. Several pathophysiologic mechanisms intersecting with each other contribute to the disease pathogenesis, including α-synuclein accumulation, mitochondrial dysfunction, neuroinflammation triggered by risk factors exposure, oxidative stress, and autophagy dysfunction[5-7].

The genetic architecture of PD is extremely complicated, with at least 20 Mendelian inherited causative genes and over 100 genetic risk loci[6,8]. For monogenic forms that affect approximately 5%-10% of PD patients, 11 autosomal dominant genes (the synuclein alpha gene, *SNCA*; the ubiquitin C-terminal hydrolase L1 gene, *UCHL1*; the leucine rich repeat kinase 2 gene, *LRRK2*; the GRB10 interacting GYF protein 2 gene, *GIGYF2*; the HtrA serine peptidase 2 gene, *HTRA2*; the VPS35 retromer complex component gene, *VPS35*; the eukaryotic translation initiation factor 4 gamma 1 gene, *EIF4G1*; the transmembrane protein 230 gene, *TMEM230*; the coiled-coil-helix-coiled-coil-helix domain containing 2 gene, *CHCHD2*; the RIC3 acetylcholine receptor chaperone gene, *RIC3*; the prosaposin gene, *PSAP*) and 9 autosomal recessive genes (the parkin RBR E3 ubiquitin protein ligase gene, *PRKN*; the PTEN induced putative kinase 1 gene, *PINK1*; the Parkinsonism associated deglycase gene, *PARK7*; the ATPase 13A2 gene, *ATP13A2*; the phospholipase A2 group VI gene, *PLA2G6*; the F-box protein 7 gene, *FBXO7*; the DnaJ heat shock protein family (Hsp40) member C6 gene, *DNAJC6*; the synaptojanin 1 gene, *SYNJ1*; the vacuolar protein sorting 13 homolog C gene, *VPS13C*) have been reported[9,10]. Despite excitement about increasing monogenic PD cases defined on a molecular basis, only several genes are well-established, responsible for autosomal dominant (*SNCA*, *LRRK2*, and *VPS35*) or recessive (*PRKN*, *PINK1*, and *PARK7*) forms of the disease[10,11]. Moreover, patients with variants in *ATP13A2*, the dynactin subunit 1 gene (*DCTN1*), *DNAJC6*, *FBXO7*, *PLA2G6*, and *SYNJ1* can present with atypical or complex parkinsonian phenotypes[4,11]. The *PLA2G6* gene (OMIM 603604) was initially reported to be responsible for Parkinson's disease 14 (PARK14, OMIM 612953) in 2009[12]. Since then, over 54 different variants have been identified in *PLA2G6*-related PD, including missense and nonsense variants, in-frame deletions, splicing variants, and frameshift changes[13].

In the present study, we described a Han Chinese patient with clinical manifestations compatible with the PARK14 phenotype, in whom two *PLA2G6* variants (NM_003560.4), c.402C>T and c.2327_2328del, were detected.

METHODS

One subject (II:1, Figure 1A) with PD from Yongzhou, Hunan, China, was included in the present study. The patient was born to non-consanguineous parents, and there was no history of similar neurological signs

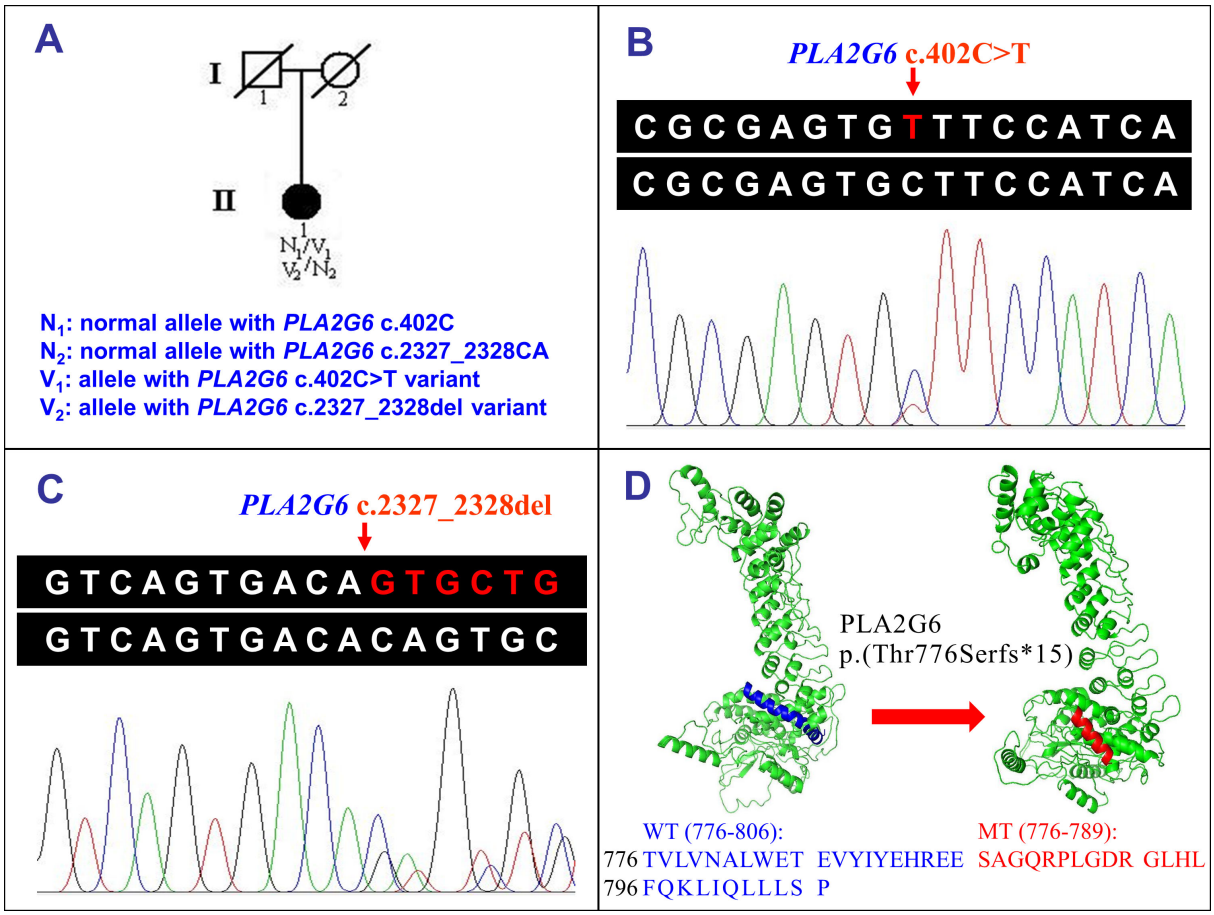

Figure 1. (A) Pedigree of a Chinese family with Parkinson's disease. A square indicates a male and circles indicate females. The fully shaded symbol indicates the affected individual and the empty symbols indicate unaffected members. Slashed symbols represent deceased members. (B, C) The sequencing for the *PLA2G6* variants, c.402C>T and c.2327_2328del, in the individual (II:1) with Parkinson's disease. (D) Cartoon models of the wild-type (left) and mutated (right) *PLA2G6* proteins shown by PyMOL. The segments of wild-type (WT) PLA2G6 protein (residues 776-806) and mutated (MT) *PLA2G6* protein caused by the variant c.2327_2328del [p.(Thr776Serfs*15], residues 776-789) are marked in the cartoon models, and the corresponding sequences are shown in the bottom. *PLA2G6*: phospholipase A2 group VI gene

in her parents (I:1 and I:2, died, Figure 1A). Neurological examination and brain magnetic resonance imaging were undertaken on the patient. Clinical data and peripheral blood sample were acquired from the patient after obtaining written informed consent for genetic analysis. This study was approved by the Institutional Review Board of the Third Xiangya Hospital, Central South University, Changsha, China, and all procedures were performed in accordance with the ethical standards of the Declaration of Helsinki.

Whole exome sequencing (WES) was performed using genomic DNA obtained from a peripheral blood sample according to a previously reported saturated phenol-chloroform extraction method[14]. The sequencing was performed at BGI-Shenzhen (Shenzhen, China). In brief, the library was prepared using the DNA nanoballs and combinatorial probe-anchor synthesis technology, and the sequencing was fulfilled in the DNBSEQ platform. DNBSEQ base-calling software was used to transform raw image files derived from sequencing into raw reads.

Generated clean data via raw data filtration by SOAPnuke (v2.1.0) were aligned to the human reference genome (GRCh37/hg19) using the Burrows-Wheeler Aligner (v0.7.17). The sequencing data alignment, base quality value correction, and variant calling were performed using Genome Analysis Toolkit (GATK, v4.1.4.1). The GATK MarkDuplicates tool was used to mark the duplicate reads. After the removal of the duplicate reads, base quality score recalibration was performed by GATK BaseRecalibrator and GATK ApplyBQSR. GATK HaplotypeCaller was used for single nucleotide polymorphisms and insertions/deletions detection. The variants were filtered out through GATK SelectVariants and GATK VariantFiltration, and annotated using the Annodb software, with reference to databases including the Single Nucleotide Polymorphism database, the 1000 Genomes Project, Exome Sequencing Project 6500, and the BGI in-house exome database. Variants whose minor allele frequency ≥ 0.01 were filtered out. The candidate variants were checked against ClinVar, the Human Gene Mutation Database, and PubMed. The global population of the Genome Aggregation Database and the Exome Aggregation Consortium database were surveyed to search for the variant frequency in the population. MutationTaster, MutationAssessor, Sorting Intolerant from Tolerant, and Polymorphism Phenotyping v2 were utilized to predict the pathogenicity of candidate variants. Synonymous variants and variants in the potential donor or acceptor splice site were predicted by Berkeley Drosophila Genome Project (BDGP) Splice Site Prediction by Neural Network tool (https://www.fruitfly.org/seq_tools/splice.html). The consensus approach meta-server from Zhang Lab (http://zhanglab.ccmb.med.umich.edu/COACH/) and PyMOL software (v2.6.0a0, Schrödinger, LLC, Portland, U.S.A.) were used to predict and perform the structural comparison of the wild-type and mutated proteins[15,16].

Primers for Sanger sequencing were designed by Primer3 (v4.1.0, https://primer3.ut.ee), including *PLA2G6*-c.402C>T-F: 5'-CCCTTCTATGAGAGCTCCCC-3', and *PLA2G6*-c.402C>T-R: 5'-CCACACAAGCAGGTACACAC-3', *PLA2G6*-c.2327_2328del-F: 5'-CCTGAGCATCCTAGGGTGAC-3', and *PLA2G6*-c.2327_2328del-R: 5'- GGGCTGAATGGACGAGGT-3'. Polymerase chain reaction (PCR) amplification and Sanger sequencing were used to verify the detected variants. Chromas software (v2.6.6) was used to align the sequencing results with the gene reference sequence.

The minigene regions encompassing whole exons 2-4 and partial introns 2-4 of the *PLA2G6* gene (NG_007094.3, NM_003560.4: c.402C>T) from genomic DNA of controls were PCR-amplified using seamless cloning strategies with two pairs of primers carrying restriction sites for BamHI/XhoI. The following were the paired primer sequences, respectively: *PLA2G6*-AF: 5'-AAGCTTGGTACCGAGCTCGGATCCACAGAGGGGGAAGACGGTGGGGCCT-3' and *PLA2G6*-AR: 5'-TTACAGGCATAGAGCCAGGGCTAAAGGTTCTCCCCATG-3', *PLA2G6*-BF: 5'-CCCTGGCTCTATGCCTGTAATCCCAGCTACTCAGGAAC-3' and *PLA2G6*-BR: 5'-TTAAACGGGCCCTCTAGACTCGAGCTGCAGCACCTGAGAATTGTCACCCT-3'. The segments including the variant sequence were obtained with mutagenesis primers of *PLA2G6*-MT-F (5'-CGCGAGTGtTTCCATCACAGCCGTATCATCAG-3') and *PLA2G6*-MT-R (5'-TGATGGAAaCACTCGCGGATCCCTAGCTCCAC-3'). PCR products were subcloned into pMini-CopGFP vector (Beijing Hitrobio Biotechnology Co., Ltd., Beijing, China) using ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China). The minigene expression plasmids were confirmed by Sanger sequencing.

For minigene assay, human embryonic kidney 293T cells (Beijing Hitrobio Biotechnology Co., Ltd., Beijing, China) were cultured to 50%-60% confluency of Dulbecco's Modified Eagle Medium (ThermoFisher Scientific, China), supplemented with 10% fetal bovine serum (PAN-Biotech Ltd, Aidenbach, Germany) in 35-mm cell culture dishes at 37°C and 5% CO_2 atmosphere. Transfection of wild-type and mutated

minigene constructs was performed using the Lipofectamine™ 2000 Transfection Reagent (ThermoFisher Scientific, China). The constructs were transiently transfected into cultured human embryonic kidney 293T cells. At 48-hour post-transfection, reverse transcription-PCR analysis using MiniRT-F (5'-GGCTAACTAGAGAACCCACTGCTTA-3') and *PLA2G6*-RT-R (5'-CTGCAGCACCTGAGAATTGTCAC-3') primers, and Sanger sequencing was performed to compare the splicing pattern of the transcripts generated from both constructs.

RESULTS

The patient (II:1, Figure 1A), a 67-year-old female, claimed to be in good health until age 65 years when she developed bradykinesia and left leg clumsiness, and later developed stiffness of the upper limbs, decreased arm swing, rest tremor in the upper limbs, and constipation. At the age of 67 years, she presented marked foot-dragging, gait disturbance, rigidity, and rest tremor. She also suffered from nocturia, significant constipation, and sleeping problems. No affective symptom was reported. Her examination at age 67 showed a masked face, speech dysfluency, hypophonia, impaired postural reflexes, decreased blink, negative Brudzinski, negative Kerning, and negative Babinski. There was bradykinesia on chewing and swallowing, and she manifested stiffness and clumsiness of movements. Physical examination revealed normal muscle strength, normal plantar responses, and increased muscle tone in all limbs, with the right side more severely affected. A sensory system, pyramidal and cerebellar examination were unremarkable. The brain magnetic resonance imaging revealed that the brain was scattered with ischemic foci. Levodopa was prescribed at a dose of 125 mg two times a day, and the response was quite good without levodopa-induced dyskinesia. The results of a detailed clinical examination of the patient are shown in Table 1.

WES generated 90 million raw reads and approximately 86.23 million clean reads after filtration. The total effective bases were 11,830.90 Mb and 99.97% were aligned to the human reference sequence. Effective bases on target were 7,133.59 Mb with an average sequencing depth of 117.99×, and the target coverage at 20× was 97.95%. A total of 130,131 single nucleotide polymorphisms and 24,837 insertions/deletions were identified, including synonymous, missense variants, nonsense variants, splicing variants, in-frame variants, and frameshift variants. After a comprehensive analysis, candidate disease-causing *PLA2G6* variants c.402C>T and c.2327_2328del were identified in the patient. Both *PLA2G6* variants were confirmed with Sanger sequencing [Figures 1B and C].

The synonymous variant, c.402C>T, p.(Cys134=), has been recorded in the Single Nucleotide Polymorphism database (rs200522242) with a low frequency in the Genome Aggregation Database (0.00011) and the Exome Aggregation Consortium database (0.00023), while it has not been reported in ClinVar, the Human Gene Mutation Database, or the literature. BDGP splice site prediction tool showed the transition c.402C>T may generate a new acceptor splice site. Electrophoresis analysis revealed that the tested mutated minigene construct produced two different transcripts, consistent with the wild-type construct. The c.2327_2328del variant, predicted to cause a frameshift leading to a premature truncation, p.(Thr776Serfs*15), was unreported in searched databases. A structural illustration of the wild-type and mutated *PLA2G6* proteins was generated [Figure 1D].

DISCUSSION

In the current study, two variants of the *PLA2G6* gene, c.402C>T and c.2327_2328del, were identified in a Chinese patient with PD. The *PLA2G6* gene, which encodes a calcium-independent group VI phospholipase $A_2\beta$ ($iPLA_2\beta$), resides on chromosome 22q13.1 and contains 17 exons for the VIA-1 transcript (NM_003560.4)[17]. Except for PD, variants in the *PLA2G6* gene were also associated with other autosomal recessive neurodegenerative disorders, including infantile neuroaxonal dystrophy, neurodegeneration with

Table 1. Clinical features of our patient with *PLA2G6* c.402C>T and c.2327_2328del variants

Item	The patient (II:1)
Sex	Female
Age at examination	67 years
Age at onset	65 years
Family history	No
Consanguineous marriage	No
Symptoms at onset	Foot dragging, gait disturbance, decreased arm swing, and left leg clumsiness
Motor features	
Bradykinesia	Yes
Rest tremor (Distribution)	Yes (Left/right hand/arm)
Rigidity	Yes
Gait disturbance	Yes
Imbalance/impaired postural reflexes	Yes
Dysarthria	Yes (Hypophonia and speech dysfluency)
Decreased blink	Yes
Levodopa-induced dyskinesia	No
Muscle tone	Increased
Muscle strength	Normal
Sensory abnormalities	No
Reflex	
Plantar reflex	Normal
Babinski sign	No
Meningeal irritation signs	No
Cognitive decline	No
Psychiatric dysfunctions	No
Sleeping disturbances	Yes (Insomnia)
Autonomic involvement	Yes (Nocturia and constipation)
Others	
Cerebellar signs	No
Seizures	No
Magnetic resonance imaging	Scattered ischemic foci in the brain
Treatment	Levodopa 250 mg/day

brain iron accumulation 2B, and hereditary spastic paraplegia[18]. Pathogenic *PLA2G6* variants may take distinct effects on iPLA$_2$β enzymatic activity, regulation, or interactions via various loss-of-function mechanisms, and therefore affect the clinical phenotypes of *PLA2G6*-associated neurodegeneration (PLAN)[13,19]. By merging data from the literature, PLAN cases harboring homozygous *PLA2G6* variants were summarized, and it showed the consistent genotype-phenotype correlation of the disease[20]. Most reported patients with *PLA2G6*-parkinsonism carry two missense variants, suggesting there is a trend wherein PARK14 is associated with the presence of missense variants.

The full length of iPLA$_2$β protein encompasses seven ankyrin repeats, a highly conserved patatin-like phospholipase domain, a proline-rich motif, a glycine-rich nucleotide binding motif, a serine lipase motif, and a proposed C-terminal Ca^{2+}-dependent calmodulin-binding domain[21,22]. However, neither of the two variants identified in this study was located in the known motif or domain. The iPLA$_2$β protein exerts a

critical effect on maintaining membrane homeostasis by phospholipid remodeling and generating lipid second messengers. It also involves insulin secretion, Ca^{2+} signaling, mitochondrial dynamics, cellular proliferation and migration, and autophagy[13,23]. Multiple isoforms of iPLA$_2$β caused by alternative splicing are associated with tissue-specific and dynamic cellular localization, different catalytic activities, and likely cellular function[24,25]. Research showed that loss of *PLA2G6* impaired store-operated Ca^{2+} signaling, which led to premature loss of dopaminergic neurons via autophagy and other pathological processes, implicating the pivotal role in neurodegeneration[26,27]. Mitochondrial dysfunction that occurs early in PLAN may lead to loss of normal iPLA$_2$β function, cell death, and neurodegeneration[28].

It is speculated that alterations of iPLA$_2$β enzyme activity may arise from distinct *PLA2G6* variants in iPLA$_2$β domains and the number of affected alleles in PD patients. The heterozygous or homozygous *PLA2G6* variant may give rise to a partial or significant decrease in its enzymatic activity[29]. No *PLA2G6*-parkinsonism variants hitherto have been reported to affect the primary structure of iPLA$_2$β that impairs its enzyme activity[13]. *In vitro,* experiments showed *PLA2G6* missense variants (p.Asp331Tyr, p.Gly517Cys, p.Thr572Ile, p.Arg632Trp, p.Leu656Val, p.Asn659Ser, and p.Leu693Val) and frameshift variant (p.Leu598Serfs*68) associated with PD could decrease iPLA$_2$β phospholipase activity[30-32]. Some *PLA2G6* variants associated with PD, including p.Arg632Trp and p.Arg747Trp, were shown not to impair the catalytic activity, but they may be involved in substrate recognition or other regulatory mechanisms of iPLA$_2$β[33,34]. The role of p.Arg741Gln was controversial, for the normal catalytic activity of recombinant iPLA$_2$β proteins was reported, while another study showed impairment of the iPLA$_2$β ability to exert a neuroprotective effect by maintaining mitochondrial function[31,33].

Synonymous variant c.1077G>A in the *PLA2G6* gene was reported to have key functions in activating a cryptic splice site leading to aberrant splicing[35]. Our synonymous variant c.402C>T was presumed to give rise to alter splice acceptor by the BDGP splice site prediction tool. However, the minigene assay showed wild-type and mutated (c.402C>T) *PLA2G6* produced the same splicing pattern, indicating that *PLA2G6* c.402C>T variant did not affect mRNA processing *in vitro*. The negative *in vitro* outcomes cannot entirely exclude the possibility of splicing alterations in affected tissues, and the plausible explanations include the cell- and tissue-specific technical issues, the methods used to characterize transcripts, and so on.

The c.2327_2328del variant of *PLA2G6* corresponding to a frameshift variant was considered as the potential pathogenic variant in the patient. The 2-bp deletion in the exon 17 of the *PLA2G6* gene is located in an undetermined functional region and might contribute to prematurely terminated *PLA2G6* mRNA and premature truncation of iPLA2β enzyme, p.(Thr776Serfs*15). We surmised that the c.2327_2328del variant may result in a truncated protein or exert a deleterious effect via nonsense-mediated decay leading to mRNA degradation, which probably plays a pathogenic role in PD combined with the c.402C>T variant, or it only increases the susceptibility to PD under the circumstances in which the c.402C>T variant may not affect natural *PLA2G6* protein function, or these two variants *in cis* may individually or collaboratively increase the PD susceptibility, implicating a haploinsufficiency mechanism.

Considering the high heterogeneity of PD, the unbiased approach of WES, along with Sanger sequencing, is effective in identifying the underlying genetic cause. Current treatments for *PLA2G6*-related PD patients are symptomatic relief, and primarily dopaminergic agents are geared towards alleviating parkinsonism and dystonia[1]. Thus, future studies to identify more *PLA2G6*-related PD patients and develop newer therapy strategies or preventive interventions based on reliable biomarkers are warranted.

In conclusion, we identified two *PLA2G6* variants in this study, which may enrich the genetic landscape of *PLA2G6*-associated parkinsonism and confirm the notion of prioritizing WES analysis in patients with PD. Further functional studies in site-specific genetic deficiency animal models are needed to assess the role of *PLA2G6* variants in PD, unravel the molecular mechanism in *PLA2G6* variants, and develop more rational drug treatments.

DECLARATIONS

Authors' contributions

Formal analysis: Deng X, Li H, Deng H, Yuan L
Investigation: Deng X, Zheng W, Yang Y, Yang Z, Song Z, Wang J, Deng H, Yuan L
Writing & original draft: Deng X, Zheng W
Writing & review and editing: Deng X, Deng H, Yuan L

Availability of data and materials

All data generated during the study are available from the corresponding author upon reasonable request.

Financial support and sponsorship

This work was supported by the National Natural Science Foundation of China (No. 81873686), Scientific Key Research Project of Health Commission of Hunan Province (No. A202303018385), Natural Science Foundation of Hunan Province (No. 2020JJ3057), the Wisdom Accumulation and Talent Cultivation Project of the Third Xiangya Hospital of Central South University (No. YX202109), and Hunan Province-level College Students' Innovative Training Plan Program (No. S2022105330510).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study was approved by the Institutional Review Board of the Third Xiangya Hospital, Central South University, Changsha, China, and all procedures were performed in accordance with the ethical standards of the Declaration of Helsinki. Clinical data and peripheral blood sample were acquired from the patient after obtaining written informed consent for genetic analysis. This study was approved by the Institutional Review Board of the Third Xiangya Hospital, Central South University, Changsha, China (No: 2018-S400).

Consent for publication

Clinical data and peripheral blood sample were acquired from the patient after obtaining a written informed consent.

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