Title

Overexpressing *PLA2G6* mutations cause symptoms of young–onset dystonia–parkinsonism type 14 and reduction in DHA levels in zebrafish model

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Abstract

Background:

Parkinson's disease (PD) is the most common neurodegenerative motor disorder,

which is currently incurable. Mutations in many genes have been demonstrated to be the primary risk factors associated with the familial or idiopathic PD; however, the mechanisms underlying these genetic mutations resulting in parkinsonism remains unclear. Phospholipase A2 group VI (PLA2G6) has been shown to regulate lipid metabolism and homeostasis in the nervous system. Previous studies have shown that point mutations in *PLA2G6* might be the risk factors associated with the young–onset of dystonia–parkinsonism type 14 (PARK14). However, limited information is available regarding its pathogenic role and the mechanism underlying its function.

Methods:

To study the role of *PLA2G6* mutations in zebrafish PARK14 models, we injected different mutation constructs of human *PLA2G6* genes and zebrafish *pla2g6* deletion constructs in the zebrafish larvae. We analyzed the locomotion behavior, performed immunohistochemistry to examine the formation of dopaminergic neurons, and identified the defective metabolites affected by *PLA2G6* mutations through metabolomics analysis.

Results:

Injection of human *PLA2G6* mutations and zebrafish *pla2g6* deletion constructs

induced symptoms such as motility defects and reduced number of dopaminergic neurons, and these symptoms resembled those observed in PARK14. These phenotypes could be rescued by treatment with L-dopa. Furthermore, the injection of two *PLA2G6* mutation constructs, D331Y and T572I, led to a decrease in the phospholipase activity of PLA2G6 and its lipid metabolites, indicating that these two mutations are the loss-of-function mutations. We further performed metabolomics analysis to identify which lipids are majorly affected by the overexpression of PLA2G6 and PLA2G6 mutants. We found that injecting D331Y or T572I mutation constructs led to higher phospholipid and lower DHA levels.

Conclusions:

D331Y and T572I injections in zebrafish were sufficient to create a PD phenotypes. In addition, D331Y and T572I are loss of function mutations and cause defective phospholipase activity and reduced the level of DHA. These results have helped us elucidate the role of *PLA2G6* mutations in PARK14 and further led to a deeper understanding of the molecular mechanisms underlying PD. The results of this study may also facilitate the development of therapeutic strategies for PD.

Keywords：PLA2G6, Parkinson's disease, PARK14, zebrafish.

Background

Parkinson's disease (PD) is the second most common neurodegenerative disorder. The main pathological cause of this disease is the progressive degeneration of the substantia nigra pars compacta dopaminergic neurons and the loss of such neurons results in a defected neural circuitry responsible for regulating voluntary movement. In advanced stages of the disease, non-motor features such as emotional and cognitive deficits also appear. Current treatments are symptomatic which mainly rely on strategies of dopamine supplement; however, because the dopaminergic neuron loss continues, the symptoms can only be temporary relieved and the disease remains incurable. (1-3)

The cause of PD is unknown but has been suggested to result from a combination of genetic and environmental factors (4). Most PD patients display symptoms in late adulthood, however, many studies showed that irregular molecular mechanisms occurring during the fetus stage can also cause the loss of dopaminergic neurons (2). Indeed, studies have indicated that genetic mutations in specific genes (named

familial PD genes, PARKs) could be the cause for familial PD (5) and can even cause neurological defects.

In 2009, Paisan-Ruiz et al. (2009) described three individuals from two unrelated families. They displayed young-adult onset of parkinsonism, dystonia, and severe cognitive decline (6) and more cases have been identified since then. This disease has been named adult-onset dystonia-parkinsonism, also known as Parkinson disease-14 (PARK14), and investigations demonstrated that PARK14 is caused by a homozygous mutation in the phospholipase A2, group VI (*PLA2G6*) gene. In addition to PARK14, mutations in the *PLA2G6* can also cause neurodegeneration with brain iron accumulation (NBIA) (7) and infantile neuroaxonal dystrophy (INAD) (8). Particularly, the symptoms of PARK14 develop during early adulthood, which leads to the hypothesis that neurodevelopmental defects during the fetus or infantile stage may be responsible for the progression of PARK14.

PLA2G6 is a calcium-independent phospholipase that is involved in the metabolism of glycerophospholipids, phospholipid remodeling, arachidonic acid release, synthesis of prostaglandins and leukotrienes, and apoptosis. PLA2G6 is highly expressed in the brain (9, 10). In addition, it catalyzes the hydrolysis of phospholipids at the sn-2

115 position, thereby producing lipid metabolites that might mediate the downstream
116 signaling pathways. As a consequence, these lipid signals regulate multiple
117 physiological and pathophysiological processes in the nervous system, hence
118 suggesting that an altered lipid signaling might contribute to the pathology of the
119 PLA2G6 mutation (11, 12). However, the molecular mechanism of PLA2G6
120 mutations, especially in PARK14, is still unknown. The presence of demyelination
121 and axonal swellings in both the central and peripheral nervous systems have been
122 observed in patients with INAD and in *PLA2G6* knockout mice (8, 13). In addition,
123 the catalytic activity of PLA2G6 is impaired in some patients with PARK14, NBIA,
124 and INAD (14, 15). These findings highlighted the role of phospholipase and lipid
125 regulation in PARK14, NBIA, and INAD pathology.
126
127 Many PLA2G6 mutations that have been identified are spread across different
128 locations on the entire PLA2G6 coding sequence. However, most of the PARK14-
129 associated *PLA2G6* mutations are not located in the critical catalytic patatin domain.
130 Therefore, the role of PLA2G6 and *PLA2G6* mutations in PARK14 is currently
131 unclear. The clinical genetic study showed that PLA2G6 D331Y mutation is
132 associated with an increased risk for early-onset PD in a Taiwanese cohort of PD
133 patients (16). In addition, an increasing number of PLA2G6 mutations have been

identified to date; hence, an efficient model to examine the neurological effects of different PLA2G6 mutations in PD is desperately required.

In this study, we aimed to investigate the pathological role of *PLA2G6* mutations in PARK14 using the zebrafish model system. To this end, we first overexpressed six human *PLA2G6* mutations in zebrafish. We demonstrated that three of the six *PLA2G6* mutations caused locomotion defects and resulted in a decrease in the number of dopaminergic neurons in zebrafish. In addition, two of the mutations, including D331Y and T572I, were found to cause defective phospholipase activity, which further lead to a reduction in the docosahexaenoic acid (DHA) level.

Materials & Methods

Ethics Statement

All experiments were performed in strict accordance with the standard guidelines for zebrafish studies and were approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC approval number: CGU106-066).

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154 Fish Maintenance

155

156 Tü (wild-type) zebrafish embryos were purchased from the Zebrafish International
157 Resource Center (Eugene, OR, USA) and were raised, maintained, and paired under
158 standard conditions. The embryos were staged according to somite numbers, hours
159 postfertilization (hpf), and days postfertilization (dpf) (17).

160

161 Generation of Constructs and Microinjection

162

163 The open reading frames of human *PLA2G6* (GenBank accession number:
164 NM_003560.3) and zebrafish *pla2g6* (GenBank accession number: NM_213097.2)
165 genes were PCR-amplified using the 2X Super Hi-Fi Taq PCR MasterMix (TOOLS,
166 Taiwan). The six *PLA2G6* mutations were generated by site-directed mutagenesis (see
167 Extended Table 1 for a complete list of the primer sequences used). All the constructs
168 were sub-cloned into the pCS2+ plasmid vector (provided by David Turner,
169 University of Michigan) and purified using the TOOLS Plasmid Mini kit (TOOLS,
170 Taiwan) to facilitate *in vitro* transcription. Capped RNAs were prepared as described
171 previously (18). All injections were performed at the one- to two-cell stages, and the

172 corresponding cRNAs were introduced into the blastomeres.

173

174 Behavioral Tests and Video Recording

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176 All tests were performed in three independent experiments. Embryonic and larval
177 behaviors were observed using a Leica Z16 APO stereomicroscope. Video images
178 were captured using an Olympus C-7070 CCD camera and analyzed using VirtualDub
179 and ImageJ (National Institutes of Health) sotwares. For in-chorion coiling
180 contraction, the total contractions of 10 embryos within 1 minute (for each test) were
181 counted and are presented as number of contractions per minute. Touch and escape
182 responses were analyzed at 48 hpf, and the embryos were gently stimulated using a
183 thin tungsten probe near the region of the trigeminal neurons. Four tactile stimuli with
184 5-second intervals were applied in batches of 10 embryos per experiment, and the
185 number of embryos that did and did not respond is expressed in percentage.

186

187 Chemical Treatment

188

189 L-dopa (Sigma-Aldrich, USA) at a concentration of 100 μM was added into the E3
190 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl2, and 0.16 mM MgSO4) 3 h

191 before performing the behavior analysis.

192

193 Immunohistochemistry

194

195 The embryos were blocked in 5% goat serum and were incubated with the antibody of
196 tyrosine hydroxylase (1/250 dilution, Merck Millipore). Fluorochrome-conjugated
197 antibody Alexa Fluor 488 (or 594) and goat anti-mouse IgG (Invitrogen) were used to
198 detect the primary antibody. Embryos were mounted using Vectashield mounting
199 medium with DAPI (Vector Laboratories, Inc.).

200

201 Detection of Dopamine Levels by HPLC

202

203 The fish were frozen in liquid nitrogen and stored at −80 °C until further use. For
204 HPLC analysis, 30-40 fish were homogenized in 65 μL of an ice-cold 0.1 N $HClO_4$
205 solution. Next, the homogenates were centrifuged at 16,100 ×g at 4 °C for 30 min and
206 filtered (pore size: 0.20 μm, Thermo Fisher Scientific, USA). Finally, to detect the
207 level of dopamine in the processed samples, a 20 μL aliquot of the supernatant was
208 injected into the HPLC system through a 5 μm C-18 column (4.6 mm × 150 mm) and
209 an electrochemical detector (BASi), and the results were analyzed using the system

Gold software (Beckman Coulter). The mobile phase buffer (0.105 % glacial acetic acid, 29.9 mM citric acid, 50 mM sodium acetate, 52.5 mM NaOH, and 1.85 mM 1-octanesulfonic acid) along with 7% methanol was used after the column was filtered and degassed. The flow rate was maintained at 1.0 mL/min and the working electrode was set at +650 mV. The total protein concentration was detected using the Bradford assay.

Phospholipase Activity

The phospholipase A2 activity of PLA2G6 was measured using a modified commercial kit that was originally designed for cPLA2 (Cayman Chemicals), as described previously (19). Briefly, the total protein was extracted and incubated in a modified calcium-free buffer (4 mM EGTA, 160 mM HEPES, pH 7.4, 300 mM NaCl, 8 mM Triton X-100, 60% glycerol, and 2 mg/mL BSA) with arachidonoyl thio-phosphatidylcholine (ARA-PC) and the synthetic substrate of phospholipase for 2 h at room temperature. PLA2G6 could hydrolyze ARA-PC without calcium and then release the free thiols. The production of free thiols was measured by adding 5,5'-Dithiobis(2-nitrobenzoic acid) and by determining the absorbance at 405 nm.

229 Extraction of Lipophilic Metabolites and Data Processing

230

231 Fishes were collected in 1.5 mL tubes, and 80% iced methanol (MeOH) was

232 immediately added into the tubes to quench any metabolic activity and extract all the

233 metabolites. The mixture was homogenized and stored at -80 °C until further use.

234 Lipid metabolites were extracted with methyl tert-butyl ether (MTBE) according to a

235 previously described method (20) with slight modifications. Briefly, the iced MeOH

236 and MTBE were sequentially added into the sample tubes (9:30, v/v) and vortexed 3

237 times for 30 s. After sonication for 15 min, iced ddH20 was added (volume was

238 obtained by mixing MTBE, MeOH, and ddH20 in a ratio of 30:9:8), and the tubes

239 were vortexed for 30 s. Following incubation at room temperature for 10 min, the

240 samples were centrifuged at 16,100 ×g for 10 min at 4 °C. The upper phase was

241 collected and dried under nitrogen gas, and stored at -80 °C until further use.

242 The chromatographic analysis was performed on a WatersAcquityTMUPLC H-Class

243 system with a flow-through-needle sample manager. The samples were separated on a

244 BEH C18 column. The column temperature was set at 60 °C, and the flow rate used

245 was 450 μL/min. Mobile phase buffers A (ACN/H2O [40/60] with 10 mM ammonium

246 acetate) and B (IPA/ACN [90/10] with 10 mM ammonium acetate) were used with a

247 gradient elution from 40 % buffer B to 99 % buffer B within 10 min. Information

regarding the mass spectrometry data, such as retention times, m/z ratios, and ion intensities, were extracted by using the MarkerLynx XS software (waters, Milford, MA), and the resulting MS data were subjected to a matrix for further analysis. Metabolites were searched against the METLIN database (available at https://metlin.scripps.edu/) and confirmed based on in-house data available from the Metabolomics Core Lab, CGU (standards based on both retention times and mass spectra). Next, the data were subjected to principal components analysis (PCA) and partial least squares discriminate analysis (PLS-DA) using the Metaboanalyst software.

Statistical Analysis

For spontaneous contraction, touch response, and axon counts, statistical analysis was performed using the unpaired Student's *t* test in Microsoft Excel 2007, and results with $P < 0.05$ are described as statistically significant. All graphs show the mean and SD and are represented as the mean ± SD in the text.

Results

PLA2G6 mutations cause locomotion defect in zebrafish

Previously, we and others have identified PLA2G6 mutations that are associated with dystonia-parkinsonism (16). We used zebrafish as a relatively efficient model to examine the role of PLA2G6 mutations and individually injected six mutated *PLA2G6* genes (Figure 1), D331Y (16, 21), G517C (7), T572I (22), R632W (7), N659S (7), and R741Q (6, 7), into zebrafish embryos.

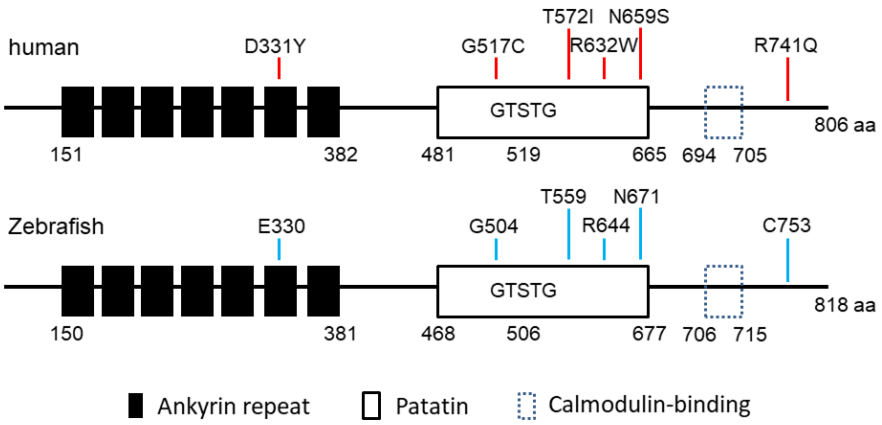


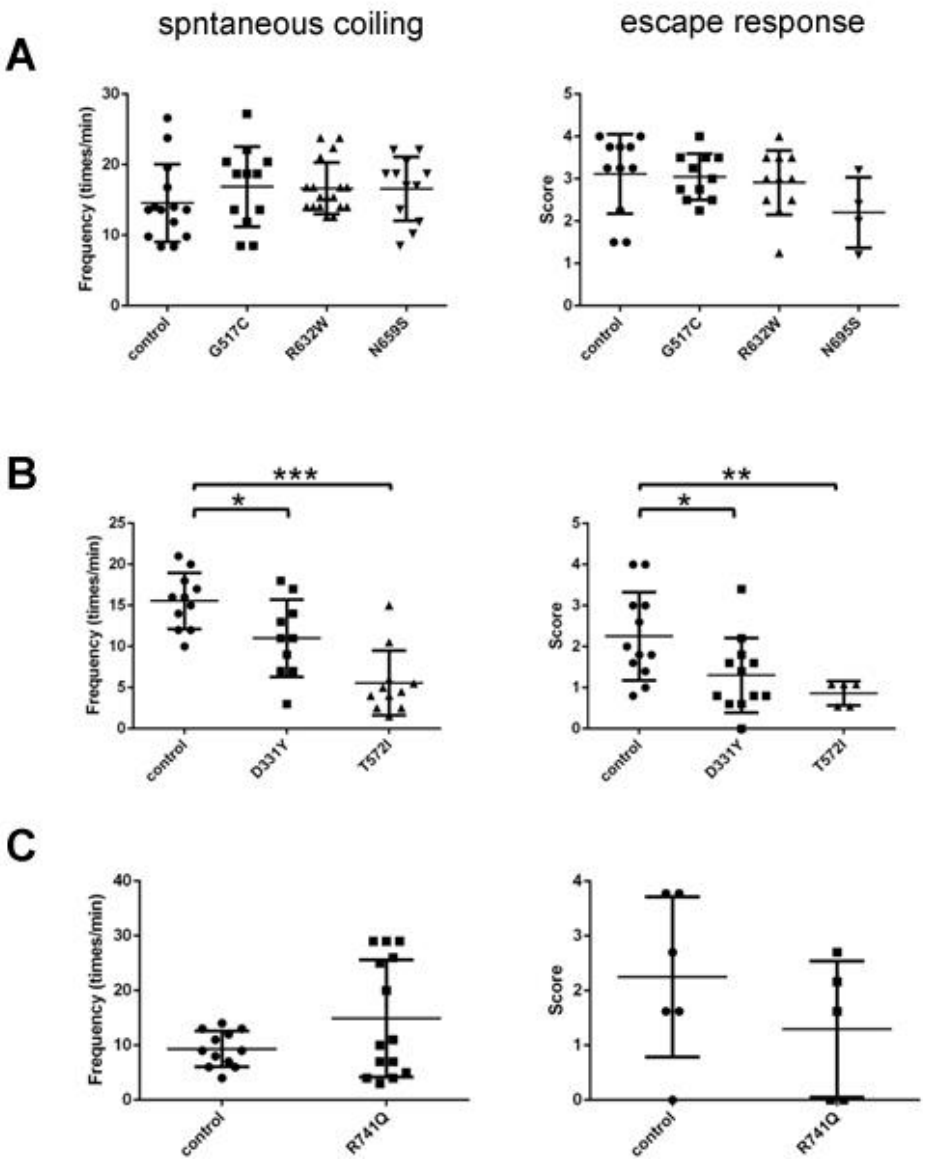
Figure 1. Schematic illustration of human PLA2G6 and zebrafish Pla2g6 functional domains and the sites of mutations

Human PLA2G6 protein consists of seven ankyrin repeats (151aa–382aa), a patatin domain (481aa–665aa), and a calmodulin-binding domain (694aa–705aa). GTSTG indicates the catalytic site present on the patatin domain. Red lines indicate the six

mutation sites in human PLA2G6, and blue lines indicate the conserved amino acids and position of the corresponding mutations on zebrafish pla2g6. aa represents amino acid.

We analyzed the locomotion behavior by examining the earliest motor behavior, spontaneous coiling, and the touch-evoke response (23, 24). Spontaneous coiling includes alternating, side-to-side metronome-like contractions of the tail, and this behavior does not require inputs from the forebrain, midbrain, or hindbrain (25-27). From 48 to 60 h postfertilization (hpf), zebrafish embryos react to the tactile stimuli with a rapid escape response (touch-evoke escape response), indicating the presence of complete afferent connections (23, 24). Injection of G517C, R632W, or N659S resulted in no substantial effects in the spontaneous coiling and the touch-evoke response of zebrafish larvae (Figure 2A). In contrast, zebrafish larvae injected with D331Y or T572I displayed lower coiling frequencies and smaller coiling angles in comparison with the controls (Figure 2B), indicating that D331Y and T572I were sufficient to cause malfunction of the spinal motor circuit. In addition, larvae injected with D331Y or T572I exhibited shorter escape distance after stimuli (Figure 2B), suggesting that the efferent connections became defective by D331Y and T572I. This result indicated that D331Y and T572I injections were sufficient to create a phenotype

that is identical that of patients with PD. Injection of R741Q caused two opposing locomotor extremes. The R741Q-injected larvae exhibited either reduced or increased frequency of spontaneous coiling (Figure 2C). They also displayed either increased or decreased swimming distance after stimuli in comparison to the controls (Figure 2C). This result suggests that R741Q injection produced a polarization of motility that resembled hypokinesia and hyperkinesia. Taken together, these studies demonstrated that different PLA2G6 mutations were capable to produce different types of motility defects in zebrafish larvae. The reduced motility resembled the hypokinesia phenotype observed in PD, while the hyperkinetic movement resembled the dystonia phenotype in PARK14. In addition, L-dopa treatment rescued movement and dopaminergic defects caused by D331Y and T572I injection (Figure 3), indicating that D331Y and T572I injections were sufficient to create a phenotype that is identical that of patients with Parkinson's disease. This further confirmed that D331Y- and T572I-injected zebrafish could be used as PARK14 models. Therefore, altering the expression of endogenous zebrafish Pla2g6 function by human PLA2G6 mutation constructs in zebrafish can be used to model motor dysfunction phenotype of PD



318

319

320 **Figure 2. D331Y, T572I and R741Q constructs cause abnormal locomotor**

321 **behavior.**

322 (A) Injection of G517C, R632W, or N659S resulted in no substantial effect in the

323 spontaneous coiling (left) and the escape response (right) of zebrafish larvae. (B) The

324 frequency of spontaneous coiling was reduced by D331Y or T572I injection (left). In

325 addition, D331Y or T572I injection cause aberrant escape responses. The injected

326 embryos displayed reduced escape distance compared with the controls (right). (C)

327 Injection of R741Q caused two different and opposite locomotor behaviors with either

328 reduced or increased frequency of spontaneous coiling (left). R741Q-injected larvae

329 exhibited either increased or decreased swimming distance after stimuli in comparison

330 with the controls (right). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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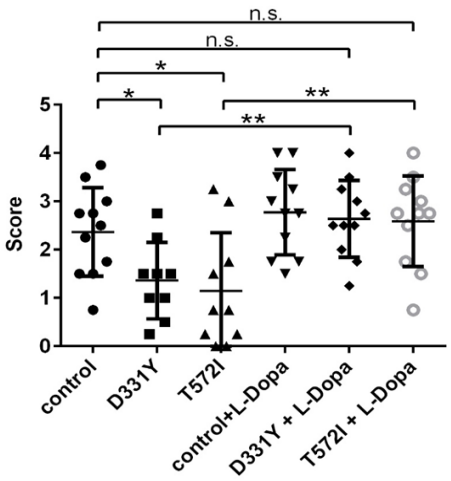

332

333 **Figure 3. L-dopa treatment can rescue aberrant escape responses due to**

334 **PLA2G6 mutation.**

335 Zebrafish larvae were incubated in 100 μM L-dopa after the injection of PLA2G6

336 mutations D331Y or T572I. Escape response analysis demonstrated that L-dopa

337 treatment can rescue escape ability in D331Y or T572I injected larvae. *, $p < 0.05$; **,

338 $p < 0.01$; ***, $p < 0.001$.

339

340 <u>PLA2G6 mutations reduce midbrain dopaminergic neuronal formation</u>

Positron emission tomography scans revealed a decreased dopamine transporter activity in the striatum (6), indicating that reduced dopamine levels are the pathogenic cause for PARK14. Accordingly, we studied the cellular defect and dopamine level by PLA2G6 mutations. We examined the formation of dopaminergic neurons in those zebrafish larvae injected with mutated *PLA2G6* genes. Injection of D331Y or T572I reduced the number of dopaminergic neurons in the ventral diencephalon (analog to substantia nigra), analyzed by immunohistochemistry with the tyrosine hydroxylase antibody (Figure 4A). The number of dopaminergic neurons also decreased following the R741Q injection (Figure 4A). In addition, the dopamine level decreased in D331Y or T572I injecting embryos (Figure 4B). According to these results, PLA2G6 mutation D331Y or T572I was sufficient to produce cellular defect that are similar to Parkinsonism phenotypes.

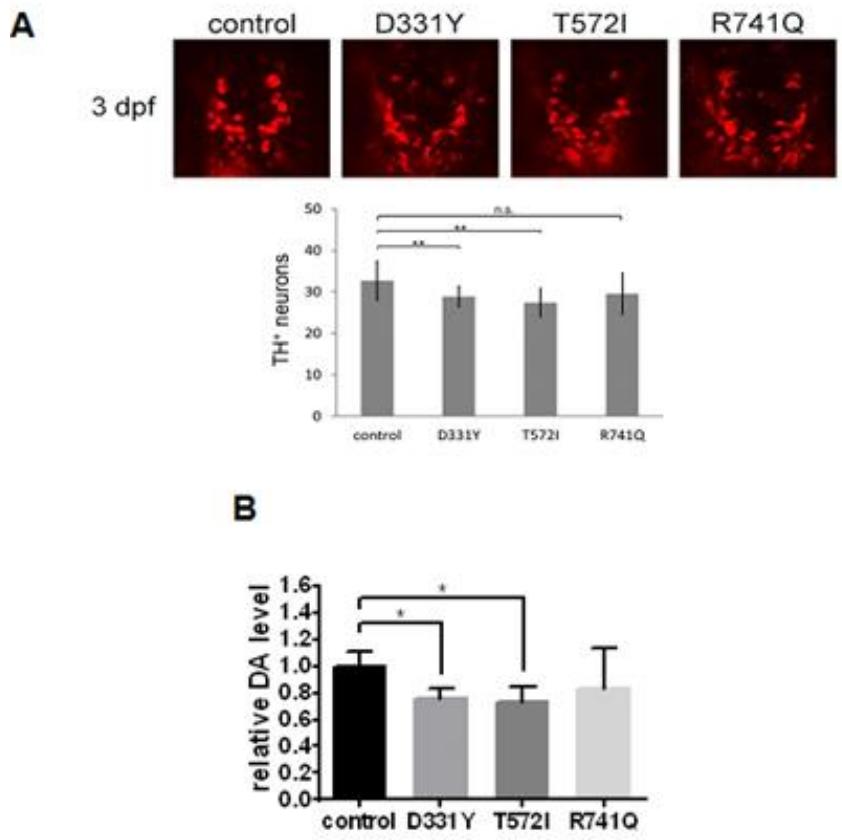

Figure 4. Injecting D331Y, T572I, or R741Q reduced the number of dopaminergic neurons and dopamine levels.

(A) Immunohistochemistry using tyrosine hydroxylase (TH) antibody showing the reduced numbers of dopaminergic neurons following injections with the D331Y, T572I, or R741Q construct. The numbers of dopaminergic neurons were counted manually accordingly to the fluorescent signals (lower panel). (B) The dopamine level decreased by the injection of D331Y or T572I, analyzed by HPLC. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s. not significant. DA, dopamine.

D331Y and T572I are loss of function mutations and cause defective phospholipase

activity

To gain insight into the structural requirements for PLA2G6 function and to further confirm whether the mutations are gain- or loss-of-function mutations, we created zebrafish *pla2g6* deletion variants lacking the ankyrin repeat (*pla2g6*$^{\Delta ANK}$), patatin (*pla2g6*$^{\Delta Patatin}$), or calmodulin-binding domain (*pla2g6*$^{\Delta Ca}$). Injection of *pla2g6*$^{\Delta Patatin}$ resulted in defective spontaneous coiling and touch-escape responses (Figure 5); these phenotypes were identical to those observed in the D331Y or T572I-injected embryos, indicating that the complete patatin domain is essential for the PLA2G6 function. In addition, this result also suggested that reduced phospholipase activity in PLA2G6 results in motility defects, because the Patatin domain has been suggested to contain phospholipase activity to cleave fatty acids from membrane lipids (28).

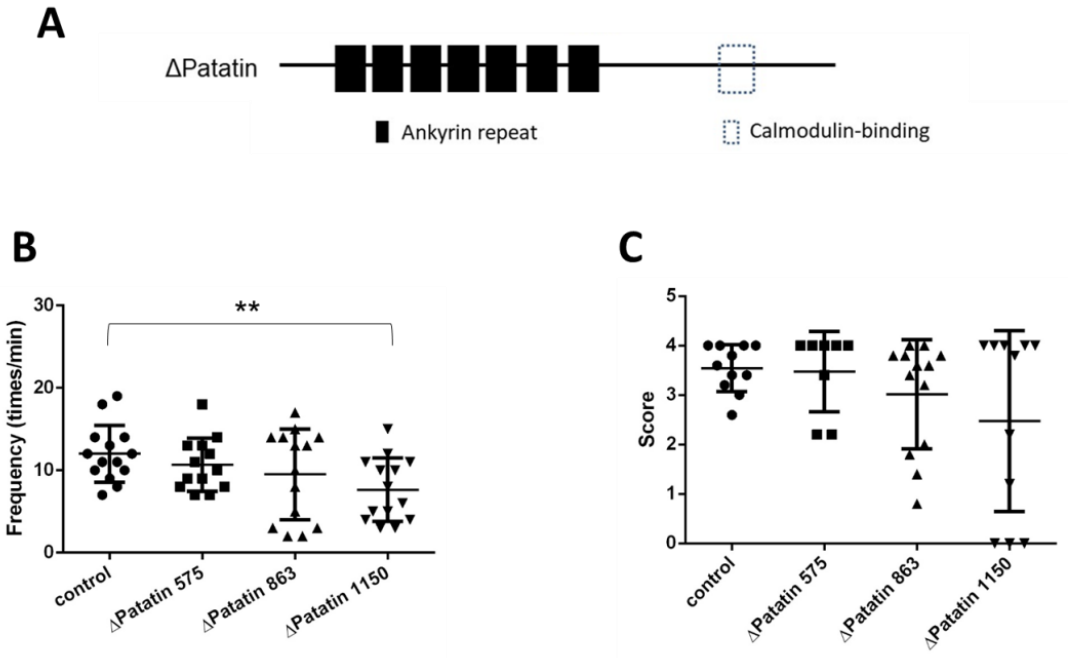

Figure 5. The patatin-deletion construct causes motility defects resembling D331Y and T572I injection in embryos.

(A) Schematic diagram of patatin-deletion construct (*ΔPatatin*, *pla2g6*$^{\Delta Patatin}$). Injection of *pla2g6*$^{\Delta Patatin}$ reduced the spontaneous coiling frequencies (B) and escape distance after stimuli (C) in a dose-dependent manner (575 ng, 863 ng, and 1150 ng).

PLA2G6 is a phospholipase that catalyzes the release of fatty acids from phospholipids. However, it is unclear whether all PLA2G6 mutations affect PLA2G6 activity. D331Y and T572I located in ankyrin repeat and patatin domains of PLA2G6, respectively, and whether these mutations affect PLA2G6 phospholipase activity is unclear. We examined the phospholipase activity and found overexpression of either human *PLA2G6* or zebrafish *pla2g6* mRNA increased the phospholipase activity (Figure 6A), on the contrary, D331Y or T572I decreased the phospholipase activity (Figure 6B). Injection of the deletion variants, *pla2g6*$^{\Delta ANK}$ or *pla2g6*$^{\Delta Patatin}$, also reduced the phospholipase activity (Figure 6C). These results further confirmed that D331Y and T572I are loss of function mutations and that lipid metabolism was defective in PARK14.

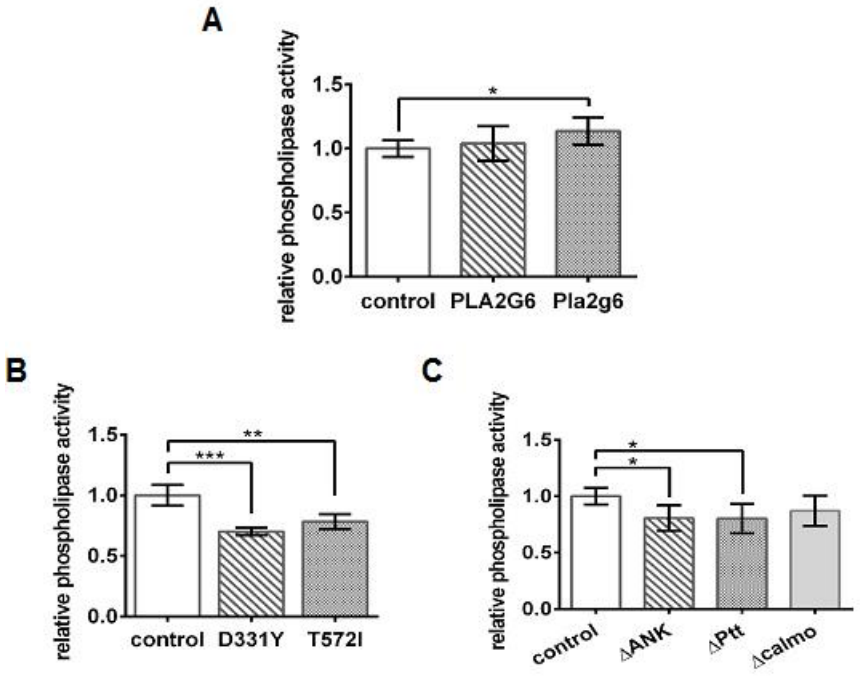

Figure 6. Injection of PLA2G6 mutations, D331Y and T572I, reduces phospholipase activity.

Phospholipase activity was investigated using a modified commercial kit. This figure demonstrates phospholipase activity was induced by human *PLA2G6* or zebrafish *Pla2g6* (A), and reduced by D331Y (B), T572I (B), *pla2g6*$^{\Delta ANK}$ (C), or *pla2g6*$^{\Delta Patatin}$ (C). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

<u>D331Y and T572I mutations reduced the level of DHA</u>

Through metabolomics, we identified the defective metabolites affected by PLA2G6 mutations in the PARK14 models by comparing lipid metabolites in the control samples with those in samples overexpressing PLA2G6 mutations (Figure 7,

Extended Table 2). The result showed that unhydrolyzed phospholipids were increased and lipid metabolites were decreased due to the injection of D331Y and T572I mutation constructs. This further confirmed that D331Y and T572I mutations altered phospholipase activity and caused a loss-of-function effect of PLA2G6 (Figure 6 and 7). Specifically, the level of docosahexaenoic acid (DHA), a free fatty acid, was decreased in larvae injected with D331Y and T572I mutations and a patatin domain deletion variant (*pla2g6*$^{\Delta Patatin}$) (Figure 7, Extended Table 2). This result suggested that D331Y and T572I mutations caused deficient phospholipase activity and reduced the level of DHA. This may subsequently cause motility and dopaminergic defects in zebrafish PARK14 models.

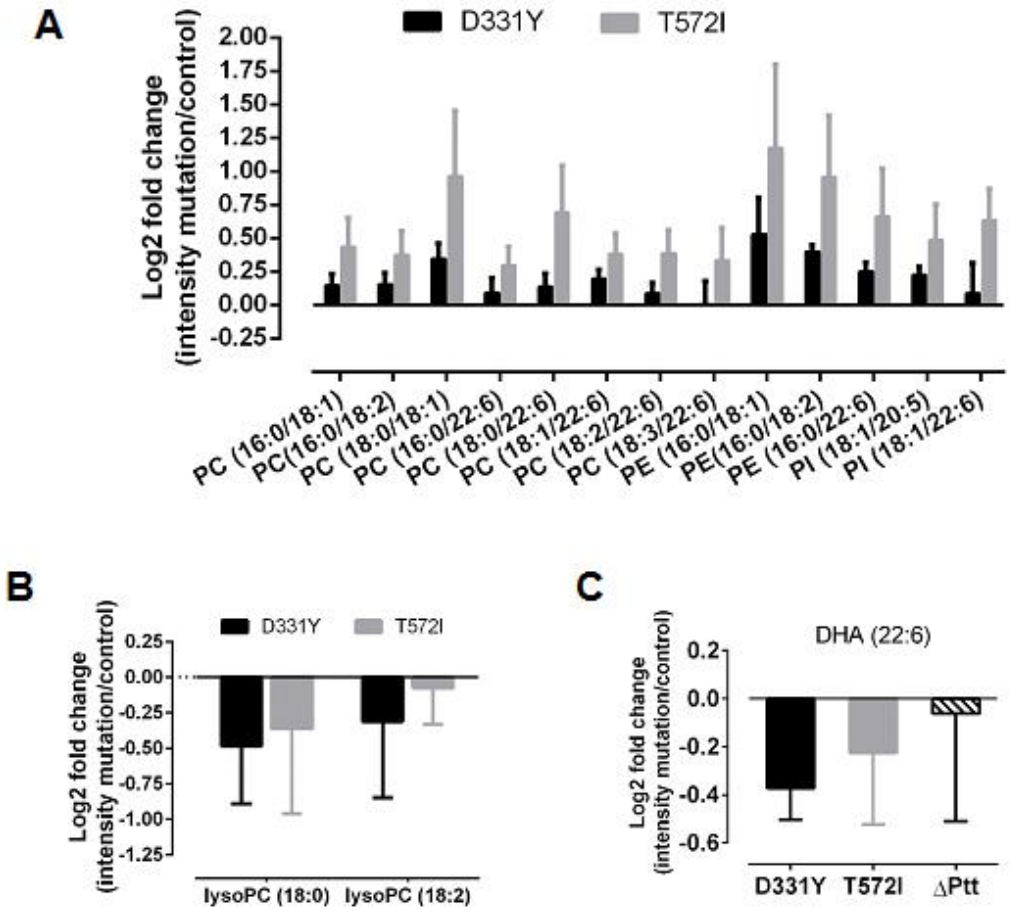

Figure 7. Lipid metabolites are altered by D331Y or T572I injection.

(A) The level of phospholipids is increased in D331Y or T572I injected embryos. PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI: phosphatidylinositol. (B) lysophospholipid, the hydrolyzed form of phospholipid, are decreased by D331Y or T572I injection. (C) The level of the free fatty acid DHA (22:6) is decreased in larvae injected with D331Y or T572I and the patatin domain deletion construct. The level of phosphatidylcholine containing one fatty acid chain (22:6) is increased in PLA2G6 mutation- and deletion construct- injected larvae. ΔPtt: patatin domain deleted *pla2g6*

construct (*pla2g6*$^{\Delta Patatin}$).

Discussion

Although previous studies have provided insights into the significant impact of genetic factors on PD, the molecular mechanism underlying PD remains largely unclear. A comprehensive analysis focusing on the biological function and interactions of PD-related genes may provide valuable information to understand the pathogenesis of PD. In this study, we examined the role of *PLA2G6* mutations using the zebrafish model system. Many *PLA2G6* mutations that have been identified to be associated with PD are spread across different locations on the entire *PLA2G6* coding sequence. However, only the D331Y mutation was able to affect the catalytic function of PLA2G6, whereas other *PLA2G6* mutations were found not to affect its catalytic activity (14). Therefore, the role of PLA2G6 and *PLA2G6* mutations in PARK14 and parkinsonism-like phenotype are currently unclear. We demonstrated that upon injecting the D331Y and T572I mutation constructs in zebrafish larvae, it induced symptoms, such as motility defect and a reduction in the number of dopaminergic neurons resembling those observed in PARK14. In addition, we confirmed that

449 D331Y and T572I were both loss-of-function mutations by comparing the phenotypes
450 that were observed upon the injection of D331Y or T572I mutation constructs with
451 the injection of the *pla2g6* deletion variant, and were found to resemble completely.
452 D331Y mutation is located in the ankyrin repeats domain, and the domains consisting
453 of ankyrin tandem repeats are known to mediate protein–protein interactions. Since
454 the phospholipase activity of PLA2G6 was rendered defective by the D331Y mutation
455 or the ankyrin repeat deletion variant, $pla2g6^{\Delta ANK}$, our result suggests that the protein–
456 protein interactions through the ankyrin repeats domain is essential for
457 PLA2G6/Pla2g6 function, and a deletion or mutation in this domain could eventually
458 result in PARK14 phenotype.
459
460 T572I mutation is located in the patatin domain, and this domain is known to be
461 responsible for the phospholipase activity. We demonstrated that both the T572I
462 mutation construct and patatin deletion variant ($pla2g6^{\Delta Patatin}$) could reduce the
463 phospholipase activity and produce PARK14 phenotype. This result indicates that the
464 defective function of the patatin domain could impair phospholipase activity and
465 produce PARK14 phenotype. However, G517C, R632W, and N659S mutations that
466 are also located in the patatin domain, neither caused the dopaminergic defect nor PD
467 symptoms following the injection of these mutation constructs into the zebrafish

468 embryos. In addition, R741Q mutation construct injection induced the polarization of
469 motility that resembled the phenotypes of hypokinesia and hyperkinesia. In previous
470 studies, it has also been demonstrated that some *PLA2G6* mutations related to
471 PARK14 do not exhibit decreased phospholipase activity (14, 15). These results
472 suggest that there could be other factors involved in the pathogenesis of PD associated
473 with these mutations. Moreover, PLA2G6/Pla2g6 may play an additional role apart
474 from that in the phospholipase activity. Several studies have previously demonstrated
475 that PLA2G6 mediate cell growth by participating in signal transduction pathways,
476 including epidermal growth factor receptor (EGFR), mitogen-activated protein kinase
477 (MAPK), mouse double minute 2 homolog (MDM2), tumor protein p53 (TP53), and
478 cyclin-dependent kinase inhibitor 1 (CDKN1A). The precise mechanism through
479 which PLA2G6 mediates these pathways is still unknown (29), and thus, the
480 pathogenic mechanism associated with the *PLA2G6* mutations may not be restricted
481 to the phospholipase activity and still remains to be identified.
482
483 PLA2G6 catalyzes the hydrolysis of phospholipids to produce the lipid metabolites.
484 Defects in the production of lipid metabolites and the downstream signaling pathways
485 caused by *PLA2G6* mutations may lead to pathophysiological conditions in the
486 nervous system, thereby resulting in PARK14. The downstream effectors associated

with PLA2G6 in PARK14 are currently unknown. In the current study, we demonstrated that the injection of D331Y or T572I mutation constructs led to alterations in the production of lipid metabolites, including an increase in phospholipid levels and a decrease in those of polyunsaturated fatty acid (PUFA) DHA. Lipid metabolites have been implicated in many neurodegenerative disorders. For example, some short-chain fatty acids (SCFAs) are sufficient to induce α-synuclein aggregation and motor deficits in PD mouse model (30); further, lipid peroxidation products of PUFA have been found to be increased in Alzheimer's disease or PD patients (31). DHA is the most abundant long-chain PUFA present in the brain and accumulating evidence in PD models indicates that it can exert neuroprotective effects (32, 33). Therefore, our results suggest that the accumulated phospholipids and reduced DHA could be the pathogenic factors responsible for PARK14. Thus, the chemicals that can catalyze the degradation of phospholipids or administration of DHA may act as potential therapeutic strategies for the treatment of PARK14, which remains to be confirmed.

Conclusions

The pathogenic role of *PLA2G6* mutations in PARK14 is currently unclear, and

particularly the underlying regulatory mechanism remains to be elucidated. We demonstrated that the overexpression of mutated *PLA2G6* genes and *PLA2G6* deletion constructs were sufficient to reduce the number of dopaminergic neurons and induce the motility defects reminiscent of parkinsonism, which partly revealed the pathogenic role of *PLA2G6* mutations and the essentiality of PLA2G6 functional domains. We also identified the *PLA2G6* mutations that were responsible for defective phospholipase activity and resulted in an increase in phospholipid levels, thereby reducing DHA levels. This may provide a potential therapeutic strategy for the treatment of PD.

Supplementary information

Additional file 1: Extended Table 1. List of primer sequences used in the present study.

Construct		primers sequence (5' to 3')
Human *PLA2G6*	Forward	gaattcgccaccatgcagttctttggcc
	Reverse	gaattctcagggtgagagcagcag

Zebrafish *pla2g6*	Forward	ctcgaggccaccatgcagttcctgggccgtattttg
	Reverse	ctcgagtcagagctgcaggagctgctggcag
Zebrafish *pla2g6*$^{\Delta ANK}$	Forward	ctcgaggccaccatgctgtgtagtgtaggtgtc
	Reverse	ctcgagtcagagctgcaggagctgctggcag
Zebrafish *pla2g6*$^{\Delta Patatin}$	Forward-1	ctcgaggccaccatgcagttcctgggccgtattttg
	Reverse-1	tactaccacttgcatgatgtcatc
	Forward-2	gtggtagtatcactgggcac
	Reverse-2	ctcgagtcagagctgcaggagctgctggcag
Zebrafish *pla2g6*$^{\Delta CB}$	Forward	ctcgaggccaccatgcagttcctgggccgtattttg
	Reverse	ctcgagtcacttcagggctttgttgaactg

Additional file 2: Extended Table 2. List of lipid metabolites altered by the *PLA2G6* mutations, D331Y and T572I.

Lipid metabolites identified in PLA2G6 mutation construct injected embryos.

Metabolites	Log2 Fold change (/control)		P-value (t.test)	VIP	Change trend[#]
	D331Y	T572I	T572I vs.C	T572I vs.C	
PC (16:0/18:1)	0.15	0.43	0.000489	10.25	Increased
PC(16:0/18:2)	0.15	0.37	0.000398	5.45	Increased
PC (18:0/18:1)	0.34	0.96	0.001793	5.26	Increased
PC (16:0/22:6)	0.09	0.29	0.000327	6.71	Increased
PC (18:0/22:6)	0.14	0.69	0.00104	4.56	Increased
PC (18:1/22:6)	0.20	0.38	0.0004	3.57	Increased
PC (18:2/22:6)	0.09	0.39	0.008881	2.59	Increased
PC (18:3/22:6)	0.00	0.33	0.003512	3.05	Increased
PE (16:0/18:1)	0.53	1.17	0.002255	3.21	Increased
PE(p-16:0/18:2)	0.40	0.96	0.000547	6.78	Increased
PE (16:0/22:6)	0.25	0.66	0.000913	5.40	Increased
PI (18:1/20:5)	0.22	0.48	0.002597	3.53	Increased
PI (18:1/22:6)	0.09	0.63	0.000238	2.45	Increased
Cer(t18:0/16:0)	0.97	1.18	2.21E-05	3.06	Increased
lysoPC (18:0)	-0.49	-0.36	0.26354	< 2	Decreased
lysoPC (18:2)	-0.31	-0.08	0.80894	< 2	Decreased
DHA	-0.37	-0.22	0.169679	< 2	Decreased

[#]Metabolite change of PLA2G6 mutation injected group compared with the control group.

Abbreviations

PD: Parkinson's disease; PLA2G6: Phospholipase A2 group VI; PARK14: dystonia–parkinsonism type 14; PARKs: familial PD genes; NBIA: neurodegeneration with brain iron accumulation; INAD: infantile neuroaxonal dystrophy; DHA: docosahexaenoic acid; hpf: hours postfertilization; dpf: days postfertilization; TH: tyrosine hydroxylase; PUFA: polyunsaturated fatty acid; SCFAs: short-chain fatty acids.

Declarations

Ethics approval and consent to participate

All experiments were performed in strict accordance with the standard guidelines for zebrafish studies and were approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC approval number: CGU106-066).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

THY: Conceptualization, formal analysis, investigation, resources, supervision, funding acquisition. **HFL**: Methodology, software, validation, formal analysis, investigation, visualization. **MLC**: Methodology, software, validation, formal analysis. **YCH**: Conceptualization, methodology, formal analysis, supervision, funding acquisition. **YZH**, Conceptualization, methodology, resources, supervision. **CSL**: Conceptualization, methodology, resources, supervision. **CCC**, Conceptualization, methodology, resources. **HYC**, Methodology, validation, visualization. **YCC**: Conceptualization, methodology, formal analysis, investigation, writing, resources, supervision, project administration, funding acquisition. All authors read and approved the final manuscript.

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