BRIEF REPORT

A Novel *PINK1* p.F385S Loss-of-Function Mutation in an Indian Family with Parkinson's Disease

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ABSTRACT: Background: Most Parkinson's disease (PD) loci have shown low prevalence in the Indian population, highlighting the need for further research.
Objective: The aim of this study was to characterize a novel phosphatase tensin homolog-induced serine/threonine kinase 1 (*PINK1*) mutation causing PD in an Indian family.
Methods: Exome sequencing of a well-characterized Indian family with PD. A novel *PINK1* mutation was studied by in silico modeling using AlphaFold2, expression of mutant PINK1 in human cells depleted of functional endogenous PINK1, followed by quantitative image analysis and biochemical assessment.
Results: We identified a homozygous chr1:20648535–20648535 T>C on GRCh38 (p.F385S) mutation in exon 6 of *PINK1*, which was absent in 1029 genomes from India and in other known databases. PINK1 F385S lies within the highly conserved Deutsche Forschungsgemeinschaft (DFG) motif, destabilizes its active state, and impairs phosphorylation of ubiquitin at serine 65 and proper engagement of parkin upon mitochondrial depolarization.
Conclusions: We characterized a novel non-conservative mutation in the DFG motif of *PINK1*, which causes loss of its ubiquitin kinase activity and inhibition of mitophagy. © 2024 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: PINK1; genome sequencing; phosphorylation; ubiquitin; mitophagy; Parkinson's disease

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Introduction

Mutations in phosphatase tensin homolog-induced serine/threonine kinase 1 gene (*PINK1*) cause autosomal recessive Parkinson's disease (PD).[1] PINK1-PD is usually of early onset, is slowly progressive, and shows excellent response to levodopa treatment.[2] The discovery of *PINK1* as a cause of familial PD, together with several other mutations, led to the identification of disturbed mitochondrial quality control as a molecular mechanism underlying PD. Most, if not all, monogenetic causes of PD have been identified in the European population, and there is a lack transferability to other ethnically diverse populations. In our study, we found a novel PINK1 mutation. The p.F385S mutation lies in the highly conserved DFG motif of PINK1, which is

important for its ATP binding and kinase activity.[3] Kinase activity is required for PINK1 to initiate the clearance of damaged mitochondria via PINK1/Parkin mitophagy.[4] Upon mitochondrial stress, PINK1 forms a dimer at the mitochondrial outer membrane (MOM) and autophosphorylates itself, enabling self-activation.[5,6] This activation frees its ubiquitin recognition site and helps in the recruitment of parkin by phosphorylation of ubiquitin at Ser65 and the ubiquitin-like domain of parkin.[7-16] PINK1 further phosphorylates proteins of the MOM, and parkin ubiquitinates them in a cascade to promote outside-in mitophagy.[17-19] Previously described mutations in PINK1 in the Caucasian population, particularly the G386A mutation, also localize in the DFG motif.[11,20] Our aim was to functionally characterize a novel PINK1 mutation causing PD identified in an Indian family.

Subjects and Methods

Monogenetic PD Cohort

Sixty families with PD were compiled over 10 years as part of Cataloging Genetic Architecture of PD and Genomic Registry of Parkinson Disease in the Movement Disorder Centre of Sree Chitra Tirunal Institute for Medical Sciences and Technology, India. All affected and nonaffected individuals were interviewed and examined by a movement disorder specialist. The ethics committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology approved the study. All participants gave written informed consent.

Exome Sequencing

Genomic DNA was extracted using the salting-out method for targeted resequencing and whole-genome analysis. Exome sequencing was performed at the Core Facility of Applied Transcriptomics and Genomics at the Institute of Medical Genetics (University of Tübingen, Tübingen, Germany). The data analysis was performed using a medical genetics sequence analysis pipeline (see Supporting Information Methods: Data S1). We first searched for known mutations in our cohorts, and to find novel PD variants, we filtered the detected variants using two main criteria: (1) the variant must be protein altering or in a splice region; and (2) the variant should have a maximum allele frequency of 0.01% in gnomAD, including subpopulations.

IndiGenomes

A publicly available database was used to search for putative variants in the control genomes ascertained from different regions of India. In brief, a total of 1029 self-declared healthy individuals underwent whole-genome sequencing to develop a comprehensive compendium of genetic variants in the Indian population.[21,22]

Molecular Modeling

We used the full-length (FL) structure of human *PINK1* predicted via AlphaFold2,[23] which is available through accession number Q9BXM7 (https://alphafold.ebi.ac.uk/entry/Q9BXM7), maintained at the European Bioinformatics Institute.[24] We assessed the effect of the *PINK1* F385S mutation through the ddg_monomer protocol[25] from the RosettaCommons molecular modeling suite (https://www.rosettacommons.org/). For further details, see Supporting Information Methods: Data S1. We calculated the statistical significance of energetic calculations through the "ranksums" method of the Python (v3.10.4) scipy (v1.7.3) and seaborn (v0.12.2) libraries.

Site-Directed Mutagenesis

Plasmid pcDNA/V5-His A with wild-type (WT) *PINK1* gene at the multiple cloning site was used to introduce the Phe385Ser (F385S) mutation by altering T>C at position 1154 of the gene with QuikChange II Site-Directed Mutagenesis Kit (200523; Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The primers used were 5′-gcagcagccagaatctgcgatcaccagcca-3′ and 5′-tggctggtgatcgcagattctggctgctgc-3′. The polymerase chain reaction, cycle parameters, and procedure were followed according to the manufacturer's instructions.

Cell Culture and Transfection

WT and PINK1 W437X HeLa cells[26] (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were maintained in Dulbecco's Modified Eagles Medium: 4.5 g/L glucose (D6429; Sigma-Aldrich) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For DNA transfection (Thermo Fisher Scientific, Waltham, MA, USA) with WT-PINK1 pcDNA/V5-His A or F385S-PINK1 pcDNA/V5-His A and WT parkin-3XFLAG pcDNA3.1, cells were seeded at least 24 h before transfecting with Lipofectamine 3000 reagent (L3000008; Invitrogen) according to the manufacturer's instructions.

Cell Culture Treatments

For half-life and stability assays, cells were treated with 100 µM cycloheximide solution (CHX; 239765; Calbiochem, Merck Chemicals GmbH, an affiliate of Merck KGaA, Darmstadt, Germany) and/or 10 µM MG132 (sc210270; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) resuspended in dimethylsulfoxide (DMSO) with an equal volume of DMSO used as a vehicle. For the stability assay, cells were pretreated with MG132 for 2 hours and maintained in the media throughout the 6-hour time course. For the mitophagy initiation assay, 40 µM carbonyl cyanide chlorophenylhydrazone (CCCP) (C2759; Sigma-Aldrich) resuspended in DMSO was used with an equal volume of DMSO as a vehicle.

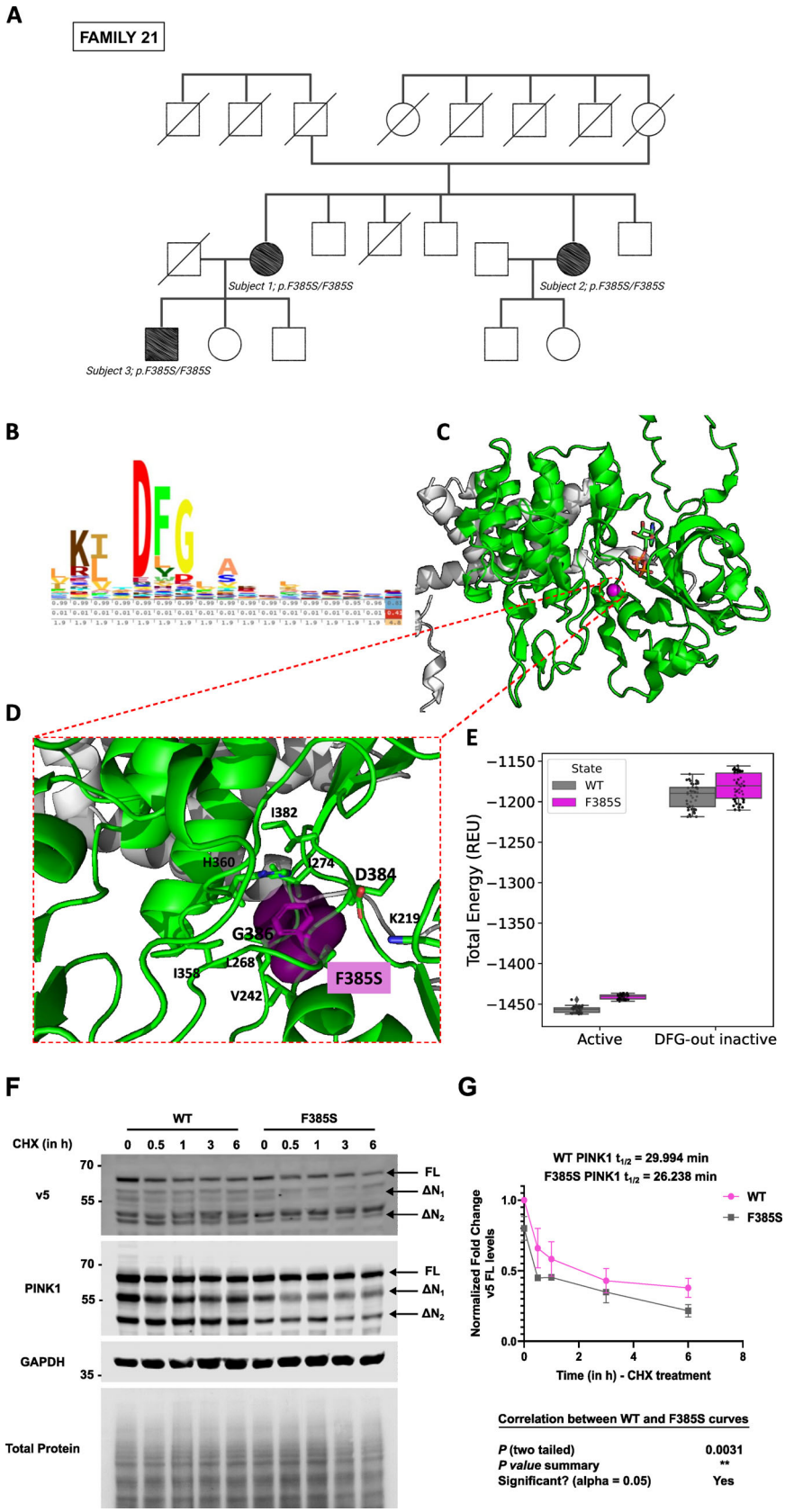


FIG. 1. Legend on next page.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde solution and washed with PBS. Permeabilization and blocking were done using 0.3% Triton X-100 + 1% bovine serum albumin and incubated for 1 hour at room temperature. Antibodies were added in 0.1% Triton X-100 + 1% bovine serum albumin overnight at room temperature inside a humidifier chamber. Primary antibodies were used at 1:1000 for Parkin (2132, rabbit; Cell Signaling Technology Europe B.V. Leiden, The Netherlands), 1:500 for v5 (sc271944, mouse; Santa Cruz), and 1:500 for single-stranded DNA binding protein (SSBP1; AF6588, sheep; R&D Systems- Bio-Techne GmbH, Wiesbaden Nordenstadt, Germany). The secondary antibodies were all used at 1:1000 and included donkey anti-sheep Alexa 488 (A-11015; Invitrogen), donkey anti-mouse Alexa 568 (A10037; Life Technologies, Thermo Fisher Scientific), and goat anti-rabbit Alexa 647 (A21245; Life Technologies). Hoechst (H3569; Molecular Devices, GmbH, Munich, Germany) was used to stain nuclei before mounting with Dako fluorescent mounting medium (S3023; Agilent).

Immunoblotting

Cells were lysed in 1% Triton X-100 + 1% SDS in PBS containing cOmplete protease inhibitor (Millipore 89, 11873580001; Sigma) and PhosStop phosphatase inhibitor (4906837001; Sigma-Aldrich). SDS-PAGE gel and protein transfer was followed by incubation with primary antibodies used at 1:500 for v5 (sc271944, mouse; Santa Cruz), 1:1000 for PINK1 (BC100-494, rabbit; Novus Biologicals, - Bio-Techne GmbH, Wiesbaden Nordenstadt, Germany), 1:1000 for Parkin (ab15954, rabbit; Abcam Limited, Cambridge, United Kingdom), 1:10,000 for GAPDH (CB1001, mouse; Sigma), and 1:500 for phospho-ubiquitin Ser65 (62802S, rabbit; Cell Signaling). Secondary antibodies were used at 1:10,000 (926-32211 and 926-68070; LI-COR Biotechnology - GmbH, Bad Homburg, Germany) and detected with Odyssey CLx (LI-COR) using Image Studio software (LI-COR). Mitochondrial fragmentation analysis used CellProfiler. For semiquantitative image analysis, CellProfiler (4.2.1 version) software was used. In all transfected conditions, a minimum of 169 cells were assessed combining all three replicates, with each replicate including between 37 and 81 cells. In nontransfected cells, a minimum of 157 cells were analyzed per replicate. In all cases, individual cells were recognized with the nuclear staining, and the cytoplasmic area was established using PINK1 and parkin staining. Nuclei were not considered as part of the cytoplasm. For details, see Supporting Information Methods: Data S1.

Parkin Translocation Analysis

Parkin localization was assessed manually using the same raw images and cell number used for SSBP1 morphology analysis (see earlier). In this study, transfected cells were classified as either positive or negative for parkin translocation only when the parkin signal colocalized with SSBP1.

Statistical Analysis and Interpretation

For statistical analyses, GraphPad Prism version 8.4.0 was used. Data are presented as mean average ± standard error of the mean (SEM) ($^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$; $^{****}P < 0.0001$; $^{ns}P > 0.05$, where ns represents nonsignificant). For details, see the figure legends and Supporting Information Methods: Data S1.

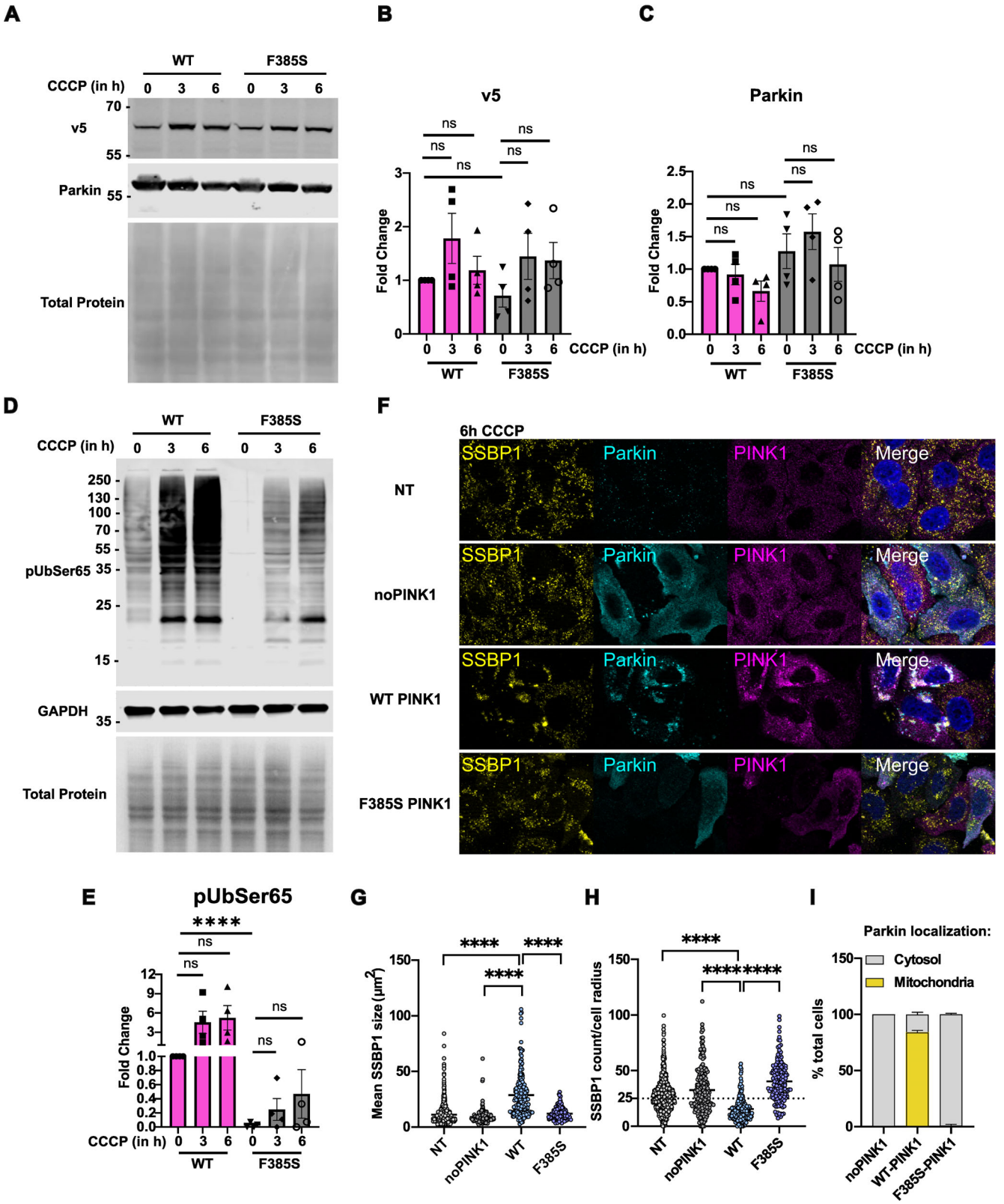
Results

Identification of a Novel *PINK1* p.F385S Mutation

For exome sequencing, the mean sequencing depth was 124×. On average, 94.82% of the 50-Mb target region was covered with at least 20× depth. The exome sequencing identified a novel missense mutation, homozygous *PINK1* p.F385S, in three affected carriers spanning two consecutive generations, but not in healthy subjects (Fig. 1A). Analysis of the sum of runs of homozygosity (ROHs) larger than 500 Kb in the three carriers showed likely consanguinity implicating a pseudodominant inheritance. When looking at the ROHs around *PINK1*, they share an ROH of 2.26 Mb at chr1:19713608–21974391 (hg38) (see Supporting Information Methods: Data S1). Using publicly available IndiGenomes and gnomad databases, the mutation was not present in the Indian and the European population,

FIG. 1. (**A**) The Indian family pedigree showed a pseudodominant mode of inheritance. (**B**) Consensus logo of the Hidden Markov Model of the Kinase domain (https://www.ebi.ac.uk/interpro/entry/pfam/PF00069/logo/) showing the conservation profile of the DFG motif. (**C**) Cartoon three-dimensional representations of the predicted full-length structure of PINK1 in complex with ATP. (**D**) Zoomed view of the contacts mediated by the F385 residue. (**E**) Bar plots showing the predicted ddG for the wild-type (WT) and F385S mutant of PINK1 predicted in its active conformation (left) and DFG-out, inactive conformation (right). (**F**) HeLa cells expressing WT or mutant (F385S) PINK1 were untreated or treated with 100 μM cycloheximide (CHX) for 0.5, 1, 3, or 6 hours. Western blot probed for V5-tagged PINK1 (v5), PINK1, and GAPDH loading control. The Western blot was stained for total protein using the Ponceau stain. Full-length PINK1, N-terminal processed PINK1 (ΔN1), and further N-terminal processed PINK1 (ΔN2) are observed. A representative Western blot is shown (n = 3). (**G**) Quantified levels of V5-tagged full-length PINK1 normalized to total protein levels at 0, 0.5, 1, 3, and 6 hours of CHX treatment. Half-lives (T1/2) of full-length WT and mutant F385S PINK1 are shown (minutes). Error bars show the standard error of the mean (SEM) (n = 3). The correlation of WT and mutant F385S PINK1 levels over time using Pearson's *r* test (2-tailed) is shown. FL, full-length PINK1. [Color figure can be viewed at wileyonlinelibrary.com]

indicating its rarity. Clinically, all affected carriers showed early-onset, typical motor symptoms and psychiatric symptoms with good response to levodopa (see Supporting Information Data S2). *PINK1* was analyzed by multiplex-ligation dependent probe amplification (MLPA), and no gene dosage change was detected.



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In Silico Modeling Predicts the PINK1 F385S Mutation to Disrupt Kinase Activation

PINK1 F385 is part of the highly conserved DFG motif, which is a master regulator of the kinase domain activation (Fig. 1B). We have modeled the functional consequences of the F385S mutation in silico using the structure of monomeric PINK1, predicted by AlphaFold2 (Fig. 1C,D). We predicted the effect of the mutation via energy calculations, which showed a higher ddG for the mutant versus the WT in its active ($P = 9.25E{-}18$) and inactive state ($P = 0.00047$) (Fig. 1E). This suggested a much greater overall destabilization of PINK1, particularly in its active state, and consequently impairment of its kinase function.

Expression and Processing of F385S Mutant PINK1

Under basal conditions, FL PINK1 is efficiently imported into mitochondria. The N-terminal mitochondrial targeting sequence is removed by mitochondrial processing proteases to yield ΔN1. PINK1 is then further N-terminally truncated to yield ΔN2, which is rapidly degraded via the proteasome.[27] Overexpressed WT PINK1-V5 showed this processing pattern: FL, 63-kDa; ΔN1, 53-kDa; and ΔN2, 45-kDa bands, which are consistent with the literature.[28,29] F385S-PINK1-V5 showed the same pattern (Fig. 1F), indicating intact processing of mutant PINK1. To determine protein half-life, we blocked translation with CHX and chased the cells for a time ranging from 0.5 to 6 hours. When probed for anti-V5 and with anti-PINK1 directly, FL-F385S-PINK1 had a slightly but significantly reduced half-life of 26.238 minutes compared with FL-WT-PINK1, which had a half-life of 29.994 minutes (Fig. 1G). To investigate protein stability, we treated the cells with CHX for 0.5 to 6 hours in the presence of MG132 followed by probing with anti-V5 and anti-PINK1 (Supporting Information Fig. S1A) and quantified the levels of FL (Fig. S1B), ΔN1 (Fig. S1C), and ΔN1/FL ratio of v5-tagged PINK1 (Fig. S1D). There were no differences in the WT and F385S-PINK1 levels, suggesting no impact of F385S mutation on PINK1 stability and processing. The reduced half-life of F385S-PINK1 observed in Figure 1G is due to slightly faster degradation of FL-F385S compared with the WT protein, as confirmed by the proteasomal inhibition with MG132. The observed half-life of FL-WT-PINK1 is in line with other studies.[28-30]

F385S-PINK1 Disrupts Phosphorylation of Ubiquitin at Ser65

To confirm that the F385S mutation abolishes PINK1 kinase activity, we assessed a well-known substrate of PINK1 in stressed cells: phospho-ubiquitin Ser65.[8,9,11] We used HeLa cells that lack endogenous parkin,[31] which were gene edited to remove functional, endogenous PINK1.[26] The PINK1-parkin system was reconstituted in the cell culture model by cotransfection of FLAG-tagged parkin ubiquitin ligase and either WT or F385S PINK1-V5. PINK1-parkin–dependent mitophagy was induced by the addition of CCCP to the culture medium. CCCP treatment led to the stabilization of WT-PINK1, with a reduction tendency of F385S-PINK1 over a time course of 6 hours (Fig. 2A–C). FLAG-parkin levels did not alter significantly in this time frame (Fig. 2A–C).

Under basal conditions (no CCCP treatment), there was no phosphorylation of ubiquitin at S65 in F385S-PINK1–expressing cells. However, when treated with CCCP, there is some residual phosphorylation of ubiquitin at Ser65 in F385S-PINK1 cells, yet still ∼90% less phosphorylated compared with WT-PINK1–expressing cells (Fig. 2D,E).

F385S-PINK1 Affects Parkin Recruitment and Mitochondrial Fragmentation

To investigate the implications of the F385S mutation on PINK1 function, we performed mitophagy induction using CCCP as a depolarizing agent. Upon cotransfection with WT-PINK1-V5 and FLAG-parkin, a strong mitochondrial phenotype was observed after 6-hour CCCP treatment, with the matrix mitochondrial marker SSBP1 appearing in big clusters instead of localized foci (Fig. 2F). In contrast, dispersed SSBP1 foci were observed upon transfection with parkin alone, as well as in the parkin and F385S-PINK1 cotransfected cells (Fig. 2F), indicating that F385S mutant PINK1 impairs initiation of PINK1-parkin–dependent mitophagy.[19]

FIG. 2. HeLa cells expressing parkin and either wild-type (WT) or mutant (F385S) PINK1 were untreated or treated with 40 μM CCCP for 3 or 6 hours. (**A**) Western blot probed for V5-tagged PINK1 (v5) and parkin. The Western blot was stained for total protein using Ponceau stain. Full-length PINK1 is shown. A representative Western blot is shown (n = 4). (**B**, **C**) Quantified fold change of V5-tagged PINK1 (B) and parkin levels (C) after CCCP treatment compared with the untreated control. Error bars show mean ± SEM (n = 4). Statistical test: ordinary one-way ANOVA with multiple comparisons using Dunnett's multiple comparison test. (**D**) Western blot probed for phospho-ubiquitin Ser65 (pUbSer65) and loading control GAPDH. The Western blot was stained for total protein using the Ponceau stain. A representative Western blot is shown (n = 4). (**E**) Quantified fold change of pUbSer65 after CCCP treatment compared with the untreated WT control. Error bars show mean ± SEM (n = 4). Statistical test: ordinary one-way ANOVA with multiple comparisons using Dunnett's multiple comparison test. (**F**) Representative images of HeLa cells transfected with parkin (blue) only or together with WT PINK1 or F385S PINK1 (pink) upon 6 hours of CCCP treatment showing SSBP1 marker of mitochondria (yellow). Scale bar: 10 μm. (**G**, **H**) Mean mitochondrial size of SSBP1 foci after 6 hours of CCCP treatment (G); normalized count of the number of SSBP1 foci per cell (H). In both panels (G, H), each dot represents a single-cell value. (**I**) Percentage of cells with mitochondrial or cytosolic parkin after 6 hours of CCCP treatment. Error bars: mean ± SEM. NT, nontransfected (n = 3). [Color figure can be viewed at wileyonlinelibrary.com]

CellProfiler image analysis allowed for an unbiased, automated quantification.[19,32] Upon expression of WT-PINK1, there was a significant increase in the mean size of SSBP1 profiles compared with nontransfected, parkin-only, and F385S-PINK1 plus parkin transfected conditions, indicating successful mitochondrial clustering as a consequence of mitophagy initiation only in WT-PINK1 conditions (Fig. 2G). Concomitantly, SSBP1 mean foci count was significantly reduced as mitochondria coalesced into mitoaggresomes only in WT-PINK1–expressing cells, again suggesting impaired mitochondrial clustering (Fig. 2H). Because translocation of active parkin to mitochondria depends on PINK1 kinase activity, we analyzed parkin localization upon mitochondrial depolarization (Fig. 2I). Strikingly, upon coexpression of parkin and WT-PINK1, parkin was found on mitochondria in 83% of the cells, whereas only 1% of the cells showed mitochondrial parkin in F385S-PINK1 conditions (Fig. 2I). Overall, our data indicate an impaired mitophagy initiation upon expression of F385S-PINK1, confirming F385S to be a loss-of-kinase-function mutation.

Discussion

Addressing the issue of global genetic disparity in genomic research, we are developing a large PD genetic catalog for the Indian population. The identification of novel loci or novel variants in known monogenic PD genes would help to understand the molecular mechanisms. Future therapeutics targeting monogenic forms of PD might not be equally generalizable for every population.[33] Further, this would also allow the possibility of linking risk variants with environmental factors in that region because they are believed to work synergistically to modify PD risk.[34] Here, in our ongoing monogenic project, we report a novel homozygous mutation in the *PINK1* gene. Interestingly, the underlying disease inheritance highlighted a pseudodominant mode in this family, a phenomenon that has been reported previously in European and North African patients with parkinsonism.[20] Although there was no consanguinity reported at the time of sampling, analysis of ROHs predicts likely consanguinity, indicating the pseudodominant mode of inheritance. The majority of the phenotypic features in this family were similar to typical PINK1 PD, with onset in the fourth decade in two affected individuals and slightly later in the third. There was sustained levodopa responsiveness, early fluctuations and dyskinesias, and the common nonmotor symptoms reported with this mutation.[35] The atypical features were cognitive involvement and the occurrence of mild dysphagia for liquids, after a decade from the onset of the disease in this family. Cognitive impairment is considered rare in PINK1 mutation carriers but was reported in 14% of 65 PINK1 mutation carriers in a recent systematic review.[35]

Duplication or deletion of PINK1 was ruled out by sequencing and MLPA, and we designed the functional experiments accordingly by expressing F385S-PINK1 in cells lacking any functional, endogenous PINK1. PINK1/Parkin mitophagy is one of the most widely studied pathways to functionally characterize the role of both PINK1 and parkin in cell culture systems, with PD-associated mutations in PINK1 having a compromised canonical mitophagy.[36] To functionally characterize F385S-PINK1, we employed this assay and found the F385S PINK1 mutation to be detrimental to PINK1 kinase activity because there is absence of phosphorylated ubiquitin at Ser65. Consequently, parkin activation is also affected, observed by reduced parkin translocation and mitoaggresome formation in F385S-PINK1 cells, which are unable to initiate mitophagy under depolarizing conditions. F385S-PINK1 has a slightly shorter half-life than WT PINK1. Thus, the reduced kinase activity of F385S mutant PINK1 could also be attributed partially to the reduced half-life of FL-PINK1. Our quantifications of PINK1 half-life were restricted to the FL form for reliability.

In summary, we identified a novel p.F385S PINK1 mutation in an Indian family. We characterized the mutation both structurally and functionally and concluded that this mutation results in a severe loss of its kinase activity. Our work provides an understanding of pathological events that are governed by PINK1 loss-of-function mutations in PD and highlights the importance of including diversity in PD genetics. ■

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Data Availability

The information on genetic variants from the Cataloging Genetic Architecture of PD cohort is available upon request from the corresponding author.

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Supporting Data

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