FBXO7 Y52C Polymorphism as a Potential Protective Factor in Parkinson’s Disease

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Abstract

Mutations in the F-box only protein 7 gene (FBXO7), the substrate-specifying subunit of SCF E3 ubiquitin ligase complex, cause Parkinson’s disease (PD)-15 (PARK15). To identify new variants, we sequenced FBXO7 cDNA in 80 Taiwanese early onset PD patients (age at onset ≤50) and only two known variants, Y52C (c.155A>G) and M115I (c.345G>A), were found. To assess the association of Y52C and M115I with the risk of PD, we conducted a case–control study in a cohort of PD and ethnically matched controls. There was a nominal difference in the Y52C G allele frequency between PD and controls (p = 0.045). After combining data from China [1], significant difference in the Y52C G allele frequency between PD and controls (p = 0.012) and significant association of G allele with decreased PD risk (p = 0.017) can be demonstrated. Upon expressing EGFP-tagged Cys52 FBXO7 in cells, a significantly reduced rate of FBXO7 protein decay was observed when compared with cells expressing Tyr52 FBXO7. In silico modeling of Cys52 exhibited a more stable feature than Tyr52. In cells expressing Cys52 FBXO7, the level of TNF receptor-associated factor 2 (TRAF2) was significantly reduced. Moreover, Cys52 FBXO7 showed stronger interaction with TRAF2 and promoted TRAF2 ubiquitination, which may be responsible for the reduced TRAF2 expression in Cys52 cells. After induced differentiation, SH-SY5Y cells expressing Cys52 FBXO7 displayed increased neuronal outgrowth. We therefore hypothesize that Cys52 variant of FBXO7 may contribute to reduced PD susceptibility in Chinese.

Introduction

Parkinson’s disease (PD) is attributable to environmental factors, whereas evidence has shown that genetic factors are also playing an important role. Molecular genetic studies have identified thirteen genes linked to rare dominant or recessive monogenic forms of PD: SNCA, Parkin, PINK1, DJ-1, LRRK2, ATP13A2, VPS35, PLA2G6, FBXO7, EIF4G1, SYN1, DNAJC6, and DNAJC13 [2,3].

Recently, the F-box only protein 7 (FBXO7) mutations have been identified in several families with early-onset parkinsonism and pyramidal tract signs. Homozygous R378G missense mutation in an Iranian kindred, homozygous nonsense mutation (R498X) in an Italian family, a Pakistan family and a Turkey family, and compound heterozygous mutations (IVS7+1G/T and T22M) in a Dutch family are unambiguously responsible for the autosomal recessive, early-onset, parkinsonian-pyramidal syndrome [4,5,6]. The presynaptic nature of the parkinsonism in the families reported by Di Fonzo et al. is shown by the dramatic abnormality of DaTSCAN SPECT, the beneficial effect of levodopa, and the presence of levodopa-induced dyskinesias, suggesting that FBXO7 mutations may be the potential genetic causes of early-onset Parkinson’s disease (EOPD) or familial PD [5]. Two missense substitutions, p.Ile87Thr and p.Asp328Arg, in a single heterozygous state, were found in two EOPD patients in Taiwan [7]. Although no pathogenetic mutations in the FBXO7 gene were detected in 135 Chinese early-onset parkinsonism patients, the PD patients showed a trend toward decrease in Y52C G allele frequency compared with the controls [1].

FBXO7, a member of the F-box-containing protein (FBP) family, encodes a protein of 522 amino acids consisting of several discrete domains: the ubiquitin-like domain, cyclin-dependent protein kinase 6 (CDK6) binding site, FBXO7/PI3 domain, F-box motif, proline-rich region, and R(ar)DP motif [5,8]. Through the interaction between the F-box and the Skp1 protein, FBPs become part of SCF (Skp1-Cullin-F-box protein) ubiquitin ligase complexes, and play roles in ubiquitin-mediated proteasomal degradation [review in [9]]. F-box proteins recruit a large number of diverse substrates to SCF complexes and allow for their ubiquitination [10]. FBXO7 promotes ubiquitin conjugation to TRAF2 (a member of the tumor necrosis factor receptor associated factor protein family with ubiquitin ligase activity) and cIAP1 (an apoptosis inhibitor possessing ubiquitin ligase activity), resulting in decreased receptor-interacting protein 1 (RIP1)
ubiquitination and lowered NF-kB signaling activity [11,12]. Strong evidence has shown that NF-kB induced neuroinflammation may be involved in development of PD [13,14]. Therefore, FBXO7 may play a role in protecting neurons from PD process. In contrast, the mutations or variations that change the function, expression, or stability of FBXO7 may confer PD risk to the subjects.

**Results**

**Mutation/variant analysis of FBXO7**

Since most of the mutations found in FBXO7 result in truncated FBXO7 protein, amino acid replacement or multiple aberrant frame-shift splice variants, we sequenced FBXO7 cDNA (Table 1) instead of genomic DNA in a cohort of ethnic Chinese patients with EOPD in Taiwan to identify previously undetected variants in EOPD cases of Chinese origin, followed by a case–control study for the identified variants. The cDNA samples contained only DNA sequences from genes that were transcribed into RNA. Thus FBXO7 cDNA fragments from 80 EOPD patients were amplified for sequence analysis. However, only two known substitutions that caused changes in the peptide sequence were identified: a c.155A>G substitution leading to an amino acid change from tyrosine to cysteine in position 52 (Y52C) in one EOPD patient (heterozygote) and a c.345G>A substitution resulting in a methionine to isoleucine change at amino acid position 115 (M115I) (rs11107) in 74 EOPD patients (40 homozygotes and 34 heterozygotes) (amino acid number according to NM_012179) (Fig. 1A). The two reported [1] variants were confirmed using PCR-restriction fragment length polymorphism (RFLP) method (Fig. 1B). Both Y52C and M115I are not evolutionarily conserved in the known mammalian homologues of the FBXO7 protein (Fig. 1C).

**Case–control study of Y52C and M115I**

A case–control study in a cohort of PD patients (n = 516, 80 EOPD patients included) and ethnically matched controls (n = 516) was conducted to assess the association of Y52C and M115I with the risk of PD (Table 2). All genotype frequencies were confirmed to be in the Hardy–Weinberg equilibrium. There was no statistically significant difference (p<0.025) in genotype or allele distribution between patients and controls for both single nucleotide polymorphisms (SNPs) examined, after correction of multiple SNP testing. However, for Y52C, the frequency of AG genotype (0.8% vs. 2.3%, p = 0.045) was notably lower in PD patients than the controls. Y52C AG genotype or G allele demonstrated a trend toward decrease in risk of developing PD (odds ratio: 0.33, 95% confidence interval: 0.09–0.95, p = 0.055–0.056). Analysis combining our patient and control subjects as well as the population in Luo’s study [1] yielded results of statistically significant difference in genotype (0.9% vs. 2.8%, p = 0.012) and allele (0.5% vs. 1.4%, p = 0.012) distribution between patients and controls. The negative association of the Y52C AG genotype or G allele with PD was significant (odds ratio: 0.32–0.33, 95% confidence interval: 0.12–0.77, p = 0.016–0.017). The identified one EOPD and three late-onset PD patients carrying Y52C presented with asymmetrical tremor, rigidity, and bradykinesia without pyramidal signs, all of whom had a good response to anti-parkinsonian medication.

**FBXO7 expression analysis**

Since the case–control study suggests that FBXO7 Y52C G allele might be a potential protective factor, we cloned the polymorphic FBXO7 cDNA, which was then expressed in HEK-293T cells to investigate the functional consequences.

The common cellular abnormality found in the PARK15 patients from the Dutch and Italian families is the depletion of the FBXO7 isoform 1 (NM_012179), which normally is located in the cell nucleus [15]. Forty-eight hours after transfection of EGFP tagged FBXO7 isoform 1 constructs, cells were analyzed by fluorescent microscopy. Although Cys52, FBXO7 protein displayed nuclear and cytosolic staining pattern similar to Tyr52, a significantly stronger green (FBXO7 fusion protein) relative to blue (nuclei staining) fluorescence signal was observed in Cys52 FBXO7 cells (1.60 vs. 2.57, p = 0.015; Fig. 2A). To further examine the transiently expressed FBXO7-EGFP fusion protein, protein blotted with FBXO7 antibody was performed. As shown in Fig. 2B, FBXO7-EGFP fusion protein in the expected size range for Tyr52 and Cys52 constructs was observed. However, the protein expression level of Cys52 FBXO7 was increased compared with the Tyr52 FBXO7 (211%, p = 0.016). The stability of Cys52 variant was further examined by a cycloheximide (200 μg/ml) chase experiment. While the Tyr52 protein was degraded to 68%, 18%, 11%, 9% and 7% left after 6, 12, 24, 36, and 48 hr of protein synthesis blocking, reduced rates of decay were observed for Cys52 variant (90%, 78%, 72%, 52%, and 21% remained, respectively) (Fig. 2C).

**Homology modeling of Cys52 FBXO7**

To understand the structure-based information of Cys52 variant in FBXO7, homology modeling of Tyr52 and Cys52 FBXO7 was performed. After energy minimization, the modeled structures for Tyr52 (WT) and Cys52 (Y52C) were shown in Fig. 3. The potential energy of Tyr52 and Cys52 mutant was –2463.854 and –2471.736 kcal/mol, indicating Cys52 FBXO7 exhibited a more stable feature than Tyr52 FBXO7. According to hydrogen-bond (H-bond) computing analysis, the H-bond interaction of Tyr and Cys52 was shown. In the Tyr52 model, Tyr52 did not form any H-bonds with adjacent residue. On the other hand, H-bond formed by the Cys52 with the Asp54 causes decrease of the local energy.
cells as compared to vector-transfected cells (100% vs. 80%, p = 0.015) (Fig. 5B). These results suggest that FBXO7 may decrease TRAF2 abundance through promoting TRAF2 ubiquitination.

Suppression or over-expression of FBXO7 affecting cell survival upon MPP⁺ treatment

To gain further insight into the possible role of FBXO7 protein involved in PD, the mitochondrial inhibitor MPP⁺ was applied to FBXO7 knockdown or over-expressing cells for 24 hours. MTT assay was performed to evaluate the cell viability. As shown in Fig. 6A, the knocked down of FBXO7 was confirmed as 76% (p = 0.005–<0.001) and 81% (p = 0.004–0.001) of FBXO7 were seen in FBXO7 siRNA-transfected HEK-293T and SH-SY5Y cells, respectively, as compared to control siRNA-transfected (106%~110%) or untransfected (100%) cells. Fig. 6B examined if suppression or over-expression of FBXO7 affects cell survival upon MPP⁺ treatment. With 300 μM MPP⁺ treatment, the cell viability of HEK-293T cells expressing FBXO7 siRNA was 61% of the untreated cells, which is significantly lower than that of untransfected (69%, p = 0.001) or control siRNA transfected (68%, p = 0.033) HEK-293T cells. A similar result was obtained in SH-SY5Y cells treated with 2 mM MPP⁺, as suppression of FBXO7 expression also significantly decreased the cell viability (64%) as compared to untransfected (73%, p = 0.024) or control siRNA transfected (74%, p = 0.013) cells. Conversely, significantly increased cell viability was observed with MPP⁺ treatment in FBXO7-EGFP over-expressing cells compared to untransfected cells (HEK-293T: 73% vs. 69%, p = 0.013; SH-SY5Y: 81% vs. 73%, p = 0.037).

SH-SY5Y cell model

To test the effect of Cys52 on neuronal phenotype, we constructed Flp-In SH-SY5Y cells with Tyr52 or Cys52 FBXO7-EGFP expression in an inducible fashion. Immunoblot analysis showed that the FBXO7 protein level was significantly increased in Cys52 cells as compared to that of Tyr52 cells after induction with doxycycline (+ Dox) for 2 days (120%, p = 0.042).

Figure 1. Variant identification and amino acid sequence alignment. (A) Chromatograms of direct cDNA sequencing of Y52C and M115I. (B) Restriction analysis of Y52C and M115I. On agarose gel, Y52C creates new PstI restriction site by mismatch PCR and leads to an additional 222 bp band, whereas M115I loses restriction by PaeI and leads to 479 bp band. (C) Evolutionary conservation of the regions of FBXO7 Y52C and M115I using the program Vector NTI.

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Table 1. Primers and conditions for PCR amplification of FBXO7 cDNA and genomic DNA.

| Test (amplified region)          | Anneal (°C) | MgCl2 (mM) | RFLP enzyme |
|----------------------------------|------------|-----------|-------------|
| cDNA sequencing                  |            |           |             |
| F: CTCTTCCCCGTTTCGCC             |            | 58/1.5    | RFLP: CTGCAG |
| R: GGAGAAGAAGGAGGAGGAGA          |            | 1955      | (240/222, 18) |
| pEGFP-N1-FBXO7 cDNA cloning      |            |           |             |
| F: AAGCTTCTTCTCTCCTTGCTCAG       | 62/1.5     | 1727      |             |
| R: ACCGGTGAGATAGCAGCCGGCC         |            | 1726      |             |
| pcDNA3.1/V5-His-FBXO7 cloning    |            |           |             |
| F: AAGCTTCTTCTCTCCTGGCTCAG       | 62/1.5     | 1726      |             |
| R: CTGAGACATGATGACAGCCGGCCATC    |            |           |             |

Y52C (TAC>TGC)                     |            |           |             |
| F: AAGCTGAGGAGGGAGGATG           | 58/1.5     | Psrl: CTGCAG |
| R: CTCCAGTGAGGAGGATGCTG          |            | (240/222, 18) |

M151I (ATG>ATA)                    |            |           |             |
| F: TCACCTGAGGATGAGAAGACC         | 58/1.5     | Pael: GCATGC |
| R: AATCGGCTGAAGCTGGAGACC         |            | (300, 179/479) |

The Psrl restriction site was created by PCR using a mismatch primer. For Y52C and M151I amplification, the underlines in the primer sequence indicate the mismatch nucleotide and polymorphic site, respectively. For cDNA cloning, the underlines in the primer sequence indicate the introduced HindIII, AgeI and XhoI restriction sites.

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(Fig. 7A). Compared to the non-induced cells (- Dox), TRAF2 protein level was significantly decreased in both Tyr52 (100% vs. 120%, p = 0.024) and Cys52 (87% vs. 128%, p = 0.022) FBXO7-EGFP expressed cells. The difference in TRAF2 abundance between Tyr52 and Cys52 FBXO7-EGFP expressed cells was also significant (100% vs. 87%, p = 0.042). These FBXO7 cells were induced for differentiation with retinoic acid [16] for 7 to 21 days. Representative fluorescence microscopy images of cells differentiated for 21 days are shown in Fig. 7B. Significantly more total outgrowth in Cys52 cells was observed compared to Tyr52 cells after differentiation for 7–21 days (131–165%, p = 0.014–<0.001).

Discussion

Up to now, only four different types of FBXO7 mutations (T22M, R378G, R498X, IVS7+1G/T) have been reported to be responsible for parkinsonian-pyramidal disease, which has been designated as the cause of PARK15 [4,5,6]. Recently, two missense substitutions (p.Ile87Thr and p.Asp328Arg) were found in two EOPD patients in Taiwan [7]. We did not detect any mutation in our EOPD patients, which is compatible with the previous results of studies showing rare pathogenic mutations of the FBXO7 gene in typical PD patients of Chinese ethnicity [1,7]. Nevertheless, our method using cDNA sequencing may miss some mutations in the non-coding and regulatory elements of the FBXO7 gene. Also variants which result in a truncated protein or nonsense mediated decay would not be identified.

Although the role of FBXO7 in neurons is still not known, because it is a part of SCF ubiquitin ligase complex, its function in the ubiquitin-mediated protein degradation is implicated [9]. Impaired ubiquitin-mediated protein degradation has been found in sporadic PD [17], autosomal dominant PARK1 [18], and autosomal recessive PARK2 [19]. Therefore, it is postulated that FBXO7 mutations may compromise the ubiquitin-proteasome function and cause neuronal dysfunction in PD. In this study, we showed that the Y52C AG genotype or G allele of FBXO7 conferred a reduced susceptibility to Chinese PD when the data from our study and Luo’s [1] were combined. However, as Y52C G allele is rare and the difference in frequency is small (0.4% in PD and 1.2% in controls), the genetic evidence is very limited and the findings may be due to chance alone. While FBXO7 mutations typically causes autosomal recessive parkinsonism with pyramidal tract signs, the Y52C AG genotype or G allele in this study appears to provide a protective effect in a dominant mode. T22M, R378G, R498X mutations resulted in decreased stability of FBXO7 protein and T22M caused loss of its nuclear activity, both of which may jeopardize the neuronal function [15]. Using the program SWISS-MODEL, we showed that the Cys52 formed H-bond with the Asp54 causing decreased local energy, which may increase the stability of the protein. The increased stability of FBXO7 protein consequent to Cys52 was further confirmed by cyclolixemide chase experiment (Fig. 2). These results suggest that Cys52 may play a protective role in PD via increasing stability of FBXO7 protein, which is in contrast to the decreased stability and loss of function caused by T22M, R378G, R498X mutations.

The study in zebrafish further suggests that FBXO7 plays an important role in the development of dopaminergic neurons and its loss of function caused by mutations may be responsible for the phenotype of PD. FBXO7 can interact with three proteins including hepatoma upregulated protein, cIAP1, and the proteasome inhibitor protein P131 [11,21,22]. FBXO7 was also reported to enhance activity of cyclin D/cdk6 that plays an important role in regulating neuronal death processes [23]. How these interactions may contribute to the pathogenesis of PD remains to be clarified.
More emerging evidence has suggested that neuroinflammation is involved in the pathogenesis of PD through inflammatory mediators such as TNFα, nitric oxide (NO), IL-6, and IL-1β [13]. NF-κB activation is required for all of these inflammatory mediators to be produced by microglial cells [14]. Recently, few NF-κB inhibitors have been applied to the therapeutic approaches of several chronic inflammatory diseases including PD [13]. Interestingly, FBXO7 protein has been shown to be a negative regulator of NF-κB signaling pathway, through binding to and ubiquitinating TRAF2 and cIAP1, which would lead to decreased RIP1 ubiquitination and NF-κB activity [12]. To further investigate the role of FBXO7 in NF-κB signaling pathway, through binding to and ubiquitinating TRAF2 and cIAP1, which would lead to decreased RIP1 ubiquitination and NF-κB activity [12].

Materials and Methods

Ethics statement

This study was performed according to a protocol approved by the Institutional Review Board of Chang Gung Memorial Hospital, and all examinations were performed after obtaining written informed consents.
A total of 516 unrelated Taiwanese PD subjects (45.0% females) were recruited from the neurology clinics of Chang Gung Memorial Hospital (CGMH). All patients were diagnosed by two neurologists specialized in movement disorders (Y.-R. Wu and C.-M. Chen) with probable idiopathic PD according to the published criteria [24], which includes substantial and sustained response to levodopa or a dopamine agonist. Subjects with prior history of multiple cerebrovascular events or other causes of parkinsonian symptoms (e.g. brain injury or tumor, encephalitis, antipsychotic medication) were excluded. The mean age at onset (AAO) of PD was 62.0 ± 11.5 years, ranging between 19 and 93 years. For juvenile PD patients (AAO < 50) (n = 80, mean age at onset 43.7 ± 0.7 years, 33.7% females), RNA was extracted using PAXgene Blood RNA Kit (PreAnalytiX). The RNA was DNase (Stratagene) treated, quantified, and reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Using polymerase chain reaction (PCR) with designed primers and conditions (Table 1), the 1955-bp amplified FBXO7 cDNA was gel purified and sequenced directly using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The identified Y52C and M115I variants were verified by genomic DNA PCR and sequencing. For

Subjects

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Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using the standard protocols. For PD patients with onset ≤ 50 (n = 80, mean age at onset 43.7 ± 0.7 years, 33.7% females), RNA was extracted using PAXgene Blood RNA Kit (PreAnalytiX). The RNA was DNase (Stratagene) treated, quantified, and reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Using polymerase chain reaction (PCR) with designed primers and conditions (Table 1), the 1955-bp amplified FBXO7 cDNA was gel purified and sequenced directly using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The identified Y52C and M115I variants were verified by genomic DNA PCR and sequencing. For
population screening, the Y52C and M115I were examined using the \textit{Pst}I and \textit{Pae}I (gain of sites) restriction enzymes, respectively (Table 1). The digested PCR products were visualized with ethidium bromide after electrophoresis in 2.2\% or 1.6\% agarose gel.

FBXO7 cDNA constructs

Using the designed primers to remove translation termination codon (Table 1), the full-length \textit{FBXO7} cDNA fragments from an individual heterozygous for Cys52 were cloned into pGEM-T Easy vector (Promega) and sequenced. The 1.7 kb \textit{Hind}III (added in the forward primer)--\textit{Age}I (added in the reverse primer)
fragments were removed from pGEM-T Easy vector and ligated into the corresponding sites of pEGFP-N1 (Clontech) to generate Tyr52 and Cys52 FBXO7 cDNA in-frame fused to the EGFP gene. The resulting EGFP-tagged FBXO7 constructs were used in transient expression studies for confocal microscopy examination, FBXO7 stability and anti-TRAF2 co-immunoprecipitation. Additionally, the HindIII-XhoI fragments containing FBXO7 were ligated into pcDNA3.1/V5-His (Invitrogen) to generate Tyr52 and Cys52 FBXO7 cDNA in-frame fused to the V5-His for Western blot analysis of NF-κB signaling pathway protein TRAF2.

Cell cultivation and transfection

Human embryonic kidney (HEK)-293T (ATCC No. CRL-11268) cells were cultivated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) in a 37°C humidified incubator with a 5% CO2 atmosphere. Cells were plated into 6-well (6×10^5/well) dishes, grown for 20 hr and transfected by the lipofection method (GibcoBRL) with EGFP-tagged FBXO7 constructs (4 μg/well). The cells were grown for 48 hr for the protein studies. To evaluate the stability of FBXO7 protein, protein synthesis inhibitor cycloheximide (200 μg/ml) was added 24 hr after transfection for 0, 6, 12, 24, 36, and 48 hr before protein preparation. For immunoprecipitation studies, proteasome inhibitor MG-132 (5 μM) was added 24 hr after transfection for 24 hr before protein preparation.

Confocal microscopy examination

For visualizing intracellular FBXO7-EGFP protein, transfected cells on coverslips were stained with 4’-6-diamidino-2-phenylindole (DAPI) to detect nuclei. The stained cells were examined for dual fluorescent imaging using a Leica TCS confocal laser scanning microscope.

siRNA transfection and cell viability assay

Human neuroblastoma SH-SY5Y cells (ATCC No. CRL-2266) were maintained in DMEM F12 supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO2. For FBXO7 knockdown, siRNA specifically targeting FBXO7 (J-013606-06, Y52C FBXO7 with Reduced PD Risk in Chinese

Figure 5. Co-immunoprecipitation of Tyr52 or Cys52 FBXO7-EGFP protein and ubiquitination of TRAF2. (A) HEK-293T cells were transiently transfected with Tyr52 or Cys52 FBXO7-EGFP construct. After 48 h, cell lysates were prepared (Input, left panel) and immunoprecipitations (IP, right panel) were performed with anti-TRAF2 antibody. Normal IgG was used as a negative control for IP. Cell lysates and immunoprecipitates were analyzed with anti-EGFP, anti-TRAF2, anti-ubiquitin or anti-actin antibody. (B) Quantification of immunoprecipitated FBXO7-EGFP, TRAF2 and ubiquitin in HEK-293T cells transiently transfected with Tyr52 or Cys52 FBXO7-EGFP construct for 2 days. Data are represented as the means ± S.D. of three separate experiments.
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Thermo Scientific) was transfected into HEK-293T (by lipofection) or SH-SY5Y cells (by 4D-Nucleofector System, Lonza). For transfection of SH-SY5Y cells, cells \((2.5 \times 10^5/48\text{-well})\) were centrifuged at \(906\) \(g\) for 10 min at room temperature. The cell pellets were resuspended in 4D-Nucleofector Solution \((100\ \mu\text{L})\) with siRNA \((100\ \text{nM})\) or FBXO7-EGFP \((250\ \text{ng})\) and CA-137 appropriate program was selected. The transfected cells were plated in 48-well plate and grown for 48 hr for protein analysis. Alternatively, after transfection for one day, cells were treated with freshly prepared mitochondrial inhibitor MPP\(^+\) \((300\ \mu\text{M} \text{ for } \text{HEK-293T} \text{ or } 2\ \text{mM} \text{ for } \text{SH-SY5Y})\) and toxic effects of MPP\(^+\) were monitored at 24 hr by MTT assay. doi:10.1371/journal.pone.0101392.g006

Figure 6. Toxic effects of MPP\(^+\) in FBXO7 suppressed or over-expressed cells. (A) HEK-293T and SH-SY5Y cells were transfected with FBXO7-specific or control siRNA. After 48 h, cell lysates were prepared and Western blot analysis was performed using FBXO7 and anti-actin (as loading control) antibodies. (B) HEK-293T and SH-SY5Y cells were transfected with siRNA (FBXO7-specific or control), FBXO7 cDNA or not \((-\)\). After 24 hr, cells were treated with MPP\(^+\) \((300\ \mu\text{M} \text{ for } \text{HEK-293T} \text{ or } 2\ \text{mM} \text{ for } \text{SH-SY5Y})\) and toxic effects of MPP\(^+\) were monitored at 24 hr by MTT assay. doi:10.1371/journal.pone.0101392.g006

Western blot analysis

Cells were lysed in hypotonic buffer \((20\ \text{mM HEPES pH 7.4, 1 mM MgCl}_2, 10\ \text{mM KCl, 1 mM DTT, 1 mM EDTA pH 8.0})\) containing the protease inhibitor mixture (Sigma). After sonication and sitting on ice for 20 min, the lysates were centrifuged at 14,000\(\times\)g for 30 min at 4\(^\circ\)C. Protein concentrations were determined using the Bio-Rad protein assay kit, with albumin as standards. Total proteins \((25\ \mu\text{g})\) were electrophoresed on 10\% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Schleicher and Schuell) by reverse electrophoresis. After being blocked, the membrane was stained with anti-FBXO7 (1:10000 dilution, GeneTex), anti-GAPDH (1:10000 dilution, GeneTex), anti-ubiquitin (1:10000 dilution, Dako) antibody. The immune complexes were detected using horseradish peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch) or goat anti-rabbit (Rochland) IgG antibody.
Immunoprecipitation

Total protein from MG-132-treated FBXO7-EGFP-transfected cells was prepared using IP lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol) containing the protease inhibitor mixture. After quantification, proteins (100 μg) were immunoprecipitated with mouse TRAF2 primary antibody (2 μg, Santa Cruz) conjugated to protein G beads (Millipore). The beads-proteins-antibody mixtures were washed three times with IP lysis buffer and the immunoprecipitated proteins were eluted by 1× SDS sample loading buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 0.005% bromophenolblue), separated on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane and probed with indicated antibodies as described.

FBXO7 SH-SY5Y cell lines generation

The SH-SY5Y-derived Flp-In host cells [28] and Flp-In T-REx System (Invitrogen) was used to generate stably induced SH-SY5Y cell lines exhibiting tetracycline-inducible expression of Tyr52 and Cys52 FBXO7. Briefly, the SH-SY5Y host cells were co-transfected with pOG44 plasmid (constitutively expressed the Flp recombinase) and pcDNA5/FRT/TO-FBXO7-EGFP plasmid according to the supplier’s instructions. These cell lines were grown in medium containing 5 μg/ml blasticidin and 100 μg/ml hygromycin. Doxycycline (dox, 5 μg/ml) was added to induce EGFP-tagged FBXO7 expression for two days. The proteins were prepared for Western blotting using antibody to FBXO7 or actin as described. Neuronal phenotypes were examined after induced differentiation with retinoid acid (10 μM) and induced expression of FBXO7 for 7 to 21 days. The morphologic differentiation of Tyr52 and Cys52 SH-SY5Y cells including total outgrowth, processes, and branches was assessed by using Metamorph microscopy automation and image analysis software (Molecular Devices).

Statistical analysis

The genotype frequency data and the expected genotypic frequency under random mating were computed and Chi-square tested for Hardy-Weinberg equilibrium using standardized formula. The genotype and allele association analysis was carried out using the Chi-square test. Odds ratios with 95% confidence intervals (95% CI) were calculated to test association between genotype/allele and disease. Given the observed Y52C G allele frequency of 0.0078 (0.0095, in combined data from Taiwan and China) and a total of 1032 (1367, combined subjects from Taiwan and China) subjects in the present study, at significance level of 0.05, we had power greater than 0.8 to identify an association when the allele genetic effect size was greater than 3.1 (2.5). Given the observed M115I G allele frequency of 0.286 and 1032 subjects in the present study, at significance level of 0.05, we had power greater than 0.8 to identify an association when the allele genetic effect was greater than 1.5.

For statistical analysis of microscopy images, immunoblots and cell viability assays, data were expressed as the means ± standard deviation (SD). Three independent experiments were performed and non-categorical variables were compared using the Student’s t-test. All p-values were two-tailed, with values of p<0.05 being considered significant.
Homology modeling

We modeled the three dimensional structures of the Tyr52 and Cys52 FBXO7 proteins by comparative methods and energy minimization using the program SWISS-MODEL [29]. The 2.9-A coordinate set for the crystal structure of human UBC protein (PDB code 2ZVO, chain A) served as the template for modeling the residue 1–79 of human FBXO7. The energy computation was done with the GROMOS96 [30] implementation of Swiss-ModelViewer. The resulting FBXO7 three-dimensional models were manipulated and rendered in PyMOL. (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrodinger, LLC).

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Conceived and designed the experiments: C-MC G-JL-C Y-JL Y-RW. Performed the experiments: I-CC Y-CH Y-LC C-HL L-CL C-ML. Analyzed the data: C-MC I-CC G-JL-C Y-JL Y-RW. Contributed reagents/materials/analysis tools: C-MC H-FJ Y-CC Y-RW. Wrote the paper: C-MC I-CC G-JL-C Y-RW.