conclusion of the above studies [31,32], i.e., the formation of cytoplasmic inclusions is not a toxic response against cell survival. Viewed from a different angle, our finding may suggest that inclusion bodies formed following proteasomal inhibition are independent of cell death.

In conclusion, we demonstrated the presence of pIκBα in LB of PD, and that similar inclusion bodies are produced in the presence of significant proteasomal dysfunction in cultured cells. Our observations in cultured cells may reflect, at least in part, the formation of LB in dopaminergic neurons of PD.

Acknowledgments

The authors thank Drs. Hideo Fujiwara and Takeshi Iwatsubo (University of Tokyo) for providing excellent advice. This study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, by the Fund for "Research for the Future" Program from the Japan Society for the Promotion of Science. This study was also supported by funds from the NH&MRC and Michael J Fox Foundation.

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Common anti-apoptotic roles of parkin and α-synuclein in human dopaminergic cells

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Received 17 April 2005
Available online 30 April 2005

Abstract

Parkin, a product of the gene responsible for autosomal recessive juvenile parkinsonism (AR-JP), is an important player in the pathogenic process of Parkinson's disease (PD). Despite numerous studies including search for the substrate of parkin as an E3 ubiquitin–protein ligase, the mechanism by which loss-of-function of parkin induces selective dopaminergic neuronal death remains unclear. Related to this issue, here we show that antisense knockdown of parkin causes apoptotic cell death of human dopaminergic SH-SY5Y cells associated with caspase activation and accompanied by accumulation of oxidative dopamine (DA) metabolites due to auto-oxidation of DOPA and DA. Forced expression of α-synuclein (α-SN), another familial PD gene product, prevented accumulation of oxidative DOPA/DA metabolites and cell death caused by parkin loss. Our findings indicate that both parkin and α-SN share a common pathway in DA metabolism whose abnormality leads to accumulation of oxidative DA metabolites and subsequent cell death.

Keywords: Parkin; Apoptosis; Antisense; Knockdown; Neuroblastoma; Synuclein dopamine metabolism; Quinone

Parkinson's disease (PD) is the second most common neurodegenerative disorder primarily caused by selective loss of dopaminergic neurons in the midbrain substantia nigra pars compacta. Familial PD has been highlighted to study the mechanisms underlying neurodegeneration in PD, although only 5–10% of patients with PD are of the familial form of PD [1,2]. To date, 10 causative genes have been mapped and cloned in familial PD by linkage studies, which have significantly enhanced our understanding of the genetic mechanisms of PD [3]. Of these genes, *parkin*, the causative gene (*PARK2*) of AR-JP, representing the most prevalent form of familial PD [4], is of special interest, because it encodes an E3 ubiquitin–protein ligase [5], which

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.04.124

covalently attaches ubiquitin to target proteins, designating them for destruction by the proteasome [6,7]. These findings suggest that impediment of *parkin* leads to deterioration of dopaminergic neurons and that PD, at least AR-JP, is caused by the failure of proteolysis mediated by the ubiquitin–proteasome system [8]. Since then, our knowledge about parkin has expanded, and indeed at present various putative substrates, e.g., CDCrel-1, synphilin-1, α-SN22 (O-glycosylated form of α-synuclein [α-SN]), Pael-R, and cyclin E have been identified [9–14]. Moreover, negative regulation of parkin E3 activity by parkin modification, such as nitration and phosphorylation, has been reported [15–17], but the pathophysiological role of parkin is still poorly understood.

One crucial issue that needs to be investigated is why AR-JP brains show severe neuronal loss with gliosis in the substantia nigra and mild neuronal loss in the locus coeruleus and why dopaminergic neurons in the substantia nigra are particularly vulnerable to the loss-of-function effect of parkin, though parkin is expressed ubiquitously throughout the brain. To define how the loss-of-function of parkin induces selective dopaminergic neuronal death in the midbrain, we interfered with endogenous parkin mRNA, a potentially suitable in vitro model of AR-JP for investigating the mechanism of selective dopaminergic neuronal death. To knock down the level of parkin in cells, we designed a full-length human parkin antisense (abbreviated as-parkin) using an adenovirus vector that has a high multiplicity of infection (moi) toward post-mitotic cells or cell lines which has neuronal characteristics and is an excellent tool to search for the effect of as-parkin on differentiated SH-SY5Y cells that exhibit features characteristic of dopaminergic neurons.

Here, we report that as-parkin selectively induced apoptosis of SH-SY5Y cells in a caspase-dependent manner. We also found that loss of parkin resulted in accumulation of endogenous L-3,4-dihydroxyphenylalanine (DOPA)- and dopamine (DA)-chromes derived from auto-oxidation of DOPA/DA-quinones, which mediates the toxic effect by covalently binding to the thiol group of proteins and consequently disintegrates cellular integrity and eventually causes cell death [18–20]. α-SN is a putative protein associated with membrane transport or signal transduction but of unknown function. α-SN gene mutations such as missense or multiplication cause familial autosomal dominant PD [21–27]. We found that forced expression of α-SN suppressed the loss of cell viability and accumulation of oxidative DOPA/DA metabolites caused by loss of parkin. Based on these findings, we propose that parkin and α-SN contribute to a common DA metabolic pathway; the impairment of which may lead to selective degeneration of dopaminergic neurons and consequently to PD.

Materials and methods

Adenoviruses. We used the adenoviral plasmid (pAdEasy-1) and the shuttle vector (p-shuttle-CMV) (Q.Bio gene). Various cDNAs used were inserted into the shuttle vector. The shuttle vector plasmid was linearized with *Pme*I. Electrocompetent *Escherichia coli* BJ5183 cells were added and electroporation was performed in 2-mm cuvettes in a Gene Pulser electroporator. Cells were inoculated onto 10-cm Petri dish containing LB-agar and 50 μg/ml kanamycin. Smaller colonies were picked and grown in 2 ml LB-broth (Sigma Chemical St. Louis, MO) containing 50 μg/ml kanamycin. Recombination was confirmed with *Pac*I. Approximately 5×10^6 cells were plated onto 10-cm culture dish. Ten micrograms of plasmid DNA linearized by *Pme*I, 12 μl FuGENE6 (Roche Molecular Systems, NJ), and 500 μl OptiMEM (Gibco-BRL) were mixed and transfected, according to the protocol provided by the manufacturer. After 7–10 days, the cells were collected by scraping off the 10-cm dish together with floating cells in the culture. The supernatant was removed after low-speed centrifugation. After sonicating the pellet, the cells were resuspended into 1 ml Dulbecco's modified Eagle's medium (DMEM) and frozen to −80 °C. In the next step, 500 μl of viral lysate was used to infect 7×10^7 cells in 15-cm dish. This process was repeated 1–3 times. Viruses were purified by CsCl banding; the final yield was 10^{10} plaque forming units.

Cells and cell culture. Human neuroblastoma cells (SH-SY5Y) and HeLa cells were obtained from American Type Culture Collection. The cells were maintained in growth medium (DMEM, Sigma, supplemented with 10% fetal bovine serum [Gibco-BRL, Gaithersburg, MD], 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 °C under 5% CO_2. SH-SY5Y cells were cultured with 100 μM of all *trans*-retinoic acid in dimethyl sulfoxide (DMSO) (Sigma R-2625) for 3 to 4 days for differentiation. The cells were infected with the antisense adenovirus at 150 moi; LacZ at 150 and 5 moi, wild and mutant α-SN adenovirus at 5 moi. Cells were collected 36 h after infection, centrifuged, and analyzed.

Western blotting. Infected or control cells were lysed in Laemmli SDS sample buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (NuPAGE, Invitrogen, San Diego, CA) and transferred onto polyvinylidene difluoride (PVDF) membrane. Western blotting was performed according to the ECL protocol provided by the supplier (Invitrogen, San Diego, CA) using specific antibodies of parkin and cleaved caspases (Cell Signaling Technology, Beverly, MA), α-SN (BD Transduction Laboratories, Lexington, KY), and β-Gal (Promega, Madison, WI).

Cell survival assay. Cells were infected with as-parkin or LacZ adenovirus and incubated for 48 h in 96-well plate. Cell viability was evaluated using the WST8, MTT reduction assay. Briefly, the solution of 0.1 mg/ml MTT in DMEM was added to each well and incubated for 2 h. The transmission was evaluated at 450 and 655 nm by 96-well microplate reader (Bio-Rad, Richmond, CA).

TUNEL assay. Terminal-deoxynucleotidyl transferase mediated d-UTP nick end labeling (TUNEL) assay was performed using formalin-fixed, ApopTag In Situ Apoptosis Detection Kits (Intergen, Purchase, NY). Fragmented DNA was labeled by fluorescein isothiocyanate (FITC) and observed under a fluorescence microscope.

Measurements of DOPA/DA-chromes. Thirty-six hours after infection, cells were solubilized in 500 μl of 1% Triton X-100 solution for 2 h and then centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant was used as cell extract and was incubated for 3 min at room temperature. After 10% TCA protein precipitation, the generation of DOPA/DA-quinones was estimated by measuring the absorbance of the incubation supernatant at 475 nm based on the formation of DOPA/DA-chromes. The amount of DOPA/DA-chromes was calculated from a standard curve constructed using known amounts of DA and 0.01 mg/ml tyrosinase. The protein concentration in the cell extracts was determined by using the BCA Protein Assay Reagent Kit

(Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

Statistical analysis. All data were expressed as means ± SEM. Differences between groups were examined for statistical significance using Dunnett's t test or Turkey's multiple t test. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results and discussion

Antisense parkin causes loss of viability of SH-SY5Y cells

We first examined the effect of knockdown of parkin on the viability of human neuroblastoma cells (SH-SY5Y). These cells contain dopaminergic machinery and can differentiate into neuronal-like phenotypes when treated for 3–5 days with retinoic acid (RA), accompanied by arrest of cell proliferation and increased dopamine metabolism [28,29]. Infection of SH-SY5Y with full-length human as-parkin adenovirus caused deterioration of cell viability, as monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The differential SH-SY5Y cell death effect was observed in the range between 50 and 250 moi titer of as-parkin adenovirus (data not shown) and thereafter we routinely used 150 moi titer (Fig. 1A, left panel). Control β-galactosidase (β-Gal) adenovirus had no effect on cell viability, although β-Gal was highly expressed in SH-SY5Y cells (Fig. 1A, right panel). Infection of cells with as-parkin caused a marked decrease of endogenous parkin protein level in differentiated SH-SY5Y cells, without altering actin level in the cells (Fig. 1A, right panel). The effect of as-parkin was abrogated, upon co-infection with sense-parkin (data not shown).

Effect of antisense parkin is cell-type specific

Intriguingly, we found that the effect of as-parkin on cell viability was much less in undifferentiated growing SH-SY5Y cells compared with differentiated cells (Fig. 1B, left panel). In addition, as-parkin did not influence cell viability of HeLa cells derived from human adenocarcinoma of the uterine cervix, which do not express parkin protein and lack the dopamine metabolic pathway (Fig. 1B, right panel). Thus, antisense knockdown of parkin exerts its effect based on the cell type, and the effects are observed in a dopaminergic neuron-specific manner, and depend on the differentiation state of dopaminergic neurons.

Antisense parkin induces apoptotic cell death

As shown in Fig. 1C, the cells appeared clear when their morphology was compared with uninfected (control) and β-Gal expressing cells. SH-SY5Y cells infected with as-parkin adenovirus showed morphological

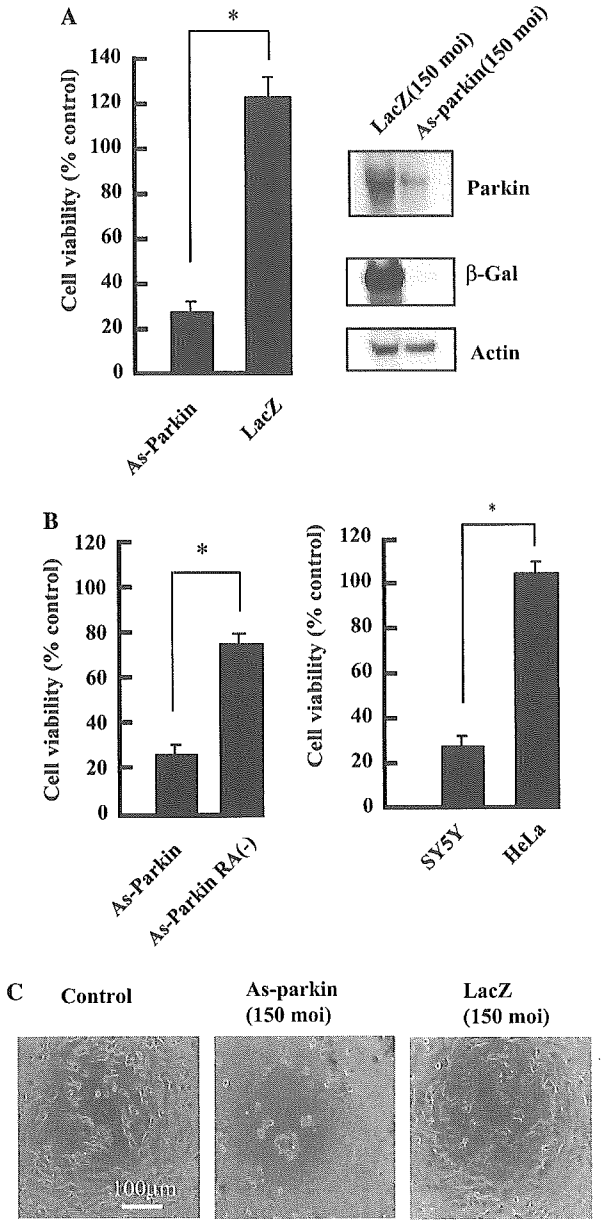

Fig. 1. Parkin knockdown is associated with loss of SH-SY5Y cell viability. (A) Effects of antisense parkin (as-parkin) and LacZ. Adenoviruses were infected for 48 h with 150 moi titers as indicated on the differentiated SH-SY5Y cells that had been pre-cultured with RA for 4 days. Cell viability was determined by the MTT assay (left panel). The results are expressed as percentage of MTT activity of uninfected cells (control). Data represent means ± SEM of 8 determinations. $^*P < 0.01$ versus control group (Dunnett's t test). Cells that had been treated for 48 h with 150 moi titers of as-parkin and LacZ adenoviruses were lysed in Laemmli SDS sample buffer, and the proteins were separated by SDS–PAGE, followed by Western blotting with antibodies against parkin, β-galactosidase (β-Gal), and actin (right panel). (B) Undifferentiated SH-SY5Y cells without treatment with RA and HeLa cells were treated for 48 h with as-parkin adenovirus. The cell viability was measured and represented as indicated. (C) Morphological changes in differentiated SH-SY5Y cells upon knockdown of parkin. The cells were infected for 48 h with as-parkin and LacZ adenovirus vectors or left uninfected (control). Note the presence of apoptotic cells. Bar, 100 μm.

changes typical of apoptosis. To determine the nature of cell death induced by as-parkin, we performed TUNEL assay. As shown in Fig. 2A, as-parkin-treated SH-SY5Y cells showed nuclear condensation and fragmentation. In contrast, these changes were rarely observed in β-Gal-expressing SH-SY5Y cells. In support

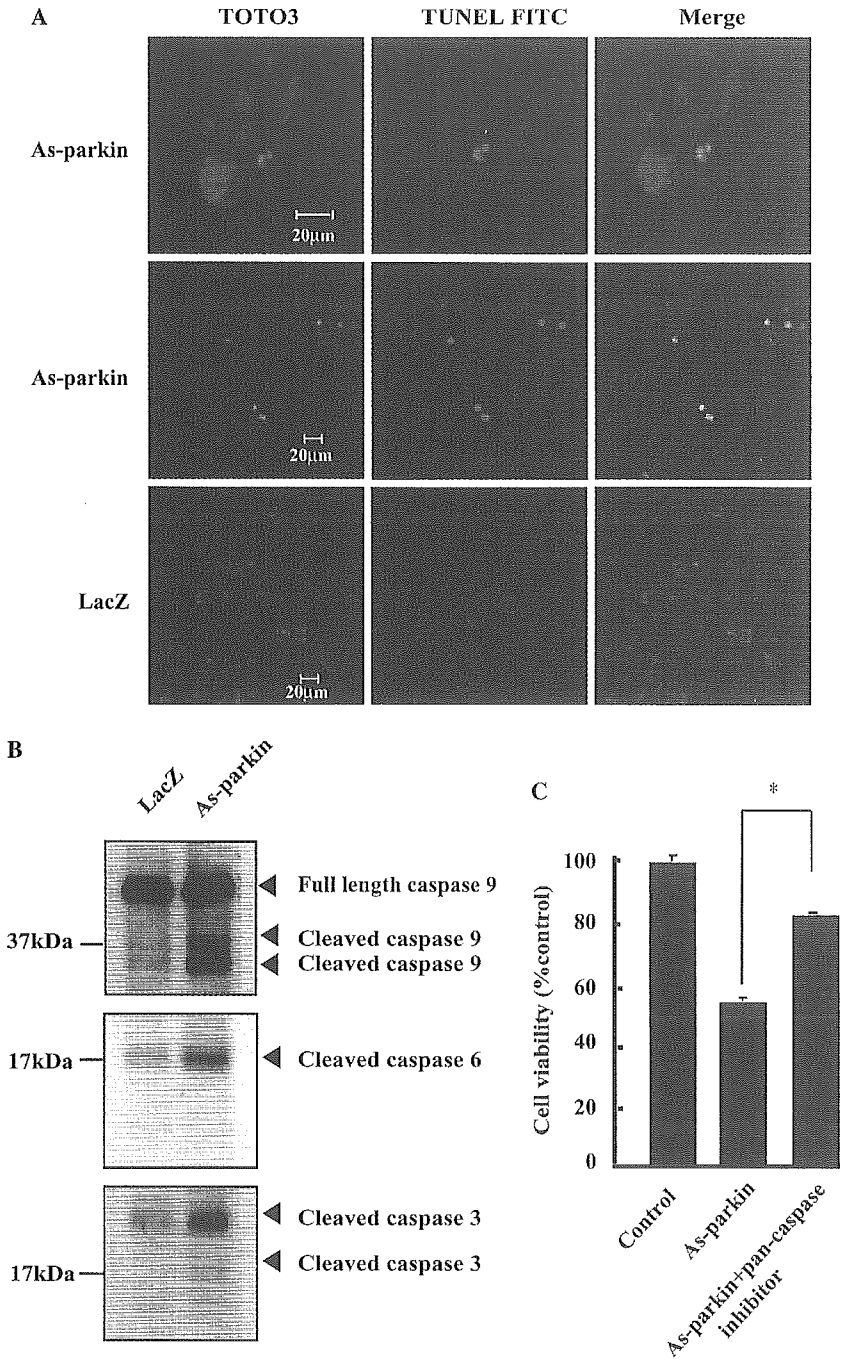

Fig. 2. Parkin knockdown induces apoptosis. (A) Detection of cells with nuclear DNA fragmentation due to parkin knockdown by TUNEL assay. Differentiated SH-SY5Y cells were treated for 48 h with as-parkin and LacZ adenoviruses (150 moi). TUNEL assay was performed to detect apoptotic cells. TUNEL-positive cells (green) were detected (TdT enzyme is labeled with FITC green fluorescence). Nuclei were counterstained with TOTO3 (red). Bar, 20 μm. (B) Activation of caspase-3, -6, and -9 by as-parkin. After infection with as-parkin and LacZ adenoviruses as for a, the cell extracts were used for Western blot analysis using antibodies against cleaved caspase-3, -6, and -9. Arrowheads on the right indicated corresponding caspases. Note that anti-cleaved caspase-3 and -6 antibodies did not react with their native forms. (C) Effects of a 'pan' caspase inhibitor on apoptosis induced by the loss of parkin. The inhibitor was added at 100 μM when cells were treated by as-parkin adenovirus as for (A). Note that the caspase inhibitor significantly blocked parkin knockdown-induced deterioration of cell viability. Data represent means ± SEM of 8 determinations. $^*P < 0.05$ versus control (uninfected) group (Dunnett's t test).

of the TUNEL findings, we also detected activation of caspase-3, -6, and -9 in SH-SY5Y cells under parkin knockdown (Fig. 2B). In addition, Western blot analysis showed cleaved poly(ADP)-ribose polymerase (PARP) in the course of as-parkin infection and a time-dependent increase of the 25-kDa cleaved fragment, confirming the activation of caspase(s) (data not shown). Further experiments showed that application of a pan-caspase inhibitor for 6 h before infection significantly prevented apoptotic cell death as determined by the MTT reduction assay (Fig. 2C). Taken together, these results suggest that as-parkin-induced SH-SY5Y cell death is likely to be mediated by activation of the caspase cascade.

Antisense parkin increases DOPA/DA metabolites

Next we examined the effect of α-SN on the viability and DOPA/DA-chrome level in differentiated SH-SY5Y cells with a reference to parkin loss. These experiments were based on previous studies describing abnormal DA metabolism in α-SN-deficient mice [30] and α-SN binding to DA-quinones [31]. For this purpose, we constructed adenovirus vectors expressing α-SN, and first tested its effect on the expression of parkin. α-SN and its PD-linked mutants (Ala30Pro and Ala53Thr) had no effect on the levels of parkin, irrespective of the treatment of as-parkin (Figs. 4A and B). It is of note that α-SN did not express at significant levels in SH-SY5Y cells under present conditions. Then, we investigated the effect of α-SN on the as-parkin-induced loss of cell viability. As shown in Fig. 4A, infection of SH-SY5Y cells by both adenovirus vectors expressing α-SN and as-parkin caused marked reduction of their cellular chrome levels and resulted in amelioration of as-parkin-induced deterioration of cell viability (Fig. 4B). Intriguingly, coinfection of cells with wild-type α-SN and as-parkin adenoviruses abrogated as-parkin-induced accumulation of DOPA/DA-chrome. However, α-SN mutants (Ala30Pro and Ala53Thr) and β-Gal expression did not reduce the generation of DOPA/DA-chrome by as-parkin. Thus, it seems that the α-SN-induced suppression of apoptosis was associated with a reduction in the DOPA/DA-chrome level in α-SN expressing SH-SY5Y cells. These results suggest that α-SN inhibits apoptosis induced by parkin knockdown by blocking the generation of DOPA/DA-chromes; i.e., DOPA/DA-quinones.

Antisense parkin-induced extensive apoptosis of differentiated dopaminergic SH-SY5Y cells but limited apoptosis of undifferentiated SH-SY5Y cells and no apoptosis of HeLa cells, indicating cell-type specificity. With regard to the cell-specific vulnerability, an important factor seems to be dopamine (DA) metabolism, which is a peculiar feature of dopaminergic neurons. Indeed, the differentiated SH-SY5Y cells retain a high DA metabolic pathway [28,29]. DA is a molecule prone to oxidation and it contributes to the generation of reactive oxygen species, which when in abundance can cause oxidative injury of various cellular components[18,20]. Indeed, abnormally high levels of these free radicals in dopaminergic neurons have been implicated as environmental factors causing not only sporadic PD but also AR-JP [32,33]. We tested the effects of as-parkin infection on the level of endogenous DOPA- and DA-chromes (DOPA/DA-chromes), which are derived from DOPA- and DA-quinones, respectively, whose metabolites could originate from cytosolic DOPA or DA oxidation [18,20]. Thus, the amounts of DOPA/DA-chromes reflect those of endogenous DOPA/DA-quinones. DOPA/DA-chrome levels were significantly high in parkin knockdown cells whereas there was no change in β-Gal expressing ones (Fig. 4A). These findings suggest that parkin knockdown-induced apoptosis is mediated by an increase in DOPA/DA-chromes.

Recently, four groups independently reported the generation of a mouse model that lacks the *parkin* gene, which display certain abnormalities of dopamine metabolism [34–37]. However, these parkin knockout mice had only subtle phenotypes exhibiting a largely normal gross brain morphology. Based on the pathologic findings, all the parkin null mice showed no neuronal loss in the SN. This is in marked contrast to our in vitro system described in this study, in which parkin knockdown induced activation of the caspase cascade and apoptosis of dopaminergic SH-SY5Y cells. Why do parkin knockout mice lack the abnormalities seen in AR-JP patients? One plausible explanation is the presence of a putative molecule(s) that suppresses the defect induced by loss-of-function of parkin, and the abundant presence of such molecule(s) in the brain should be linked to the pathogenesis of PD. Here, we propose that α-SN is the molecule that compensates for the loss of parkin, since α-SN prevented apoptotic cell death induced by as-parkin. In this regard, Western blot analysis showed that the dopaminergic SH-SY5Y cells did not express α-SN at significant levels (Fig. 3A, lanes 2 and 4), which is in marked contrast to the high abundance of dopaminergic neurons in vivo [38]. Regardless of the compensatory role of α-SN for the loss-of-function of parkin in the AR-JP, α-SN probably cannot cope with the accumulation of toxic molecules in the absence of parkin and thus apoptotic neuronal death perhaps occurs gradually, leading to degeneration of dopaminergic neurons and consequently the development of early-onset PD. We provide the first evidence for the anti-apoptotic role of α-SN and its involvement in the common pathway of parkin.

To date, several studies have demonstrated that α-SN exerts protective effects against various cellular stresses such as oxidative damage and related apoptosis of neurons [39,40]. Considering the reason why muta-

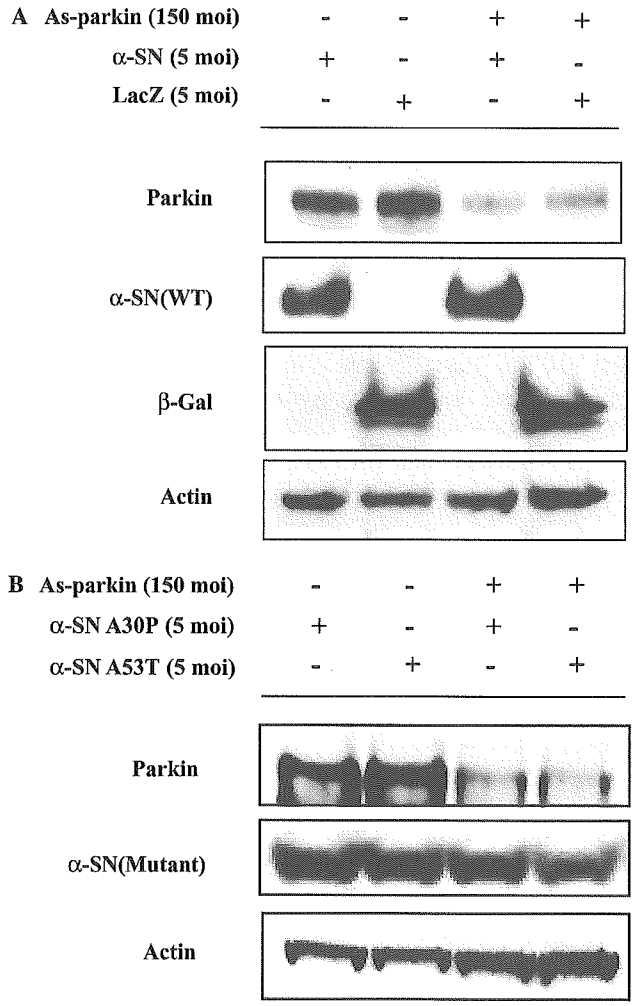

Fig. 3. Expression levels of parkin, α-SN, and LacZ. The expression levels of parkin, wild, and mutant α-SN, and LacZ in SH-SY5Y cells treated for 48 h with adenoviruses were monitored by Western blot analysis.

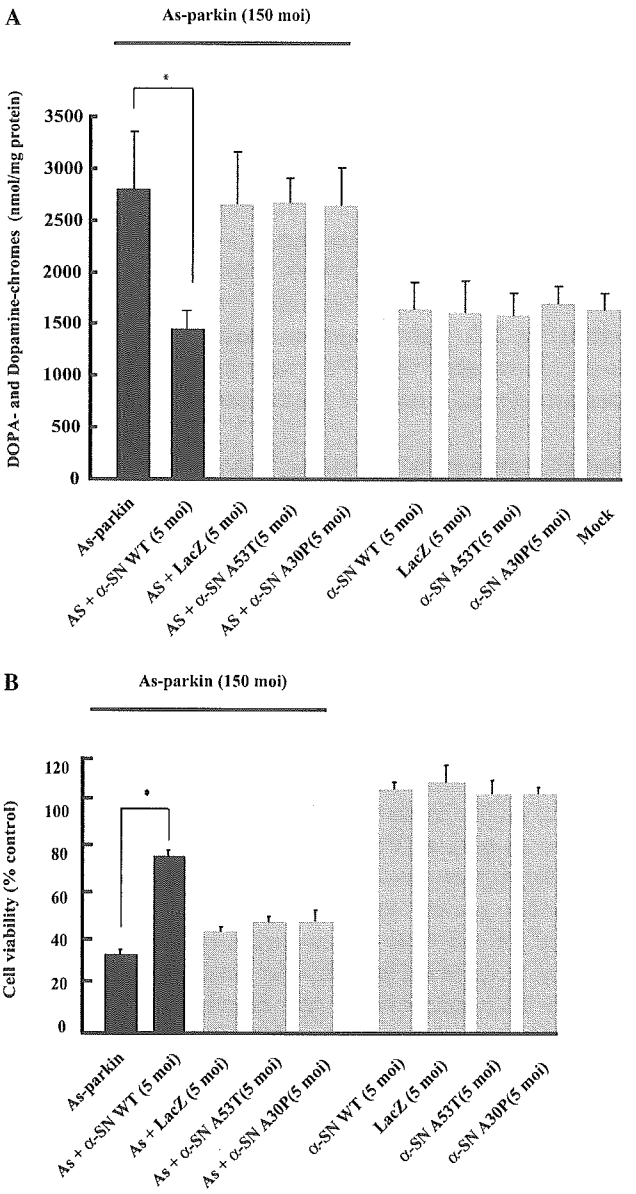

Fig. 4. α-Synuclein inhibits parkin knockdown-induced apoptosis and accumulation of DOPA- and DA-quinones. (A) Cellular level of DOPA/DA-chromes. After the differentiated SH-SY5Y cells were treated for 36 h with as-parkin, wild, and mutant α-SN, LacZ, and adenoviruses, cellular DOPA/DA-chromes were measured. Note the profound decrease of DOPA/DA-chromes in α-SN-expressing SH-SY5Y cells. Data are means ± SEM of 10 determinations. $^*P < 0.05$ versus control group (Turkey's multiple t test). (B) Effects of overexpression of wild and mutant α-SN on as-parkin-induced deterioration of cell viability. Differentiated SH-SY5Y cells were treated for 48 h with as-parkin adenovirus. Cells were coinfected with LacZ and α-SN adenovirus (5 moi) and at 150 moi titers of as-parkin adenovirus. The cell viability was measured and represented as in Fig. 1A (left panel).

tion of α-SN causes familial PD, it is clear that this type of disease is due to the gain-of-toxic function of the α-SN mutants with missense mutations, differing from the neuroprotective roles of the wild-type α-SN. In addition, α-SN proteins with disease-causing missense mutations tend to generate protofibrils [31,41], suggesting that protein misfolding including α-SN plays a key role in the pathogenesis of PD. In contrast, at high concentrations, it oligomerizes to β-pleated sheets known as protofibrils (i.e., fibrillar polymers with amyloid-like characteristics). Indeed, multiplication of α-SN has been reported in the autosomal dominant form of PD, indicating that overproduction of this protein affects the cellular damages. In this regard, there is a discrepancy between the protective role of α-SN in the present study and combination of PD and α-SN multiplication. This could be explained by appropriate physiological level of synuclein [40]. Thus, in patients with α-SN multiplication, the copy numbers of this gene may be related to the clinical severity of PD; patients with triplicate α-SN show dementia with Lewy bodies [24]; while those with duplicate levels do not show dementia [26,27].

It remains unclear why dopaminergic neurons of the substantia nigra are selectively vulnerable to the loss of parkin in AR-JP patients. In the present study, we provided a clue for this enigmatic puzzle. Considering the specificity of the lesions in PD, it is possible that the high oxidative state associated with DA metabolism may cause deterioration of dopaminergic neurons. The mechanism underlying increased oxidative stress may involve DA itself, because oxidation of cytosolic DOPA/DA may be deleterious to neurons. Indeed, DA causes apoptotic cell death as evident by morphological nuclear changes and DNA fragmentation [42–44]. In this regard, we showed here that as-parkin directed loss of parkin leads to abnormality of DOPA/DA metabolism, which resulted in the generation of DOPA/DA-quinones in SH-SY5Y cells. Thus, DA and its metabolites seem to exert cytotoxicity mainly by generating highly reactive quinones through auto-oxidation. On the other hand, the toxicity of DOPA and DA is due to the generation of reactive oxygen species that could disrupt cellular integrity, causing cell death. However, the reason for the production of oxidative DOPA/DA-metabolites following loss of parkin is not clear at present.

Our results showed for the first time that loss of parkin leads to death of differentiated dopaminergic cells in vitro. This cell-based experiment enhances our understanding of the pathophysiology of PD and could be potentially useful for drug screening. Our results also showed that α-SN and parkin are involved in DA metabolism and that aberrant regulation of DA is accompanied by accumulation of oxidative DOPA/DA metabolites.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Acta Neuropathol (2005) 109: 256–262
DOI 10.1007/s00401-004-0937-9

REGULAR PAPER

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Expression of 8-oxoguanine DNA glycosylase (OGG1) in Parkinson's disease and related neurodegenerative disorders

Received: 7 June 2004 / Revised: 31 August 2004 / Accepted: 30 September 2004 / Published online: 17 November 2004

Abstract Oxidative stress including DNA oxidation is implicated in Parkinson's disease (PD). We postulated that DNA repair enzymes such as 8-oxoguanosine DNA glycosylase (OGG1) are involved in the PD process. We performed immunohistochemical and biochemical studies on brains of patients with PD and those of patients with progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) as disease controls, and control subjects. We found higher expression levels of mitochondrial isoforms of OGG1 enzymes in the substantia nigra (SN) in cases of PD. Furthermore, Western blot analysis revealed high OGG1 levels in the SN of the patients with PD. Our results indicate the importance of oxidative stress within the susceptible lesions in the pathogenesis of PD.

Keywords 8-Oxoguanosine DNA glycosylase · Mitochondrial DNA · Parkinson's disease · Progressive supranuclear palsy · Corticobasal degeneration

Introduction

The primary cause of Parkinson's disease (PD) is still unknown; however, oxidative stress and mitochondrial dysfunction have been implicated as major contributors to neuronal death in the substantia nigra (SN) [1, 11, 21, 27, 28, 29, 32, 33]. Reactive oxygen species (ROS) are highly reactive and oxidize nucleic acids, increasing the frequency of mutations in DNA. 8-Oxoguanosine (8-oxoG) is one of the oxidized forms of guanine. Because 8-oxoG mispairs with adenine and cytosine, 8-oxoG induces the occurrence GC:CG to T:A transversion mutation. There are various error-avoiding mechanisms in organisms against oxidative DNA damage [20, 31]. MutM, one of the DNA repair enzymes found in *Escherichia coli*, removes 8-oxoG paired with cytosine in DNA [19]. The human OGG1 gene encodes an 8-oxoG DNA glycosylase (hOGG1), which is a functional homolog of MutM [4, 23]. In human tissues, there are two major isoforms of hOGG1; hOGG1-1a and hOGG1-2a [23]. Whereas hOGG1-1a is located in the nucleus, hOGG1-2a is located on inner mitochondrial membrane, on which mitochondrial DNA (mtDNA) is situated [23]. The hOGG1-2a plays important roles in repairing errors caused by oxidative stress in mtDNA [23]. In the present study, we examined immunohistochemically and biochemically the expression of hOGG1-2a in human brains of normal subjects, patients with parkinsonism including PD, progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD).

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Materials and methods

Patients and brain tissues (paraffin-embedded sections)

We examined autopsied brains of seven patients with PD, four with PSP, four with CBD, and seven aged-matched control subjects. The pathological diagnosis was confirmed in all patients and aged-matched control subjects by the Department of Neurology, Juntendo University School of Medicine. The clinical profiles of the patients are summarized in Table 1. To determine whether the disease duration of PD influenced the expression of hOGG1-2a, we divided PD patients into two groups by disease duration. We defined disease duration less than

Table1 clinical summary of all subjects (*ALS* amyotrophic lateral sclerosis,*CBD* corticobasal degeneration, *CML* chronic myelocytic leukaemia,*Fr* fresh frozen brain, *P* paraffin-embedded section,*PD* Parkinson's disease, *PN* pontine nucleus,*PSP* progressive supranuclear palsy, *SN* substantia nigra)

Case	Diagnosis	Age	Sex	Duration (years)	Postmortem delay (h)	Cause of death	Sample
1	Control	65	F	-	1.5	ALS	P
2	Control	38	M	-	10	Malignant lymphoma	P
3	Control	82	F	-	1.5	Cerebral infarction	P
4	Control	60	M	-	5.5	Liver cirrhosis	P
5	Control	67	M	-	3.5	CML	P
6	Control	55	M	-	11	Gastric cancer	P
7	Control	81	M	-	2.5	Cerebral infarction	P
8	Control	89	M	-	2.5	Lung cancer	Fr (SN1, PN1)
9	Control	66	M	-	5	Gallbladder cancer	Fr (SN2, PN2)
10	Control	57	M	-	5	Chronic renal failure	Fr (SN3, PN3)
11	Control	48	F	-	5.5	SLE	Fr (SN4)
12	Control	70	F	-	2.5	Laryngeal cancer	Fr (SN5)
13	PD	72	F	5	< 24	Lung haemorrhage	P
14	PD	51	M	11	3.5	Pneumonia	P
15	PD	65	F	4.5	2	Asthma	P
16	PD	74	M	17	1	Liver cirrhosis	P
17	PD	37	F	7	< 24	Unknown	P
18	PD	71	F	13	8	Pneumonia	P
19	PD	63	M	12	20	Unknown	P
20	PD	56	F	17	6	Pneumonia	Fr (SN6)
21	PD	71	M	Unknown	< 24	Unknown	Fr (SN7)
22	PD	75	M	11	5	Pneumonia	Fr (SN8)
23	PD	85	F	13	5	Pneumonia	Fr (SN9)
24	PD	66	M	10		Pneumonia	Fr (SN10)
25	PSP	71	F	13	13	Lung cancer	P
26	PSP	85	F	3	3.5	Pneumonia	P
27	PSP	77	M	6	12	Pulmonary embolism	P
28	PSP	69	M	4	6.5	Pneumonia	P
29	PSP	86	F	6	< 12	Pneumonia	Fr (PN4)
30	PSP	74	M	6	3	Pneumonia	Fr (PN5)
31	CBD	61	M	8	8	Pneumonia	P, Fr (PN6)
32	CBD	81	F	8	9	Pneumonia	P
33	CBD	59	F	3	4.5	Ileus	P
34	CBD	65	M	6	9	Pneumonia	P, Fr (PN7)

10 years as short-duration group and over 10 years as long-duration group. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine.

Antibodies

A rabbit polyclonal anti-hOGG1-2a antibody was prepared as described previously [23]. The antibody recognizes the C terminus of hOGG1-2a and its specificity has been confirmed [23]. Anti-TOM40, cytochrome oxidase subunit I, tyrosine hydroxylase (TH), and GAPDH antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA), Molecular Probes (Eugene, OR), Calbiochem (La Jolla, CA) and Chemicon (Temecula, CA), respectively.

Immunohistochemistry and immunofluorescence for hOGG1-2a

The deparaffinized sections were microwaved in phosphate buffer (Antigen Retrieval Citra, BioGenex, San Ramon, CA) for antigen retrieval. Endogenous peroxidases were quenched by incubation with 3% hydrogen peroxide. After incubating the slides with blocking solution (10% normal goat serum), sections were treated overnight at 4°C with an anti-hOGG1-2a antibody. These sections were incubated with biotinylated anti-rabbit IgG from goat (DAKO, Carpinteria, CA; 1:100). After incubation with streptavidin conjugated to horseradish peroxidase (HRP) (DAKO; 1:200), we treated sections with biotinyl tyramide and hydrogen peroxide. Sections were then incubated with streptavidin conjugated to HRP and visualized with 3',3'-diaminobenidine (DAB). Double immunofluorescence was performed with anti-hOGG1-2a and anti-cytochrome oxidase subunit I antibodies, or with anti-hOGG1-2a and TH antibodies. These sections were treated with Alexa fluor goat anti-rabbit IgG (Molecular Probes; 1:200) and Alexa fluor goat anti-mouse IgG (Molecular Probes; 1:300). Signal was observed under Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

Semiquantitative analysis

For semiquantitative analysis, microscopic photographs of the whole SN and pontine nuclei (PN) were prepared.

To minimize the influence of neuronal loss on semiquantitative analysis, we selected specimens containing at least 100 remaining neurons as described previously [33]. All sections obtained from the enrolled subjects and used for this analysis fulfilled this criterion. Two independent observers blinded to the clinical information counted the numbers of hOGG1-2a-positive neurons in SN and PN. Differences between groups were examined for statistical significance using one-way ANOVA. A P value less than 0.05 was considered significant.

Subcellular fractionation

Fresh frozen brain including midbrain (five PD and five control subjects) and pons (two PSP, two CBD and three control subjects) were obtained (Table 1). Approximately 0.5 g of frozen brain blocks were placed into 3.5 ml ice-cold homogenization buffer [0.32 M sucrose and 4 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH, pH 7.4] and homogenized using a Potter-Elvehjem homogenizer (9 up-and-down strokes, 900 rpm) in the presence of a mixture of protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics, Penzberg, Germany). Nuclear, mitochondrial, microsomal, and cytosolic fractions were obtained using the methods described previously [15, 29].

Western blot analysis of hOGG1-2a

Each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) microporous membrane (Bio-Rad, Hercules, CA) using transfer buffer (40 mM CAPS, 30 mM TRIS, and 15% methanol). The transferred membrane was blocked with 5% skim milk and incubated overnight with primary antibodies at 4°C. After incubation with HRP-conjugated secondary antibodies, the reaction was visualized using a chemiluminescence reagent. The intensity was analyzed by LAS-1000plus (Fuji film, Tokyo, Japan).

Results

Regional expression of hOGG1-2a

Control subjects

In the control subjects, hOGG1-2a immunoreactivity was rarely observed in any brain region examined including cortex, basal ganglia, SN (Fig. 1A), and PN (Fig. 1E), and never observed in glial cells. However, the number of hOGG1-2a-positive neurons in SN increased with age (Fig. 2A). Western blot analysis also showed that expression of hOGG1-2a increased with age in control subjects (Fig. 3A, C). These results are consistent with those reported previously [5, 7].

Parkinson's disease

All PD patients showed moderate to severe neuronal loss in the SN. While the remaining nigral neurons of short-duration PD group showed intense cytoplasmic immunostaining with granular pattern for hOGG1-2a (Fig. 1B), those of the long-duration group did not show intense cytoplasmic immunostaining. Double-immunofluorescence staining for hOGG1-2a and TH showed immunoreactivity for hOGG1-2a in TH-positive neurons of the SN (Fig. 1I–K), suggesting increased hOGG1-2a in dopaminergic neurons of the SN. Lewy bodies did not stain with hOGG1-2a. There was no immunoreactivity in the nuclei. Interestingly, the immunoreactivity was barely seen in the cortex, basal ganglia, and PN (Fig. 1F). There was no immunoreactivity in glial cells. Semiquantitative analysis showed that the percentage of hOGG1-2a-positive neurons in the SN was higher in PD than in aged-matched control, PSP, and CBD cases. Repeated paired analyses with Bonferroni's correction showed significant differences between PD and aged-matched control ($P < 0.05$), but no significant differences between PSP, CBD and aged-matched control ($P > 0.05$) (Fig. 2B). Semiquantitative analysis also showed that the expression of hOGG1-2a was significantly higher in the short-duration PD group relative to the aged-matched control. In the long-duration PD group, the number of hOGG1-2a-positive neurons was slightly higher, albeit statistically insignificant from the aged-matched control (Fig. 2C). Western blot analysis demonstrated up-regulation of hOGG1-2a in the SN of PD compared with age-matched controls. The level of hOGG1-2a in SN of PD was 1.6- to 2.9-fold higher than age-matched controls (cases 9, 10, 12) (Fig. 3B).

Progressive supranuclear palsy and corticobasal degeneration

In PSP and CBD patients, severe neuronal loss was noted in basal ganglia and SN but not in PN. Immunoreactivity for hOGG1-2a was observed in limited regions such as PN (Fig. 1G, H). Immunostaining showed cytoplasmic granular pattern without nuclear staining in pontine neurons, but no obvious immunoreactivity in the cortex, basal ganglia, and SN (Fig. 1C, D). Glial cells including oligodendrocytes and astrocytes were barely immunoreactive in PSP and CBD. Semiquantitative analysis showed that the percentage of hOGG1-2a-positive neurons in PN was higher in PSP and CBD than aged-matched control and PD. Repeated paired analyses with Bonferroni's correction showed significant differences between PSP, CBD and aged-matched control ($P < 0.05$) (Fig. 2D). Western blot analysis showed 3.9- to 4.9- and 2.8- to

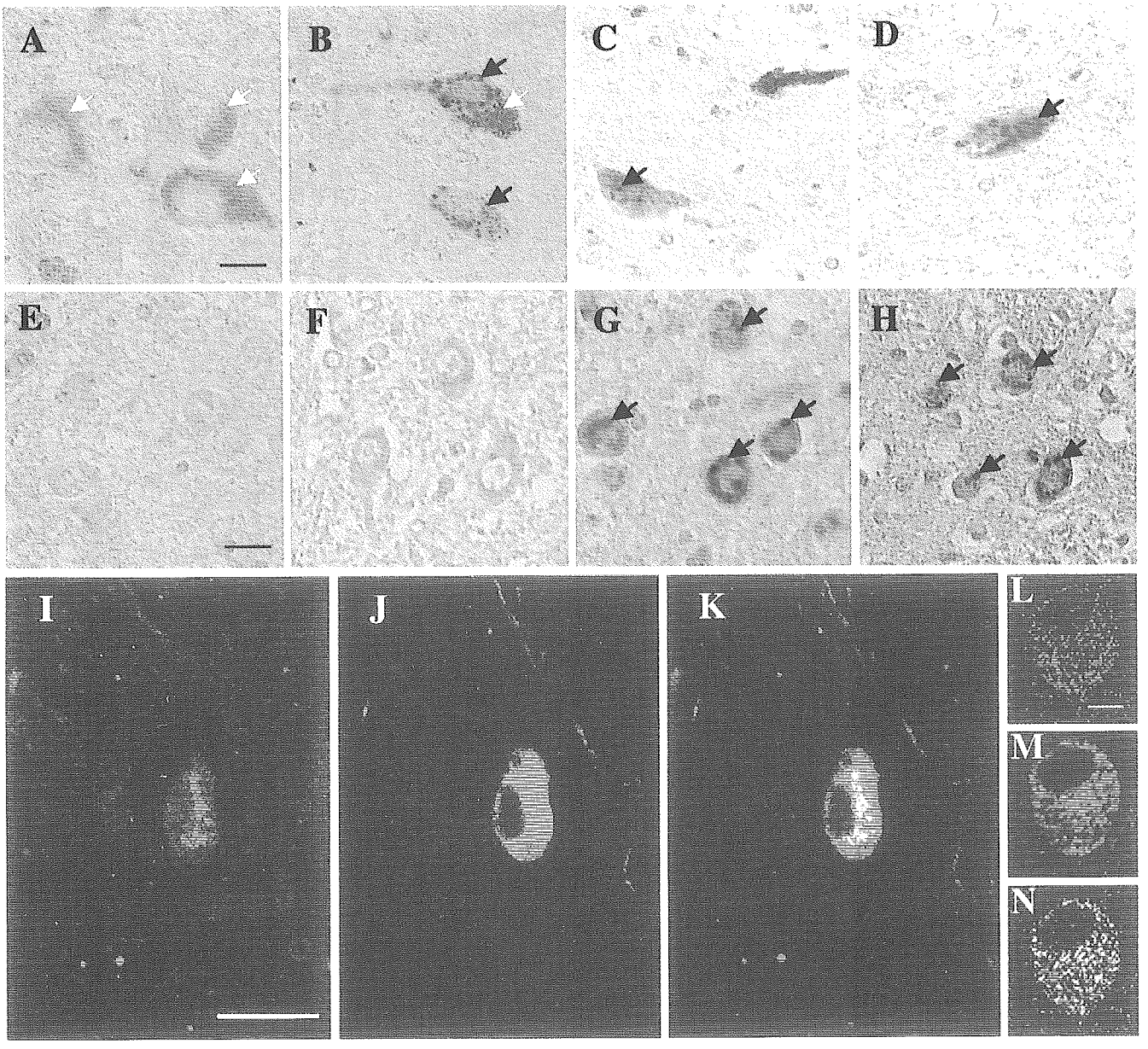

Fig. 1 Immunohistochemistry for hOGG1-2a in the SN (**A–D**) and PN (**E–H**) in representative subjects. **A**, **E** Control; **B**, **F** PD; **C**, **G** PSP; **D**, **H** CBD. Note the granular staining for hOGG1-2a (*arrow*) in the cytoplasm of SN neurons in the PD patient. Neuromelanin (*white arrow*). Note also the granular staining for hOGG1-2a in the cytoplasm of pontine neurons in PSP and CBD (*arrows*). **I–N** Neurons in SN of PD patient double stained with anti-hOGG1-2 antibody (*red* in **I**), anti-TH antibody (*green* in **J**) and merge (**K**), or with anti-hOGG1-2 antibody (*red* in **L**), anti-cytochrome oxidase subunit I antibody (*green* in **M**) and merge (**N**). Note the granular pattern of hOGG1-2a expression in the cytoplasm in TH-positive neurons and colocalization with cytochrome oxidase subunit 1 (*hOGG* human 8-oxoguanine DNA glycosylase, *SN* substantia nigra, *PN* pontine nuclei, *PD* Parkinson's disease, *PSP* progressive supranuclear palsy, *CBD* corticobasal degeneration, *TH* tyrosine hydroxylase). *Bars* **A–H** 10 μm; **I–K** 20 μm; **L–N** 10 μm

5.8-fold higher expression of hOGG1-2a in the pons of PSP and CBD than age-matched controls (casea 9, 10) (Fig. 3C, D), respectively.

Subcellular localization of hOGG1-2a

Since immunohistochemical studies could not distinguish between the precursor and processed forms of hOGG1-2a (the former but not the latter possesses a mitochondrial targeting signal consisting of 23 amino acid residues [23]), we performed subcellular fractionation study using SN from PD patients. hOGG1-2a is initially translated as a 43-kDa precursor molecule with the mitochondria targeting signal at the N-terminal end. After translocation into the mitochondria, the 43-kDa precursor is processed to a 40-kDa mature hOGG1-2a. As expected, we detected 40- and 43-kDa bands in mitochondrial and cytosolic fractions, respectively. However, the signal in the mitochondrial fraction was 1.4-fold stronger than in cytosolic fraction (Fig. 3E). We used TOM40 as the mitochondria marker, which is located in the outer membrane of the mitochondria [14,

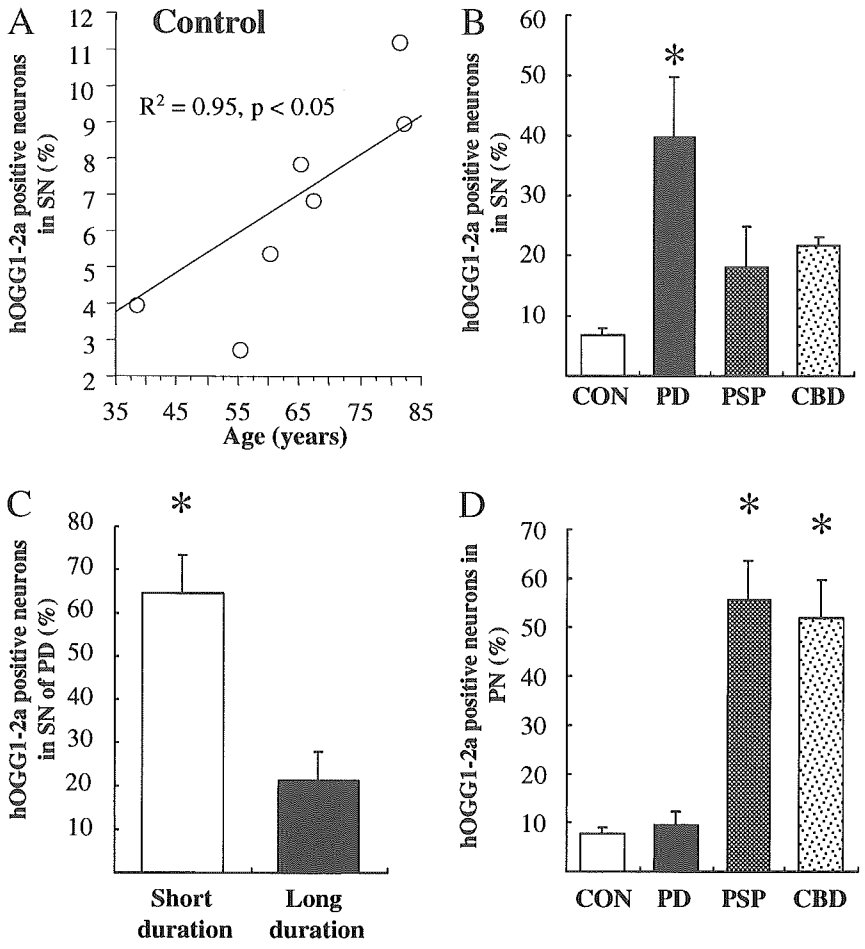

Fig. 2 Results of semiquantitative analysis. **A** Age-dependent increase in the percentage of hOGG1-2a-positive neurons in SN of control subjects. There was a significant correlation with age ($P < 0.05$). **B, D** Results of semiquantitative analysis of hOGG1-2a in SN and PN. The percentages of hOGG1-2a-positive neurons (mean ± SEM) were significantly higher in SN of PD and PN of PSP and CBD than the control (*CON*) (*$P < 0.05$, one-way ANOVA and Bonferroni's correction). **C** Percentage of hOGG1-2a-positive neurons in short- and long-duration PD groups (mean ± SEM). The percentage of hOGG1-2a-positive neurons was significantly higher in the short-duration group than in long-duration group (*$P < 0.05$)

25]. There was no signal for hOGG1-2a in the nuclear fraction.

We also performed double immunostaining with anti-hOGG1-2a and anti-cytochrome oxidase subunit I antibodies. Immunofluorescence for anti-hOGG1-2a antibody also showed a granular pattern in the cytoplasm of nigral neurons (Fig. 1L). Part of hOGG1-2a molecules were colocalized with cytochrome oxidase I, suggesting that hOGG1-2a that colocalized with cytochrome oxidase I in the mitochondria is the 40-kDa processed molecule from the 43-kDa precursor (Fig. 1L–N).

Discussion

Mitochondria are intracellular organelles in which ATP is synthesized, and such synthesis requires oxygen. mtDNA is more vulnerable to oxidative stress than nuclear DNA [18, 26], because mtDNA is located in the inner mitochondria membrane in which electron transport chain generates ROS. Since mtDNA has no intron, mtDNA mutations could lead to amino acid replacement that could induce mitochondrial dysfunction(s). Therefore, mtDNA repair enzymes are important to maintain mitochondrial functions.

In the present study, we demonstrated the up-regulated expression of hOGG1-2a, one of mtDNA repair enzymes, in dopaminergic neurons in the SN of PD brains, especially in the short-duration group but not in the long-duration group of the disease, indicating a different time course of compensatory mechanism of mtDNA oxidation. We have previously reported that MutT homolog (MTH1), an enzyme known to play an important role in controlling spontaneous mutagenesis in mtDNA, was up-regulated in the SN neurons of PD brains but not in other related neurodegenerative disorders such as multiple system atrophy [29]. Thus, overexpression of such repair enzymes could be a common event in the process of PD. What does hOGG1-2a up-regulation mean? The most plausible explanation is that hOGG1-2a is up-regulated secondarily to mtDNA oxidative damage to protect neurons form mutagenesis. Indeed, overexpression of hOGG1 within the mitochondria enhances the repair of mtDNA errors and rescues the cells from oxidative stress [9, 24].

Apart from PD, we detected intense hOGG1-2a immunoreactivity only in PN, while we could not detect hOGG1-2a immunoreactivity in the regions of severe neuronal loss, such as SN and frontal cortex, in PSP and CBD brains. Why were there regional differences in the expression of hOGG1-2a in such diseases? It is possible

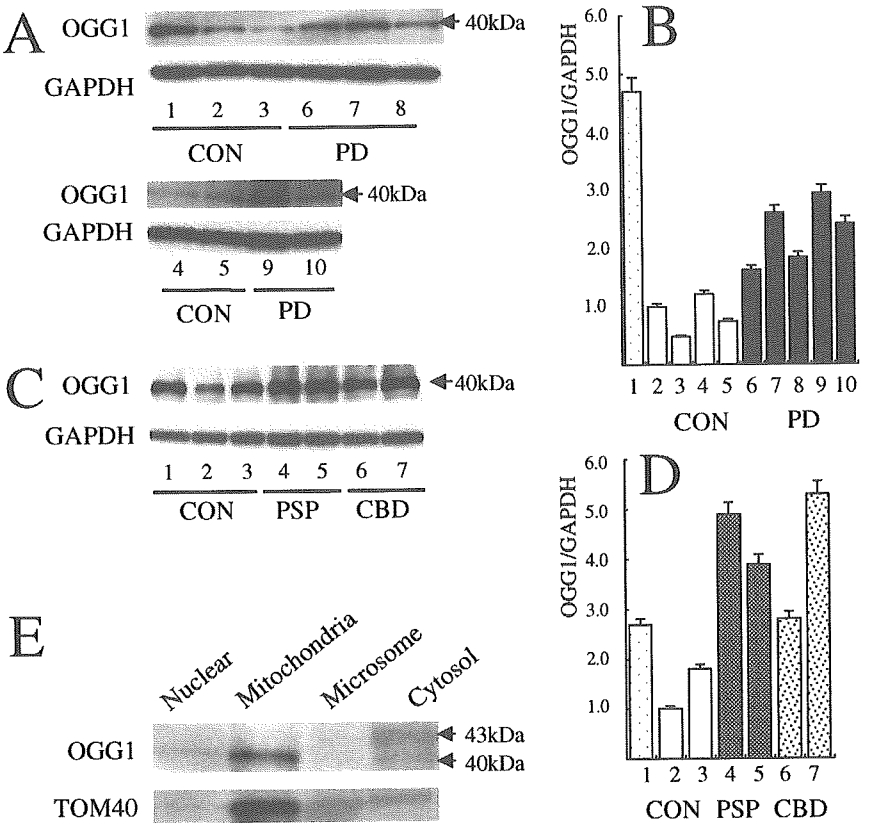

Fig. 3 **A** Immunoblotting of hOGG1-2a in midbrain sections of an elderly control subject (*lane 1*), younger control subject (*lane 4*), three control subjects age-matched for patients with PD (*lanes 2, 3, 5*), and five PD patients (*lanes 6–10*). **B** Quantitative analysis of hOGG1-2a expression levels normalized to GAPDH in control (*CON*) and PD. **C** Immunoblotting of hOGG1-2a in the pons of an elderly control subject (*lane 1*), two control subjects aged-matched for PSP and CBD patients (*lanes 2* and *3*), two PSP patients (*lanes 4* and *5*) and two CBD patients (*lanes 6* and *7*). **D** Quantitative analysis of hOGG1-2a expression levels normalized to GAPDH in control, PSP and CBD. **E** Analysis of subcellular fractions in the midbrain of PD patient

that the topographic differences reflect the course of the compensatory mechanism for mtDNA oxidation as observed in the different stages of PD. PSP and CBD are progressive neurodegenerative disorders characterized by extensive neuronal degeneration in multiple subcortical regions such as basal ganglia and brainstem nuclei [8, 16]. Among the vulnerable regions, PN is one of the affected lesions. In PSP, the appearance of neurofibrillary tangles (NFTs) and occasional neuronal loss are noted in PN [8, 17]. On the other hand, there is almost no neuronal loss and NFTs in PN of CBD [8, 10]. The characteristics of CBD in PN are tau inclusions in glia and cell processes [8]. However, abnormal tau and NFTs were found in pontine neurons in CBD [22], suggesting such neurons are also affected. Although the pathological findings in PN are different between PSP and CBD, our results showed that the expression pattern of hOGG1-2a in PSP was similar to that in CBD, suggesting both disorders have some common neurodegenerative mechanism. The immunoreactivities for hOGG1-2a in the PN of PSP and CBD indicated that oxidative stress occurred in the PN neurons. Ample evidence indicates that mitochondrial impairment and oxidative stress are associated with NFTs formation and neurodegeneration [2]. Thus, mitochondrial dysfunctions and oxidative stress might be related to the pathogenesis of PSP and CBD [2, 3, 6, 30]. Indeed, complex I activity was reduced in a cybrid line that originated from PSP patients [30]. Although there is no report of mitochondrial dysfunction in CBD so far, considering the common biological background including tauopathies, it is possible that mitochondrial dysfunction is involved in the pathogenesis of CBD.

In the control subjects, the proportion of hOGG1-2a-positive neurons increased with age. In addition, Western blot analysis revealed high expression of OGG1-2a in the normal control at the age of 89 years. In human brain of patients older than 70 years, the amount of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in mtDNA is 15-fold greater than in patients < 70 years of age [18]. In other human organs (heart and diaphragm), 8-OHdG levels in mtDNA also increased exponentially with age [12, 13]. These results suggest that oxidative stress is increased significantly in old age rather than young age. The significant increase in hOGG1-2a in old age is consistent with response to oxidative stress associated with aging.

The up-regulation of mtDNA repair enzymes might overcome the oxidative insults in the brain. Furthermore, it is conceivable that up-regulation of mtDNA repair enzymes could be a potentially beneficial target for gene therapy aiming at neuroprotection in PD [9, 24]. Although the primary cause of neurodegeneration remains to be elucidated, our results indicate the importance of oxidative stress and mitochondrial dysfunction in the pathogenesis of neurodegenerative disorders.

In conclusion, we showed here that hOGG1-2a is up-regulated in limited areas of the brain lesion, i.e., SN of PD patients and PN of PSP and CBD patients. This selectivity suggests that up-regulation of hOGG1-2a is a

secondary response to neurodegenerative process, probably due to oxidative stress in the mitochondria.

Acknowledgement The authors thank Dr. Makiko Iijima for the technical assistance.

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Clinicogenetic study of *PINK1* mutations in autosomal recessive early-onset parkinsonism

Abstract—The authors performed *PINK1* mutation analysis of 51 families with autosomal recessive Parkinson disease (ARPD). They found two novel *PINK1* mutations: one was a homozygous deletion (13516-18118del) and the other a homozygous missense mutation (C388R). Clinically, the patients with the deletion had dementia. Thus, early-onset PD with dementia may be considered *PINK1*-linked parkinsonism. Furthermore, patients with *PINK1* mutations form 8.9% of *parkin*- and *DJ-1*-negative ARPD families.

NEUROLOGY 2005;64:1955–1957

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To date, six genes have been identified as the causative genes for familial forms of Parkinson disease (PD). All the causative genes are proven causes of PD except for *UCH-L1*. The *alpha-synuclein*[1] and *PARK8*[2] are the causative genes for autosomal dominant PD, and the *parkin*,[3] *DJ-1*,[4] and *PINK1*[5] are the causative genes for autosomal recessive PD (ARPD). Among the monogenic forms of PD, mutations of *parkin* have been detected in approximately 50% of families with ARPD.[6] In contrast, *DJ-1* mutations are rare in ARPD.[7] Recently, *PINK1* was detected as the causative gene for PARK6 in Italian and Spanish families.[5] We recently reported six novel point mutations in *PINK1* in Japanese, Israeli, Philippine, and Taiwanese families.[8] Thus, this mutation appears to be distributed worldwide. In the present study, we performed extensive mutation analyses for *PINK1* in 51 families with ARPD negative for *parkin* and *DJ-1* mutations.

Methods. Blood samples and clinical information were obtained from the neurologists. Diagnosis of PD was made by the participating neurologists. We investigated 51 ARPD families (56 patients; male 28, female 28, aged 9 to 80 years, mean 47 years) from nine countries including 26 Japanese, 11 Canadian, 5 Taiwanese, 4 Israeli, 1 Tunisian, 2 Korean, 1 Turkish, and 1 Bulgarian. In the present study, the subjects were either from families of consanguineous marriages or at least two affected siblings in the same generation, and we also included a single patient with early-onset parkinsonism with homozygosity in *PARK6* region in haplotype analysis. The study was approved by the Ethics Review Committee of Juntendo University. Blood samples for genetic analysis were collected after obtaining informed consent from 56 patients from 51 families. DNA was prepared using standard methods. None of the subjects had mutations in *parkin* and *DJ-1*.

We investigated 56 patients from 51 families for *PINK1* mutations.[3,8] For sequence analysis, the coding exons of *PINK1* were amplified by PCR using published primers.[8] We also performed direct sequencing of all coding exons of *DJ-1*. Dideoxy cycle sequencing was performed with Big Dye Terminater Chemistry (Applied Biosystems, Foster City, CA). This was followed by exon sequencing on ABI377 and 310 automated DNA sequence analyzers (Applied Biosystems). We used the following primer to detect the breakpoint of deletion involving exons 6 to 8: forward 5'-AGACAGAATCTTGCTTTGTTGC-3', reverse 5'-TGGTTCTCC-CTAACGTCTCCT-3'.

Results. We found two novel *PINK1* mutations. The first mutation was a homozygous exonic deletion involving exons 6 to 8. In this family (Family A), no consanguinity was reported. We performed the mutation analysis based on the homozygosity of the haplotype analysis in this gene region. Subsequently, we identified the breakpoint of the deletional mutation (figure 1). The second mutation was a novel point mutation. A homozygous missense mutation (C388R) in exon 6 was detected in all the affected members of the family (Family B) (figure 2). The mutation is highly conserved across species. We did not find the same mutations in 300 chromosomes from normal Japanese population.

Clinically, all three patients were of a young age at onset (table) and had parkinsonism that showed good response to levodopa. All had hyperreflexia but no remarkable autonomic disturbances. Patient A1 with the deletional mutation showed long disease duration, sleep benefit, and dystonia at onset, similar to patients with *parkin* mutations.[6] Patient A1 also had various psychological disorders including dementia, depression, and hallucinations. Sleep benefit was observed in Patient B2, but not in Patient B1. Patients B1 and B2 with the missense mutation showed lack of dystonia at onset and psychological disorders.

Discussion. To our knowledge, all the *PINK1* mutations reported so far have been point mutations. In the case of *parkin*, exonic deletion mutations are more frequent than point mutations among the Japanese.[7] This relatively high mutation rate of the de-

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Supported in part by the Ministry of Education, Science, Sports, and Culture of Japan and by the Fund for "Research for the Future" Program from the Japan Society for the Promotion of Science.

Received December 30, 2004. Accepted in final form March 1, 2005.

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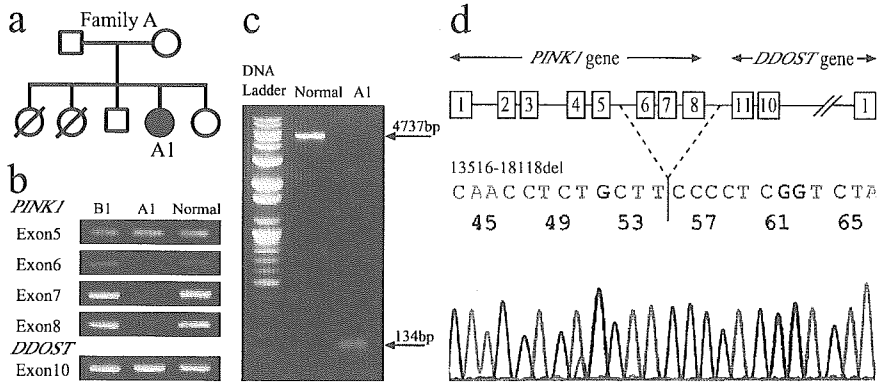

Figure 1. (a) Pedigree structure of Family A. (b) Patient A1 had exons 6 to 8 deletion. The DDOST *gene, which is located next to the* PINK1 *gene, showed no deletional mutation. Patient B1 and a normal control had no deletion. (c) Exons 6 to 8 PCR products: 4,737 bp, normal; 134 bp, exons 6 to 8 deleted. (d) Analysis of the sequences of the breakpoint regions revealed the exon 6 to 8 deletion. The sequenced deletion junctions did not reveal extensive homology such as repetitive elements to the deletion end point.*

letions may be related to the giant size of the 1.4-Mb *parkin* gene.[7] Considering the structure of *PINK1* spanning 18 kb,[5] *PINK1* deletional mutations may be more infrequent than *parkin* deletional mutations. The frequency of deletion formation correlates with the extent of homology between the short repeated sequences, although other factors may be involved. In our case, the deletion junctions sequenced did not reveal extensive homology such as repetitive elements to the deletion end point. A recent report identified a higher number of patients carrying a single heterozygous mutation in mostly sporadic early-onset parkinsonism (5%) than controls (1%).[9] Thus, the heterozygous deletional mutation may be a risk factor or it is possible that some patients with sporadic early-onset parkinsonism may have a single heterozygous deletion including this deletion. In this regard, it is important to look for the breakpoint not only to elucidate the mechanism of deletion but also to screen the deletions using PCR methods. Based on the semiquantitative analysis to detect deletions, conventional PCR methods are not suitable. Therefore, the information on the breakpoints allows us to detect the heterozygous deletion using conventional PCR methods.

We also found a novel point mutation in exon 6 of *PINK1*. The deletional mutation and the point mutation were located in the putative serine/threonine kinase domain of *PINK1*. Mutations in this region of *PINK1* may be important for the pathogenesis, as loss of function at this domain may affect the kinase activity or substrate recognition.[5]

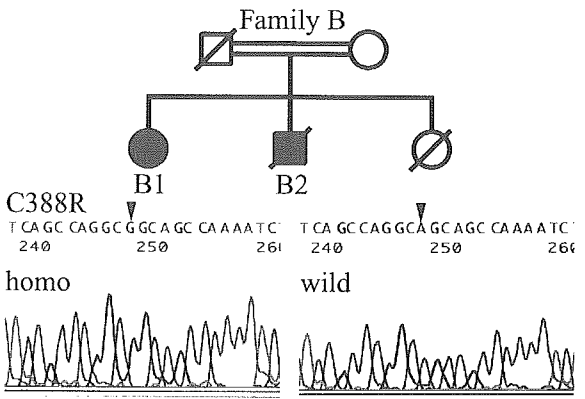

Figure 2. Pedigree and chromatograms of Family B illustrate missense mutation in a brother and a sister. A homozygous missense mutation (C388R) was found in exon 6. Complementary sequences are presented for exon 6.

Table *Clinical features of the three patients with* PINK1 *mutation*

	PINK1 mutation		
	13516-18118del homozygous Patient A1	C388R homozygous Patient B1	C388R homozygous Patient B2
Country	Japan	Japan	Japan
Consanguinity	−	+	+
Age at onset, y	38	39	44
Disease duration, y	25	16	5
Sex	F	F	M
Resting tremor	+	+	−
Rigidity	−	+	+
Bradykinesia	+	+	+
Postural instability	+	+	+
Frozen gait	+	+	−
Clinical response to levodopa	+	+	+
Wearing off	+	−	−
"On"/"off"	+	−	−
Asymmetry at onset	+	+	Not clear
Incontinence	−	−	−
Urinary urgency	−	−	−
Levodopa-induced dyskinesia	−	+	−
Sleep benefit	+	−	+
Dystonia at onset	+	−	−
Hyperreflexia	+	+	+
Dementia	+	−	−
Depression	+	−	−
Hallucination	+	−	−
Other psychosis	−	−	−

Young age at onset, parkinsonism, and good response to levodopa were noted in all three patients.

Because it is difficult to distinguish *PINK1*-positive ARPD from the *PINK1*-negative one,[8] a genetic approach is required for accurate diagnosis. In this study, the clinical manifestations of these three patients almost resembled those of patients with *parkin* mutations, although some features were different. For example, the age at onset was a little later than that of patients with *parkin* mutations. Patients B1 and B2 showed lack of dystonia at onset. Adding to our previous study, two of the 12 (17%) patients with PD with a *PINK1* point mutation (E417G and Q239X/R492X) showed dystonia at onset.[8,10] In a previous study of 101 patients with *parkin* mutation, dystonia at onset was noted in 42% of the patients, while dystonia was noted in 22% of the 85 patients without *parkin* mutation.[6] The lack of dystonia might help us to distinguish *PINK1*-positive ARPD from *parkin*- or *DJ-1*–positive ARPD.[8] In addition to dystonia, Patient A1 developed dementia. In contrast, patients with *parkin* mutations rarely develop dementia. Taken together with our previous study, the frequency of dementia in patients with *PINK1* mutations was 15.4% (2/13). Another patient who developed dementia had a nonsense mutation (R246X) in exon 3.[8,10] No patients with missense *PINK1* mutations had dementia so far.[8,10] Thus, in addition to the deletional mutation described in the present study, the defect of the putative serine/threonine kinase domain, including the 3'-terminal of *PINK1*, may be related to a more severe disease compared with missense mutations. However, further studies are needed to make any definite conclusion on the genetic-clinical correlation.

By combining our previous study, *PINK1* mutations were found in eight of 90 (8.9%) *parkin*- and *DJ-1*-negative ARPD families.[8,10] *PINK1* mutations appear to be the second most common in ARPD after *parkin*. However, the frequency of the mutation is not high enough to account for the majority of *parkin*-negative ARPD, and our results indicate that as many as 40% of our families were negative for *parkin*, *PINK1*, and *DJ-1*.

Acknowledgment

The authors thank the patients, their families, and all the participants.

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