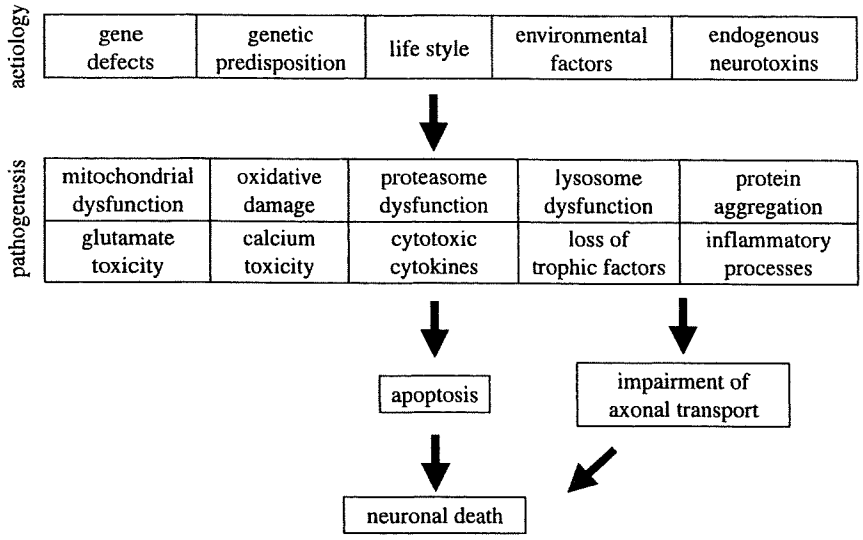

Figure 1. Aetiological and pathogenetic factors of PD. Aetiology refers to the cause of the disease and pathogenesis represents molecular events that lead nigral neurons to death.

4. NEUROTOXIN-BASED MODELS OF PD

The following substances have been used in producing animal models of PD, i.e. 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-tetrahydropyridine (MPTP), mitochondrial complex I-inhibitors such as rotenone, and α-synuclein overexpression. Systemic administration of MPTP produces selective degeneration of the dopaminergic neurons in SN in both humans and animals (Davis *et al.* 1979; Burns *et al.* 1983; Langston *et al.* 1983). We were interested in how MPTP killed nigral neurons. MPTP is oxidized by glial monoamine oxidase B to 1-methyl-4-phenylpyridinium ion (MPP^+; Chiba *et al.* 1984), which kills nigral neurons (Chiba *et al.* 1985; Javitch *et al.* 1985). We noted structural similarity between MPP^+ and NAD^+, which is an important cofactor of respiratory enzymes. We thought that MPP^+ might inhibit the activities of mitochondrial NAD^+-linked respiratory enzymes. Two groups reported inhibition of complex I by MPP^+ (Nicklas *et al.* 1985; Ramsay *et al.* 1986). We independently made the same observation (Mizuno *et al.* 1987*a*). Furthermore, we found inhibition of the α-ketoglutarate dehydrogenase complex of the mitochondrial tricarboxylic acid cycle by MPP^+ (Mizuno *et al.* 1987*b*). This enzyme synthesizes succinate from α-ketoglutarate; succinate is an electron donor for complex II of the electron transport chain. Thus dual inhibition of the activities of complexes I and II would impair deleteriously the electron transport and ATP synthesis. Inhibition of the electron transport induces oxidative damage by increasing the formation of reactive oxygen species.

5. AETIOLOGY AND PATHOGENESIS OF PD

We thought that mitochondrial function might be impaired in PD. Schapira *et al.* (1989) reported decreased activity of complex I in SN of PD. We found a decrease in complex I proteins by immunoblotting (Mizuno *et al.* 1989) and by immunohistochemistry (Hattori *et al.* 1991). We also found a decrease in the amount of α-ketoglutarate dehydrogenase complex in SN of PD by immunohistochemistry (Mizuno *et al.* 1994). Thus biochemical changes in PD were essentially similar to those of the MPTP-induced Parkinsonism.

Oxidative damage is also an important factor for nigral neuronal death; increase in iron (Youdim *et al.* 1989), increase in lipid peroxides (Dexter *et al.* 1989), decrease in glutathione (Sofic *et al.* 1992), increase in hydroxynonenal-modified proteins (Yoritaka *et al.* 1996) and increase in 8-hydroxy-deoxy guanine (Shimura-Miura *et al.* 1999) were reported in SN of PD. Reactive oxygen species impair mitochondrial proteins, further aggravating mitochondrial function. Ultimate outcomes are the dissipation of mitochondrial membrane potential and the release of cytochrome *c* into cytoplasm activating the apoptotic cascade. Apoptotic nigral neuronal death has been postulated in PD (Mochizuki *et al.* 1996; Anglade *et al.* 1997). The interaction of genetic predisposition and environmental factors is believed to trigger mitochondrial dysfunction and oxidative damage in sporadic PD. Additional aetiological as well as pathogenetic factors are summarized in figure 1.

6. DYSFUNCTION OF PROTEIN DEGRADATION IN PD

In recent years, dysfunction of protein degradation has emerged as an important contributor to nigral neuronal death in PD. Impaired protein degradation is likely to follow mitochondrial dysfunction and oxidative damage. In the presence of oxidative stress or mutated proteins, the folding process of proteins may be impaired resulting in an increase in misfolded proteins. Misfolded proteins are generally cytotoxic and have to be removed by protein degradating systems. In eukaryotic cells, the ubiquitin–proteasome system and the autophagy–lysosomal pathway are two major protein degradation systems. The ubiquitin system consists of three enzymes, i.e. the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase; these enzymes work

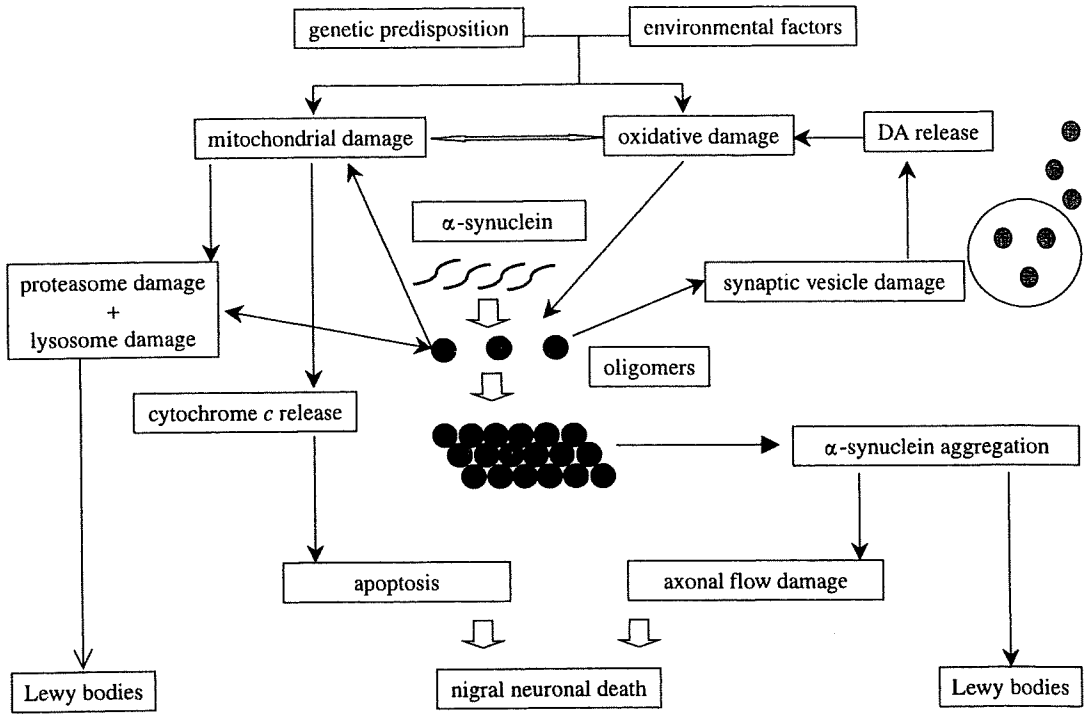

Figure 2. Schematic presentation of nigral neuronal death in PD. In sporadic PD, interaction of genetic predisposition and environmental risk factors is believed to initiate the pathological cascade. In familial PD due to *SNCA* mutations, mutant α-synuclein probably initiates the pathological process. DA, dopamine.

together to transfer ubiquitin molecules to target proteins that have to be metabolized by the 26S proteasome, which is an ATP-dependent proteolytic enzyme (Tanaka *et al.* 2004). Proteins with four or more than four ubiquitin molecules attached to lysine 48 residue of ubiquitin are recognized by the 26S proteasome. The 26S proteasome predominantly degrades short-lived nuclear and cytosolic proteins and misfolded proteins in the endoplasmic reticulum (Rubinsztein 2006).

The autophagy–lysosome pathway is able to degrade oligomers and aggregates of proteins as well as intracellular organelles (Yorimitsu & Klionsky 2005). In this pathway, double membrane-bound autophagosomes are generated by the elongation of small membranous structures, which encircle proteins to be digested. Then the autophagosome fuses with the lysosome to form the autophagosomes–lysosome. Then acidic hydrolases within the lysosome digest proteins that were incorporated into autophagosomes (Rubinsztein 2006).

What evidence do we have to suggest the dysfunction of protein degradation in PD? First of all, presence of Lewy bodies is strong evidence of impaired protein degradation. Lewy bodies consist of aggregated proteins and α-synuclein is the major component (Spillantini *et al.* 1997). Further interestingly, missense mutations of the *α-synuclein* gene (*SNCA*) cause autosomal dominant familial PD (Polymeropoulos *et al.* 1987; Krueger *et al.* 1998; Zarranz *et al.* 2004). α-Synuclein is a neuron-specific protein expressed predominantly in presynaptic membranes and the nucleus (Maroteaux *et al.* 1988). It is natively unfolded without a significant amount of secondary structure consisting of 140 amino acids (Weinreb *et al.* 1996). Thus the aggregation of α-synuclein emerged as one of the most important processes in nigral degeneration in PD. The mutant α-synuclein has increased tendency for self-aggregation (El Agnaf *et al.* 1998). α-Synuclein is mainly located in the lipid raft in membranes (Fortin *et al.* 2004; Kubo *et al.* 2005) and this localization appears to be important in the trafficking of α-synuclein and its final localization in presynaptic and synaptic vesicular membranes.

α-Synuclein is degraded by both autophagy and the proteasome; however, mutant forms of α-synuclein and oligomers are dependent on the autophagy–lysosome pathway for their clearance (Webb *et al.* 2003). Wild-type α-synuclein is translocated into lysosomes for degradation by the chaperone-mediated autophagy pathway; however, mutant A53T and A30P proteins can bind to the chaperon-mediated autophagy-pathway receptor on the lysosomal membrane, but act as uptake blockers inhibiting their own degradation and that of other proteins (Cuervo *et al.* 2004). In sporadic PD, dysfunctions of both ubiquitin–proteasome and autophagy–lysosome systems appear to be present. As 26S proteasome is an ATP-dependent enzyme, dysfunction of mitochondria will compromise its function. Furthermore, oxidative stress enhances oligomer formation of α-synuclein. Thus formed oligomers impair membrane structures (Volles & Lansbury 2002) such as synaptic vesicles and mitochondria, and further increase oxidative stress and mitochondrial dysfunction. In this way, vicious cycles will be formed within nigral neurons leading to neuronal death (figure 2). Decrease in the 26S proteasomal activity was reported in PD (McNaught *et al.* 2003).

We have only indirect evidence of lysosomal dysfunction in PD. Activation of lysosomal functions

were reported by treating cell lines with Parkinsonism-inducing neurotoxins, such as overexpression of mutant α-synuclein (Stefanis *et al.* 2001), or proteasomal inhibitors (Ding *et al.* 2003; Rideout *et al.* 2004). Other indirect evidence came from studies on the association of Gaucher disease and PD. Gaucher disease is an autosomal recessive lysosomal lipid storage disease caused by mutations of a lysosomal enzyme, glucocerebroside β-glucosidase. Gaucher disease and its carrier state appear to be risk factors for PD (Tayebi *et al.* 2003; Ahron-Peretz *et al.* 2004; Goker-Alpan *et al.* 2004, 2006; Lwin *et al.* 2004; Sato *et al.* 2005; Kono *et al.* 2007). Furthermore, the recently identified *ATP13A2*, the disease gene for *PARK9*-linked PD (Kufor-Rakeb syndrome), encodes a lysosomal membrane protein (Ramirez *et al.* 2006). These two observations indicate the importance of lysosomal function for the maintenance of nigral neurons.

7. PROGRESS IN FAMILIAL PD

Thirteen chromosome loci have been identified to be linked to familial forms of PD (table 1). As *PARK1* and *PARK4* represent the same locus, the number of the familial forms is 12.

(a) PARK1- *and* PARK4-*linked PD*

PARK1- and *PARK4*-linked PD is an autosomal dominant one caused by mutations of the α-synuclein gene (*SNCA*); *PARK1* is caused by missense mutations and *PARK4* by multiplications of *SNCA*.

Three missense mutations, i.e. A53T (Polymeropoulos *et al.* 1997), A30P (Krüger *et al.* 1998) and E46K (Zarranz *et al.* 2004), duplications (Chartier-Harlin *et al.* 2004; Ibanez *et al.* 2004; Nishioka *et al.* 2006; Fuchs *et al.* 2007) and triplications (Singleton *et al.* 2003; Farrer *et al.* 2004) of *SNCA* are known. Missense mutations are very rare; A53T is limited to families with Greece origin; only one German family with A30P mutation and one Spanish family with E46K mutation are known. Multiplications of *SNCA* appear to be more common. Singleton *et al.* (2003) reported triplication of *SNCA* in a large kindred (Iowanian family). The triplication involved the 1.5 Mb region; exons of the adjacent genes on both side of *SNCA* were also triplicated. The amount of protein expressed would be doubled. Duplications of *SNCA* (Chartier-Harlin *et al.* 2004; Ibanez *et al.* 2004; Nishioka *et al.* 2006) were also reported. In the recently reported Swedish–American family (Fuchs *et al.* 2007), patients in the Swedish branch had duplication and those in the American branch (Farrer *et al.* 2004) had triplication. They suggested unequal recombination and unequal crossing over as the potential mechanisms for duplication and triplication, respectively (Fuchs *et al.* 2007).

There is a clinico-genetic correlation. E46K mutation and triplications are associated with Parkinsonism and dementia, and the age of onset is younger than the other mutations; neuropathological changes are those of diffuse Lewy body disease. A30P mutation is usually not associated with dementia. Duplication usually does not cause dementia but it can happen (Nishioka *et al.* 2006; Fuchs *et al.* 2007). A53T mutation may cause dementia and cortical Lewy bodies are reported (Golbe *et al.* 1990).

Table 1. Familial forms of PD. (AD, autosomal dominant; AR, autosomal recessive; LB, Lewy bodies; SP, sporadic.)

type	loci	genes	Inh	LB
PARK1	4q21–23	*α-synuclein* (*SNCA*)	AD	+
PARK2	6q25.20–27	*parkin* (*PRKN*)	AR	±
PARK3	2p13	*unknown*	AD	+
PARK4	4q21–23	*α-synuclein* (*SNCA*)	AD	+
PARK5	4p14	*UCH-L1*	AD	
PARK6	1p35–36	*PINK1*	AR	+
PARK7	1p36	*DJ-1*	AR	
PARK8	12p11.2–q13.1	*LRRK2*	AD	±
PARK9	1p36	*ATP13A2*	AR	
PARK10	1p32	*unknown*	SP	
PARK11	2q36–37	*unknown*	AD	
PARK12	Xq21–25	*unknown*	SP	
PARK13	2p13	Omi/HtrA2	AD?	

Regarding the pathogenesis of *PARK1*-linked PD, increased tendency for oligomer and aggregate formations of mutant α-synuclein is likely to be the cause (El Agnaf *et al.* 1998; Fredenburg *et al.* 2007). In duplication and triplication, increased amount of normal α-synuclein is probably predisposing nigral neurons for oligomer and aggregate formations. Recently, two groups independently reported that *SNCA* polymorphic mutations are significant risk factors for sporadic PD (Mueller *et al.* 2005; Mizuta *et al.* 2006); some of those polymorphic mutations were associated with increased α-synuclein expression. Thus the molecular mechanism of nigral degeneration is similar between *SNCA*-mutated and sporadic PDs.

Regarding the toxicity of oligomers, Volles & Lansbury (2002) reported that protofibrillar Ala30Pro and Ala53Thr had greater permeabilizing activities per mole than the wild-type protein. The leakage of vesicular contents induced by protofibrillar α-synuclein exhibited a strong preference for low-molecular mass molecules like dopamine, suggesting a pore-like mechanism for permeabilization.

(b) PARK2-*linked PD*

PARK2-linked PD is an autosomal recessive young onset PD. Clinical features were first described by Yamamura *et al.* in 1973. The usual age of onset is between 20 and 40, but it can be before 10 years and above 60 years. When the age of onset is young, dystonic features and sleep benefits are characteristic symptoms; sleep benefit represents temporal improvement in Parkinsonism after a sleep or nap. They respond well to L-dopa; however, they will soon develop motor fluctuations. Pathologically, SN undergoes severe neuronal loss and gliosis; the locus coeruleus is much less severely involved. Usually no Lewy bodies are seen (Takahashi *et al.* 1994; Mori *et al.* 1998), although rare Lewy body positive cases were reported (Farrer *et al.* 2001).

We identified the disease gene as follows. While we were doing an association study between the genetic polymorphism of the manganese superoxide dismutase

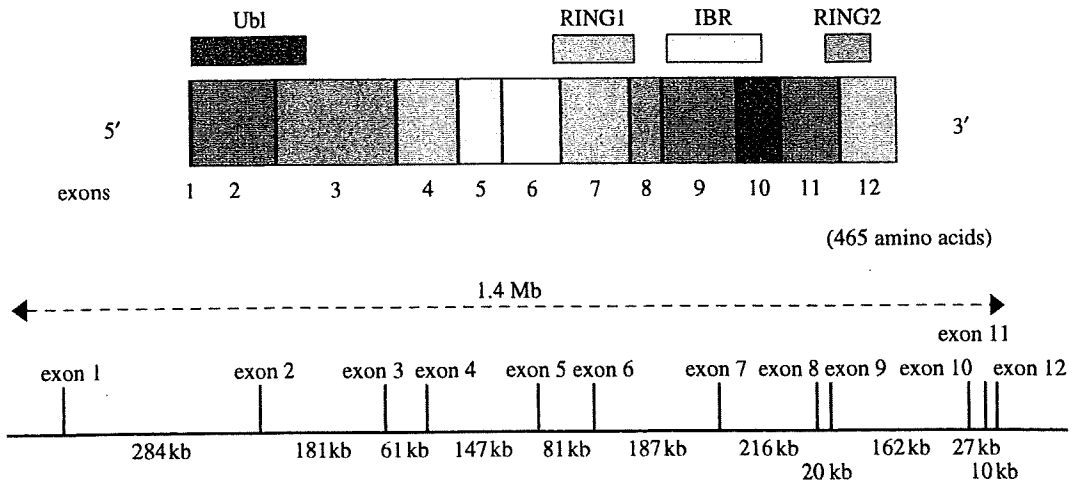

Figure 3. A schematic of the *parkin* gene. The coding region consists of 12 exons. In the amino terminal region, ubiquitin-like domain is indicated as 'Ubl', and in the carboxyl terminal side, two RING finger motives are indicated as 'RING1' and 'RING2'. RING, rare interesting gene; IBR, in between RINGs.

gene (*sod2*) and sporadic PD (Shimoda-Matsubayashi *et al.* 1996), we found a family that appeared to be linked to the *sod2* locus, which had been mapped to the telomeric region of the long arm of chromosome 6. We did linkage analysis on 13 similar families and mapped the disease locus to the long arm of chromosome 6 near the *sod2* locus (6q25.2–27; Matsumine *et al.* 1997).

While we were doing linkage analysis on additional families, we found a patient who showed deletion of one of the microsatellite markers (D6S305) that we were using in the linkage analysis (Matsumine *et al.* 1998). We thought that this microsatellite marker might be located within the disease gene. By screening the Keio BAC library (Asakawa *et al.* 1997) using D6S305, we cloned a cDNA consisting of 2960 base pairs, of which 1395 base pairs constituted the open reading frame (Kitada *et al.* 1998). As this was a novel gene, we named it *parkin*. The total size of *parkin* was 1.4 Mb, the second largest gene after *dystrophin*. The number of exons was 12. The gene product consisted of 462 amino acids. There were unique structures in parkin protein (figure 3). There was 30% homology to ubiquitin in the amino terminal domain and there were two RING-finger-like motifs in the carboxyl half of the protein. RING stands for rare interesting gene and RING-like structures have been found in proteins with ubiquitin-ligase activity (Lorick *et al.* 1999). By northern blot, parkin messengers were ubiquitously expressed including the systemic organs (Kitada *et al.* 1998).

Reported mutations in *parkin* now exceed 100 (Hattori *et al.* 1998; Abbas *et al.* 1999; Klein *et al.* 2000; Kann *et al.* 2002; Khan *et al.* 2003; Hedrich *et al.* 2004). Not only exonic deletions but also missense and nonsense mutations and multiplications of exons were reported.

We thought that parkin might be related to the ubiquitin–proteasome system (UPS); in fact the parkin protein had ubiquitin-protein ligase activity (Shimura *et al.* 2000). Since then many parkin-interacting proteins have been reported; the following are substrate candidates: CDCrel-1 (Zhang *et al.* 2000), which is believed to be negatively regulating transmitter release; glycosylated α-synuclein (Shimura *et al.* 2001); synphilin-1 (Chung *et al.* 2001), which is an α-synuclein-interacting protein; PAEL-receptor (Imai *et al.* 2001), which is an endoplasmic reticulum protein; p38 (Corti *et al.* 2003); polyglutamine (Tsai *et al.* 2003); α- and β-tubulins (Ren *et al.* 2003); cycline-E (Staropoli *et al.* 2003); SEPT5_v2 (Choi *et al.* 2003), which is also known as cell division-control protein-2; DJ-1 (Moore *et al.* 2005); RanBP2 (Um *et al.* 2006), which is a protein localized in the cytosolic filament of the nuclear core complex; and protein-1 (Ko *et al.* 2006). Also, parkin-regulatory proteins have been reported, i.e. CHIP (Imai *et al.* 2002), which is a chaperone; HSP-70 (Imai *et al.* 2002); Rpn10 subunit (Sakata *et al.* 2003); BAG5 (Kalia *et al.* 2004); Nrdp1/FLRF (Zhong *et al.* 2005); LRRK2 (Smith *et al.* 2005); and 14-3-3η (Sato *et al.* 2006*a*,*b*). In addition, non-lysine 48-related ubiquitylation substrates were reported. Lysine-63 polyubiquitylation is believed to be related to endocytosis, DNA repair, translation, iκB activation, DNA silencing, virus budding, protein sorting and protein trafficking (Tanaka *et al.* 2004). Recently, Lim *et al.* (2005) reported that synphilin-1 was polyubiquitylated at lysine 63 residue of ubiquitin. Other lysine sites are mono-ubiquitylated. Despite vast number of parkin-interacting proteins, there is no immunohistochemical proof of accumulation of above parkin-interacting proteins in autopsied patients.

Parkin-knockout (KO) mice do not show nigral neuronal loss or striatal dopamine deficiency (Goldberger *et al.* 2003; von Coelln *et al.* 2004; Perez & Palmiter 2005; Ko *et al.* 2005; Sato *et al.* 2006). What were reported are only subtle changes in dopaminergic functions. von Coelln *et al.* (2004) found some loss of neurons in the locus coeruleus and reduced startle response. Ko *et al.* (2005) found an increase in the amount of the aminoacyl-tRNA synthase cofactor p38 in the midbrain/hindbrain region of both young and old parkin-null mice. They postulated that p38 is a substrate of parkin as E3 ligase. They further showed that overexpression of p38 in the SN in mice lead to loss of dopaminergic neurons. They analysed the level of p38 in the cortical regions of the patients with parkin mutations by Western blotting; they found an increase in p38. We studied striatal dopamine receptors by *ex*

vivo autoradiography. In *parkin*-KO mice, both striatal D1 and D2 receptor bindings were significantly increased when compared with wild mice. Midbrain dopamine content was increased in KO mice. Increase in D1 and D2 receptor bindings in the striatum would indicate reduction in dopamine release; increase in dopamine in nigral neurons would cause oxidative stress.

Ved *et al.* (2005) found increased sensitivity of mitochondria to complex I inhibitors such as rotenone, fenperoximate, pyridaben or stigmatellin in their *parkin*-KO *Caenorhabditis elegans*; they observed similar effects by overexpressing α-synuclein, or knocking down *DJ-1*. Further interestingly, *parkin*-KO *Drosophila* produced by Creene *et al.* (2003) exhibited reduced lifespan, locomotor defects and male sterility. Locomotor defects were due to muscle degeneration with mitochondrial damage consisting of disruption and disintegration of the cristae. There was no neuronal loss in the brain including dopaminergic neurons in these flies. Pesah *et al.* (2004) also reported similar findings. Then Clark *et al.* (2006*a*,*b*) and Park *et al.* (2006) made *PINK1*/*parkin* double KO *Drosophila*. Both groups reported that overexpression of parkin rescued PINK1-KO-induced muscle damage; but PINK1 overexpression could not rescue parkin-KO-induced damage. Furthermore, they showed interaction of parkin and PINK1. They concluded that parkin was functioning in the downstream of PINK1 in a common pathway to keep mitochondrial integrity. Their flies also showed dopaminergic neuronal degeneration.

At a cellular level, Machida *et al.* (2005) constructed a parkin-knockdown cell line using SH-SY5Y cells, which showed apoptotic cell death. Furthermore, they found increase in the auto-oxidized forms of L-dopa and dopamine (Dopa chrome+dopamine chrome), suggesting the presence of anti-oxidative property in parkin. We reported a profound accumulation of iron in the SN of a *parkin*-mutated patient (Takanashi *et al.* 2001). Taken together, oxidative stress appears to be a pathogenetic pathway common to *PARK2*-linked and sporadic PDs.

(c) PARK6-*linked PD*

PARK6-linked PD is another form of young onset autosomal recessive PD. The age of onset is slightly older than that of *PARK2*, i.e. from 32 to 48 years (Valente *et al.* 2001). Therefore, dystonic features and sleep benefit are uncommon. Affected patients show L-dopa-responsive Parkinsonism. The disease gene was identified as *PINK1 (PTEN-induced kinase 1*; Valente *et al.* 2004*a*,*b*). *PTEN* stands for *phosphatase with tensin homology*. *PINK1* has eight exons and cDNA spans 1.8 kb. It encodes a protein with 581 amino acids. The protein is ubiquitously expressed including brain and systemic organs. Interestingly, it is a mitochondrial protein located in the matrix and the intermembrane space. It has a serine/threonine protein kinase domain. However, its function is not known (Valente *et al.* 2004).

Many missense and nonsense mutations have been reported (Hatano *et al.* 2004; Healy *et al.* 2004; Rohe *et al.* 2004; Valente *et al.* 2004; Li *et al.* 2005). In contrast to *parkin*, most of the *PINK1* mutations reported are either missense or nonsense mutations, although one family with a large deletion mutation is known (Li *et al.* 2005); this deletion involved exons 6–8 homozygously.

Regarding the molecular mechanisms of nigral neuronal death, as PINK1 has a protein kinase domain as LRRK2 has, dysfunction in some phosphorylation reactions may be important in the pathogenesis. PINK1 is inducible by PTEN and *PTEN* is an oncogene mutated in many neoplastic cells (Li *et al.* 1997). As oncogenesis and degeneration are the opposite sides of eukaryotic cell fate, elucidation of the function of PINK1 in relation to PTEN is an interesting topic.

(d) *Do heterozygotes for* parkin *or* PINK1 *mutations develop PD?*

In autosomal recessive diseases, usually both alleles should have a mutation to show the disease. But in cases of *PINK1* (Valente *et al.* 2004; Bonifati *et al.* 2005; Fung *et al.* 2006; Criscuolo *et al.* 2006; Toft *et al.* 2007) as well as *parkin* mutations (Hedrich *et al.* 2002; Khan *et al.* 2002; West *et al.* 2002; Olivieira *et al.* 2003; Clark *et al.* 2006*a*,*b*; Schlitter *et al.* 2006), at times only one mutation can be found (heterozygote). The question is, how do they get the disease? There are several possibilities. First of all, the second mutation may be localized in a place that is difficult to find by the currently available methods. The second possibility is the haploinsufficiency (West *et al.* 2002); here the amount of normal gene product is not sufficient to keep nigral neurons alive. But usually in autosomal recessive diseases, only one normal gene is sufficient to prevent the disease; parents (usually carriers) of a patient are normal in most of the cases. The third possibility is the interaction at the protein level; the mutated protein might interfere with the functions of normal protein (dominant-negative effect); but this possibility has not been proved. Finally, single heterozygous state might be acting as a risk factor for sporadic PD (Schlitter *et al.* 2006). Another interesting observation in *parkin* heterozygotes was made by Olivieira *et al.* (2003) who reported that mutations in the first RING finger domain tended to be heterozygotic and associated with later age of onset.

(e) PARK7-*linked PD*

PARK7-linked PD is another young onset PD. Clinical features are very similar to those of *PARK2*-linked PD (Duijin *et al.* 2001); the age of onset is usually 20–40 years. Some atypical features such as psychiatric symptoms (anxiety attacks; Dekker *et al.* 2003), and short statue and brachydactyly (Dekker *et al.* 2004) have been reported. The disease gene was identified as *DJ-1* (Bonifati *et al.* 2003), which had been cloned by Nagakubo *et al.* (1997). The size of *DJ-1* is 24 kb with eight exons encoding a protein consisting of 189 amino acids. *PARK7*-linked PD is very rare (Bonifati *et al.* 2003; Hague *et al.* 2003; Hering *et al.* 2004).

The function of DJ-1 protein is not well known. The active form of DJ-1 is a dimer of monomeric DJ-1. DJ-1 is a cytoplasmic protein; however, it can translocate into the mitochondria. It has a strong anti-oxidative property (Nagakubo *et al.* 1997; Abou-Sleiman *et al.*

2003; Canet-Aviles *et al.* 2004; Moore *et al.* 2005) that depends on its cysteine residue at 106, which undergoes oxidation to form a disulphide bond (Canet-Aviles *et al.* 2004). Downregulation of endogenous DJ-1 protein of the neuronal cell line by siRNA was reported to enhance the cell death induced by oxidative stress, ER stress and proteasome inhibition, but not by pro-apoptotic stimulus (Yokota *et al.* 2003). The Leu166-Pro mutant DJ-1 protein has a reduced anti-oxidative activity (Takahashi-Niki *et al.* 2004). DJ-1 protein expression is increased upon oxidative stress induced by paraquat (Mitsumoto *et al.* 2001). As nigral neurons are exposed to high oxidative stress owing to the presence of dopamine, DJ-1 may be acting as a strong anti-oxidative protein. As mutant DJ-1 was reported to interact with parkin (Moore *et al.* 2005), parkin might be acting as E3 ligase to remove mutated DJ-1.

(f) PARK8-*linked PD*

PARK8-linked PD is an autosomal dominant PD linked to the centromeric region of chromosome 12 (Funayama *et al.* 2002). Clinical features were described back in 1978 on a large Japanese family (Nukada *et al.* 1978); clinical features are essentially similar to those of sporadic PD, except for slightly earlier onset of age. Dementia is not a common feature but it is known to occur (Wszolek *et al.* 1997).

The disease gene was identified as *lrrk2* (Paisan-Ruiz *et al.* 2004; Zimprich *et al.* 2004); *lrrk2* is a huge gene encompassing 144 kb, consisting of 7449 bp and encoding a protein consisting of 2517 amino acids, and has 51 exons. The carboxyl half of the LRRK2 contains several functional domains such as ANK (ankyrin-repeat domain), LRR (leucine-repeat-rich), ROC (Ras of complex proteins), COR (carboxy terminal of ROC), MAPKKK (mitogen activated protein kinase kinase kinase) and WD domain that is rich in tryptophan and aspartate repeats. Pathogenetic mutations are concentrated in these functional domains.

PARK8-linked PD is now believed to be the most common form of autosomal dominant familial PD and 20 missense or nonsense mutations have been reported (Paisan-Ruiz *et al.* 2004; Zimprich *et al.* 2004; Aasly *et al.* 2005; Di Fonzo *et al.* 2005; Funayama *et al.* 2005; Hernandez *et al.* 2005; Kachergus *et al.* 2005; Nichols *et al.* 2005; Paisan-Ruiz *et al.* 2005; Mata *et al.* 2006). *lrrk2* Mutations were also found in some of the apparently sporadic PD patients (Gilks *et al.* 2005). One of the polymorphic mutations, G2385R, is a genetic risk factor for sporadic PD in Asian populations (Di Fonzo *et al.* 2006; Funayama *et al.* 2007; Tan *et al.* 2007).

Four different neuropathologies were reported within the same family (Wszolek *et al.* 2004); one of their patients showed brain stem-type Lewy body disease, the second showed diffuse-type Lewy body disease, the third accumulation of tau in the remaining nigral neurons and the last simple nigral atrophy. This observation tells us the difficulty of defining a disease by neuronal inclusions.

Function of LRRK2 is not well known. The ROC domain is able to bind GTP but it does not have GTPase activity, but GTP binding is essential for the MAPKKK domain to exert kinase activity (Ito *et al.* 2007); some of the mutant LRRK2 have increased kinase activity (Gloeckner *et al.* 2006). Other functional domains are believed to be important in protein–protein interactions (Zimprich *et al.* 2004). LRRK2 also interacts with other familial PD proteins; Smith *et al.* (2005) reported interaction of LRRK2 with parkin through the ROC domain; however, the interaction with parkin did not enhance polyubiquitylation of LRRK2.

Recently, Hatano *et al.* (2007) made a detailed observation on the intracellular distribution of LRRK2. It was found to be present in Golgi apparatus, plasma membrane, synaptic vesicles and particularly in the lipid rafts; presence in the lipid rafts suggests that LRRK2 is probably involved in signal transduction, membrane trafficking and cytoskeletal organization (Brawn & London 1998). Biskup *et al.* (2006) also reported the presence of LRRK2 in membrane structures, such as lysosomes, endosomes, transport vesicles and mitochondria. In this regard it is interesting to note that α-synuclein is also expressed in the presynaptic membranes and lipid rafts (Fortin *et al.* 2004; Kubo *et al.* 2005).

(g) PARK9-*linked PD*

PARK9-linked PD is an autosomal recessive disorder characterized by L-dopa-responsive Parkinsonism, supranuclear gaze palsy, pyramidal sign and dementia; it is also called as Kufor-Rakeb syndrome; the name of the initial Jordanian family with this disorder (Najim Al-Din *et al.* 1994). Age of onset was very early, between 11 and 16 years. MRI showed significant atrophy of the globus pallidus and the pyramids, as well as generalized brain atrophy in later stages. Some of them developed facial–faucial–finger mini-myoclonus, visual hallucinations and oculogyric dystonic spasm (Williams *et al.* 2005).

Hamprhire *et al.* (2001) performed linkage analysis on this Kufor-Rakeb family and mapped the disease locus to the short arm of chromosome 1 at 1p36 with a maximum LOD score of 3.6, the hot spot for autosomal recessive familial PD. The disease gene was identified as *ATP13A2* (Ramirez *et al.* 2006), which is a lysosomal membrane protein with an ATPase domain; exact function is still unknown. It is interesting to note that mutations of a lysosomal membrane protein can induce nigral degeneration, suggesting the importance of lysosomes for the maintenance of the integrity of nigral neurons.

(h) *Other forms of familial PD*

PARK3-linked PD is an autosomal dominant PD. Clinical features are essentially similar to those of sporadic PD with the age of onset between 36 and 89 (Gasser *et al.* 1998). Patients from two out of six families reported in that literature developed dementia. Autopsy findings in two families showed nigral degeneration and neurofibirillary tangles in cortical neurons.

PARK5-linked PD is an autosomal dominant PD. Only one family is reported (Leroy *et al.* 1998). Clinical features are similar to those of sporadic PD with the age of onset from 49 to 50. The disease gene was reported as ubiquitin carboxyl-terminal hydrolase-L1 (*UCH-L1*; Leroy *et al.* 1998). Ile93Met missense mutation

was found in the affected members. Deletion of exons 7 and 8 in mouse *UCH-L1* causes gracile axonal dystrophy (*gad* mouse); this is an autosomal recessive condition characterized by axonal degeneration and formation of spheroid bodies in motor and sensory nerve terminals (Saigho *et al.* 1999).

UCH-L1 is an enzyme that cleaves carboxy-terminal peptide bond of polyubiquitine chains. Thus UCH-L1 is an ubiquitin-recycling enzyme. UCH-L1 is a neuron-specific enzyme. Catalytic activity of Ile93Met-mutated UCH-L1 was reported to be half of the wild enzyme (Leroy *et al.* 1998). Thus the supply of ubiquitin for 26S proteasome may be reduced with this mutation.

PARK10 was found by genome-wide scanning. Hicks *et al.* (2002) studied 51 Icelandic families with more than one PD patient; they analysed 117 patients and 168 of their unaffected relatives using 781 micro-satellite markers. Allele-sharing, model-independent analysis of their results showed linkage to a region on chromosome 1p32 with a LOD score of 4.9. They designate this region *PARK10*. The disease gene has not been identified yet. Clinical features are essentially similar to those of sporadic PD and the mean age of onset was 65.8 years.

PARK11 was also found by genome-wide scanning; Pankratz *et al.* (2003*a*,*b*) screened 85 families with a very strong family history of PD and found an evidence of linkage to the long arm of chromosome 2 (LOD = 4.9). Clinical features are essentially similar to those of sporadic PD with the mean age of onset at 58 years.

PARK12 was also found by genome-wide scanning on sporadic PD; Pankratz *et al.* (2003*a*,*b*) screened 277 families without a strong family history of PD and detected linkage to the long arm of chromosome X (LOD = 3.2).

PARK13-linked PD was reported to have a mutation (G399S) in *Omi/HtrA2* (Strauss *et al.* 2005); they found G399S mutation in four German sporadic PD patients. They also identified a novel A141S polymorphism that was associated with PD ($p < 0.05$). Both mutations resulted in defective activation of the protease activity of Omi/HtrA2. Further studies are necessary before concluding that this is a new familial PD-inducing protein. A cDNA of HtrA2 was first isolated by Faccio *et al.* (2000); it encoded a protein (Omi) consisting of 458 amino acids and had homology to bacterial HtrA endoprotease and had a PDZ domain. Omi mRNA was expressed ubiquitously, and the gene was localized on human chromosome 2p12 near the *PARK3* locus; however, *PARK3*-linked PD patients did not have this mutation. Omi/HtrA2 is a nuclearly encoded mitochondrial protein localized in the intermembrane space. Further interestingly, it has a serine protease domain and it binds to apoptosis-inhibiting protein upon release into the cytoplasm (Suzuki *et al.* 2001). G399S mutant Omi/HtrA2 induces mitochondrial dysfunction associated with altered mitochondrial morphology and cells over-expressing G399S mutant Omi/HtrA2 are more susceptible to stress-induced cell death than wild-type (Strauss *et al.* 2005).

Finally, there are many families in which linkage analysis failed to show linkage to any one of the known loci that are associated with familial PD. By elucidating the functions of familial PD proteins, pathogenesis of sporadic PD will be better understood.

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In conclusion, COMT inhibition combined with LD/DDI improves absorption of a coadministered salt probably due to a COMT inhibition induced basic environment in gastrointestinal membranes. This improves dissolution and absorption of acids and salts. Thus it may enhance absorption of LD itself.[2,4,5]

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Familial Parkinsonism with Digenic *Parkin* and *PINK1* Mutations

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Abstract: **To clarify the genetic correlation between *parkin* and *PINK1*, we screened for *PINK1* mutations in 175 parkinsonism patients with *parkin* mutations. We detected two sibling pairs and one sporadic patient carrying both *parkin* and *PINK1* mutations. The age at onset of Parkinsonism of patients with the digenic mutations was lower than that of patients with the same *parkin* mutation alone. In addition, two of three patients carrying both *parkin* and *PINK1* mutations had schizophrenia. These findings indicate that *PINK1* mutation might modify *parkin* mutation-positive Parkinsonism, and *PINK1* mutations might**

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be associated withpsychiatric disorders. © 2008 Movement Disorder Society

Key words: Parkinson's disease; *parkin*; *PINK1*; digenic; psychiatric disorder

Parkinson's disease (PD) is one of the most frequent neurodegenerative disorders caused by loss of dopaminergic neurons in the substantia nigra, which results in decreased dopamine availability in the striatum. Although most cases with PD are sporadic, several genes are associated with the monogenic forms of Parkinsonism and related disorders. Identification of the causative genes and their functions in these rare forms of the disease can provide tremendous insights into the pathogenesis of PD and opens up new areas of medical research on this disease.

Parkin [MIM 602544; *PARK2*] and *PTEN-induced putative kinase 1* (*PINK1*) [MIM 608309; *PARK6*] have been reported as the causative genes of *PARK2*- and *PARK6*-linked autosomal recessive parkinsonism (ARP), respectively.[1] Intriguingly, several lines of evidence suggest that heterozygous mutations of *parkin* and *PINK1* could play a role in the development of parkinsonism despite the fact that they were originally identified as the responsible genes for ARP.[2,3] In addition, *parkin* and *PINK1* mutations might be associated with psychiatric disorders.[1,4,5] Thus, these results suggest the importance of these genes in sporadic PD as well as psychiatric disorders, in addition to ARP.

Recent biochemical and morphological studies using *Drosophila melanogaster* suggest that Parkin and PINK1 are involved, through a common pathway, in maintenance of mitochondrial function and that PINK1 acts upstream of Parkin.[6,7] Thus, it is possible that reduced activities of both gene products significantly lower the threshold of nigral degeneration compared with loss of activity of either Parkin or PINK1 alone.

In the present study, we screened for *PINK1* mutations in Parkinsonism patients with *parkin* mutations and detected patients with both *PINK1* and *parkin* mutations. Clinicogenetic analysis revealed that the presence of *PINK1* mutation in addition to *parkin* mutation could hasten the disease process.

PATIENTS AND METHODS

Subjects

This study was approved by the ethics review committee of Juntendo University School of Medicine. All subjects gave informed and written consent before participation. We selected patients with one- (single heterozygous, n = 19; 19 probands), and two- (homozygous or compound heterozygous, n = 156; 119 probands) *parkin* mutation(s). All patients were screened for *parkin* mutations by PCR, direct sequencing, and gene dosage analyses of all exons. The mean age at onset was 40.6 ± 17.6 years (±SD, range 18–75; one *parkin* mutation) and 27.9 ± 9.9 years (range 6–61; two *parkin* mutations). Among the total of 175 patients, 130 (74.3%) had family histories of Parkinsonism, and 149 (85.1%) were Asian (133 Japanese, 6 Chinese, 6 Korean, and 4 Taiwanese). The remaining were 15 Israelis, 3 Americans, 2 Tunisians, 2 Greeks, 1 Canadian, 1 German, 1 Iraqi, and 1 Moroccan.

Genetic Analyses

Genomic DNA samples were sequenced for all exons and splice junctions of *PINK1* using BigDye Terminator v1.1 Cycle Sequencing kit and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Only patients with heterozygous *PINK1* mutation were also screened by gene dosage analyses of all exons of *PINK1* by real-time PCR using TaqMan probes and ABI PRISM 7700 Sequence Detector (Applied Biosystems). Microsatellite markers flanking PARK2 and PARK6 loci were genotyped by PCR using fluorescence labeled primers, 3130 Genetic Analyzer, and GeneMapper software (Applied Biosystems). PCR, sequencing, and real-time PCR were used standard methods and published primers and probes.[8]

RESULTS

We identified a novel heterozygous mutation (p.R58-V59insGR) in exon 1 of *PINK1* in a pair of Japanese siblings with homozygous *parkin* mutations (p.T175PfsX2: Fig. 1; Family A, A3 and A4). These mutations were absent in 300 Japanese normal chromosomes, indicating that the mutations might be pathogenic. We also detected the same heterozygous *PINK1* mutation in one of the unaffected parents who had heterozygous *parkin* p.T175PfsX2 mutation (Fig. 1; Family A, A1). Another heterozygous *PINK1* mutation (p.R407Q) in exon 6 was detected in a pair of Chinese siblings with compound heterozygous *parkin* mutations (p.C441R and p.A138GfsX7: Fig. 1; Family C). The p.R407Q mutation of *PINK1* was reported previously in one Taiwanese patient with PD, but was absent in 188 Taiwanese control chromosomes.[9] We

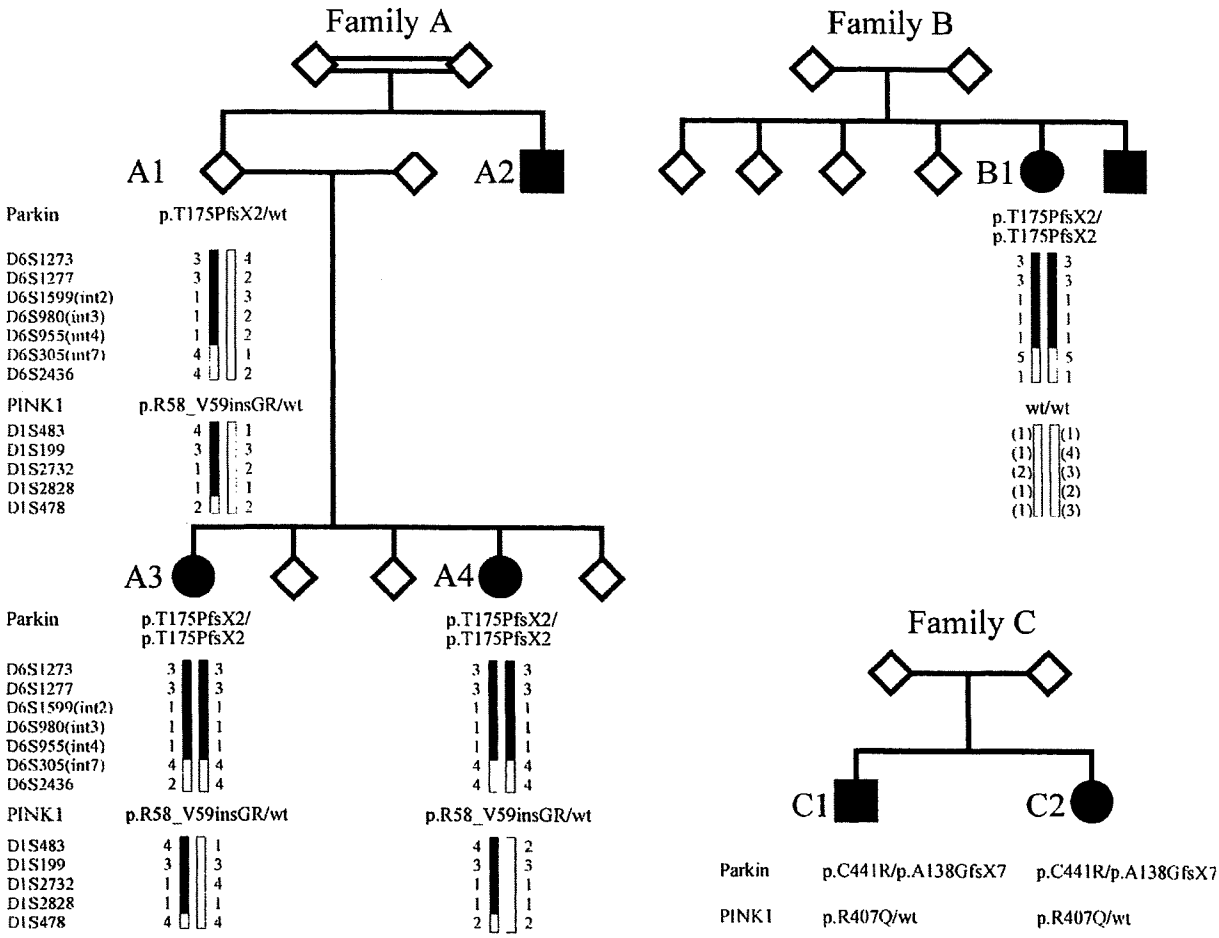

FIG. 1. Pedigrees of families analyzed in this study. Solid bars indicate shared disease haplotype. DNA of Patient A2 was not available. The haplotypes with undetermined phases in proband B-B1 are shown in parentheses. Int, intron.

did not detect this mutation in 300 Japanese normal chromosomes.

Next, we screened mutations of *PINK1* in patients who had heterozygous *parkin* mutation. We detected a patient with sporadic PD with heterozygous *PINK1* mutation (p.E476K) and heterozygous *parkin* mutation (p.P437L: Table 1; Patient D), which were absent in 300 Japanese normal chromosomes. In addition, we performed gene dosage analyses of *PINK1* for subjects who were identified with a single heterozygous mutation of the gene. No exonic rearrangements in *PINK1* were detected in any of the subjects.

We found one patient (Patient B1) from the original sample series who had homozygous *parkin* p.T175 PfsX2 mutation (the same mutation in Patients A3 and A4) but no *PINK1* mutation. Haplotype analyses of PARK2 and PARK6 loci in families A and B revealed a common haplotype in PARK2, but not in PARK6 locus (Fig. 1). The p.T175PfsX2 mutation was absent in 108 normal chromosomes from the Kyusyu region in Japan (families A and B originated from Kyusyu region). These results suggest that p.T175PfsX2 mutation of *parkin* spread from a single founder. With regard to the clinical features, the age at onset in patients of family A who had both homozygous *parkin* mutation (p.T175PfsX2) and heterozygous *PINK1* mutation (p.R58-V59insGR) was more than 10 years earlier than that in Patient B1 who had only homozygous *parkin* mutation (Table 1). In addition, the age at onset was significantly lower in patients with both two *parkin* and one *PINK1* mutations (Patients A3, A4, C1, and C2) compared with the only two *parkin* mutations ($P = 0.025$, Student's t-test). Interestingly, two of the three patients with PD of family A had nondrug-

TABLE 1. *Clinical features of patients*

Patient	A2	A3	A4	B1	C1	C2	D
Origin	Japan	Japan	Japan	Japan	Hong Kong	Hong Kong	Morocco
Age at onset	15	12	20	30	18	18	35
Disease duration	38	25	9	36	22	17	18
Sex	M	F	F	F	M	F	M
Resting tremor	+	+	+	−	−	−	+
Rigidity	+	+	+	NA	+	+	+
Bradykinesia	+	+	−	+	+	+	+
Postural instability	+	+	−	−	+	+	−
Frozen gait	−	+	−	+	−	−	+
Clinical response to levodopa	+	+	+	+	+	+	+
Wearing off	−	+	−	+	+	+	+
On off	−	+	−	+	+	+	+
Asymmetry at onset	+	+	+	−	−	−	+
Incontinence	−	−	−	−	−	−	−
Urinary urgency	−	−	−	NA	−	−	+
Levodopa-induced dyskinesia	+	+	−	+	NA	+	+
Sleep benefit	−	−	−	+	+	+	NA
Dystonia at onset	−	+	−	−	+	+	+
Hyperreflexia	−	−	−	−	+	+	−
Dementia	−	+	−	NA	−	−	−
Depression	−	−	−	−	−	−	+
Hallucination	+	+	−	+	−	−	−
UPDRS III (on/off)	20/NA	32/NA	NA	15/34	NA	NA	NA
Other psychosis	sch	sch	−	−	−	−	−
Special comment	−	−	−	−	−	−	RLS, RBD, facial dyskinesia with grimacing, severe dysarthria from onset

sch, schizophrenia; UPDRS, unified Parkinson's disease rating scale (motor score) in on and off condition; NA, not applicable or not available; RLS, restless legs syndrome; RBD, REM sleep behavior disorder; +, present; −, absent.

induced schizophrenia with hallucination. None of the patients in this cohort other than family A had schizophrenia. In addition, Patient B1 had hallucination and Patient D had depression.

DISCUSSION

In the present study, we set out to investigate whether Parkin and PINK1 could influence each other in patients with PD, based on the reports that Parkin and PINK1 share a common pathway using *Drosophila* models.[6,7] We identified digenic mutations of *parkin* and *PINK1* and found that *PINK1* mutation could modify the clinical course of *parkin* mutation-positive parkinsonism. Our results suggest that a single heterozygous mutation of *PINK1* might act not only as a susceptibility gene[3] but also as a modifier gene, in the pathogenesis of PD.

The relatively high frequency of *PINK1* heterozygous mutation identified in the present study (2.2% in PD vs. 0% in controls) is similar to that reported in a recent study (1.2% in PD vs. 0.4% in controls).[3] These results suggest that *PINK1* heterozygous mutation might also increase the risk of development of PD in patients who have mutations in other PD genes. Considering Patient D (Table 1), heterozygous *PINK1* p.E476K mutation was reported previously in three patients and two control subjects.[3,10] In addition, heterozygous p.P437L of *parkin* was found at the same frequency in patients and control subjects,[11] whereas none of Japanese 300 normal chromosomes harbored these mutations in the present study. This could represent differences based on ethnicity. Observation of patients carrying single nucleotide polymorphisms in both *parkin* and *PINK1* might be somewhat related to the position of mutated amino acids, the type of mutation, and one or more of the other gene mutations. On the other hand, the presence of asymptomatic carrier with the digenic mutations (family A-A1) also indicates the role of heterozygous mutation of *PINK1* in disease modification and suggests that other factors such as aging and environment are required for the development of the disease.

Based on recent reports, asymptomatic carriers of heterozygous *parkin* or *PINK1* mutations exhibit low ^{18}F-dopa uptake in the putamen on positron emission tomography.[12,13] These studies suggest that heterozygous mutation of *parkin* or *PINK1* gradually impairs the function of dopaminergic neurons. Interestingly, our patients of Family A, B, and D also developed

psychiatric disorders. Previous studies also reported that some *parkin* and *PINK1* mutations, even though heterozygous mutations, could be related to levodopa-responsive parkinsonism and psychiatric clinical pictures.[1,4,5] In this regard, our results might further indicate that *parkin* and *PINK1* mutations could be involved in psychiatric disorders not only singularly but also in combination. Furthermore, additional heterozygous *PINK1* mutation could hasten the age at onset of the disease. Combining the previous reports, our results emphasize that some heterozygous *PINK1* mutations might be related to the development of PD.[3,10] However, further genetic and functional analyses are required before one can make definite conclusions.

Intriguingly, digenic mutations of *PINK1-DJ-1* and *parkin-LRRK2* have recently been reported.[14,15] Screening for digenic or more mutations in responsible genes for familial PD could lead to the elucidation of the molecular pathway involved in nigral degeneration. In this regard, the mitochondrion is a good target for elucidating the pathogenesis of PD since Parkin, PINK1, and DJ-1 could be related to the mitochondrial function/dysfunction. Indeed, several studies highlighted the role of ARP gene products in maintaining mitochondrial function and in the pathogenesis of PD. Our results and these findings suggest that, multigenic mutation screening and analyses for interactions among related gene products could help enhance our understanding of the pathogenesis of PD.

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Mutation Analyses in Amyotrophic Lateral Sclerosis/ Parkinsonism–Dementia Complex of the Kii Peninsula, Japan

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Abstract: To clarify the genetic background of amyotrophic lateral sclerosis (ALS)/parkinsonism–dementia complex (PDC) of the Kii peninsula, Japan (Kii ALS/PDC), we performed extended mutation analyses of three patients with pathologically diagnosed Kii ALS/PDC. Direct sequencing analyses were performed in 19 genes, including ALS/frontotemporal lobar degeneration (FTLD)-related genes (*SOD2, SOD3, ALS2/alsin, SMN1, PGRN, ANG, VEGF, VCP, VAPB, DCTN1, CHMP2B,* and *TARDBP* or *TDP-43*), tauopathy-related gene (*GSK3*β), and parkinsonism-related genes (*alpha-synuclein, LRRK2, parkin, DJ-1, PINK1,* and *ATP13A2*). Gene dosage analyses were conducted in screening of *MAPT, alpha-synuclein, TDP-43* (or *TARDBP*), *GSK3*β, and *parkin*. We found no mutation in the 19 genes. We found a homozygous nonsynonymous SNP (*ALS2/alsin* V368M) shared by all the three patients. Gene dosage was normal in *MAPT, alpha-synuclein, TDP-43, GSK3*β, and *parkin*. The present findings, together with a previous negative study on *MAPT* and *SOD1* mutation, further elucidated the lack of causative mutations in all exons, exon–intron boundaries, or some rearrangements of the reported major causative or susceptible genes related to ALS, FTLD, parkinsonism, synucleinopathy, TDP-43 proteinopathy, and tauopathy. However, the familial aggregation and lack of any environment factors suggest that Kii ALS/PDC is caused by other yet unidentified genetic factors. © 2008 Movement Disorder Society

Key words: Kii ALS/PDC; amyotrophic lateral sclerosis; parkinsonism; dementia; genetics

The Western Pacific amyotrophic lateral sclerosis (ALS)/parkinsonism–dementia complex (PDC) is a progressive and fatal neurodegenerative disorder with high incidence among the indigenous people of three areas on the Pacific volcanic belt; Chamorros on Guam and Mariana Islands, Papuans in the coastal plain of West New Guinea, and Japanese in the Kii peninsula of Japan.[1] Clinically, ALS and PDC occur in isolation or in combination. Neuropathologically, ALS and PDC on Guam and Kii are characterized by abundant neurofibrillary tangles (NFTs) throughout the entire central nervous system, most markedly in the brainstem and temporal lobe, together with selective involvement of the upper and lower motor neurons.[2,3] Most, but not all, investigators consider ALS and PDC to be different manifestations of a single disease entity (ALS/PDC).[1,4]

In the 1980s, the disappearance of high incidence of ALS and marked decline in PDC were reported in Guam possibly related to changes in the environment and westernization of the lifestyle of Chamorros.[5] Although various environment factors, such as consumption of cycad and fruit bats, and deficiency of various minerals, have been suspected in Chamorros of Guam,[6–8] none has been experimentally verified so far. With regard to genetic factors, although *Tau (MAPT)*[9]

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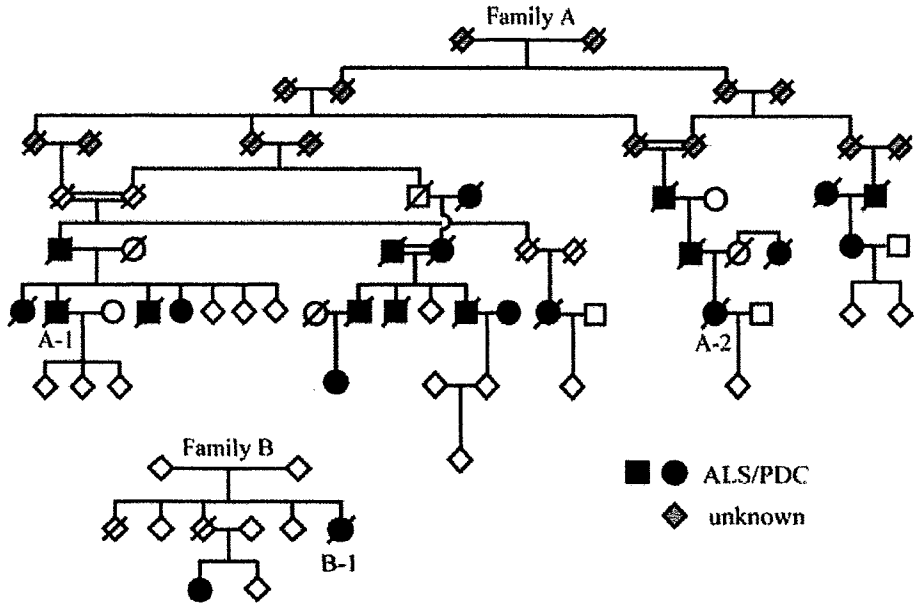

FIG. 1. Family trees (Family A and Family B) of patients with ALS/PDC from the Kii peninsula. Squares, men; circles, women; solid symbols, patients with amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC); open symbols, healthy individuals; gray symbols, unknown (precise information was not available); /(slash mark), deceased. The sexes are concealed (diamond symbols) to safeguard the confidentiality of the family members.

might be a modifier gene that increases the risk for Guam ALS, Guam PDC, and Guam neurodegenerative disorders in the presence of other unidentified gene(s) or by regulating *Tau* expression,[10,11] no causative mutation in *Tau* was detected in both Guam and Kii ALS/PDC with abundant NFTs pathology.[4] In addition, a previous genome-wide association study could not identify a single gene locus for Guam PDC, suggesting a geographic disease isolate with a complex genetic, genetic/environmental, or purely environmental etiology.[12] On the other hand, other studies proposed a mixture of other factors in the pathogenesis of ALS/PDC on Guam, including prolonged exposure to an environment severely deficient in Ca^{2+} and Mg^{2+} concurrent with a susceptibility genotype of *TRPM7* T1482I allele,[13] as well as neurotoxicity associated with β-methylamino-L-alanine in the cycads.[6]

In contrast to Guam ALS/PDC, high average annual incidence rates (417.9/100,000 in 1995–1998, unpublished data) of ALS/PDC in Hohara area of Kii continuing even after dramatic changes in foods and drinking water, and the much higher aggregation in the same family with a family history of approximately 80% in patients with Kii (40% in Guam ALS/PDC) strongly suggest major contribution of genetic factors.[11,14,15] And no customs of eating cycad or fruit bats exist in Japanese people living in Kii. Thus, further genetic analyses for Kii ALS/PDC might help disclose the pathogenesis of ALS/PDC.[4,16] Backed with this background, we performed mutation analysis of genes related to ALS, frontotemporal lobar degeneration (FTLD), tauopathy, and parkinsonism, and gene dosage analyses of *MAPT*, *alpha-synuclein*, *TDP-43* (or *TARDBP*), *GSK3*β, and *parkin* in three Kii patients with neuropathologically verified ALS/PDC.

METHODS

Patients

The study was approved by the Ethics Review Committees of Mie and Juntendo Universities. We analyzed DNA samples of three patients with Kii ALS/PDC from two families in the Kii peninsula. Consanguinity was seen in Family A but not in Family B (Fig. 1). The clinical diagnoses were based on features of typical ALS and PDC occurring singularly or in combination as shown in Table 1. The clinical diagnosis was verified as ALS/PDC in each patient at postmortem examination. All patients showed cardinal neuropathological findings of ALS/PDC including abundant NFTs associated with loss of nerve cells in the cerebral cor-

TABLE 1. *Clinical features of three patients (A-1, A-2, and B-1) with ALS/PDC of the Kii peninsula*

	A-1	A-2	B-1
Suspected mode of inheritance	AD	AD	AD?
Age at onset (yr)	70	52	70
Duration of the illness (yr)	7	8	6
Sex	M	F	F
Clinical presentation	ALS with Dementia	PDC with ALS	PDC
Dementia	+	+	+
Psychosis	−	−	−
Resting tremor	−	−	+
Bradykinesia	−	+	+
Rigidity	−	+	+
Gait disturbance	+	+	+
Asymmetric sign at onset	−	+	+
Clinical response to levodopa	NA	+	−
Hoehn-Yahr stage (best on stage)	0	4.5	5
Hyperreflexia	−	+	+
Babinski's sign	−	+	+
Bulbar palsy	+	+	+
Respiratory failure	+	−	−
Amyotrophy	+	+	−
Fasciculation	+	+	−
Sensory disturbance	−	−	−
Orthostatic hypotension	−	−	−
Incontinence	−	+	+
Urinary urgency	−	−	−

ALS/PDC, amyotrophic lateral sclerosis/parkinsonism-dementia complex; AD, autosomal dominant; F, female; M, male; NA, not available; +, present; −, absent.

tex and brainstem, loss of anterior horn cells of the spinal cord, together with degeneration of pyramidal tract, and loss of Betz cells in the motor cortex. NFTs with neuronal loss were prominent in the medial temporal lobe without senile plaques.[4,17,18] Blood samples for genetic analysis and clinical information were collected after obtaining informed consent from the participants.

Genetic Analysis

Genomic DNA samples were isolated from peripheral blood using standard protocols. They were amplified by polymerase chain reaction (PCR) for each exon and sequenced for all exons and splice junctions of 19 genes (*SOD2*,[19] *SOD3*,[20] *ALS2/alsin*,[21,22] *SMN1*,[23] *PGRN*,[24,25] *ANG*,[26] *VEGF*,[27] *VCP*,[28] *VAPB*,[29] *DCTN1*,[30] *CHMP2B*,[31] *TDP-43*,[32] *GSK3*β,[33] *alpha-synuclein*,[34] *LRRK2*,[35] *parkin*,[36] *DJ-1*,[37] *PINK1*,[38] and *ATP13A2*[39]) using BigDye Terminator v1.1 Cycle Sequencing kit and 310 and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Gene dosage analyses of exons 1, 9, 10, 13 of *MAPT*, exon 3 of *alpha-synuclein*, exon 3 of *TDP-43*, exon 5 of *GSK3*β, and all exons of *parkin* were performed by real-time PCR using TaqMan probes and ABI PRISM 7700 Sequence Detector (Applied Biosystems). We used β-actin or β-globin as an internal standard for each real-time PCR. We used the primers and probes prepared by "Custom TaqMan Genomic Assays" (Applied Biosystems). Sequences of the primers and probes, and conditions of PCR, sequencing, and real-time PCR are available upon request to the corresponding author or the first author.

RESULTS

Genetic Studies

Direct sequencing of all exons and splice junctions of the 19 genes (*SOD2*, *SOD3*, *ALS2/alsin*, *SMN1*, *PGRN*, *ANG*, *VEGF*, *VCP*, *VAPB*, *DCTN1*, *CHMP2B*, *TDP-43*, *GSK3*β, *alpha-synuclein*, *LRRK2*, *parkin*, *DJ-1*, *PINK1*, and *ATP13A2*) revealed no mutations that were shared by all three patients. A homozygous non-synonymous SNP (*ALS2/alsin* V368M: rs3219156) was detected in all three patients. This SNP showed a high allele frequency in the dbSNP database of normal Asian population (http://www.ncbi.nlm.nih.gov/SNP/) and all our 100 controls of healthy Japanese population had homozygous V368M. Gene dosage was normal in exons 1, 9, 10, 13 of MAPT, exon 3 of *alpha-synuclein*, exon 3 of *TDP-43*, exon 5 of *GSK3*β, and all exons of *parkin*.

DISCUSSION

In families with Kii ALS/PDC, many affected members in more than two generations have been described, with age at onset of 57-63 (mean 60.0) years for Kii ALS and 53-74 (mean 66.5) years for Kii PDC.[1] Anticipation has not been observed. Some unaffected siblings of parents with ALS/PDC were identified.[4] No marked gender differences in prevalence have been seen. These patterns suggest autosomal dominant inheritance with low penetrance rather than autosomal recessive one. Considering this genetic background and the clinicopathological features, previous studies on *MAPT* and *SOD1* mutations as well as *APOE* polymorphism of Alzheimer's disease (AD), CYP2D6B of Parkinson's disease (PD) and polymorphic dinucleotide repeats in *MAPT* intron of progressive supranuclear palsy were reported to be negative.[1,4] In this study, for further clarification of genetic factors, we extended candidate gene analyses for variants in coding regions