As an early disease onset is frequent in familial PD, we undertook in this study to estimate the prevalence of known genetic forms of Parkinsonism in a typical Australian population (Queensland) by screening a subset of early-onset cases, derived from a large movement disorders clinic in Brisbane, Australia.

2. Methods

2.1. Sampling frame

Patients were derived from a case series of 950 patients with a diagnosis of PD seen in one specialist movement disorders practice in Brisbane, between 2000 and 2005. Informed consent was obtained from all participating patients.

2.2. Patient selection

Patients were included in the study if they (1) received a diagnosis of probable PD according to stringent clinical and neurological criteria; (2) exhibited onset of symptoms ≤50 years; and (3) had been seen at the clinic between 2001 and 2005.

2.3. Patient ethnicity

Patients in this sample were in the majority (95%) of European extraction. Two patients reported Australian aboriginal ancestry, one case was of New Zealand Maori extraction and one patient reported Asian ancestry.

2.4. Screening methodology

DNA was extracted from peripheral blood according to the standard methods for use in gene-dosage studies. A whole genome amplification of the original DNA was performed prior to sequencing studies. The entire coding region of the *SNCA* (MIM 163890), *PRKN* (MIM 602544), *DJ-1* (MIM 602533) and *PINK1* (MIM 698309) genes, and exon 41 of the *LRRK2* gene (MIM 609007) were screened by direct sequencing using standard methods. All exons of *PRKN* were examined for gene-dosage abnormalities using TaqMan based methods. Details have been given elsewhere [2,3]. Primers and TaqMan probes used in the quantitative PCR amplification analysis are listed in Table 1. *UCHL1* (*PARK5*) was excluded from the study for reason of its extreme rareness; the importance of the gene in PD is still unclear. For similar reasons, no attempt was made to identify mutations in the *ATP13A2* (*PARK9*) gene, that has been identified as causative for rare cases of Kufor Rakeb disease.

3. Results

Seventy-four patients met the inclusion criteria. Demographic data are shown in Table 2. In this sample, males were slightly over-represented ($n = 44$, 59.5%). A self-reported family history of PD existed for 30 patients (40.5%). Of these, 13 each had a first- or a second-degree relative with PD and four patients reported a more distant relative affected by the condition. Among the first-degree relatives, there were six affected fathers and four affected mothers. More specific unambiguous inheritance patterns could not be ascertained for any of the patients. The average age of onset was 42.4 ± 5.7 years.

Screening identified mutations in five patients with putative genetic disease (Table 3 and Fig. 1). Two patients carried *PRKN* mutations (c.34G > C and c.1289G > A), the first leading to a heterozygous p.G12R amino acid change in exon 2, and the second to a homozygous p.G430D amino acid change in exon 12.

Table 1
Primers and TaqMan probes used in the *PARK2* analysis

Primer name	Forward (5′–3′)	Reverse (5′–3′)	Probe
Parkin Ex1(MGB)	CGCAGCCGCCACCTA	GGCGCAGAGAGGCTGTAC	Fam-CCCAGTGACCATGATAGGTA-MGB
Parkin Ex2	CACAGTCCAGTCATTCCTCAGC	GTTCAACTCCAGCCATGGTTTC	Fam-CCTTCCCTGCGAAAATCACACCGCAT-Tamra
Parkin Ex3	GAGGACTGAGCTGCTGAGG	AGAGCATTGTTCACATTGTGC	Fam-TCGTCGCCTCCAGTTGCATTCAT-Tamra
Parkin Ex4	TCTTCTCCAGCAGGTAGATCAATC	TGCTGACACTGCATTTCCTTAC	Fam-ATGTGTATTGCAAAGGCCCCTGTCAAT-Tamra
Parkin Ex5(MGB)	CCCAAAGGGTCCATCTTGCT	ACTAGTCCCAGGGCAGTGT	Fam-ACCACTCATCCGGTTTGG -MBG
Parkin Ex6	TAGAGGAAAAATGAGCAGCCG	CGTAATGCAAGTGATGTTCCGA	Fam-AGCACACCCCACCTCTGACAAGGAAAT-Tamra
Parkin Ex7	TGCCGATCATTGAGTCTTGTCA	CCAGTTGCCTTTCCACACTGA	Fam-AGTGGAAACAGTCTAAGCAAATCACGTGGCT -Tamra
Parkin Ex8	ATTCTTCTTTCCAACAGCTGGC	ATGACAGTCTGATGCAGCCTTT	Fam-CCCAACTCCTTGATTAAAGAGCTCCATCACT-Tamra
Parkin Ex9	TTTTGCAGTACAACCGGTACCA	AGCAAACAAGGACAGGAACACA	Fam-AGTATGGTGCAGAGGAGTGTGTCCTGCAT-Tamra
Parkin Ex10	CCAAATGCAACCTAATGTCCC	TGGAGGAATGAGTAGGGCATTC	Fam-AGTGCAGTGCCGTATTTGAAGCCTCAT-Tamra
Parkin Ex11	AGCTGAGATTAAACGCCTTTCC	TTTTTCCACTGGTACATGGCAG	Fam-CTTTGTTTCCCCAGGCCTACAGAGTCGGAT-Tamra
Parkin Ex12	GTTTTCCAGGTACTTGCTGCG	AAGGTAGACACTGGGTATGCTCC	Fam-ACCACACCTTTGTTTTCTGCCCCCT-Tamra

Table 2
Patient demographic data

Total number of patients, *n* (%)	74 (100.0)
Male, *n* (%)	44 (59.5)
Female, *n* (%)	30 (40.5)
Age at onset (years)	
All[a]	42.4 ± 5.7 (26–50)
Male[a]	42.4 ± 6.3 (26–50)
Female[a]	42.5 ± 4.9 (35–49)
Age at examination (years)	
All[a]	58.0 ± 8.1 (40–78)
Male[a]	58.9 ± 8.6 (40–77)
Female[a]	56.5 ± 7.2 (43–78)

[a] Data given as mean ± SD (range).

One patient possessed a heterozygous p.G411S mutation resulting from a c.1231G > A mutation in exon 6 of the *PINK1* gene.

Two individuals were found to be heterozygous for the common p.G2019S mutation in *LRRK2*.

No alpha-synuclein or *DJ-1* variants were observed. The results are summarized in Table 4.

The previously reported *PRKN* (p.S167N) and *PINK1* (p.Q115L) polymorphisms were also identified (data not shown).

Gene copy assays were performed for the *PRKN* gene only. The possibility that exonic or intronic rearrangements and deletions have occurred in the other *PARK* genes cannot be excluded.

4. Discussion

The importance of genetic factors for the aetiology of PD has been debated controversially for a long time. Longitudinal twin studies argued for a genetic element contributing to the condition whereas other cross-sectional studies could find no evidence for inheritance [4,5]. In the meantime, at least five genes have been identified that are implicated in the

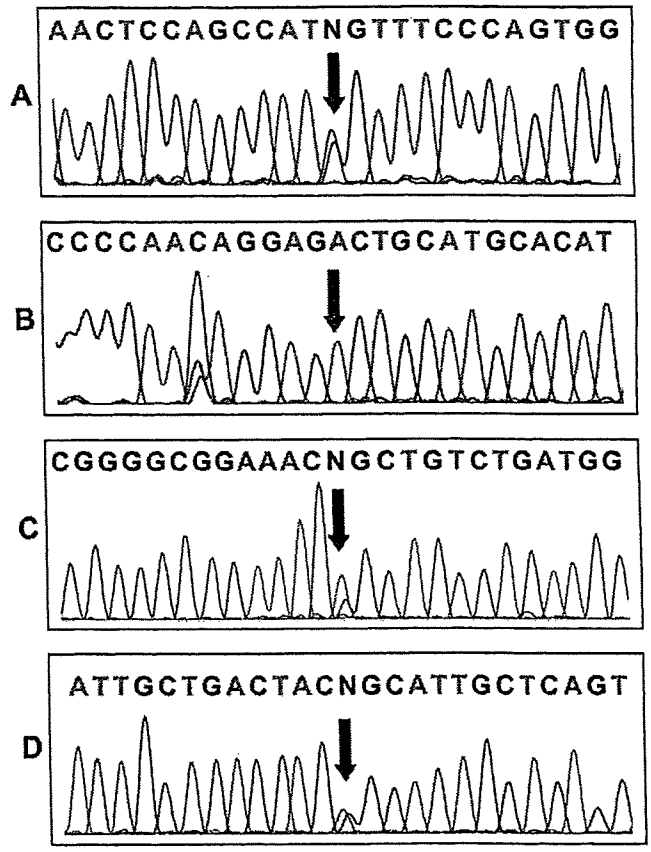

Fig. 1. Electropherogram of parts of the *PRKN* (A, B), *PINK1* (C) and *LRRK2* sequence, showing mutated sites, indicated by arrows. A. Heterozygous *PRKN* mutation p.G12R (c.34G > C, exon 2); B. Homozygous *PRKN* mutation p.G430D (c.1289G > A, exon 12); C. Heterozygous *PARK6* mutation p.G411S (c.1231G > A, exon 6); D. Heterozygous *PARK8* mutation p.G2019S (c.6055G > A, exon 41).

Table 3
Detected mutations

Gene and ID	Mutations	Family history of PD	Age at onset
PARK1 (alpha-synuclein)	None detected		
PARK2 (PRKN)			
10782	p.G12R (c.34G > C: exon 2) heterozygous	Negative	40
12238	p.G430D (c.1289G > A: exon 12) homozygous	Negative	30
PARK6 (PINK1)			
11280	p.G411S (c.1231G > A: exon 6) heterozygous	Positive: Uncle, grandmother, cousin	26
PARK7 (DJ-1)	None detected		
PARK8 (LRRK2)			
10002	p.G2019S (c. 6055G > A: exon 41) heterozygous	Positive: Aunt	49
12248	p.G2019S (c. 6055G > A: exon 41) heterozygous	Positive: Father, Mother	46

Table 4
Clinical characteristics of mutation carriers

10782	Female, aged 48 years at consultation. Initial symptoms: rigidity, loss of dexterity and dystonia of right foot. Good response to levodopa. No family history of PD
12238	Female, aged 35 years at consultation. Initial symptoms: right hand tremor and dystonia of right foot. No family history of PD. Good response to 100 mg levodopa b.i.d. suffers from depression, requiring treatment
11280	Male, aged 39 years at consultation. Initial symptoms were speech problems and loss of dexterity at age 26 years. Family history of Parkinson's disease: paternal grandmother, uncle and cousin
10002	Female, aged 58 years at consultation. Initial symptoms: unilateral tremor and gait disturbance at age 49 years. Family history: aunt. Currently, well responding to treatment with 100 mg levodopa t.d.s.
12248	Male, aged 58 years at consultation. Initial symptoms: cramping, "turning in" of right leg and gait disturbances. Family history: father and mother (father subsequently found to carry the *LRRK2* G2019S mutation). Currently well responding to treatment with 200/50 Madopar and 5 mg Artane t.d.s

development of a monogenic form of PD (*PARK1, 2, 6, 7,* and *8*). These monogenic forms of the condition may mimic clinically sporadic PD but generally (though not exclusively, with the probable exception of *LRRK2*) appear at an earlier age of onset. In this study, we therefore screened a cohort of 74 PD patients with age of onset earlier than 50 years, taken from a case series of 950 patients, for monogenic disease.

The first gene implicated in the development of PD was *SNCA*, coding for the alpha-synuclein protein (*PARK1*). Three point mutations and gene multiplications leading to familial Parkinsonism have been reported, although these mutations are considered rare and are estimated to contribute <1% of monogenic cases of PD. In our cohort, no mutations in this gene were detected, in agreement with statistical expectations with respect to the sample size.

Mutations in the *PRKN* gene (*PARK2*) were first found to be causative for autosomal recessive juvenile Parkinsonism in Japanese families [6]. The frequency of *PARK2* mutations has been estimated to be as high as 40–50% in early-onset disease [7–9] and 10–20% in sporadic cases [7,9–11]. In our study, two patients (2.7% of all subjects screened) possessed *PRKN* mutations (Table 3). Notably, no confirmed exon-dosage abnormalities were observed. This number is lower than that reported in several comparable studies, which report *PRKN* mutations to be present in between 10.4 and 18.0% of early-onset cases [9,10,12,13]. Our data is comparable to the reported 3.8% frequency of *PRKN* mutations in patients screened in a cohort of 313 North American PD cases [14]. The ability to detect *PRKN* mutations depends on factors such as sample size, ethnic extraction, inclusion criteria for cases and the methods used for mutation detection. Of the two identified *PRKN* mutations reported in the current study, one has been reported previously (p.G430D) [12]. To the best of our knowledge, there have been no previous reports of the p.G12R variant. The functional significance of these sequence variants remains to be established. This particular *PRKN* mutation (G12R) is predicted to be 'possibly damaging' (PolyPhen, http://coot.embl.de/PolyPhen/) and the amino acid in this position is highly conserved through *Bos taurus*, *Sus scrofa*, *Rattus norvegicus*, *Mus musculus*, *Gallus gallus* and *Danio rerio*. The mutation has not been found in a cohort of 170 European control subjects, comparable in ethnicity to subjects in our study [15]. *PRKN* mutations are generally presumed to be recessive, so the possibility that additional non-coding region sequence variants or additional factors contributing to the disease outcome in these individuals cannot be ruled out. There is a growing body of evidence that heterozygous *PRKN* mutations can be pathogenic and may be causative for disease [7,9,16]. In a recent report from Denmark, 10 out of 87 patients screened possessed putative disease-causing *PRKN* mutations; eight of these mutations were heterozygous in nature [13]. It has also been proposed that a heterozygous genotype may lead to a comparatively later age of onset. Our data are not necessarily consistent with this argument. Our mutation carriers developed symptoms well before the age of 45 years at 40 and 30 years of age, respectively (Table 4).

PARK6 (*PINK1*) mutations have been reported in 3–15% of early-onset recessive Parkinsonism cases while 5% of sporadic cases reportedly carry a single heterozygous *PINK1* mutation [17–19]. An estimated contribution of <1% for *PINK1* mutations to familial PD is probably more realistic [20,21]. Statistically, the one *PINK1* mutation carrier identified in our study accounts for 1.4% of the cohort. This mutation leading to a p.G411S amino acid substitution has been previously described in at least five PD patients [22–24]. Whether this particular mutation in its heterozygous form is causative for the disease phenotype is a matter of speculation. Interestingly, all five patients reported to date were heterozygous for this mutation, and no homozygous case has been described so far. Given that the amino acid change occurs within the kinase domain, in a sequence highly conserved in vertebrates, and that it has not been observed in normal subjects despite considerable investigation [22,23] it seems reasonable to assume that the mutation at least contributes significantly to the development of Parkinsonism. As no assays covering large genomic rearrangements for the *PINK1* gene were carried out in this study, the possibility that such rearrangements occurred in this particular patient cannot absolutely be ruled out.

No *PARK7* (*DJ-1*) mutations were detected in our study, consistent with previous studies that suggest that <2% of early-onset cases of PD carry coding region mutations in *PARK7* [25].

Two of our patients carried the common *LRRK2* p.G2019S mutation in exon 41 (2.7%, Table 3). Funayama et al. linked the *PARK8* locus to chromosome 12 in 2002 [26]. It has subsequently been found that *LRRK2* mutations constitute the most frequent form of monogenic PD. The p.G2019S mutation occurs in more than 2% of North American and English patients [27,28], and is found in >10% of North African, Ashkenazi Jewish and Portuguese populations [29–31]. The mutation falls within an activation segment of the MAPKKK domain, changing a highly conserved glycine at the start of the activation loop. Alternatively, it has been proposed that a reduction in kinase enzyme activity may be caused through changes in the magnesium-binding loop or by introduction of new phosphorylation sites. A recent Australian study, that did not include any of the subjects investigated in the current report, identified eight of 830 PD patients (1%) with this mutation.

4.1. Conclusions

Our data suggest that approximately 7% of early-onset PD cases seen in Queensland movement disorders clinics have mutations involving known *PARK* genes. However, whether these mutations were disease causing in all patients must remain open. The number of mutations found will increase as additional causal genes are identified from current gene-hunting strategies.

References

[1] Van Den Eeden SK, Tanner CM, Bernstein AL, Fross RD, Leimpeter A, Bloch DA, et al. Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. Am J Epidemiol 2003;157:1015–22.

[2] Kobayashi T, Matsumine H, Zhang J, Imamichi Y, Mizuno Y, Hattori N. Pseudo-autosomal dominant inheritance of *PARK2*: two families with parkin gene mutations. J Neurol Sci 2003;207:11–7.
[3] Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the parkin gene cause autosomal recessive juvenile Parkinsonism. Nature 1998;392:605–8.
[4] Tanner CM, Ottman R, Goldman SM, Ellenberg J, Chan P, Mayeux R, et al. Parkinson disease in twins: an etiologic study. JAMA 1999;281:341–6.
[5] Piccini P, Burn DJ, Ceravolo R, Maraganore D, Brooks DJ. The role of inheritance in sporadic Parkinson's disease: evidence from a longitudinal study of dopaminergic function in twins. Ann Neurol 1999;45:577–82.
[6] Takahashi H, Ohama E, Suzuki S, Horikawa Y, Ishikawa A, Morita T, et al. Familial juvenile Parkinsonism: clinical and pathologic study in a family. Neurology 1994;44:437–41.
[7] Hedrich K, Marder K, Harris J, Kann M, Lynch T, Meija-Santana H, et al. Evaluation of 50 probands with early-onset Parkinson's disease for parkin mutations. Neurology 2002;58:1239–46.
[8] Klein C, Hedrich K, Wellenbrock C, Kann M, Harris J, Marder K, et al. Frequency of parkin mutations in late-onset Parkinson's disease. Ann Neurol 2003;54:415–6. author reply 6–7.
[9] Lucking CB, Durr A, Bonifati V, Vaughan J, De Michele G, Gasser T, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. N Engl J Med 2000;342:1560–7.
[10] Periquet M, Latouche M, Lohmann E, Rawal N, De Michele G, Ricard S, et al. Parkin mutations are frequent in patients with isolated early-onset Parkinsonism. Brain 2003;126:1271–8.
[11] Khan NL, Graham E, Critchley P, Schrag AE, Wood NW, Lees AJ, et al. Parkin disease: a phenotypic study of a large case series. Brain 2003;126:1279–92.
[12] Sun M, Latourelle JC, Wooten GF, Lew MF, Klein C, Shill HA, et al. Influence of heterozygosity for parkin mutation on onset age in familial Parkinson disease: the GenePD study. Arch Neurol 2006;63:826–32.
[13] Hertz JM, Ostergaard K, Juncker I, Pedersen S, Romstad A, Moller LB, et al. Low frequency of parkin, tyrosine hydroxylase, and GTP cyclohydrolase I gene mutations in a Danish population of early-onset Parkinson's Disease. Eur J Neurol 2006;13:385–90.
[14] Lincoln SJ, Maraganore DM, Lesnick TG, Bounds R, de Andrade M, Bower JH, et al. Parkin variants in North American Parkinson's disease: cases and controls. Mov Disord 2003;18:1306–11.
[15] Lesage S, Lohmann E, Tison F, Durif F, Durr A, Brice A. Rare heterozygous parkin variants in French early-onset Parkinson's disease patients and controls. J Med Genet 2007.
[16] Chen R, Gosavi NS, Langston JW, Chan P. Parkin mutations are rare in patients with young-onset Parkinsonism in a US population. Parkinsonism Relat Disord 2003;9:309–12.
[17] Hatano Y, Li Y, Sato K, Asakawa S, Yamamura Y, Tomiyama H, et al. Novel *PINK1* mutations in early-onset Parkinsonism. Ann Neurol 2004;56:424–7.
[18] Hatano Y, Sato K, Elibol B, Yoshino H, Yamamura Y, Bonifati V, et al. *PARK6*-linked autosomal recessive early-onset Parkinsonism in Asian populations. Neurology 2004;63:1482–5.
[19] Bonifati V, Rohe CF, Breedveld GJ, Fabrizio E, De Mari M, Tassorelli C, et al. Early-onset Parkinsonism associated with *PINK1* mutations: frequency, genotypes, and phenotypes. Neurology 2005;65:87–95.
[20] Healy DG, Abou-Sleiman PM, Gibson JM, Ross OA, Jain S, Gandhi S, et al. *PINK1* (*PARK6*) associated Parkinson disease in Ireland. Neurology 2004;63:1486–8.
[21] Rogaeva E, Johnson J, Lang AE, Gulick C, Gwinn-Hardy K, Kawarai T, et al. Analysis of the *PINK1* gene in a large cohort of cases with Parkinson disease. Arch Neurol 2004;61:1898–904.
[22] Abou-Sleiman PM, Muqit MM, McDonald NQ, Yang YX, Gandhi S, Healy DG, et al. A heterozygous effect for *PINK1* mutations in Parkinson's disease? Ann Neurol 2006;60:414–9.
[23] Zadikoff C, Rogaeva E, Djarmati A, Sato C, Salehi-Rad S, St George-Hyslop P, et al. Homozygous and heterozygous *PINK1* mutations: considerations for diagnosis and care of Parkinson's disease patients. Mov Disord 2006;21:875–9.
[24] Toft M, Myhre R, Pielsticker L, White LR, Aasly JO, Farrer MJ. *PINK1* mutation heterozygosity and the risk of Parkinson's disease. J Neurol Neurosurg Psychiatr 2007;78:82–4.
[25] Hedrich K, Djarmati A, Schafer N, Hering R, Wellenbrock C, Weiss PH, et al. *DJ-1* (*PARK7*) mutations are less frequent than parkin (*PARK2*) mutations in early-onset Parkinson disease. Neurology 2004;62:389–94.
[26] Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F. A new locus for Parkinson's disease (*PARK8*) maps to chromosome 12p11.2-q13.1. Ann Neurol 2002;51:296–301.
[27] Nichols WC, Pankratz N, Hernandez D, Paisan-Ruiz C, Jain S, Halter CA, et al. Genetic screening for a single common *LRRK2* mutation in familial Parkinson's disease. Lancet 2005;365:410–2.
[28] Gilks WP, Abou-Sleiman PM, Gandhi S, Jain S, Singleton A, Lees AJ, et al. A common *LRRK2* mutation in idiopathic Parkinson's disease. Lancet 2005;365:415–6.
[29] Ozelius LJ, Senthil G, Saunders-Pullman R, Ohmann E, Deligtisch A, Tagliati M, et al. *LRRK2* G2019S as a cause of Parkinson's disease in Ashkenazi Jews. N Engl J Med 2006;354:424–5.
[30] Lesage S, Durr A, Tazir M, Lohmann E, Leutenegger AL, Janin S, et al. *LRRK2* G2019S as a cause of Parkinson's disease in North African Arabs. N Engl J Med 2006;354:422–3.
[31] Bras JM, Guerreiro RJ, Ribeiro MH, Januario C, Morgadinho A, Oliveira CR, et al. G2019S dardarin substitution is a common cause of Parkinson's disease in a Portuguese cohort. Mov Disord 2005;20:1653–5.

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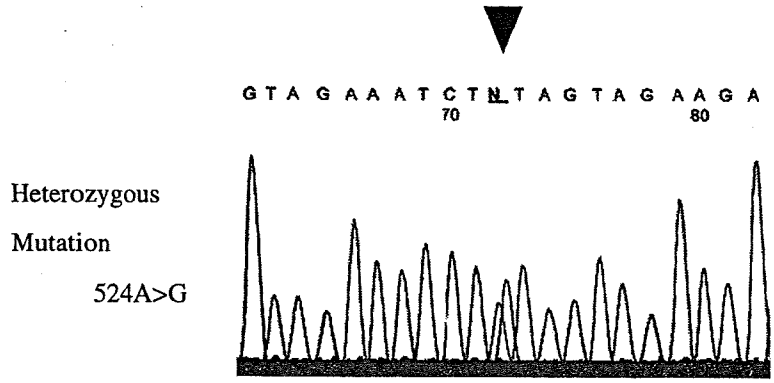
Letter to the Editor

A new mutation in the *GCH1* gene presents as early-onset Parkinsonism

The *GCH1* gene encodes GTP cyclohydrolase I (GCH1), which catalyzes the first step (GTP to 7, 8-dihydroneopterin-triphosphate) in the biosynthesis of tetrahydrobiopterin (BH4). This enzyme is rate-limiting and deficiency causes Segawa disease, characterized by hereditary progressive dystonia with marked diurnal fluctuation (HPD/DRD) and autosomal recessive GCH-deficient hyperphenylalanemia. Conventionally, the first symptoms of DRD develop before 10 years of age, and few cases of adult onset have been described [1,2]. To date, over one hundred kinds of the *GCH1* mutation have been reported to lower enzymatic activity, which has also been observed in mononuclear blood cells of juvenile Parkinsonism [3]. However, no reports have described only Parkinsonism occurring with a *GCH1* gene mutation.

Herein, we highlight a 33-year-old Chinese male computer engineer who developed resting tremor, and noted difficulty in skillful movement when manipulating objects during computer work. The patient showed a Myerson's sign, tremor in the left arm and leg at rest, and sluggish gait with anteversion posture. Neither autonomic nervous system dysfunction nor dystonic movement was apparent. Blood examination and magnetic resonance imaging (MRI) of the brain showed no abnormality. Based on the clinical presentation, the patient was diagnosed with early-onset Parkinsonism in Yahr stage 2 for which levodopa was started. Levodopa improved clinical symptoms, disease progression was slow and no dyskinesia appeared. At age 41 years (8 years after onset), medications were 250 mg of levodopa/carbidopa, 0.75 mg of cabergoline



Homo sapiens	A	R	I	V	E	I	**Y**	S	R	R	L
Mus musculus	A	R	I	V	E	I	**Y**	S	R	R	L
Rattus norvegicus	A	R	I	V	E	I	**Y**	S	R	R	L
G. gallus	A	R	I	V	E	I	**Y**	S	R	R	L
S. cerevisiae	A	R	L	A	E	M	**Y**	A	R	R	L
K. lactis	A	R	L	A	E	M	**Y**	A	R	R	L
E. gossypii	A	R	L	A	E	M	**Y**	A	R	R	L

Fig. 1. The arrowhead indicates point mutation c.524A > G (p.Y175C). This mutation was not detected in 192 chromosomes. The tyrosine position (Y) is preserved in various species.

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and 2.5 mg of selegiline hydrochloride. Daily life and job were well-managed. When medication effects disappeared, the patient displayed tremor in the left upper limb at rest, left-sided rigidity, frozen gait and disability in skillful movement of fingers. Notably, his father was reported to have exhibited resting tremor of the left upper limb.

Parkin gene analysis was negative; however, sequence analysis of the *GCH1* gene identified a heterozygous mutation (c.524 A > G; p.Y175C) in exon 4 (Fig. 1).

The father was subsequently found to carry the same mutation. This mutation was also not observed in 96 healthy control subjects and this tyrosine residue is conserved across species supporting the pathogenicity of p.Y175C (Fig. 1). The GCH1 enzyme activity was measured in the proband according to the methods previously reported by Ichinose et al. [3] at 1.80 pmol/h/mg protein (normal control, 4.17 pmol/h/mg protein). Next, concentrations of neopterin and biopterin in cerebrospinal fluid were measured. Both neopterin and biopterin are metabolic products of GTP. Concentrations were 13.3 pmol/ml for neopterin and 5.2 pmol/ml for biopterin (normal, around 22–32 pmol/l for neopterin and 21–26 pmol/l for biopterin) [4].

Neopterin concentrations in the cerebrospinal fluid of patients with dopa-responsive–dystonia (DRD) due to *GCH1* mutation are reportedly <10 pmol/ml. Therefore, in the present patient neopterin concentration was not as low as that in DRD, and was similar to that in patients with Parkinsonism. Differences among *GCH1* gene mutation positive patients may be influenced by the position of mutated amino acids, the type of mutation, and one or more of the other gene mutations. To elucidate genotype-phenotype correlations, examination of abnormalities in the *GCH1* gene in patients who present with only Parkinsonism is important and may contribute to better understand the pathogenesis of this syndrome.

Conflict of interest

The author has no conflicts of interest.

References

[1] Ichinose H, Ohye T, Takahashi E, Seki N, Hori T, Segawa M, et al. Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene. Nat Genet 1994;8:236–42.
[2] Segawa M, Nomura Y, Nishiyama N. Autosomal dominant guanosine triphosphate cyclohydrolase I deficiency (Segawa Disease). Ann Neurol 2003;54:S32–45.
[3] Ichinose H, Ohye T, Yokochi M, Fujita K, Nagatsu T. GTP cyclohydrolase 1 activity in mononuclear blood cells in juvenile Parkinsonism. Neurosci Lett 1995;190:140–2.
[4] Fujishiro K, Hagihara M, Takahashi A, Nagatsu T. Concentrations of neopterin and biopterin in the cerebrospinal fluid of patients with Parkinson's disease. Biochem Med Metab Biol 1990;44:97–100.

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Programmed cell death-2 isoform1 is ubiquitinated by parkin and increased in the substantia nigra of patients with autosomal recessive Parkinson's disease

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Substantia nigra

ABSTRACT

Mutations in *parkin* gene are responsible for autosomal recessive Parkinson's disease (ARPD) and its loss-of-function is assumed to affect parkin ubiquitin ligase activity. Accumulation of its substrate may induce dopaminergic neurodegeneration in the substantia nigra (SN) of ARPD. Here, we show that parkin interacts with programmed cell death-2 isoform 1 (PDCD2-1) and promotes its ubiquitination. Furthermore, accumulation of PDCD2-1 was found in the SN of ARPD as well as in sporadic PD, suggesting that common failure of the ubiquitin–proteasome system is associated with neuronal death in both ARPD and sporadic PD.

Structured summary:
MINT-6805975, MINT-6806032, MINT-6806051, MINT-6806070: *PDCD2* (uniprotkb:Q16342) *physically interacts* (MI:0218) with *Parkin* (uniprotkb:O60260) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6805947: *Parkin* (uniprotkb:O60260) *physically interacts* (MI:0218) with *PDCD2* (uniprotkb:Q16342) by *two hybrid* (MI:0018)
MINT-6806000: *PDCD2* (uniprotkb:Q16342) *physically interacts* (MI:0218) with *ubiquitin* (uniprotkb:P62988) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

Mutations in the *parkin* gene are linked to autosomal recessive Parkinson's disease (ARPD) [1]. Parkin functions as an E3 ubiquitin (Ub) ligase in the ubiquitin–proteasome system (UPS) [2], which maintains intracellular protein quality. The E3 Ub ligase attaches Ub to specific substrate(s), as well as activates E1 and conjugating E2 enzymes. Polyubiquitinated proteins are degraded by the 26S proteasome. Homozygous mutations of the *parkin* gene result in loss-of-function and result in failure of the UPS. In this context, degeneration of dopaminergic neurons in the substantia nigra (SN) of ARPD is presumed to be induced by accumulation of protein substrate(s). Thus, identification of the substrate(s) for parkin is important for understanding the mechanism of neurodegeneration of ARPD.

Programmed cell death-2 (PDCD2) protein was isolated from a human fetal lung cDNA library and characterized as highly homologous to *Rp-8*, a protein associated with apoptosis [3–5]. PDCD2 exists in two isoforms [6]. The PDCD2 isoform 1 (PDCD2-1) comprises 344 amino acids residues containing the MYND zinc-finger domain [4,6]. In present study, we found that parkin interacts with PDCD2-1 and controls its degradation. We also demonstrated high levels of PDCD2-1 in the SN of patients with ARPD and those with sporadic Parkinson's disease (sPD).

2. Materials and methods

2.1. Yeast two-hybrid screening

The parkin ubl-linker (1-238) cDNA was linked to the cDNA for the GAL4-DNA binding domain in pGBD-ubl-linker, and yeast PJ69-2A cells were transformed with pGBD-ubl-linker, and subsequently with the library of SH-SY5Y cDNAs fused to the cDNA for the GAL4 activation domain (GAL4AD). The transformed yeast were selected and confirmed as described by the manufacturer (Clontech, Shiga, Japan).

2.2. Plasmids and antibodies

The full-length and mutant parkins were cloned into 3XMyc-pcDNA3.1(+) vector at the *Bam*HI site. Mutagenesis to introduce

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point mutations was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The pCMV/3XFLAG-PDCD2-1 was prepared by polymerase chain reaction (PCR) using appropriately designed primers with restriction site (*Bam*HI). The PCR product was inserted into the p3XFLAG-CMV™ vector (Sigma–Aldrich, St. Louis, MO). HA-Ub was a kind gift from Prof. Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo). Rabbit polyclonal PDCD2 antibody was kindly provided by Prof. Philip A. Sharp (Massachusetts Institute of Technology, Cambridge, MA) [7]. Rabbit polyclonal anti-parkin and mouse monoclonal GAPDH antibodies were obtained from Cell Signaling Technology (Beverly, MA) and Chemicon (Temecula, CA), respectively. Monoclonal (9E10), horseradish peroxidase (HRP)-conjugated monoclonal (9E10), and polyclonal (A-14) anti-Myc, HRP-conjugated anti-FLAG (M2), biotin-conjugated anti-HA (3F10) antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA), Sigma–Aldrich, and Roche (Mannheim, Germany), respectively.

2.3. Cell culture and transfection

COS1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml) at 37 °C under 5% CO_2. COS1 cells were transiently transfected with expression vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol supplied by the manufacturer.

2.4. Immunoprecipitation (IP) and western blotting

Twenty-four hours after transfection, the cells were washed with phosphate-buffered saline (PBS) and then lysed on ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, and 10% glycerol) containing protease inhibitor (Complete Mini EDTA-free, Roche Diagnostics, Penzberg, Germany) for 20 min. Lysates were centrifuged at 4 °C at 15000 rpm. The protein concentration in the supernatant was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). The supernatant fractions were incubated with the primary antibodies and then treated with protein G sepharose 4 fast flow (Amersham, Sweden). Immunoprecipitates were washed three times with lysis buffer then boiled in 3X SDS loading buffer (30 μl) for 5 min. Each sample (10 μl) was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The separated proteins were transferred onto a polyvinylidene difluoride microporous membrane (Bio-Rad, Hercules, CA) using transfer buffer (40 mM CAPS, 30 mM Tris, and 15% MeOH). The transferred membrane was blocked with 5% skim milk and incubated overnight with the primary antibody at 4 °C. After incubation with HRP-conjugated secondary antibodies, the reaction was visualized using a chemiluminescence reagent (ECL-plus, Amersham). The intensity of the signal was analyzed by LAS-1000plus (Fuji film, Tokyo).

Table 1
Clinical summary of the subjects included in the study.

Case	Diagnosis	Age	Sex	Duration	Mutation
1	Control	45	Female	–	–
2	Control	57	Male	–	–
3	Control	70	Female	–	–
4	sPD	66	Male	10	–
5	sPD	81	Female	13	–
6	sPD	62	Male	12	–
7	ARPD	63	Male	30	Exon 3 deletion
8	ARPD	68	Male	35	Exon 3 deletion
9	ARPD	62	Male	38	Exon 4 deletion

2.5. Ubiquitination assay

COS1 cells were transfected with 3XMyc-parkin, 3XFLAG-PDCD2-1, and HA-Ub in various combinations. After 24 h, the cells were incubated in a medium with 50 μM MG132 (Peptide Institute Inc., Osaka, Japan) for additional 30 min. The cell lysate was immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) and products were analyzed with biotin-conjugated anti-HA, HRP-conjugated anti-FLAG, and HRP-conjugated anti-Myc antibodies.

2.6. Brain samples

Fresh frozen brains were obtained from the Department of Neurology, Juntendo University School of Medicine. The subjects were three patients with ARPD, three with sPD, and three control subjects. Table 1 provides a summary of the clinical profile of the subjects. The diagnosis was confirmed by neuropathological examination of the brain tissues as sPD, and excluding neurodegenerative disorders in control subjects. Gene analysis of *parkin* was preformed as reported previously [1]. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine. Approximately, 0.5 g of frozen midbrain were placed into 3.5 ml ice-cold homogenisation buffer [DTT, 50 mM Tris–HCl (pH 7.4), 0.1% SDS, 1% Triton X 100, and 1% sodium deoxycholate] and homogenized in the presence of a mixture of protease inhibitors (Complete Mini EDTA-free). The protein concentration in each sample was determined using the DC protein assay kit (Bio-Rad).

3. Results and discussion

3.1. Identification of PDCD2-1 as a parkin-binding protein

To identify parkin-binding protein, we screened the SHSY-5Y cDNA library using the ubl-linker parkin by the yeast two-hybrid method. PDCD2-1 was isolated as a potential clone that can interact with parkin (data not shown). Co-immunoprecipitation analysis was performed to confirm the interaction between parkin and PDCD2-1. The 3XFLAG-PDCD2-1 was co-transfected with either 3XMyc-parkin or 3XMyc control plasmid into COS1 cells. After lysis of the transfected cells, the supernatant (1 mg protein) was pulled-down with anti-Myc antibody (A-14). Immunoprecipitates were boiled in 3XSDS loading buffer (30 μl) and then each sample (10 μl) was subjected to SDS–PAGE, followed by western blotting with HRP-conjugated anti-FLAG antibody. The 3XFLAG-PDCD2-1 band was only detected in the sample with 3XMyc-parkin, but not in the 3XMyc plasmid (Fig. 1A). When co-immunoprecipitation was performed in reverse with anti-FLAG M2 affinity gel, only the 3XMyc-parkin band was noted in protein extracts with 3XFLAG-PDCD2-1 (Fig. 1B). These results suggest that wild parkin interacts with PDCD2-1.

3.2. PDCD2-1 binds to parkin at two sites

Parkin is a 465-amino acid protein, consisting of Ub-like domain (UBL) in the N-terminal and two RING finger motifs towards the C-terminal. Between the two RINGs, there is an in-between-RING (IBR) structure comprising a RING-box (Fig. 1C). To investigate the PDCD2-1 binding site(s) of parkin, we constructed various truncated and point mutants of parkin tagged with 3XMyc (Fig. 1C). Various mutants of 3XMyc-parkin and 3XFLAG-PDCD2-1 were co-transfected into COS1 cells. The supernatant (1 mg protein) was then immunoprecipitated with anti-Myc antibody (A-14), and each sample (10 μl) was subsequently subjected to SDS–PAGE, and western blotting with HRP-conjugated anti-FLAG

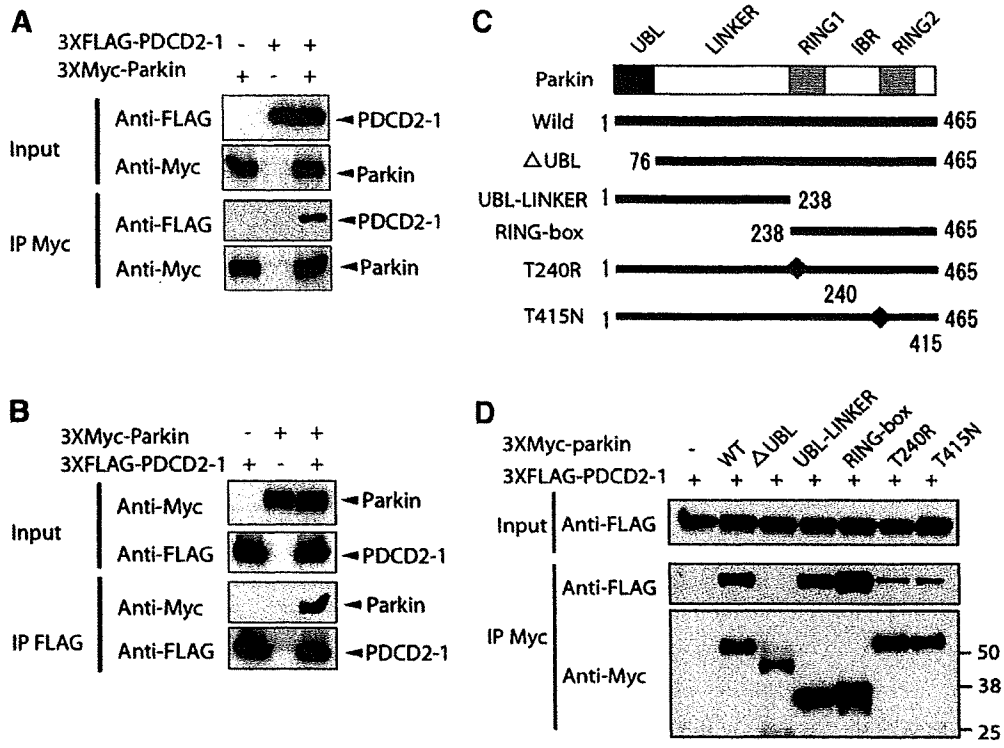

Fig. 1. Parkin interacts with PDCD2-1. (A) Lysates (1 mg protein) from COS1 cells co-transfected with 3XMyc-parkin and 3XFLAG-PDCD2-1 were subjected to immunoprecipitation (IP) with anti-Myc antibody followed by western blot analysis with HRP-conjugated anti-FLAG and anti-Myc antibodies. To confirm proper expression level of the transiently transfected protein, input lysates were also analyzed by western blotting with HRP-conjugated anti-FLAG and anti-Myc antibodies. (B) In reverse analysis, lysates (1 mg protein) from COS1 cells co-transfected with 3XMyc-parkin and 3XFLAG-PDCD2-1 were subjected to immunoprecipitation with anti-FLAG M2 affinity gel followed by Western blotting with HRP-conjugated anti-FLAG and anti-Myc antibodies. Input lysates were also analyzed by western blotting with HRP-conjugated anti-FLAG and anti-Myc antibodies. (C) The structure of parkin protein. Schematic representation of wild-type and various mutant parkins. UBL: Ub-like domain; IBR: inbetween RING structure. (D) Interaction between mutant parkin and PDCD2-1. Lysates (1 mg protein) from COS1 cells co-transfected with various 3XMyc tagged parkin domain constructs or control plasmid, together with 3XFLAG-PDCD2-1, were subjected to IP with anti-Myc antibody. IP products and input lysates were analyzed by western blotting with HRP-conjugated anti-FLAG and anti-Myc antibodies.

antibody. Truncated parkin lacking the UBL domain did not interact with PDCD2-1 (Fig. 1D, lane 3), suggesting that the UBL domain is necessary for such interaction. Furthermore, PDCD2-1 interacted with the RING-box domain (Fig. 1D, lane 5). However, in the presence of the LINKER domain, no interaction was noted between UBL-domain-negative parkin and RING-box domain as evident by the similarity of the amount of FLAG in lane 2 to that in lane 4 (Fig. 1D). Thus, the linker domain may structurally block the bind-

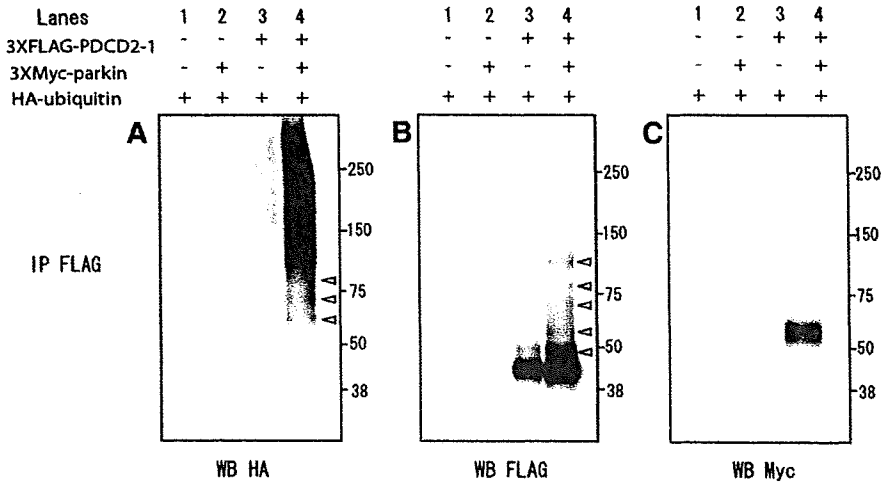

Fig. 2. Parkin ubiquitinates PDCD2-1. COS1 cells transfected with 3XFLAG-PDCD2-1, 3XMyc-parkin, and HA-Ub, were treated with MG132 for 30 min, and lysates (2 mg protein) were immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitated products of the ubiquitination assays were detected with biotin-conjugated anti-HA (A), HRP-conjugated anti-FLAG (B) and HRP-conjugated anti-Myc (C) antibodies. The ubiquitination of PDCD2-1 was promoted with transfection of parkin. White *arrowheads* indicate ubiquitination of PDCD2-1.

ing to RING-box. Interestingly, the ARPD causing missense mutation within the RING-box such as T240R and T415N, showed reduced binding to PDCD2-1 (Fig. 1D).

3.3. Ubiquitination and degeneration of PDCD2-1 are promoted by parkin

Next, we used the ubiquitination assay to determine whether PDCD2-1 is a substrate of parkin. COS1 cells were co-transfected with various combinations of 3XFLAG-PDCD2-1, 3XMyc-parkin, and HA-Ub. After treatment of cells with MG132 for 30 min, they were lysed and each supernatant (2 mg protein) was immunoprecipitated with anti-FLAG M2 affinity gel. Immunoprecipitates were boiled in 3XSDS loading buffer (30 μl) and then each sample (10 μl) was subjected to SDS–PAGE, followed by western blotting with biotin-conjugated anti-HA, HRP-conjugated anti-FLAG, and HRP-conjugated anti-Myc antibodies. Ubiquitination of PDCD2-1 was detected by western blotting with biotin-conjugated anti-HA and HRP-conjugated FLAG antibodies. As shown in Fig. 2A–C, ubiquitination of PDCD2-1 was promoted by transfection of 3XMyc-parkin. Mild ubiquitination of PDCD2-1 was noted without transfection of 3XMyc-parkin (lane 3, Fig. 2A–C). These results may reflect the influence of endogenous parkin in COS1 cells. To examine whether parkin promotes PDCD2-1 degradation, COS1 cells were transfected with a fixed amount of 3XFLAG-PDCD2-1 and increasing amounts of 3XMyc-parkin. The total amounts of transfected plasmids were adjusted with 3XMyc plasmid to avoid the influence of transfection, and the experiment was performed three times. As is shown in Fig. 3A and B, the amount of co-transfected parkin modulated the level of PDCD2-1 in a dose-dependent manner; the relative value of PDCD2-1 decreased to approximately 60% at the highest dose of parkin.

3.4. Accumulation of PDCD2 in SN of ARPD and sPD

In the next step, we examined the level of PDCD2-1 in the SN of patients with ARPD and sPD and control subjects. The brain tissues were homogenized followed by western blotting with rabbit polyclonal anti-PDCD2 antibody. As shown Fig. 4A–C, the amount of PDCD2-1 was higher in all ARPD and sPD compared with the control subjects. Furthermore, PDCD2-1 level was lower in Cases 2 and 5 with high amount of parkin, compared with those with low amount of parkin Fig. 4A–C.

We demonstrated in the present study that PDCD2-1 is a parkin substrate. PDCD2-1 is expressed ubiquitously in various human tissues including the brain [4]. Previous reports described the involvement of PDCD2-1 in various processes such as apoptosis, inflammation, and cell proliferation [6,8–10]. Although the role of PDCD2-1 in the central nervous system remains to be elucidated, the expression of Rp-8, which is homologous to PDCD2-1, increases during cell death in rodent cerebellum [5]. Furthermore, antisense knockdown of parkin causes apoptosis of SH-SY5Y cells [11] and overexpression of parkin protects SH-SY5Y cells against apoptosis

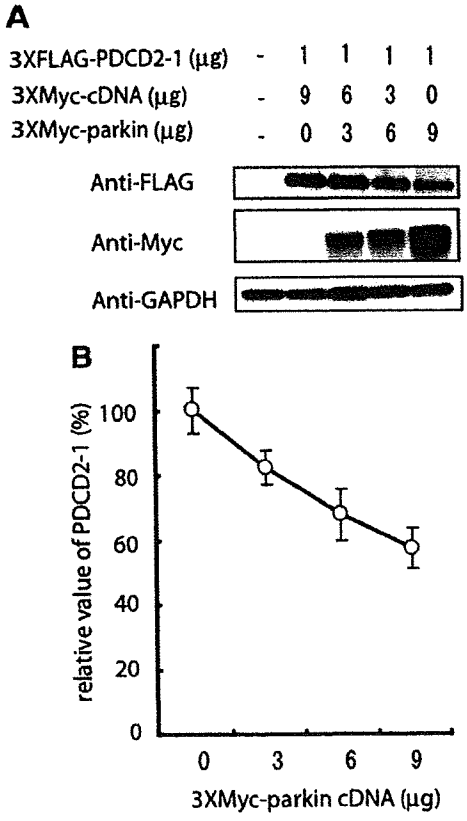

Fig. 3. Degradation of PDCD2 depends on parkin. (A) Lysates prepared from COS1 cells transfected with 3XFLAG-PDCD2-1 and with 3XMyc-parkin. The amount of transfected 3XFLAG-PDCD2-1 was fixed at 1 μg while the amount of transfected 3XMyc-parkin was increased to 0, 3, 6, and 9 μg. The total amounts of transfected plasmids were adjusted with 3XMyc plasmid to avoid the influence of transfection. (B) Data are expressed as mean ± S.E.M. Note the fall in the level of 3XFLAG-PDCD2-1 with increased expression of 3XMyc-parkin.

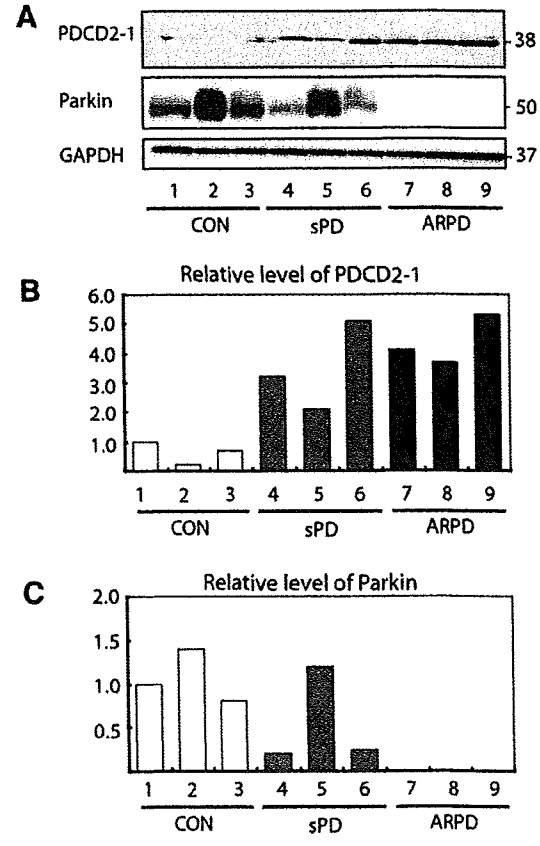

Fig. 4. (A) Quantitative analysis of PDCD2-1 and parkin levels normalized to GAPDH in control subjects, sPD, and ARPD. Homogenates of human midbrain from control subjects ($n = 3$), sPD ($n = 3$), and ARPD ($n = 3$) were immunoblotted with anti-PDCD2 (top panel) and anti-parkin (middle panel) antibodies. (B) PDCD2-1 levels were significantly higher in sPD and ARPD than control subjects. The levels of PDCD2-1 in the SN of sPD and ARPD were 2.2 to 5.1- and 3.8 to 5.7-fold higher than the control, respectively. (C) Expression of parkin was noted in control and sPD, but not in ARPD.

induced by dopamine or 6-hydroxydopamine [12]. These findings suggest that parkin is associated with apoptosis and its anti-apoptotic protective effect is dependent on its E3 activity.

Another important finding of this study was the increased levels of PDCD2-1 in the SN of ARPD and sPD. To date, several studies demonstrated that parkin is modified after translation [13,14]. Both in vivo and in vitro experiments have shown that parkin is *S*-nitrosylated and that such *S*-nitrosylation serves to inhibit the E3 ligase activity and protective function of parkin [13,14]. Furthermore, *S*-nitrosylated parkin is increased in sPD [13,14]. These results suggest the pathogenesis of sPD could also involve failure of parkin activity in the UPS. The UPS may be an important and common pathway of nigral neuronal death in both ARPD and sPD.

References

[1] Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392, 605–608.

[2] Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. and Suzuki, T. (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin–protein ligase. Nat. Genet. 25, 302–305.

[3] Owens, G.P., Hahn, W.E. and Cohen, J.J. (1991) Identification of mRNAs associated with programmed cell death in immature thymocytes. Mol. Cell Biol. 11, 4177–4188.

[4] Kawakami, T., Furukawa, Y., Sudo, K., Saito, H., Takami, S., Takahashi, E. and Nakamura, Y. (1995) Isolation and mapping of a human gene (PDCD2) that is highly homologous to Rp8, a rat gene associated with programmed cell death. Cytogenet. Cell Genet. 71, 41–43.

[5] Owens, G.P., Mahalik, T.J. and Hahn, W.E. (1995) Expression of the death-associated gene RP-8 in granule cell neurons undergoing postnatal cell death in the cerebellum of weaver mice. Brain Res. Dev. Brain Res. 86, 35–47.

[6] Chen, Q., Qian, K. and Yan, C. (2005) Cloning of cDNAs with PDCD2(C) domain and their expressions during apoptosis of HEK293T cells. Mol. Cell Biochem. 280, 185–191.

[7] Scarr, R.B. and Sharp, P.A. (2002) PDCD2 is a negative regulator of HCF-1 (C1). Oncogene 21, 5245–5254.

[8] Baron, B.W., Anastasi, J., Thirman, M.J., Furukawa, Y., Fears, S., Kim, D.C., Simone, F., Birkenbach, M., Montag, A., Sadhu, A., Zeleznik-Le, N. and McKeithan, T.W. (2002) The human programmed cell death-2 (PDCD2) gene is a target of BCL6 repression: implications for a role of BCL6 in the down-regulation of apoptosis. Proc. Nat. Acad. Sci. USA 99, 2860–2865.

[9] Minakhina, S., Druzhinina, M. and Steward, R. (2007) Zfrp8, the *Drosophila ortholog* of PDCD2, functions in lymph gland development and controls cell proliferation. Development 134, 2387–2396.

[10] Chen, Q., Yan, C.Q., Liu, F.J., Tong, J., Miao, S.L. and Chen, J.P. (2008) Overexpression of the PDCD2-like gene results in inhibited TNF-alpha production in activated Daudi cells. Human Immunol. 69, 259–265.

[11] Machida, Y., Chiba, T., Takayanagi, A., Tanaka, Y., Asanuma, M., Ogawa, N., Koyama, A., Iwatsubo, T., Ito, S., Jansen, P.H., Shimizu, N., Tanaka, K., Mizuno, Y. and Hattori, N. (2005) Common anti-apoptotic roles of parkin and alpha-synuclein in human dopaminergic cells. Biochem. Biophys. Res. Commun. 332, 233–240.

[12] Jiang, H., Ren, Y., Zhao, J. and Feng, J. (2004) Parkin protects human dopaminergic neuroblastoma cells against dopamine-induced apoptosis. Human Mol. Genet. 13, 1745–1754.

[13] Chung, K.K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, V.L. and Dawson, T.M. (2004) *S*-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. Science 304, 1328–1331.

[14] Yao, D., Gu, Z., Nakamura, T., Shi, Z.Q., Ma, Y., Gaston, B., Palmer, L.A., Rockenstein, E.M., Zhang, Z., Masliah, E., Uehara, T. and Lipton, S.A. (2004) Nitrosative stress linked to sporadic Parkinson's disease: *S*-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. Proc. Nat. Acad. Sci. USA 101, 10810–10814.

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Mutation analysis for *DJ-1* in sporadic and familial parkinsonism: Screening strategy in parkinsonism

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ABSTRACT

DJ-1 mutations cause autosomal recessive parkinsonism (ARP). Although some reports of *DJ-1* mutations have been published, there is lack of information on the prevalence of these mutations in large-scale studies of both familial and sporadic parkinsonism. In this genetic screening study, we analyzed the distribution and frequency of *DJ-1* mutations by direct nucleotide sequencing of coding exons and exon–intron boundaries of *DJ-1*, in 386 *parkin*-negative parkinsonism patients (371 index cases: 67 probands of autosomal recessive parkinsonism families, 90 probands of autosomal dominant parkinsonism families, 201 patients with sporadic parkinsonism, and 13 with unknown family histories) from 12 countries (Japan 283, China 27, Taiwan 22, Korea 22, Israel 16, Turkey 5, Philippines 2, Bulgaria 2, Greece 2, Tunisia 1, USA 2, Ukraine 1, unknown 1). None had causative mutation in *DJ-1*, suggesting *DJ-1* mutation is very rare among patients with familial and sporadic parkinsonism from Asian countries and those with other ethnic background. This is in contrast to the higher frequencies and worldwide distribution of *parkin*- and *PINK1*-related parkinsonism in ARP and sporadic parkinsonism. Thus, after obtaining clinical information, screening for mutations in (1) *parkin*, (2) *PINK1*, (3) *DJ-1*, (4) *ATP13A2* should be conducted in that order, in ARP and sporadic parkinsonism, based on their reported frequencies. In addition, haplotype analysis should be employed to check for homozygosity of 1p36, which harbors a cluster of causative genes for ARP such as *DJ-1*, *PINK1* and *ATP13A2* in ARP and sporadic parkinsonism, especially in parkinsonism with consanguinity.

To date, four genes, *parkin* (*PARK2*) [16], *PINK1* (*PARK6*) [30], *DJ-1* (*PARK7*) [4], and *ATP13A2* (*PARK9*) [25] have been identified as the causative genes for familial autosomal recessive parkinsonism (ARP). *Parkin*, *PINK1*, and *DJ-1* mutations cause levodopa-responsive parkinsonism with or without dystonia at onset and psychiatric problems [16,30,4,20,31,11,19,17]. On the other hand, *ATP13A2* mutations cause rare atypical parkinsonism with multisystemic neurodegeneration [25,23]. Mutations in *parkin* have been detected in approximately 50% of patients with ARP and 15% of patients with sporadic early-onset parkinsonism [20]. The reported frequency of *PINK1* mutations in ARP is approximately 4.5% [19]. Single heterozygous *PINK1* mutations were reported in 5% of patients with sporadic early-onset parkinsonism [31]. In some ethnic groups, the incidence of *DJ-1* mutations have been estimated to be not more than 1–2% [1,9,7,6,12,14], although further large studies are needed.

In patients with ARP, it is important to determine the best screening method that can detect mutations in these genes. In addition, the roles of single heterozygous mutations of *parkin*, *PINK1*, *DJ-1*, and *ATP13A2* in familial parkinsonism and sporadic Parkinson's disease (PD) remain unclear. However, to our knowledge, there is currently a lack of information about the frequency of mutations, including single heterozygous mutation, for *DJ-1* in both familial and sporadic parkinsonism, especially in large population samples. In this study, we report the results of mutation analysis for *DJ-1* in a large number of patients with familial and sporadic parkinsonism and discuss the best strategies for genetic screening.

Blood samples and clinical information on 386 patients with parkinsonism (371 index cases: autosomal recessive = 67, autosomal dominant = 90, sporadic = 201, and unknown = 13) from 12 countries (Japan, 283; China, 27; Taiwan, 22; Korea, 22; Israel, 16; Turkey, 5; Philippines, 2; Bulgaria, 2; Greece, 2; Tunisia, 1; USA, 2; Ukraine, 1; and unknown, 1) were obtained from each neurologist (Table 1). In this study, we classified the mode of inheritance as autosomal recessive (families with consanguineous marriages or at least two affected siblings in only one generation) and autosomal

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Table 1
Details of 386 analyzed patients with parkinsonism.

	Total number of analyzed patients	Age at onset (years) (for analyzed patients)*
Sporadic parkinsonism	201 (males 113; females 88)	35.3 ± 10.6 (7–80)
Familial parkinsonism	185 (170 probands + 15 relatives) (males 90, females 95)	46.2 ± 15.2 (9–80)
ARP	74 (67 probands)	49.6 ± 14.9
ADP	97 (90 probands)	44.3 ± 14.9
No clear hereditary information	14 (13 probands)	41.8 ± 16.4
Total	386 (371 index patients = 201 sporadic patients + 170 probands)	40.5 ± 14.0

ARP: autosomal recessive parkinsonism; ADP: autosomal dominant parkinsonism.
* Data are mean ± SD (range).

dominant (one or more affected members in each consecutive two generations). The diagnosis of parkinsonism was established by the participating neurologists. The majority of patients had typical parkinsonism with good clinical response to levodopa. The study was approved by the ethics review committee of Juntendo University And blood samples were collected after obtaining informed consent from all participants. DNA was prepared by using standard methods. None of the participants had *parkin* mutation in the coding regions and exon–intron boundaries and no gene dosage abnormality, as confirmed in our previous study (unpublished data).

For sequence analysis, six coding exons and exon–intron boundaries of *DJ-1* were amplified by PCR using published primers and conditions [4]. Dideoxy cycle sequencing was performed with Big Dye Terminater Chemistry (Applied Biosystems, Foster City, CA). This was followed by nucleotide sequencing on ABI377, 310, and 3130 automated DNA sequence analyzers (Applied Biosystems).

We found no heterozygous or homozygous missense/nonsense mutations in the coding exons and no causative mutations in exon–intron boundaries of *DJ-1*. We also found a non-synonymous p.R98Q variant, which had been reported to be a polymorphism [13].

This extended large study identified no pathogenic *DJ-1* mutations in 386 patients (mainly Asian) with familial or sporadic parkinsonism by direct sequencing. Our cohort was more selected than the overall PD population, since all *parkin*-positive patients were excluded. In this regard, the lack of *DJ-1* mutations, especially among the rest of approximately 50% of autosomal recessive cases without *parkin* mutation, is even more striking. Furthermore, our study identified the lack of a single heterozygous *DJ-1* missense or nonsense mutation in a large number of patients with sporadic and familial parkinsonism. However, the role of single heterozygous mutation of causative genes for ARP such as *DJ-1*, *parkin*, *PINK1*, and *ATP13A2*, remains intriguing because multiple rare variants might cause the disease. In the 386 patients, the conventional PCR method showed no homozygous *DJ-1* deletion in contrast to *parkin*. Accordingly, as a screening strategy in parkinsonism, gene dosage study for *DJ-1* appears less important than *parkin*, although multiplication or heterozygous exonic deletion cannot be ruled out. Although other mutations in the promoter region or intron of *DJ-1* could exist, our large study suggests that *DJ-1* mutation is rare in Asian or Japanese as well as in parkinsonism patients of other ethnic origin.

In several cohort studies, the frequency of *DJ-1* mutation has also been reported to be lower than that of *parkin* or *PINK1* mutation [30,4,20,31,11,19,1,9,7,6,12]. Because of the rare and characteristic phenotype of *PARK9*-linked parkinsonism, we did not screen for *ATP13A2* (which contains 29 exons) in this study [25,23]. Thus, we propose that, after checking the clinical features carefully, one should screen for mutations in the following order: (1) *parkin*, (2) *PINK1*, (3) *DJ-1*, and (4) *ATP13A2* in ARP in Asian and worldwide populations [20,19,17,12]. Combining our previous data with previous reports indicate that the frequencies of causative mutations in *parkin*, *PINK1*, and *DJ-1* are approximately 50%, 4.5%, 0% in our ARP cohort, respectively [20,19,17]. Conversely, our data further suggests the existence of more yet unknown causative genes for ARP.

The locus of *DJ-1* is 1p36 [32], which harbors a cluster of other causative genes for ARP such as *PINK1* and *ATP13A2* around the small chromosomal region. Therefore, checking the homozygosity of this locus by haplotype analysis is a fruitful strategy for genetic analysis in ARP and especially in parkinsonism with consanguinity. Although mutations in *DJ-1* have not yet been identified in sporadic parkinsonism patients, analyses of *DJ-1*, *PINK1*, and *ATP13A2* should be considered even in sporadic parkinsonism patients (especially in patients with consanguinity or homozygosity in locus 1p36). Indeed, although patients with *ATP13A2* mutation seem to be quite rare, we applied the above strategy and could identify the first Japanese patient with a novel homozygous *ATP13A2* mutation, who was the sole parkinsonism patient without other affected family members and with consanguinity [23].

Interestingly, the locus of TDP-43 (*TARDBP*), which is associated with TDP-43 proteinopathy and amyotrophic lateral sclerosis (ALS), is located in the same small chromosomal region 1p36 [22,27]. Surprisingly, *DJ-1* was also reported to cause parkinsonism-dementia-ALS complex [3]. Moreover, DJ-1 colocalizes with tau inclusions linking parkinsonism to dementia [26]. Subjects with the ALS/parkinsonism-dementia complex (ALS/PDC) of Guam or Kii have been reported to have tau and TDP-43 pathology [18,10], although no mutations were identified [29]. The roles of genes clustering around 1p36 in neurodegenerative disorders needs further investigation.

Furthermore, in parkinsonism, digenic or trigenic mutations (multigenic mutations) or functional interactions among the causative genes for ARP have been reported, such as *DJ-1-PINK1* and *parkin-PINK1* [28,24,5,8]. Thus, *DJ-1* could play some roles in certain common pathways of neurodegeneration via oxidative stress and mitochondrial dysfunction [15]. Although *DJ-1* was initially described as a ubiquitously expressed protein and an oncogene related to the pathogenesis of cancer [21], recent evidence of its neuroprotective roles in neurodegenerative disorders and stroke has been reported [2]. Accordingly, loss of *DJ-1* function could lead to various diseases.

In conclusion, despite the large cohort of both familial and sporadic parkinsonism, no causative *DJ-1* mutations were identified in our patients. These results could reflect the crucial importance of homoeostasis and ability to survive lethal events from the early stages of life. Therefore, further genetic analyses of *DJ-1* and studies of DJ-1 functions might provide important insights into human diseases and survival.

Acknowledgments

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References

[1] P. Abou-Sleiman, D. Healy, N. Quinn, N. Wood, The role of pathogenic *DJ-1* mutations in Parkinson's disease, Ann. Neurol. 54 (2003) 283–286.

[2] H. Aleyasin, M.W. Rousseaux, M. Phillips, R.H. Kim, R.J. Bland, S. Callaghan, R.S. Slack, M.J. During, T.W. Mak, D.S. Park, The Parkinson's disease gene DJ-1 is also a key regulator of stroke-induced damage, Proc. Natl. Acad. Sci. USA 104 (2007) 18748–18753.

[3] G. Annesi, G. Savettieri, P. Pugliese, M. D'Amelio, P. Tarantino, P. Ragonese, V. La Bella, T. Piccoli, D. Civitelli, F. Annesi, B. Fierro, F. Piccoli, G. Arabia, M. Caracciolo, I.C. Cirò Candiano, A. Quattrone, DJ-1 mutations and parkinsonism-dementia-amyotrophic lateral sclerosis complex, Ann. Neurol. 58 (2005) 803–807.

[4] V. Bonifati, P. Rizzu, M.J. van Baren, O. Schaap, G.J. Breedveld, E. Krieger, M.C. Dekker, F. Squitieri, P. Ibanez, M. Joosse, J.W. van Dongen, N. Vanacore, J.C. van Swieten, A. Brice, G. Meco, C.M. van Duijn, B.A. Oostra, P. Heutink, Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism, Science 299 (2003) 256–259.

[5] I.E. Clark, M.W. Dodson, C. Jiang, J.H. Cao, J.R. Huh, J.H. Seol, S.J. Yoo, B.A. Hay, M. Guo, Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin, Nature 441 (2006) 1162–1166.

[6] L.N. Clark, S. Afridi, H. Mejia-Santana, J. Harris, E.D. Louis, L.J. Cote, H. Andrews, A. Singleton, F. Wavrant De-Vrieze, J. Hardy, R. Mayeux, S. Fahn, C. Waters, B. Ford, S. Frucht, R. Ottman, K. Marder, Analysis of an early-onset Parkinson's disease Cohort for *DJ-1* Mutations, Mov. Disord. 19 (2004) 796–800.

[7] A. Djarmati, K. Hedrich, M. Svetel, N. Schäfer, V. Juric, S. Vukosavic, R. Hering, O. Riess, S. Romac, C. Klein, V. Kostic, Detection of *Parkin* (*PARK2*) and *DJ1* (*PARK7*) mutations in early- onset Parkinson disease: *Parkin* mutation frequency depends on ethnic origin of patients, Hum. Mutat. 23 (2004) 525.

[8] M. Funayama, Y. Li, T.H. Tsoi, C.W. Lam, T. Ohi, S. Yazawa, E. Uyama, R. Djaldetti, E. Melamed, H. Yoshino, Y. Imamichi, H. Takashima, K. Nishioka, K. Sato, H. Tomiyama, S. Kubo, Y. Mizuno, N. Hattori, Familial parkinsonism with digenic parkin and PINK1 mutations, Mov. Disord. 23 (2008) 1461–1465.

[9] S. Hague, E. Rogaeva, D. Hernandez, C. Gulick, A. Singleton, M. Hanson, J. Johnson, R. Weiser, M. Gallardo, B. Ravina, K. Gwinn-Hardy, A. Crawley, P. St George-Hyslop, A. Lang, P. Heutink, V. Bonifati, J. Hardy, A. Singleton, Early-onset Parkinson's disease caused by a compound heterozygous *DJ-1* mutation, Ann. Neurol. 54 (2003) 271–274.

[10] M. Hasegawa, T. Arai, H. Akiyama, T. Nonaka, H. Mori, T. Hashimoto, M. Yamazaki, K. Oyanagi, TDP-43 is deposited in the Guam parkinsonism-dementia complex brains, Brain 130 (2007) 1386–1394.

[11] Y. Hatano, Y. Li, K. Sato, S. Asakawa, Y. Yamamura, H. Tomiyama, H. Yoshino, M. Asahina, S. Kobayashi, S. Hassin-Baer, C.S. Lu, A.R. Ng, R.L. Rosales, N. Shimizu, T. Toda, Y. Mizuno, N. Hattori, Novel *PINK1* mutations in early-onset parkinsonism, Ann. Neurol. 56 (2004) 424–427, Erratum in: Ann. Neurol. 56 (2004) 603.

[12] K. Hedrich, A. Djarmati, N. Schäfer, R. Hering, C. Wellenbrock, P. Weiss, R. Hilker, P. Vieregge, L. Ozelius, P. Heutink, V. Bonifati, E. Schwinger, A. Lang, J. Noth, S. Bressman, P. Pramstaller, O. Riess, C. Klein, *DJ-1 (PARK7)* mutations are less frequent than *Parkin (PARK2)* mutations in early-onset Parkinson disease, Neurology 62 (2004) 389–394.

[13] K. Hedrich, N. Schäfer, R. Hering, J. Hagenah, A. Lanthaler, E. Schwinger, P. Kramer, L. Ozelius, S. Bressman, G. Abbruzzese, P. Martinelli, V. Kostic, P. Pramstaller, P. Vieregge, O. Riess, C. Klein, The R98Q variation in DJ-1 represents a rare polymorphism, Ann. Neurol. 55 (2004) 145.

[14] P. Ibáñez, G. Michele, V Bonifati, E Lohmann, S. Thobois, P Pollak, Y. Agid, P Heutink, A Dürr, A. Brice, French Parkinson's Disease Genetics Study Group, Screening for DJ-1 mutations in early onset autosomal recessive parkinsonism, Neurology 61 (2003) 1429–1431.

[15] P. Jenner, Oxidative stress in Parkinson's disease, Ann. Neurol. 53 (suppl. 3) (2003) S26–S38.

[16] T. Kitada, S. Asakawa, N. Hattori, H. Matsumine, Y. Yamamura, S. Minoshima, M. Yokochi, Y. Mizuno, N. Shimizu, Mutations in the *parkin* gene cause autosomal recessive juvenile parkinsonism, Nature 392 (1998) 605–608.

[17] R. Kumazawa, H. Tomiyama, Y. Li, Y. Imamichi, M. Funayama, H. Yoshino, F. Yokochi, T. Fukusako, Y. Takehisa, K. Kashihara, T. Kondo, B. Elibol, S. Bostantjopoulou, T. Toda, H. Takahashi, F. Yoshii, Y. Mizuno, N. Hattori, Mutation analysis of the PINK1 gene in 391 patients with Parkinson's disease, Arch. Neurol. 65 (2008) 802–808.

[18] S. Kuzuhara, Y. Kokubo, R. Sasaki, Y. Narita, T. Yabana, M. Hasegawa, T. Iwatsubo, Familial amyotrophic lateral sclerosis and parkinsonism-dementia complex of the Kii Peninsula of Japan: clinical and neuropathological study and tau analysis, Ann. Neurol. 49 (2001) 501–511.

[19] Y. Li, H. Tomiyama, K. Sato, Y. Hatano, H. Yoshino, M. Atsumi, M. Kitaguchi, S. Sasaki, S. Kawaguchi, H. Miyajima, T. Toda, Y. Mizuno, N. Hattori, Clinicogenetic study of PINK1 mutations in autosomal recessive early-onset parkinsonism, Neurology 64 (2005) 1955–1957.

[20] C.B. Lücking, A. Dürr, V. Bonifati, J. Vaughan, G. De Michele, T. Gasser, B.S. Harhangi, G. Meco, P. Denèfle, N.W. Wood, Y. Agid, A. Brice, French Parkinson's Disease Genetics Study Group, European consortium on genetic susceptibility in Parkinson's disease. Association between early-onset Parkinson's disease and mutations in the parkin gene, N. Engl. J. Med. 342 (2000) 1560–1567.

[21] D. Nagakubo, T. Taira, H. Kitaura, M. Ikeda, K. Tamai, S.M. Iguchi-Ariga, H. Ariga, DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras, Biochem. Biophys. Res. Commun. 231 (1997) 509–513.

[22] M. Neumann, D. Sampathu, L. Kwong, A. Truax, M. Micsenyi, T. Chou, J. Bruce, T. Schuck, M. Grossman, C. Clark, L. McCluskey, B. Miller, E. Masliah, I. Mackenzie, H. Feldman, W. Feiden, H. Kretzschmar, J. Trojanowski, V. Lee, Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis, Science 314 (2006) 130–133.

[23] Y.P. Ning, K. Kanai, H. Tomiyama, Y. Li, M. Funayama, H. Yoshino, S. Sato, M. Asahina, S. Kuwabara, A. Takeda, T. Hattori, Y. Mizuno, N. Hattori, *PARK9*-linked parkinsonism in Eastern Asia: mutation detection in *ATP13A2* and clinical phenotype, Neurology 7 (2008) 1491–1493.

[24] J. Park, S.B. Lee, S. Lee, Y. Kim, S. Song, S. Kim, E. Bae, J. Kim, M. Shong, J.M. Kim, J. Chung, Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin, Nature 441 (2006) 1157–1161.

[25] A. Ramirez, A. Heimbach, J. Grundemann, B. Stiller, D. Hampshire, L.P. Cid, I. Goebel, A.F. Mubaidin, A.L. Wriekat, J. Roeper, A. Al-Din, A.M. Hillmer, M. Karsak, B. Liss, C.G. Woods, M.I. Behrens, C. Kubisch, Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 p-type ATPase, Nat. Genet. 38 (2006) 1184–1191.

[26] P. Rizzu, D.A. Hinkle, V. Zhukareva, V. Bonifati, L.A. Severijnen, D. Martinez, R. Ravid, W. Kamphorst, J.H. Eberwine, V.M. Lee, J.Q. Trojanowski, P. Heutink, DJ-1 colocalizes with tau inclusions: a link between parkinsonism and dementia, Ann. Neurol. 55 (2004) 113–118.

[27] J. Sreedharan, J. Sreedharan, I.P. Blair, V.B. Tripathi, X. Hu, C. Vance, B. Rogelj, S. Ackerley, J.C. Durnall, K.L. Williams, E. Buratti, F. Baralle, J. de Belleroche, J.D. Mitchell, P.N. Leigh, A. Al-Chalabi, C.C. Miller, G. Nicholson, C.E. Shaw, TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis, Science 319 (2008) 1668–1672.

[28] B. Tang, H. Xiong, P. Sun, Y. Zhang, D. Wang, Z. Hu, Z. Zhu, H. Ma, Q. Pan, J.H. Xia, K. Xia, Z. Zhang, Association of PINK1 and DJ-1 confers digenic inheritance of early-onset Parkinson's disease, Hum. Mol. Genet. 15 (2006) 1816–1825.

[29] H. Tomiyama, Y. Kokubo, R. Sasaki, Y. Li, Y. Imamichi, M. Funayama, Y. Mizuno, N. Hattori, S. Kuzuhara, Mutation analyses in amyotrophic lateral sclerosis/parkinsonism-dementia complex of the Kii peninsula, Japan Mov. Disord. 23 (2008) 2344–2348.

[30] E.M. Valente, P.M. Abou-Sleiman, V. Caputo, M.M. Muqit, K. Harvey, S. Gispert, Z. Ali, D. Del Turco, A.R. Bentivoglio, D.G. Healy, A. Albanese, R. Nussbaum, R. González-Maldonado, T. Deller, S. Salvi, P. Cortelli, W.P. Gilks, D.S. Latchman, R.J. Harvey, B. Dallapiccola, G. Auburger, N.W. Wood, Hereditary early-onset Parkinson's disease caused by mutations in *PINK1*, Science 304 (2004) 1158–1160.

[31] E.M. Valente, S. Salvi, T. Ialongo, R. Marongiu, A.E. Elia, V. Caputo, L. Romito, A. Albanese, B. Dallapiccola, A.R. Bentivoglio, PINK1 mutations are associated with sporadic early-onset parkinsonism, Ann. Neurol. 56 (2004) 336–341.

[32] C. van Duijn, M. Dekker, V. Bonifati, R. Galjaard, J. Houwing-Duistermaat, P. Snijders, L. Testers, G. Breedveld, M. Horstink, L. Sandkuijl, J. van Swieten, B. Oostra, P. Heutink, PARK7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36, Am. J. Hum. Genet. 69 (2001) 629–634.

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Excitatory amino acid transporter 2 associates with phosphorylated tau and is localized in neurofibrillary tangles of tauopathic brains

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ABSTRACT

Phosphorylated tau (p-tau) is the principal component of neurofibrillary tangles, a pathological hallmark, and likely plays a neurotoxic role in tauopathies including Alzheimer's disease (AD), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD). We subjected brains from autopsy cases of AD, PSP, and CBD to a variety of immunohistochemical, immunoblotting, and pull-down assays. In this study, we show that excitatory amino acid transporter 2 (EAAT2) preferentially interacted with phosphorylated tau and was localized in neurofibrillary tangles in the brains of such patients. These results strongly indicate that EAAT2 acts in tauopathy-related neurodegeneration, and abnormalities in glutamate transport play an important role in the pathogenesis of tauopathies.

Structured summary:
MINT-7148349, MINT-7148361:*TAU* (uniprotkb:P10636) *physically interacts* (MI:0914) with *EAAT2* (uniprotkb:P43004) by *pull-down* (MI:0096)
MINT-7148372, MINT-7148384:*TAU* (uniprotkb:P10636) *physically interacts* (MI:0914) with *EAAT2* (uniprotkb:P43004) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Tau is a microtubule-associated protein mainly expressed in neurons where it plays a role in the assembly and stability of the microtubule network. Tau is also strongly linked to several neurodegenerative disorders as the principle pathological component of filamentous inclusions called neurofibrillary tangles (NFTs) found in the brains of Alzheimer's disease (AD) patients and in a variety of diseases collectively called "tauopathies", including progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) [1]. Although these diseases are sporadic, the tau gene can harbor mutations leading to diverse clinical phenotypes collectively grouped under frontotemporal dementia with parkinsonism linked to chromosome 17 [2].

Phosphorylated tau (p-tau) has been identified as a major protein component of NFTs [3,4]. Considerable evidence supports that p-tau represents the pathological entity in tauopathies, including the observation that increased p-tau causes neuronal cell death [5]. Because phosphorylation releases tau from microtubules and tau in NFTs is phosphorylated, kinases have been viewed suspiciously for a possible pathogenetic role. In vivo evidence for interaction with tau exists for cyclin-dependent kinase-5 (cdk5)/p25 and glycogen synthase kinase-3β (GSK-3β). Tau phosphorylation by cdk5 in mice [6] and GSK-3β in flies [7] can cause or accelerate NFT formation in vivo with an attendant worsening of neurodegeneration. Moreover, as endogenous tau was phosphorylated, aggregated tau accumulated, and neurofibrillary pathology developed progressively in these animals.

In light of such evidence, analyzing the molecular complex of p-tau and mechanism of p-tau-induced cell death is important for

Abbreviations: AD, Alzheimer's disease; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; EAAT2, excitatory amino acid transporter 2; NFTs, neurofibrillary tangles; GSK-3β, glycogen synthase kinase-3β; p-tau, phosphorylated tau; GLT1, glutamate transporter

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understanding the pathogenesis of tauopathies. In previous studies, we showed that CHIP and HSP27 interact with p-tau, decrease its amounts, and protect against p-tau-induced neurotoxicity [8,9]. In this study, we provide evidence that excitatory amino acid transporter 2 (EAAT2) is a molecular complex of p-tau. EAAT2 is a glutamate transporter and its dysfunction is tightly linked to neurodegenerative disorders [10]. We also demonstrate that EAAT2 preferentially interacts with p-tau rather than dephosphorylated tau and is localized within NFTs of patients with AD, PSP, and CBD.

2. Materials and methods

2.1. Antibodies

The EAAT2 polyclonal antibody, also known as HS5, which is an antibody to the 20-mer sequence (TEGANNMPKQVEVRNHDSHL) at the N-terminal domain of human EAAT2, was generated and characterized as described previously [11]. The anti-tau antibodies used included tau antibodies, anti-phosphorylated tau antibodies, and anti-non-phosphorylated tau antibodies. The tau antibody used was a monoclonal mouse tau antibody (Sigma, St. Louis, MO, USA), which recognizes all tau isoforms. The anti-phosphorylated tau antibodies used included AT8 (Pierce, Rockford, IL, USA), which recognizes phosphorylated S202 and T205 residues, and PHF1 [12,13], which recognizes phosphorylated S396 and S404 residues. Tau 1 [14] was used as an anti-non-phosphorylated tau antibody, which recognizes non-phosphorylated S199, S202, and T205 residues. Rabbit anti-EAAT2 antibody H85 and goat anti-EAAT2 antibody N19 were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Table 1
Clinical data of all autopsied subjects.

Case	Age	Sex	Postmortem delay (h)
Normal 1	66	Male	5
Normal 2	70	Female	2.5
Normal 3	81	Male	2.5
AD1	66	Male	3.5
AD2	72	Female	3
AD3	76	Male	5
AD4	81	Female	4.5
PSP1	69	Male	6.5
PSP2	85	Female	3.5
CBD1	61	Male	8
CBD2	81	Female	9

2.2. In vitro phosphorylation

Active recombinant GSK-3β (Upstate Biotechnology, Lake Placid, NY, USA) and Cdk5/p25 (Cosmo Bio Co. Ltd., Tokyo, Japan) were incubated in a reaction mixture containing 2 mM ATP, 20 mM HEPES (pH 6.8), 10 mM $MgCl_2$, and 10 mM $MnCl_2$ with recombinant His-tau (Sigma). Tau phosphorylation sites were confirmed by the phosphorylated tau-specific antibodies PHF1 (S396 and S404) and AT8 (S202 and T205).

2.3. Brain tissue samples

Brain tissue was obtained by autopsy from four AD (AD1, 2, 3 and 4), two CBD (CBD1 and 2), two PSP (PSP1 and 2), and three normal cases (normal 1, 2 and 3). Autopsies were performed by authorized pathologists to confirm diagnosis or other clinical imperatives. Informed consent was obtained from the next-of-kin in all cases. Diagnoses were performed by viewing the pathological sections. Age, sex, and postmortem delay are summarized in Table 1.

2.4. Immunopurification and immunoblotting

Immunopurification, immunoblotting, and pull-down assays were performed as described previously [8,9]. Temporal cortex specimens (2 g) were homogenized in lysis buffer containing 1% Triton-X 100 and sedimented at 20 000×*g* for 1 h at 4 °C. Supernatants were incubated with protein A/G (Pierce) bound to the tau monoclonal antibody (Sigma) or HS5 for 2 h at 4 °C. SDS buffer (4×) was added to lysates and immunoprecipitates. SDS–PAGE and immunoblotting were performed as described previously [11,12]. The intensity was analyzed using a chemiluminescence image analyzer Las-1000 Plus (Fuji film, Tokyo, Japan). The value was compared to that of AD1.

2.5. Pull-down assays

Either His-tau or phosphorylated His-tau-coupled nickel beads were added to human brain homogenates incubated at 4 °C for 1 h with shaking. We added 4× SDS to release precipitated proteins from the beads. The eluted fraction was transferred to a new tube after centrifugation and 0.1 N Tris–HCl buffer (pH 8.5) was added.

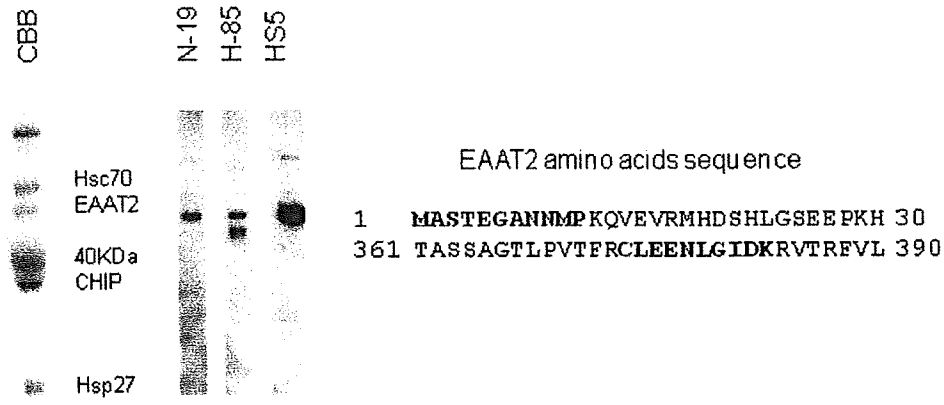

Fig. 1. Excitatory amino acid transporter 2 (EAAT2) interacts with phosphorylated tau. Human brain homogenates were passed over nickel beads coupled to His-tau that had been phosphorylated by GSK-3β and Cdk5/p25. Eluates were separated by SDS–PAGE and stained with Coomassie brilliant blue (CBB) or immunoblotted with antibodies to EAAT2. The peptide sequence (bold) obtained from the 62-kDa band was consistent with the human EAAT2 protein sequence (NCB Accession # NP 004162).

2.6. Immunohistochemistry

Immunohistochemical studies were performed as described previously [13]. Sections (10-μm thick) of formalin-fixed, paraffin-embedded tissue from the medial temporal cortex were incubated with 70% formic acid for antigen activation followed by immunostaining with EAAT2 antibodies. By confocal microscopy, AT8 (Pierce), which specifically recognizes p-tau, was visualized by a secondary antibody conjugated to Texas Red and EAAT2 by a secondary antibody conjugated to FITC.

2.7. Extraction of sarkosyl-insoluble proteins

Sarkosyl-insoluble proteins were extracted from the temporal cortex as described previously [14]. Extracts were divided into

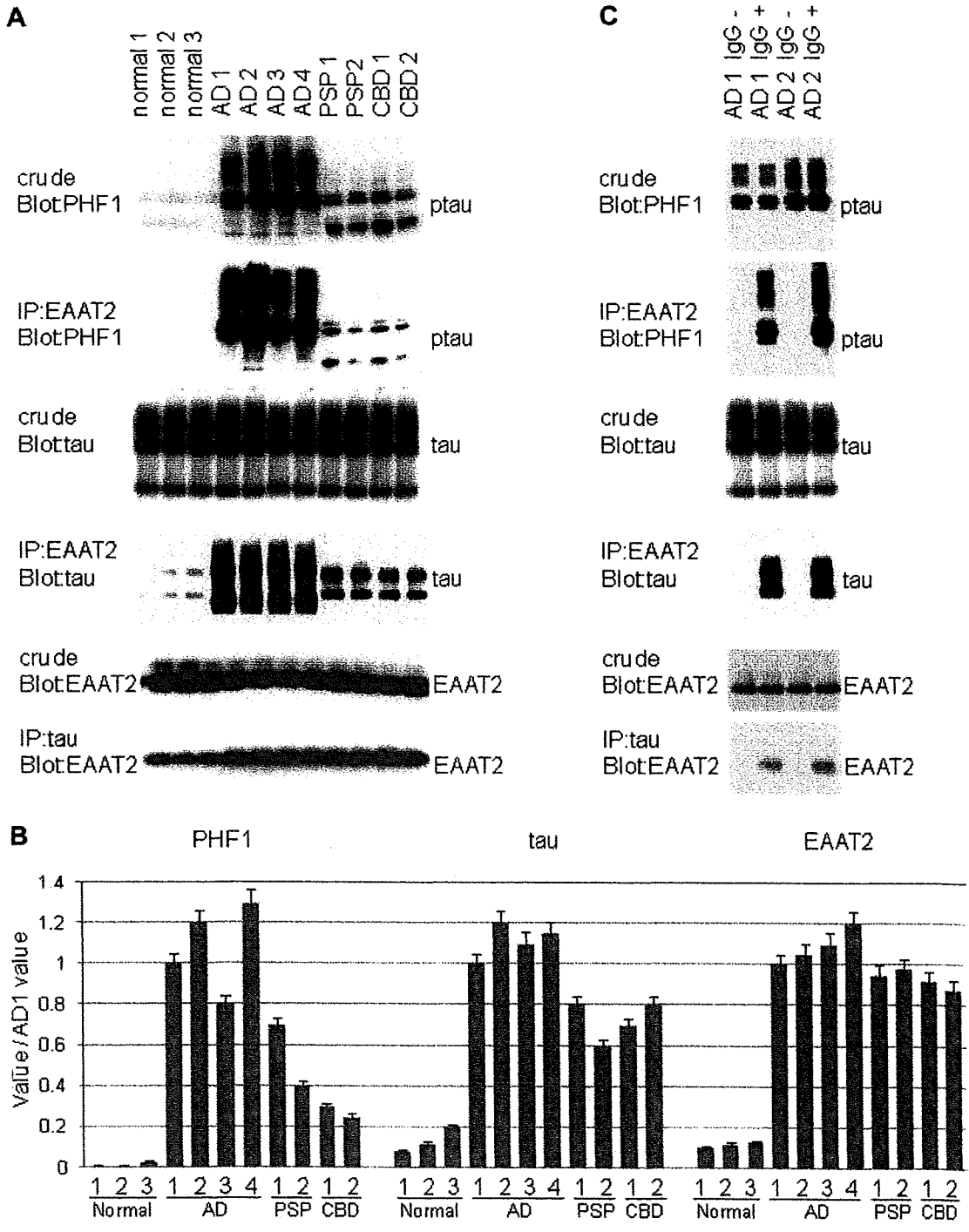

Fig. 2. (A) Brain homogenates of the temporal cortex from four Alzheimer's disease (AD), two progressive supranuclear palsy (PSP), two corticobasal degeneration (CBD), and three normal cases were immunoprecipitated with HS5 polyclonal EAAT2 or monoclonal tau antibodies. Precipitates were analyzed by PHF1, monoclonal tau, or HS5 polyclonal EAAT2 antibodies. (B) Quantitative analysis of immunoprecipitated tau and EAAT2 levels standardized against the amount of protein detected in relevant crude homogenates. Values are normalized to the value of AD1. Data are presented as mean S.D. (C) Analysis of antibody specificity for immunoprecipitation.

TBS-soluble, 1% Triton-X 100-soluble, sarkosyl-soluble, and sarkosyl-insoluble fractions.

3. Results

We sought to identify molecular complexes associated with tau when it is phosphorylated at sites that are phosphorylated in AD (Ser-202, Thr-205, Ser-396, and Ser-404). Human brain homogenates were passed through an affinity column with nickel beads coupled to a recombinant four-repeat His-tau phosphorylated by Cdk5/p25 and GSK-3β, and associated proteins were eluted with 1 M NaCl. Eluted proteins were separated by SDS–PAGE and several bands were stained with Coomassie Brilliant Blue (CBB). A 62-kDa CBB band was identified by mass spectrometry that contained the sequences MASTEGANNMP and CLEENLGIDK, which exactly matched the human EAAT2 sequence (Fig. 1). The sequence did not, however, match the human EAAT1, EAAT3, EAAT4, and EAAT5 sequences. This 62-kDa molecule was also immunoreactive to various anti-EAAT2 antibodies, including N19 (Santa Cruz Biotechnology), H85 (Santa Cruz Biotechnology), and the rabbit polyclonal anti-EAAT2 antibody HS5 (Fig. 1).

To examine the interaction between EAAT2 and p-tau, we conducted coimmunoprecipitation of homogenates from the frozen temporal cortex obtained from 4 AD, 2 PSP, 2 CBD, and 3 normal brains. We used HS5 for immunoprecipitation followed by immunoblotting with PHF1, a monoclonal antibody that recognizes pathological tau phosphorylated at residues Ser-396 and Ser-404, and a monoclonal tau antibody that recognizes all tau isoforms (Fig. 2A). Multiple tau bands of 30–60 kDa were recognized by the monoclonal tau antibody in all cases, whereas p-tau bands were detected only in crude extracts of AD, PSP, and CBD brains. As expected, only faint bands were recognized by PHF1 in normal brains. P-tau coprecipitated with the anti-EAAT2 antibody HS5 from AD, PSP, and CBD brains but not from normal brains. Larger amounts of tau coprecipitated with HS5 from AD, PSP, and CBD brains than those from normal brains (Fig. 2B). Coprecipitates of the anti-EAAT2 antibody were not stained with the tau1 antibody, which only recognized dephosphorylated tau (data not shown). Reverse coimmunoprecipitation experiments showed that larger amounts of EAAT2 coprecipitated with the monoclonal tau antibodies from AD, PSP, and CBD brains than those from normal brains. Thus, the tau protein that immunoprecipitated with the EAAT2 antibody was mainly p-tau. We performed immunoprecipitations in the absence or presence of an antibody for immunoprecipitations for determining antibody specificity for immunoprecipitation. We did not detect immunoreactivities in all immunoprecipitation experiments conducted in the absence of antibodies for immunoprecipitates (Fig. 2C).

To further determine the specificity of interaction between p-tau and EAAT2, we incubated nickel beads coupled either to recombinant His-tau, which was phosphorylated, or to a dephosphorylated control. Considerably larger amounts of EAAT2 were precipitated by p-tau than by dephosphorylated tau in all cases, suggesting that EAAT2 preferentially interacted with p-tau compared to dephosphorylated tau (Fig. 3A). The same amount of recombinant p-tau precipitated larger amounts of EAAT2 from AD, PSP, and CBD brains than from normal brains (Fig. 3B). These results suggest that p-tau more preferentially associates with EAAT2 in tauopathic brains than normal brains.

Interaction of EAAT2 with p-tau suggested that EAAT2 may localize in NFTs. In the midtemporal cortex, EAAT2 labeling occurred in large numbers of neuronal NFTs in all AD brains (Fig. 4A, B, and E), and in glial NFTs in PSP (Fig. 4C) and CBD brains (Fig. 4D). There was weak staining of glia and no obvious staining of neurons except for NFTs in AD, PSP, and CBD cases when the

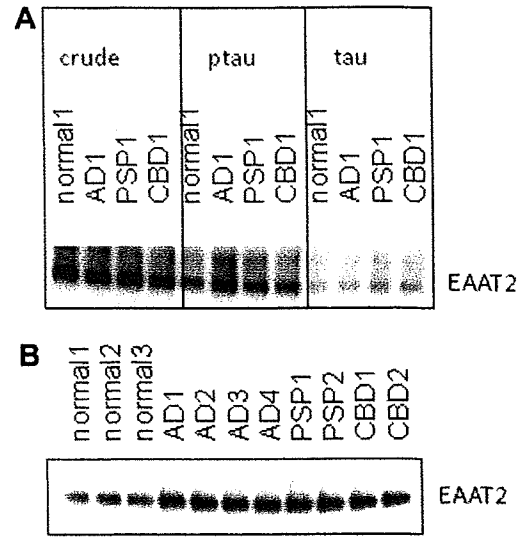

Fig. 3. Ability of EAAT2 to interact with phosphorylated tau (p-tau) was examined by a pull-down assay in which human brain homogenates were passed over 20 μL nickel beads coupled to His-phosphorylated tau or His-tau. Precipitated proteins were analyzed by the EAAT2 antibody.

samples were incubated with 70% formic acid. HS5 weakly stained ordinarily expressed glial EAAT2 but not NFTs when slides were incubated without 70% formic acid. Confocal microscopic examinations showed that EAAT2 was partially colocalized with p-tau, which was recognized by AT8 in NFTs of AD, PSP, and CBD brains (Fig. 4F–Q).

Immunoblots showed the expected serial extraction of tau with tau immunoreactivity in the sarkosyl-insoluble/formic acid-soluble fraction in AD patients but not in controls (Fig. 5A). Remarkably, EAAT2 and high-molecular-weight EAAT2 were also present in this fraction (Fig. 5A). EAAT2 was present in this fraction only in AD, PSP, and CBD brains (Fig. 5B).

4. Discussion

We have shown that EAAT2 interacts with p-tau in biochemical and histochemical studies. Immunoprecipitation studies indicated that EAAT2 preferentially associated with p-tau compared to dephosphorylated tau. A pull-down assay using recombinant tau or p-tau revealed that p-tau preferentially combined with EAAT2 from AD, PSP, or CBD brains compared to controls. Immunohistochemical studies showed that not only neuronal EAAT2 in AD brains but glial EAAT2 in PSP and CBD brains also colocalized with p-tau in NFTs. This suggests that EAAT2 may have been caught up with p-tau as tangles formed. Furthermore, we found high-molecular-weight EAAT2 in the sarkosyl-insoluble fraction. This suggests that EAAT2 may be oligomerized such as α-synuclein [15] and β-amyloid [16], which are major components of inclusions in neurodegenerative disorders.

EAATs are essential proteins for glutamate and aspartate uptake from the synaptic cleft into glias and neurons [17]. Several EAATs have been isolated and characterized [18]. EAAT1 is primarily localized in glia of the cerebellum and retina. EAAT2 is specifically located on astrocytes in non-diseased brains and is quantitatively dominant in the cortex [19]. EAAT2 is responsible for a majority of glutamate clearance in the adult forebrain [18]. A link among glutamate transporter dysfunction, increased extracellular glutamate levels, and the onset of excitotoxic neuronal damage has been established in AD and other neurodegenerative

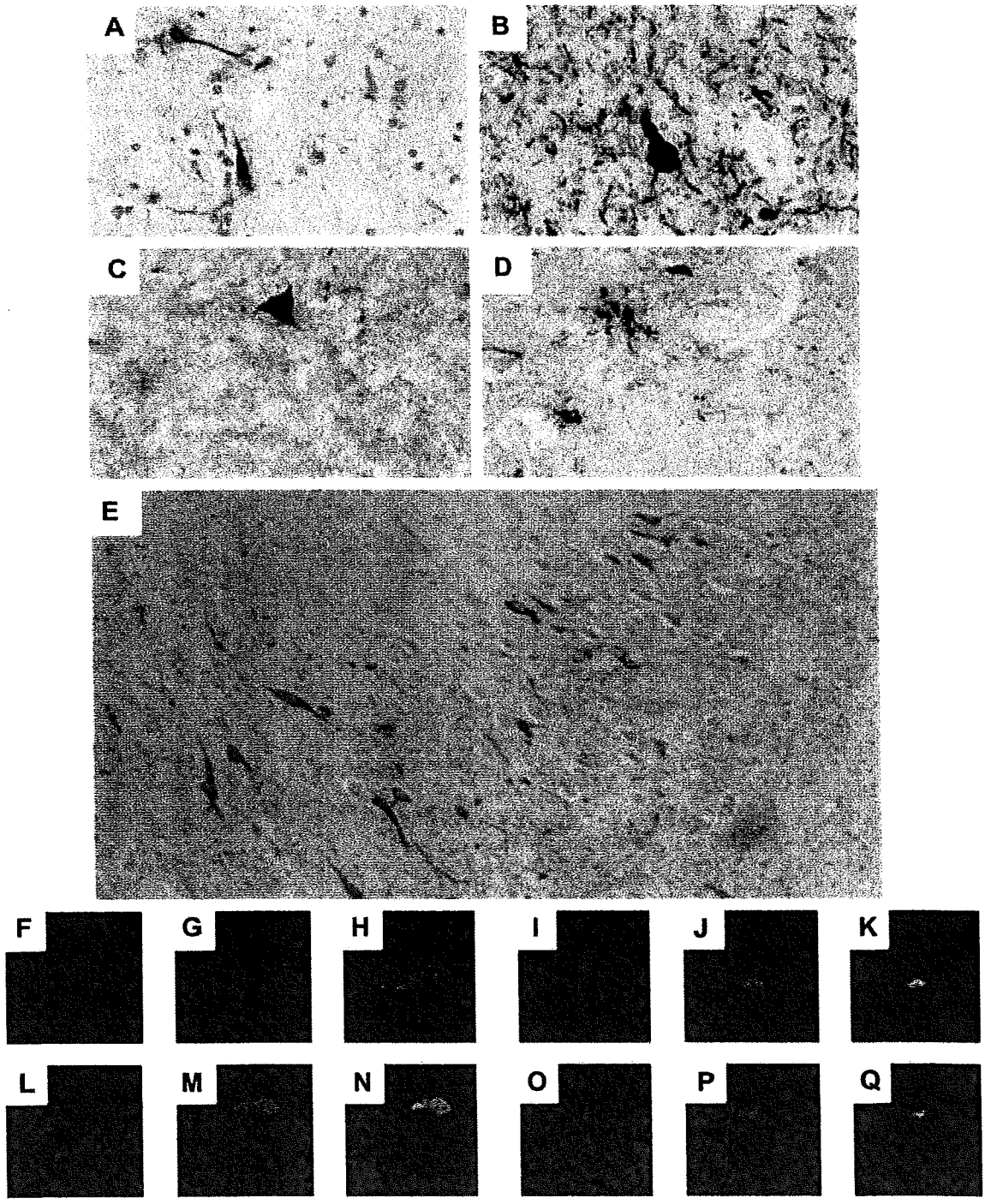

Fig. 4. Immunohistochemical analysis of EAAT2 in tauopathic specimens. (A–E) Neurofibrillary tangles (NFTs) in CA1 immunoreactive for EAAT2. Neuronal NFTs immunoreactive for EAAT2 in patients with AD (A, B, ×400) (E, ×100). Glial NFTs immunoreactive for EAAT2 in patients with PSP (C) or CBD (D, ×400). AD (F–K), PSP (L–N), and CBD (O–Q) double-labeling for EAAT2 (F, I, L and O) (green) or p-tau (G, J, M and P) (red). Merged images (H, K, N and Q) show the colocalization of p-tau and EAAT2. There is an overlap of the epitopes in some NFTs.

disorders [20]. Ablation of EAAT2 or glutamate transporter 1 (rodent homolog, GLT1) in mice has a significant effect on the development of pathogenic conditions and neurodegeneration [21]. The glial transport of excitatory amino acids in AD is deficient and the number of EAAT2-expressing cortical astrocytes is decreased [22].

In AD cases, expression of a glutamate transporter not normally found in neurons could imply that abnormal EAAT2 expression precedes and causes tangle formation in the affected neurons. Pull-down assays with recombinant p-tau showed that EAAT2 not only from AD but also from PSP and CBD brains preferentially interacted with p-tau. Thus, even physiologically expressed glial EAAT2 may be pathologically modified and subsequently bind with p-tau in pathological glia of PSP and CBD brains. This implies that neuronal and glial dysregulation of glutamate transport is strongly linked to the pathogenesis of tauopathies.

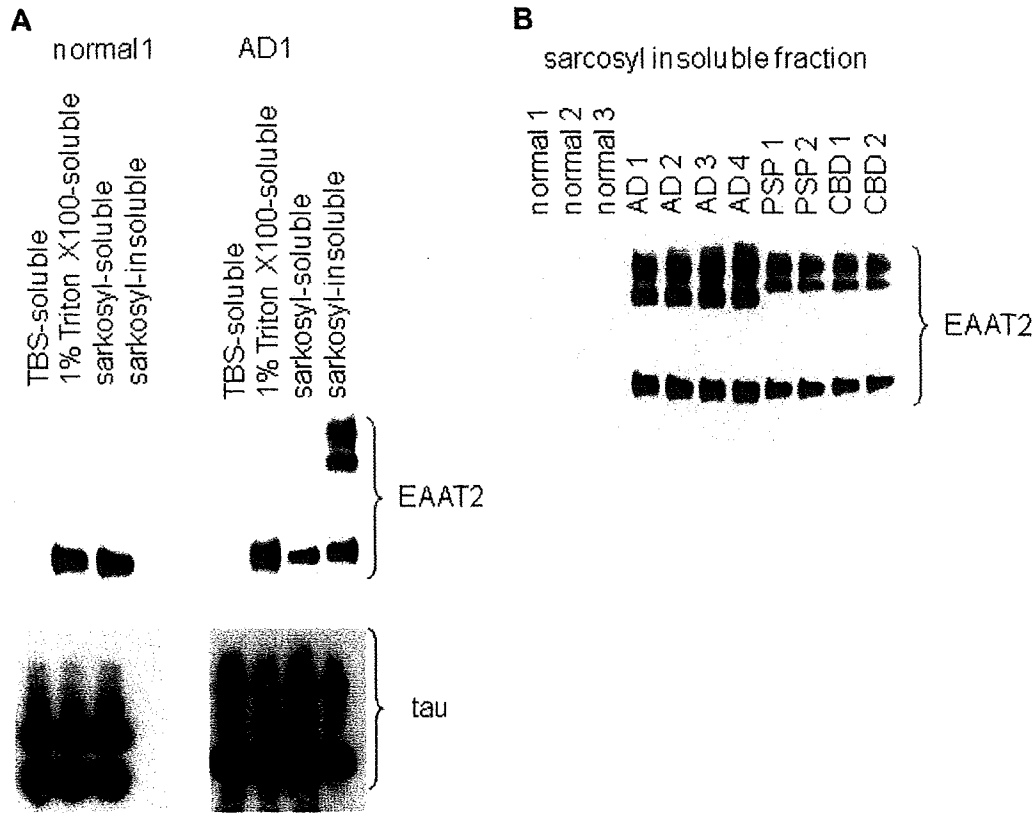

Fig. 5. Serial extractions of the temporal cortex were prepared from AD, PSP, CBD, and normal brains. (A) Preparation of supernatants and TBS-soluble, 1% Triton-X 100-soluble, sarkosyl-soluble, and sarkosyl-insoluble fractions are described in the Methods section. (B) Anti-EAAT2 immunoblots of sarkosyl-insoluble fraction from homogenates from AD, PSP, CBD, and normal brains.

Recently, it was shown that EAAT1 [23] and EAAT2 [24] were detected in NFT-bearing neurons but were not localized in NFTs of AD patients by immunohistochemical studies. Our results showed that EAAT2 is localized in NFTs. The difference between our results and those of previous studies is because of the different antibodies and immunohistochemical methods used. In our study, NFTs were not stained with our EAAT2 antibody due to the absence of using 70% formic acid to activate the antigens. Our EAAT2 antibody might be suitable for the detection of insoluble EAAT2 in immunohistochemical studies.

We therefore conclude that glutamate transport is altered in tauopathies with aberrant and physiological expression of EAAT2, and speculate that these changes occur before, but are related to, neurofibrillary pathology. These findings strongly implicate glutamate-mediated toxicity as an important mechanism in tauopathy-related neurodegeneration.

Acknowledgments

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References

[1] Buée, L., Bussière, T., Buée-Scherrer, V., Delacourte, A. and Hof, P.R. (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res. Rev. 33, 95–130.
[2] Hutton, M. (2000) Molecular genetics of chromosome 17 tauopathies. Ann. NY Acad. Sci. 920, 63–73.
[3] Trojanowski, J.Q. and Lee, V.M. (1995) Phosphorylation of paired helical filament tau in Alzheimer's disease neurofibrillary lesions: focusing on phosphatases. FASEB J. 9, 1570–1576.
[4] Kosik, K.S. (1990) Tau protein and neurodegeneration. Mol. Neurobiol. 4, 171–179.
[5] Geschwind, D.H. (2003) Tau phosphorylation, tangles, and neurodegeneration: the chicken or the egg? Neuron 40, 457–460.
[6] Noble, W. et al. (2003) Cdk5 is a key factor in tau aggregation and tangle formation in vivo. Neuron 38, 555–565.
[7] Jackson, G.R., Wiedau-Pazos, M., Sang, T.K., Wagle, N., Brown, C.A., Massachi, S. and Geschwind, D.H. (2002) Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in Drosophila. Neuron 34, 509–519.
[8] Shimura, H., Schwartz, D., Gygi, S.P. and Kosik, K.S. (2004) CHIP-Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival. J. Biol. Chem. 279, 4869–4876.
[9] Shimura, H., Miura-Shimura, Y. and Kosik, K.S. (2004) Binding of tau to heat shock protein 27 leads to decreased concentration of phosphorylated tau and enhanced cell survival. J. Biol. Chem. 279, 17957–17962.
[10] Rothstein, J.D., Jin, L., Dykes-Hoberg, M. and Kuncl, R.W. (1993) Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc. Natl. Acad. Sci. USA 90, 6591–6595.
[11] Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S. and Selkoe, D.J. (2001) Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science 293, 263–269.
[12] Greenberg, S.G., Davies, P., Schein, J.D. and Binder, L.I. (1992) Hydrofluoric acid-treated tau PHF proteins display the same biochemical properties as normal tau. J. Biol. Chem. 267, 564–569.
[13] Shimura, H., Hattori, N., Kubo, S., Yoshikawa, M., Kitada, T., Matsumine, H., Asakawa, S., Minoshima, S., Yamamura, Y., Shimizu, N. and Mizuno, Y. (1999) Immunohistochemical and subcellular localization of Parkin protein: absence of protein in autosomal recessive juvenile parkinsonism patients. Ann. Neurol. 45, 668–672.
[14] Wang, Q., Woltjer, R.L., Cimino, P.J., Pan, C., Montine, K.S., Zhang, J. and Montine, T.J. (2005) Proteomic analysis of neurofibrillary tangles in Alzheimer disease identifies GAPDH as a detergent-insoluble paired helical filament tau binding protein. FASEB J. 19, 869–871.
[15] Goldberg, M.S. and Lansbury Jr., P.T. (2000) Is there a cause-and-effect relationship between alpha-synuclein fibrillization and Parkinson's disease? Nat. Cell Biol. 2, E115–E119.
[16] Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J. and Selkoe, D.J. (2002) Naturally secreted oligomers of amyloid