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ORIGINAL CONTRIBUTION

Parkin Mutations and Early-Onset Parkinsonism in a Taiwanese Cohort

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Background: Loss of function of the parkin gene (*PRKN*) is the predominant genetic cause of juvenile and early-onset parkinsonism in Japan, Europe, and the United States.

Objectives: To evaluate the frequency of *PRKN* mutations in Taiwanese (ethnic Chinese) patients with early-onset parkinsonism and to explore genotype-phenotype correlations.

Design: Clinical assessment included medical, neurologic, and psychiatric evaluation. Genomic DNA sequencing and quantitative polymerase chain reaction were performed to identify *PRKN* mutations. Gene expression was examined in patient lymphoblastoid cell lines, in which *PRKN* mutations were identified.

Patients: Forty-one Taiwanese patients with early-onset parkinsonism (aged <50 years at onset).

Results: Four of 41 probands had *PRKN* mutations. One proband had compound heterozygous mutations, with a *PRKN* exon 2 deletion and an exon 7 G284R substitution. The phenotype resembled typical Parkinson disease. Three patients were mutation carriers. One proband had *PRKN* exon 2 and exon 3 deletions in the same allele. However, this patient's phenotype was that of classic "parkin-proven" autosomal recessive juvenile parkinsonism, characterized by symmetrical foot dystonia at onset, gait disturbance, diurnal change, and very slow progression. The 2 remaining carriers had novel heterozygous exon 11 R396G substitutions. Patients with *PRKN* mutations were younger at onset than those without mutations, and they required a lower dose of levodopa despite longer disease duration.

Conclusions: Mutations in *PRKN* are a rare cause of early-onset parkinsonism in Taiwanese individuals. The overall mutation frequency, adjusted for age at onset, was comparable with that reported for white cohorts; however, the point mutations identified seem to be population specific.

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THE PREDOMINANT GENETIC cause of early-onset parkinsonism (aged <50 years at onset) in Japan, Europe, and the United States is recessive homozygous or compound heterozygous mutation of the parkin gene (*PRKN*). Many studies suggest that 18% to 49% of early-onset disease can be ascribed to loss of parkin function in these populations (reviewed by Mata et al[1]). Exonic deletions/duplications of *PRKN* are generally de novo, whereas many of the missense mutations discovered in European and North American populations seem to originate from common European founders.[2,3]

Taiwan's population history encompasses indigenous Austronesian peoples and early Chinese settlers, known as the Hakka, originating from the Hunan province approximately 1500 years ago. *Benshengren* settlers originated from the Fujian province during the Ming dynasty (1366-1644). During the 17th century, the Portuguese, Dutch, and Spanish maintained colonial interests in Taiwan for several decades. However, ethnic Chinese immigration increased 7-fold during the subsequent 150 years of Manchu rule on the Chinese mainland. More recent history includes the Treaty of Shimonoseki, which ceded Taiwan to Japan from 1895 to 1945. Most recently, ethnic Chinese *Waishengren* immigrants settled in Taiwan starting in 1949.[4] Taiwan's diverse population history suggests that known and novel *PRKN* mutations may be prevalent in patients with early-onset parkinsonism. Herein, we report the first comprehensive evaluation of the clinical features and frequency of *PRKN* mutations in Taiwanese patients with early-onset parkinsonism living on the island.

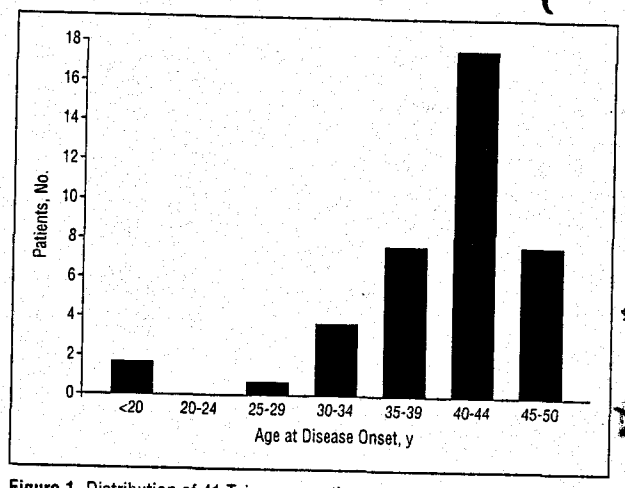

Figure 1. Distribution of 41 Taiwanese patients with early-onset parkinsonism by age at disease onset.

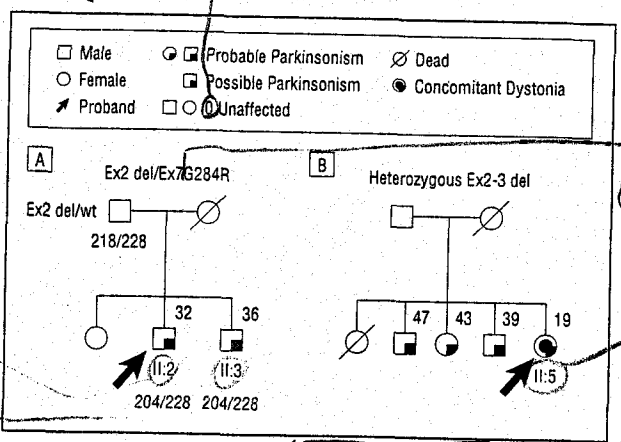

Figure 2. Pedigrees of probands II:2 (family A) (A) and II:5 (family B) (B). Age at symptom onset is shown above each affected individual. In family A, allele sizes are shown for D6S305; the exon 2 deletion (Ex2 del) segregates with the 228–base pair allele, whereas the G284R missense mutation can be inferred to segregate with the 204–base pair allele.

METHODS

Taiwanese (ethnic Chinese) patients with early-onset parkinsonism (symptomatic onset at age <50 years) were recruited from the Movement Disorder Clinics of the National Taiwan University Hospital (**Figure 1**). Informed consent was obtained according to an ethically approved protocol before the study. A neurologist who specializes in movement disorders (R.-M.W.) evaluated all patients in Taiwan, where they currently reside. All patients met the criteria for possible or probable Parkinson disease (PD), including the presence of at least 2 (possible PD) or 3 (probable PD) of the 4 cardinal features (resting tremor, bradykinesia, rigidity, and postural instability with asymmetrical onset), with a substantial and sustained response to treatment with levodopa or a dopamine agonist.[5]

Clinical evaluations included a history of the present illness, a family history, a medical history, and a review of systems, with an emphasis on movement disorder, psychiatric illness, and cognitive function. Patient evaluations included the Unified Parkinson's Disease Rating Scale, the Hoehn and Yahr stage, the Folstein Mini-Mental State Examination, and a standard neurologic examination.[6] Patients with atypical features or evidence of secondary parkinsonism caused by other neurologic diseases or known drugs or toxins were excluded. Assessment of possible secondary parkinsonism or atypical PD included neuroimaging (head computed tomography or magnetic resonance imaging) and additional laboratory tests (including tests for Wilson disease and for trinucleotide repeat expansions in the ataxin-2 and ataxin-3 genes).

For each proband identified as having a *PRKN* mutation, the living relatives were contacted according to an approved protocol, and informed consent was obtained for further clinical and genetic studies. Follow-up was performed for the nuclear families of probands II:2 and II:5 (**Figure 2**); 2 additional family A members were included. Neither family C nor family D had a family history of PD or psychiatric disorder. Forty-seven Taiwanese patients with typical late-onset PD and 50 control subjects without signs of neurologic disease were also recruited to assess the frequency of any *PRKN* point mutation identified.

For genetic analysis, a 10-mL venous blood sample was collected for Epstein-Barr virus transformation, providing a source of lymphoblastoid cells from which messenger RNA and DNA were isolated. Spectrophotometry and electrophoresis were used to assess the quality of DNA extracted. A genetic marker, D6S305, in *PRKN* intron 6 underwent genotyping for family members as previously described (Figure 2).[7] In addition, quantitative polymerase chain reaction and sequencing of genomic DNA were carried out to assay for *PRKN* exonic deletion/duplication. For patients in whom *PRKN* mutations were identified, total RNA was extracted from Epstein-Barr virus–transformed lymphoblastoid cell lines using TRIzol reagent (Invitrogen Corp, Carlsbad, Calif), and complementary DNA (cDNA) was prepared by reverse transcription (Gibco-BRL; Invitrogen Corp). The cDNA was sequenced using published primers and established methods (**Figure 3**).[7] Base variants are labeled from the ATG start of protein translation (GenBank Accession No. AB009973).

RESULTS

SUMMARY OF CLINICAL FINDINGS

Included in this study were 41 probands of Taiwanese (ethnic Chinese) descent, with a mean±SD age at onset of 40.0±6.4 years (range, 19-48 years) and a mean±SD disease duration of 8.0.±5.3 years (range, 1-19 years). In the overall sample, 7 (17%) of 41 patients had a family history of PD. Of the 7 probands with familial parkinsonism, 3 had affected siblings with disease onset before age 40 years, consistent with autosomal recessive inheritance. In 1 patient, disease onset was consistent with autosomal dominant inheritance, with a parent affected by parkinsonism. The remaining 3 index patients had 3 relatives diagnosed as having PD. Of the 41 patients with parkinsonism, consanguinity was noted for only 1 patient, who was initially seen for hemiparkinsonism (rigidity and bradykinesia without tremor) at age 30 years, but without a family history of PD.

In the affected cohort, there were 25 men and 16 women (male-female ratio, 1.6:1). Regarding clinical manifestations, most patients (38 [93%] of 41) had an asymmetrical onset of symptoms. Dystonic features, sleep benefit, and diurnal variation in symptoms were noted in 44% (18/41), 28% (11/39), and 16% (6/38) of patients with early onset, respectively. Resting tremor was less common than rigidity and bradykinesia either initially (41% [17/41] vs 73% [30/41]) or as a cardinal symptom during the disease course (49% [20/41] vs 88% [36/41]). Psychiatric symptoms of depression and anxiety were seen in 24% (10/41) and 27% (11/41) of the index pa-

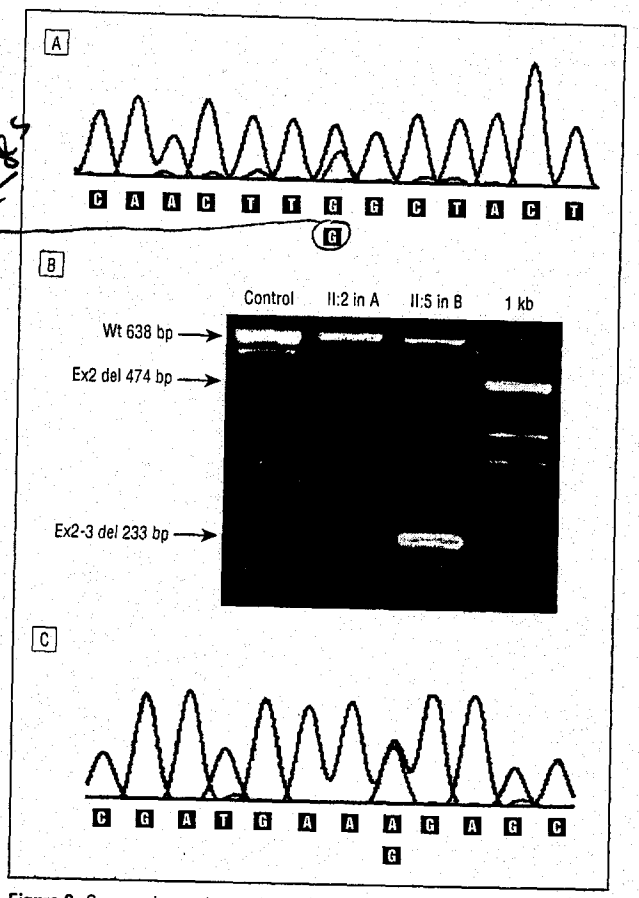

Figure 3. Sequencing and complementary DNA (cDNA) analysis. A, Nucleotide sequence for the exon 7 (Ex7) 951 G→C transversion (G284R) for the proband in family A. Primers and methods available on request. B, An agarose gel of polymerase chain reaction (PCR)–amplified cDNA products for a control and the family A and B probands. Messenger RNA (mRNA) and cDNA were obtained from lymphoblastoid cell lines; PCR used Ex1 forward primer 5′ACCTACCCAGTGACCATGATAG (anneals 5′ adjacent to Ex2) and Ex6 reverse primer 5′AATTCTGCACTAGTCCCAG (anneals ~14-bp 5′ of Ex6 spanning the Ex5-6 splice junction) to amplify a 638-bp fragment. Faint amplification is noted for a 474-bp Ex2 deleted product in family A proband II:2. In contrast, a 233-bp band, corresponding to an Ex2-3 deleted mRNA, robustly amplifies from family B proband II:5. The identity of both products has been confirmed by sequencing. C, Nucleotide sequence for the Ex11 1186 A→G transition (R396G) for family C. The cDNA was amplified and sequenced from probands C and D. Assay details available on request. The nucleotide sequence for the Ex11 1186 A→G transition (R396G) is shown; the chromatogram A→G indicates that mutant and wild-type genes are expressed. Assay methods available on request.

tients, respectively. The mean±SD Unified Parkinson's Disease Rating Scale motor score was 10.5±5.5 in the on state and 20.8±8.2 in the off state, and the mean Hoehn and Yahr stage was 1.8 in the on state and 2.9 in the off state. Dyskinesia and wearing-off phenomenon, related to levodopa therapy and possibly disease duration, were observed in 59% (24/41) and 56% (23/41) of patients, respectively. Similar clinical observations have been noted for early-onset parkinsonism in Western populations.[8,9]

A summary of the clinical features of the 4 probands with early-onset parkinsonism, identified as having *PRKN* mutations, and their affected siblings is given in the **Table**. Disease transmission was consistent with autosomal recessive inheritance in 2 probands (Figure 2). The clinical features of proband II:2 and his sibling II:3 in family A were that of idiopathic PD, but with a slow progressive course. Brain imaging for dopamine transporters (^{99m}TcTRODAT-1 [a specific ligand] single-photon emission computed tomography) in family A proband II:2 indicated severe degeneration of the nigrostriatal pathway, most pronounced in the putamen and consistent with changes observed in sporadic PD. Family B proband II:5 had a history of major depression and was initially considered to have "dopa-responsive dystonia" owing to diurnal fluctuation in foot dystonia and remarkable improvement in gait when taking low doses of levodopa (50 mg twice daily). The clinical presentations of probands C II:7 and D II:2 resembled sporadic PD, but with earlier onset. The mean±SD age at onset in the 4 patients with 1 or more *PRKN* mutations was 33.7±11.0 years (range, 19-44 years), which was younger than that of the 37 patients without *PRKN* mutations (mean±SD, 40.6±5.5 years; range, 29-48 years; $P \leq .04$). The mean±SD duration of parkinsonism was 11.5±5.8 years (range, 5-20 years; n=4), which was longer than that in patients without *PRKN* mutations (mean±SD, 7.6±5.1 years; range, 1-19 years; n=37; $P \leq .007$), suggesting slower disease progression. However, despite longer disease duration, the mean±SD dose of levodopa required by these individuals for benefit was typically less than that prescribed to patients with early onset without *PRKN* mutations (287±103 mg/d vs 532±352 mg/d; $P \leq .001$).

SUMMARY OF GENETIC FINDINGS

The *PRKN* gene was comprehensively evaluated for 41 patients with early-onset parkinsonism. All 12 exons of the gene were assessed for genomic copy number, and findings were verified using appropriate controls who tested positive for specific deletions or duplications of the parkin gene. Complete deletion of exon 2 (Ex2 del) was identified in proband II:2 in family A. Subsequent sequencing revealed an Ex7 951 G→C transversion, leading to a glycine to arginine amino acid substitution at position 284 (Ex7 G284R). Before evaluation of the *PRKN* gene, first-degree relatives of proband II:2 (family A) noted to have a family history of parkinsonism were clinically examined. These family members subsequently underwent genotyping for chromosome band 6q25-27 inheritance using D6S305, a microsatellite marker in intron 6, and were assessed for the presence of *PRKN* Ex2 del/Ex7 G284R mutations. The Ex2 del mutation was contributed by the father, and the Ex7 G284R substitution segregated with the maternal allele along with the 204–base pair allele of D6S305, as seen in the proband and his affected sibling (Figure 2A).

Proband II:5 in family B had complete deletion of Ex2 and Ex3 (Ex2-3 del). Analysis of messenger RNA and cDNA revealed that both exonic deletions were in *cis*, affecting only 1 allele (Figure 3B). Sequencing of cDNA and genomic DNA, and quantitative assessment of copy number, showed that the wild-type allele was normally expressed. Two other patients, in families C and D, had heterozygous Ex11 1186 A→G transitions, leading to arginine to glycine amino acid substitutions at position 396 (Ex11 R396G). The A→G transition is shown in the cDNA, indicating that mutant and wild-type genes are expressed (Figure 3C).

The Ex7 951 G→C (G284R) and Ex11 1186 A→G (R396G) missense mutations were not present in 50 Tai-

Table. Clinical Characteristics of the Individuals With *PRKN* Mutations

Clinical Feature	Patient*				
	Family A II:2	Family A II:3	Family B II:5	Family C	Family D
Sex	M	M	F	M	M
Age at onset/examination/ levodopa treatment, y	32/44/40	36/41/NA	19/39/33	40/50/49	44/49/48
Disease duration, y	12	5	20	10	5
Family history of PD	+	+	+	–	–
Cardinal symptoms of early-onset parkinsonism					
Tremor, rest/posture	–/–	+/–	–/–	+/–	–
Rigidity	+	+	+	+	+
Bradykinesia	+	+	+	+	+
Postural instability	–	–	+	–	+
Dystonia	–	–	+	–	–
Hoehn and Yahr stage (on/off)	2/3	1	2/3	1/3	2/3.5
UPDRS III (on/off)	9/16	NP/4	11/NP	13/21	13/26
Initial symptoms					
Asymmetrical onset	+ (Leg)	+ (Leg)	– (Legs)	+ (Leg)	+ (Leg)
Resting tremor	–	+	–	–	–
Rigidity	+	+	+	+	+
Bradykinesia	+	+	+	+	+
Foot dystonia	–	–	+	–	–
Hyperreflexia/Babinski sign	+/–	+/–	+/–	–	–
Psychiatric symptoms	–	+	+	–	
Depression/anxiety	–/–	–/+	+/+ (Committed suicide)	–/–	–/–
Sleep benefit/diurnal change	+/+	–/–	+/+	–/–	+/–
Response to levodopa therapy	Remarkable	Untreated	Remarkable	Good	Remarkable
Antiparkinsonian treatment at the time of study	Levodopa, 150 mg/d; benserazide; amantadine hydrochloride; selegiline hydrochloride; biperiden	None	Levodopa, 300 mg/d; carbidopa	Levodopa, 300 mg/d; benserazide; ropinirole hydrochloride, 3 mg/d; amantadine hydrochloride	Levodopa, 400 mg/d; ropinirole hydrochloride, 2 mg/d
Levodopa therapy complications	–/–	–	+/+	–/–	–/+
Dyskinesia/motor fluctuation			Levodopa, 100 mg, induced chorea in the legs at initial treatment		

Abbreviations: NA, not applicable; NP, not performed; PD, Parkinson disease; UPDRS, Unified Parkinson's Disease Rating Scale; +, yes; –, no.
*Probands II:2 and II:3 in family A had mutations identified in both *parkin* alleles; family B proband II:5, family C, and family D had 1 allele mutated.

wanese controls. However, 1 of the 47 subjects with idiopathic PD with late-onset disease was identified as a carrier of the Ex7 G284R mutation. This individual also had a neighboring intron 6 D6S305 204–base pair allele, indicative of linkage disequilibrium and suggesting that the variant may originate from a common founder (Figure 2). There are no published microsatellite markers adjacent to Ex11. Common, polymorphic amino acid substitutions were also identified in 4 patients with early onset, including heterozygous and homozygous Ex4 S167N substitutions in probands II:2 in family A and II:5 in family B, respectively.

COMMENT

To our knowledge, this is the first comprehensive evaluation of the *PRKN* gene in a Taiwanese (ethnic Chinese) series with early-onset parkinsonism; moreover, this is the first detailed description of the phenotype associated with "parkin-proven" disease in the Taiwanese population. Three complementary methods, including quantitative polymerase chain reaction for *PRKN* genomic deletion/duplication and sequencing in genomic DNA and cDNA, ensured that all variants were identified. Screening for *PRKN* mutations has previously been reported in clinic-based populations of Chinese patients with early-onset parkinsonism (aged <50 years at onset). One study[10] identified a *PRKN* mutation rate of 14% in 35 patients (aged <50 years at onset), and another study[11] found no homozygous deletions or point mutations in 25 samples (aged <49 years at onset). However, the methods used would have overlooked heterozygous *PRKN* deletions or duplications in these Chinese patients.

The overall frequency of *PRKN* mutations in this Taiwanese cohort of patients with early-onset parkinsonism was 6% (5 of 82 alleles): 2% (1/41) were compound het-

erozygotes and 7% (3/41) were carriers. Adjusting for age at onset (Figure 1), our data are in agreement with those of past studies of sporadic early-onset PD in European and North American cohorts (reviewed by Mata et al[1]).

The clinical phenotype of patients with *PRKN* gene mutations was variable, even for siblings with identical mutations and a shared environment. For example, disease in family A proband II:2 expressed asymmetrical rigidity and bradykinesia but without tremor. In contrast, his affected sibling showed a prolonged course of unilateral resting tremor of the great and second toes of the left lower extremity. The clinical features of family B proband II:5 closely resemble dopa-responsive dystonia, with early-onset symmetrical foot dystonia, shuffling gait, and postural instability. In addition, her symptoms included obvious diurnal fluctuations, with marked benefit from sleep. Psychiatric features (major depression) preceded the onset of movement disorder and remained prominent; the patient committed suicide after a disease duration of 20 years. Similar behavioral and psychiatric manifestations have been highlighted in other families with *PRKN* mutations[7,12] and in idiopathic PD[13] and warrant careful evaluation and treatment.

It is valuable to thoroughly document the symptoms of parkin-proven disease, especially in patients with missense mutations because they may have different phenotypic consequences to truncating mutations, depending on the domain of the protein affected.[1] Family A proband II:2 is a compound heterozygote with *PRKN* Ex2 del/Ex7 G284R. De novo Ex2 deletions have been widely reported, whereas the Ex7 G284R substitution has been reported only once in an ethnic Chinese patient, consistent with a founder effect.[1,10] The Ex7 G284R substitution is the fourth missense mutation (in addition to R256C, R275W, and R275Y) to be described in the RING1 domain (from amino acids 238 to 293 of the parkin protein) and provides an additional tool with which to assess the function of RING1. Previous postmortem evaluation of compound heterozygous patients with missense mutations in or adjacent to the RING1 domain have demonstrated alternate pathologic conditions, including Lewy bodies[14] and tauopathy, with or without neurofibrillary tangles.[15,16] These studies extend original observations[17,18] of predominant nigral neuronal loss, made in patients with homozygous truncating mutations. Consistent with a dominant negative effect, RING1 mutations R275W and R256C have been shown to alter the localization of parkin protein in transfected cells,[19] and clinically, Ex 7 R275W is associated with a more severe phenotype.[20]

In family B proband II:5, the *PRKN* Ex2-3 del was in *cis*, affecting only 1 allele of the gene. The other allele was expressed normally, and no additional mutations were identified. The Ex2-3 deleted messenger RNA is abundantly expressed in lymphoblastoid tissue, may be translated in-frame to produce a 330–amino acid parkin isoform, and would lack the N-terminal ubiquitin-like domain that mediates parkin binding to the Rpn10 subunit of the 26S proteasome.[21] Consequently, a 330–amino acid parkin isoform may conceivably be more deleterious than a simple loss-of-function mutation. Family B proband II:5 was also an Ex4 N167 homozygote, a genotype that may contribute to her onset of parkinsonism at age 19 years. Parkin Ex4 S167N substitutions have previously been associated with risk of idiopathic PD.[22] However, the evidence remains equivocal even in Asian populations.[23,24] The Ex11 R396G substitutions in families C and D are novel and may be specific to the Taiwanese population; despite comprehensive analysis of the gene, no other mutation was identified in these patients with early-onset disease. Again, expression of the wild-type allele seems to be normal in these sporadic cases. At this time, it is unclear whether the Ex11 R396G substitution is pathogenic or a rare but harmless variant. Given the lack of family history, we postulate the latter; functional analysis may help clarify the pathogenicity of *PRKN* point mutations.[19]

Findings from twin studies suggest that susceptibility to early-onset parkinsonism (aged <50 years at onset) is consistent with a genetic etiology,[25] and the incidence of PD in the Taiwanese population closely approximates that reported in Western nations.[26] In Europe, in hospital referral series, *PRKN* mutations account for 18% of sporadic PD and 49% of familial disease (aged <45 years at onset).[1,27] In the present study, we show that the frequency of *PRKN* mutations is similar across Asian and white populations. We are confident that no exonic point mutations, splice mutations, or deletions/duplications have been overlooked because genomic DNA, messenger RNA, and cDNA were examined for each case. Our screening methods were sufficiently sensitive to identify known and novel parkin mutations Ex7 G284R and Ex11 R396G, both of which may be specific to the Taiwanese population. However, referral bias and the small sample size remain limitations of this and many previous studies.

Given the wide range of clinical symptoms in parkin-proven disease, a genetic diagnosis of *PRKN* mutations should be considered as part of an evaluation for early-onset or familial parkinsonism. Because mutations in the *PRKN* gene explain only a small proportion of disease in this cohort, additional risk factors must now be identified.

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