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E. K. Tan · H. Shen · J. M. M. Tan · K. L. Lim · S. Fook-Chong ·
W. P. Hu · M. C. Paterson · V. R. Chandran · K. Yew · C. Tan ·
Y. Yuen · R. Pavanni · M. C. Wong · K. Puvan · Y. Zhao

Differential expression of splice variant and wild-type *parkin* in sporadic Parkinson's disease

Abstract *Background:* Altered splicing of *parkin* under cellular stress could lead to changes in gene expression and altered protein activity. The causative role of *parkin* in sporadic Parkinson's disease (PD) is unknown. *Objectives:* We described a *parkin* splice variant (SV) in the substantia nigra and leukocytes of sporadic PD patients.

E.K. Tan and H. Shen have equally contributed to this work.

E. K. Tan (✉) · H. Shen · K. Yew · C. Tan · R. Pavanni ·
M. C. Wong · K. Puvan
Department of Neurology, Singapore General Hospital,
Outram Road,
Singapore, 169608, Singapore
e-mail: gnrtek@sgh.com.sg
Fax: +65-6220-3321

E. K. Tan · J. M. M. Tan · K. L. Lim · R. Pavanni ·
M. C. Wong · K. Puvan
National Neuroscience Institute,
Singapore, Singapore

E. K. Tan · H. Shen
SingHealth Research,
Singapore, Singapore

J. M. M. Tan · K. L. Lim
Neurodegeneration Research Laboratory,
Singapore, Singapore

S. Fook-Chong · V. R. Chandran · Y. Zhao
Department of Clinical Research, Singapore General Hospital,
Singapore, Singapore

W. P. Hu
Department of Obstetrics and Gynaecology,
Singapore General Hospital,
Singapore, Singapore

M. C. Paterson
Division of Cellular and Molecular Research,
National Cancer Center,
Singapore, Singapore

Y. Yuen
Department of Health Screening, Singapore General Hospital,
Singapore, Singapore

Using a case control methodology, we investigated the exon 4 SV (E4SV) and wild-type *parkin* expression in the leukocytes of sporadic PD patients and healthy individuals. *Methods/Results:* We identified a *parkin* E4SV in the substantia nigra and leukocytes of sporadic PD patients and controls by reverse transcriptase–polymerase chain reaction (PCR). The exon 4 (122 bp) deletion resulted in a reading frame shift over the junction of exons 3–5 and a stop codon (tga) 17 bp downstream from exon 3. The translated truncated protein was associated with a total loss of the two-RING finger functional domain. Utilizing TaqMan real-time PCR with probes located across the junction of exons 3–4 or 3–5, we demonstrated an over-expression of E4SV/wild-type *parkin* ratio in the leukocytes of sporadic PD patients compared to age-, gender-, and race-matched controls ($p<0.0005$). A multivariate regression analysis demonstrated that the ratio of E4SV/wild-type *parkin* expression increased with age in PD patients, but this was not observed in the controls ($p< 0.0005$). *Conclusion:* The relative expression of E4SV/wild type *parkin* was increased in sporadic PD compared to healthy controls. Based on our observations, further functional studies to determine the pathophysiologic role of E4SV in sporadic PD patients will be of importance.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized by the loss of dopaminergic cells in the substantia nigra pars compacta and the presence of Lewy bodies. *Alpha-synuclein*, *parkin*, *UCH-L1 (Ubiquitin-C terminal hydrolase-L1)*, *DJ-1*, *PINK1*, and *LRRK2 (leucine-rich repeat kinase 2* mutations have been found in familial forms and/or early onset PD [1–8]. Polymorphic variants of genes involved in xenobiotism or gene-environmental interaction may also modulate the risk of PD [9–11].

The *parkin* gene has 12 exons, encoding 465 amino acids, spanning 1.3 Mb of genomic DNA. *Parkin* functions as a E3-ubiquitin ligase, with a UBL domain N′

terminus and a two-RING finger domains [7, 8]. Mutations in the *parkin* gene is the most common cause of familial and early onset PD, accounting for 50% of the familial cases and more than 70% in those sporadic cases with age of onset <20 years [12]. It has been thought that *parkin*-linked PD is recessively inherited, where a deleterious alteration is presumed on both alleles and heterozygous carriers are unaffected. However, some studies suggest that more than half of the total number of cases identified with a *parkin* mutation had only a single heterozygous mutation [13, 14]. Patients having a loss of one *parkin* allele may suffer from haploinsufficiency as a consequence of a reduced expression and enzymatic activity, resulting in a greater risk for disease [15, 16]. Despite the heterogeneity of *parkin* mutations (missense, truncating, duplication, exonic rearrangement, etc.), there is a lack of genotype–phenotype correlation, suggesting presence of other genetic or epigenetic modulation. The role of *parkin* in sporadic PD has not been clarified, although variability of the promoter region and, consequently, altered gene expression has been implicated [16].

Alternative RNA splicing may be used as a mechanism of gene regulation [17]. The primary transcript can be processed in different ways by alternative usage of exons. Altered regulation and splicing of genes either under oxidative stress or due to mutations in the splicing sites could lead to significant changes in gene expression and altered protein activity [17]. There is currently little information on the prevalence, type, and significance of splicing of the *parkin* gene in the literature. A *parkin* exons 3-4-5 (447 bp; 149 AA) splice variant (E3-4-5SV) was found in human leukocytes [18], and a *parkin* exon 5 (84 bp; 28 AA) splice variant (E5SV) was found in brain [4]. The reading frame was maintained over the junction of exons 2–6 or exons 4–6. The translated protein would have the two-RING functional domain.

In this study, we identified a *parkin* splice variant in the substantia nigra and leukocytes of sporadic PD patients, and using a case control methodology, investigated the exon 4 SV (E4SV)/wild-type *parkin* expression in the leukocytes of sporadic PD patients and healthy individuals.

Materials and methods

Subjects

The PD patients and healthy controls were recruited from our movement disorder clinics and health screening unit as previously described [19]. All subjects were examined by the authors and gave informed consent for their blood samples for genetic research. The work received approval from the ethics committee of our institution. There were 80 PD and 80 controls with mean age of 56.0±11.0 and 53.0±8.0 years comprising 60 and 55% men, respectively. All study subjects were ethnic Chinese.

Extraction of total RNA and DNase treatment

Total RNA from human peripheral leukocytes was isolated with erythrocyte lysis buffer (Qiagen) and RNAgents total RNA isolation system (Promega). Total RNA from HEK 293 and neuroblastoma SH-SY5Y cells was isolated directly with RNAgents total RNA isolation system (Promega). Total RNA from human substantia nigra was purchased from Ambion. To remove DNA contamination from the total RNA preparation, DNase digestion was performed following the manual instructions (Invitrogen).

RT-PCR analysis

For cDNA synthesis, 500 ng of total RNA was reverse transcribed using oligo-dT primers following the manual instructions of the "First Strand cDNA Synthesis Kit" (Invitrogen).

Primers at exons 2 and 5 (forward: 5′-gaggaatgactg gactgtgc-3′; reverse: 5′-ctagtcccagggcagtgtg-3′) were used for amplification of *parkin* normal fragment and *parkin* splice variant. A 20-µl polymerase chain reaction (PCR) contained 0.2 µl cDNA, 1 µl DMSO, 2 µl 10× PCR buffer (Qiagen), 1.5 mM magnesium chloride, 0.25 µM of each primer, 0.4 µM dNTP mix, and 0.5 U HotstarTaq DNA polymerase (Qiagen). Thermal cycler parameters were 35 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min, with preheat at 95°C for 15 min and last extension at 72°C for 5 min. Electrophoretical separation of the reverse transcriptase (RT) PCR fragments was performed on ethidium bromide-stained 2% agarose gels running at 100 mA for 40 min.

Nucleotide sequence analysis of all 12 exons of *parkin* including the intron–exon boundary regions

The 12 exons of *parkin* including the intron–exon boundary region were amplified following the conditions previously described [4]. The PCR products were visualized on ethidium bromide-stained 2% agarose gel. The PCR products were subjected to direct nucleotide sequence analysis using automated DNA sequencers (Applied Biosystems).

Gene dosage analysis of *parkin* exons 3 and 4

Gene dosage analysis was performed using the ABI Prism 7700 sequence detections systems (Applied Biosystems). β-globin was used as internal control, and the primers and TaqMan probe were purchased from Applied Biosystems. Primers and TaqMan probe for exon 3 (forward primer: 5′-GGATCAGCAGAGCATTGTTCAC-3′, TaqMan probe: 5′-TTGTGCAGAGACCGTGGAGAAAAGGT-3′, reverse

primer: 5′-GCCTCCAGTTGCATTCATTTCT-3′) and primers and Taq Man probe for exon 4 (forward primer: 5′-CAA AGGCCCCTGTCAAAGA-3′, TaqManprobe: 5′-CTCAG GGTACAGTGCAGCACCTGC-3′, reverse primer: 5′-GGT CAAGGTGAGCGTTGC-3′) were synthesized according to previous published literature [20]. The cycle number at which each PCR reached a significant threshold (C_T) during the log phase of the amplification was used as a quantitative measure of the target product. The value calculated from C_T for exons 3 and 4 was normalized using those for the β-globin gene.

Quantitative PCR using real-time TaqMan–PCR methods

Quantitative PCR was performed using the real-time TaqMan–PCR method. In this method, AmpliTaqMan DNA polymerase extends the primer and displaces the TaqMan probe through its 5′- to 3′- exonuclease activity. The probes were labeled with a reporter fluorescent dye [6-carboxy-fluorescein dye (FAM)] at the 5′ end and a quencher fluorescent dye (TAMARA) at the 3′ end. The expression level of β-actin gene was used as an endogenous control, and a TaqMan probe for β-actin was procured from Applied Biosystems.

Primers and TaqMan probes for real-time PCR

The primers and TaqMan probes were designed according to published sequence of the *parkin* mRNA(AB009973). Specific primers were used to amplify the wild-type (forward: 5′-cactgacagcaggaaggactcac-3′ and reverse: 5′-cttt gcaatacacataaaagctgttgta-3′) and splice variant (forward: 5′-cactgacagcaggaaggactcac-3′ and reverse: 5′-ccgtttggaat taaaacatcatc-3′) alleles. TaqMan probes were designed at the junction of exons 3–4 (wild-type *parkin*: 5′-tctacctgctg gacttc-3′) or exons 3–5 (E4SV: 5′-atggaccctggacttc-3′). The reaction mixture contained 25×TaqMan mixture (Applied Biosystems), the appropriate primer set, the TaqMan probe, and the cDNA template in a total volume of 50 μl. The thermal cycling protocol consisted of a minute at 95°C and 10 min at 95°C, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. TaqMan–PCR was analyzed using a Model 7700 Sequence Detector (Applied Biosystem). The quantity of the target mRNA was calculated using the Sequence Detector software package version 1.6.3 (Applied Biosystem). The starting quantity of each target mRNA was normalized to that of the β-actin endogenous control mRNA, allowing for measurement of the relative expression level of each RNA species with the comparative C_T method (ABI user's manual). For control TaqMan studies, we prepared constructs for both wild-type and splice variant alleles in the plasmid vector DNA3.1 (Qiagen). The specificity of the primers and TaqMan probes for the wild-type and splice variant alleles was confirmed using three templates: the wild-type allele only, a mixture of wild-type and splice variant alleles at a 1:1 ratio, and the splice variant allele only.

Results

Identification of a *parkin* splice variant from the human peripheral leukocytes and brain by RT-PCR

During the screening of *parkin* gene mutations in our patients, we identified a *parkin* SV, E4SV. RT-PCR amplified a major 470 bp and a minor fragment around 340 bp from the leukocytes and substantia nigra of PD patients with primers in exons 2 and 5 (Fig. 1). DNA sequence analysis confirmed that the former (470 bp) corresponded to exons 3–5 and the latter (340 bp) contained the exon 4-spliced variant transcript. A 122-bp deletion corresponding to exon 4 (nucleotide, 514–635 bp; residues, 139–178 AA) resulted in exon 3-derived sequence being directly connected to exon 5-derived sequence (Fig. 2). The reading frame was shifted over the junction and a stop codon (tga) appeared just 17 bp downstream from exon 3. A similar splicing was also found from leukocytes in the controls without neurological disease, suggesting that this is a common splice variant.

Quantitative PCR using real-time TaqMan–PCR method

The expression of wild-type *parkin* and *parkin* SV was discriminated using the protocol described in Materials and methods.
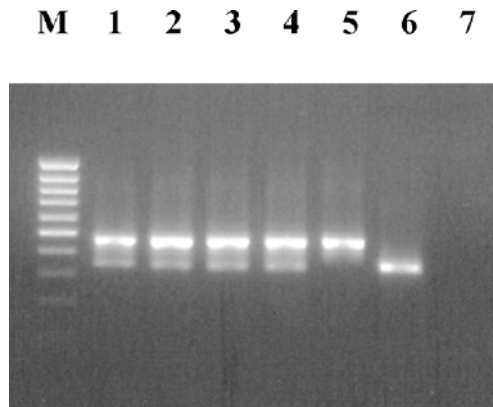

Fig. 1 Abnormal splicing of *parkin*: cDNA derived from the human leukocytes (normal control, lane 1; PD, lane 3) and substantia nigra (normal control, lane 2; PD, lane 4) was amplified with primers in exons 2 and 5. Six microliters of product underwent electrophoresis on a 1.5% agarose gel. *Lane M* is a 100-bp DNA ladder (MBI Fermantas). A duplex banding pattern is observed in *lanes 1*, *2*, *3*, and *4*. The normal size is about 470 bp, the size of *parkin* spliced variant is about 340 bp. The PCR products from cDNA clones of wild-type *parkin* (*lane 5*) and E4SV (*lane 6*) were used as positive control. *Lane 7* is a negative control without cDNA template
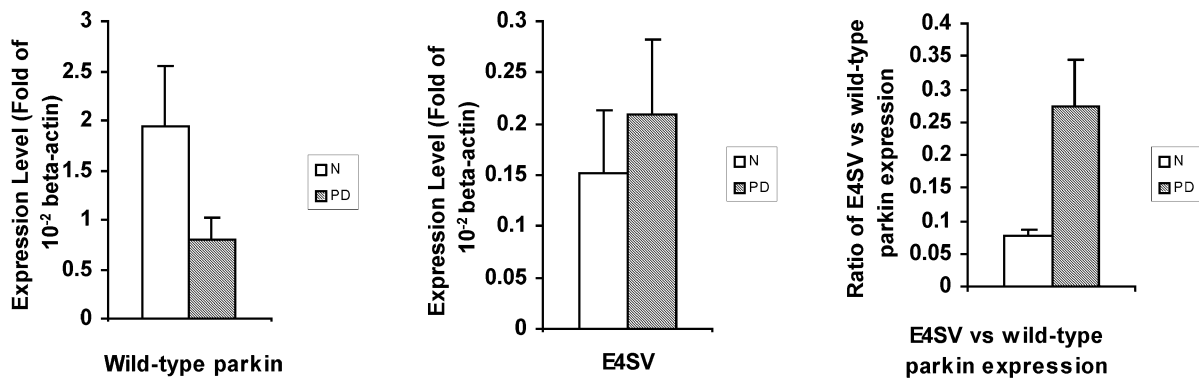
A
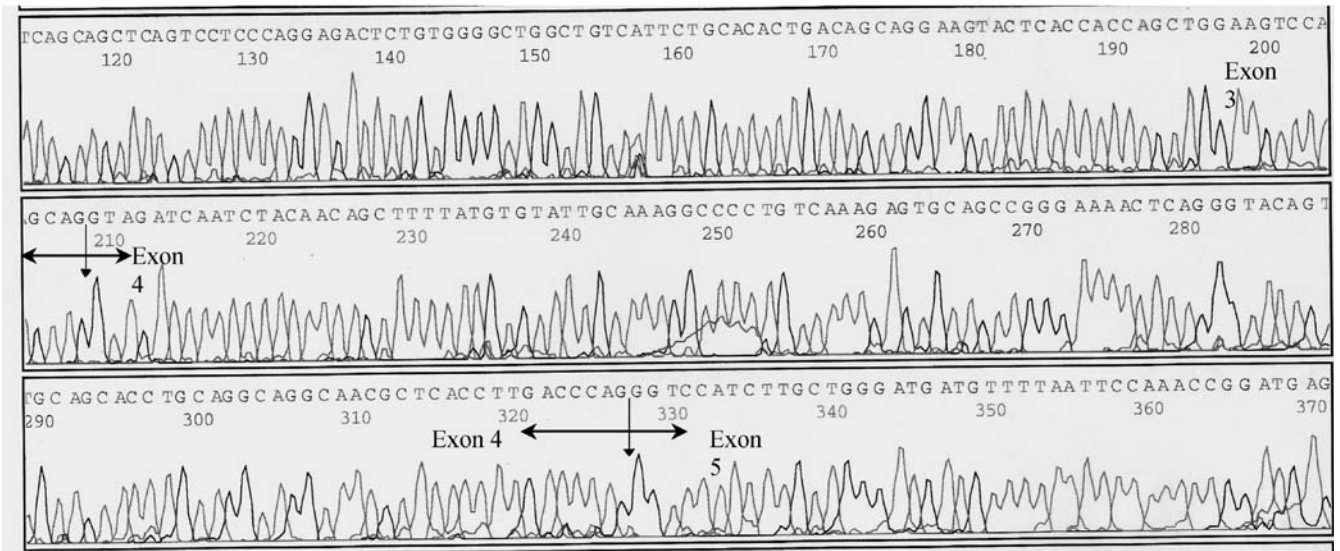
B
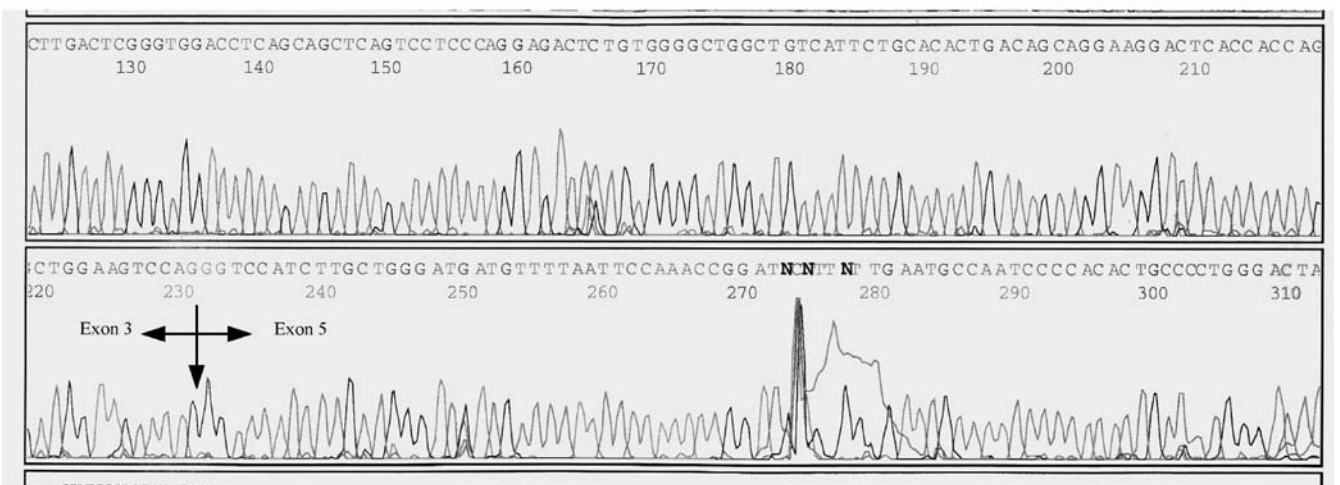
Fig. 2 Sequence analysis of the RT-PCR products. The 470-bp fragment corresponds to exons 3–5 (**a**). The 340-bp fragment corresponds to exons 3–5 with exon-4 (122 bp) deletion (**b**)


A   B   C

Fig. 3 Real-time PCR assays of *parkin* mRNA levels in the human leukocytes from PD patients and normal controls. Expression of wild-type *parkin* (**a**) and E4SV (**b**), $p < 0.05$; ANOVA. Ratio of E4SV/wild-type *parkin* expression higher in PD, $p < 0.0005$; ANOVA (**c**). Raw data were normalized to beta-actin. *Bars* correspond to mean±SEM

Expression of wild-type *parkin* and *parkin* SV
in leukocytes from the controls and PD patients

Expression analysis of the relative levels of *parkin* SV and
wild-type *parkin* was performed using TaqMan real-time–
PCR method. Total RNA samples were extracted from
leukocytes of 80 PD patients and 80 controls. All 80 PD
patients were previously screened negative for *parkin* gene
mutations based on sequence analysis (including exon 4
and its exon–intron boundaries). Although E4SV is a com-
mon splice variant, E4SV expression may be influenced by
a small likelihood of gene dosage multiplication in exons 3
and 4. However, no abnormalities on gene dosage studies
of exons 3 and 4 were found in our study subjects.

The results demonstrated that expression level of wild-
type *parkin* was lower in PD patients ($p<0.05$), whereas
the expression level of E4SV increased modestly (Fig. 3a,b).
These changes in the expression levels of wild-type *parkin*
and E4SV in PD patients led to a significantly increased
ratio of E4SV to wild-type *parkin* expression ($p<0.0005$)
(Fig. 3c). To examine for confounding or predictive factors
of the observed difference, a logistic regression analysis of
the following variables: age, gender, disease group (PD or
control), interaction of age and disease group, and inter-
action of gender with disease group, was carried out with
ratio of *parkin* SV to wild-type *parkin* expression as the
outcome measure. None of the variables confounded our
findings except the interaction of age with the disease group.
This meant that for any given age, there was a significant
difference in the ratio of E4SV/wild-type *parkin* between
PD and controls ($p<0.0005$). The ratio of E4SV/wild-type
parkin increased with age in PD patients, but this was not
observed in the controls (Fig. 4).
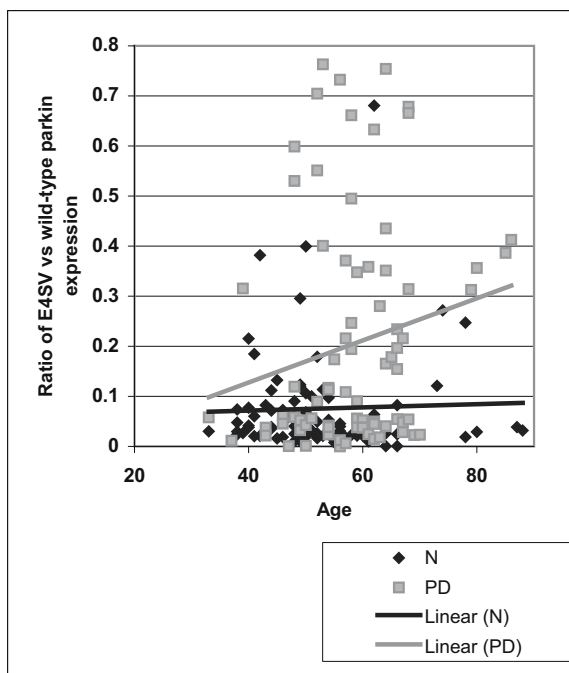
Fig. 4 The ratio of E4SV vs. wild-type *parkin* expression increased
with age in PD patients ($p<0.0005$)

Validation of mRNA analysis

Following our previous protocol [21], the gene expression
analysis was repeated for a random sample of 36 PD and 36
controls over a 2-month interval. The median fold expres-
sion of wild-type *parkin* for PD (original vs duplicate) was
0.2438 vs 0.2121 ($p>0.05$), and for controls 0.3527 vs
0.3184 ($p>0.05$). The median fold expression of E4SV for
PD (original vs duplicate) was 0.0237 vs 0.0205 ($p>0.05$),
and for controls 0.0183 vs 0.0178 ($p>0.05$). The median
fold expression of E4SV vs wild-type *parkin* for PD
(original vs duplicate) was 0.0612 vs 0.0598 ($p>0.05$), and
for controls 0.0383 vs 0.0326 ($p>0.05$).

Discussion

There is little published information on the prevalence,
type, and role of *parkin* splicing in PD. One *parkin* splice
variant lacking 84 bases was found during the cloning of
human *parkin* [4] This variant corresponds to exon 5
encoding amino acid residues 179–206 that reside within
the unique segment. It is postulated that this splicing could
alter *parkin* function as deletion of exon 5 in the *parkin*
gene has been found in autosomal recessive PD [22]. A
parkin SV involving splicing of exons 3–5 was found in
leukocytes, but not in brain tissue [18]. Other workers have
also isolated a novel *parkin* SV in mouse that lacks the
C-terminal RING box with a predicted molecular mass of
28 kD [23]. Using antibodies directed against Parkin UBL
domain, a predominantly 50 kD *parkin* in mouse tissues
and another product similar to the predicted size of the
mouse *parkin* variant described by Kitada [23] has been
isolated [24]. This 22-kD N-terminal *parkin* fragment,
containing the UBL domain, has some semblance to the
fragment released by caspase cleavage [25]. More recently,
other splice variants have also been found in developing
mouse and humans [26, 27].

In this study, we identified a *parkin* E4SV in the SNc and
leukocytes in sporadic patients and healthy controls. The
E4SV appears to be ubiquitous inasmuch as it could be
found in both brain and leukocytes. There was an increase
of E4SV and a significant reduction in wild-type *parkin*
expression, with a net result of increased E4SV/wild-type
expression ratio in PD patients compared to healthy con-
trols. The E4SV we have identified corresponds theoreti-
cally to a genomic heterozygous exon 4 deletion, leading to
a truncated protein devoid of the RING-functional do-
mains, hence with total loss of enzymatic activity. Homo-
zygous or heterozygous exon 4 deletions have been
described in PD patients, and the heterozygous exon 4
deletion was associated with the disease [12, 15]. At any
given age, there was a significant difference in the ratio of
E4SV/wild-type *parkin* between PD and controls. The ratio
of E4SV/wild type increased with age in PD patients, but
this was not observed in the controls. This is interesting in
light of the increased risk of PD with age, and the ex-
pression of E4SV/wild type may predispose to PD in
certain at-risk individuals. It has been demonstrated that

there is a shift in *parkin* solubility with aging, suggesting an age-dependent modification of *parkin* or its substrates [28]. It is possible that E4SV may also be a factor in these interactions. The relative amounts of the E4SV and wild type *parkin* could modulate the steady-state dynamics of *parkin* substrates and proteasomal functioning. Alternatively, the relative expression of E4SV/wild type may act via the mechanism of haploinsufficiency in risk association. This may provide an explanation why some *parkin* mutations (e.g., a 40-bp deletion in exon 3) manifest a varied mode of inheritance (both recessive and dominant) in familial cases [29, 30].

Although we demonstrated different E4SV/WT *parkin* expression in PD compared to controls, the exact mechanism governing skipping of exon 4 has not been clarified. We sequenced the exon–intron boundaries (between 36 to 50 bp) in our PD patients but did not find any mutations, particularly in the 5′ splice site. It is possible that the altered splice site choice is enriched in PD and with advancing age, but whether this is modulated by specific splicing factors is currently unclear. The stop codon may cause nonsense decay of the mRNA transcript. However, as all the PD patients have been treated with levodopa and other dopaminergic agents, we could not conduct a subset analysis of the effect of drugs on the potential nonsense decay.

In conclusion, we identified a *parkin* E4SV in the substantia nigra and leukocytes of sporadic PD and controls. The relative expression of E4SV/wild type *parkin* was increased in sporadic PD patients compared to controls. Based on our observations, further functional studies to determine the pathophysiologic role of E4SV in sporadic PD patients are warranted.

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