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Assessing the prevalence of *PINK1* genetic variants in South African patients diagnosed with early- and late-onset Parkinson's disease

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ABSTRACT

Mutations in the *PINK1* gene are the second most common cause after *parkin* of autosomal recessive early-onset Parkinson's disease (PD). PINK1 is a protein kinase that is localized to the mitochondrion and is ubiquitously expressed in the human brain. Recent studies aimed at elucidating the function of PINK1, have found that it has neuroprotective properties against mitochondrial dysfunction and proteasomally-induced apoptosis. In the present study, we aimed to investigate the prevalence of *PINK1* genetic variants in 154 South African PD patients from all ethnic groups. Mutation screening was performed using the High-Resolution Melt technique and direct sequencing. A total of 16 sequence variants were identified: one known homozygous mutation (Y258X), two heterozygous missense variants (P305A and E476K), and 13 polymorphisms of which five were novel. No homozygous exonic deletions were detected. The novel P305A variant was found in a female patient of Black Xhosa ethnicity who has a positive family history of the disease and an age at onset of 30 years. This variant has the potential to modulate enzymatic activity due to its location in the kinase domain. This is the first report on mutation screening of *PINK1* in the South African population. Results from the present study showed that point mutations and homozygous exonic deletions in *PINK1* are not a common cause of PD in the South African population.

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1. Introduction

The *PINK1* (PTEN-induced putative kinase 1) gene (*PARK6*; OMIM 608309) encodes a 581 amino acid protein kinase that is localized to the mitochondrion and is ubiquitously expressed in the human brain [1,2]. It contains an N-terminal mitochondrial-targeting motif and a highly conserved serine/threonine kinase domain. Mutations in *PINK1* are the second most common cause after *parkin* of autosomal recessive early-onset Parkinson's disease (PD), a common and incurable progressive neurodegenerative disorder that is characterized by motor impairments involving resting tremors, bradykinesia, postural instability and rigidity. Atypical PD clinical features, such as psychiatric disturbances, dystonia at onset and sleep benefit, have been observed in PD patients with *PINK1* mutations [3,4].

The involvement of *PINK1* in PD was first discovered in 2004 with the identification of homozygous point mutations in families of Italian and Spanish origin [1]. Since then, missense mutations, genomic rearrangements and truncating mutations have been identified in diverse populations. It has been shown that the frequency of *PINK1* mutations ranges from 1% to 8% in patients of different ethnicities [5–7]. The discovery of the involvement of *PINK1* in PD was the first evidence that a kinase signaling pathway may be important in the pathogenesis of dopaminergic nigral cell death. Also, it provided a molecular link between the mitochondria and neurodegeneration in PD [1,8]. Mitochondrial dysfunction is thought to play an integral role in the pathogenesis of PD [9].

PINK-deficient Drosophila has been shown to display mitochondrial defects leading to degeneration of flight muscles and loss of dopaminergic neurons [10–12]. PINK1 is thought to have neuroprotective properties since overexpression in neuroblastoma cells is associated with a decrease in the susceptibility of the cells to

Abbreviations: AAO, age at onset; HRM, high-resolution melt; LRRK2, Leucine-rich repeat kinase 2; MLPA, multiplex ligation-dependent probe amplification; PCR, polymerase chain reaction; PD, Parkinson's disease; PINK1, PTEN-induced putative kinase 1.

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neurotoxin-induced cell death [1,13]. The mechanism of PINK1's suggested ability to prevent cell death is by the inhibition of release of cytochrome C from mitochondria and maintenance of the mitochondrial membrane potential [1,13,14]. PINK1 phosphorylates mitochondrial proteins in response to cellular stress and in so doing protects against mitochondrial dysfunction [1]. It has been reported that PINK1 phosphorylates TNF receptor-associated protein 1, which causes the cells to be protected against oxidative stress-induced apoptosis [15]. Parkin and PINK1 are thought to function in the same pathway in maintaining mitochondrial integrity and function, with PINK1 functioning upstream from parkin [11,16].

Currently, the majority of *PINK1* mutations are distributed throughout the serine/threonine kinase domain. Some of these mutations have been reported to cause reduction in enzymatic activity [17,18] and could affect substrate recognition as well. The mutations that are located outside the domain might affect protein stability, which could indirectly influence kinase activity. In the present study, we conducted genetic mutation screening, in order to determine the prevalence of *PINK1* genetic variants in South African patients diagnosed with early- and late-onset PD.

2. Materials and methods

2.1. Study participants

The study was approved by the Committee for Human Research at the University of Stellenbosch, South Africa (Protocol number 2002/C059). A total of 154 unrelated PD patients from all South African ethnic groups were recruited with informed consent. They were recruited from the Movement Disorders clinic at Tygerberg Hospital, Cape Town, as well as from the Parkinson's Association of South Africa. The patients were examined by a movement disorder specialist (JC) and met the UK Parkinson's Disease Society Brain Bank Research criteria for diagnosis of PD [19]. Inclusion criteria of patients in this study were early-onset and/or a positive family history of PD.

The ethnic breakdown was as follows: 35.3% Caucasian, 30.9% South African Afrikaner, 19.8% mixed ancestry (defined in Bardien et al., 2009 [20]), 11.8% Black, and 2.2% Indian. The average age at onset (AAO) of the study group was 52 years ± 12.83. Among the patients, 65 (48%) were $\leqslant$50 years old at onset of the disease (age range 17–50 years) and 71 (52%) were >50 years old at onset (age range 51–77). The percentage of males in our study group was 62%. The percentage of patients with positive family history was 36% and both autosomal recessive and autosomal dominant patterns of inheritance were present. Seventy-two mixed ancestry control samples (male = 72%) and 54 black control samples (male = 57%) were recruited from unrelated blood donors at the South African Western Province Blood Transfusion Service blood collection clinics. The controls had been 'de-identified' and had not been clinically assessed for signs of PD.

2.2. Genetic analysis

For each study participant, a blood sample was collected for genetic analysis and genomic DNA was extracted using established methods. Polymerase chain reaction (PCR) primers were designed for each of the eight exons of the *PINK1* gene using Primer3 software. Primer sequences are available from the authors on request. PCR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, USA). The 25 μl reactions contained 10 ng template DNA, 10 pmol of each primer, 75 μM dNTPs (Promega, USA), 1.5 mM $MgCl_2$, 1× NH_4 buffer (Bioline, UK), 5% DMSO (for selected primer sets), 2 μM SYTO9 fluorescent dye (Invitrogen, USA) and 0.25 U of BIOTAQ DNA polymerase (Bioline, UK). The PCR cycling parameters were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at different temperatures (ranging from 55 to 61 °C) according to each primer set for 30 s, extension at 72 °C for 45 s, and a final extension step at 72 °C for 7 min.

Thereafter, the PCR products were subjected to high-resolution melt (HRM) analysis. For this, the samples underwent a melt from 75 to 95 °C rising by 0.1 °C each step on a Rotor-Gene 6000 analyzer (Corbett Life Sciences, Australia). In HRM, double stranded DNA dissociates into single stranded DNA as the temperature increases up to 95 °C, during which the shift in fluorescence is monitored. The thermal denaturation profile that is produced is characteristic of a specific PCR product and is dependent on its sequence length, base and GC content [21,22]. The samples that showed different HRM profiles when compared to profiles produced by the wild-type sample were sequenced in order to characterize the genetic variant. Direct sequencing was performed using the BigDye Terminator Sequence Ready Reaction kit version 3.1 (Applied Biosystems) and analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). The primers used for sequencing were the same as those used to generate the PCR products for HRM analysis. For eighteen of the samples, mutation screening was performed using direct sequencing of all eight exons and not the HRM method. The frequency of selected sequence variants was determined in ethnically matched control samples.

3. Results

The genomic DNA of 154 South African PD patients were screened for mutations in *PINK1* using the HRM method and direct sequencing. No homozygous exonic deletions were detected. A total of 16 sequence variants were identified (Table 1A and B) of which one (Y258X) is a known mutation that had previously been

Table 1A
Known and putative mutations identified in *PINK1* in South African PD patients.

Exon	Variant	Zygosity	Number of patients/ ethnicity[a]	AAO (yrs)	Family history	Protein domain	Frequency in control chromosomes	Previously reported/ reference
3	Y258X (TAC-TAA)	Homozygous	1/Indian (68.10)	37	+	Kinase domain	0/130	[23,37]
4	P305A (CCT-GCT)	Heterozygous	1/Black (42.03)	30	+	Kinase domain	2/108 (1.9%)	Novel
7	E476K (GAG-AAG)	Heterozygous	1/Mixed ancestry (63.69)	48	–	Kinase domain	2/144 (1.4%)	[3,5,6,8]

[a] Patient sample number shown in brackets; AAO, age at onset; N/A, not applicable.

Table 1B
Polymorphisms identified in *PINK1* in South African PD patients.

Location	Variant	Ethnicity of patients with variant	Frequency in patients (n = 154)
5′-UTR	−35 C > T	Afrikaner	1
Exon 1	L63L	Afrikaner	6
Exon 1	Q115L	Afrikaner	1
Intron 1	IVS1-7 A > G	Afrikaner, Caucasian, mixed ancestry, Indian and Black	30
Intron 1	IVS1-65 C > G	Afrikaner	1
Intron 4	IVS4 + 56 G > T	Black	1
Intron 4	IVS4 + 72 G > C	Indian	1
Exon 5	A340T	Afrikaner, Caucasian, mixed ancestry, Indian and Black	10
Intron 6	IVS6 + 43 C > T	Afrikaner and Caucasian	4
Intron 7	IVS7-8 T > G	Afrikaner, Caucasian, mixed ancestry, Indian and Black	18
Exon 8	N521T	Afrikaner, Caucasian, mixed ancestry, Indian and Black	10
3′-UTR	*37 A > T	Afrikaner, Caucasian and mixed ancestry, Indian and Black	24
3′-UTR	*43 G > A	Mixed ancestry and Indian	2

identified using the Multiplex Ligation-dependent Probe Amplification method (MLPA) [23]. The homozygous Y258X mutation (Fig. 1A) was found in one patient of Indian ethnicity and was also present in both of her affected siblings. Although her duration of illness at the time of assessment was 30 years, her symptoms were relatively well controlled on only 500 mg of levodopa daily. She experienced occasional freezing, and had mild dyskinesias affecting the legs. There were no features of autonomic dysfunction,

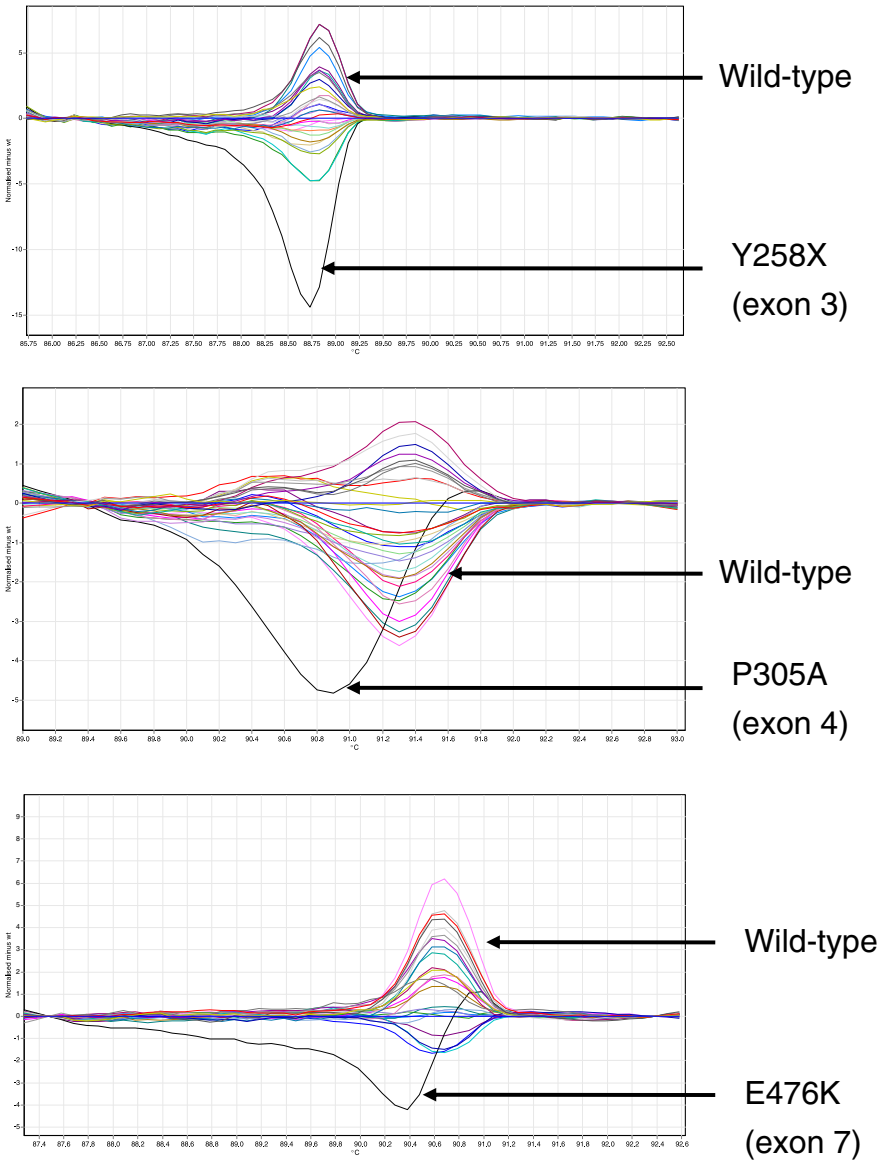

Fig. 1. Difference graphs produced by the high-resolution melt technique illustrating that sequence variants can be distinguished from wild-type alleles. Melt curves of the (A) previously reported Y258X mutation, (B) novel P305A variant, (C) previously reported E476K variant.

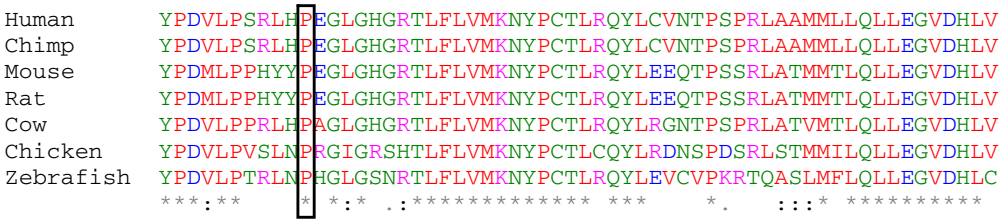

Fig. 2. Sequence alignment (using ClustalW) of *PINK1* amino acid sequences of human (NP_115785.1), chimp (ENSPTRP00000000500), mouse (NP_081156.2), rat (XP_216565.2), cow (NP_001093171.1), chicken (XP_423139.2), and zebrafish (NP_001008628.1).The box indicates the position of the novel P305A variant. Identical amino acids are indicated by asterisks.

but she did self-report impairment of memory for recent events. Detection of Y258X by HRM in the present study showed the efficacy of this mutation screening method in detecting sequence variants.

Two other sequence variants (P305A, E476K) were also identified as well as 13 polymorphisms. The novel P305A variant (Fig. 1B) was identified in a heterozygous state in a female patient of Black Xhosa ethnicity. This patient had a positive family history of the disease and an AAO of 30 years. However, the variant was not present in the proband's affected sibling who had an AAO of 45 years. P305A is located within an evolutionarily conserved region of the gene (Fig. 2). PolyPhen analysis (http://genetics.bwh.harvard.edu/pph/) revealed that this variant is 'probably damaging' although it was found in 1.9% of control chromosomes.

The E476K variant (Fig. 1C) was identified in a heterozygous state in a female patient of mixed ancestry who had an AAO of 48 years and no family history of the disease. This variant has been previously reported in a number of studies [3,5,6,8]. PolyPhen analysis revealed that E476K is 'benign' and in the present study it was found in 1.4% of control chromosomes. This variant is not located in an evolutionarily conserved region of PINK1 (data not shown).

All three of these variants occur in the functional serine/threonine kinase domain of PINK1. The P305A variant occurs within the third kinase insert domain [24], which is suggested to contain regulatory motifs important for kinase activity and substrate selectivity [25,26]. Y258X and E476K are located in the second kinase insert domain and eleventh kinase subdomain, respectively [24].

PINK1 was found to be polymorphic in the South African population although very few pathogenic mutations were found. Out of the 13 polymorphisms detected, 5 were novel variants and included 5′UTR-35 C > T, IVS4 + 56 G > T, IVS7-8 T > G, 3′UTR + 37 A > T, and 3′UTR + 43 G > A. The IVS1-7 A > G polymorphism had the highest frequency (22%) in the study group compared to the other variants.

4. Discussion

We report the results of genetic mutation screening of the *PINK1* gene which was conducted on PD patients from all South African ethnic groups. The study participants had previously been screened for mutations in the *parkin* gene [20] and the G2019S mutation in the *LRRK2* gene [27]. For parkin, only two patients with mutations were found (both homozygous whole exon deletions) and only four individuals were found to harbor the G2019S mutation. These results indicate that *parkin* and *LRRK2* are not a common cause of PD in South African patients and the *PINK1* gene was therefore investigated.

In the present study one previously identified mutation (Y258X) and two missense variants (P305A and E476K) were identified as well as 13 polymorphisms. The novel P305A variant was found in one PD patient of Black Xhosa ethnicity who had a positive family history of the disease. The clinical features of PD in Black individuals have been reported to be atypical and that it is a late-onset akinetic-rigid syndrome [28]. However, the individual harboring the P305A variant has typical clinical features of PD but she had an earlier onset and a more severe phenotype than her affected sibling. The P305A variant, due to its location in the serine/threonine kinase domain, has the potential to influence the function of PINK1 by affecting its autophosphorylation ability [29], and it is predicted to be 'probably damaging' by PolyPhen analysis. However, the fact that P305A was not present in the proband's affected sibling as well as the fact that it was found in 1.9% of ethnic matched control chromosomes suggests that it is a non-pathogenic polymorphism.

The E476K variant has previously been found in heterozygous form in four unrelated PD patients [3,5,6,30] but has also been found at very low frequencies in control subjects [5,8]. This variant has been shown to impair mitochondrial membrane potential after cellular stress induced by proteasomal inhibition [8]; however, its pathogenic status is currently equivocal. In the present study E476K was observed at a frequency of 1.4% in control chromosomes, which together with the fact that this residue is evolutionarily poorly-conserved and is also predicted by PolyPhen analysis to be 'benign', indicates that it may be a non-pathogenic polymorphism.

HRM detected 13 polymorphisms in the present study. The previously reported IVS1-7 A > G variant had the highest prevalence in the study group compared to the other polymorphisms. This variant might have an effect on splicing of the gene; however, further studies are necessary to investigate this. The presence of the IVS1-7 A > G variant has been shown to decrease the AAO in patients exposed to various environmental risk factors [31]. Since we do not have comprehensive data on environmental exposures for all our study participants, this finding could not be verified in the present study. However, preliminary analysis showed that the average AAO for patients with (44.1 ± 9.9 years; 30/154) and without (52.7 ± 12.5 years; 124/154) the IVS1-7 A > G variant was significantly different ($p < 0.001$; *t*-test).

A few recent studies have reported either no *PINK1* mutations in PD patients from Brazil [32], China [33] and Portugal [34] or only a few heterozygous variants of unknown pathogenic significance in patients from Australia [35] and India [36]. These findings are in contrast to the high frequencies observed in earlier studies. This may reflect that the contribution of this gene varies according to ethnicity or that the frequency of *PINK1* mutations is not as common as was previously thought. Therefore, innovative bioinformatic *in silico* and wet-bench experimental strategies are urgently needed to identify novel disease-causing genes for PD.

5. Conclusion

Results from the present study showed that point mutations, small insertions or deletions, and homozygous exonic deletions in *PINK1* are not a common cause of PD (<1%) in the South African population. Therefore, it is proposed that as yet unknown genes are

responsible for PD in this population. Further studies are necessary to determine whether the P305A variant could possibly alter or modulate the enzymatic activity of PINK1.

Conflict of interest

None declared.

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