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Prevalence of *GJB2* causing recessive profound non-syndromic deafness in Japanese children

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ABSTRACT

Objective: *GJB2* (gap junction protein, beta 2, 26 kDa: connexin 26) is a gap junction protein gene that has been implicated in many cases of autosomal recessive non-syndromic deafness. Point and deletion mutations in *GJB2* are the most frequent cause of non-syndromic deafness across racial groups. To clarify the relation between profound non-syndromic deafness and *GJB2* mutation in Japanese children, we performed genetic testing for *GJB2*.
Methods: We conducted mutation screening employing PCR and direct sequencing for *GJB2* in 126 children who had undergone cochlear implantation with congenital deafness.
Results: We detected 10 mutations, including two unreported mutations (p.R32S and p.P225L) in *GJB2*. We identified the highest-frequency mutation (c.235delC: 44.8%) and other nonsense or truncating mutations, as in previous studies. However, in our research, p.R143W, which is one of the missense mutations, may also show an important correlation with severe deafness.
Conclusion: Our results suggest that the frequencies of mutations in *GJB2* and *GJB6* deletions differ among cohorts. Thus, our report is an important study of *GJB2* in Japanese children with profound non-syndromic deafness.

1. Introduction

People with any degree of sensory impairment may encounter problems such as discrimination within the education system or when looking for work, and a reduced life expectancy. Sensorineural hearing loss (SNHL) is the most common sensory impairment in developed societies [1,2], where one child in 1000 presents at birth with severe or profound deafness [3].

Recent advances in human genetics have indicated that more than half of congenital SNHL cases involve a genetic factor [4]. In 75–80% of genetic cases, SNHL is the result of autosomal recessive inheritance, and both parents have normal hearing [5]. Mutations of *GJB2* are the most frequent cause of autosomal recessive non-syndromic deafness. Indeed, previous studies have shown that *GJB2* mutations account for up to 50% of non-syndromic deafness cases [6]. Hearing-impaired subjects with biallelic *GJB2* mutations range widely but most commonly follow a severe to profound and non-progressive pattern [7–9]. About 100 different *GJB2* mutations have been reported globally [the Connexin-Deafness homepage: http://davinci.crg.es/deafness/], and these mutations show a relatively high local dependence (founder effect). A high prevalence of c.35delG has been found among Caucasians; c.235delC among Eastern Asians, including Japanese [10–13]; c.167delT among Ashkenazi Jews [14]; p.R143W among certain Africans [15]; and p.W24X among Indians [16,17] and European Gypsies [18–20]. Some recent reports have indicated a genotype-phenotype correlation: children with two truncating mutations, such as c.35delG or c.235delC, are profoundly deaf, while children with a truncating and missense mutation, or two missense mutations, show better hearing [9,21,22]. Since improved speech performance after cochlear implantation in early childhood is usually observed in hearing-impaired subjects with *GJB2* mutations [23], the genetic testing of newborn babies will provide useful prognostic information when selecting appropriate treatment for such children.

In the present study, to clarify the frequency and genotype-phenotype correlation of *GJB2* mutations in children with profound non-syndromic deafness, we performed genetic testing for *GJB2* mutations involving 119 Japanese children who had undergone cochlear implantation with congenital deafness.

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2. Materials and methods

2.1. Subjects

We enrolled 119 Japanese children, who were unrelated to each other, with non-syndromic deafness for genetic analysis. Of these, 107 were sporadic cases (with only one affected individual in the family); the remaining 12 patients were autosomal recessive cases (with normal hearing parents and at least two affected children). The study sample consisted of 70 males (58.8%) and 49 females (41.2%). All of their hearing impairment levels were severe (71–95 dB) to profound (>95 dB); impairments were detected between 0 and 3 years old. All children had undergone cochlear implantation at Tokyo Medical University School of Medicine.

All cases underwent otoscopic examination and audiometric testing. Subjective tests of hearing acuity were assessed based on the auditory brain-stem response (ABR) and auditory steady-state response (ASSR) in infants and children. Behavioral observation audiometry (BOA) was used as a subsidiary measure to ABR and ASSR. A detailed history was taken to exclude other possible causes of deafness (such as neonatal complications, bacterial meningitis or other infections, use of ototoxic medication, or head trauma). Extended pedigrees were elicited from each family to exclude interfamilial relations. Temporal bone computed tomography was used in children to exclude any anomalies. The control group was carefully chosen to determine the carrier frequency, and consisted of 150 unrelated individuals with normal hearing.

Informed consent was obtained from the parents or guardians when necessary, and these were approved by the Ethical Committees of Juntendo University School of Medicine.

2.2. Genetic analysis

All samples from the children and normal controls were extracted from peripheral blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Germantown, MD, USA). The coding region of *GJB2* was amplified from DNA samples by the polymerase chain reaction (PCR) using the primers *GJB2*-F 5′-GTGTGCATTCGTCTTTTCCAG-3′ and *GJB2*-R 5′-GCGACTGAGCCTTGACA-3′. PCR products were sequenced using the PCR primers and sequence primers *GJB2*-A 5′-CCACGCCAGCGCTCCTAGTG-3′ and *GJB2*-B 5′-GAAGATGCTGCT GCTTGTGTAGG-3′. The sequencing reaction products were electrophoresed on an ABI Prism 310 Analyzer (Applied Biosystems). When no mutation or a single heterozygous mutation in *GJB2* was confirmed, we performed the multiplex PCR assay and direct sequencing for the coding region of *GJB6*. Multiplex PCR was carried out according to the method of Del Castillo et al. [24] to confirm the presence of the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) deletions in *GJB6*.

Samples with no mutation or a single heterozygous mutation in *GJB2* and *GJB6* were analyzed for the gene dosage using real-time quantitative PCR (qPCR) to detect exon rearrangements in *GJB2* and *GJB6*. qPCR was performed with TaqMan Gene Expression Assays (Hs00269615_s1 for *GJB2*, and Hs00272726_s1 for *GJB6*, Applied Biosystems) and the 7500 Fast Real-Time PCR System (Applied Biosystems).

We obtained blood samples from the family which had one of two unreported mutations, pP225L, and the unreported one was confirmed as follows. The samples were subjected to mutation screening by PCR and direct sequencing for *GJB2*. The PCR product was subcloned into pCR 2.1 vecto-TOPO by TOPO TA cloning (Invitrogen, Carlsbad, CA, USA), and independent subclones were sequenced employing M13forward (5_-TTGTAAAACGACGGCCAG) and reverse (5_-ACACAGGAAACAGCTATG) primers. The sequence data using in this study have been submitted to the GenBank databases under accession numbers X65361, AB098335, NM_000816, and NM_001037.

2.3. Statistical analysis

A *Z*-test was used to calculate the difference in the allele frequency. In all statistical analyses, *P*-values of 0.01 or less were considered significant.

3. Results

3.1. Mutation screening of GJB2

GJB2 mutations were found in 45 of the 119 affected individuals, and, of these, 35 patients were homozygous or compound heterozygous (29.4%). *GJB2*-related deafness patients, who had two *GJB2* mutant alleles, were found in 7 of 12 familial cases (58.3%), and there were 28 of 107 sporadic cases (26.2%). Eight mutations, including two unreported ones (p.R32S and p.P225L), were identified in these patients (Table 1). Three mutations were truncating mutations [one was a nonsense mutation (p.Y136X), and two were frameshifts (c.235delC and c.176-191del)]. The remaining five were missense mutations (p.R143W, p.G45E, p.T86R, p.R32S, and p.P225L). Among these mutations, c.235delC was the most frequent. The c.235delC mutation accounted for 52.9% (37 of 70) of the *GJB2*-mutated alleles (Table 1).

We identified 10 subjects who had three or more mutations. All of them had p.G45E and p.Y136X, including one homozygous child. TA cloning and sequencing of subcloned PCR products revealed that all subjects had both mutations in the same allele (data not shown). G45E accompanied with Y136X has been reported as a pathogenic mutation in previous reports, especially in Japanese patients [11,25], although it remains unclear which mutation is more related to the pathogenicity.

We compared the allele frequency for each mutation with that in Ohtsuka's study [25] (Fig. 1). The frequency of c. 235delC and three mutations (p.R143W, p.G45E/Y136X, and c.176-191del) in this study were significantly different from that in Ohtsuka's study ($P < 0.01$). While the p.V37I mutation was reported to be the second most frequent autosomal recessive deafness allele in Asian countries [11,12], the present subjects did not follow this pattern.

In one subject, we identified a missense mutation, p.P225L, which has not previously been reported (Fig. 2). The sister and father of the proband had this mutation, while they showed a normal hearing function. The mother, with a normal hearing function, showed no mutation at this site, while she revealed only heterozygous p.G45E/Y136X mutation as a known pathogenic mutation of *GJB2*. The sequencing results of TA cloning further confirmed the existence of the pP225L nonsense mutation in this patient. We also identified another unreported mutation, p.R32S, in another subject. The patient had p.R32S/p.G45E/Y136X mutations. The amino acid positions of two unreported mutations

Table 1
Mutations identified in the Cx26 gene, *GJB2* (NG_008358.1), in child cases of congenital deafness.

Nucleotide change	Amino acid change	Allele (%)
c.235delC	p.Leu79CysfsX3	37 (52.9)
c.427C>T	p.Arg143Trp(p.R143W)	15 (21.4)
c.134G>A/c.408C>A	p.Gly45Glu/p.Tyr136X(p.G45E/Y136X)	10 (14.3)
c.176_191del	p.Gly59AlafsX18	4 (5.7)
c.257C>G	p.Thr86Arg(p.T86R)	2 (2.9)
c.94C>A	p.Arg32Ser[a](p.R32S)	1 (1.4)
c.674C>T	p.Pro225Leu[a](p.P225L)	1 (1.4)
Total mutations		70 (100)

[a] Novel mutations detected in this study.

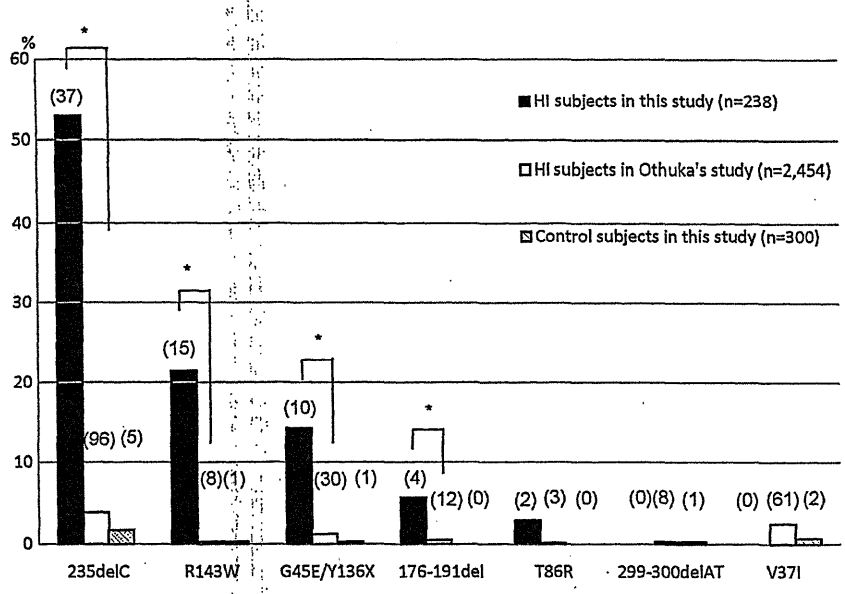

Fig. 1. Allele frequency for each mutation in three groups. A Z-test was used to assess the difference in frequency. Note the *P*-value of <0.01 between the two deafness groups for c.235delC, p.R143W, p.G45E/Y136X, and c.176-191del. *$P < 0.01$.

(p.R32S and p.P225L) are highly conserved among various species, and we did not detect any of these mutations in 300 chromosomes in normal Japanese controls.

4. Discussion

In this study, *GJB2*-related deafness patients accounted for 29.4% of non-syndromic deafness cases. This frequency was less than in a previous report, which pointed to a frequency of around 50% [6]. Familial cases were twice as prevalent as sporadic cases. In most of the previously reported studies, the prevalence of *GJB2* mutations was significantly higher in familial non-syndromic deafness than in sporadic cases [7,26,27]. The frequent mutations of *GJB2* (c.235delC, p.R143W, p. G45E/Y136X, and c.176-191del) in this study were partly different from previous reports [25]. It is assumed that all of our subjects had severe to profound deafness, as they had received cochlear implants, whereas Ohtsuka's subjects had mild to profound deafness and included heterozygous mutations. A few studies have confirmed that some genotypes are correlated with clinical phenotypes in *GJB2*-related deafness. Further, truncating mutations are associated with a greater degree of deafness than non-truncating mutations [9,21,22]. For this reason, three of these cases might be truncating mutations. In contrast, p.R143W mutation was previously implicated in an extraordinarily high prevalence of profound deafness in Ghana [15,28] and Caucasians [9]. This missense mutation may also show an important correlation with severe deafness in Japan. On the other hand, an effect of geography on the allele frequency may have been present, because most of our subjects were from a different area compared to a previous report [25].

The relation between p.V37I mutation of *GJB2* and SNHL is controversial. While some reports suggest that this mutation is

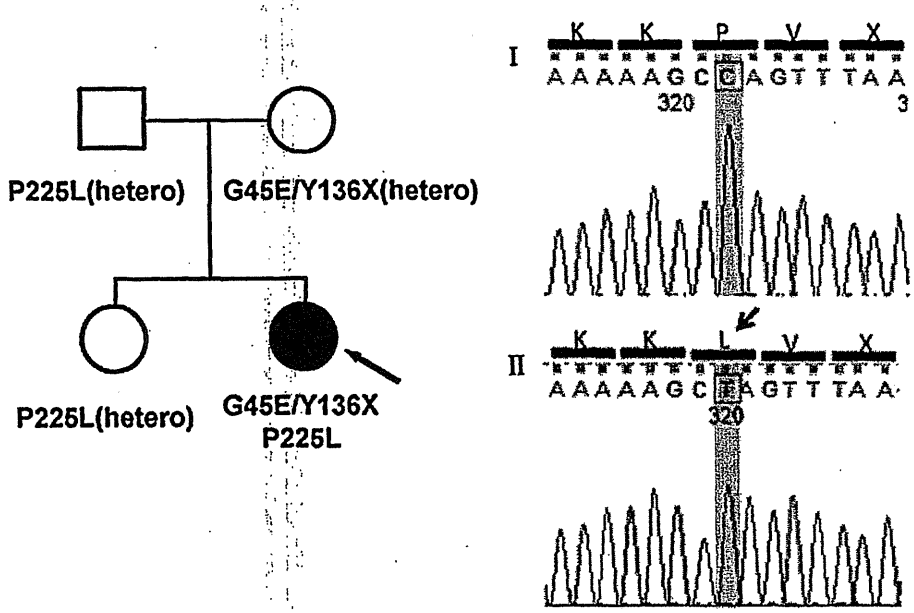

Fig. 2. (A) The pedigree and PCR direct sequencing results for the family; the arrow indicates the proband. (B) The sequencing results on TA cloning. Genomic PCR products were subcloned into a plasmid vector and sequenced separately (see Section 2). The sequences from independent clones are shown in the above two examples. I shows wild-type sequence, whereas II shows mutated sequence in which the proline residue is changed to leucine. Three of 8 subclones showed a missense mutation similar to that in II.

more common among individuals of Asian ancestry [11,12,29], others suggest that homozygous p.V37I is associated with slight/mild hearing loss [22,30,31]. In this study, no cases of homozygous p.V37I were observed. These findings support that this mutation is associated with mild hearing loss, because all of our subjects showed severe deafness.

The two unreported *GJB2* mutations, p.R32S and p.P225L, were not detected in normal hearing controls. These appeared in amino acid residues that were highly conserved. Additionally, three types of mutation were seen in arginine as the thirty-second amino acid, such as p.R32C, p.R32L, and p.R32H. Therefore, R32 is thought to be a mutation "hot spot." Thus, it is likely that these are pathological mutations, rather than rare or functionally neutral polymorphic changes. On the other hand, the mutation site of p.P225 located at the C-terminus of Connexin26 has not previously been reported. As the C-terminus region of connexins is thought to be an important region for intracellular molecular signaling and interaction with scaffolding proteins and the cytoskeleton [32–34], p.P225L mutation found in this study may affect important intracellular molecular networks to maintain the normal function of the cochlear gap junction.

5. Conclusion

In conclusion, this study identified significant genotypic features of Japanese children with profound non-syndromic deafness. Further research is required covering a broader range of genes in the subjects in this study with either single heterozygous or no mutation, in order to better understand the epidemiology of deafness in Japan.

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Review

Cell PRESS

Genetic mutations and functions of PINK1

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Parkinson's disease (PD) is the second most common neurodegenerative disease. Mutations in *PINK1* (*PARK6*) are the second most frequent cause of autosomal recessive, young-onset PD, after *parkin* (*PARK2*). PINK1 (a kinase with an N-terminal mitochondrial targeting sequence) provides protection against mitochondrial dysfunction and regulates mitochondrial morphology via fission/fusion machinery. PINK1 also acts upstream of parkin (a cytosolic E3 ubiquitin ligase) in a common pathway. Recent studies have described PINK1/parkin function in the maintenance of mitochondrial quality via autophagy (mitophagy). PINK1/parkin-mediated mitophagy provides new insights into the etiology of PD and could be a suitable target for new treatment of PD. In this review, we discuss the molecular genetics and functions of PINK1, which could be key factors in novel rational therapy for sporadic PD as well as *PINK1*-linked PD.

Parkinson's disease (PD)

PD is the second most common neurodegenerative disease worldwide after Alzheimer's disease. The prevalence of PD increases with age, and is estimated to be ~1% in those aged > 65 years [1]. The major clinical features are: (i) motor symptoms (called 'parkinsonism'), which include resting tremor, rigidity, bradykinesia (slowness in executing movement) and postural instability; and (ii) non-motor symptoms (e.g. cognitive dysfunction, autonomic nervous system dysfunction, sleep disorders). Pathological features include pronounced loss of dopaminergic neurons in the substantia nigra pars compacta and eosinophilic cytoplasmic inclusion containing α-synuclein aggregates (known as Lewy bodies) in the remaining dopaminergic neurons. No treatment is available to suppress the progression of cell death, and the goal of current therapies is only to alleviate symptoms.

The pathogenesis of PD remains unclear, although mitochondrial dysfunction due to oxidative stress has been proposed to play a major part [2]. Most cases of PD are sporadic, but ~5–10% of PD cases are hereditary. Several genes (e.g. *α-synuclein, parkin, PTEN-induced putative kinase 1* (*PINK1*), *DJ-1, leucine rich repeat kinase 2* (*LRRK2*)) have been identified as causative genes for familial Parkinson's disease (FPD) [3]. *PINK1*-linked PD (*PARK6*-linked PD) is the second most common autosomal recessive young-onset PD, after *parkin*-linked PD (*PARK2*-linked PD). Initial studies suggested that PINK1 provided protection against mitochondrial dysfunction [4–6]. However, the exact function of PINK1 remains unclear. Recent evidence also suggests that PINK1 plays a part in mitochondrial quality control via autophagy machinery, in collaboration with parkin (a cytosolic E3 ligase). In this review, we analyze the recent work published on PINK1 function, which can be a key factor in novel rational therapy for sporadic PD as well as *PINK1*-linked PD.

Clinical characteristics of PINK1-linked PD

Clinical features

The clinical features of *PINK1*-linked PD include parkinsonism associated with a good response to levodopa (the precursor to the dopamine), frequent occurrence of levodopa-induced dyskinesias, and infrequent occurrence of dystonia at onset, similar to those of sporadic PD. The only distinctive features are the earlier age of onset and slower progression [7]. The age of onset of *PINK1*-linked PD is around the early thirties [8,9], whereas that of sporadic PD is after the age of 60 years[1]. Unlike *parkin*-linked PD, hyperreflexia and sleep benefit are not common in *PINK1*-linked PD. However, some patients with *PINK1*-linked PD exhibit foot dystonia at onset and sleep benefit, mimicking those with *parkin*-linked PD. Others with *PINK1*-linked PD show atypical clinical features associated with psychiatric problems and dementia, both of which are rare in patients with *parkin*-linked PD.

Pathological features

As mentioned above, accumulation of Lewy bodies is the pathological hallmark of sporadic PD. Lewy bodies were also detected in the brain of a *PINK1*-linked PD patient with compound heterozygous mutations (c.1252_1488 del and c.1488 + 1G > A) [10], whereas they are absent in the brains of *parkin*-linked PD patients [11,12]. Other pathological changes seen in the PD patient with the compound heterozygous *PINK1*-mutations include neuronal loss in the substantia nigra pars compacta accompanied with astrocytic gliosis and moderate microgliosis. No apparent cell loss, Lewy bodies, or abnormal neurites are seen in the locus ceruleus. However, pathological examination has been reported in only one case of *PINK1*-linked PD, so further pathological studies are needed to determine the association between Lewy bodies and the pathogenesis of *PINK1*-linked PD.

PINK1

Molecular structure

The *PINK1* gene contains 8 exons spanning ~1.8 kilobases and encodes a 581–amino acid protein. The transcript is

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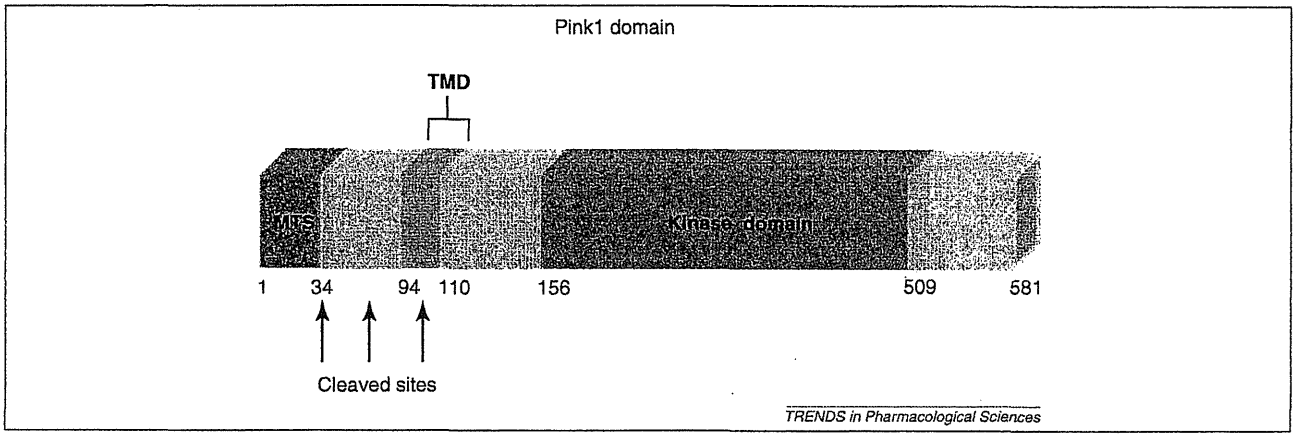

Figure 1. Putative functional domains and motifs of PINK1. Possible cleaved sites consist of the end of mitochondrial targeting sequence (MTS), sites between MTS and the transmembrane domain (TMD), and sites in the TMD.

ubiquitously expressed and predicted to encode an N-terminal 34–amino acid mitochondrial targeting sequence (MTS), a transmembrane domain (TMD) (residues 94–110) and a highly conserved protein kinase domain (residues 156–509) showing a high degree of homology to the serine/threonine kinases of the Ca^{2+}/calmodulin family [13] (Figure 1). It is important to understand the topology and subcellular distribution of PINK1 when considering PINK1 functions. PINK1 is a mitochondrial membrane integral protein whose kinase domain localizes in the outer mitochondrial membrane and is accessible from the cytoplasm. The TMD is crucial for anchoring PINK1 to the mitochondrial membrane and to ensure that the kinase domain faces the cytoplasm [14]. Subcellular fractionation shows that overexpressed PINK1 is localized in the mitochondria and cytoplasm [15], although the localization of endogenous PINK1 is not clear. This is because PINK1 is so rapidly turned over under basal conditions [16,17] that the expression level of PINK1 is very low and no antibodies against endogenous PINK1 are available. A chimeric protein consisting of a fluorescence protein fused to the N-terminus of PINK1 localizes to the mitochondria. Thus, the putative MTS of PINK1 is sufficient for its mitochondrial localization [18]. PINK1 translocated to the mitochondria by MTS is processed at several sites, such as 34 and/or 77 amino acids at the N-terminus and another site in the TMD. PINK1 mainly includes the full-length form (~63 kDa) and the cleaved form (~55 kDa) [15,18–20] (Figure 1).

Human genetics

PINK1 (PARK6) was identified in 2004 as a causative gene of autosomal recessive young-onset PD [13]. Since then, several mutations have been identified in PD patients in Europe and Japan [21]. With regard to the mode of inheritance (e.g. recessive form), loss of PINK1 function is proposed as the mechanism of *PINK1*-linked PD. The estimated prevalence of PINK1 mutations in different ethnicities is 1–8% of familial or young-onset PD [22]. Approximately 50 pathogenic mutations (missense mutations, genomic rearrangements, truncating mutations) have been identified in diverse populations. Most of the mutations are observed in the serine/threonine kinase domain, suggesting that loss of kinase activity plays a crucial part in the pathogenesis of *PINK1*-linked PD. Although the genotype–phenotype correlation has not been confirmed, the mean age at onset in patients with single heterozygous mutations is higher than that in patients with homozygous mutations [9,23]. Homozygous mutations in *PINK1* invariably cause *PINK1*-linked PD, whereas heterozygous mutations have been suggested to be a susceptibility factor for sporadic PD [24].

PINK1 function

Kinase activity

As mentioned above, PINK1 is a serine/threonine kinase protein. Several studies have reported that pathogenic mutations in PINK1, such as p.K219A, p.G309D, p.L347P, p.D362A, p.D384A, p.G386A, p.G409 V, p.E417G, are associated with reduced kinase activity [5,15,18,25]. Furthermore, the C-terminus of PINK1 regulates its kinase activity, although there is controversy over whether it up-regulates or down-regulates [18,25] activity [25]. Mutations in the PINK1 C-terminus cause early-onset parkinsonism [26]. Therefore, we have to consider the effect of the C-terminus on kinase activity to be significant. TNF receptor-associated protein 1 (TRAP1), a mitochondrial chaperone, has been identified as a PINK1 substrate. PINK1 might provide protection against oxidative stress-induced apoptosis by the phosphorylation of TRAP1 [5]. Another candidate substrate of PINK1 is parkin. The linker region of parkin is phosphorylated by PINK1, and parkin phosphorylated by PINK1 promotes its mitochondrial translocation [27]. The activity of parkin E3 ligase functions to catalyze the K63-linked polyubiquitination of IKK-γ, which is a critical step in the cytoprotective signaling pathway that activates NF-κB, a ubiquitously expressed transcription of several pro-survival genes [18,28]. PINK1 is thought to modulate the phosphorylation status of another mitochondrial protein, Omi/HtrA2 (a gene product for *PARK13*), possibly through indirect mechanisms [6]. Rictor, a specific component of mammalian target of rapamycin complex 2 (mTORC2), is phosphorylated by overexpression of PINK1. Enhanced Akt through activation of mTORC2

provides cytoprotection [20,29]. These reports corroborate the fact that kinase activity has crucial roles in the pathogenesis of *PINK1*-linked PD.

Interaction with other PD-associated genes

PINK1-deficient *Drosophila* and *PINK1*-linked PD patients show very similar phenotypes, in contrast to flies and patients whose symptoms are caused by *parkin* mutations. Moreover, overexpression of parkin rescues the phenotype of *PINK1*-deficient *Drosophila*, but not *vice versa* [30,31]. However, Omi/HtrA2 is not an essential component of the PINK1/parkin pathway in *Drosophila* [32]. In cultured cells, PINK1 knockdown phenotypes are also rescued by overexpression of parkin, but not *vice versa* [33]. Parkin stabilizes PINK1 through direct interaction [34]. These results suggest that PINK1 functions upstream of parkin in a common pathway. Furthermore, parkin, PINK1, and DJ-1 form a ubiquitin E3 ligase complex that promotes the degradation of unfolded proteins [35]. In *Drosophila*, DJ-1 can rescue the consequences of PINK1 loss (except for infertility), but not the consequences of parkin loss. Furthermore, parkin cannot rescue DJ-1 loss, suggesting that DJ-1 may not be directly downstream of PINK1 [26,36]. In human neuroblastoma cells, parkin protects against the loss of DJ-1 and, although DJ-1 does not alter *PINK1*-deficient mitochondrial phenotypes, DJ-1 is active against rotenone-induced damage in the absence of PINK1 [27,37]. These findings indicate that DJ-1 works in parallel to the PINK1/parkin pathway to maintain mitochondrial function.

PINK1 knockdown causes proteasome dysfunction, accompanied by increased α-synuclein aggregation [38]. *PINK1*-deficient *Caenorhabditis elegans* exhibits a reduced length of mitochondrial cristae, increased sensitivity to paraquat (a herbicide which causes oxidative stress and parkinsonism) and defective axonal outgrowth of a pair of canal-associated neurons. In the absence of LRRK2, all these phenotypic aspects can be suppressed [39]. Furthermore, in *Drosophila*, overexpression of LRRK2 potentiates the bristle loss phenotype of PINK1 [40]. These results suggest that loss of LRRK2 suppresses the PINK1 phenotype. Taken together, evidence suggests that dysfunction of several causative gene products might contribute to the pathogenesis for PD through a common pathway (Figure 2).

Mitochondrial regulation

Mitochondrial function: In cultured cells, overexpression of PINK1 confers resistance to toxins against mitochondria such as staurosporine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone, and PINK1 expression provides resistance to MPTP-induced dopaminergic neuronal loss in mice [4,41]. Consistent with these reports, reduction of PINK1 levels by RNA interference (RNAi) in cultured cells resulted in enhanced cell death in the presence of MPTP and rotenone [42]. In this regard, *PINK1*-deficient animal models and model-derived cells have provided important information on the endogenous function of PINK1. *PINK1*-deficient *Drosophila* showed degeneration of flight muscles, male sterility, and dopaminergic neuronal cells accompanied by mitochondrial abnormality, and shared phenotypic similarity with *parkin*-deficient *Drosophila* [30,31]. These models exhibit decreases in mitochondrial membrane potential ($\Delta\Psi m$), mitochondrial DNA, complex I, ATP, and an increased proportion of swollen mitochondria and susceptibility to apoptotic stimuli. Concomitant defects in synaptic function are also observed [43]. These results suggest that

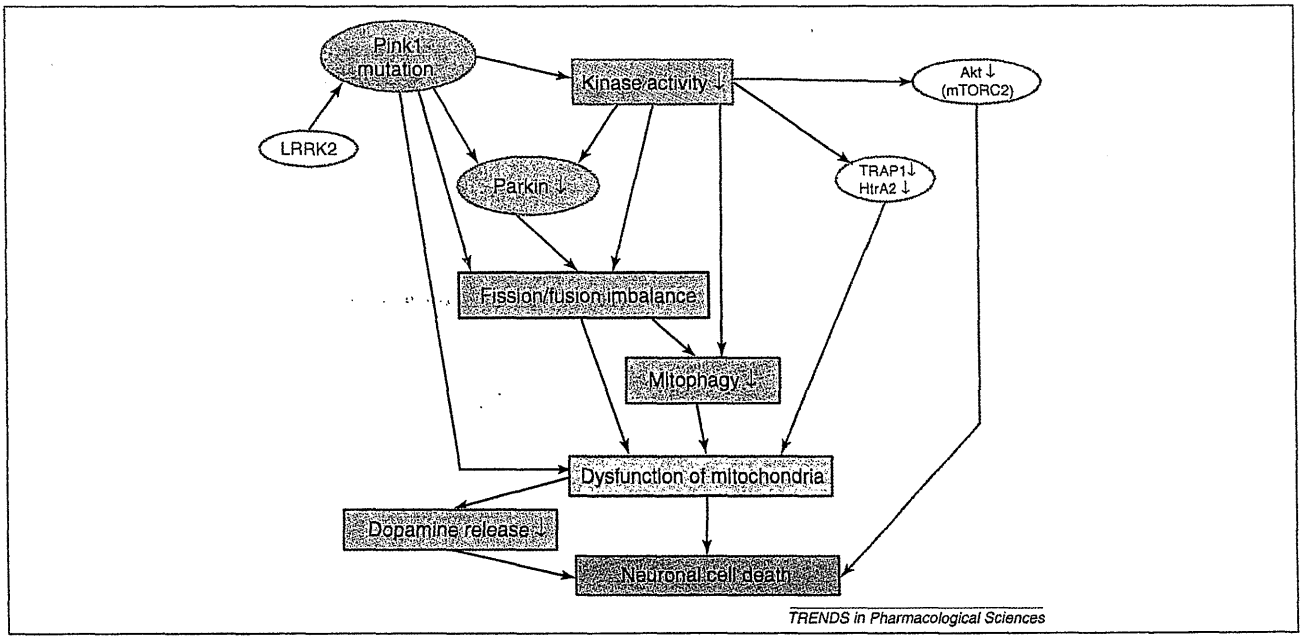

Figure 2. Proposed mechanism of *PINK1*-linked PD. Loss of PINK1 on mitochondria inhibits parkin functions and induces mitochondrial fission/fusion imbalance, which in turn causes impaired mitophagy, resulting in mitochondrial dysfunction. The kinase activity of PINK1 is important for mitophagy. Decreased dopamine release due to mitochondrial dysfunction induces neuronal cell death. Furthermore, TRAP1 and HtrA2 are not activated under reduced PINK1 kinase activity, leading to mitochondrial dysfunction. Reduced PINK1 kinase activity also results in failure of activation of Akt, leading to decreased cytoprotective function. LRRK2 exacerbates loss of PINK1.

loss of PINK1 can cause dopaminergic neuronal cell death due to functional defects in mitochondria.

In contrast, *PINK1*- and *parkin*-deficient mice do not exhibit major abnormalities, whereas absence of PINK1 causes several deficits, including: (i) reduced synaptic dopamine release and plasticity in the striatum [44]; (ii) impaired mitochondrial respiration in the striatum at 3–4 months of age and in the cerebral cortex at 2 years [45]; and (iii) progressive weight loss and selective reduction of locomotor activity for spontaneous movements in old age [46]. These findings indicate that the mitochondria in *PINK1*-deficient models are more vulnerable to aging. Primary cultured neurons derived from *PINK1*-deficient mice showed increased intracellular calcium levels and vulnerability, with subsequent excess production of reactive oxygen species (ROS), decreased glucose availability, loss of $\Delta\Psi m$ and defects of complex I, causing pathological opening of the mitochondrial permeability transition pore [47]. In *PINK1*-deficient mouse embryonic fibroblasts (MEFs), $\Delta\Psi m$ and cellular ATP levels are lower than in wild-type MEFs. However, mitochondrial proton leak (which reduces membrane potential in the absence of ATP synthesis) is not altered by loss of PINK1. Instead, low activity of the respiratory chain (which produces membrane potential oxidizing substrates using oxygen) has been observed [48]. These results suggest that decreased $\Delta\Psi m$ caused by loss of PINK1 is not due to proton leak, but to respiratory chain defects [39].

Similar to *parkin*-deficient mice, *PINK1*-deficient mice do not show prominent phenotypes. However, results from *PINK1*-deficient mice indicate that dysfunction of mitochondrial proteins (e.g. complex I) and aging are important in the pathogenesis of *PINK1*-linked PD as well as in sporadic PD.

Samples obtained from patients with hereditary PD can provide important information regarding the pathogenesis of PD. Skin fibroblasts of patients homozygous for the p.G309D *PINK1* mutation show: (i) a mild decrease in complex I activity and a trend of superoxide elevation [49]; (ii) fragmented mitochondrial morphology [33]; and (iii) low expression of parkin and selective vulnerability to proteasomal stress-triggered caspase activation [41,50].

In samples obtained from mice and PD patients, as well as from cultured cells, loss of PINK1 causes mitochondrial dysfunction and vulnerability to cell death.

Mitochondrial morphology: In mammalian cultured cells (with the exception of COS7 cells), PINK1 knockdown phenotype shows fragmented mitochondria [33,51]. In human neuroblastoma cells transduced with a PINK1 shRNA lentivirus, the activity of dynamin-related protein 1 (Drp1), which is controlled by phosphatase calcineurin, enhances the effects of PINK1 upon mitochondrial morphology [52]. In contrast, transgenic *Drosophila* with PINK1 promotes mitochondrial fission in dopaminergic neurons, whereas complete ablation of PINK1 leads to excessive fusion [53]. Fis1 (mitochondrial fission 1 protein) may act in-between PINK1 and Drp1 in controlling mitochondrial fission [53]. Heterozygous loss-of-function mutations of Drp1 are largely lethal in a *PINK1* or *parkin* mutant background. Conversely, the degeneration of flight muscle and mitochondrial morphological changes which result from mutations in *PINK1* and *parkin* are strongly suppressed by increasing the dosage of the Drp1 gene and by heterozygous loss-of-function mutations in OPA1 and Mfn2. In pseudopupil analyses, an eye phenotype associated with increased activity of the PINK1/parkin pathway is suppressed by perturbations that reduce mitochondrial fission but enhanced by perturbations that reduce mitochondrial fusion [54,55]. These results suggest that the PINK1/parkin pathway promotes mitochondrial fission, and that the loss of mitochondrial and tissue integrity in *PINK1* and *parkin* mutants is due to reduced mitochondrial fission in *Drosophila*. The cortical neurons of *PINK1*-deficient mice show reduced fission and increased aggregation of mitochondria only under stress [46]. Dopaminergic neuronal rat cells with a *PINK1* mutation (p.L347P) show mitochondrial fragmentation and dysfunction, which can be prevented by inhibitors of mitochondrial division [47,56].

There is controversy over whether PINK1 modulates mitochondrial fission or fusion. Fragmented mitochondria in *parkin* and/or *PINK1*-deficient *Drosophila* S2 cells are observed at day 2 after double-stranded RNA (dsRNA) treatment. At days 3 and 4 after dsRNA treatment, a dense network of fine thread-like mitochondria is observed. *PINK1*-deficient primary mouse hippocampal neurons show a decrease in the length of mitochondria and an increase in mitochondrial fragmentation. The mitochondrial phenotype observed in *parkin*- and *PINK1*-deficient cells can be rescued morphologically and functionally by increased expression of a dominant negative mutant of Drp1 [51]. Considering the discrepancy between cellular and fly models with low PINK1 expression, we might observe an acute manifestation of *parkin* or *PINK1* knockdown in cultured cells and the chronic phenotype influenced by compensatory effects in adult *Drosophila* (although the mitochondrial morphological change might be dependent upon cell lines). Otherwise, PINK1 might regulate and maintain a balance between fission and fusion depending upon certain conditions (e.g. phase, stress).

Mitochondrial fission and fusion are highly regulated processes that are critical for the maintenance of mitochondria (especially in neurons). Imbalance of mitochondrial fission and fusion machineries has increasingly been linked to neurodegeneration [57]. Based on this concept, PINK1 has a crucial role in the pathogenesis of PD.

Mitophagy: Recent studies have provided new insights about the PINK1/parkin pathway. It has been reported that parkin is translocated to depolarized mitochondria and that the parkin-labeled mitochondria are subsequently eliminated by autophagy (mitophagy) [58]. Several studies on this pathway were subsequently reported. Endogenous PINK1 is not detected under basal conditions because it is rapidly degraded [16]. However, reduction of $\Delta\Psi m$ induced by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, results in gradual accumulation of endogenous PINK1 (full-length form) on mitochondria [17,59]. Interestingly, clearance of CCCP results in immediate disappearance of the accumulated endogenous PINK1 in the presence and absence of cycloheximide (an inhibitor of protein biosynthesis) by interfering with the translocation step of protein synthesis.

Likewise, CCCP treatment does not alter PINK1 mRNA levels [17]. These results suggest that PINK1 is stabilized by reduced $\Delta\Psi$m and subsequently accumulates on depolarized mitochondria. By contrast, parkin is not translocated to the mitochondria in *PINK1*-deficient CCCP-treated MEFs, and subsequent mitochondrial clearance is also completely blocked, indicating that PINK1 is indispensable for parkin translocation to depolarized mitochondria. Parkin E3 ligase activity is suppressed under basal conditions, although the activity is increased in mitochondria with low $\Delta\Psi$m. PINK1 accumulation induces recruitment of parkin to the depolarized mitochondria, and subsequently the mitochondria are eliminated by mitophagy. These processes are inhibited by pathogenic mutants of PINK1 or parkin [16,17,59]. Furthermore, elimination of parkin-labeled mitochondria is blocked in *Atg5*- or *Atg7*-deficient MEFs [58,59]. Moreover, MTS, kinase activity of PINK1, and the linker domain of parkin are indispensable for PINK1/parkin-mediated mitophagy [17,52,59].

In cells with normal $\Delta\Psi$m, expression of PINK1 on the outer mitochondrial membrane or overexpression of PINK1 induces translocation of parkin to the mitochondria [60,61]. Furthermore, valinomycin- and hydrogen peroxide (H_2O_2)-induced stress result in mitochondrial localization of parkin in skin fibroblasts of healthy controls but not in those of PD patients with PINK1 mutations (p.Q456X) [56,62]. Valinomycin, but not H_2O_2, reduces $\Delta\Psi$m. Taken together, it seems that PINK1 expression on the mitochondria, rather than low $\Delta\Psi$m, is indispensable for parkin translocation to the mitochondria.

The mitochondrial inner membrane rhomboid protease presenilin-associated rhomboid-like protein (PARL) mediates cleavage of PINK1 in the TMD in a $\Delta\Psi$m-dependent manner. In the absence of PARL, full-length PINK1 accumulates on the outer mitochondrial membrane, where it recruits parkin to the impaired mitochondria. Thus, the role of PARL in the PINK1/parkin pathway appears to include facilitating the rapid degradation of PINK1 by mediating the cleavage of PINK1 [19,20,63].

PINK1/parkin-mediated mitophagy involves the formation of linkage-specific polyubiquitin chains (K27 and K63) and requires the ubiquitin-autophagy adaptor p62/SQSTM1. VDAC1 is a mitochondrial target of parkin-dependent K27 ubiquitination, and VDAC1 ubiquitination in neuronal cells is dependent upon functional parkin. Because VDAC1 is a component of the mitochondrial permeability transition pore (mPTP), which is involved in apoptosis, a parkin-dependent, timely mitophagic clearance may prevent the release of pro-apoptotic factors from damaged mitochondria under physiological conditions [58,64]. By contrast, p62 is reported to be necessary for mitochondrial aggregation but not mitophagy, and mitochondrial-associated proteins other than VDAC1 and VDAC3 are K63-polyubiquitinated in a parkin-dependent manner [65,66]. Another candidate parkin substrate on the mitochondria is mitofusin (MFN), which is involved in mitochondrial fusion. *Drosophila* Marf (a fly MFN ortholog), which is localized on the outer surface of mitochondria, is ubiquitinated by parkin and accumulates in *parkin* mutants [67,68]. Mammalian cells contain two types of MFN: MFN1 and MFN2. Treatment of cultured cells with CCCP results in ubiquitination of MFN1 and MFN2 in a PINK1/parkin-dependent manner [63,69]. Moreover, parkin-ubiquitinated MFN1 and MFN2 are degraded by the proteasome. The hexametric AAA-type ATPase, p97, is required downstream of PINK1 and parkin to promote the proteasomal turnover of ubiquitinated MFN. Parkin-promoted MFN degradation prevents refusion of damaged mitochondria with healthy mitochondria [64,70]. Fission followed by selective fusion segregates dysfunctional mitochondria and permits their removal by autophagy [71]. These reports suggest that mitochondrial fission may be a key factor in PINK1/parkin-mediated mitophagy. However, there is controversy over the substrates of parkin, making further investigations necessary.

PINK1/parkin-mediated mitophagy model

Based on the collective results of the studies mentioned above, we propose the model illustrated in Figure 3 for PINK1/parkin-mediated mitophagy. PINK1 accumulated on the mitochondrial outer membrane due to the low $\Delta\Psi$m or certain mitochondrial insults recruit parkin to the mitochondria. Parkin E3 ligase activity is subsequently activated and ubiquitinates MFN on the mitochondrial outer membrane. Ubiquitinated MFN assembles p97 and the complex is degraded by proteasome. Mitochondria lacking MFN, which cannot fuse with other healthy mitochondria, can be a target for mitophagy. The model advances our understanding of the pathogenic process of PD and the role of the direct associations between parkin and mitochondria, as well as between the PINK1/parkin pathway and autophagy. Mitochondrial dysfunction is also considered to be one of the main causes of the sporadic form of PD, and impaired autophagy leads to neurodegenerative diseases such as PD. However, to fully understand the molecular mechanisms of this pathway, further details are needed. For example, how does PINK1 recruit parkin to the damaged mitochondria? What is the mechanism responsible for parkin activation in the mitochondria? There is controversy over whether PINK1 phosphorylates parkin in this pathway [16,17,27,28,61,66]. PINK1 kinase activity is essential for parkin translocation to mitochondria [17,59]. Therefore, it is a feasible and attractive hypothesis that parkin is directly phosphorylated by PINK1. However, further investigations are needed.

Other candidate PINK1 substrates that recruit parkin or function as parkin receptors on the outer mitochondrial membrane also need to be investigated. In yeast, Atg32 (which has no known metazoan homolog) is identified as an outer mitochondrial membrane protein necessary for mitophagy [72,73], and contains a conserved WXXI/L/V motif for interaction with Atg8. Recent studies reported that another outer mitochondrial membrane protein, Nix (which has no yeast homolog and contains a WXXL-like motif), is crucial for PINK1/parkin-mediated mitophagy [68,74]. Does endogenous parkin contribute to mitophagy? Most reports on this pathway are conducted by overexpressing parkin. It remains unclear at this stage whether parkin deficiency or parkin partial knockdown down-regulates mitophagy. Furthermore, there is no information on whether $\Delta\Psi$m of the brain is decreased in patients with

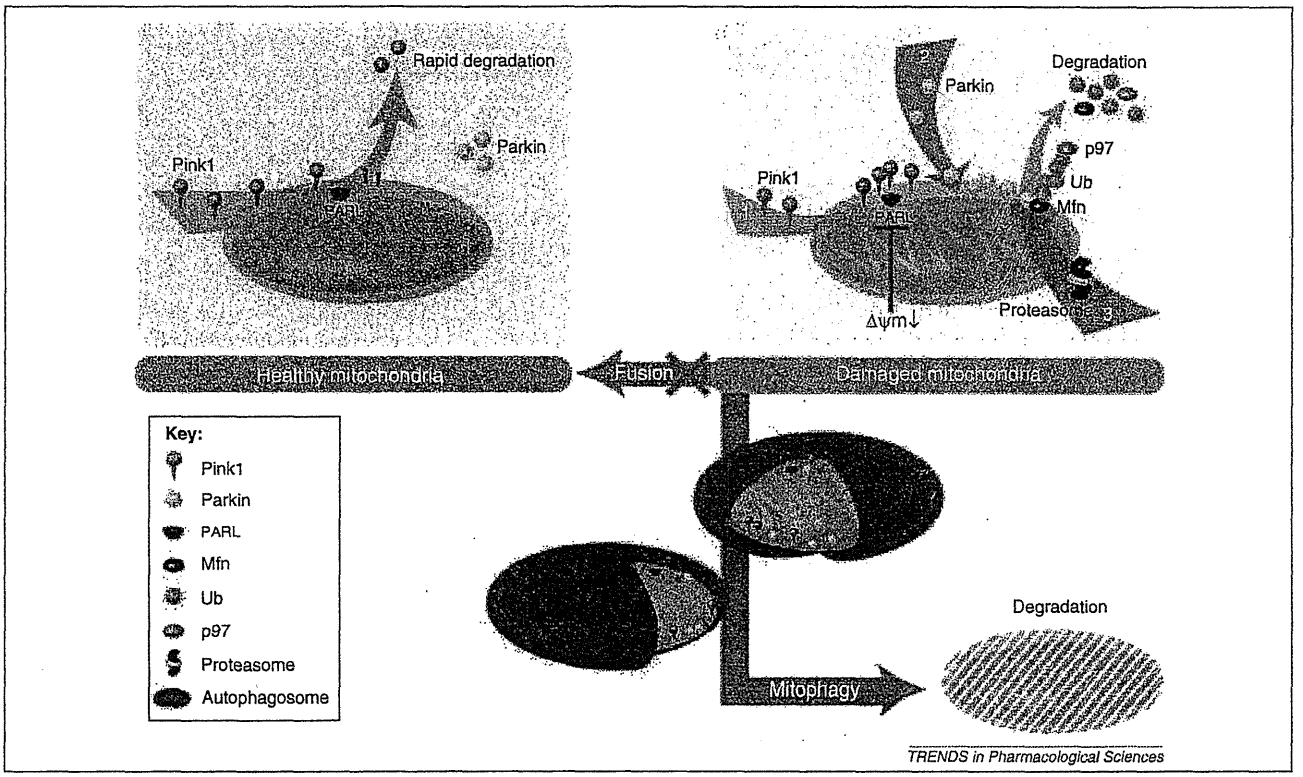

Figure 3. Proposed model of PINK1/parkin-mediated mitophagy. PINK1 on mitochondria is processed by PARL, which localizes in the mitochondrial inner membrane under basal conditions. The processed PINK1 is rapidly degraded by the proteasome. In the presence of a low membrane potential or certain insults to the mitochondria, PINK1 accumulates in the mitochondrial outer membrane. This results in translocation of parkin to the damaged mitochondria and subsequent activation of parkin-E3 ligase. Activated parkin ubiquitinates mitofusin (MFN) on the mitochondrial outer membrane. This leads to the assembly of p97 on ubiquitinated MFN and proteasomal degradation. Mitochondria lacking MFN cannot fuse with other healthy mitochondria and become a target for mitophagy.

sporadic PD. As mentioned above, *PINK1*-deficient mice and MEFs as well as PINK1 knockdown cells show decreased ΔΨm. However, skin fibroblasts derived from neither the nonsense (p.Q456X) nor the missense (p.V170G) mutation show significantly low ΔΨm [75]. There is no model of CCCP-induced human parkinsonism, unlike with MPTP-and rotenone-induced parkinsonism. In this regard, there is no evidence for translocation of parkin to mitochondria in cells treated with MPTP or rotenone. CCCP treatment does not reflect a human physiological condition. Therefore, there is a limitation in discussing the pathogenesis of PD using models based on CCCP treatment.

Is there any association between PINK1/parkin-mediated mitophagy and α-synuclein? As discussed above, α-synuclein is a major component of Lewy bodies (the hallmark of sporadic PD) and is considered to be a key protein in the pathogenesis of sporadic PD. There is no direct association between PINK1/parkin-mediated mitophagy and α-synuclein, although wild-type and mutants of α-synuclein are degraded by chaperone-mediated autophagy and macroautophagy, respectively [76,77]. Studies demonstrated that α-synuclein inhibits autophagy [71,78], and that inhibition of mitochondrial fusion by α-synuclein is rescued by PINK1 and parkin [72,73,79]. To develop a novel therapeutic strategy for sporadic PD as well as *PINK1*- and *parkin*-linked PD, it is necessary to investigate the relationship between aggregation of α-synuclein and dysfunction of PINK1/parkin-mediated mitophagy.

Concluding remarks

PD is a progressive neurodegenerative disease for which the frequency increases with age. As 'developing nations' face rapidly aging populations, the prevalence of PD is expected to rise. PD patients in the advanced stage require great support from families and the medical community. However, curative therapies to suppress the progression are lacking. Therefore, it is important to elucidate the pathogenesis and establish novel and rational treatments. PINK1 provides valuable clues regarding the molecular pathogenesis of PD because the pathomechanism for sporadic PD probably has certain common pathways with that of *PINK1*-linked PD. Based on the available information on PINK1, we propose the model shown in Figure 2 for the mechanisms involved in the development of *PINK1*-linked PD. PINK1 mainly protects the mitochondria via mitophagy with parkin, and may subsequently suppress neuronal cell death due to reduced dopamine release. However, PINK1 may have a cytoprotective function by activating Akt in the cytoplasm. We expect that a compound which inhibits this pathway could be established as a novel treatment for not only *PINK1*-linked PD but also sporadic PD. In particular, PINK1/parkin-mediated mitophagy (i.e. mitochondrial quality control via autophagic machinery that includes the combination of PINK1 and parkin) could be viewed as the foundation for the design of novel therapies for PD, although several issues remain unresolved. If we could determine the precise mechanism of mitophagy which eliminates abnormal mitochondria, we

might regulate the clearance of deleterious mitochondria with new chemicals which target this pathway to maintain cell integrity and subsequently suppress cell death. However, further studies are required to elucidate the exact pathomechanism and develop effective therapies for hereditary PD and sporadic PD.

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Association of *LRRK2* exonic variants with susceptibility to Parkinson's disease: a case–control study

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Summary

Background The leucine-rich repeat kinase 2 gene (*LRRK2*) harbours highly penetrant mutations that are linked to familial parkinsonism. However, the extent of its polymorphic variability in relation to risk of Parkinson's disease (PD) has not been assessed systematically. We therefore assessed the frequency of *LRRK2* exonic variants in individuals with and without PD, to investigate the role of the variants in PD susceptibility.

Methods *LRRK2* was genotyped in patients with PD and controls from three series (white, Asian, and Arab–Berber) from sites participating in the Genetic Epidemiology of Parkinson's Disease Consortium. Genotyping was done for exonic variants of *LRRK2* that were identified through searches of literature and the personal communications of consortium members. Associations with PD were assessed by use of logistic regression models. For variants that had a minor allele frequency of 0·5% or greater, single variant associations were assessed, whereas for rarer variants information was collapsed across variants.

Findings 121 exonic *LRRK2* variants were assessed in 15 540 individuals: 6995 white patients with PD and 5595 controls, 1376 Asian patients and 962 controls, and 240 Arab–Berber patients and 372 controls. After exclusion of carriers of known pathogenic mutations, new independent risk associations were identified for polymorphic variants in white individuals (M1646T, odds ratio 1·43, 95% CI 1·15–1·78; p=0·0012) and Asian individuals (A419V, 2·27, 1·35–3·83; p=0·0011). A protective haplotype (N551K-R1398H-K1423K) was noted at a frequency greater than 5% in the white and Asian series, with a similar finding in the Arab–Berber series (combined odds ratio 0·82, 0·72–0·94; p=0·0043). Of the two previously reported Asian risk variants, G2385R was associated with disease (1·73, 1·20–2·49; p=0·0026), but no association was noted for R1628P (0·62, 0·36–1·07; p=0·087). In the Arab–Berber series, Y2189C showed potential evidence of risk association with PD (4·48, 1·33–15·09; p=0·012).

Interpretation The results for *LRRK2* show that several rare and common genetic variants in the same gene can have independent effects on disease risk. LRRK2, and the pathway in which it functions, is important in the cause and pathogenesis of PD in a greater proportion of patients with this disease than previously believed. These results will help discriminate those patients who will benefit most from therapies targeted at LRRK2 pathogenic activity.

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Introduction

Parkinson's disease (PD) is generally thought of as a late-onset sporadic disorder. Nevertheless, genetic insights are helping to define the molecular causes of PD and have provided new models for the development of neuroprotective interventions. Mutations in the leucine-rich repeat kinase 2 gene (*LRRK2*) are now recognised as the most common genetic determinant of familial and sporadic PD.[1] *LRRK2* has 51 exons and encodes the 2527 aminoacid protein LRRK2, which has five conserved domains, including a Roc (Ras in complex proteins, Rab GTPase) domain and a catalytic core common to both tyrosine and serine-threonine kinases.

Pathogenic *LRRK2* variability has been identified by sequencing probands with familial parkinsonism, with results confirmed and occasionally extended within community or clinically-based patient–control series.[2–6] Seven definite pathogenic *LRRK2* mutations (encoding LRRK2 N1437H, R1441C, R1441G, R1441H, Y1699C, G2019S, and I2020T) have been described.[7,8] These mutations can be relatively common in patients from some ethnic origins, but are rare in ethnically matched controls. LRRK2 R1441G has been identified in more than 8% of patients with PD originating from the Basque region of northern Spain,[9] and LRRK2 G2019S has been reported in 30% of Arab–Berber patients

with PD.[10,11] *LRRK2* polymorphisms with more than 1% minor allele frequency have also been associated with PD in Asia, with the estimated attributable risk often dependent on ethnic origin. LRRK2 R1628P and G2385R have each been recorded in 3–4% of individuals who are of Chinese descent and roughly double the risk of PD.[12–15]

However, most *LRRK2* variants have not been systematically studied. *LRRK2* might harbour more variants that are important determinants of PD pathogenicity and clinical risk. To address this possibility, with the Genetic Epidemiology of Parkinson's Disease (GEO-PD) Consortium, we assessed the frequency of *LRRK2* exonic variants in people with and without PD, and assessed the role of the variants in disease susceptibility.

Methods

Participants and procedures

All 35 GEO-PD sites (hospitals and centres), representing 22 countries and six continents, were invited to participate in this study. Patients were diagnosed by use of either the Gelb or the UK Parkinson's Disease Society Brain Bank criteria (the exclusion criterion of more than one affected relative was not included).[16,17] Controls at each site were healthy individuals who were not related to the patients; not all controls were given a detailed neurological examination but all were asked about any previous diagnosis or family history of a neurological disorder. All biological samples were gathered after ethics approval had been obtained from the Mayo Clinic Institutional Review Board Committee, and were used in accordance with the terms of the written informed consent provided by the participants.

LRRK2 exonic variants were identified through searches of available literature up to April 1, 2010, from personal communications with consortium members, and from in-house sequencing studies that had identified novel variants (unpublished data; table 1). DNA was sourced from blood and was stored in a –20°C freezer. All samples were de-identified with an anonymous code from each site and only a minimal clinical dataset. Data were collected in batches but analysed as a single dataset. Genotyping was done on a MassArray iPLEX platform (Sequenom, San Diego, CA, USA) at the Mayo Clinic neurogenetics laboratory, FL, USA (except for the groups from Paris, France, and Antwerp, Belgium, who supplied genotype data and positive control genomic DNA[2,3]); all primer sequences are provided in the webappendix pp 1–4). Eight iPLEX variant combinations were used to incorporate 123 *LRRK2* coding variants (table 1). Positive control DNA was run for each variant; in the absence of a positive genomic control DNA, a synthetic positive control DNA sequence was generated by use of mismatch-primer PCR. A χ^2 test followed by Bonferroni correction was used to test for deviation from the Hardy–Weinberg equilibrium (HWE) in controls for

	Exon	Accession number	cDNA	Aminoacid	Domain
chr12:38905228	1	..	28G>A	E10K	..
chr12:38905349	1	rs2256408	149G>A	R50H	..
chr12:38905627	2	rs72546335	155C>T	S52F	..
chr12:38905696	2	rs75054132	224G>A	A75A	..
chr12:38915703	4	rs33995463	356T>C	L119P	..
chr12:38915711	4	rs41286468	364T>C	L122L	..
chr12:38918058	5	rs10878245	457T>C	L153L	..
chr12:38918147	5	rs35517158	546A>G	K182K	..
chr12:38920612	6	rs112794616	632C>T	A211V	..
chr12:38920663	6	rs56108242	683G>C	C228S	..
chr12:38923625	7	rs28365216	713A>T	N238I	..
chr12:38923737	7	rs72546315	824C>T	H275H	..
chr12:38929923	8	rs17490713	867T>C	N289N	..
chr12:38929949	8	rs57355477	893T>C	A298A	..
chr12:38929992	8	rs41286466	936G>T	A312A	..
chr12:38931342	9	rs78501232	1000G>A	E334K	..
chr12:38931397	9	rs36016791	1055delC	A352fsX357	..
chr12:38931430	9	rs72546336	1088A>G	N363S	..
chr12:38931438	9	rs113065049	1096G>A	V366M	..
chr12:38933053	11	rs34594498	1256C>T	A419V	..
chr12:38937411	12	rs35847451	1383C>T	S461S	..
chr12:38939594	13	rs75711334	1464A>T	L488L	..
chr12:38939673	13	rs34090008	1543insG	P514fsX529	..
chr12:38943875	14	rs35328937	1561A>G	R521G	..
chr12:38943944	14	rs79996249	1630 A>G	K544E	..
chr12:38943967	14	rs7308720	1653C>G	N551K	..
chr12:38954669	15	rs77424631	1647G>A	G558G	..
chr12:38958002	17	rs78154388	1987T>C	S663P	..
chr12:38958037	17	rs72546319	2022A>C	V674V	..
chr12:38958213	17	rs35611877	2198insA	L708fsX718	Ankyrin
chr12:38958223	18	..	2134A>G	M712V	Ankyrin
chr12:38958236	18	..	2147C>T	A716V	Ankyrin
chr12:38958256	18	rs10878307	2167A>G	I723V	Ankyrin
chr12:38963966	19	rs34410987	2264C>T	P755L	Ankyrin
chr12:38964080	19	rs35173587	2378G>T	R793M	Ankyrin
chr12:38964130	19	rs72546337	2428A>G	I810V	Ankyrin
chr12:38964183	19	rs76890302	2481T>C	S827S	Ankyrin
chr12:38967530	20	..	2611A>G	K871E	..
chr12:38973693	21	rs58559150	2769G>C	Q923H	..
chr12:38973713	21	..	2789A>G	Q930R	..
chr12:38974935	22	rs17519916	2830G>T	D944Y	..
chr12:38974962	22	rs7966550	2857T>C	L953L	..
chr12:38975535	23	rs75148313	2918G>A	S973N	..
chr12:38975635	23	rs113217062	3018A>G	I1006M	LRR
chr12:38975638	23	rs55783828	3021C>T	S1007S	LRR
chr12:38978415	24	rs111341148	3200G>A	R1067Q	LRR
chr12:38978502	24	rs76535406	3287C>G	S1096C	LRR
chr12:38978548	24	rs78365431	3333G>T	Q1111H	LRR
chr12:38978557	24	rs35808389	3342A>G	L1114L	LRR
chr12:38979194	25	rs34805604	3364A>G	I1122V	LRR
chr12:38979281	25	rs74985840	3451G>A	A1151T	LRR
chr12:38979324	25	..	3494T>C	L1165P	LRR

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	Exon	Accession number	cDNA	Aminoacid	Domain
(Continued from previous page)					
chr12:38982935	26	..	3574A>G	I1192V	LRR
chr12:38984073	27	rs72546324	3647A>G	H1216R	LRR
chr12:38984109	27	rs80179604	3683G>C	S1228T	LRR
chr12:38984109	27	rs60185966	3683G>T	S1228I	LRR
chr12:38985860	28	rs4640000	3784C>G	P1262A	LRR
chr12:38988536	29	rs77018758	3960G>C/T	R1320S	..
chr12:38988550	29	rs72546338	3974G>A	R1325Q	..
chr12:38988687	29	rs17466213	4111A>G	I1371V	Roc
chr12:38988701	29	rs28365226	4125C>A	D1375E	Roc
chr12:38989178	30	rs7133914	4193G>A	R1398H	Roc
chr12:38989214	30	rs72546327	4229C>T	T1410M	Roc
chr12:38989243	30	rs113589830	4258G>A	D1420N	Roc
chr12:38989254	30	rs11175964	4269G>A	K1423K	Roc
chr12:38989275	30	rs111435410	4290C>T	A1430A	Roc
chr12:38989294	30	rs74163686	4309A>C	N1437H	Roc
chr12:38990503	31	rs33939927	4321C>T	R1441C	Roc
chr12:38990503	31	rs33939927	4321C>G	R1441G	Roc
chr12:38990504	31	rs34995376	4322G>A	R1441H	Roc
chr12:38990505	31	rs112998035	4323C>T	R1441R	Roc
chr12:38990506	31	..	4324G>C	A1442P	Roc
chr12:38990519	31	rs74681492	4337C>T	P1446L	Roc
chr12:38990530	31	rs111501952	4348G>A	V1450I	Roc
chr12:38990569	31	rs35363614	4387InsA	R1462fsX1468	Roc
chr12:38990584	31	..	4402A>G	K1468E	Roc
chr12:38990630	31	rs113431708	4448G>A	R1483Q	Roc
chr12:38994045	32	rs35507033	4541G>A	R1514Q	COR
chr12:38994128	32	rs33958906	4624C>T	P1542S	COR
chr12:38994170	32	rs17491187	4666C>A	L1556I	COR
chr12:38995335	33	rs721710	4793T>A	V1598E	COR
chr12:39000067	34	..	4838T>C	V1613A	COR
chr12:39000101	34	rs1427263	4872C>A	G1624G	COR
chr12:39000112	34	rs33949390	4883G>C	R1628P	COR
chr12:39000140	34	rs11176013	4911A>G	K1637K	COR
chr12:39000166	34	rs35303786	4937T>C	M1646T	COR
chr12:39000168	34	rs11564148	4939T>A	S1647T	COR
chr12:39000188	34	rs111503579	4959A>G	L1653L	COR
chr12:39001183	35	rs35801418	5096A>G	Y1699C	COR
chr12:39001350	35	rs79909111	5163A>G	S1721S	COR
chr12:39002106	36	rs11564176	5173C>T	R1725X	COR
chr12:39002116	36	..	5183G>T	R1728L	COR
chr12:39002116	36	rs145364431	5183G>A	R1728H	COR
chr12:39002455	37	rs111910483	5385G>T	L1795F	COR
chr12:39002527	37	rs10878371	5457T>C	G1819G	COR
chr12:39003324	38	..	5605A>G	M1869V	COR
chr12:39003325	38	rs35602796	5606T>C	M1869T	COR
chr12:39003329	38	..	5610G>T	L1870F	COR
chr12:39003339	38	..	5620G>T	E1874X	COR
chr12:39015100	39	rs77428810	5822G>A	R1941H	MAPKKK
chr12:39020430	41	..	6016T>C	Y2006H	MAPKKK
chr12:39020449	41	rs34015634	6035T>C	I2012T	MAPKKK
chr12:39020469	41	rs34637584	6055G>A	G2019S	MAPKKK

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each site. Direct DNA sequencing was used to confirm genotyping for all variants with a frequency of less than 0·3% (n<50 carriers).

Statistical analysis

All analyses were undertaken separately for the patients in the white, Asian, and Arab–Berber series. For common variants with a minor allele frequency of 0·5% or greater, single variant associations with PD were assessed by use of fixed-effects logistic regression models, in which genotypes were dichotomised as presence versus absence of the minor allele (dominant model), because *LRRK2* mutations cause an autosomal dominantly inherited form of PD and homozygotes for many of the variants are rare; additive models were also assessed. Models were adjusted for site in the white and Asian series. Sensitivity of results to the use of random-effects models was also assessed.[18] Odds ratios (ORs) and 95% CIs were estimated. Between-site heterogeneity was assessed with likelihood ratio tests for variant by site interaction in a logistic regression analysis, and also by estimation of the I^2 statistic (a measure of the proportion of total variation in ORs between sites due to heterogeneity beyond chance).[19]

For variants with a minor allele frequency of less than 0·5% (rare variants), although we estimated the proportion of carriers separately in patients and controls, no statistical tests were used to evaluate associations with PD because of insufficient power. Instead, we collapsed information for rare variants, acknowledging that this has the potential limitation of mixing groups of variants with protective and risk effects, and evaluated the association between the presence of any rare variant and PD in a logistic regression analysis adjusted by site.[20] In an exploratory analysis, when collapsing data across variants, we also used the Sorts Intolerant From Tolerant (SIFT) prediction program[21] to assess only those substitutions predicted to be not tolerated.

Haplotype analysis was done by use of score tests for association with adjustment for site;[22] haplotypes of less than 0·5% frequency were not assessed. Any patient with a copy of the minor allele for any of the pathogenic variants that were noted in the study population (R1441C, R1441H, or G2019S) was excluded from all disease-association analyses to prevent confounding by the pathogenic variants; these patients were not excluded for any other portion of the analysis. Linkage disequilibrium between variants was assessed by use of r^2 values in study controls, separately for each series. Single variant associations with age at onset were assessed with linear regression models, adjusting for site in the white and Asian series; regression coefficients and 95% CIs were estimated.

We adjusted for multiple testing by use of the single-step minP method,[23] with 10 000 within-site permutations of outcome labels to assess the level of significance that controls the family-wise error rate at

5%. After this adjustment, in the logistic regression disease-association analysis p≤0·0033 was judged to be significant in the white series and p≤0·0038 in the Asian series, whereas in the linear regression age at onset association analysis p≤0·0035 was judged to be significant in the white series and p≤0·0037 in the Asian series. The adjusted significance cutoff levels differed between the white and Asian series because of the different number of tests undertaken in each series, and the different correlation structures between variants within them. For the fairly small Arab–Berber series, no adjustment for multiple testing was made, and as such the results were judged to be exploratory. All statistical analyses were done by use of SAS software (version 9.2) or S-Plus (8.0.1).

Role of the funding source

The funding agencies did not play any part in the design of the study, collection, analysis, or interpretation of data, writing of the report, or the decision to submit the report for publication. The principal investigators (OAR and MJF) had access to all the data in this study. The corresponding author had final responsibility for the decision to submit.

Results

Data were gathered from June, 2008, to October, 2010. 23 sites from the GEO-PD Consortium, representing 15 countries and five continents, agreed to participate in this study and contributed clinical data from 8611 patients with PD and 6929 controls. We studied individuals in three series: white (6995 patients and 5595 controls), Asian (1376 patients and 962 controls), and Arab–Berber (240 patients and 372 controls). Table 2 shows the demographics for each series, and webappendix p 5 shows the sample size breakdown for each site. 123 *LRRK2* variants were selected for genotype analysis, but two (R793M and L2466H) did not assay by use of iPLEX and were dropped from the study. The other 121 variants were genotyped in the entire patient–control series (n=15 540); genotyping was successful in all individuals. Call rates for all genotypes in the series were greater than 95%. Deviation from HWE in the controls for each site (all p>0·05) was noted for LRRK2 N2081D in the Norwegian series and was attributable to two patients with a rare homozygous genotype; all patients were retained in the analysis. However, N289N and P1262A were excluded from the analysis of the Arab–Berber series because of significant variation from HWE due to an increased number of rare minor allele homozygotes, which might have been attributable to the consanguineous nature of the population.

Four of 121 *LRRK2* exonic variants were nonsense, 89 missense, and 28 silent. 48 variants, including four of the seven known pathogenic mutations, were not identified in the 15 540 patients and controls. For most of the variants, the pair-wise linkage disequilibrium was weak (r^2<0·3), with higher values noted with D' because of the low minor allele frequency for many of these variants (webappendix pp 6–17).

	Exon	Accession number	cDNA	Aminoacid	Domain
(Continued from previous page)					
chr12:39020473	41	rs35870237	6059T>C	I2020T	MAPKKK
chr12:39020505	41	rs78029637	6091A>T	T2031S	MAPKKK
chr12:39026899	42	rs111739194	6187delCTCTA	L2063X	MAPKKK
chr12:39026953	42	rs33995883	6241A>G	N2081D	MAPKKK
chr12:39028521	43	rs10878405	6324G>A	E2108E	MAPKKK
chr12:39028553	43	rs12423862	6356C>T	P2119L	MAPKKK
chr12:39031648	44	rs111691891	6422C>T	T2141M	..
chr12:39031736	44	rs34869625	6510C>A	G2170G	WD40
chr12:39031792	44	rs35658131	6566A>G	Y2189C	WD40
chr12:39036195	46	rs12581902	6782A>T	N2261I	WD40
chr12:39043509	48	rs113511708	7067C>T	T2356I	WD40
chr12:39043595	48	rs34778348	7153G>A	G2385R	WD40
chr12:39043597	48	rs33962975	7155A>G	G2385G	WD40
chr12:39043610	48	rs79546190	7168G>A	V2390M	WD40
chr12:39044912	49	rs78964014	7183G>A	E2395K	WD40
chr12:39044916	49	rs111272009	7187InsGT	T2356fsX2360	WD40
chr12:39044919	49	rs3761863	7190C>T	M2397T	WD40
chr12:39044953	49	rs60545352	7224G>A	M2408I	WD40
chr12:39047081	50	..	7397T>A	L2466H	WD40
chr12:39047119	50	rs55633591	7435A>G	N2479D	WD40

Chr12=chromosome 12. Roc=Ras in complex. COR=C-terminal of Ras. MAPKKK=mitogen-activated protein kinase kinase kinase. LRR=leucine-rich repeat.

Table 1: **LRRK2 exonic variants investigated in the study**

	Patients	Controls
White series	n=6995	n=5595
Age (years)	69 (12; 18–107)	65 (15; 19–107)
Men	4036 (58%)	2669 (48%)
Age at onset (years)	58 (12; 18–96)	NA
Asian series	n=1376	n=962
Age (years)	63 (13; 20–91)	59 (11; 23–98)
Men	681 (49%)	319 (33%)
Age at onset (years)	54 (12; 20–89)	NA
Arab–Berber series	n=240	n=372
Age (years)	66 (12; 27–87)	58 (11; 31–92)
Men	116 (48%)	190 (51%)
Age at onset (years)	57 (13; 20–82)	NA

Data are mean (SD; range) or number (%), unless otherwise indicated. Information about sex was not available for six patients and eight controls in the Asian series, and 16 patients and 249 controls in the white series. Information about age was not available for eight patients and eight controls in the Asian series, 482 patients and 289 controls in the white series, and six patients and four controls in the Arab–Berber series. Information about the age at onset was not available for 14 patients in the Asian series and 801 patients in the white series. 71 controls in the Taiwan case–control series overlapped with a previous study of R1628P.[15] NA=not applicable.

Table 2: **Characteristics of participants**

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		White series				Asian series				Arab–Berber series			
	Aminoacid	MA	MAF	OR (95% CI)	p value	MA	MAF	OR (95% CI)	p value	MA	MAF	OR (95% CI)	p value
rs2256408	R50H	G	+	+	+	..	..	..	..	G	1·7%	2·05 (0·82–5·14)	0·13
rs10878245	L153L	T	39·6%	0·98 (0·91–1·06)	0·57	C	31·2%	1·04 (0·88–1·23)	0·65	C	47·1%	0·81 (0·55–1·19)	0·28
rs34594498	A419V	T	+	+	+	T	1·9%	2·27 (1·35–3·83)	0·0011	..	..	..	..
rs7308720	N551K	G	6·7%	0·88 (0·79–0·98)	0·025	G	11·9%	0·73 (0·60–0·89)	0·0017	G	8·0%	0·83 (0·49–1·39)	0·47
rs10878307	I723V	G	7·4%	0·94 (0·84–1·04)	0·23	G	1·1%	1·36 (0·74–2·49)	0·32	G	9·0%	1·09 (0·68–1·75)	0·71
rs34410987	P755L	..	..	..	..	T	0·6%	0·56 (0·27–1·18)	0·13	..	..	..	..
rs58559150	Q923H	C	+	+	+	..	..	..	..	C	0·9%	0·62 (0·13–2·99)	0·55
rs7966550	L953L	C	12·8%	0·98 (0·90–1·07)	0·66	C	17·6%	0·80 (0·66–0·95)	0·012	C	12·4%	0·92 (0·60–1·41)	0·70
rs77018758	R1320S	..	..	..	..	T	1·2%	1·20 (0·69–2·11)	0·51	..	..	..	..
rs17466213	I1371V	G	+	+	+	G	+	+	+	G	0·5%	4·45 (0·81–24·56)	0·086
rs7133914	R1398H	A	6·6%	0·89 (0·80–0·99)	0·034	A	11·5%	0·73 (0·59–0·89)	0·0020	A	8·7%	1·00 (0·61–1·64)	1·00
rs11175964	K1423K	A	6·6%	0·83 (0·74–0·92)	0·0006	A	11·5%	0·75 (0·62–0·92)	0·0064	A	5·4%	0·42 (0·21–0·86)	0·011
rs35507033	R1514Q	A	0·9%	1·13 (0·85–1·49)	0·41	..	..	..	..	A	+	+	+
rs33958906	P1542S	T	2·8%	0·90 (0·77–1·06)	0·21	..	..	..	..	T	1·0%	2·27 (0·72–7·13)	0·16
rs1427263	G1624G	C	34·7%	1·06 (0·98–1·14)	0·15	A	46·7%	0·92 (0·77–1·11)	0·40	C	31·7%	0·96 (0·67–1·39)	0·84
rs33949390	R1628P	C	+	+	+	C	1·2%	0·62 (0·36–1·07)	0·087	..	..	..	..
rs11176013	K1637K	A	45·0%	1·02 (0·94–1·11)	0·60	G	44·6%	0·96 (0·80–1·16)	0·68	A	46·0%	1·07 (0·70–1·63)	0·76
rs35303786	M1646T	C	1·6%	1·43 (1·15–1·78)	0·0012	..	..	..	..	C	+	+	+
rs11564148	S1647T	A	29·9%	0·93 (0·86–1·00)	0·048	A	28·3%	0·97 (0·82–1·15)	0·73	A	27·6%	0·81 (0·55–1·19)	0·29
rs10878731	G1819G	T	45·2%	1·06 (0·98–1·15)	0·16	C	43·3%	0·99 (0·83–1·19)	0·95	T	46·2%	1·07 (0·70–1·64)	0·75
rs33995883	N2081D	G	2·6%	1·24 (1·05–1·47)	0·013	G	+	+	+	G	4·7%	0·92 (0·49–1·73)	0·79
rs10878405	E2108E	A	31·4%	0·96 (0·89–1·03)	0·27	A	29·6%	1·01 (0·85–1·20)	0·92	A	28·1%	0·75 (0·51–1·10)	0·14
rs35658131	Y2189C	G	+	+	+	..	..	..	..	G	1·1%	4·48 (1·33–15·09)	0·012
rs3477838348	G2385R	..	..	..	..	A	3·3%	1·73 (1·20–2·49)	0·0026	..	..	..	..
rs33962975	G2385G	G	15·7%	0·97 (0·89–1·06)	0·49	G	1·8%	0·96 (0·62–1·49)	0·85	G	8·4%	1·14 (0·7–0 1·83)	0·60
rs3761863	M2397T	C	34·4%	1·06 (0·98–1·14)	0·17	C	43·9%	0·88 (0·73–1·05)	0·16	C	39·8%	1·33 (0·85–2·07)	0·21

ORs and p values result from logistic regression models, where adjustment was made for the site in the Asian and white series. ORs correspond to the presence of the MA. After adjustment for multiple testing, p≤0·0038 was judged to be significant in the Asian series, and p≤0·0033 was judged to be significant in the white series. No adjustment for multiple testing was made in the Arab-Berber series, for which p≤0·05 was judged to be significant. MA=minor allele. MAF=MA frequency. OR=odds ratio. +=a variant with a MAF of less than 0·5% and therefore not included in the logistic regression analysis. ..=a variant not noted in the series.

Table 3: **Common single *LRRK2* variant associations with Parkinson's disease**

Table 3 shows the results of the disease-association analysis of single *LRRK2* variants. In the white series, significant associations with PD were noted for K1423K and M1646T. Figure 1 shows the country-specific ORs and 95% CIs for the risk factor M1646T. The between-site heterogeneity was low for M1646T (I^2=0%, p=0·44) and moderate for K1423K (I^2=34%, p=0·069) in the white series.

In the Asian series, significant associations with PD were noted for LRRK2 A419V, N551K, R1398H, and G2385R (table 3). Figure 2 and figure 3 show the country-specific ORs and 95% CIs for A419V and G2385R, and for the N551K-R1398H-K1423K haplotype; between-site heterogeneity was very low for each of these associations in the Asian series (all I^2=0%, all p≥0·42, webappendix p 18). Notably, LRRK2 R1628P was not associated with PD in the Asian series (table 3), with a non-significant protective effect noted for this variant in the Taiwanese series (minor allele frequency 3·8%, OR 0·56, 95% CI 0·32–1·01; p=0·054). Although not significant, the predicted risk effect for R1628P was noted in the South Korean series, particularly at the Seoul site (0·2%, 2·47, 0·28–22·15; p=0·42). R1628P was not noted in the Japanese series. The previously suggested association of S1647T with PD in Asian populations[14] was not supported by the results of our study (0·97, 0·82–1·15; p=0·73).

In an exploratory analysis of the small Arab–Berber series, significant associations (p≤0·05, without correction for multiple testing) with PD were noted for K1423K and Y2189C (table 3). Larger Arab–Berber series are needed to confirm these associations.

For patients with available information (95%), results for the analysis of the association of single variants with disease in each series remained similar after adjustment for age and sex (webappendix p 19) and by use of an additive model (webappendix p 20). Effect sizes were also similar after simultaneous adjustment for other variants that were significantly associated with PD in a particular series, and after adjustment for R1628P in the Asian series in which a previous association had been shown (webappendix p 21), providing evidence that these associations are independent of one another. With a random-effects model for the white and Asian series, results were generally similar though slightly weaker (webappendix p 18) than those obtained with a fixed-effects model.

Haplotype analysis showed a significant overall association with disease in the series of white (p=0·0016) and Asian (p=2x1^{-24}) individuals, but was non-significant in the Arab–Berber series (p=0·056). Haplotype associations seemed to be attributable to the variants independently implicated in disease (webappendix pp 22–24). When the three series were assessed together, LRRK2 N551K, R1398H, and K1423K, which are in strong linkage disequilibrium and constitute a common (>5% frequency) haplotype, were associated with a protective effect (combined OR 0·82, 95% CI 0·72–0·94; p=0·0043; figure 3).

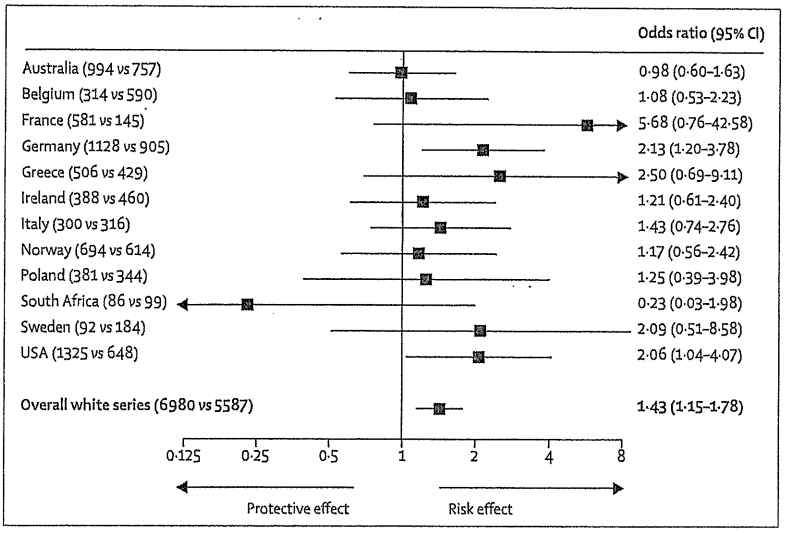

Figure 1: Forest plot of LRRK2 variant M1646T in individuals with versus without Parkinson's disease in the white series

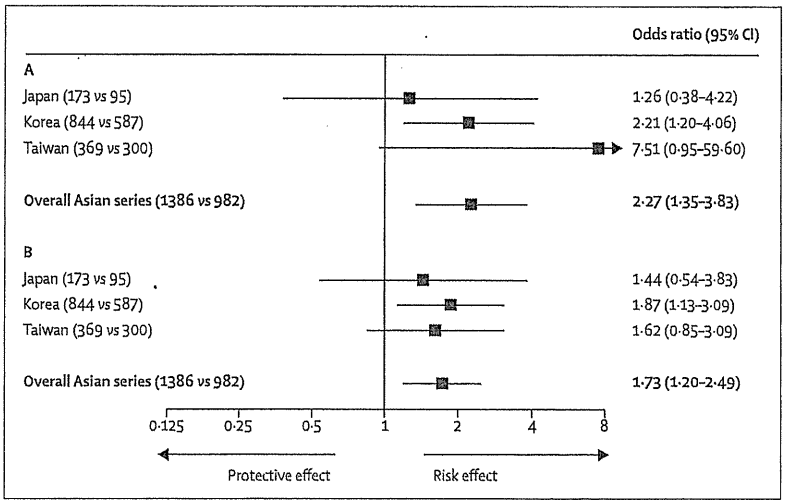

Figure 2: Forest plots of LRRK2 variants A419V (A) and G2385R (B) in individuals with versus without Parkinson's disease in the Asian series

Results of all common single variant associations with age at onset are shown on webappendix p 25. We did not identify any associations that withstood multiple testing correction in the white and Asian series. In the Arab–Berber series, L153L was associated with age at onset roughly 4 years earlier (p=0·038), which needs confirmation in larger samples.

Table 4 provides a descriptive summary of rare variants (minor allele frequency <0·5%) in patients and controls in each series. The pathogenic variant R1441H was noted in an Asian patient, R1441C in only ten patients from the white series, and G2019S in all three series (table 4). The

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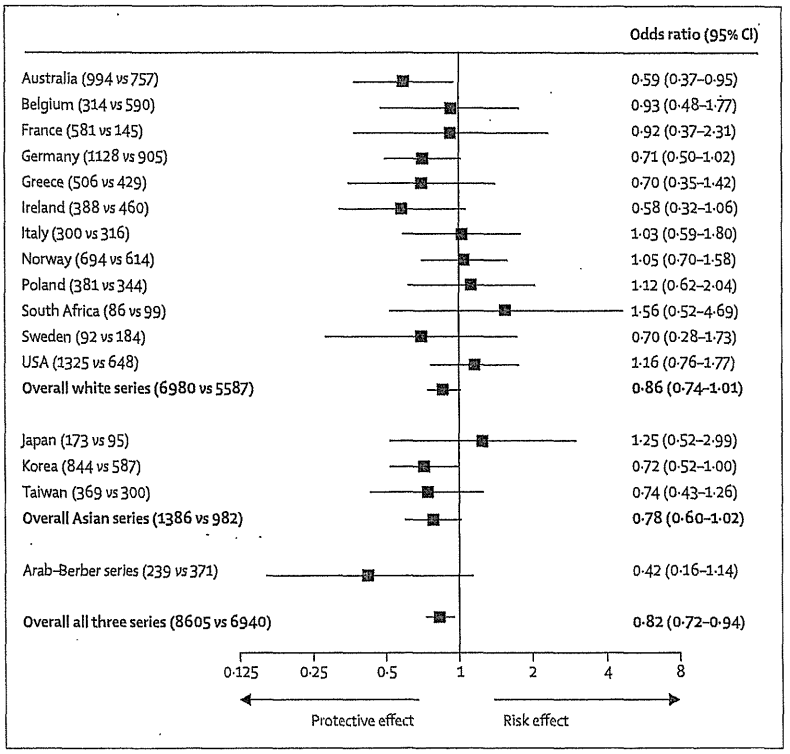

Figure 3: Forest plot of protective LRRK2 haplotype N551K-R1398H-K1423K in individuals with versus without Parkinson's disease in the white, Asian, and Arab-Berber series

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median age of the eight control carriers of G2019S was 64 years (range 48–76 years). Due to the strong confounding potential of these three variants on disease-association analyses, any patient with a copy of these risk alleles was excluded from the analysis. Other possible rare risk variants (E334K, R1325Q, and T1410M) and protective variants (A221V and A1151T) with differences in frequency between patients with PD and controls were noted. When data for all rare variants were combined, the presence of any rare variant was not associated with PD in the white series (OR 1·01, 95% CI 0·81–1·25; p=0·95), Asian series (1·03, 0·57–1·85; p=0·92), or Arab–Berber series (0·78, 0·28–2·20; p=0·64). Additionally, no association was noted in the white series (0·89, 0·55–1·43; p=0·62), Asian series (1·05, 0·37–2·99; p=0·93), or Arab–Berber series (no PD cases, two [<1%] controls, Fisher's exact p=1·00) when the data were combined only for those variants predicted by use of the SIFT program to be not tolerated.[24] Webappendix p 26 provides a summary of variants for which there were no carriers in any of the three series.

Discussion

The results of our study, one of the largest so far of the genetics of PD, show that a single gene, *LRRK2*, harbours many rare and common variants that confer susceptibility to PD in diverse populations (panel). Although population stratification is an inherent caveat of this type of large-scale collaborative effort (and a potential limitation of the present study in the absence of genome-wide population control markers), these findings exemplify the confluence and independent effects of rare and common variations on gene loci that have a major effect in shaping both familial and sporadic disease.

About a third of variants we assessed were not identified in any study participant. These included four previously documented pathogenic mutations (LRRK2 N1437H, R1441G, Y1699C, and I2020T), showing that they are rare mutations in the population samples we assessed. 26 variants were recorded at a frequency greater than 0·5% in any of the three series, and only 13 were noted at a frequency greater than 0·5% in all three series. This finding draws attention to the importance of studying genetic variability in large samples and in different ethnic groups, because frequencies and genetic effects might vary substantially.[26]

The newly identified associations warrant further discussion. M1646T in the COR (C-terminal of Ras) domain of LRRK2 was identified in the white series, and the effect was consistent in many countries (figure 1). This variant was not identified in participants of Asian descent and was rare in the series of Arab–Berber participants. LRRK2 A419V was consistently more common in patients than in controls in Asian sites (figure 2). Although we cannot exclude the possibility of a non-coding element in linkage disequilibrium, the N-terminal region of the protein seems functionally relevant to disease development. LRRK2 M1646T is the first common-risk factor to have been identified in white populations, whereas A419V is now the third risk factor reported to be specific to individuals of Asian ancestry, along with R1628P and G2385R.[12,14,15] LRRK2 R1628P was not significantly associated with risk in our Asian series. This variant was common only in the Taiwanese series, in which a non-significant protective effect was noted. Our inability to replicate the previously reported risk effect of R1628P is likely to be due to a combination of the low frequency of this variant, natural sampling variation, and population heterogeneity, in view of the results of previous studies of ethnic Han Chinese populations (of note, G2385R did show association).[14,15]

The identification of a common three-variant haplotype (N551K-R1398H-K1423K) that seems to act in a protective manner (figure 3) is also important. It suggests that the reduced penetrance that is noted in patients with *LRRK2*-associated parkinsonism might be due to variants acting in cis or trans with the pathogenic variant and that LRRK2 activity can be exploited to modify symptom onset in patients. Any future therapeutic strategies that lower risk in *LRRK2*-associated parkinsonism might protect against symptomatic onset in idiopathic PD.[14,27] The previous report[14] of a protective effect with N551K and R1398H showed a reduced kinase

		White series		Asian series		Arab-Berber series	
	Aminoacid	Patients (n=6995)	Controls (n=5595)	Patients (n=1376)	Controls (n=962)	Patients (n=240)	Controls (n=372)
rs2256408	R50H	7 (0·10%)	1 (0·02%)	..	..	+	+
rs75054132	A75A	..	..	..	..	0	1 (0·27%)
rs33995463	L119P	21 (0·31%)	23 (0·44%)	..	..	0	2 (0·55%)
rs41286468	L122L	5 (0·08%)	7 (0·13%)	..	..	..	..
rs112794616	A211V	4 (0·06%)	11 (0·21%)	..	..	0	1 (0·27%)
rs56108242	C228S	2 (0·03%)	2 (0·04%)	..	..	..	..
rs28365216	N238I	..	..	3 (0·22%)	2 (0·22%)	..	..
rs72546315	H275H	3 (0·04%)	2 (0·04%)	..	..	1 (0·43%)	0
rs17490713	N289N	1 (0·01%)	2 (0·04%)	..	..	NA	NA
rs41286466	A312A	26 (0·38%)	15 (0·28%)	1 (0·7%)	0	0	4 (1·10%)
rs78501232	E334K	14 (0·21%)	4 (0·07%)	..	..	..	..
rs113065049	V366M	1 (0·02%)	0	..	..	..	..
rs34594498	A419V	5 (0·07%)	3 (0·06%)	+	+	..	..
rs35847451	S416S	12 (0·18%)	16 (0·29%)	..	..	..	..
rs75711334	L488L	1 (0·01%)	0	..	..	..	..
rs79996249	K544E	2 (0·03%)	2 (0·04%)	..	..	..	..
rs78154388	S663P	2 (0·03%)	2 (0·04%)	..	..	..	..
rs72546319	V674V	0	2 (0·04%)	..	..	0	1 (0·27%)
rs58559150	Q923H	1 (0·01%)	2 (0·04%)	..	..	+	+
rs75148313	S973N	1 (0·01%)	2 (0·04%)	..	..	..	..
rs113217062	I1006M	1 (0·01%)	0	..	..	..	..
rs76535406	S1096C	0	2 (0·04%)	..	..	..	..
rs35808389	L1114L	5 (0·07%)	1 (0·02%)	..	..	..	..
rs74985840	A1151T	1 (0·01%)	5 (0·09%)	..	..	..	..
rs80179604	S1228T	5 (0·07%)	4 (0·07%)	..	..	..	..
rs4640000	P1262A	1 (0·01%)	1 (0·02%)	..	..	NA	NA
rs72546338	R1325Q	10 (0·15%)	3 (0·06%)	4 (0·29%)	1 (0·11%)	..	..
rs17466213	I1371V	7 (0·10%)	4 (0·07%)	1 (0·07%)	0	+	+
rs72546327	T1410M	5 (0·07%)	1 (0·02%)	..	..	..	..
rs113589830	D1420N	1 (0·01%)	0	..	..	..	..
rs111435410	A1430A	2 (0·03%)	1 (0·02%)	..	..	..	..
rs112998035	R1441R	..	..	1 (0·07%)	0	..	..
rs33939927*	R1441C	10 (0·15%)	0	..	..	..	..
rs34995376*	R1441H	..	..	1 (0·07%)	0	..	..
rs74681492	P1446L	..	..	10 (0·74%)	6 (0·62%)	..	..
rs111501952	V1450I	..	..	2 (0·15%)	1 (0·11%)	..	..
rs113431708	R1483Q	1 (0·01%)	0	..	..	..	..
rs35507033	R1514Q	+	+	..	..	0	1 (0·27%)
rs33949390	R1628P	7 (0·10%)	0	+	+	..	..
rs35303786	M1646T	+	+	..	..	3 (1·25%)	2 (0·54%)
rs111503579	L1653L	2 (0·03%)	1 (0·02%)	4 (0·30%)	9 (0·93%)	..	..
rs79909111	S1721S	1 (0·02%)	1 (0·02%)	..	..	..	..
rs263192805	R1728H	1 (0·01%)	3 (0·05%)	..	..	..	..
rs35602796	M1869T	5 (0·07%)	2 (0·04%)	..	..	..	..
rs77428810	R1941H	2 (0·03%)	1 (0·02%)	..	..	..	..
rs34637584*	G2019S	48 (0·71%)	3 (0·06%)	1 (0·07%)	1 (0·11%)	72 (30·25%)	4 (1·10%)
rs111739194	L2063STOP	1 (0·02%)	2 (0·04%)	..	..	..	..
rs33995883	N2081D	+	+	2 (0·15%)	0	+	+
rs34869625	G2170G	20 (0·30%)	21 (0·39%)	..	..	1 (0·60%)	0
rs35658131	Y2189C	1 (0·01%)	2 (0·04%)	..	..	+	+
rs113511708	T2356I	7 (0·1%)	5 (0·09%)	..	..	..	..

(Continues on next page)

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