INVESTIGATION OF GENETIC LOCI ASSOCIATED WITH PARKINSON'S DISEASE AND THE FUNCTIONAL EFFECT OF LRRK2 MUTATIONS

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ABSTRACT

Parkinson's disease (PD) is a progressive movement disorder which results in bradykinesia, rigidity, resting tremors and postural instability. The role of genetics in modulating the risk of developing PD has been highlighted through genome wide association studies (GWAS) and candidate gene screening. This thesis explores the extent to which variants identified through studies in Caucasian populations are also relevant to the Malaysian PD population. Fourteen variants spanning several different loci including the *HLA-DRA*, *PARK16* and *GAK/DGKQ* loci and two genes, *GRN* as well as *LRRK2* were screened. The *HLA-DRA* locus had a protective association with an odds ratio (OR) of 0.76 while the variants in *PARK16* had OR values between 0.71 and 0.86. An association with *GRN* was not detectable in our Malaysian cohort but did show a significant risk association when a meta-analysis was performed with other ethnically matched PD cohorts. Five *LRRK2* mutations (G2385R, R1628P, A419V, N551K and R1398H) were screened. The G2385R and R1628P mutations were found to be risk factors. We excluded A419V as a risk factor, and determined it as a rare variant in the Malaysian population. The N551K and R1398H mutations showed significant protective effects with OR values of 0.623 and 0.699 respectively. As the postulated protective role of N551K and R1398H has not been fully characterised functionally, we sought to further investigate this effect. Constructs of LRRK2 mutations (G2019S, N551K and R1398H) were transfected into human neuroblastoma (SH-SY5Y) cells. In cell viability assays, cells carrying the N551K and R1398H mutations were found to confer greater protection when exposed to cellular stress under hydrogen peroxide treatment compared to G2019S. As a kinase, LRRK2 is known to activate pathways that are triggered by cellular stress, initiating a cascade of cell death. In keeping with this, cells carrying the risk factor G2019S mutation showed higher kinase activity and lower cell viability, while the protective factor R1398H had the lowest kinase activity and

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higher cell viability. In addition, although R1398H is located on a domain that is responsible for GTPase activity, our data suggests that there is no significant effect on the GTP binding ability. Although cells expressing protective factor N551K showed high cell viability, the kinase and GTP binding activity was unaltered suggesting that it may use alternative pathway to confer protection. Collectively, this thesis represents the first investigation into genetic loci for PD in Malaysia and has revealed some insight into how selected variants within *LRRK2* may influence protective mechanisms within neurons when exposed to cellular stress. Furthermore, while we are not yet at the stage of being able to provide diagnostic testing for late onset PD, the establishment of a Malaysian PD DNA bank achieved as part of this project will provide an invaluable resource for further genetic studies on the contribution of newly identified loci in our cohort.

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ABSTRAK

Penyakit Parkinson (PD) ialah penyakit gangguan pergerakan secara progresif yang menghasilkan simptom-simptom seperti bradykinesia, ketegaran, gegaran anggota ketika berehat dan ketidakstabilan postur badan. Penyakit Parkinson pernah dipercayai berpunca daripada kesan pendedahan kepada alam persekitaran, tetapi peranan genetik kini lebih diketengahkan melalui kajian mengaitkan seluruh genom dan saringan calon-calon gen. Tesis ini meneroka sejauh mana varian-varian genetik yang dikenalpasti melalui kajian di dalam golongan PD Kaukasian adalah juga relevan kepada golongan PD di Malaysia. Empat belas varian dari lokus-lokus *HLA-DRA*, *PARK16*, *GAK/DGKQ* dan dua gen, *GRN* dan *LRRK2* telah disiasat di dalam golongan PD Malaysia. Lokus HLA-DRA didapati berperanan sebagai faktor genetik yang melindungi daripada terjadinya penyakit PD dengan nisbah kemungkinan ('odds ratio', OR) bernilai 0.76 manakala varian-varian di dalam lokus *PARK16* mempunyai nilai OR di antara 0.71 dan 0.86 (faktor perlindungan). Varian di dalam gen *GRN* pula merupakan faktor risiko hanya apabila dikira secara meta-analisis dengan hasil kajian daripada negara lain, dan analisa dengan kalangan pesakit PD Malaysia sahaja tidak menunjukkan keputusan yang signifikan. Lima mutasi (G2385R, R1628P, A419V, N551K dan R1398H) di dalam gen *LRRK2* telah disiasat. Mutasi G2385R dan R1628P dikenalpasti sebagai faktor risiko. Kami telah mengecualikan mutasi A419V sebagai faktor risiko, dan menetapkan ia sebagai varian yang jarang berlaku di dalam golongan penduduk Malaysia. Mutasi N551K dan R1398H dikenalpasti sebagai faktor perlindungan dengan masing-masing mempunyai nilai OR 0.62 dan 0.69. Memandangkan peranan faktor perlindungan N551K dan R1398H tidak diketahui dengan sepenuhnya, kami telah menyiasat dengan lebih lanjut tentang peranan mereka dalam aspek ini. Konstruk *LRRK2* yang mempunyai mutasi G2019S, N551K dan R1398H telah dijana dan ditransfekasikan ke dalam sel-sel neuroblastoma manusia (SH-SY5Y). Apabila sel-sel

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tersebut diberi tekanan selular melalui pendedahan kepada hidrogen peroksida, sel-sel yang mempunyai mutasi N551K dan R1398H didapati lebih terlindung daripada akibat tekanan selular ini berbanding dengan mutasi G2019S. LRRK2 merupakan sebuah enzim kinas yang boleh mengaktifkan turutan selular yang mencetuskan kematian sel-sel. Selaras dengan ini, sel-sel G2019S menunjukkan tahap aktiviti kinas yang lebih tinggi dan bilangan sel-sel hidup yang rendah, manakala sel-sel R1398H menujukkan tahap aktiviti kinas yang paling rendah dan bilangan sel-sel hidup yang lebih tinggi. Di samping itu, walaupun mutasi R1398H terletak pada domain protin yang bertanggungjawab untuk aktiviti GTPase, data kami menunjukkan bahawa mutasi ini tidak memberi kesan ke atas keupayaan protin LRRK2 untuk mengikat molekul GTP. Untuk sel N551K pula, walaupun ujian bilangan sel-sel hidup bagi mutasi ini menunjukkan kesan perlindungan, tetapi tahap aktiviti kinas dan keupayaan mengikat GTP tidak berubah, menandakan bahawa ia mungkin menggunakan laluan alternatif untuk memberikan kesan perlindungan tersebut. Secara keseluruhannya, tesis ini merupakan siasatan pertama ke atas peranan lokus-lokus genetik untuk PD di Malaysia dan telah mendedahkan bagaimana beberapa variasi dalam *LRRK2* boleh mempengaruhi mekanisme perlindungan di dalam sel neuron apabila terdedah kepada tekanan selular. Tambahan lagi, walaupun kita belum berupaya menyediakan ujian diagnostik untuk PD yang bermula di usia lewat, tetapi penubuhan bank DNA pesakit PD di Malaysia yang tercapai melalui projek ini akan menyediakan sumber yang tidak ternilai untuk kajian genetik pada masa yang akan datang.

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LIST OF SYMBOLS AND ABBREVIATIONS

4E-BP	:	4E-binding protein
ArfGAP1	:	ADP-Ribosylation factor GTPase activating protein 1
ArhGEF7	:	Rho guanine nucleotide exchange factor 7
BAG	:	Bcl2-associated anthanogene
bp	:	base pair
CI	:	confidence interval
CO_2	:	carbon dioxide
COR	:	C-terminal of Ras of complex protein
DMEM	:	Dulbecco's Modified Eagle Medium
DNA	:	deoxyribonucleic acid
DTT	:	Dithiothreitol
DVL	:	Dishevelled
EDTA	:	ethylenediaminetetraacetic acid
EGFP	:	enhanced green fluorescence protein
EOPD	:	Early onset PD
ExoI	:	Exonuclease1
Fzl	:	Frizzled receptor
GAK	:	Cyclin G-associated kinase
GERP	:	Genomic Evolutionary Rate Profiling
GST	:	glutathione S-transferase
GWAS	:	genome wide association study
Hsp70	:	heat shock protein 70
HWE	:	Hardy-Weinberg equilibrium
Hz	:	Hertz

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IPTG	:	isopropyl-β-D-1-thiogalactopyranoside
JIPs	:	JNK-interacting proteins
JNK	:	c-Jun N-terminal kinase
Kb	:	kilo base pair
LRP5/6	:	lipoprotein receptor-like proteins 5 and 6
LRRK2	:	leucine-rich repeat kinase repeat
MAF	:	minor allele frequency
MAPKKK	:	mitogen-activated protein kinase kinase kinase
MBP	:	myelin basic protein
$MgCl_2$	:	magnesium chloride
miRNA	:	microRNA
MKK	:	mitogen-activated protein (MAP) kinase kinase
MPTP	:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	:	messenger ribonucleic acid
NaCl	:	sodium chloride
$NaVO_3$	:	Sodium orthovanadate
OR	:	odds ratio
ORF	:	open reading frame
PCR	:	polymerase chain reaction
PD	:	Parkinson's disease
Rab7L1	:	RAB7, member RAS oncogene family-like 1
Rac1	:	Ras-related C3 botulinum toxin substrate 1
RCLB	:	red cell lysis buffer
RGS2	:	regulator of G protein signalling 2
RNA	:	ribonucleic acid
ROC	:	Ras of complex protein

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rpm	:	revolutions per minute
S.E.M.	:	standard error of the mean
SNP	:	single nucleotide polymorphism
UPDRS	:	Unified Parkinson's Disease Rating Scale
UTR	:	untranslated region
V	:	Voltage

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CHAPTER 1: INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (Tanner & Goldman, 1996). It is a chronic and progressive disease with motor and non-motor symptoms primarily affecting older individuals over the age of 60, which are largely sporadic in nature. Approximately 10% of patients have a clear family history of PD, and the disease may appear at a younger age in these cases. It is more common in men than women (Van Den Eeden et al., 2003). PD is a complex disease that results from the interaction of multiple genetic loci and environmental factors (Farrer, 2006).

1.1 History

Although there have been earlier descriptions dating from 1000BC that provide observations suggestive of PD, James Parkinson was the first to medically describe PD as a neurological syndrome in 1817 (Factor & Weiner, 2007). Parkinson described the core symptoms such as tremors, shuffling and postural instability. However, it was insufficient to cover all aspects of this disorder (Goetz, 2011). Jean-Martin Charcot played an important role in further expanding and characterising certain distinct symptoms of PD such as bradykinesia. Charcot was also the first to suggest the term 'Parkinson's disease' and differentiated this disorder from other disorders that presented with tremors (Goetz, 2011). Subsequent to that, Hoehn and Yahr introduced a staging system that helped assess the severity of the disease that is still widely used today (Goetz et al., 2004).

Most PD cases occur above the age of 60 (known as late onset PD, LOPD), however there are cases that occur below the age of 40 and these are usually classified as early onset PD (EOPD). Approximately 20% of EOPD cases have a history of movement disorders in their family. There are slight differences in the clinical manifestations of

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EOPD and LOPD cases (Mehanna et al., 2014). In EOPD cases, the initial symptoms were more likely to be rigidity and painful cramps while in the LOPD cases, gait instability was more common. A common clinical manifestation that was found in both these groups was tremor. The EOPD cases were also more likely to develop certain non-motor symptoms such as depression and treatment-related problems such as motor fluctuations, dyskinesia and dystonia.

The underlying mechanism of PD was determined through an accidental discovery when patients who were administered an antipsychotic drug (reserpine) developed parkinsonism. In 1957, Carlsson discovered that reserpine depletes both serotonin and dopamine, and at that time dopamine was a relatively unknown neurotransmitter. Carlsson discovered that administering L-dopa intravenously to mice and rabbits could reverse the parkinsonism effects of reserpine (Carlsson et al., 1957). Dopamine was later found to be the main neurotransmitter in the striatum of the basal ganglia (Bertler & Rosengren, 1959; Sano et al., 1959) and brain autopsies of PD patients showed that the substantia nigra of the basal ganglia had severe loss of dopaminergic neurons (Scatton et al., 1983). The link between the damage in the substantia nigra and PD was further established when a group of young patients were found to have typical PD symptoms after self-administration of a narcotic derivative, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that selectively damages this part of the brain (Snyder & D'Amato, 1986).

1.2 Dopamine and the basal ganglia

The basal ganglia plays a key role in modulating motor control and is also thought to be involved in the brain's reward circuitry. The basal ganglia consists of three main regions, the corpus striatum, subthalamic nucleus and substantia nigra (Table 1.1). The corpus striatum consists of three major components namely the striatum (that consists of

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caudate nucleus and putamen), nucleus accumbens (also known as the ventral striatum) and globus pallidus (that can be subdivided into the internal and external region). The substantia nigra can be subdivided into the pars compacta and the pars reticulata.

Table 1.1: Components of the basal ganglia

Basal ganglia	**Corpus striatum**	**Striatum**
		- Caudate nucleus
		- Putamen
		Nucleus accumbens
		Globus pallidus
		- Internal
		- External
	Subthalamic nucleus	
	Substantia nigra	
	- Pars compacta	
	- Pars reticulata	

The basal ganglia is located in deep within the cerebral hemisphere and forms an intricate network with the cortex and thalamus. The cortical neurons project to the striatum to signal two pathways, the direct and indirect pathway (summarised in Figure 1.1).

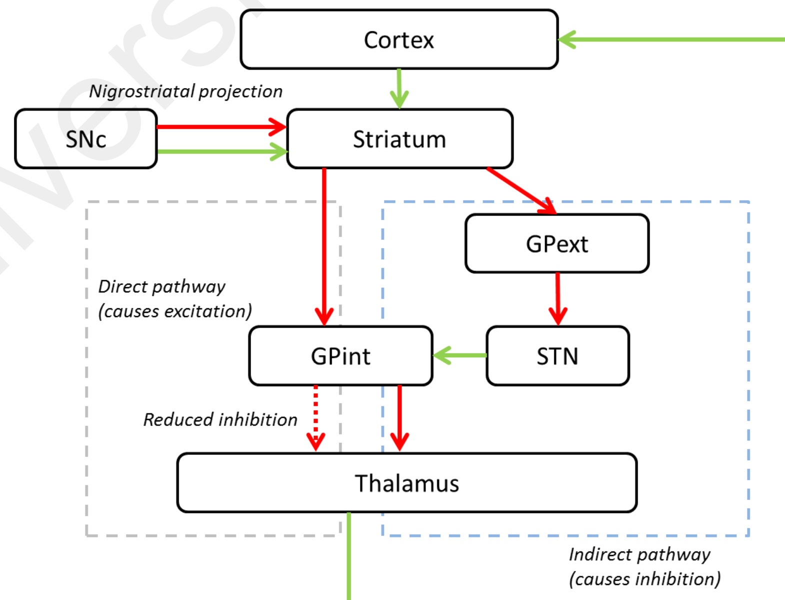

Figure 1.1: A summary of the basal ganglia circuit. The green arrows indicate excitatory effect while the red arrows represent inhibitory effect. Legend: SNc - substantia nigra pars compacta; GPint - globus pallidus internal; GPext - globus pallidus external; STN - subthalamic nucleus.

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The direct pathway starts with the excitatory projections of the cortical cells into the striatum. The excitation of striatum inhibits the firing of internal globus pallidus that results in the activation of motor cortex through the thalamus. While in the indirect pathway, the excitation of striatum neurons inhibits the external globus pallidus and subthalamic nucleus which in turn excites the internal globus pallidus. The excitation of internal globus pallidus inhibits the thalamus and therefore results in a motor cortex inhibition. The nigrostriatal projection that consists of the projection from the substantia nigra pars compacta to the striatum modulates both the direct and indirect pathway. It increases the excitatory effect of the direct pathway through the D1 receptors in the striatum and simultaneously able to reduce the inhibitory effect of the indirect pathway through the D2 receptors in the striatum. The neurons in the substantia nigra pars compacta produce dopamine. Dopamine plays an important role in movement coordination and reward-motivated behaviour (Wise, 2004).

In PD, this system is perturbed by the death of dopaminergic neurons in the substantia nigra pars compacta (Damier et al., 1999; Hornykiewicz, 2006). Fearnley and colleague showed neuronal loss in PD brains were concentrated in the pars compacta and the neurodegeneration is ten times greater as compared to a normal ageing brain (Fearnley & Lees, 1991). Figure 1.2 depicts the depigmentation in the substantia nigra of a PD brain. As PD brains are deprived of dopamine, the direct pathway is inhibited (motor cortex inhibited) and the inhibitory effect on the indirect pathway is in overdrive resulting in a total inhibition of movement. This is seen in the rigidity and difficulty in initiating movement in PD patients. Motor symptoms become evident when more than 70% of neurons in the substantia nigra are lost (Cheng et al., 2010).

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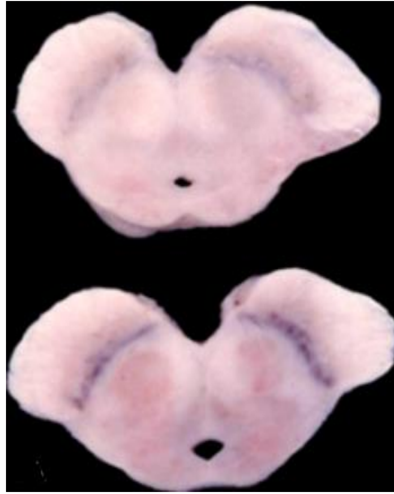

Figure 1.2: A cross section of the midbrain showing depigmentation of the substantia nigra in a PD brain (upper) in contrast to a normal brain (lower). Adapted from Fahn et al., 2013.

1.3 Diagnosis and treatment

A definite diagnosis of PD can only be confirmed upon post mortem examination of the brain. A study reviewing the clinical and pathological diagnosis of PD patients showed that the diagnosis of trained neurologists are >90% accurate (Hughes et al., 2001). Upon post mortem examination, PD is defined pathologically by the presence of Lewy bodies (LB) and degeneration of dopaminergic neurons (Marsden & Jenner, 1980). LB are abnormal cytoplasmic aggregations of proteins that primarily consists of α-synuclein, a presynaptic nerve terminal protein whose association to PD has been established (Figure 1.3). LB are predominantly found in sites where there is neuronal loss such as the substantia nigra. The number of LB found in mild to moderate neuron loss in the substantia nigra is higher than in patients with severe neuron loss suggesting that LB could precede neuron loss (Wakabayashi et al., 2007). However, using the presence of LB alone as a pathological definition of PD is problematic as LB can be detected in asymptomatic individuals (Markesbery et al., 2009) and a subset of EOPD patients with *PARKIN* mutations did not have LB (Morrison, 2003).

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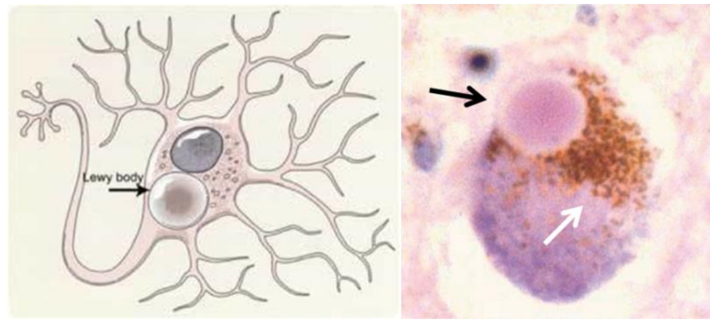

Figure 1.3: The picture on the left is a schematic diagram showing the location of the Lewy body and the picture on the right is a section of the substantia nigra containing neuromelanin (white arrow) and the black arrow indicate Lewy bodies in the cytoplasm of the neuron. Adapted from Hartmann, 2004.

The most effective and widely used treatment for PD currently is Levodopa. Levodopa (also known as 3,4-dihydroxyphenylalanine or L-dopa) is a precursor of dopamine that can cross the blood brain barrier. Levodopa is converted to dopamine through the enzyme dopa decarboxylase (Goetz, 2011). This increases the dopamine levels in the brain hence reducing PD symptoms (Carlsson et al., 1957). Other kinds of treatment options are surgical such as pallidotomy or deep brain stimulation. However these options are only explored in patients with severe or disabling motor fluctuations or dyskinesias. Pallidotomy is a procedure that lesions the globus pallidus in order to prevent over-inhibition to the thalamus, while deep brain stimulation is a procedure that implants electrodes into the affected area of the brain.

1.4 Causes of PD

The occurrence of PD is largely idiopathic and there are many hypotheses as to the underlying cause. Several of which are discussed below.

1.4.1 Environmental factors

Many epidemiological studies suggest an important role of environmental toxicants such as solvents, pesticides, metals and other pollutants as risk factors for developing PD (Goldman, 2014). Chronic exposure to pesticides can increase the risk of developing

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PD (Goldman, 2014; Lee et al., 2012). Evidence comes from studies on pesticides such as rotenone and paraquat which are analogs of a drug of abuse known as MPTP.

The self-administration of MPTP led to an important discovery of a causative agent of severe nigral damage and parkinsonism (Langston et al., 1983). MPTP crosses the blood brain barrier freely and is converted to MPP+, a proximate toxin that is taken up by the mitochondria. MPP+ causes the inhibition of Complex I and interferes with oxidative phosphorylation in the mitochondria (Przedborski et al., 2004). The perturbation of oxidative phosphorylation leads to excessive generation of free radicals as well as reduced ATP synthesis. Reduced ATP synthesis leads to lipid peroxidation and membrane disruption that finally results in cell death. Mitochondria are constantly transported throughout the axons and dendrites as synaptic transmission is dependent on high levels of ATP (Perier & Vila, 2012). Therefore the disruption in mitochondria function especially in the neurons could cause both cell death and impairment of the synaptic transmission.

A recent study pooling together 46 studies worldwide also further established that the use of pesticides and herbicides such as DDT, malathion, increases the risk of developing PD by 1.6 times (Goldman, 2014). However, environmental toxicants are likely to cause a small subset of cases as there are no geographical 'hotspots' of PD cases. In Malaysia, there are no obvious reports of PD clusters occurring despite being a country that is actively involved in both agriculture and manufacturing where there are high levels of toxicants present. Apart from these toxicants, chemicals such as food preservatives, colourings, flavourings as well as, well-water poisoning are important environmental factors that cause a chronic exposure (Chade et al., 2006). As dopaminergic neurons are particularly susceptible to oxidative stress, these toxicants may cause the selective loss of the cells in the brain.

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1.4.2 Traumatic head injury

Generally in PD, head injuries that are severe enough to cause a concussion or post concussive syndrome (such as personality change, amnesia, dizziness, visual problems, headache lasting more than two days post trauma) are considered. A study investigating the role of head injuries (that resulted in loss of consciousness, physician-diagnosed concussions, hospitalisation, fractured skulls, intracranial haemorrhage, convulsions or post-concussive syndromes), found a risk association between head injury and PD (OR 1.57, p=0.00001) (Jafari et al., 2013). This means that an individual with a head injury as described above is 1.5 times more likely to develop PD. One possible mechanism that has been hypothesised is that head injury increases the risk of PD through neuroinflammation. The impact on the head could cause the blood-brain barrier to be leaky resulting in leukocyte infiltration and microglia activation (Chodobski et al., 2011). When microglias are activated, they release proinflammatory cytokines and superoxides that are harmful to the dopaminergic neurons as they are especially susceptible to oxidative stress (Nagatsu & Sawada, 2005).

1.4.3 Infectious disease

The high incidence of gastric ulcers and other gastrointestinal problems in PD patients has ignited research into gastrointestinal health and PD. In particular, the bacterium *Helicobacter pylori* (HP) has been a prime suspect since serological HP tests were found to be more frequently positive in PD patients compared to controls (Charlett et al., 1999; Nielsen et al., 2012). Furthermore, PD patients with HP infection appear to have worse motor symptoms compared to PD patients without HP infection (Tan et al., 2015). HP infection is able to cause chronic systemic infection and generally persists throughout life unless specific eradication therapy is administered. It has been hypothesized that the infection triggers the activation of microglia through circulating proinflammatory cytokines or leukocytes that are able to enter the brain because of a

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compromised blood-brain barrier (Qian et al., 2010). Nevertheless the involvement of HP as a cause of PD is still not fully established. A large scale meta-analysis involving 202 studies to identify risk factors of PD could not identify an association between PD and HP infection (Noyce et al., 2012).

1.4.4 Genetics

Several discoveries have indicated a strong genetic component for PD, whereby several genes are linked to monogenic forms of EOPD including *SNCA, PARKIN, LRRK2, PINK1, DJ1,* and *ATP13A2* (Klein & Westenberger, 2012), Figure 1.4. *SNCA* and *LRRK2* have been implicated in autosomal-dominant forms while *PARKIN, PINK1, DJ-1* and *ATP13A2* are associated with autosomal recessive forms. EOPD accounts for up to 15% of the PD population.

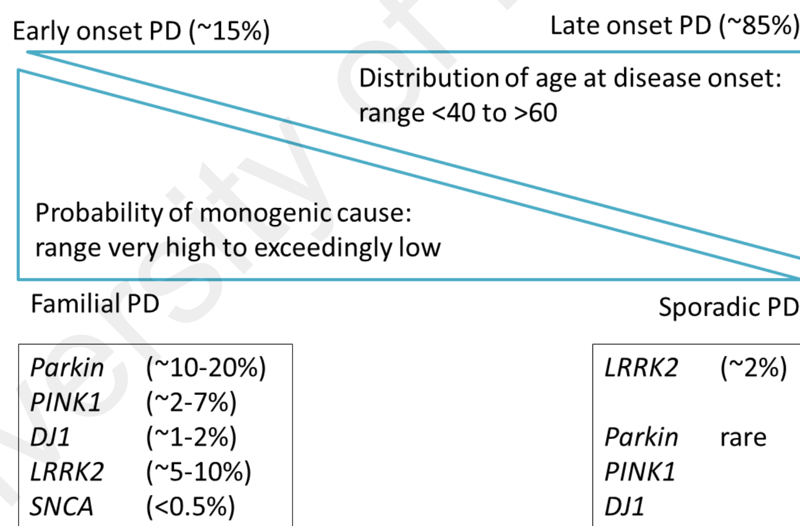

Figure 1.4: Patients with a younger age of onset have higher frequency of monogenic causes while it is rare to find apparent monogenic forms of late onset PD. Adapted from Klein et al., 2006.

1.4.4.1 Early-onset PD genetics

(a) ***SNCA***

SNCA (or α-synuclein) is a presynaptic protein that is expressed throughout the mammalian brain whose function is still unclear but is potentially involved in synaptic plasticity and neurotransmitter release (Bendor et al., 2013). Mutations in *SNCA* were

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first linked to an Italian kindred with autosomal dominant PD (Polymeropoulos et al., 1997). Point mutations, and less frequently, copy number variations have been reported (Klein & Westenberger, 2012; Lesage & Brice, 2009). Mutations in *SNCA* can cause misfolding, protein aggregation and inhibit proteosomal function (Bekris et al., 2010; Goedert, 2001).

(b) ***PARKIN***

Mutations in *PARKIN* were first identified in a Japanese family (Kitada et al., 1998) and are associated with autosomal recessive forms of PD. There are over 887 *PARKIN* mutations and it accounts for up to 77% of a particular form of EOPD which develops below the age of 30, and for 10-20% of EOPD as a whole (Klein & Westenberger, 2012; Lucking et al., 2000). Parkin is a cytosolic protein that is involved in E3 ubiquitin ligase. It conjugates ubiquitin to the lysine residues of targeted protein (Yoshii et al., 2011). Lee and colleagues showed that Parkin is able to induce mitophagy by promoting the ubiquitination of dysfunctional mitochondria while mutant Parkin was not able to detect and ubiquitinise defective mitochondria (Lee et al., 2010).

(c) ***PINK1***

The phosphotase and tensin homolog (PTEN)-induced kinase 1 (*PINK1*) gene has been linked to autosomal recessive PD (Valente et al., 2004). Overall, mutations in *PINK1* are reported to have a prevalence of between 2-9% in EOPD population throughout the world (Healy et al., 2004; Li et al., 2005; Tan et al., 2006). *PINK1* has a mitochondrial-targeting motif as well as a serine/threonine kinase domain. Most of the reported mutations are missense mutations in the highly conserved kinase domain implicating the role of kinase activity in PD (Li et al., 2005). PINK1 is localised in mitochondrial matrix and the intermembrane space. Wildtype PINK1 is able to protect

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the cells from stress-induced mitochondrial dysfunction and apoptosis, however mutant PINK1 cells are more susceptible to stress (Petit et al., 2005).

(d) ***DJ1***

Mutations in *DJ1* are very rare (1-2% of autosomal recessive EOPD) and are mostly missense mutations or deletions (Bonifati et al., 2003). DJ1 is a cytoplasmic protein that is able to translocate into the mitochondria under stress (Canet-Avilés et al., 2004; Junn et al., 2009). The oxidative stress-induced translocation of DJ1 from the cytosol to the mitochondria is part of its protective function against oxidative stress, suggesting that wildtype DJ1 could possibly function as a 'stress sensor' within cells (Junn et al., 2009).

(e) ***ATP13A2***

Mutations in *ATP13A2* causes Kurof-Rakeb syndrome, a form of autosomal recessive EOPD with rapid disease progression accompanied with dementia and pyramidal signs (Ramirez et al., 2006). *ATP13A2* encodes for a lysosomal membrane protein with 10 trans-membrane domain and an ATPase domain. Impaired ATP13A2 has been implicated in altered lysosomal function which causes accumulation of α-synuclein (Usenovic et al., 2012).

(f) ***LRRK2***

Leucine rich repeat kinase 2, (*LRRK2*) is a well-studied PD gene and is associated with early and late forms of PD. Mutations within *LRRK2* have been found to be equally present in both the EOPD and LOPD (Hedrich et al., 2006). Further details on *LRRK2* involvement in LOPD are covered in Chapter 4 and the functional involvement of LRRK2 is covered in the section 1.5.

In summary, research on PD genetics through studying families with EOPD has highlighted key pathways in mitochondrial activity, kinase activity and stress response.

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Nonetheless, EOPD only accounts for approximately 15% of all PD cases and there is a need to understand what is driving PD in the LOPD cases, so that better early diagnosis and intervention can be developed to delay or ultimately halt the progression of this disease.

1.4.4.2 Late-onset PD genetics

Studying LOPD genetics requires a different approach than studying EOPD. In LOPD, loci or genetic variants that possibly act as 'risk or susceptibility' factors for developing PD at a later age are investigated. To identify these genetic loci in unrelated individuals, large cohorts are required for statistical analysis and are typically discovered either through linkage analysis, candidate gene screening or genome wide association studies (GWAS). Currently there are more than 22 loci as well as 11 associated genes that have been identified (Corti et al., 2011; Nalls et al., 2014) including *UCHL1, LRRK2, PARK16, GAK* and *HLA-DRA* (Bekris et al., 2010; Hamza et al., 2010; Satake et al., 2009).

(a) ***Use of GWAS to identify susceptibility loci for complex diseases***

GWAS are conducted by comparing the frequency of common variants between case and control cohorts to determine whether particular variants are more frequent in the cases, thus assuming an association between that variant (and that genetic locus) with the disease. In most cases, the variant is just a marker for the genetic locus, and is not the causal mutation. Thus genes within a genetic interval that are in linkage disequilibrium with the variant will be screened for mutations. The measurement of association of a variant is statistically determined, as the analysis takes into account the frequency of the 'alternate allele' at that location between cohorts, inferring that a higher frequency in cases correlates with an association with that variant. As such, large cohorts are often used for better accuracy of statistical analysis. In a single GWAS,

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many different genetic loci may be identified indicating multiple possible susceptibility factors which fits into the ongoing theory that late-onset complex diseases are polygenic and likely to be driven by small perturbations in multiple genetic pathways, which may be further affected by external factors.

GWAS studies have been successful in identifying disease loci for many diseases including breast cancer and diabetes (Michailidou et al., 2013). Follow up studies are essential in determining the true association of the genetic loci identified through this approach and whether these loci are common susceptibility loci in populations across the world.

For PD, several GWAS linked loci have been identified including *PARK16*, *GAK/DGKQ* and *HLA-DRA*. Further description of the loci considered in this thesis is outlined in chapter 3.

(b) ***Candidate gene screening approach***

Candidate gene screening unlike GWAS, screens selected genes based on prior knowledge of their biological functions. The rationale behind this is to identify if a specific allelic variation could directly impact the function of the gene and hence lead to the phenotype observed. The selection of the gene is based on the known function, physiological and biological relevance to the disease. In this thesis, the *GRN* and *LRRK2* gene were considered.

i. GRN

The *GRN* gene encodes an 88-kDa secreted growth factor, progranulin which is involved in multiple physiological functions including wound healing, tumour growth, and embryonic brain development (Chang et al., 2013a). Mutations in *GRN* have been implicated in frontotemporal lobar degeneration where these patients exhibit

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parkinsonism (Rademakers et al., 2008). A SNP in the 3' untranslated region (UTR) of *GRN* was recently implicated in Taiwanese PD cases (Chang et al., 2013a). In addition to that, a 50% reduction of GRN function gave rise to neuronal cell death in substantia nigra (Shankaran et al., 2008). Mateo and colleagues in 2013 showed that PD patients had significantly lower levels of progranulin in the serum as compared to controls suggesting that there could be a possible protective mechanism inferred by progranulin against PD (Mateo et al., 2013). Delivery of progranulin protected nigrostriatal neurons in mice from MPTP toxicity (Van Kampen et al., 2014), further implicating its role in PD.

ii. LRRK2

Leucine-rich repeat kinase 2 (*LRRK2*) is the most extensively studied PD gene because it is associated with both EOPD and LOPD. Interestingly, mutations within *LRRK2* tend to be ethnic-specific, for example G2019S is found in Caucasian, Ashkenazi Jews and North African Arabs cases but not in the Asian PD population (Di Fonzo et al., 2006; Ozelius et al., 2006). There are currently 127 reported exonic mutations in *LRRK2* (discussed further in Chapter 4), a few of which have been established as pathogenic (G2019S, I2020T, Y1699C/G and R1441C/G/H), (PD Mutation Database, Bekris et al., 2010).

LRRK2 consists of seven domains namely armadillo (ARM), ankyrin (ANK) region, N-terminal leucine-rich repeat (LRR) domain, a ROC (Ras of complex protein) domain that shares sequence homology to the Ras-related GTPase superfamily, a COR (C-terminal of Roc) domain, a mitogen-activated protein kinase kinase kinase (MAPKKK) domain, and a WD40 domain. This 286kDa cytoplasmic protein has also been associated with the outer mitochondrial membrane (Paisán-Ruiz et al., 2013). The function of this protein in the cell is unclear however the ROC and kinase domain have

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shown GTPase and kinase activities respectively (Li et al., 2014). As this thesis reports the functional aspects of certain LRRK2 mutations, what is currently known about LRRK2 function is reviewed in the next section (Section 1.5).

1.5 Current research on the functional aspects of LRRK2

Despite almost a decade since it was first linked to PD, the function of LRRK2 is still not clearly understood. The challenge in understanding its function is compounded by the fact that it is a large multidomain protein and that it has two enzymatic activities, namely the GTPase and kinase activities. PD researchers across the world have used many different models to study the function of LRRK2 including cell lines, *Caenorhabditis elegans*, *Drosophila melanogaster*, Zebrafish (*Danio rerio*), rodents and recently, induced pluripotent stem cells (iPSC) from PD patients (Nguyen et al., 2011).

Figure 1.5 summarises the interactions of LRRK2. LRRK2 has been found to have multiple cellular roles such as interaction with the cytoskeleton, synaptogenesis, within the mitochondria and also involved in activating the stress-induced pathway. In summary, most of the interactions of LRRK2 reviewed below suggest that LRRK2 either acts as a scaffold protein or its interactions are dependent on its GTPase and kinase function.

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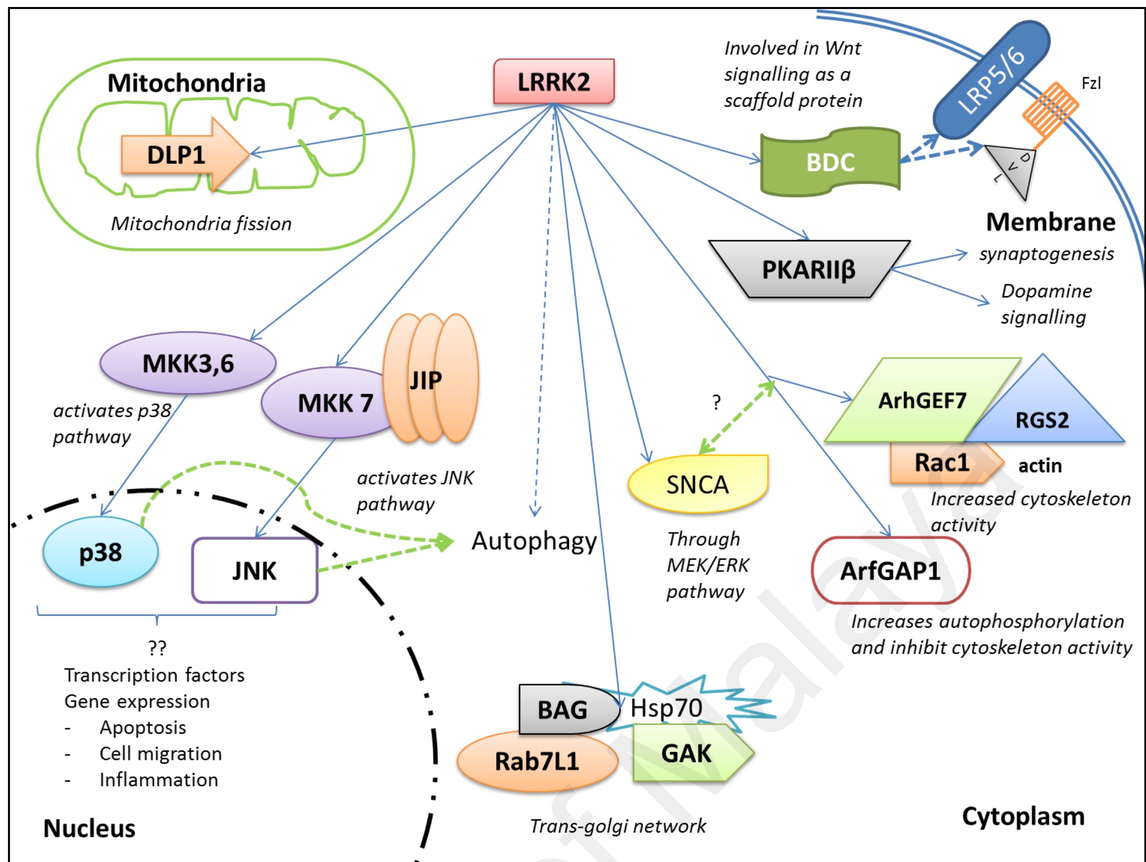

Figure 1.5: An overview of LRRK2 interactions

1.5.1 LRRK2 interaction with the cytoskeletal proteins

The regulation of cytoskeletal proteins is vital to cells especially in neurons for development, trafficking, synapse formation and maintenance. Neurite outgrowth is achieved through the dynamic network of actin and microtubules. LRRK2 interacts with cytoskeletal proteins such as actin and tubulin (Parisiadou & Cai, 2010). In neurons and fibroblast carrying G2019S (an established risk factor) mutation, increased amount of F-actin was reported (Parisiadou et al., 2009). Caesar and colleagues proposed that the increased amount of F-actin was to compensate the depolymerising effect of G2019S on actin (Caesar et al., 2015). The same study also showed that the sensitivity towards actin depolymerising stimuli was increased in mutant LRRK2 in an age-dependent manner.

LRRK2 has been shown to co-localise with tubulin in cells and primary hippocampal neurons (Parisiadou & Cai, 2010). Mutant LRRK2 has also shown to inhibit the protrusion of microtubules resulting in decreased neurite outgrowth. The possible mechanism is through the kinase and GTPase activity of LRRK2 together with the interaction of key players such as RGS2 (regulator of G protein signalling 2), ArhGEF7, Rac1 and ArfGAP1 (Chan et al., 2011; Stafa et al., 2012). RGS2 interacts with LRRK2 to rescue neurite shortening, concurrently ArhGEF7 a protein that increases LRRK2 GTPase activity induces LRRK2 neurons to increase neurite length. Both these proteins reduce LRRK2 kinase activity (Boon et al., 2014). In contrast, ArfGAP1 increases LRRK2 autophosphorylation and inhibits neurite outgrowth *in vitro*.

It is unknown why neuron death is specific to dopaminergic neurons in PD despite LRRK2 being expressed in other cell types. Figure 1.6 depicts LRRK2's interaction with other proteins to affect neurite outgrowth.

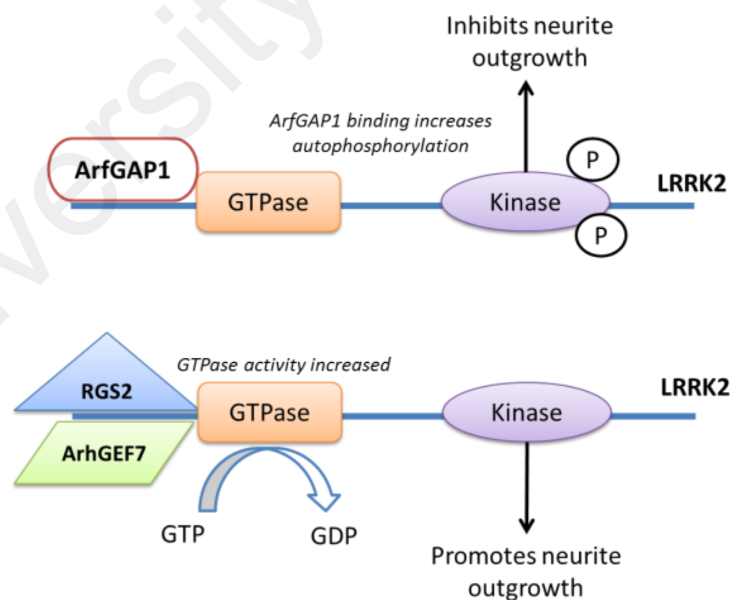

Figure 1.6: Schematic diagram of LRRK2 involvement in neurite outgrowth

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1.5.2 The role of LRRK2 in the Wnt signalling pathway

LRRK2 has been shown to interact with lipoprotein receptor-like proteins 6 (LRP6) and dishevelled (DVL), key players of the Wnt signalling pathway (Berwick & Harvey, 2013). Wnt signalling is highly conserved and controls numerous events such axon guidance, neuronal maintenance, synapse formation as well as embryonic development (Komiya & Habas, 2008). DVL, β-catenin, glycogen synthesis kinase 3 (GSK3) and LRRK2 have been shown to interact together to form the β-catenin destruction complex (BDC) (Berwick & Harvey, 2013). In the absence of LRRK2, the BDC is not formed and the β-catenin accumulation leads to gene transcription, highlighting LRRK2's role in this pathway (Figure 1.7).

Exposure to toxins that result in dopaminergic cell death (such as 6-hydroxydopamine, 6-OHDA) can alter expression of components within the Wnt signaling pathway (Ohnuki et al., 2010; Wei et al., 2013). In addition, the development of dopaminergic neurons in the midbrain has been shown to be Wnt dependent. Induced Wnt5a expression in the ventral midbrain neural stem cells have shown to be able to generate 10-fold more dopamine neurons, further implicating this pathway and PD (Verma et al., 2014).

LRRK2 mutation (R1441C/G and Y1699C) showed decreased binding ability with DVL and LRP6, hence interrupting β-catenin accumulation (Berwick & Harvey, 2012; Sancho et al., 2009). In addition as the Wnt signalling plays a key role in neuronal patterning, it is tempting to speculate that mutant LRRK2 could cause subtle defects in the neuron network hence making it more susceptible to cell death.

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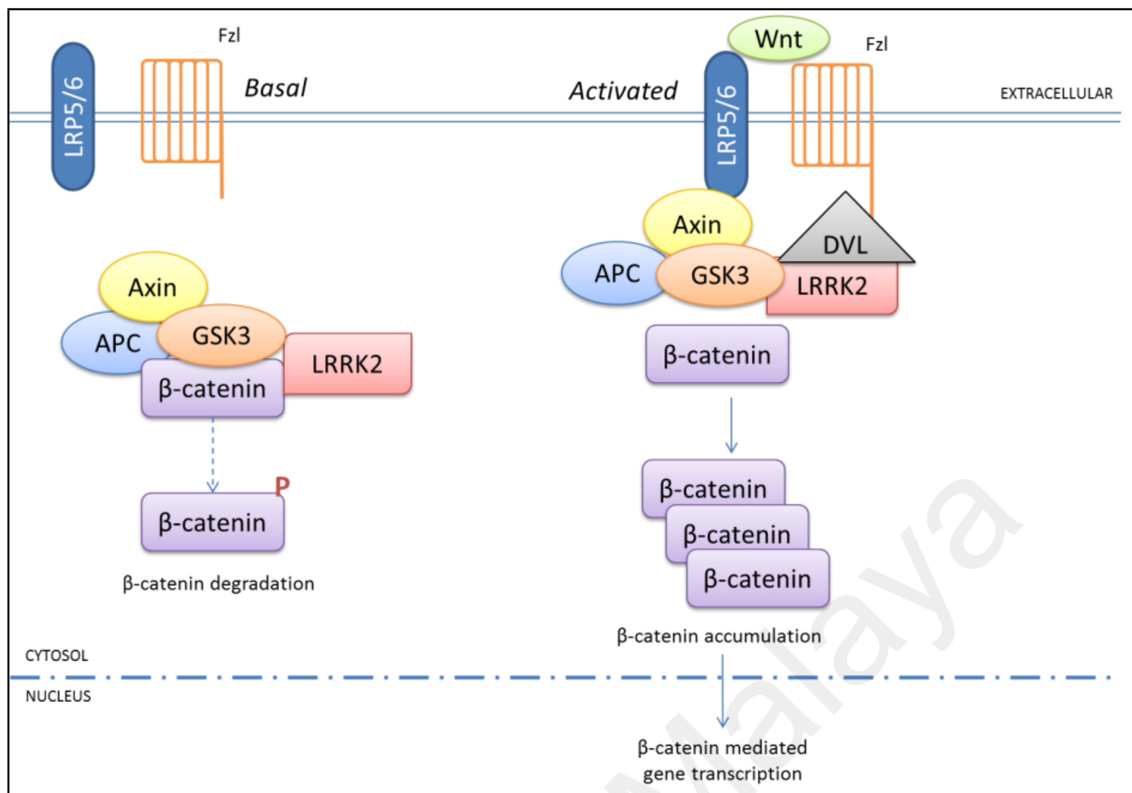

Figure 1.7: The Wnt canonical pathway and LRRK2 involvement. APC, adenomatous polyposis coli; DVL, dishevelled; Fzl, frizzled receptor; GSK3, glycogen synthase kinase 3; JNK, c-Jun n-terminal kinase; LRP5/6, low density lipoprotein receptor-related protein 5/6.

1.5.3 The role of LRRK2 in synaptogenesis

As explained in Section 1.2, the neurons within the striatum are important in the direct and indirect excitatory pathway. LRRK2 has been shown to be expressed in these neurons (Mandemakers et al., 2012). Altered dendritic spines morphology and synaptic transmission in the striatum neurons were observed in knockout LRRK2. This is similar to what is observed in PD brains suggesting LRRK2's role in synaptogenesis may lead to PD (Parisiadou et al., 2014).

In keeping with this hypothesis, a recent study implicated LRRK2 in synaptogenesis through the regulation of protein kinase A (PKA) (Li et al., 2011) in this pathway. The ROC domain of LRRK2 has been shown to selectively bind to PKARIIβ, a PKA subunit. PKA in turn regulates Cofilin, a critical regulator of spine formation. Knock-

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out LRRK2 mice showed significant increase in the spine head diameter and increased phosphorylated cofilin, suggesting that LRRK2 negatively regulates the spine formation (Parisiadou et al., 2014) and confines PKARIIβ to the dendritic shafts. This restriction causes a decrease in the spine area and is also necessary for the regulation of other PKA domain activity during excitatory synaptic transmission. The LRRK2 R1441C mutation in the ROC domain, showed weakened PKARIIβ binding and increased amounts of PKARIIβ in the dendritic spines (Muda et al., 2014). This proposes that LRRK2 plays a role in synaptogenesis and altered synaptic transmission due to compromised synapses.

1.5.4 The role of LRRK2 interacting proteins

Out of the seven LRRK2 domains, four domains have protein-protein interaction motifs and studies have shown that LRRK2 binds to proteins involved in the Golgi complex and α-synuclein.

In the trans-Golgi network, LRRK2 interacts with Rab7L1 (RAB7, member RAS oncogene family-like 1), cyclin G-associated kinase (GAK) and Bcl2-associated anthanogene (BAG) (Beilina et al., 2014). In particular, Rab7L1 has a single Ras-like domain that has a GTP binding ability which allows LRRK2 binding (Wang et al., 2014). As discussed in chapter 3, GAK is associated with LOPD and is differentially expressed in PD brains (Grünblatt et al., 2004). Meanwhile, BAG5 binds to the COR domain of LRRK2 and in turn, GAK binds to BAG5 via the Hsp70. Thus, LRRK2 forms a complex within these proteins to promote trans-Golgi network clearance. Figure 1.8 depicts a graphical representation of the complex.

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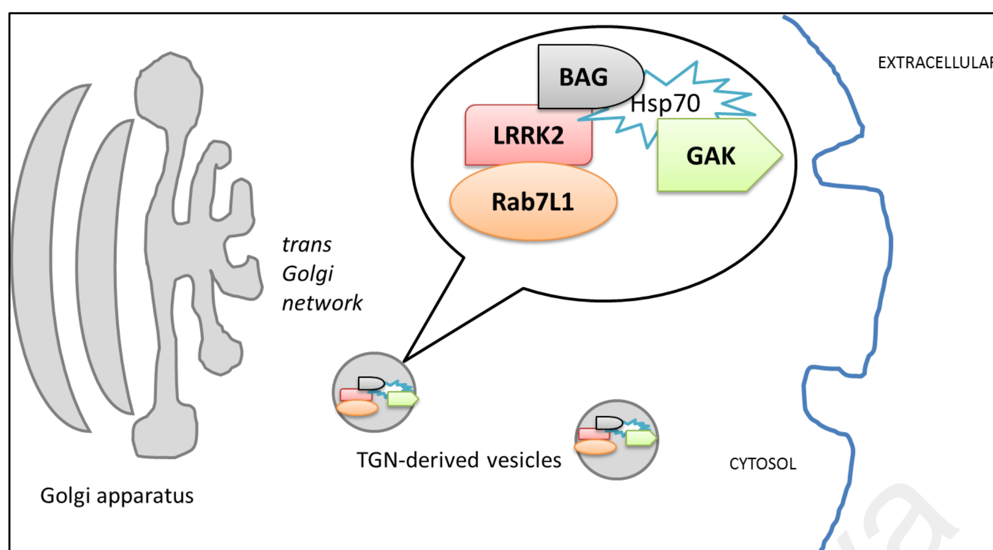

Figure 1.8: Schematic diagram of LRRK2 interaction with other proteins found to promote trans-Golgi network (TGN) clearance

Besides interacting with proteins that are involved in the Golgi network, LRRK2 has been shown to interact with α-synuclein (Guerreiro et al., 2015). However, it is still unknown if both these proteins bind directly to one another or through other protein-protein binding. In other studies as reviewed in Boon et al., 2014, LRRK2 is able to up-regulate α-synuclein's transcription through the MEK/ERK activation (Boon et al., 2014).

1.5.5 The role of LRRK2 in JNK and p38 pathways in stress-induced neurodegeneration

Both JNK and p38 belong to the MAPK (mitogen-activated protein kinase) family that are activated through phosphorylation in the presence of stress as well as inflammatory cytokines. The activation of JNK and p38 can alter gene expression and lead cell either towards apoptosis, cell migration or inflammation based on the type of stress.

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The kinase domain in LRRK2 is homologous to the receptor interacting kinase (RIP) family of proteins. The RIP protein regulates stress kinase cascade and responses to death receptors through the binding of MKKs (mitogen-activated protein kinase kinase). LRRK2 is able to activate the p38 and JNK pathway through the binding of LRRK2 to MKK3, 6 and 7 (Hsu et al., 2010) and JNK-interacting proteins (JIP).

1.5.6 The role of LRRK2 in mitochondrial function

There is increasing evidence that support the idea that oxidative stress causes dopaminergic cell death in PD. The mitochondria are key free radical producers and it controls important function such as calcium homeostasis and initiation of cell-death pathways.

Compromised mitochondria dynamics in relation to fusion and fission activities have also been implicated in PD. Fusion events take place when the cells are under stress while fission occurs when the cells are not under stress. Fission segregates possible damaged mitochondria. In two independent studies, LRRK2 was shown to interact with both Fis1 and Dynamin-like protein-1 (DLP1), two critical proteins in mitochondria fission (Niu et al., 2012). Studies with the LRRK2 K1347A mutation (located in the GTP-binding domain) and the D1994A mutation (resulting in a complete loss of kinase activity) had less interaction with DLP1 suggesting that this interaction is mediated through a GTPase dependent pathway and involves kinase activity (Henchcliffe & Beal, 2008; Wang et al., 2012).

C. elegans mutants carrying G2019S did not show protection against mitochondrial dysfunction induced by rotenone and paraquat (Saha et al., 2009). Studies conducted on SHSY-5Y cell lines as well as primary cortical neurons showed that LRRK2 increased mitochondria fragmentation especially in the presence of G2019S and R1441C mutants.

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1.5.7 The role of LRRK2 as a kinase

The kinase domain in LRRK2 is located between residues 1879-2138 (Figure 1.9). Every kinase has an activation loop consisting of a 20-35 residue sequence between the conserved tripeptide motifs of DF/YG and APE motif. The established risk factor G2019S is located on the N-terminal of the DF/YG motif and the higher kinase activity seen with G2019S is thought able to cause neurotoxic effects (Smith et al., 2006).

The kinase function of LRRK2 has been shown to regulate a number of events such as the neurite outgrowth (as briefly discussed in Section 1.5.1 and 1.5.5). As a kinase, LRRK2 can function as an enzyme that transfers the terminal phosphate (ɤ position) of ATP to a target protein (substrate), which in turn can initiate downstream signalling cascades such as the JNK and p38 pathways.

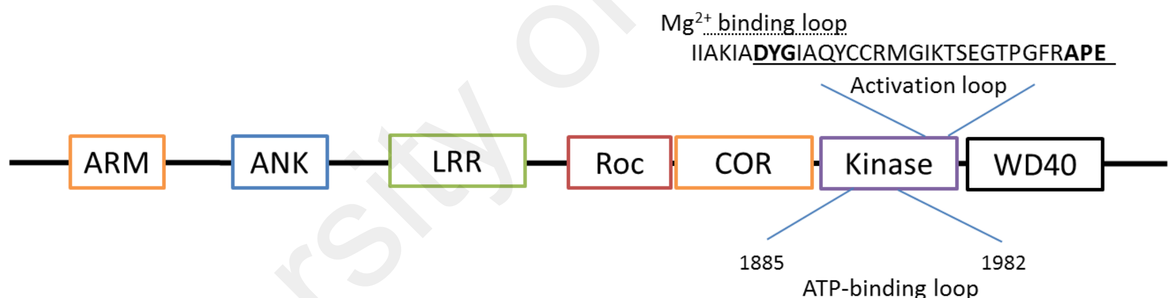

Figure 1.9: Schematic diagram of LRRK2 showing the location of the activation loop and the ATP-binding pocket of LRRK2. The glycine-rich loop that functions as the ATP-binding pocket of LRRK2 is located between residues 1885-1982, while residues 2017-2043 are the activation loop of LRRK2

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1.6 Research questions

In Malaysia, it is estimated that 15,000 to 20,000 individuals suffer from PD at any given time (Malaysian Parkinson Disease Association, http://www.mpda.org.my/). The advancement of the medical practice has greatly improved the life expectancy of the elderly in Malaysia, where the average life expectancy is 74.5 years (World Health Organisation, 2014), and the projected number of individuals above 60 will be approximately 15% of the total population (Malaysian Department of Statistics, http://pqi.stats.gov.my/searchBI.php) in 2035. Hence, with the increase in the number of elderly individuals, an increase in the number of PD patients can be expected. Dorsey et al., 2007 projected that by 2030, there will be a 100% increase in PD cases. Therefore, there is a need to identify if the PD genes implicated in other populations are also the cause of PD in Malaysian population, so that same drugs or treatment will be equally effective in this population.

In this thesis, we sought to investigate the association of two genes; *LRRK2* and *GRN* (a total of 6 variants) and three GWAS loci (*HLA-DRA*, *GAK/DGKQ* and *PARK16* locus) (8 variants) in the Malaysian population as it is an understudied population. It is important to validate reported loci/variants in different populations across the world, as in PD-linked loci there is evidence of ethnic-specific inheritance for some of the polymorphic variants (Bekris et al., 2010). The identified susceptible variants can be used to increase the accuracy of risk prediction and help improve disease diagnosis.

Although the functional work thus far gave us insights into how the risk mutations such as G2019S and R1441C could render a pathogenic role onto LRRK2, it does not provide us with data that is reflective of what is happening in Asian populations as these mutations are not common in these ethnic groups. Therefore this thesis also aims to

understand the possible functional effects of selected LRRK2 mutations which are associated with Asian PD.

1.6.1 Hypothesis and objectives

This thesis hypothesises that the presence of particular genetic variants can increase or decrease susceptibility for developing Parkinson's disease and, the effect of the genetic variants can be observed at a cellular level.

The objectives of this project are as below:

1. To investigate the association between GWAS-linked loci (*HLA-DRA*, *PARK16* and *GAK/DGKQ*) and PD in the Malaysian population.
2. To explore the association of the *GRN* variant with the Malaysian PD population
3. To investigate the susceptibility of selected mutations within *LRRK2* in the Malaysian PD population.
4. To examine the protective effect of N551K and R1398H mutations in *LRRK2* function in the following aspects
   i) Cell viability in the presence of cellular stress
   ii) Kinase activity
   iii) GTP binding

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CHAPTER 2: MATERIALS AND METHODS

The first part of this chapter describes the methods in the genetic analysis of the variants studied (section 2.1 to 2.6) while the second part describes the methods used for the functional aspect of the work (section 2.7 onwards)

2.1 Ethics approval

This project was approved by the University of Malaya Ethics committee (MERC 732.5) and the National Medical Research Register (NMRR-10-935-7179). The purpose of the project was explained to each subject and a written consent was obtained. Twenty millilitres of peripheral blood was collected in EDTA tubes by trained phlebotomists. In the event a trained phlebotomist was not available, mouthwash or saliva was obtained from the subject. The ethnic history for three generations was obtained in order to avoid racial admixture as some analyses were ethnic based.

2.2 Selection criteria

2.2.1 Parkinson's disease patients

Subjects suffering from PD were examined by neurologists and diagnosed based on the United Kingdom Brain Bank Criteria (Table 2.1). Briefly, the criteria include bradykinesia presented with muscular rigidity, 4-6 Hz resting tremor or postural instability, and the patients were responsive to Levodopa. Subjects were recruited from nine collection sites throughout Malaysia (4 sites in the Klang valley, 2 sites in Penang and one site each in Terengganu, Perak and Sarawak).

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Table 2.1: The diagnosis criteria of PD adapted from the United Kingdom Brain Bank Criteria (Hughes et al., 1992).

Step 1: Diagnosis of Parkinsonian Syndrome
• Bradykinesia • At least one of the following (muscular rigidity, 4-6Hz rest tremor, postural instability not caused by primary visual, vestibular, cerebellar or proprioceptive dysfunction)
Step 2: Exclusion criteria for PD
• History of repeated strokes • History of repeated head injury • History of definite encephalitis • Oculogyric crises • Neuroleptic treatment at onset of symptoms • Sustained remission • Strictly unilateral features after 3 years • Supranuclear gaze palsy • Cerebellar signs • Early severe autonomic involvement • Early severe dementia with disturbances of memory, language and praxis • Babinski sign • Presence of cerebral tumour or communication hydrocephalus on imaging study • Negative response to large doses of levodopa in absence of malabsorption • MPTP exposure
Step 3: Supportive prospective positive criteria for PD
Three or more required for diagnosis of definite PD in combination with step one • Unilateral onset • Rest tremor present • Progressive disorder • Persistent asymmetry affecting side of onset most • Excellent response to levodopa (70-100%) • Clinical course of ten years or more

2.2.2 Unaffected controls

The controls consisted of individuals who were over 50 years old, free from any neurological conditions and without a family history of PD. It was important to match the age of the control cohort as closely to the PD cohort in order to avoid population stratification.

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2.3 Deoxyribonucleic acid (DNA) extraction

2.3.1 DNA extraction from blood

The DNA of 1,096 PD patients and 418 controls were extracted from blood for the genetic analysis. DNA was obtained from leukocytes using the phenol-chloroform extraction method (Miller et al., 1988). Twenty millilitres of blood was decanted into a 50ml tube and topped up with 30ml of red cell lysis buffer (0.32M sucrose, 10mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 1% Triton X-100). The solution was mixed by gentle inversion and incubated on ice for 5 minutes with occasional mixing. The tube was centrifuged at a speed of 3,700 rpm for 10 minutes at 10°C. The supernatant was discarded and the pellet was reconstituted in 40ml of red cell lysis buffer. The tube was centrifuged again at 3,700 rpm for 10 minutes at 10°C. The supernatant was discarded again and the pellet was reconstituted in 400µl of lysis buffer (consisting of 0.1M NaCl and 2% SDS in Tris-EDTA) and 20µl of 20mg/ml Proteinase K (Thermo Fisher Scientific). The pellet was incubated overnight at 37°C and shaken at 80 rpm to digest the pellet.

After overnight incubation, 200µl of 4M NaCl was added to the digested pellet and vortexed for 30s. One millilitre of phenol-chloroform (Amresco) was added to the tube and mixed vigorously till a milky appearance was observed. The solution was transferred into a sterile 1.5ml tube and centrifuged at 13,000 rpm for 25 minutes, 10°C. The aqueous phase was transferred into a new 1.5ml tube where 900µl of cold absolute ethanol was added. White strands of DNA precipitation were observed when the tube was inverted. The tube was then centrifuged at 13,000 rpm for 5 minutes, after which the supernatant was discarded.

The washing step of the pellet involved the addition of 500µl 70% ethanol and the tube was spun at 13,000 rpm for 3 minutes. The supernatant was discarded and the

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DNA pellet was dried using a DNA vacuum (Heto Holten DNA Mini Centrifugal Evaporator) for 5 minutes. DNA was solubilised in 100µl of sterile water. The concentration of DNA was determined using Nanodrop 2000 (Thermo Scientific) and a working DNA stock of 20ng/µl was prepared for every sample.

2.3.2 DNA extraction from mouthwash

Subjects were told to rub their cheeks against their teeth for 15s before rinsing their mouth with 10ml sterile 0.9% saline provided in 50ml sterile tubes. The cheek cells in the mouthwash were pelleted by spinning the tube at 3,000 rpm for 15 minutes at 10°C. The supernatant was discarded, the pellet was washed with 25ml of Tris-EDTA buffer. The tube was centrifuged again at 3,000 rpm for 15 minutes at 10°C. The supernatant was discarded and 400µl of lysis buffer (consisting of 0.1M NaCl and 2% SDS in Tris-EDTA) and 35µl of 20mg/ml proteinase K was added to the cell pellet. The solution was mixed vigorously and incubated overnight at 37°C, while continuously shaken at 80 rpm.

After overnight incubation, 450µl of phenol-chloroform was added into the tube and the solution was mixed vigorously till a milky appearance was observed. The solution was centrifuged at 3,500 rpm for 10 minutes. The aqueous phase was transferred into a new 1.5ml tube and another 450µl of phenol-chloroform was added into the tube. The solution was mixed vigorously and centrifuged at 13,000rpm for 25 minutes. The aqueous phase was transferred into a new 1.5ml tube where 45µl of 4M NaCl and 900µl of cold absolute ethanol was added. Upon gentle mixing, whitish strands of DNA were visible. The tube was centrifuged at 13,000 rpm for 5 minutes and supernatant discarded. The DNA pellet is washed in the similar way as described for the DNA extraction from blood. One hundred and eighty five PD patients as well as 171 control samples were extracted from mouthwash for the genetic analysis.

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2.4 Genotyping the candidate SNPs by Taqman SNP assays

Single nucleotide polymorphisms (SNPs) examined in this study were genotyped using Taqman® allelic discrimination assays. Genotyping was carried out on a 7500 Fast Real-Time PCR machine (Applied Biosystems) using standard protocols as recommended by the manufacturer. The genotyping was conducted 'blind' to disease status on all the samples. Below are the fourteen SNPs examined in this study.

i) ***HLA-DRA***, rs3129882, G>A

ii) ***PARK16*** (rs947211, G>A; rs823128, A>G; rs823156, A>G; rs11240572, C>A; rs16856139, C>T)

iii) ***GAK/DGKQ*** (rs11248051, C>T; rs1564282, C>T)

iv) ***GRN***, rs5848, C>T

v) ***LRRK2*** [G2385R (rs34778348), G>A; R1628P (rs33949390), G>C; A419V (rs34594498), C>T; N551K (rs7308720), C>G; R1398H (rs7133914), G>A]

2.5 Error determination by Sanger sequencing

Genotypes were confirmed by polymerase chain reaction (PCR) and Sanger sequencing in a random subset of individuals to determine the error rate for each of the Taqman SNP assays listed in Section 2.4. The primers and annealing temperature used are stated in Appendix A. The PCR products were electrophoresed on 1.5% agarose gels containing 3X GelRed (Biotum) at 100V for half an hour, and the amplicons were purified using Exonuclease1 (ExoI) enzyme (Thermo Scientific). Cycle sequencing was conducted on purified PCR products to incorporate fluorescent dideoxynucleotide for the detection on a capillary sequencer. Sample preparation for Sanger sequencing is described in the section below.

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The genotype obtained from the electropherogram (Appendix J) was compared with the genotype that was scored through the Taqman® SNP genotyping assay (an example of the genotype scored through SNP genotyping can be found in Appendix D). If the genotypes did not match, this was considered to be a 'false genotype call' and calculated as an 'error rate'. The formula used to calculate the error rate was as below:

$$Error\ rate\ (\%) = \frac{Number\ of\ false\ genotype\ calls}{Total\ number\ of\ chromosomes\ screened} \times 100\%$$

2.5.1 PCR product purification by ExoI

PCR product clean-up was done using the Exonuclease1 (ExoI) enzyme (Thermo Scientific). ExoI degrades excess single-stranded primers present in the PCR product. This was carried out by adding 5µl of PCR product to 0.5µl of ExoI (20 U/µl) as well as 1µl of FastAP (Thermo Scientific) and incubated in a thermocycler for 15 minutes at 37°C followed by another 15 minutes at 80°C to terminate the reaction.

2.5.2 Cycle sequencing and purification of cycle sequencing products

Cycle sequencing was carried out using BigDye® Terminator v3.1 (Applied Biosystems) and its recommended protocol provided by the manufacturer. The purification method used for the cycle sequencing product was ethanol, EDTA and sodium acetate precipitation. A detailed protocol of the purification step was also provided by the manufacturer. Once the sample was purified and dried, 12µl of Hi-Di^{TM} Formamide (Applied Biosystems) was used to reconstitute the samples before loading onto the capillary electrophoresis (3100 Genetic Analyser, Applied Biosystems). The electropherograms were analysed using an open-source software, Chromas Lite.

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2.6 Statistical analysis of the genetic data

The Fisher's exact test was performed to verify whether the SNP genotypes conformed to the Hardy–Weinberg equilibrium (HWE). Odds ratio (OR) and the corresponding 95% confidence interval (CI) were used to estimate the effect of the individual SNPs in the development of PD. The precision of the calculated OR is estimated by the 95% CI, where a large CI indicates a low level of precision of the OR and vice versa. Analyses were done using R version 2.11.1 (http://www.Rproject.org) and an open-source software (OpenEpi).

2.6.1 Odds ratio analysis

The odds ratio (OR) is a measure of the association between the mutant allele (exposure) and developing PD (outcome). An OR of more than 1.0 indicates a risk association while an OR of below 1.0 indicates a protective association. An OR equal to 1.0 indicates that the exposure does not affect the risk of developing PD.

2.6.2 Logistic regression analysis

Logistic regression analyses the relationship between a categorical or binary dependent variable (controls or cases) and multiple independent variables (such as alleles, race or gender). This regression also estimates the probability of an event occurring (developing PD). Below are some of the models of how the alternative ('mutant') allele can be associated with developing PD.

a) Dominant

A dominant model is when inheriting one allele is sufficient to increase the risk of developing the disease.

b) Recessive

Recessive model in a disease is when the risk of the disease is present only when both alleles are present.

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c) Additive

Additive modelling in disease association studies is when the risk of an allele is increased by b (an arbitrary value) for heterozygotes and $2b$ for homozygotes.

2.6.3 Meta-analysis

Meta-analyses were carried out separately for each SNP for *PARK16* and *GAK* locus. The effect of these SNPs on PD risk was evaluated using logistic regression to obtain pooled estimates of Odds Ratio (OR) and 95% Confidence Interval (CI) (Bagos et al., 2007). The most plausible gene effect mode is determined without assuming *a priori* a genetic model to avoid multiple comparisons.

To calculate the logistic regression, wildtype alleles were designated as A and the variants 'B'. Parameters θ_2 and θ_3 are $\log OR_{AB/AA}$ and $\log OR_{BB/AA}$ respectively, as defined by the following logistic regression model (Bagos et al., 2007):

$$\log it(\pi_{ij}) = \alpha_i + \theta_2 z_{i2} + \theta_3 z_{i3},$$

where π_{ij} is the disease risk for j^{th} genotype in the ith study, and z_{i2} and z_{i3} are dummy variables indicating genotypes AB, and BB respectively. The appropriate genetic model was determined based on the following relationship between θ_2 and θ_3 (Bagos et al., 2007; Minelli et al., 2005):

1. No association: $\theta_2 = \theta_3 = 0$ ($OR_{AB/AA} = OR_{BB/AA} = 1$);
2. Recessive model: $\theta_2 = 0$ ($OR_{AB/AA} = 1$) and $\theta_3 \neq 0$ ($OR_{BB/AA} \neq 1$);
3. Dominant model: $\theta_2 \neq 0$, $\theta_3 \neq 0$ and $\theta_2 = \theta_3$ ($OR_{AB/AA} = OR_{BB/AA} \neq 1$);
4. Multiplicative codominant model:

   $\theta_2 \neq 0$, $\theta_3 \neq 0$ and $2\theta_2 = \theta_3$ ($OR^2{}_{AB/AA} = OR_{BB/AA}$).

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Once the best genetic model was identified, the three genotypes were collapsed into two groups to obtain the pooled results (Bagos et al., 2007; Minelli et al., 2005) for dominant (*AB*+*BB* vs. *AA*) and recessive (*BB* vs. *AA*+*AB*) models, or estimation of per-allele risk if multiplicative codominant model is more suitable. Sensitivity analyses were done to evaluate the effect of single study on the overall pooled results by excluding one study every time. The statistical analyses were carried out using METAGEN (http://bioinformatics.biol.uoa.gr/~pbagos/metagen/) and STATA version 11 (Stata Corporation, College Station, TX, USA). All statistical evaluations were made assuming a two-sided test with significance level of 0.05.

2.7 Generation of LRRK2 constructs carrying the wildtype, G2019S, N551K, R1398H and R1441C mutations

2.7.1 Site-directed mutagenesis

LRRK2 constructs containing single mutations (N551K, c.1653C>G or R1398H, c.4193G>A or R1441C, c.4323C>T or G2019S, c.6055G>A) were generated by PCR-mediated site-directed mutagenesis (Stratagene) using the LRRK2 wildtype plasmid DNA as the starting template. The sequence of the whole constructs was confirmed by DNA sequencing using primers stated in Appendix B before any downstream application was conducted.

The desired mutations were individually introduced using the QuickChange® Site-Directed Mutagenesis kit (Stratagene). Essentially, the reaction consisted of the plasmid DNA and two oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the plasmid DNA, were extended during temperature cycling by *PfuTurbo*® DNA polymerase which is a high fidelity polymerase. *PfuTurbo*® DNA polymerase was used to ensure the incorporation of nucleotides would be less error-prone.

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Incorporation of the oligonucleotide primers generated a mutated DNA fragment with staggered nicks. Following temperature cycling, the product was treated with DpnI to digest the parental DNA template. The DpnI endonuclease (target sequence: 5´-Gm6A/TC-3´) was specific for methylated DNA and was used to select the newly synthesised DNA that contains the desired mutation. DNA isolated from *E. coli* strains are dam methylated and therefore susceptible to DpnI digestion. The nicked vector DNA containing the desired mutations was then transformed into competent cells.

The site-directed mutagenesis was carried out based on the manufacturer's recommendation. The mutagenesis reaction had three main parts (Mutant strand synthesis, DpnI digestion and transformation).

2.7.1.1 Mutant strand synthesis

Primers for the site-directed mutation reactions were designed using the PrimerX software available online (http://www.bioinformatics.org/primerx/). Table 2.2 lists the primer sequence used to introduce the mutations on the wildtype *LRRK2* plasmid DNA.

Table 2.2: Primer sequences used to induce the desired mutations

Mutant	**Primer sequence (5' to 3')** Mutation introduced is underlined	**Melting temperature**
N551K	F-GGTCCTAGCAGCTTTGAAGAGGTTCATTGG R-GGATTTCCAATGAACCTCTTCAAAGCTGCTA	79.0°C
R1398H	F-GTGGGATTTTGCAGGTCATGAGGAATTCTATAGTAC R-GTACTATAGAATTCCTCATGACCTGCAAAATCCCAC	77.0°C
R1441C	F-CTTCAATATAAAGGCTTGCGCTTCTTCTTCCCCTGTG R-CACAGGGGAAGAAGAAGCGCAAGCCTTTATATTGAAG	78.0°C
G2019S	F-CAAAGATTGCTGACTACAGCATTGCTCAGTACTGC R-GCAGTACTGAGCAATGCTGTAGTCAGCAATCTTTG	78.0°C

A 50µl PCR reaction containing the reagents in Table 2.3 was prepared and placed in a thermocycler that was programmed to denature at 95°C for 1 minute, followed by 18 cycles of 95°C for 50s, 79°C for 50s and 68°C for 15 minutes, ending with a final extension of 68°C for 7 minutes.

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Table 2.3: Site-directed mutagenesis reaction mixture

Reagent	Volume (µl)
Nuclease free water	35.5
10x Reaction buffer	5.0
Plasmid DNA (5ng/µl)	2.0
Forward primer (100ng/µl)	1.25
Reverse primer (100ng/µl)	1.25
dNTP mix	1.0
QuickSolution	3.0
PfuUltra HF DNA polymerase (2.5 U/µl)	1.0

2.7.1.2 Removal of parental DNA by DpnI digestion

One microlitre of DpnI restriction enzyme (10U/µl) was added in to the amplified product. The solution was mixed thoroughly and incubated at 37°C for an hour.

2.7.1.3 Transformation into competent cells

Forty five microlitres of *XL10-Gold®* Ultracompetent cells was aliquoted into a pre-chilled 1.5ml tube. Two microlitres of DpnI-treated DNA was then transferred into the tube containing the ultracompetent cells. The transformation mixture was gently mixed and incubated on ice for 30 minutes, after which the mixture was heat-pulsed at 42°C for 30s. The tubes were incubated on ice for 2 minutes. Five hundred microlitres of SOC media (Invitrogen) was added into the tube and incubated at 37°C for 1 hour while shaken at 225–250 rpm. Approximately 50µl of the mixture was streaked on an agar plate containing ampicillin (Sigma-Aldrich), and the plate was incubated overnight at 37°C. Only successfully transformed colonies would grow as the plasmid carried an ampicillin resistant gene. Single colonies were selected and cultured in 5ml of LB broth at 37°C for 16 hours and subjected to plasmid DNA extraction using QIAprep Spin Miniprep kit (Qiagen).

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2.7.2 Site-directed mutagenesis validation

The plasmid DNA was then subjected to validation before any downstream application of the plasmid was carried out to ensure the integrity of the plasmid and mutation induced. Primers were designed using the mRNA LRRK2 sequence to check the position of the mutation introduced. Table 2.4 shows the primers that were used to validate the mutations generated in the plasmid. A PCR reaction containing 16.35µl of water, 2.5µl of 10x PCR Buffer, 1.5µl of $MgCl_2$, 0.5µl 10mM of dNTP, 0.5µl of 20mM forward and reverse primers, 2.0µl of DMSO, 0.15µl of Taq and 1.0µl of plasmid DNA was prepared. The reaction was placed in a thermocycler that was programmed to denature at 95°C for 3 minutes, cycle 35 times at 95°C for 30s, 58°C for 45s and 72°C for 1 minute followed by a final extension step at 72°C for 10 minutes. The samples were then purified and sequenced.

Table 2.4: Primers for site-directed mutagenesis validation

Mutant	Primer sequence (5' to 3')	Annealing temperature (^{o}C)	Product size (bp)
N551K	F- GGCGCTTCGAGCTATTTTAC R- TTGGTCATCTGGATACATCTGC	58	292
R1398H	F- TGCAGCAATTAATGAAAACCA R- GCTTCATGGCATCAACTTCA	58	246
R1441C	F- CGAGCATTGTACCTTGCTGTC R- GGTGGCATTCACAAAGTGGT	59	240
G2019S	F- ACCCTACAGCACAGGATTGC R- CATTTCCTCTGGCAACTTCA	58	238

2.7.3 Plasmid DNA extraction for transfection into mammalian cells

Plasmid DNA extraction was performed using PureLink® HiPure Plasmid Filter purification kit (Invitrogen) based on the manufacturer's protocol.

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2.7.3.1 Preparation and lysis of bacterial cell culture

A single colony that has been picked and grown in 2ml of LB broth for 5 hours, was refreshed in 200ml LB broth and incubated overnight at 37°C, 220rpm. The bacterial culture was harvested using a fixed angle rotor centrifuge at 6,000 x g for 12 minutes. The supernatant was discarded and 10ml of Resuspension buffer (R3) was added to resuspend the cell pellet. The mixture was vortexed to ensure complete reconstitution of the pellet. Ten millilitres of Lysis buffer (L7) was added and the tube was inverted gently for 3-5 times and incubated for 4 minutes at room temperature. The reaction was stopped by adding 10ml of Precipitation buffer (N3) followed by gentle inversion for 10-15 times. The lysate was then applied to the filter column that has been equilibrated with 30ml of Equilibrium buffer (EQ1). The solution in the filter column was allowed to drain by gravity flow.

2.7.3.2 Washing, elution and purification of plasmid DNA

Once the flow of the lysate was complete, the inner filtration cartridge was discarded and the column was washed with 50ml of wash buffer (W8). The solution was allowed to drain completely by gravity flow. A sterile 50ml centrifuge tube for elution was placed under the column where 15ml of Elution buffer (E4) was added to elute the DNA that has bound to the column. Once elution was complete, the column was discarded. The plasmid DNA was precipitated by the addition of 10.5ml of isopropanol. The tube was mixed and centrifuged at 4,000 rpm for 30 minutes at 4°C. The supernatant was carefully removed and discarded. One millilitre of 70% ethanol was used to reconstitute the DNA pellet and transferred into a 1.5ml tube. The tube was spun at 13,000 rpm for 5 minutes after which the supernatant was discarded. The pellet was dried and reconstituted in 0.5ml sterile water. The DNA was quantified using Nanodrop 2000 (Thermo Scientific) and stored at -20°C.

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2.8 Plasmid constructs for LRRK2 functional assays

2.8.1 GFP-tagged LRRK2 constructs

The full length wildtype *LRRK2* plasmid construct was a generous gift from Professor Tan Eng King's lab in Singapore. The full 9kb coding sequence was cloned into the pEGFP vector, which tagged LRRK2 with GFP at the C terminus (Figure 2.1). The total size of the plasmid is approximately 13kb and the sequence was verified to be free from any in-frame stop codons.

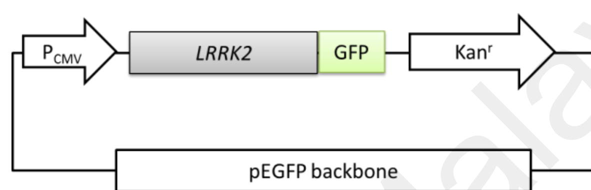

Figure 2.1: Graphical representation of the GFP tagged LRRK2 plasmid. Legend: P_{CMV} - Cytomegalovirus promoter; GFP - green fluorescence protein; Kan^r - kanamycin resistant gene.

2.8.2 Wildtype and mutant constructs of the GST-tagged ROC-COR domain

The GTPase activity of LRRK2 was investigated by synthesising a recombinant protein from *E. coli*. A glutathione S-transferase (GST) tag was used because initially a GTP hydrolysis assay was also planned and it required soluble protein, however this hydrolysis assay was not performed due to time constraints (discussed further in Chapter 5, Section 5.5). The ROC-COR domain followed by an in-frame stop codon (4,972bp) was inserted in the BamHI and XhoI site on the pGEX-5X-1 vector that has an N terminal GST tag (Figure 2.2).

The ROC-COR template was amplified using the primers stated in Table 2.5 with *Pfu* ultraII polymerase (Agilent) based on the manufacturer's protocol. The PCR product was electrophoresed on a 1% gel to ensure successful amplification followed by PCR product purification using the QIAquick PCR purification kit (Qiagen).

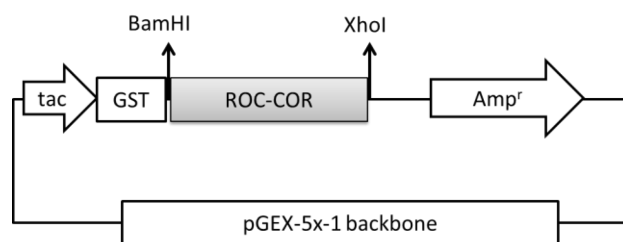

Figure 2.2: Graphical representation of the GST-tagged ROC-COR vector. Legend: Amp^r - ampicillin resistant gene; ROC-COR - the ROC-COR domain of LRRK2; tac - tac promoter; BamHI and XhoI are restriction enzyme sites on the vector that were used to insert the gene of interest.

Table 2.5: Primers used for ROC-COR domain amplification

Primer name	Primer sequence (5' to 3')	Melting temperature
BamHIF-Roc	GAA GGT CGT GGG ATC CCC AAC CGA ATG AAA CTT ATG ATT G	55.0^{o}C
Cor-XhoIR	GAT GCG GCC GCT CGA GTT CAA ATT CCA ACT CAT	55.0^{o}C

2.8.2.1 Digestion & ligation

In separate tubes, the PCR product and the pGEX-5X-1 vector were digested using BamHI and XhoI for 4 hours at 37°C. The digested samples were purified after electrophoresis in a 1% gel and the purified products were ligated using T4 Ligase for 2 hours at room temperature.

2.8.2.2 Transformation into DH5α cells

Five microlitres of the ligated product was added to 50µl of competent DH5α cells (Invitrogen) that had been thawed on ice. This mixture was incubated on ice for 30 minutes after which it was heat shocked for 30s at 42°C and placed on ice again for 2 minutes. Nine hundred millilitres of SOC (Invitrogen) media was added to the mixture and allowed to shake for an hour at 37°C before it was plated on an Ampicillin agar plate overnight at 37°C.

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The colonies that grew were expanded in 4ml LB broth and DNA extraction was carried out using QIAprep Spin Miniprep kit (Qiagen). The plasmid DNA was then digested with BamHI and XhoI for an hour at 37°C as an initial screening to check if the cloning was successful. Colonies that yielded two bands at the expected band size on a 1% gel after digestion were sequenced with primers stated in the Appendix to ensure in-frame start and stop codons. The R1398H and R1441C mutants were introduced using the QuickChange® Site-Directed Mutagenesis kit (Stratagene) as described in section 2.7.1. The plasmids carrying the desired mutants were then transformed into BL-21 cells for protein extraction as described in section below.

2.9 Recombinant protein purification

The recombinant protein was purified based on the protocol described by Harper & Speicher, 2011 with slight variations (Harper & Speicher, 2011). Briefly, a single colony from the BL-21 transformation was expanded in a 30ml LB broth supplemented with 100µg/ml ampicillin overnight at 37°C shaking at 220 rpm. The culture was then refreshed with 170ml of LB broth and incubated at 37°C till the optical density at 600nm (OD_{600}) was 0.5-0.7 (approximately after 1 hour of shaking at 220 rpm). A final concentration of 1mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) was added into the culture to induce protein production. The culture was incubated for 4 hours at 37°C, 220 rpm. The cells were harvested by centrifugation at 4,000 rpm, 10 minutes at 4°C. The supernatant was discarded and the pellet was gently dislodged before the addition of 10ml sodium chloride-Tris-EDTA (STE) buffer (10mM Tris HCl pH8.0, 1mM EDTA, 150mM NaCl) supplemented with protease inhibitor (Roche). Once the pellet has been completely dissolved by vortex, 0.1mg/ml lysozyme was added followed by gentle inversion and 20 minutes incubation on ice. Ten millimolar dithiothreitol (DTT) and 3% sarkosyl (N-Lauroylsarcosine sodium salt, Sigma) was

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added into the mixture to help increase the solubility of the recombinant protein. The tube was gently inverted 3-5 times to ensure proper mixing of the reagents.

The mixture was sonicated on ice for 2 minutes with 20s intervals, for 3 times. The sample was subjected to sonication to aid in the breaking of the bacteria cell wall and release of the recombinant protein. However as the sonication process generates heat, the samples were kept submerged in ice to avoid denaturation. In addition, care was taken to ensure that the sonicator tip was fully submerged into the mixture to avoid frothing as this can also cause denaturation of the recombinant protein. The cell debris was separated from the recombinant protein by centrifugation at 4,000 rpm $4^{o}C$ for 30 minutes. The supernatant was carefully transferred into a fresh 15ml tube, and 3% Triton X-100 was added to help the recombinant protein bind to GST beads (GE Healthcare). The GST beads which had been prewashed in HEPES pH 7.0 buffer (12.5mM HEPES, 150mM NaCl, 2mM Na_2HPO_4) and reconstituted to be 50% slurry in 120µl was added to the 15ml tube. The recombinant protein was allowed to bind to the GST beads overnight at $4^{o}C$ rolling.

The beads were harvested by spinning the 15ml tube for 5 minutes at $4^{o}C$ at 4,000 rpm. The supernatant was discarded and the beads were transferred to a 1.5ml tube. The beads were washed three times with 1ml HEPES buffer pH7.0. Recombinant proteins were eluted from the beads by the addition of 300µl of freshly prepared elution buffer and rolling for 1 hour at $4^{o}C$. The elution buffer consisted of 20mM reduced L-glutathione, 50mM Tris pH8.8, 150mM NaCl, 1% Triton-X 100 and 1% glycerol. The tube was spun at 10,000 rpm for 2 minutes. The supernatant containing the purified recombinant protein was transferred into a fresh tube. The purified proteins were snap-frozen as 5% glycerol stocks and stored in $-80^{o}C$ till further analysis. The samples were aliquoted into a few tubes to avoid repetitive freeze and thaw cycles.

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2.10 Cell culture maintenance

2.10.1 SHSY-5Y cells

SHSY-5Y cells were maintained in Dulbecco's Modified Eagle Medium supplemented with Nutrient Mixture F-12 (DMEM/F12), Minimum Essential Media (MEM) nonessential amino acids, 100 units/ml of Penicillin, 100ug/ml Streptomycin and 10% fetal bovine serum incubated at 37°C with 5% CO_2. SHSY-5Y cells freezing media consisted of complete media supplemented with 10% sterile DMSO.

2.10.2 HEK-293T cells

HEK-293T cells were maintained in Dulbecco's Modified Eagle Medium supplemented with MEM nonessential amino acid, 100 units/ml of Penicillin, 100µg/ml Streptomycin and 10% fetal bovine serum incubated at 37°C with 5% CO_2. HEK-293T cells freezing media consists of complete media supplemented with 10% sterile DMSO. The HEK-293T cells were transfected using Turbofect (Fermentas) based on the manufacturer’s protocol. Cells were harvested 24 hours after transfection for biochemical assays.

2.11 Generation of stable cell lines of the wildtype and mutant LRRK2 constructs

The SHSY-5Y cells were sub-cultured in 10cm petri dishes. The cells were transfected individually with the various mutated constructs using NeuroFECT transfection reagent (Gelantis) in the ratio of 5:1 to DNA. The optimised DNA concentrations used during transfection was 6µg for the LRRK2 constructs and 2µg for the empty vector. After 24 hours, G418 (PAA Laboratories) was added in the complete media to a final concentration of 400µg/ml to select for cells that are successfully transfected. Cells that did not take up the construct die as they do not have the G418

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resistant gene. The selection was carried out for approximately 2 weeks till most of the cells were washed off the bottom of the dish, leaving colonies of stable cells behind.

Single colonies were selected and transferred into a 24-well plate containing selection media. When the wells were confluent, the cells were sub-cultured into 3 wells of a 6-well plate (one well was used for passaging and two for screening). All clones of interest were kept and sub-cultured from the 6-well plate into two 10cm dishes: one used for freezing as Passage 0 and another for further passaging. The early passages were periodically frozen down.

2.11.1 Determining LRRK2 expression

The cells from two wells of a 6-well plate was trypsinised and combined. The cells were spun down at 1,000 rpm for 5 minutes after which the supernatant was discarded and the cells were rinsed with 1x PBS. The cells were pelleted again and RNA was extracted using the RNeasy mini kit (Qiagen) based on the manufacturer's protocol. The concentration of the sample was measured and standardised to 200ng in 10µl of nuclease free water. The cDNA was synthesised using High capacity cDNA reverse transcription kit (Applied Biosystems) based on the manufacturer's protocol. The cDNA was firstly amplified accordingly with the primers in Table 2.4 and sequenced to ensure that the colony was carrying the right mutation. In addition, gene expression assays were carried out using Taqman® gene expression assay. Beta-actin (Hs99999903_m1) was used as the internal control and the LRRK2 probe used was Hs0041194_m1. The fold change was calculated based on the formula below, where ΔCT = the difference between the CT value of LRRK2 and beta-actin; ΔΔCT = the difference of ΔCT between the sample and the control (empty vector).

$$Fold\ change = 2^{-\Delta\Delta CT}$$

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2.12 Proteomic analysis for the downstream kinase assay in transiently transfected cells with the wildtype and mutant constructs

HEK-293T cells were seeded onto 10cm dishes and transfected with individual 20µg GFP tagged LRRK2 plasmid DNAs using Turbofect as recommended by the manufacturer. Twenty four hours post transfection, cells were harvested on ice and lysed in 500µl immunoprecipitation (IP) buffer [25mM HEPES pH 7.3, 150mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 20mM β-glycerophosphate, 1mM $NaVO_3$, 5% glycerol, 0.25% Triton-X supplemented with 100nM Calyculin A (LC labs) and 1X protease inhibitor (Merck, Protease Inhibitor Cocktail III)]. The lysate was homogenised and spun at 13,000 rpm for 10 min at 4^oC. The supernatant was then transferred into a new tube. GFP-Trap® beads (Chromotek) were added to the supernatant and incubated for 4 hours on a shaker at 4^oC. Beads were washed three times with the IP buffer with 5-minute shaking intervals. The protein was eluted by boiling the beads in 2X sample buffer (Biorad) for 10 minutes. The IP and total lysate was resolved in 7% SDS-PAGE and transferred onto a PVDF membrane using wet transfer for 2 hours at 100V. The membrane was subjected to Western blotting using anti-GFP (Santa Cruz, sc-9996) (1:2,000 for Figure 5.2, Figure 5.3, Figure 5.4 and Figure 5.6); anti-phosT1503 (Abcam) (1:1,000 for Figure 5.9) and secondary antibody tagged with HRP at 1:4,000 dilution. The membrane was visualised using enhanced chemiluminescence reagents (Milipore) on an X-ray film. ImageJ was used to quantitate the protein levels.

2.13 Cell viability assay

SHSY-5Y cells were seeded into 24 wells with a seeding density of 40,000 cells per well. Cells were then transfected with constructs carrying the desired mutants using TurboFect transfection reagents (Thermo Scientific) on the following day. The transfected cells were then divided into two groups: one treated with 40µM hydrogen

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peroxide (H_2O_2) in sodium pyruvate free media for 24 hours and the other group is incubated for 24 hours in sodium pyruvate free media. The cell viability assay was then conducted using CellTitre One Solution Cell Proliferation Assay (Promega) based on the manufacturer's protocol.

2.14 Kinase assays to determine the kinase activity of LRRK2 mutants

The kinase assay was set up in a total volume of 30µl with immunoprecipitated LRRK2 protein in 20mM HEPES pH 7.3, 15mM MgCl2, 25mM NaCl, 5mM EGTA, 20mM β-glycerophosphate, 0.025% Triton-X, 1.25µCi (ɤ-^{32}P) ATP, 15µM ATP and 12µg of MBP (Sigma) as the substrate. The reaction was incubated at 30°C for 20 minutes. The reaction was stopped by the addition of 2X loading buffer and heating samples at 100°C for 10 minutes. Samples were loaded on a gradient (7%-12%) SDS-PAGE gel to be able to resolve LRRK2 (260kDa) and retain MBP (18kDa) within the same gel. After samples were transferred onto a nitrocellulose membrane, the membrane was exposed to a phosphoscreen to allow the detection of the radiolabeled protein.

2.15 GTPase activity to determine the GTP binding of LRRK2 mutants

2.15.1 GTP binding of full length LRRK2 constructs

HEK-293T cells that had been transfected for 24 hours were harvested on ice and lysed in lysis buffer [containing 25mM HEPES pH 7.3, 150mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 20mM β-glycerophosphate, 1mM $NaVO_3$, 5% glycerol, 0.25% Triton-X supplemented with 100nM Calyculin A (LC labs) and 1X protease inhibitor (Merck, Protease Inhibitor Cocktail III)]. The lysate was homogenised and spun at 13,000rpm for 10 min at 4°C. The supernatant was then transferred into a new tube. Approximately 30µl of the lysate was boiled in 2X loading buffer followed by heating at 70°C for 10

minutes (input lysate). Twenty five microlitres of guanosine 5'-triphosphate-agarose (GTP beads, Sigma-Aldrich) was added to the tube and allowed to bind for 2 hours under rotation at 4°C. The beads were then washed three times with the lysis buffer and once with 1X PBS after which the GTP-bound proteins were eluted in 2X loading buffer followed by heating at 70°C for 10 minutes. The GTP-bound proteins and the input lysates were resolved by SDS-PAGE and probed with anti-GFP for Western blot analysis. The ratio of LRRK2 present in the GTP-bound proteins and input lysates was obtained for analysis.

2.15.2 GTP binding of the ROC-COR LRRK2 constructs

Two hundred and fifty nanograms of total purified ROC-COR domain protein with different mutants was diluted in 500µl of buffer (containing 25mM HEPES pH 7.3, 150mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 20mM β-glycerophosphate, 1mM $NaVO_3$, 5% glycerol, 0.1% Triton-X and 0.1% BSA). Thirty microlitres of the input protein was boiled in 2X loading buffer (input protein). Twenty microlitres of GTP beads (Sigma-Aldrich) was added to the remaining input protein and incubated for 2 hours on a shaker at 4°C. At the end of the incubation, the beads were spun down at 1,000 rpm for 2 minutes. Beads were washed twice with 3-minute shaking intervals. The protein was eluted by boiling the beads in 2X sample buffer (Biorad) for 10 minutes. The beads and input protein was resolved by 10% SDS-PAGE and transferred onto a PVDF membrane using wet transfer for 1 hour at 100V. The membrane was subjected to Western blotting using anti-GST antibodies (Santa Cruz). The membrane was visualised and analysed using similar methods to Section 2.12.

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CHAPTER 3: GENETIC LOCI ASSOCIATED WITH PARKINSON'S DISEASE

In this chapter, three GWAS-linked loci (*HLA-DRA*, *PARK16* and *GAK/DGKQ*) and the *GRN* gene were considered. Each section provides an introduction to the loci, followed by the results and discussion. All methods used in this chapter have been described in detail in Section 2.3 and 2.4. All SNPs screened in this chapter were in Hardy-Weinberg equilibrium (HWE) by means of Fisher's exact test unless stated otherwise. Before each SNP were screened, the mean age of the controls and cases were calculated to ensure that the controls were older than the cases. The rationale behind this was that since the type of PD we were studying was late-onset PD and older individuals are more likely to develop it, we wanted to ensure that we did not inadvertently include younger individuals in our control cohort who may develop PD at an older age. Generally in our cohort, the controls were older than cases. The mean age and standard deviation of the cohort examined for each locus are included in Appendix C. Based on our cohort, more males developed PD compared to females (males with PD=56.5%; p=0.0023) when a Fisher's exact test was conducted, coherent with the trend observed in PD (Wooten et al., 2004). The sample sizes screened for each SNP may differ as different loci were screened at different time-points during the study, as newer loci were discovered (Appendix F). The detection rate using the Taqman® genotyping assays for all the SNPs was 100% (error rate 0%). An example of how genotypes were scored by analysing the allelic discrimination assay plots can be found in Appendix D.

3.1 *HLA-DRA* locus

HLA-DRA is a major histocompatibility complex which has a role in immune responses and inflammation (Gruen & Weissman, 1997). From a pathogenic angle, it can be hypothesised that *HLA-DRA* may be involved in PD pathogenesis as recent reviews has implicated uncontrolled inflammation as one of the possible aetiology of

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PD (Mosley et al., 2012; Nagatsu & Sawada, 2005), although these reviews did not directly implicate *HLA-DRA*.

A GWAS conducted by Hamza and colleagues in 2010 on 3,986 Americans of European ancestry identified an association of the *HLA-DRA* locus with late onset PD (Hamza et al., 2010). They found that the G allele of the rs3129882 variant was a risk factor (OR 1.26, $p<0.001$) especially in male patients with LOPD. While, a recent study conducted on an Iranian population established a protective association with the A allele (OR 0.65; $p=0.002$), (Jamshidi et al., 2014).

However, these findings were not replicable in a northern Spanish population (2,606 individuals), Dutch population consisting of 2,796 individuals or in a Swedish population of 1,147 individuals (Mata et al., 2011; Ran et al., 2013; Simon-Sanchez et al., 2009). In addition, Puschmann and colleagues in a combined Caucasian cohort consisting of 2,618 Polish, Irish and American individuals found that G homozygotes conferred protection against PD (OR 0.75; $p=0.006$), which contrasted with the original report of a risk association by Hamza et al., 2010 (Puschmann et al., 2011).

In an Asian context, the Hamza study was not replicable, as studies on a Chinese PD population from mainland China revealed that the A allele is associated with a risk effect, with an OR of 1.37, $p=0.02$ (Guo et al., 2011) and no association was found in a Taiwanese PD population (Chiang et al., 2012).

The variable result seen in the various populations studied may be attributed to the highly polymorphic nature of the *HLA* region (Trowsdale et al., 1985). The MAF in the Caucasian population ranged between 0.40-0.46 while in the Asians, the MAF reported ranged between 0.29-0.37 suggesting that there is some variation between the two populations.

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3.1.1 Results and discussion

To uncover the possible HLA-PD link in the Malaysian population, 493 controls and 462 PD cases of different races were screened (Table 3.1).

Table 3.1: Analysis of rs3129882 within the *HLA-DRA* in the Malaysian cohort. The median age, together with range of controls and cases for the Malaysian cohort are 59 years (29-86 years old) and 57 years (21-90 years old) respectively.

	PD cases			Control		
Genotype	**Malay**	**Chinese**	**Indian**	**Malay**	**Chinese**	**Indian**
Wildtype (G/G)	75	113	16	67	101	17
Heterozygous mutant (G/A)	53	99	40	51	140	50
Homozygous mutant (A/A)	17	31	18	16	28	23
Allelic frequency (%)						
Wildtype (G)	70.0	66.9	48.6	69.0	63.6	46.7
Mutant (A)	30.0	33.1	51.4	31.0	36.4	53.3

When the Malaysian cohort was analysed on its own with all the races combined, a protective trend with the A allele was observed with an OR 0.78, however this association was not significant (p=0.08), Table 3.1. Additional samples consisting of predominantly Chinese samples from Singapore (420 controls and 405 cases), were added to our cohort (Table 3.2).

Table 3.2: Singaporean data on rs3129882 that was included into the analysis. The median age, together with range of controls and cases are 62 years (39-88 years old) and 65 years (39-89 years old) respectively.

	PD cases			Control		
Genotype	**Malay**	**Chinese**	**Indian**	**Malay**	**Chinese**	**Indian**
Wildtype (G/G)	-	191	4	3	177	3
Heterozygous mutant (G/A)	-	161	7	-	181	6
Homozygous mutant (A/A)	-	42	-	-	48	2
Allelic frequency (%)						
Wildtype (G)	-	68.9	68.2	100	65.9	54.5
Mutant (A)		31.0	31.8	-	34.1	45.5

When the three races in the Malaysian and Singaporean cohort was analysed together, the rs3129882 had an OR of 0.81 (p=0.03) suggesting that the A allele has a protective association with PD in the combined cohort. Although the combined analysis

of all three races showed significance, the genotypic trend of this variant in the Indians was different (MAF 0.5 in cases and controls) as compared to the Chinese and Malays (MAF approximately 0.3). The Malay cohort consisting of Malaysian and Singaporean samples were too small to conduct a statistical analysis to yield a conclusive association.

Therefore, the Chinese was separated and analysed on its own. Table 3.3 summarises the genotype data in the Chinese cohort. In the Chinese, the A allele showed a protective association with OR 0.76 and *p*-value of 0.018, similar to the association seen in the Iranian population (Jamshidi et al., 2014).

Table 3.3: Frequency of genotypes and allele polymorphisms for the rs3129882 variant among Chinese PD cases and controls in our combined cohort (Malaysia and Singapore)

Genotype	PD Cases	Controls	Odds Ratio
GG	304	278	Reference
GA	260	321	0.74 (0.58, 0.94)
AA	73	76	0.87 (0.59, 1.27)
Common (G) allele	868	877	-
Minor (A) allele	406	473	-
Odds ratio (OR)	-	-	0.76 (0.61, 0.96)
p-value	-	-	**0.018**

Table 3.4 summarises the findings from studies on relevant Asian ethnicities. It can be seen that there are a number of contradictory findings even when groups of the same Chinese ethnicity were analysed. To attempt to explain these findings, we compared the reported MAF of the A allele in the controls in the various studies. In studies that found risk associations, the MAF was 0.29 (Guo et al., 2011) and 0.28 (Zhou et al., 2014), with both populations based in mainland China. In studies that found no association, the MAF was 0.368 (Chiang et al., 2012) and 0.37 (Lin et al., 2013). In fact, our study is the only study so far in the Asian population that has shown significant protective association and the MAF in our cohort was 0.35. We observed that the MAF in the HapMap Han Chinese (China) database was 0.384.

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Judging from the MAF values, it is possible that there was a selection bias of controls in the study by Guo et al., 2011. Guo’s and Zhou’s results were not replicable by a Taiwanese population (OR 0.94, p=0.446) or in our cohort (Chiang et al., 2012; Guo et al., 2011; Zhou et al., 2014).

In view of this discrepancy, Ma and colleagues conducted a meta-analysis on existing reports up to 2014, where they found that this variant does not associate with the Chinese population and this was also seen in another meta-analysis by Zhu et al., 2015 (but in this case irrespective of ethnicity) (Ma et al., 2014; Zhu et al., 2015). However, when studies reporting a risk association and protective association are combined together, the effect may be cancelled out and mask studies that have a modest association as seen in this study. Different geographical origin may also alter the effect of the variant towards the susceptibility of PD as there could be environmental factors that are contributing to the risk as seen in the two China studies (Guo et al., 2011; Zhou et al., 2014). Figure 3.1 depicts the OR of all the Asian studies on the *HLA-DRA* locus.

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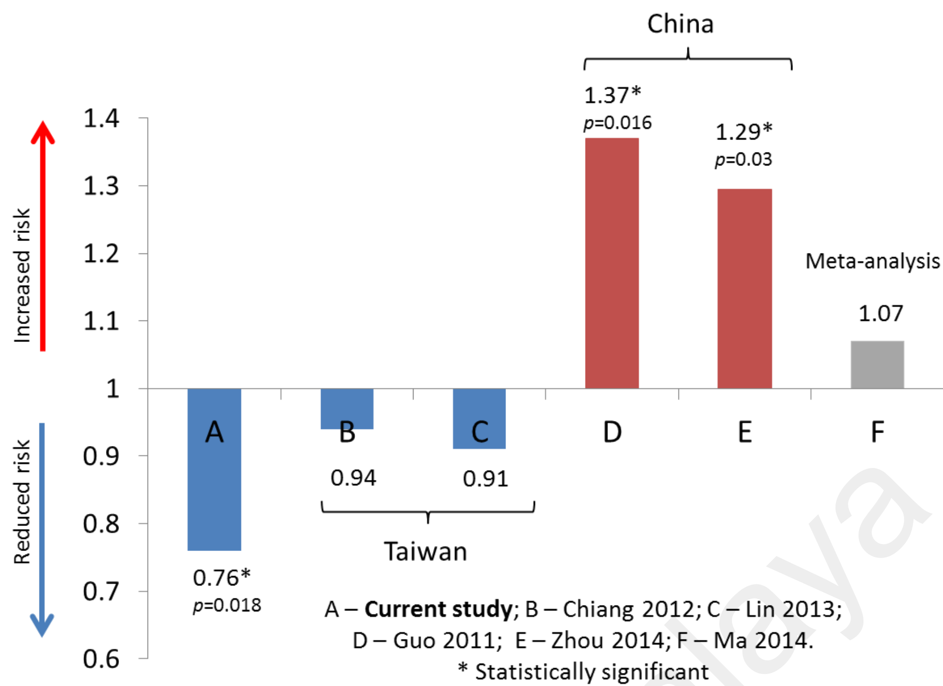

Figure 3.1: Graphical representation of the Asian studies done on rs3129882. Blue indicate reduced risk, red indicates increased risk while grey indicates borderline association.

Apart from contributing to the knowledge of the effect of rs3129882 the Chinese, the work described here is the first study exploring the genetic association of rs3129882 in Malays and Indians. Based on our data, the Indians have a very different genotypic trend for this locus. The minor allele is present at almost a similar frequency as the major allele in the Indians. Although the Malays had similar genotypic trend as the Chinese, the size of the Malay cohort was not large enough to establish a significant association on its own.

Table 3.4: Summary of published data on rs3129882 across all ethnic groups.

Author	Population	Results	Comment
Hamza et al., 2010	Caucasians (American of European ancestry) 2,000 PD 1,986 controls	Minor allele G 0.46 PD 0.40 controls	G allele increases risk of PD OR 1.32; *p*=2.9 x 10^{-8}
Edwards et al., 2010	Caucasian 604 PD 619 controls	Minor allele G 0.44 PD 0.40 controls	Borderline association OR 1.17; *p*=0.06
Mata et al., 2010	Spanish 1,445 PD 1,161 controls	Minor allele G 0.43 PD 0.42 controls	No association OR 1.01; *p*=0.88
Puschmann et al., 2011	Caucasian 1,313 PD 1,305 controls	Minor G allele 0.41 PD 0.42 controls	GG allele protective OR 0.75; *p*=0.006
Simon-Sanchez et al., 2011	Caucasian (Dutch) 772 PD 2,024 controls	Not indicated	No association OR 1.07; *p*=0.312
Guo et al., 2011*	Chinese 284 PD 258 controls	Minor A allele 0.359 PD 0.291 controls	A allele confers increased risk of PD OR 1.37; *p*= 0.016
Chiang et al., 2012*	Taiwanese 538 PD 532 controls	Minor allele A 0.35 PD 0.37 controls	No association OR 0.94 for A allele *p*=0.446
Combined Chiang and Guo (as in Chiang)	Chinese (Mainland and Taiwan) 822 PD 790 Controls	Minor A allele 0.36 PD 0.34 controls	No association OR 1.06; *p*=0.47
Current study*	Chinese 637 PD 675 controls	Minor A allele 0.32 PD 0.35 controls	**A allele is protective OR 0.76; *p*=0.018**
Lin et al., 2013*	Chinese (Taiwan) 448 PD 452 controls	Minor A allele 0.34 PD 0.37 controls	A allele shows a protective trend but not significant OR 0.91; *p*=0.18
Ran et al., 2013	Swedish 511 PD 636 controls	Not mentioned 0.41 PD 0.41 controls	No association OR 1.00; *p*=0.99
Zhou et al., 2014*	Chinese (China) 323 PD 345 controls	Minor A allele 0.33 PD 0.28 controls	A allele risk OR 1.294; *p*=0.03
Ma et al., 2014 (meta-analysis of *)	Chinese 2,230 PD 2,262 controls	Minor A allele 0.34 PD 0.34 controls	No association OR 1.03; *p*=0.74
Jamshidi et al., 2014	Iran 520 PD 520 controls	Minor A allele 0.44 PD 0.48 controls	A allele protective OR 0.65; *p*=0.002
Zhu et al., 2015	11,951 PD 11,902 controls	Meta-analysis of all the above	No association OR 1.043; *p*=0.198

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3.2 *PARK16* locus

In 2009, two new susceptibility loci were identified for PD; *PARK16* on chromosome 1q32 and *BST1* on chromosome 4p15 in a Japanese PD cohort (Satake et al., 2009). In Satake's study, seven single nucleotide polymorphisms (SNPs) in the *PARK16* locus (rs16856139, rs823128, rs823122, rs947211, rs823156, rs708730 and rs11240572) were found to be risk factors for PD (OR 1.23-1.5; $p< 5\times10^{-7}$). The *BST1* locus was not considered in this thesis as a study in a neighbouring country showed that there was no association found (Tan et al., 2010a).

These *PARK16* SNPs are in linkage disequilibrium with five genes (*SLC45A3*, *NUCKS1*, *RAB7L1*, *SLC41A1*, and *FLJ32569*), (Satake et al., 2009). Reports have implicated several of these genes with PD. *SLC41A1* in magnesium homeostasis (Kolisek et al., 2008) which has been linked to amyotrophic lateral sclerosis-parkinsonism, and *RAB7L1* has been shown to interact with *LRRK2* (MacLeod et al., 2013). The positions of the genes in relation to the SNPs are depicted in Figure 3.2.

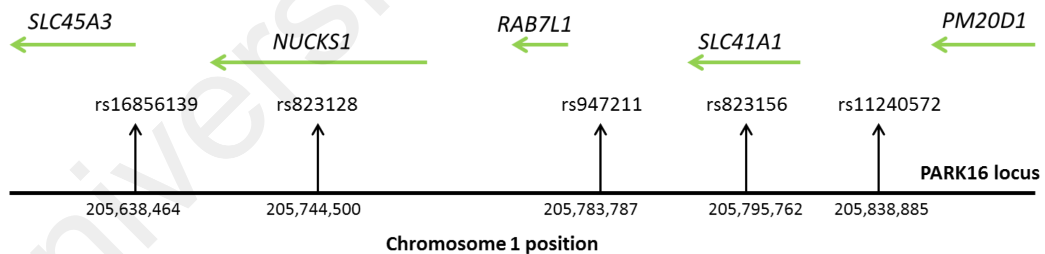

Figure 3.2: Relative position of the genes in relation to SNPs in *PARK16* on chromosome 1

However, replication studies have not been able to reach the same risk determination. In European, Jewish and Chilean populations, rs823128, rs823156 and rs11240572 of *PARK16* SNPs were associated with a reduced risk of PD (Liu et al., 2011a; Ramirez et al., 2011; Soto-Ortolaza et al., 2013). Similarly, the rs823128 was associated with a reduced risk of PD in a Taiwanese population (Vilariño-Güell et al., 2010) while another replication study on Chinese PD cases found three of *PARK16* SNPs (rs823128,

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rs823156 and rs11240572) significantly reduced the risk of PD (Tan et al., 2010a). More recently, similar protective associations with a number of *PARK16* SNPs have also been reported in three independent Chinese PD populations (Chang et al., 2013b; Chang et al., 2011; Yan et al., 2011). To date there has not been any data on the *PARK16* locus in the Malaysian population therefore we screened this population to determine the contribution of *PARK16* SNPs in modulating the risk of developing PD.

Although *PARK16* has seven SNPs, only five were screened as two SNPs (rs708730 and rs823122) are in close linkage disequilibrium (LD) and can be represented by rs823156 and rs823128, respectively, as indicated in a previous study in a closely-related population (Tan et al., 2010a). Figure 3.3 shows a table taken from Tan et al., 2010 showing that the two SNPs are in LD.

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Figure   Selection of genome-wide association study single nucleotide polymorphisms (SNPs)

Gene	SNP	Position No.	SNPs selected for genotyping	r2
BST1	rs11931532	26	rs11931532_1	>0.8
BST1	rs12645693	37		
BST1	rs4538475	62		>0.8
BST1	rs4698412	59	rs4698412_1	
PARK8	rs1994090	16	rs1994090_1	
PARK8	rs2046932		rs2046932_1	>0.8
PARK8	rs4768212			
PARK8	rs2708453	65		>0.8
PARK8	rs7304279	54	rs7304279_1	
PARK16	rs11240572		rs11240572_1	
PARK16	rs16856139	7	rs16856139_1	
PARK16	rs708730	85		>0.8
PARK16	rs823156	78	rs823156_1	
PARK16	rs823122	46		
PARK16	rs823128	42	rs823128_1	>0.8
PARK16	rs947211	70	rs947211_1	
PARK1	rs11931074	5	rs11931074_1	>0.8
PARK1	rs3857059	25		
PARK1	rs6532194	130	rs6532194_1	
PARK1	rs894278	76	rs894278_1	

Only 1 SNP from each shaded box was selected for analysis as the 2 SNPs in each shaded box are in close linkage disequilibrium (r^2 >0.8).

Figure 3.3: Excerpt from Tan et al., 2010a showing that rs708730 and rs823156 as well as the rs823122 and rs823128 are in LD as shown by r^2 values that are >0.8

3.2.1 Results and discussion

Five SNPs were screened at this locus in a total cohort of 825 PD cases and 507 controls. Similar to what was seen at the *HLA-DRA* locus, the Indians in our cohort showed a very different genotypic pattern from the other ethnicities. Since our Indian cohort was small (100 cases and 93 controls), we removed the Indians from the analysis. When the Malays and Chinese were analysed independently (Table 3.5), similar genotypic trend was observed. Therefore the Malays and Chinese cohort were analysed together from this point forth for the five SNPs in this locus (725 PD cases and 414 controls).

Table 3.5: Summary of the *PARK16* locus when analysed according to ethnicity

PARK16 **SNPs**	**Malay (MAF cases, controls)**	**Chinese (MAF cases, controls)**
rs947211	OR 0.66; *p*=0.081 (0.42 case, 0.38 ctrl)	**OR 0.50; *p*=0.0004** (0.37 case, 0.45 ctrl)
rs823128	No association (0.14 case, 0.14 ctrl)	No association (0.11 case, 0.11 ctrl)
rs823156	No association (0.24 case, 0.24 ctrl)	No association (0.17 case, 0.20 ctrl)
rs11240572	No association (0.18 case, 0.18 ctrl)	**OR 0.38; *p*= 0.0443** (0.14 case, 0.17 ctrl)
rs16856139	No association (0.15 case, 0.14 ctrl)	No association (0.13 cases, 0.14 ctrl)

Out of the five SNPs, only rs947211 showed an association in a protective manner with our combined cohort (OR 0.57, *p*=0.0003), (Table 3.6). Detailed genotypic data for each SNP is included in Appendix E. As four SNPs did not show an association in our cohort (Table 3.6), we then investigated if a larger sample size would have an effect. Genotypic data from published reports on *PARK16* in ethnically matched populations data were combined with ours to perform a pooled analysis (Chang et al., 2013b; Chang et al., 2011; Tan et al., 2010a; Vilariño-Güell et al., 2010; Yan et al., 2011). Interestingly, when pooled together, three (rs823128, rs823156 and rs11240572) out of the remaining four SNPs showed a significant protective association. The fourth SNP,

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rs16856139 showed a protective trend but was not statistically significant (Table 3.6). The pooled analysis revealed that the G alleles at rs823128 (OR 0.84, *p*=0.0155) and rs823156 (OR 0.79, *p*=0.0001) as well as the A allele at rs11240572 (OR 0.79, *p*=0.0002) could reduce the risk of developing PD. The forest plots of each SNP are depicted in Figure 3.4-Figure 3.8.

Table 3.6: Summary of *PARK16* results, when analysis was done in our cohort and when published studies were pooled with our cohort

PARK16 SNP	Independent analysis	Pooled analysis (Chang et al., 2013b; Chang et al., 2011; Tan et al., 2010a; Vilariño-Güell et al., 2010; Yan et al., 2011)
rs947211	OR 0.57 (*p*=0.0003)	OR 0.71, *p*=0.0001
rs823128	No association	OR 0.84, *p*=0.02
rs823156	No association	OR 0.79, *p*=0.0001
rs11240572	No association	OR 0.79, *p*=0.02
rs16856139	No association	OR 0.86, *p*=0.09

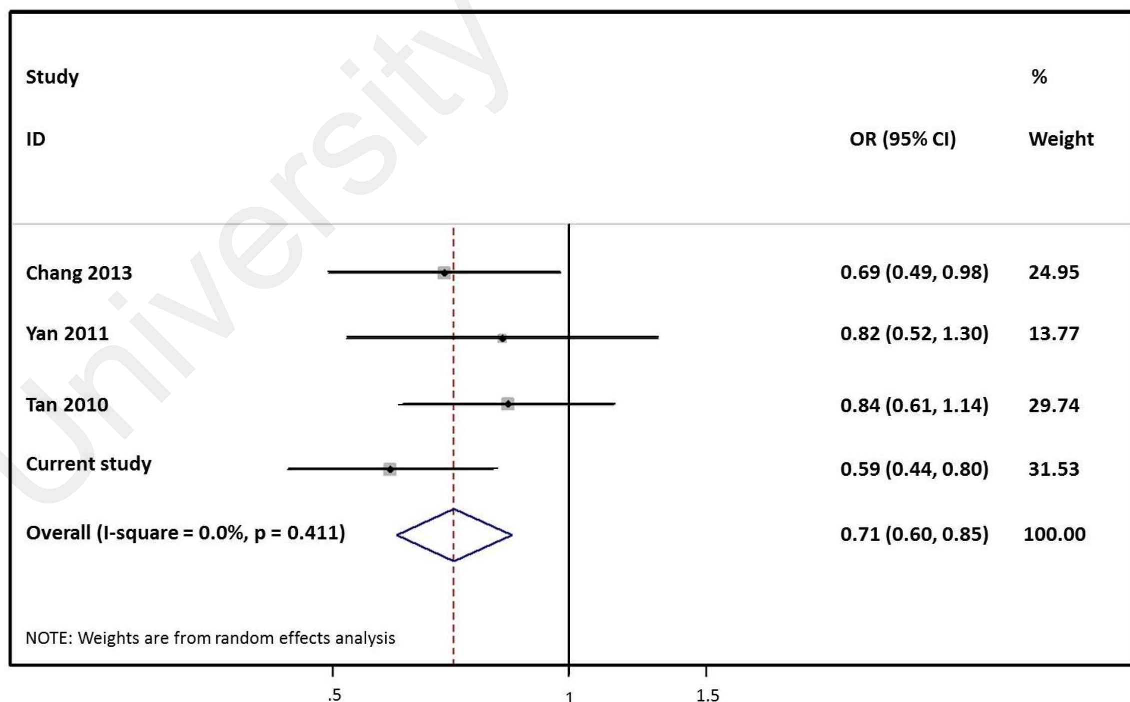

Figure 3.4: Forest plot of the pooled analysis at rs947211

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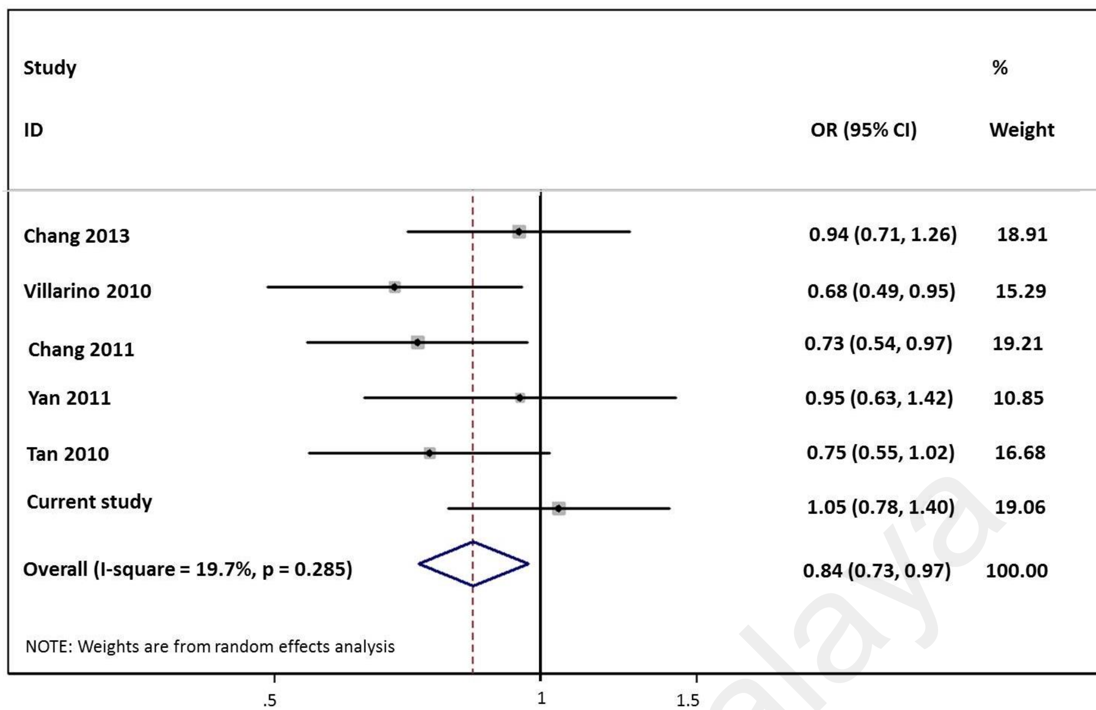

Figure 3.5: Forest plot of the pooled analysis at rs823128

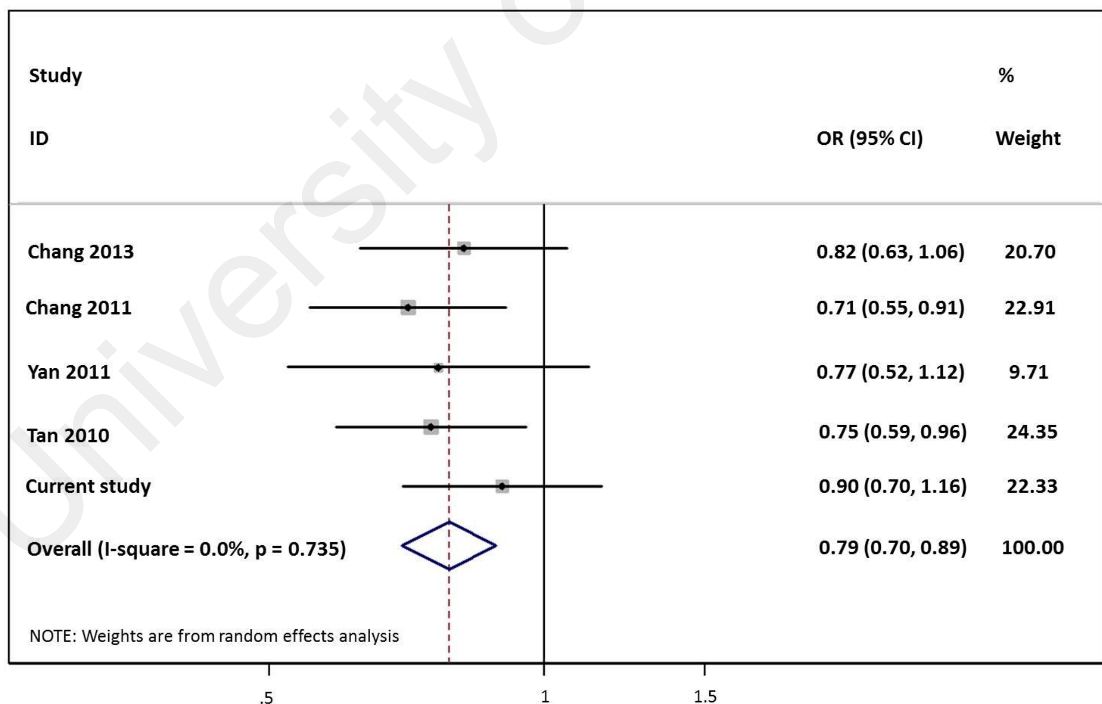

Figure 3.6: Forest plot of the pooled analysis at rs823156

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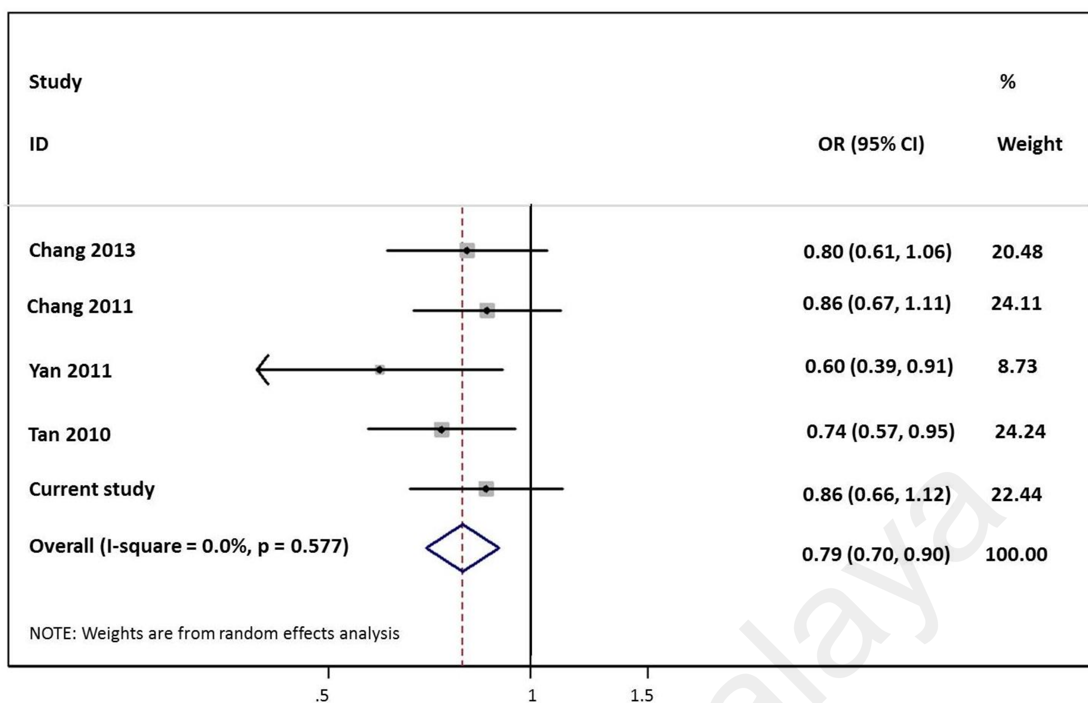

Figure 3.7: Forest plot of the pooled analysis at rs11240572

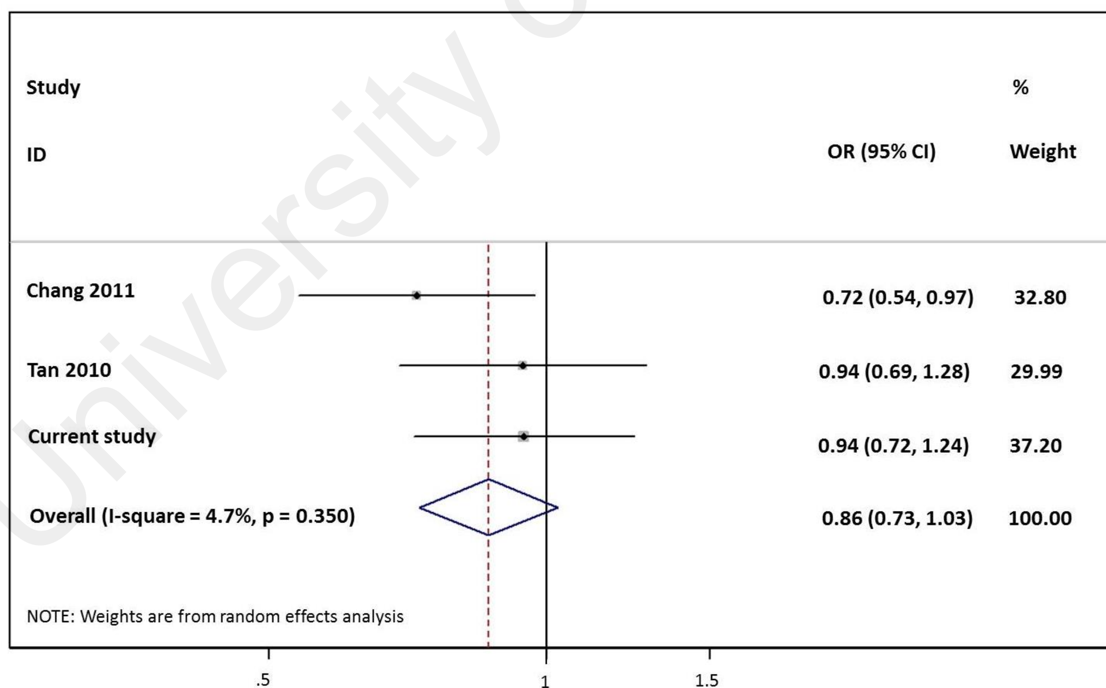

Figure 3.8: Forest plot of the pooled analysis at rs16856139

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In our cohort, rs947211 had the strongest association as we could detect its association even in a modest sample size of 1,139 individuals. This SNP is located 8.5kb upstream of *RAB7L1*, which encodes the RAB7L1 protein that is involved in vesicle trafficking and in maintaining the integrity of the trans-Golgi network (Beilina et al., 2014). *LRRK2*, the established PD gene, has been shown to interact with this protein (MacLeod et al., 2013). We postulate that the rs823128, rs823156 and rs11240572 SNPs have a moderate effect with our population as the protective association could only be detected in a much larger cohort of 5,250 individuals.

Apart from Satake et al., 2009, all the other Asian studies on *PARK16*, including our study, show a protective trend with the G allele at rs823128 (Chang et al., 2013b; Chang et al., 2011; Tan et al., 2010a; Vilariño-Güell et al., 2010; Yan et al., 2011). The conflicting findings between Satake et al., 2009 and the other Asian studies may be due to ethnic-specific effects of *PARK16*, population stratifications that exist in the cohorts, environmental factors that modulate the risk of developing PD, or limited sample size and statistical power. The various Asian studies and *PARK16* SNPs that have been studied is summarised in Appendix G.

A recent paper looking at linkage disequilibrium (LD) using SNPs obtained from the Singapore Genome Variation Project has indicated that the LD pattern around the rs947211 SNP was similar to the pattern seen in the Caucasian and Asian, compared to the other *PARK16* SNPs (Li et al., 2015a). This finding is coherent with our results where a similar effect is seen with the rs947211 SNP in our Malaysian cohort with other ethnically close populations as listed in Appendix G.

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3.3 *GAK* locus

Two SNPs within the *GAK/DGKQ* locus (rs11248051 and rs1564282) were found to be associated with PD through a GWAS conducted by Pankratz et al., 2009 on PD patients with affected first degree relatives. This was replicable in other Caucasian (Hamza et al., 2010) and Asian populations (Chinese from China and Taiwan) (Li et al., 2012a; Pankratz et al., 2012; Rhodes et al., 2011; Tseng et al., 2013) but not in a Spanish population (Mata et al., 2011).

Rs11248051 and rs1564282 are located within introns 28 and 30 respectively of the cyclin-G-associated kinase (*GAK*) gene on chromosome 4. Cyclin G-associated kinase (GAK) is a 160 kDa serine/threonine kinase. GAK plays a role in microtubule growth around chromosomes during spindle formation (Tanenbaum et al., 2010). Recently, GAK has been reported to modify α-synuclein expression levels as GAK binds to the main lysosomal enzyme (pre-cathepsin D) that is involved in α-synuclein degradation, and reduced GAK function is able to enhance α-synuclein mediated toxicity (Dumitriu et al., 2011). Interestingly, *GAK* is one of the 137 genes that are differentially expressed in PD, with a 1.56-fold change in expression in the substantia nigra pars compacta of PD patients as compared to controls (Grünblatt et al., 2004). GAK also binds indirectly to LRRK2 in the trans-golgi network (Beilina et al., 2014). The identification of a secondary independent risk variant within the *GAK/DGKQ* locus by a recent study further implicates the role of *GAK* as a candidate PD gene (Nalls et al., 2014).

3.3.1 Results and discussion

A total of 1,023 PD cases and 524 controls were screened for these SNPs in our cohort, however no association was observed for both the SNPs.

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3.3.1.1 rs1564282, NC000004.12:g.858525C>T

A combined analysis of the three ethnic groups in the Malaysian cohort yielded no association for this SNP (OR 1.018, *p*=0.93; Table 3.7). When analysed according to ethnic groups, the T allele of rs1564282 indicated that it was associated in a protective manner in the Malays (OR 0.27, *p*=0.03). The non-significant association in the Chinese in our cohort is similar to what has been found in the Taiwanese and Singaporean studies (Lin et al., 2013; Tseng et al., 2013). This is in contrast with the risk association reported in a Chinese cohort in mainland China (Chen et al., 2013; Li et al., 2012a).

Table 3.7: Analysis of rs1564282 shows no significant association (OR 1.018; *p*=0.93)

	PD cases			Control		
Genotype	**Malay**	**Chinese**	**Indian**	**Malay**	**Chinese**	**Indian**
Wildtype (C/C)	194	457	79	101	223	55
Heterozygous mutant (C/T)	97	126	51	47	51	31
Homozygous mutant (T/T)	7	9	6	7	5	4
Allelic frequency (%)						
Wildtype (C)	81.4	87.8	76.8	80.3	89.0	78.3
Mutant (T)	18.6	12.2	23.2	19.7	11.0	21.7

As there were other published data for rs1564282 in the Chinese (Chen et al., 2013; Li et al., 2012a; Lin et al., 2013; Tseng et al., 2013), a pooled analysis with our Chinese data was conducted. When pooled analysis was performed on this SNP, we found a risk association in the Chinese (OR 2.02, *p*=0.009; Table 3.8) with the T allele. A similar risk association was also found in a recent meta-analysis (Ma et al., 2015). Figure 3.9 depicts the forest plot of the pooled analysis done on the Chinese population.

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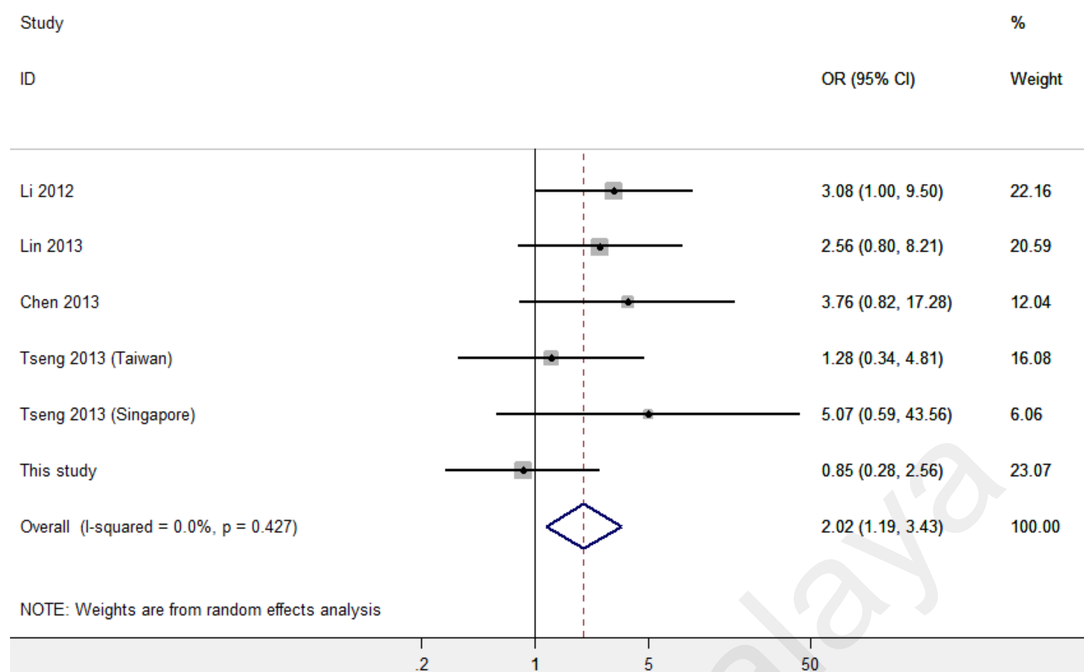

Figure 3.9: Forest plot of the pooled analysis for rs1564282 done in the Chinese

3.3.1.2 rs11248051, NC_000004.11:g.858332C>T

When all the ethnic groups were analysed together, the OR obtained was 1.018 (p=0.87). When the data were analysed individually according to ethnicity, no association was found. The genotypic data is included in Appendix H.

In order to investigate whether a larger sample size would affect the significance, we obtained genotypic data from published reports on rs11248051 in ethnically matched populations (Lin et al., 2013; Zhou et al., 2014), and performed a pooled analysis with our Chinese data (Table 3.8). When pooled analysis was performed on rs11248051, we found a risk association with this SNP (OR 1.30, p=0.008). Figure 3.10 shows the forest plot for the pooled analysis done in the Chinese. Further analysis was not possible for the Malay or Indian cohorts as no other published data was available.

Our current study on the *GAK* locus suggests that the rs11248051 and rs1564282 are risk factors in the Chinese population, though a much larger sample size was needed to ascertain the association.

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Table 3.8: Pooled analysis of *GAK* locus and PD in the Chinese cohort

Genotype **rs1564282**	Controls	Cases
C/C	2,127	2,384
C/T	507	661
T/T	20	52
Total	2,654	3,097
OR and *p*-value	2.02 (95% CI 1.19-3.43); *p*=0.009	
rs11240851		
C/C	871	1,040
C/T	161	281
T/T	45	40
Total	1,077	1,361
OR and *p*-value	1.31 (95% CI 1.07-1.60); *p*=0.008	

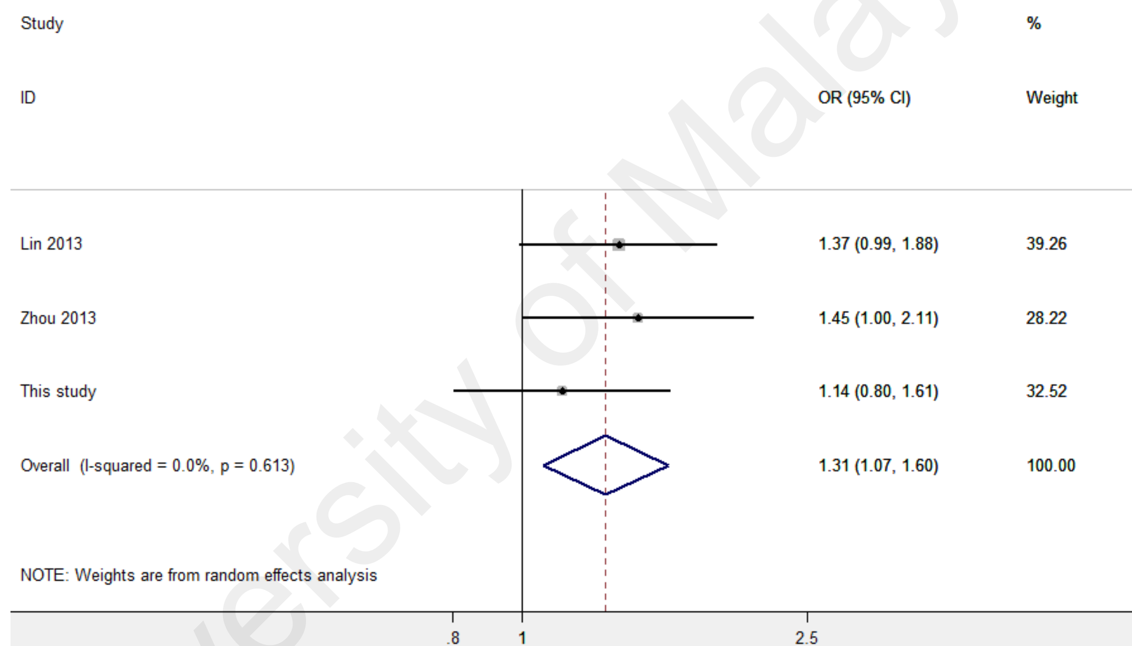

Figure 3.10: Forest plot of the pooled analysis for rs11248051 in the Chinese

Based on the Genomic Evolutionary Rate Profiling (GERP) scores for rs1564282 and rs11248051 (0.88 and -1.21 respectively), the wildtype allele of rs1564282 is predicted to be more conserved across species as compared to rs11240851. To date, the rs1564282 SNP in this locus is the first variant examined in this thesis to show an association in the Malay PD population, despite the relatively small sample size (n=296). Interestingly, the effect of the SNP in the Malay with the T allele was a

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protective association which differed from the pooled Chinese data which showed a risk association. Table 3.9 summarises published data on this locus.

Table 3.9: Summary of published data on the *GAK* locus

Study	Population	rs11248051 (MAF cases, controls)	rs1564282 (MAF cases, controls)
Pankratz et al., 2009	Caucasian 935 PD, 895 controls	OR 1.70 (*p*<0.0001) (0.13,0.09)	OR 1.70 (*p*<0.0001) (0.13,0.09)
Hamza et al., 2010	Caucasian 2,000 PD; 1,986 controls	OR 1.32 (*p*<0.0001) (0.12,0.09)	
Mata et al., 2011	Spanish 1,445 PD; 1,161 controls	OR 1.05 (*p*=0.60) (0.09,0.08)	
Simon-Sanchez et al., 2011 (Netherlands)	Caucasian 841 PD; 2,082 controls		OR 1.31 (*p*<0.0001) MAF NA
Spencer et al., 2011	Caucasian 1,705 PD; 5,175 controls		OR 1.17 (*p*=0.02) (0.10,0.09)
Rhodes et al., 2011	Caucasian 273 PD, 306 controls		OR 1.61 (*p*=0.01) (0.13,0.09)
Li et al., 2012 (China)	Han Chinese 812 PD, 762 controls		OR 1.34 (*p*=0.017) (0.13,0.10)
Lin et al., 2013	Taiwanese 448 PD, 452 controls	OR 1.37 (*p*=0.03) (0.14,0.11)	OR 1.28 (*p*=0.13) (0.13,0.08)
Chen et al., 2013	China 376 PD, 277 controls		OR 1.50 (*p*=0.02) (0.13,0.09)
Tseng et al., 2013 *cohort from Li	Chinese 1,683 PD; 1,646 controls Taiwan 483 PD, 495 controls Singapore 388 PD, 389 controls China from Li 2012		OR 1.18 (*p*=0.04) (0.12,0.10) OR 0.96 (*p*=0.79) (0.11,0.11) OR 1.06 (*p*=0.71) (0.11,0.10)
Zhou et al., 2014	Chinese 323 PD, 345 controls	OR 1.52 (*p*=0.02) (0.13;0.10)	
Current study	Total 1,026 PD, 524 controls	OR 1.018 (p>0.05) (0.16;0.15)	OR 1.018 (p>0.05) (0.15;0.15)
	Chinese 592 PD, 279 controls	(0.12,0.11)	(0.12,0.11)
	Malays 298 PD, 155 controls	(0.19,0.20)	OR 0.27 (*p*=0.03) (0.15,0.20)
	Indians 136 PD, 90 controls	(0.24,0.22)	(0.23,0.22)
Pooled analysis	Chinese	1,361 PD; 1,077 controls OR 1.31, *p*=0.008	3,097 PD; 2,654 controls OR 2.02, *p*=0.009

3.4 *GRN* gene

The *GRN* encodes a secreted growth factor progranulin. Mutations in *GRN* have been implicated with frontotemporal lobar degeneration and 50% reduction in the function of GRN causes neuronal cell death in this region (Eriksen & Mackenzie, 2008; Rademakers et al., 2008). Patients with frontotemporal lobar degeneration present with parkinsonism features which suggests a possible overlap of pathways between frontotemporal lobar degeneration and PD (Le Ber et al., 2007).

An independent study by Mateo et al., 2013 also found that PD patients had significantly lower levels of progranulin in the serum as compared to controls suggesting that progranulin may infer protection against PD (Mateo et al., 2013). This is further established by the delivery of progranulin that was able to protect nigrostriatal neurons from MPTP toxicity in mice (Van Kampen et al., 2014).

A recent study suggested that rs5848, c.*78C>T, a SNP in the 3'-UTR of the *GRN* gene was implicated in Taiwan PD cases (Chang et al., 2013a). This association was stronger in females with 1.59 fold increased risk of developing PD. However this association was not observed in a Caucasian population (Jasinska-Myga et al., 2009). The wildtype allele for rs5848 has a high GERP score of 2.11, suggesting its importance as it is relatively conserved across species.

Although the location of the 3'-UTR SNP does not immediately hint to a pathogenic role, it is known that the 3'-UTR of genes contain multiple recognition sequences for mRNA stability and transport as well as binding sites for microRNAs (miRNAs). Thus polymorphisms within this region can cause miss regulation of the expression of this gene. Interestingly, the microRNA link has been addressed in relation to rs5848 as it has been reported to be within the binding site for miR-659. *In silico* analyses predicted a

stronger binding (hence lower *GRN* expression) of miR-659 to the *GRN* 3'-UTR containing the risk T-allele compared with the wildtype C-allele (Rademakers et al., 2008).

3.4.1 Results and discussion

We sought to determine the association of rs5848 in our cohort, and a total of 1,013 PD cases and 522 controls were screened. Table 3.10 shows the genotypic data of rs5848 in our cohort. Statistical analysis suggests that there is no significant association with our PD cases (OR 1.14, p=0.26). We then conducted a pooled analysis with Chang et al., 2013 and a significant association was found in the Chinese (OR 1.27, p=0.01).

Table 3.10: The *GRN* rs5848 SNP which was reported to alter the risk of developing PD is not seen in our cohort although there was a weak trend towards risk (OR 1.14; *p*=0.2556).

	PD cases			Control		
Genotype	**Malay**	**Chinese**	**Indian**	**Malay**	**Chinese**	**Indian**
Wildtype (C/C)	109	222	58	57	118	48
Heterozygous mutant (C/T)	133	289	60	72	113	35
Homozygous mutant (T/T)	50	75	17	28	44	7
Allelic frequency (%)						
Wildtype (C)	60.1	62.5	65.1	59.2	63.5	72.7
Mutant (T)	39.9	37.5	34.8	40.8	36.5	27.2

As Chang et al., 2013 showed a risk association with the T allele and particularly with the females in their cohort (OR 1.59, p=0.004), we investigated this in our cohort. When the females from all ethnic groups were analysed together, we did not observe any association (OR 1.29, p=0.127). However female Indian PD cases showed a significant risk association (OR 2.29, p=0.01), which was interesting when considering the small sample size of these individuals. A bigger cohort should be studied to confirm this association. Table 3.11 summarises the studies on *GRN* rs5848.

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Table 3.11: Summary of studies done on *GRN* rs5848

Study	Population	Findings
Jasinka et al., 2009	Caucasian 771 PD, 642 controls	T minor allele (OR 1.06, *p*=0.478) 0.322 PD, 0.309 control
Chang et al., 2013	Taiwan Chinese 573 PD, 490 controls	T minor allele (OR 1.25, *p*=0.014) 0.372 PD, 0.322 control In females, T allele OR 1.59, *p*=0.004
Current study	Malaysian 1,013 PD, 522 controls	T minor allele (OR 1.14; *p*=0.2556) 0.378 PD, 0.362 control In females, T allele OR 1.29, *p*=0.127
Pooled analysis	Chinese 1,159 PD, 765 control	OR 1.27; *p*=0.01 0.373 PD, 0.338 control

3.5 Conclusions

In conclusion, the *HLA-DRA* locus and rs947211 in *PARK16* are protective variants in our cohort. The four other SNPs in *PARK16* (rs823128, rs823156, rs11240572 and rs16856139) show significant protective association with PD when analysed with other Asian populations, indicating that there is likely to be a protective effect. The pooled analysis for the two SNPs in *GAK* showed a risk association but independently in our cohort, no association was found. Intriguingly, the Malay cohort showed significant protective association with rs1564282 when analysed independently. The same allele had two opposing effect in the different race. The association in the Malays for rs1564282 ought to be further tested in a larger cohort before a conclusive association can be made. Based on the current data on rs1564282, it is not possible to conclude with a single SNP the possible mechanism involved. More SNPs in this locus should be screened to better understand how this single allele can produce an opposing effect in the different races. For the other ethnic groups, we hypothesise that the SNPs in the *GAK* locus have a more moderate effect as a larger sample size was needed to establish an association (only when our cohort was pooled with other studies).

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The *GRN* rs5848 SNP revealed a risk association when pooled analysis of the Chinese cohort and published data was conducted. A risk association with the female Indian PD patients was also identified.

The work done further emphasises the ethnic specific inheritance of the GWAS-linked loci and gene studied in this chapter as well as the importance of validating the reported variants. In the Malaysian cohort, the 3 loci and *GRN* gene discussed in this chapter does not conform to the Caucasian studies except the *GAK* locus. The use of these variants for PD prediction in the Malaysian population should be done cautiously especially in the Malay and Indian population. The Malay and Indian sample collection is still at its infancy and therefore a definite association between the SNPs and these cohorts is not possible at this stage. The *HLA-DRA* and rs947211 of *PARK16* that are associated in a protective manner are better choices for PD case prediction in Malaysia as compared to the *GAK* or *GRN*.

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CHAPTER 4: GENETIC ANALYSIS OF *LRRK2* MUTATIONS

The earlier chapter investigated the association between several loci (*HLA-DRA*, *PARK16*, *GAK* and *GRN*) with PD in our cohort. Here we discuss the results from our investigation into *LRRK2*. Since Funamaya et al., 2007 first reported the association between *LRRK2* and LOPD through linkage analysis, many subsequent studies have corroborated this result. Multiple *LRRK2* mutations have been reported in Caucasian, Arabs and Jewish PD populations (Funayama et al., 2007; Ozelius et al., 2006; Ross et al., 2011). In Asian populations, several groups in Singapore, Taiwan and China have also explored the *LRRK2* association with PD (Lu et al., 2008; Tan et al., 2007a; Tan et al., 2010b). This firmly puts *LRRK2* as a common susceptibility locus in PD populations across the world.

4.1 Introduction

The *LRRK2* gene consists of 51 exons which encodes for a protein with 2,527 amino acids, 286 kDa. Figure 4.1 depicts *LRRK2*, the various domains within it, and some of the mutations that have been reported in PD patients. A detailed discussion of LRRK2 function and its known role in PD pathogenesis is outlined in Chapter 1, Section 1.5.

Based on the PD Mutation Database, there are 127 reported *LRRK2* mutations, some of which are shown in Figure 4.1 (Lesage & Brice, 2009). Several mutations such as the R1441C/G/H, Y1699C, and G2019S have been reported in the Spanish, Africans, Caucasian and Middle Eastern populations but not in the Asian populations (Ohta et al., 2011; Wu-Chou et al., 2013). The G2019S mutation accounts for 40% of North African Arabs as well as 20% of Ashkenazi Jews PD cases but it is not present in any Asian (Japanese and Chinese) populations studied thus far (Bekris et al., 2010; Correia Guedes et al., 2010; Lesage et al., 2006).

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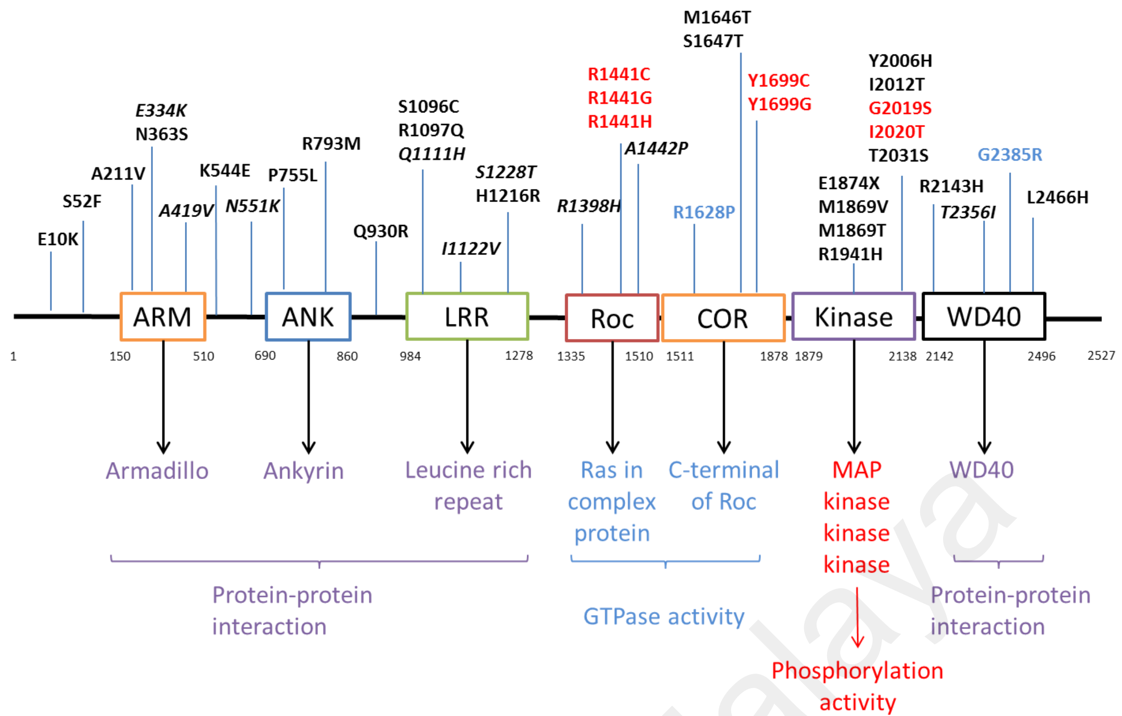

Figure 4.1: Schematic diagram of *LRRK2*, its functional domains and its sequence changes. Number under the protein indicates the boundaries of each domain. Proven pathogenic mutations are shown in red, risk factors are shown in blue, and mutations that have been reported in more than one population are italics. Adapted from Lesage et al., 2009.

Given that there appear to be some ethnic-specific *LRRK2* mutations, it is necessary to determine the *LRRK2* variation profile in the Asian PD population. Most Asian studies thus far have involved cohorts of Chinese ethnicity mainly based in China or Taiwan. There are only two papers on the Malays reported thus far from a Singaporean study (Tan et al., 2008b; Tan et al., 2007b) which examined G2385R and R1628P. There is still a large gap in our knowledge of the involvement of the *LRRK2* mutations in the Malaysian PD population. In the following section, we present data on five *LRRK2* mutations which were screened (G2385R, A419V, R1628P, R1398H and N551K). The demographic features of the samples screened can be found in Appendix F. The error rate for all the *LRRK2* mutations screened was 0% (calculated as described in Section 2.5). Appendix I shows an example of the allelic discrimination plots obtained from the screening of A419V, N551K and R1398H mutations in this chapter.

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4.2 G2385R (c.7153G>A, rs34778348)

The G2385R mutation has been reported to be the most common 'risk variant' in the Chinese, Japanese and Koreans but not in Caucasians (An et al., 2008; Kim et al., 2010; Tan et al., 2007a). Most of the Asian studies have shown that individuals with this mutation have a two-fold increased risk of developing PD. This pathogenic effect may be mediated by the fact that G2385R is located in the WD40 domain of LRRK2, which is involved in forming stable protein-protein interactions.

4.2.1 Results and discussion

A total of 695 PD cases and 507 controls were screened. In the Chinese cohort, the PD cases had a minor allelic frequency (MAF) of 0.04, and controls 0.02. Meanwhile it was rare in the Malays (MAF <0.005, present only in two PD patients) and it was absent in the Indians (Table 4.1), which was also seen in Tan et al. 2007.

Table 4.1: Genotypic data of G2385R in the Malaysian cohort

	PD cases			Control		
Genotype	**Malay**	**Chinese**	**Indian**	**Malay**	**Chinese**	**Indian**
Wildtype (G/G)	198	361	101	135	266	94
Heterozygous mutant (G/A)	2	32	-	1	11	-
Homozygous mutant (A/A)	-	1	-	-	-	-
Allelic frequency (%)						
Wildtype (G)	99.5	95.6	100	99.7	98.0	100
Mutant (A)	0.5	4.3	-	0.3	2.0	-

Taking into consideration the rarity of the mutation in Malays and Indians, only the Chinese cohorts were analysed further. We found that the minor allele was present almost two folds higher in the PD cases as compared to the controls, and statistical analysis showed that the G2385R is a risk variant in the Malaysian Chinese cohort (OR 2.22, p=0.019). Our findings are in keeping with other Asian populations such as the Taiwanese, Chinese, Japanese and Singaporean where the risk of developing PD is also approximately two-fold (Table 4.2).

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Table 4.2: Summary of G2385R studies in Asian populations

Study	Asian country	Sample size	Results
G2385R (c.7153G>A), rs34778348			
Di Fonzo et al., 2006	Taiwan	608 PD, 373 controls	OR 2.24 (*p*=0.004)
Fung et al., 2006	Taiwan	305 PD, 176 controls	OR 17.00 (*p*=0.0002)
Farrer et al., 2006	Taiwan	410 PD, 335 controls	OR 2.24 (*p*=0.014)
Funamaya et al., 2006	Japan	448 PD, 457 controls	OR 2.60 (p=1.24x10^{-4})
Tan et al., 2007	Singapore	495 PD, 494 controls	OR 2.14 (*p*=0.014)
Tan et al., 2007	Non-Chinese Asians Malays, Indians	98 PD, 173 controls 66 PD, 133 controls	Malays OR 1.78 (*p*=0.3) Indians – monomorphic
An et al., 2008	Mainland China	600 PD, 334 controls	OR 3.94 (*p*<0.01)
Zabetian et al., 2009	Japan	601 PD, 1628 controls	OR 1.96 (*p*<0.001)
Miyake et al., 2010	Japan	229 PD, 358 controls	OR 2.06
Kim et al., 2010	Korea	923 PD, 422 controls 119 YOPD 814 LOPD	Combined OR 1.83 (*p*=0.017) YOPD OR 2.28 (*p*=0.098) LOPD OR 1.81 (*p*=0.022)
Wu-Chou et al., 2013	Taiwan	914 PD, 618 controls	OR 1.4 (*p*=0.089)
Current study	Malaysia Chinese	695 PD, 507 controls 394 PD, 277 controls	OR 2.22 (*p*=0.013) OR 2.22 (*p*=0.019)

This effect of this mutation in conferring a greater susceptibility to develop PD could be due to an abnormal WD40 domain. The WD40 domain exists in various eukaryotic proteins and has a range of functions including signal transduction, RNA processing, vesicle trafficking as well as cytoskeleton assembly (Xu & Min, 2011). LRRK2 has been shown to regulate synaptic vesicle trafficking through the interaction of its WD40 domain with presynaptic protein (*N*-ethylmaleimide sensitive factor, synaptic vesicle glycoprotein 2A, synapsin and syntaxin 1) that are key components of the synaptic trafficking (Piccoli et al., 2014). Therefore in theory mutations in the WD40 domain may interfere with this ability to bind to synaptic vesicles. Deletions of the WD40 domain show a decrease in autophosphorylation activity and reduced neurotoxic effects in cell lines as well as primary neuronal cultures (Jorgensen et al., 2009). When expressed in HEK-293T and primary cortical cultures, cells carrying the G2385R

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mutation were more susceptible to oxidative stress and more prone to apoptosis (Tan et al., 2007a; West et al., 2007).

In addition, since the LRRK2 protein is a kinase, studies have been conducted to understand how the G2385R mutation affects the kinase activity. *In vitro* kinase studies on G2385R have yielded contradicting results. Some groups have shown a decrease in kinase activity (Jaleel et al., 2007; Rudenko et al., 2012) while others showed no significant changes (West et al., 2007) although these three studies used comparable methods and starting material. Studies on zebrafish mutants have also been inconclusive. A deletion of the WD40 domain of LRRK2 in zebrafish caused locomotive defects and PD-like dopaminergic neuron loss (Sheng et al., 2010) but this was later refuted by Ren et al, 2011. Therefore although genetic studies have clearly implicated G2385R in PD, the actual mechanism through which it mediates this is still under investigation.

4.3 A419V (c.1256C>T, rs34594498)

A large case control study consisting of 2,338 Asian patients and controls suggested A419V to be the third risk variant amongst Asians (Ross et al., 2011). This mutation was reported to have an OR of 1.26 in their Japanese cohort, 2.21 in the Korean cohort and 7.51 in the Taiwanese cohort. In contrast, various independent studies have shown that A419V was either rare (found in a frequency below 1%) or monomorphic in Han Chinese PD patients from Taiwan and Singapore (Di Fonzo et al., 2006; Tan et al., 2010b; Wu-Chou et al., 2013; Wu et al., 2012).

4.3.1 Results and discussion

The A419V mutation was screened in our cohort of 404 PD cases and 426 controls. Compared to G2385R which had a higher MAF (0.04), the MAF for A419V in the

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Chinese cohort was rare (0.002). This mutation was absent (monomorphic) in Malays and Indians (Table 4.3).

Table 4.3: Genotypic data of A419V

Genotype	PD cases Malay	PD cases Chinese	PD cases Indian	Control Malay	Control Chinese	Control Indian
Wildtype (C/C)	122	222	59	110	233	80
Heterozygous mutant (C/T)	-	1	-	-	3	-
Homozygous mutant (T/T)	-	-	-	-	-	-
Allelic frequency (%)						
Wildtype (C)	100	99.8	100	100	99.4	100
Mutant (T)	-	0.2	-	-	0.6	-

No subject was homozygous for this mutation. Only one Chinese PD patient was positive compared to three Chinese controls; MAF 0.002 (cases) against MAF 0.006 (controls), yielding an OR of 0.35 (*p*=0.624). The PD patient positive for A419V was diagnosed at 45 years and had no family history of PD. The median age of the 3 controls was 61 years, indicating that it was not a case of undetected PD in the controls, taking into consideration the PD patient with A419V had already developed this disease at a much younger age. This result contrasts sharply with the report by Ross and colleagues, in which an OR of 7.51 was found in their Taiwanese Chinese cohort, despite having a comparable ethnic group (i.e., Han Chinese). While we acknowledge that our sample size is relatively small, our finding of a low MAF is similar to that reported recently by Wu and colleagues with over 3,000 samples, suggesting that the MAF in our cohort is representative of the ethnic group as a whole (Wu et al., 2012).

Based on the current published findings (Table 4.4) describing A419V in Chinese PD patients, two papers reported that the A419V was monomorphic in their cohort (Tan et al., 2010b; Wu-Chou et al., 2013). However, two papers (Di Fonzo et al., 2006; Wu et al., 2012) showed that the OR value was below 1.

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Table 4.4: Summary of A419V studies in Asian populations

Study	Asian country	Sample size	Results
A419V (c.1256C>T), rs34594498			
Di Fonzo et al., 2006	Taiwan	608 PD, 373 controls	No association
Tan et al., 2010	Singapore	250 PD, 250 controls	Monomorphic
Ross et al., 2011	Japan Korea Taiwan	173 PD, 75 controls 844 PD, 587 controls 369 PD, 300 controls	Japan OR 1.26 Korea OR 2.21 Taiwan OR 7.51 Combined OR of 2.27 (*p*=0.0011)
Li et al., 2012	Han Chinese from China	729 PD, 585 controls	EOPD OR 14.89 (*p*=0.0005) LOPD OR 2.37 (*p*=0.17) Combined OR 4.14 (*p*=0.003)
Wu et al., 2012	Taiwan, Singapore and China	1517 PD, 1487 controls	No association Taiwan: OR 0.67 (*p*=0.57) Singapore: OR 0.98 (*p*=0.98) China: OR 1.56 (*p*=0.56) Combined: 0.98 (*p*=0.96)
Wu-Chou et al., 2013	Taiwan	621 PD, 439 controls	Monomorphic
Li et al., 2015	Mainland China	500 PD, 574 controls (181 EOPD, 150 controls) (319 LOPD, 424 controls)	EOPD OR 10.40 (*p*=0.027) LOPD OR 1.70 (*p*=0.319) Combined OR 2.57 (*p*=0.025)
Current study	Malaysia	404 PD, 426 controls	OR 0.33 (*p*=0.624)

Out of the eight studies on A419V in Asian PD thus far, five suggest that there is no association with the exception of Ross et al., 2011, Li et al., 2012 and Li et al., 2015. The association seen by Li and colleagues (Li et al., 2012 and Li et al., 2015) appears to be primarily driven by the occurrence of this mutant in early-onset PD cases. This is because, when the EOPD cases were removed from their cohort, the LOPD cases in these cohorts did not yield significant risk association (Li et al., 2012; Li et al., 2015). As discussed by Wu et al., 2012 and Wu-Chou et al., 2013, it is unclear why there is

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such a large discrepancy between these studies. One factor may be due to population stratification whereby cases and controls were not selected properly. There may also be specific environmental factors that are unique to Taiwan and not present in other populations, or possible gene-environment interactions that have not been evaluated.

We performed a combined analysis of five Chinese studies (Di Fonzo et al., 2006; Tan et al., 2010b; Wu-Chou et al., 2013; Wu et al., 2012) and an OR value of 1.05 (p=0.763) was obtained suggesting no association with A419V. This combined cohort consisted of 3,219 cases and 2,785 controls as compared to Ross and the two Li studies (1,598 cases and 1,459 controls). The combined cohort size of the five studies should be regarded as being representative of the A419V mutation in the Chinese ethnic group. The analysis strongly suggests that A419V mutation is not a risk factor in LOPD in Chinese PD patients. Although the meta-analysis on all the published Chinese data conducted by Li et al., 2015 suggests A419V to be a risk factor (OR 2.07), we call for cautious interpretation of this data as the risk factor may be primarily contributed by the EOPD cases.

As there are no other studies that have examined A419V in Malay and Indian PD cases, we were unable to perform a meta-analysis for these groups. Our current study, which is the first report in these ethnic groups, suggests that A419V is unlikely to be a common cause of PD in the Malays and Indians.

4.4 R1628P (c.4883G>C, rs33949390)

R1628P was first reported in 2005 (Mata et al., 2005) and confirmed by a series of studies in Asians (Lu et al., 2008; Ross et al., 2008; Tan et al., 2008a; Yu et al., 2009; Zhang et al., 2009). In a study on the Chinese, R1628P was determined to be the second common genetic risk factor for PD (Ross et al., 2008). The cohort in that study

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consisted of three independent Asian Chinese centres (2 centres in Taiwan and 1 in Singapore) with an OR of 1.84 (*p*=0.006). Other independent studies in Singapore and Thailand, also showed a similar risk trend (OR 2.5 and 3.4) (Pulkes et al., 2011; Tan et al., 2008a). However, in the Japanese population, the R1628P was absent (Zabetian et al., 2009). There is currently only one study that looked at the distribution of R1628P in non-Asian population, specifically Arabs and Caucasians. In both these populations, the R1628P were absent or rare. In the same study, based on an Asian cohort consisting of Taiwanese, Korean and Japanese populations, the result suggested that R1628P has a protective association with PD (OR 0.62, *p*=0.087) (Ross et al., 2011).

4.4.1 Results and discussion

A total of 698 PD cases and 510 controls were screened in our cohort. R1628P was found to be a risk factor with an OR 1.23 (*p*=0.05). Compared to G2385R and A419V, R1628P was more common in all three ethnic groups. Mutant alleles were observed to be present at higher frequencies in ethnic Chinese (MAF 0.03) followed by Malays (MAF 0.02). No homozygotes were found in this cohort. Table 4.5 summarises the genotyping results for R1628P.

Table 4.5: Genotypic data of R1628P

	PD cases			Control		
Genotype	**Malay**	**Chinese**	**Indian**	**Malay**	**Chinese**	**Indian**
Wildtype (G/G)	189	366	100	130	260	96
Heterozygous mutant (G/C)	11	30	2	6	18	-
Homozygous mutant (C/C)	-	-	-	-	-	-
Allelic frequency (%)						
Wildtype (G)	97.3	96.2	99.0	97.8	96.8	100
Mutant (C)	2.7	3.8	1.0	2.2	3.2	-

Our findings concur with published data in the Asian populations (Table 4.6), but our conclusions are at odds with the Ross' 2011 study. The protective association observed in their study is probably due to the pooling of the Japanese cohort together with their

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Taiwanese and Korean population. As Zabetian and colleagues in 2009 have established that this mutation is not present in the Japanese population, including the Japanese in their analysis would have skewed the data towards a protective association.

The R1628P is located in the C-terminal of Roc (COR) domain of *LRRK2* and is conserved across species, highlighting the importance of this arginine residue in protein function (Lu et al., 2008). The COR domain is thought to aid in the ROC-COR domain dimerisation. However, the functional role of R1628P is still unknown, although *in silico* analysis predicts that the substitution of a highly basic polar arginine to a neutral nonpolar proline abolishes the formation of two β-sheets within the COR domain (Cardona et al., 2014; Ross et al., 2008). Compounding this effect, the R1628P is predicted to be located at the interface of the ROC domain that is responsible for the GTPase activity. The removal of the positive charge and two β-sheets could alter the dimerisation and make the interaction of the COR and ROC domain especially difficult, altering its GTPase activity.

Table 4.6: Summary of R1628P studies in Asian populations

Study	Asian country	Sample size	Results
R1628P (c.4883G>C), rs33949390			
Lu et al., 2008	China	834 PD, 543 controls	OR 2.13 (p=0.004)
Tan et al., 2008	Singapore	246 PD, 243 controls	OR 2.5 (p=0.046)
Ross et al., 2008	Taiwan, Singapore		
	Wu RM	484 PD, 341 controls	OR 2.15 (p=0.025)
	Wu YR	345 PD, 316 controls	OR 1.39 (p=0.179)
	EK Tan	250 PD, 250 controls	OR 2.20 (p=0.163)
	Combined	1079 PD, 907 controls	OR 1.84 (p=0.006)
Zabetian et al., 2009	Japanese	631 PD, 320 controls	Monomorphic
Yu et al., 2009	Mainland China	328 PD, 300 controls	OR 2.68 ($p<0.05$)
Zhang et al., 2009	Mainland China	600 PD, 459 controls	OR 3.14 ($p<0.01$)
Kim et al., 2010	Korea	384 PD, 384 controls	OR 2.98 (p=0.32)
Pulkes et al., 2011	Thai	154 PD, 156 controls	OR 3.25 (p=0.021)
Ross et al., 2011	Japan, Korea and Taiwan	1,386 PD, 982 controls	OR 0.62 (p=0.087)
Wu-Chou et al., 2013	Taiwan	747 PD, 461 controls	OR 2.1 (p=0.005)
Current study	Malaysia	698 PD, 507 controls	OR 1.23 (p=0.054)

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4.5 N551K (c.1653C>G, rs7308720)

N551K was first described in 2006 (Paisán-Ruíz et al., 2006). Despite being described for more than 9 years, currently there are only three other studies investigating the association between this mutation and PD. All three suggest a protective association both in the Asian and Caucasian populations (Ross et al., 2011; Tan et al., 2010b; Wu et al., 2013).

4.5.1 Results and discussion

A total of 523 PD cases and 491 controls were screened. Table 4.7 summarises the genotypic data. When the three ethnic groups were analysed together, the mutant allele is present approximately two folds higher in the controls than in cases (MAF 0.09 and 0.06 respectively), and has an OR of 0.623 (*p*=0.007). When analysed separately, the protective association is seen in the Malay (OR 0.44, *p*=0.02) and Chinese (OR 0.70, *p*=0.05) ethnic groups. A protective association was found in the Indians but it was not statistically significant (OR 0.57, *p*=0.4), this could be due to the small sample size.

Table 4.7: Genotypic data of N551K

	PD cases			Control		
Genotype	**Malay**	**Chinese**	**Indian**	**Malay**	**Chinese**	**Indian**
Wildtype (C/C)	155	239	72	113	214	81
Heterozygous mutant (C/G)	13	38	4	18	54	8
Homozygous mutant (G/G)	-	2	-	2	1	-
Allelic frequency (%)						
Wildtype (C)	96.1	92.5	97.4	91.7	89.6	95.5
Mutant (G)	3.9	7.5	2.6	8.3	10.4	4.5

Table 4.8 summarises published data on N551K. The mutant allele is relatively common in all three ethnic groups with a MAF of 0.08, 0.10 (Chinese PD cases, controls), 0.04, 0.08 (Malay PD cases, controls) and 0.03, 0.05 (Indian PD cases, controls) respectively.

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Table 4.8: Summary of published data on N551K

Study	**Country/Population**	**Sample size**	**Results**
N551K (c.1653C>G), rs7308720			
Tan et al., 2010 (Han Chinese)	Singapore Taiwan China	250 PD, 250 controls 293 PD, 299 controls 628 PD, 510 controls 1363 PD, 1251 controls	OR 0.60 (*p*=0.019) OR 0.62 (*p*=0.021) OR 0.91 (*p*=0.570) OR 0.74 (*p*=0.004)
Ross et al., 2011	Caucasian Asian Arab-Berber	6995 PD, 5595 controls 1376 PD, 962 controls 240 PD, 372 controls	OR 0.88 (*p*=0·025) OR 0.73 (*p*=0·0017) OR 0.83 (*p*=0·47)
Wu et al., 2013	Taiwan	573 PD, 503 controls	OR 0.87 (*p*=0.413)
Current study	Malaysia	523 PD, 491 controls	OR 0.623 (*p*=0.007)

The N551K mutation is not within any domain of the LRRK2 protein but has been shown to be conserved across species (Figure 4.2) and has been shown to be in linkage disequilibrium with R1398H (Tan et al., 2010).

Figure 4.2: The amino acid at position 551, highlighted with the red box is conserved across species (obtained from UCSC Genome browser)

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4.6 R1398H (c.4193G>A, rs7133914)

R1398H was also first described in the same study as N551K in 2006 (Paisán-Ruíz et al., 2006). There are only five studies on this mutation to date and unlike the other three mutations (G2385R, A419V and R1628P) considered in this chapter, the R1398H have been shown a protective association in both the Asian and Caucasian studies (Ross et al., 2011; Tan et al., 2010b; Wu-Chou et al., 2013; Wu et al., 2013).

4.6.1 Results and discussion

Table 4.9 summarises the genotypic data for the 523 PD cases and 491 controls screened for R1398H. When the whole cohort was analysed together, an OR value of 0.699 (*p*=0.036) was obtained, suggesting that R1398H has a protective effect. When analysed according to ethnicity, the protective association was seen in the Malays (OR 0.50; *p*=0.05) but it was not as apparent in the Chinese (OR 0.80; *p*=0.15) and Indians (OR 0.57, *p*=0.4).

Table 4.9: Genotypic data of R1398H

Genotype	PD cases			Control		
	Malay	Chinese	Indian	Malay	Chinese	Indian
Wildtype (G/G)	155	233	71	115	215	80
Heterozygous mutant (G/A)	13	45	5	16	53	8
Homozygous mutant (A/A)	-	1	-	2	1	1
Allelic frequency (%)						
Wildtype(G)	95.6	91.5	96.7	92.5	89.8	93.6
Mutant (A)	4.4	8.6	3.3	7.5	10.2	5.4

This result is coherent with the published data as summarised in Table 4.10. The mutant allele was found to be almost two-fold in the controls as compared to the cases (MAF 0.09 and 0.06 respectively).

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Table 4.10: Summary on published data on R1398H

Study	Country/Population	Sample size	Results
R1398H (c. 4193G>A), rs7133914			
Tan et al., 2010 Han Chinese	Singapore Taiwan China Combined	250 PD, 250 controls 293 PD, 299 controls 628 PD, 510 controls 1363 PD, 1251 controls	OR 0.64 (*p*=0.038) OR 0.64 (*p*=0.033) OR 0.90 (*p*=0.566) OR 0.75 (*p*=0.005)
Ross et al., 2011	Caucasian Asian Arab-Berber	6995 PD, 5595 controls 1376 PD, 962 controls 240 PD, 372 controls	OR 0.89 (*p*=0.034) OR 0.73 (*p*=0.0020) OR 1.00 (*p*=1.00)
Wu et al., 2013	Taiwan	573 PD, 503 controls	OR 0.84 (*p*=0.24)
Wu-Chou et al., 2013	Taiwan	647 PD, 444 controls	OR 0.89 (*p*=0.45)
Current study	Malaysia	523 PD, 491 controls	OR 0.699 (*p*=0.036)

A linkage analysis of G2385R, R1628P, N551K and R1398H indicated that both the N551K and R1398H are in linkage disequilibrium (D'=0.959, r^2=0.906) similar to what has been suggested by Tan et al., 2010 (Figure 4.3). The linkage analysis was carried out using Haploview 4.2.

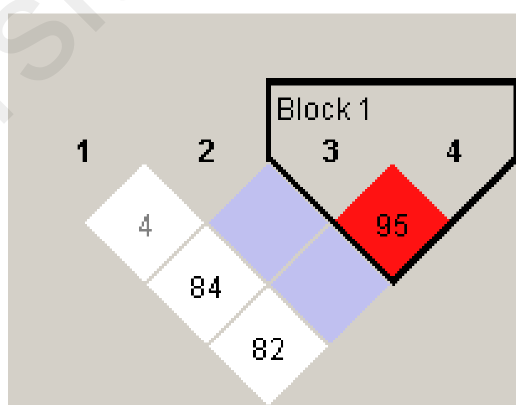

Figure 4.3: LD plot of the four *LRRK2* mutations generated using Haploview 4.2. Legend: 1-G2385R; 2-R1628P; 3-N551K; 4-R1398H; squares without numbers represent D' values of 1.0; all numbers within the box represent the D' value expressed as a percentile; red squares represent log of the odds (LOD) score for linkage disequilibrium of ≥ 2, blue squares represent D'=1 but LOD<2, and white squares represent LOD<2 and D'<1.0.

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The R1398H mutation is located in the Ras-of-complex (ROC) GTPase domain. The GTPase domain of LRRK2 binds guanine nucleotides via a phosphate-binding motif (P-loop) region (amino acid 1341 to 1348) and can hydrolyse GTP. The hydrolysis of GTP also involves a catalytic Switch II motif (amino acid 1394-1398 of LRRK2). The critical GTPase activity in the majority of small GTPase is dependent on the residue at position 1398 and the wildtype amino acid is conserved across species (Figure 4.4). It is interesting to identify if the R1398H is able to confer protection in the cellular level as seen genetically. To date, functional data on this mutation has not been reported.

Human	G	R	E	E	F	Y	S	T	H	P
Chimp	G	R	E	E	F	Y	S	T	H	P
Rhesus	G	R	E	E	F	Y	S	T	H	P
Mouse	G	R	E	E	F	Y	S	T	H	P
Rat	G	R	E	E	F	Y	S	T	H	P
Dog	G	R	E	E	F	Y	S	T	H	P
Opossum	G	R	E	E	F	Y	S	T	H	P

Figure 4.4: The amino acid at position 1398, highlighted with the red box is conserved across species (obtained from UCSC Genome browser)

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4.7 Conclusions

In summary, the G2385R and R1628P mutations are risk factors in the Malaysian cohort, increasing the risk of developing PD by 1.2 to 2 folds. We also observed that in our control cohort, there was a small subset of individuals (2%) that carried the G2385R mutation and 4% that carried R1628P mutation but had not developed PD. This suggests that there could be other factors involved in compensating the susceptibility towards stress or other triggers are needed to exacerbate the cell death. We ruled out A419V as a risk factor while we have shown that N551K and R1398H are protective factors. Figure 4.5 is a graphical summary of the results in this chapter. The functional mechanism of the N551K and R1398H is still unclear. In understanding how the mutation affects the protein function, therapeutic targets can be identified to help halt or slow down disease progression.

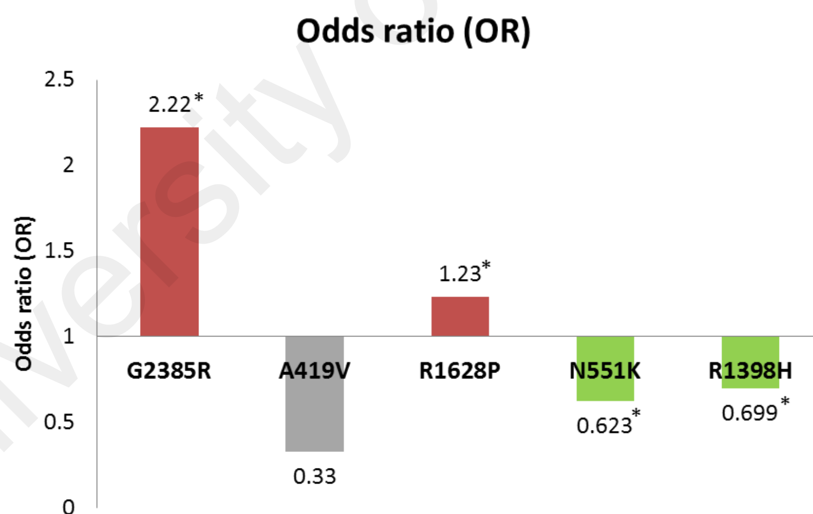

Figure 4.5: Graphical summary of the *LRRK2* genetic results. The asterisks represent statistical significance. The red bar represents a risk association, green bar represents protective association while the grey bar represents no association.

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CHAPTER 5: STUDYING THE FUNCTIONAL EFFECTS OF N551K AND R1398H LRRK2 MUTATIONS

The function of LRRK2 is still not fully understood despite being the most extensively studied PD gene. In particular, its exact role in causing the dopaminergic cell death in the substantia nigra is still under investigation by various labs. The complexity of understanding the function of LRRK2 lies partly in the large size of the protein as well as presence of two enzymatic domains within LRRK2 - the GTPase and kinase domains. Labs across the world have focused on studying the effects of G2019S and other risk mutations in cells, *Drosophila*, zebrafish as well as mice. Not many studies have looked at the genetically protective mutations to understand its mechanism. In this chapter the functional properties of N551K and R1398H are examined to uncover their possible protective mechanism.

Similar to other kinases, LRRK2 has shown autophosphorylation activity *in vitro*. Autophosphorylation changes the kinase activity by repositioning the activation loop as well as change the binding ability of ATP or substrates. Within the activation loop of LRRK2, there are 3 potential autophosphorylation sites (T2031, S2032 and T2035) (Gilsbach & Kortholt, 2014). However, there are a number of LRRK2 autophosphorylation sites in the ROC domain. This domain controls the GTPase activity suggesting a possible intra molecule regulation of the kinase activity. Figure 5.1 depicts the currently known LRRK2 autophosphorylation sites.

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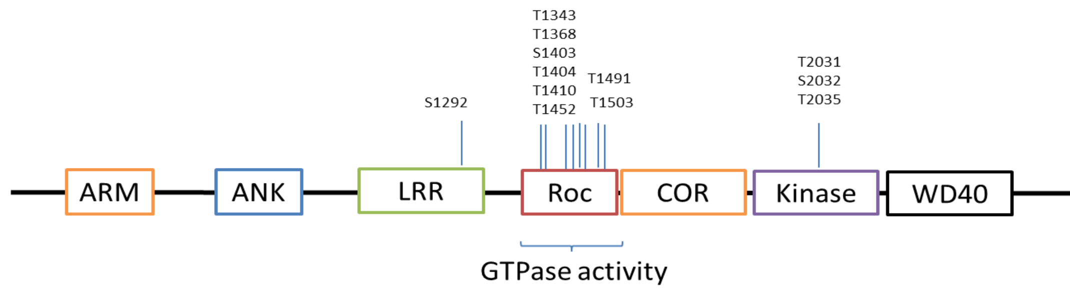

Figure 5.1: LRRK2 autophosphorylation sites clustered in the GTPase responsible domain

5.1 Rationale of selecting which LRRK2 mutations and functional effects to study

As shown in Chapter 4, N551K and R1398H were found to have a significant protective effect in the Malaysian cohort (OR 0.699 and OR 0.623; $p<0.05$ respectively). The N551K is not within any domain but is in LD with R1398H (Chapter 4, Figure 4.3). R1398H is located in the ROC domain, which is responsible for the GTPase activity. To investigate the protective effect, we examined their effects on cell viability, as well as the kinase and GTPase activities.

The ability to counter the effects of cellular stress as mediated by hydrogen peroxide (H_2O_2) was examined. H_2O_2 was used to induce stress in cells as it mimics the oxidative stress that occurs in the dopaminergic neurons of the substantia nigra (Chun et al., 2001). Oxidative stress has been shown to be one of the contributing factors of dopaminergic cell death resulting in PD (Jenner, 2003). In the brain, the oxidation of dopamine produces quinones, monoamines and especially hydrogen peroxide, agents that give rise to oxidative stress increasing the cell's susceptibility to cell death (Fahn & Cohen, 1992). Other chemicals such as MPTP, rotenone, paraquat and 6-hydroxydopamine (6-OHDA) can be used to induce oxidative stress through various pathways. MPTP, rotenone, 6-OHDA cause oxidative stress through the inhibition of mitochondria complex 1 (Przedborski et al., 2004). We chose H_2O_2 to induce stress

since it is a chemical that is naturally occurring within the cell and would be more physiologically relevant than exogenous chemicals. Exposure to exogenous chemicals as a cause of PD is rare and only in isolated populations. However, there are some limitations involved when using H_2O_2 as it degrades easily. To overcome this, we freshly prepared H_2O_2 just before addition to the cells as well as using pyruvate free media since pyruvate is able to protect the cells from the effect of H_2O_2 (Desagher et al., 1997). The G2019S was used as a positive control in the experiments as it has shown to be functionally pathogenic across many labs.

Human neuroblastoma cell lines (SH-SY5Y) were used for the cell viability assay because these cells are dopaminergic cells that could mimic the dopaminergic neurons in the substantia nigra that are lost in PD brains. In addition, a review on 60 articles using SH-SY5Y as an *in vitro* model for PD showed that it is a widely accepted cell model for dopaminergic neurons (Xie et al., 2010). A recent study looking at the SH-SY5Y genomics also showed that most of the genes that belong to the major PD pathways were present in these cells further emphasising its suitability for the cell viability assay (Krishna et al., 2014).

The neurotoxic effect of G2019S can be attributed to the fact that G2019S is within the kinase domain of LRRK2 which then causes an increase in the kinase activity (Smith et al., 2006). G2019S confers a toxic gain of function effect on LRRK2 which is able to activate the MAPK pathway (West et al., 2007). However, while the LRRK2's kinase activity has been proposed to be dependent on the GTPase activity, the abnormal kinase activity of G2019S is not due to impaired GTP binding and hydrolysis activity (Biosa et al., 2012). The R1441C was included in the experiments as a control for the GTPase activity since the mutation is also located on the ROC domain and also because it has been reported to have decreased GTPase activity (Lewis et al., 2007).

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5.2 LRRK2 constructs and expression

5.2.1 LRRK2 constructs cloned into the pCDNA3.1 vector had poor expression and low transfection efficiency

A full length LRRK2-GFP tagged construct in pCDNA 3.1 vector was first utilised in the experiments. Mutations were generated as described in Chapter 2, Section 2.7.1. However, the transfection efficiency and expression for this vector was low in both the SH-SY5Y cells as well as human embryonic kidney cells (HEK-293T), despite being a highly transfectable cell line. To overcome this problem, stable cell lines were generated for wildtype, G2019S, N551K and R1398H in SH-SY5Y cells as described in Chapter 2 Section 2.11. However, Western blot analysis showed that there was no significant expression of LRRK2 in the stable cell lines (Figure 5.2).

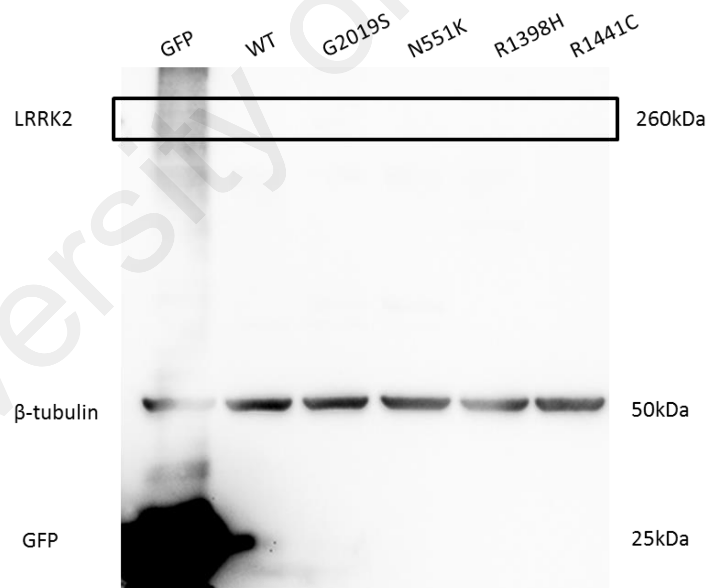

Figure 5.2: Western blot of the stable cell lines revealed no detectable GFP tagged LRRK2 (~260kDa). Legend: GFP-green fluorescent protein; WT-wildtype.

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5.2.2 pEGFP is a better expression vector for LRRK2

A new set of constructs was prepared from a full length LRRK2 construct in pEGFP vector kindly shared by Professor Tan Eng King's lab in Singapore. The various mutations were generated and the expression levels were examined through Western blot analysis (Figure 5.3). The LRRK2 expression was higher than pCDNA 3.1 vector and the expression of the various mutations had comparable levels. The mutations were confirmed by sequencing each construct. The electropherogram of selected constructs can be found in Appendix K.

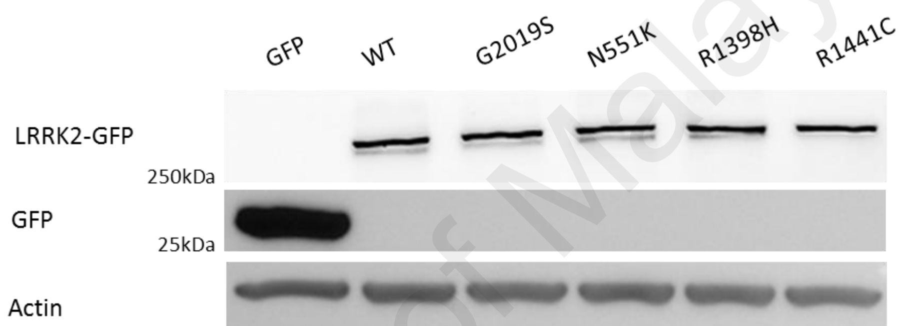

Figure 5.3: All pEGFP construct LRRK2 mutants transfected in HEK-293T cells were expressing LRRK2 (~260kDa).

Figure 5.4 shows the difference in LRRK2 expression between the initial pCDNA 3.1 vector compared to the pEGFP vector. Since the expression level of LRRK2 using the pEGFP vector was much higher, all subsequent experiments were done using transient transfection.

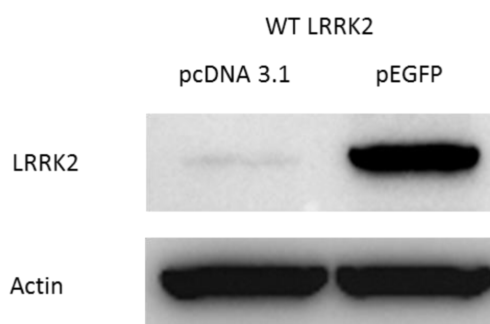

Figure 5.4: Western blot comparing the two different plasmid used with equal loading. The pEGFP showed a better expression as compared to the pcDNA 3.1

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To further quantify the transfection efficiency of the LRRK2 pEGFP vector in HEK-293T cells, the transfected cells were examined by flow cytometry (Beckman Coulter Cyan ADP). The excitation wavelength was at 488 nm and emission filter was set to 530/40 nm to detect the GFP signal. Figure 5.5 depicts the flow cytometry data comparing the transfection efficiency between the pCDNA 3.1 and pEGFP vectors in HEK-293T cells. Untransfected HEK-293T cells were used as the negative control. The pEGFP vector had better transfection efficiency (33.6%) as compared to the pCDNA 3.1 vector (14.3%).

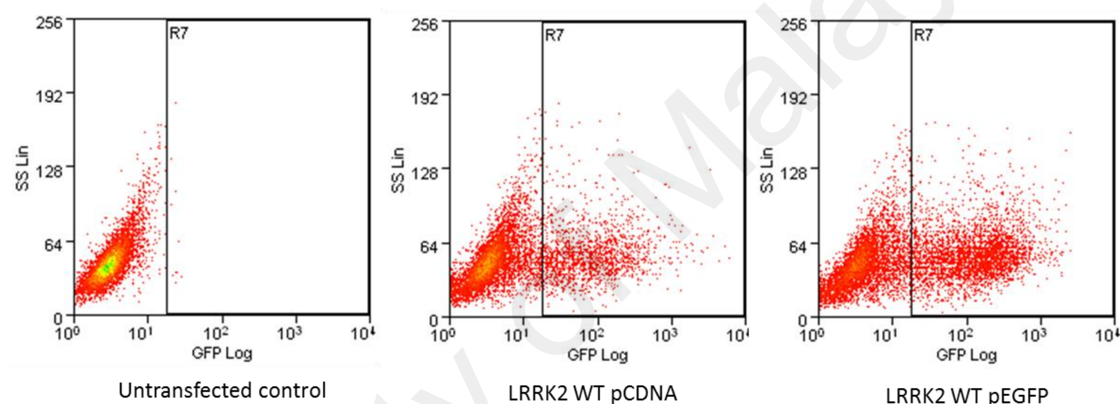

Figure 5.5: Flow cytometry data comparing the transfection efficiency of the pCDNA3.1 and pEGFP vector in HEK-293T cells. The LRRK2 WT pEGFP had a higher transfection efficiency rate as compared to the WT pCDNA3.1 (33.6% vs 14.3%).

5.3 R1398H and N551K have greater cell viability when exposed to cellular stress

The cell viability assay was conducted as described in Chapter 2, Section 2.13 on SH-SY5Y cell lines. In section 5.2, since the pEGFP vector was more efficient and had better expression, the expression level was also examined in SHSY-5Y cells. The expression level of LRRK2 transfected into SH-SY5Y cells was high and comparable with the expression obtained in the HEK-293T cells (Figure 5.6).

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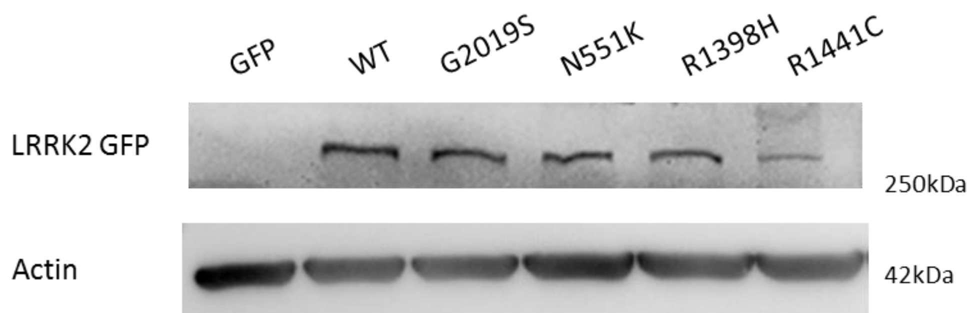

Figure 5.6: A representative Western blot to screen for LRRK2 expression level in transfected SH-SY5Y cells prior to the cell viability assay. GFP band for the empty vector is not shown. Legend: GFP-green fluorescent protein; WT-wildtype.

Each experiment was conducted with triplicates and repeated five times. As expected, the cells transfected with G2019S showed a significant decrease in the number of viable cells when treated with H_2O_2 (p=0.05). The cells carrying R1398H and N551K respectively had a significant increase in the number of viable cells as compared to G2019S when treated with H_2O_2 (p=0.01), (Figure 5.7).

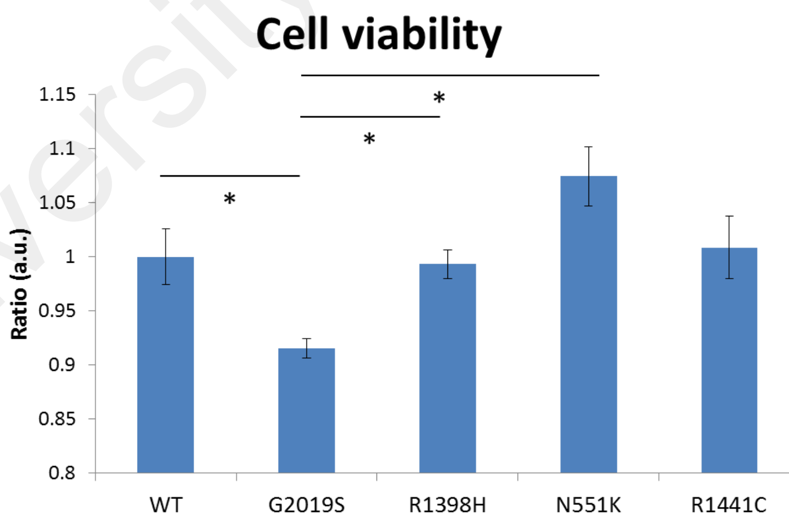

Figure 5.7: Cell viability assay results across the different mutations plotted in ratio to wildtype (WT), n=5. R1398H and N551K had significantly higher viability as compared to G2019S (*p*=0.01). Legend: Error bars represent ± S.E.M.; *-statistically significant; a.u- arbitrary unit.

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Thus, the R1398H and N551K mutations are able to protect against cellular stress induced by H_2O_2, and supports our genetic finding of a protective effect. Although R1441C has been reported to be a genetic risk factor (Nuytemans et al., 2008), we did not see this effect in the cell viability assay.

5.4 LRRK2 kinase activity

The role of kinase activity in LRRK2 function has been studied by various groups (Lewis, 2012; Liu et al., 2011b; Smith et al., 2006; West et al., 2007). Only G2019S has consistently been shown to mediate neurotoxicity through an increased kinase activity. Other mutations have shown variable results. A mutation within the ROC domain of LRRK2 (T1348) that disrupts GTP-binding can abolish the kinase activity of the protein. However, kinase dead mutants show intact GTP binding, suggesting that GTP binding regulates the kinase activity (Ito et al., 2007). As GTPase activity is controlled by the ROC domain and R1398H is located within this domain, we sought to investigate if this mutation affects the kinase activity.

The natural substrates of LRRK2 is still largely unknown however there are a few studies which have suggested that MBP, 4E-BP, MKKs, β-tubulin, α-synuclein, peroxiredoxin 3, Akt1 and ArfGAP1 are LRRK2 substrates (Ray & Liu, 2012). To study the *in vitro* phosphorylation of LRRK2, myelin basic protein (MBP) was used as the substrate. MBP is a generic substrate that has been widely used to study LRRK2 *in vitro* kinase activity (Lewis, 2012; Lewis et al., 2007; MacLeod et al., 2006; Tan et al., 2010b; West et al., 2007).

The effect of the mutations on the autophosphorylation activity of LRRK2 was also examined. Autophosphorylation is a means through which a kinase regulates itself. Autophosphorylation causes change to the kinase activity by repositioning the activation

loop as well as altering the binding ability of ATP or substrates. The antibody for the autophosphorylation site at T1503 was selected since a recent paper described its ability to modify the kinase activity (Webber et al., 2011). A triple kinase dead, TKD mutant (carrying K1906A, D1994A and D2017A) was used as a negative control for the antibody. These mutations abolishes the kinase activity at three critical sites: disruption of the highly conserved ATP binding loop and conserved D residue at position 1994 found in all known kinase protein as well as the disruption of Mg^{2+} binding site.

Immunoprecipitation (IP) was carried out to pull down LRRK2 from the cell lysate of the transiently transfected cells. This was done to ensure that the kinase reaction was measuring the kinase activity of only LRRK2.

5.4.1 Kinase activity is decreased in R1398H and unchanged for N551K when MBP is the LRRK2 substrate

All the relevant constructs of LRRK2 mutants and controls were expressed in HEK-293T cells and immunoprecipitated before the kinase activity was conducted. The experiment was repeated four times. The average and fold change when comparing activity levels was calculated after the background was removed.

The ability of the various LRRK2 mutants to phosphorylate MBP is summarised in Figure 5.8. G2019S had the highest kinase activity while the R1398H had the lowest kinase activity. The N551K and R1441C had almost similar kinase activity as the wildtype. Although R1441C is a genetic risk factor, an increase of kinase activity was not observed as in the case of G2019S.

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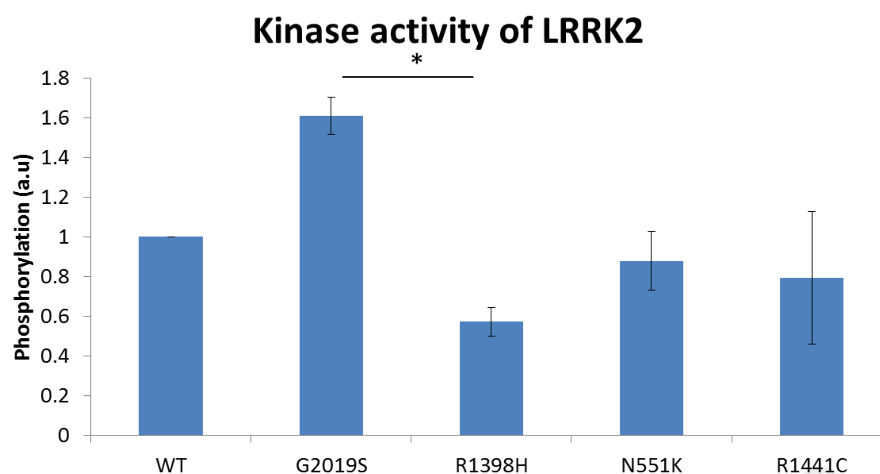

Figure 5.8: MBP substrate phosphorylation by LRRK2 mutants normalised against wildtype (WT), n=4. Error bars represent ± S.E.M. Legend: *- statistically significant; a.u- arbitrary unit.

5.4.2 Autophosphorylation activity at T1503 is increased for R1398H

The kinase reaction for autophosphorylation differed slightly where the substrate was not included in the set up (Chapter 2, Section 2.14). We investigated if there could be changes to the autophosphorylation site in the various mutations. Since there is no validated downstream substrate, the autophosphorylation sites could be an indicative of its kinase activity. As LRRK2 has many autophosphorylation sites, we examined the site to which was readily available to us and had been recently described to modify the kinase activity (Webber et al., 2011).

The autophosphorylation at site T1503 was examined by using antibody that is specific to this phosphorylation site. Figure 5.9 shows a Western blot picture of the autophosphorylation activity at T1503 for the different mutations. The negative control, TKD showed no phosphorylation at T1503 indicating that the antibody is detecting phosphorylated protein. R1398H showed the highest and a three-fold increase in autophosphorylation at T1503 (p=0.03). The G2019S also showed an increase in autophosphorylation activity at T1503 that was approximately 1.5 fold higher (p=0.03). Similar to the MBP phosphorylation, autophosphorylation of N551K and R1441C was

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similar to wildtype. The experiment was repeated three times, the amount of autophosphorylation was normalised against the LRRK2 expression and the fold change against wildtype was calculated (Figure 5.10).

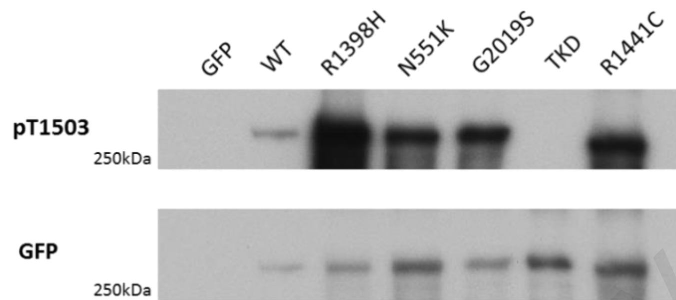

Figure 5.9: Western blot of one of the autophosphorylation activity at T1503. Legend: GFP-green fluorescent protein; TKD-triple kinase dead; WT-wildtype. (n=3)

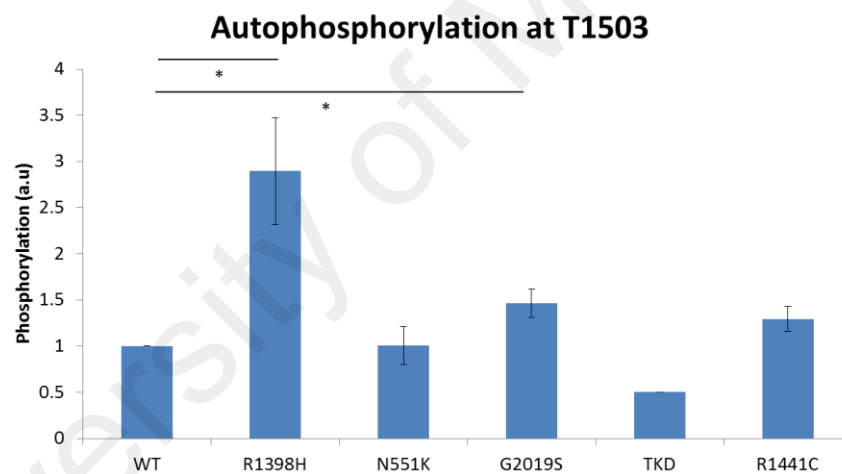

Figure 5.10: Autophosphorylation activity at T1503, normalised against wildtype (WT), n=3. Error bars represent ± S.E.M. Legend: TKD-triple kinase dead; * - statistically significant.

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5.5 GTPase activity of LRRK2

The ROC domain is consistently observed to be present together with a unique 300-400 amino acid long tandem COR domain. The COR domain consists of the highly conserved N-terminal that interacts with the ROC domain and the less conserved C-terminal that functions as a dimerization platform. The ROC domain of LRRK2 is related to the Ras/GTPase superfamily (Bosgraaf & Van Haastert, 2003). The ROC domain has a conserved sequence for GDP/GTP-binding motifs (G-box motifs) that contains a P-loop, a switch I and II motif, as well as a G4 and G5 motif, similar to Ras/GTPase family (Figure 5.11). The G-box motifs are involved in GTP binding and hydrolysis function. The P-loop binds to α and β phosphate of guanine as well as interacts with the Mg^{2+} ion in the guanine binding pocket. The switch II motif is responsible for the hydrolysis of GTP although at a slow rate.

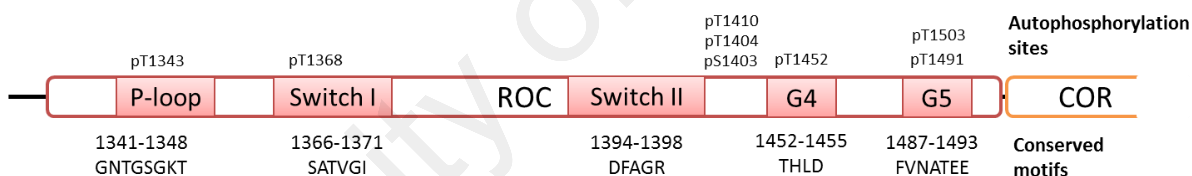

Figure 5.11: The ROC domain of LRRK2 and the conserved motifs involved in GTPase activity within this domain

LRRK2 has shown both GTP binding and hydrolysis activity. Mutations introduced on the conserved P-loop (K1347A and T1348N) inhibited GTP binding (Ito et al., 2007) while mutations in the switch II motif (R1398L) showed enhanced GTP hydrolysis (Biosa et al., 2012) and R1441C showed decreased GTP hydrolysis (Lewis et al., 2007). Since the R1398H mutation is on the ROC domain, we sought to identify the effect of the mutation on GTPase activity.

Initially GTP hydrolysis assays were planned and preparations of relevant constructs were made including the ROC-COR domain constructs to study R1398H. The

constructs needed to be tagged with GST as the GTP hydrolysis assays require the LRRK2 protein to be in a soluble, purified form. The smaller ROC-COR construct is also easier to be expressed in the bacterial expression system, enabling more protein to be harvested and analysed.

To study the effect of the LRRK2 mutations on GTPase activity, the GTP binding ability was first tested as described in (Chapter 2; Section 2.15) for both the full length LRRK2 and the ROC-COR constructs. Given that only the ROC-COR domain was studied, just the wildtype, R1398H and R1441C mutants were generated. The R1441C was included as a positive control for the GTP hydrolysis assay as this mutation has shown a decreased activity (Lewis et al., 2007).

5.5.1 Generation of the GST-tagged ROC-COR domain constructs

The ROC-COR domain was cloned into pGEX-5x1, a vector that has an N-terminal GST tag. The detailed protocol of the cloning has been described in Chapter 2, Section 2.8.2. Figure 5.12 shows the digested product of the cloned vectors, as an initial screening to check if the cloning was successful. After which the undigested products were sequenced to ensure that the mutation induced were correct and the coding was in-frame.

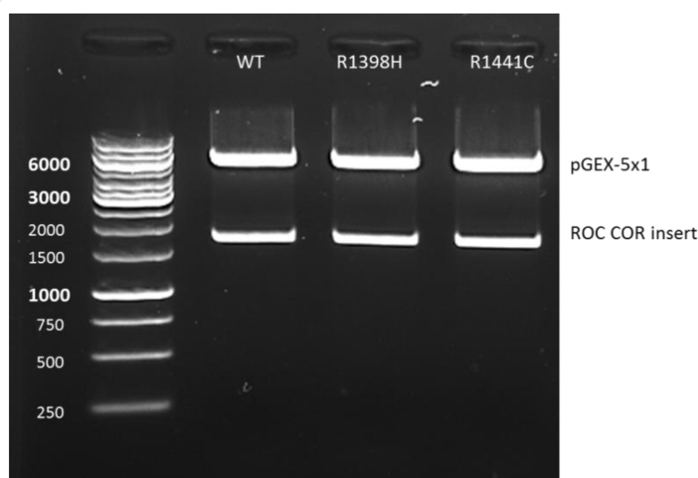

Figure 5.12: Gel electrophoresis of the digested ROC-COR pGEX-5x1 vector. The size of the ROC-COR insert is 1,638bp, which corresponds to the digested product.

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Once the sequence of the plasmids were confirmed, the plasmid was then transformed into BL-21 competent cells for protein expression (Chapter 2, Section 2.9). Figure 5.13 is a stained Coomasie blue gel that indicates that the eluted purified protein was obtained at the expected size. Although we followed a previously reported (Harper & Speicher, 2011) protocol for protein elution with the standard elution buffer condition of 20mM reduced L-glutathione, no proteins were eluted. Therefore, the elution buffer was optimised to a mixture containing 20mM reduced L-glutathione, 50mM Tris pH8.8, 150mM NaCl, 1% Triton-X 100 and 1% glycerol.

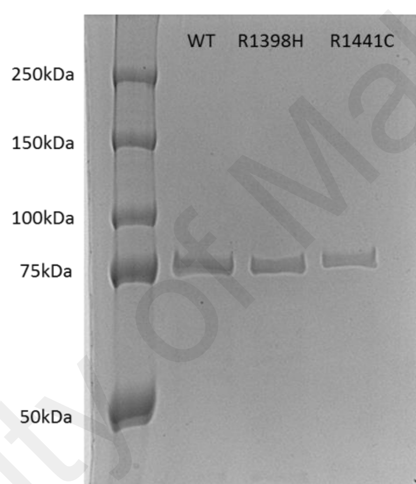

Figure 5.13: Purified recombinant protein on a 6% SDS-Page gel stained with Coomasie blue. The expected size of the recombinant protein is ~78kDa.

5.5.2 Unaltered GTP binding in the LRRK2 mutations examined

The GTP binding assay was performed with full length LRRK2 (GFP tagged) and a shorter ROC-COR GST-tagged fragment as described in Chapter 2, Section 2.15. Figure 5.14 shows the Western blot for the full length LRRK2 GTP binding assay. Analysis of this preliminary data suggests that the binding ability is unaltered for the various mutations (Figure 5.15). This data concurs with what has been published so far on the G2019S and R1441C (Biosa et al., 2012; Lewis et al., 2007). We note that the

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Western blot result for the GTP binding can be improved by using more LRRK2 protein as starting material, which may enable further evaluation of this result.

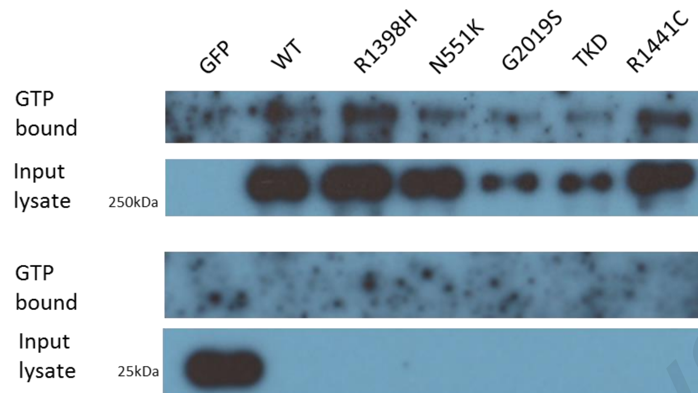

Figure 5.14: Preliminary data of the GTP binding assay in the various LRRK2 mutations. Legend: GFP-green fluorescent protein; TKD-triple kinase dead; WT-wildtype.

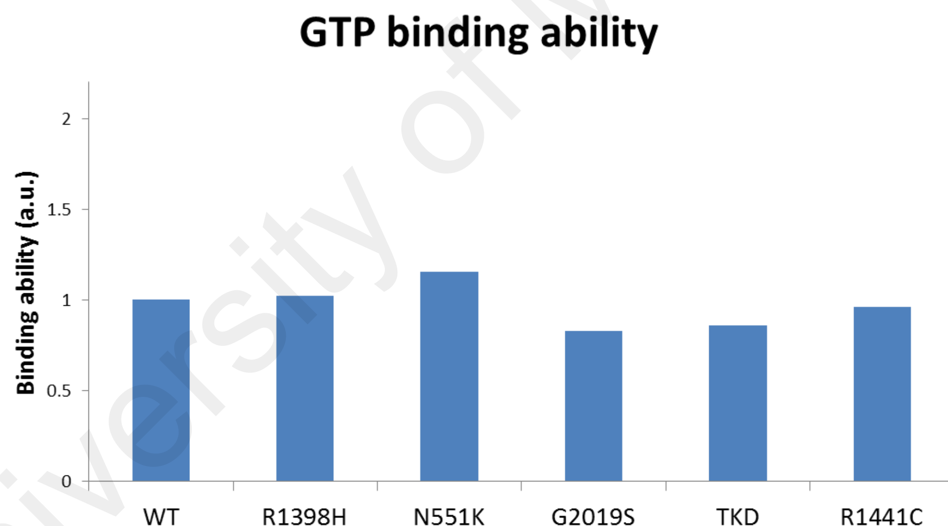

Figure 5.15: Analysis of the GTP binding assay across the LRRK2 mutations examined showed an unaltered binding ability, normalised against wildtype.

Since LRRK2 is a large and complex protein, expressing it in bacteria will produce very low yield of protein. Therefore the domain that is responsible for the GTPase activity was used. Both the ROC and COR domain was cloned into a bacteria expression system to produce soluble protein for the GTPase activity assays.

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The GTP binding with the ROC-COR recombinant protein required optimisation as native GST was also binding non-specifically to the GTP beads. The non-specific binding was overcome by the addition of 0.1% BSA and using 250ng starting material. The recombinant ROC-COR protein was still able to bind to the GTP beads under this condition (Figure 5.16). This experiment was repeated three times and we found that the binding was not affected by the mutations (Figure 5.17), similar to what was observed when full length LRRK2 was used (Figure 5.14 & Figure 5.15).

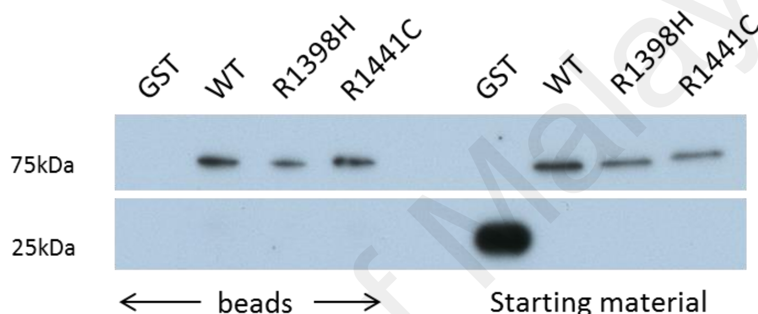

Figure 5.16: GTP binding ability of the ROC-COR domain in the various mutations. Legend: GST- glutathione S-transferase; WT-wildtype.

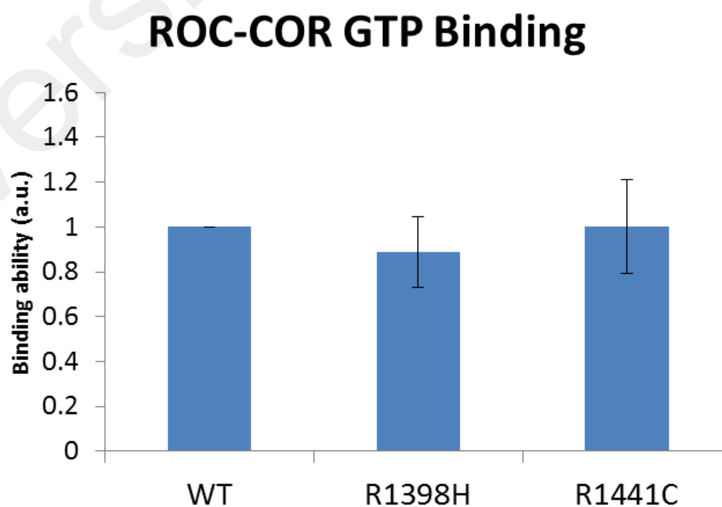

Figure 5.17: Analysis of ROC-COR GTP binding. Error bars represent ± S.E.M.

5.6 Discussion

In this chapter, we have provided supporting evidence of the protective effect of R1398H and N551K in relation to their response to H_2O_2 induced cellular stress. We also confirmed that the G2019S mutant, a known risk factor, conferred the least viability.

In addition, we also demonstrated that the increase in viability for R1398H correlated with a decrease in MBP phosphorylation, indicating a lower kinase activity. The low kinase activity supports the observation that the R1398H mutant was more resistant towards cellular stress. This concurs with Smith et al., 2006 who reported that the high kinase activity of G2019S mediates its neuronal toxicity. West et al., 2005 showed that there was no significant difference of MBP phosphorylation in the R1441C mutant while Lewis et al., 2007 showed that there was an increase. Unlike the G2019S whose increased kinase activity is reproducible in labs across the world, R1441C has shown variable results. As the N551K is not located within any functional domain, it could be possible that the protective mechanism conferred is not through the kinase pathway.

The autophosphorylation activity at T1503 however, shows an unexpected result where the autophosphorylation at this point is increased by approximately 3 folds for R1398H. In general, autophosphorylation promotes the kinase activity of the protein. However, there are proteins whose autophosphorylation can cause kinase inhibition as seen in death-associated protein kinase (Shoshat et al., 2001). There has been no solid evidence that autophosphorylation of LRRK2 causes kinase inhibition apart from what we have seen here.

There are many different reasons why an increase in the autophosphorylation activity at T1503 was observed. Firstly, the autophosphorylation activity could be due to the conformational change brought about the R1398H mutant that exposes the T1503 site to

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be readily phosphorylated. Therefore the increased autophosphorylation seen may not be an overall indication of higher kinase activity but rather due to the conformational change. An example of this is the R1441C that causes a conformational change to the ROC domain (Li et al., 2009) and the autophosphorylation site at T1503 was shown to be increased (Webber et al., 2011). The overall kinase activity of R1441C however, has been shown to be variable (Lewis et al., 2007; West et al., 2007).

The autophosphorylation activity of R1398H can be confirmed by firstly probing for phospho-serine/threonine antibody to check if the overall autophosphorylation of LRRK2 is increased or is this increase specific to the 1503 site. Besides that, a bioinformatics prediction of the protein using the protein crystal structure will be able to confirm the kind of conformational change brought about by the R1398H.

A noteworthy point to also consider is that the autophosphorylation of T1503 signal was only detectable after an *in vitro* kinase reaction was conducted suggesting that the autophosphorylation at this point may not be occurring physiologically. This can be tested by either detecting T1503 autophosphorylation in LRRK2 expressed *in vivo*.

Another possibility of the unexpected result is that the phosphorylation at T1503 could be causing the protein to be constantly in a GTP-bound state, and the R1398H mutation that sits on the switch II motif may cause an increase in GTP hydrolysis activity that in turns decreases kinase activity.

We also showed that the GTP binding was not altered among the different mutations. No evident change in GTP binding was observed when a shorter fragment was used suggesting that the other domains within LRRK2 did not contribute or hinder GTP binding. The kinase dead mutant also showed binding ability, similar to Ito et al., 2007. Our findings support the possibility of intra molecular regulation between the kinase

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and GTPase activity. When the ROC-COR domain of LRRK2 is compared to other GTPase protein, LRRK2 is predicted to have a very low GTP binding and hydrolysis activity (Webber et al., 2011). The low GTPase activity may poise a more dynamic downstream kinase activity reinforcing the idea that changes to the GTPase activity primarily regulates the LRRK2 kinase activity.

In conclusion, this chapter gives insights into the effect of the R1398H mutation on LRRK2 protein function. Neuronal cells carrying this mutation were more resistant to cellular stress and the kinase activity was decreased but had equal GTP binding ability as the wildtype LRRK2 protein. Further work to investigate how R1398H affect GTP hydrolysis should be done to better understand its role in regulating LRRK2 kinase activity.

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CHAPTER 6: CONCLUSIONS & FUTURE WORK

6.1 Establishment of a Malaysian PD DNA bank

To our knowledge, this is the first initiative in Malaysia to collect and establish a PD DNA bank. To date, the Malaysian database consists of 1,568 PD cases and 654 controls, with approximately 144 cases with reported family history, collected over a period of 6 years from various medical centres throughout Malaysia. Close to 60% of our PD cohort consists of Chinese ethnicity followed by 30% of the Malays and the rest are of Indian ethnicity. These numbers are not a representation of which ethnicity was more prone to PD, but rather because main collection sites were located in areas that were more prone to be visited by a particular ethnic group. Efforts were made to also include medical centres (for patients) and community centres that are frequented by Malays and Indians but the collection rate was poor. The ethnic distribution of non-affected controls was also similar to the PD cohort as the collection site was similar. Despite efforts to enrich the sample size, we faced a challenge in recruiting more healthy elderly individuals as they were less willing to contribute their DNA sample for research.

6.2 Genetic overview for the GWAS-linked loci considered in this thesis

We sought to uncover if known genetic susceptibility loci were also of relevance to Malaysian PD patients as there is no existing knowledge on the genetic susceptibility loci in this population. As the average life expectancy in Malaysia increases, the number of PD cases in Malaysia will also see a rise since the risk of PD increases with age. With healthcare moving towards personalised medicine, there is a need to identify if the genes implicated in other populations are relevant in Malaysia so that same drugs or treatment can be shared and will be effective.

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At the time work on this thesis started, there were three GWAS-linked loci known which were *HLA-DRA*, *PARK16* and *GAK*. Since we added new loci to be screened as they were published, the sample size for a particular variant may differ, for example the *HLA-DRA* examined 462 PD patients while the *GRN* (a later addition) examined 1,013 PD patients.

Of the three GWAS loci that were screened in this thesis, only the *HLA-DRA* and rs947211 within *PARK16* locus were associated in our population with a reduced risk, contrary to what was reported in the Caucasian population. There seems to be a distinct pattern of susceptibility between the different races, especially between the Caucasians and the Asians. This could be possible as both the Caucasian and Asians are in different clusters of the phylogenetic tree (Rosenberg et al., 2002). However in our study, we have also observed conflicting results within same ethnic group as seen in the *HLA-DRA* locus. Although the same ethnic was considered, the association of this locus was different in the Chinese from Taiwan and the Chinese from China. We postulate that because PD is a complex disease, there could be environmental factors that could be contributing to the discrepancy observed between the same ethnicity with different geographical origin.

While we performed allelic discrimination assays for our variants, the next step would be to perform more in depth genetic analysis on our PD samples given that advancements in next generation sequencing have made it more economically possible to do so. In a recent large scale meta-analysis of GWAS study that comprised 108,990 subjects, six new loci (*SIPA1L2*, *INPP5F*, *MIR4697*, *GCH1*, *VPS13C* and *DDRGK1)* were associated with PD (Nalls et al., 2014). These loci are the next potential candidate genes that could be screened in the existing Malaysian database to confirm their relevance in our population.

6.3 The Malay and Indian genetics results

(a) ***Largest Malay population study***

This is the largest study conducted so far on the Malay population. Only two small scale studies involving approximately 150 PD patients screening mutations within *LRRK2* (G2385R and R1628P) have been conducted (Tan et al., 2008b; Tan et al., 2007b). Apart from that, there are hardly any other studies that have looked at the genetic susceptibility to PD in Malays. Based on results obtained from this thesis, the Malays had a similar genotypic distribution as the Chinese. This was corroborated by a study that showed the Chinese and Malays are close in the phylogenetic tree (Hatin et al., 2011). Although the Malays and Chinese are closely related and it is possible to predict the association using the Chinese population, we call for cautious extrapolation of data on mainland Chinese.

(b) ***First GWAS-linked loci screening in the Indians***

In this thesis, we also considered a modest cohort of 130 Indian PD patients. This is the first initiative thus far taken to screen the GWAS-linked loci in the Indians, both in Malaysia and globally. We observe that the genotypic distribution for this ethnicity differed from the Chinese and Malays in the loci screened. While it is not possible to draw a conclusion on the genetic susceptibility for this ethnic at this stage as the sample size is currently small, we acknowledge the need for continuous efforts to enlarge the sample size. PD-linked genetic loci in the Indian population are greatly understudied. Only two other PD genes (*PINK1* and *PARKIN*) have been considered so far with a relatively small cohort (Biswas et al., 2006; Biswas et al., 2010). While in *LRRK2*, to date there is only one study that has looked at six *LRRK2* mutations (G2019S, R1441C/G/H, I2012T and I2020T) in a group of 800 Indian PD patients (Punia et al., 2006). They found that only a female PD patient had a heterozygous mutant for G2019S and the other mutations were absent in the Indian cohort. Other studies found G2385R

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and R1628P to be absent in a small cohort consisting of approximately 60 Indian PD patients (Tan et al., 2008b; Tan et al., 2007b).

6.4 LRRK2 results overview

6.4.1 G2019S and R1441C are not relevant in the Malaysian population

The established risk factor within *LRRK2*, G2019S was not screened in this thesis as a set of 90 Malaysian PD patients as well as 70 controls was previously screened in our lab and in other studies have indicated that this mutation is absent in Asians (unpublished data, Chua JY). The R1441C was first identified in a large family from Western Nebraska (Zimprich et al., 2004) and was subsequently found in families in the United States of America, Italy as well as Belgium (Nuytemans et al., 2008). Similar to G2019S, R1441C is absent in Asians (Correia Guedes et al., 2010; Li et al., 2015c; Punia et al., 2006; Sanyal et al., 2010; Tan et al., 2010b; Tan et al., 2005; Vijayan et al., 2011) thus for these reasons, we have chosen to omit the genetic screening of these two mutations in the Malaysian population.

6.4.2 First large scale LRRK2 genetic screening in Malaysia

Out of the five LRRK2 mutations screened, four mutations were significantly associated with our PD population. The A419V was not associated in our population or in the other populations that considered Chinese LOPD cases despite being suggested to be the Asian third risk variant by Ross and colleagues in 2011 (Di Fonzo et al., 2006; Tan et al., 2010a; Wu-Chou et al., 2013; Wu et al., 2012). The only two studies that showed a risk association considered EOPD cases (Li et al., 2015b; Li et al., 2012b) suggesting that A419V may be associated with EOPD rather than LOPD. The G2385R and R1628P were risk factors, each conferring a risk of 2-fold and 1.2-fold, respectively. R1398H and N551K are in LD and show a significant protective association in our population, as well as Caucasian populations (Ross et al., 2011).

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As most studies focused on the risk mutations, we set out on the second aim of the project which was to identify the functional effect brought about by the two 'protective' mutations. We focused on three functional aspects, firstly the viability of cells carrying the mutation in the presence of stress, the kinase activity as well as the GTP binding ability since R1398H is located in the ROC domain of LRRK2 that is responsible for GTP-binding and hydrolysis activity.

6.4.3 New pathophysiological insights on the neuroprotective effect by N551K and R1398H

The cells carrying the N551K and R1398H mutations had better viability than cells that were carrying the risk mutation (G2019S), consistent with other effects observed. Studies have shown that G2019S causes a decrease in neurite outgrowth both in cell lines as well as primary neurons (Dächsel et al., 2010; MacLeod et al., 2006; Parisiadou et al., 2009; Plowey et al., 2008; Ramonet et al., 2011). In G2019S Drosophila mutants, loss of dopaminergic neurons, mitochondrial disruption and impaired locomotor abilities have been reported (Ng et al., 2012). This mutation has also shown an increased kinase activity that mediates its neurotoxicity (Smith et al., 2006), coherent with what we have observe in our experiments.

(a) ***N551K does not alter LRRK2's kinase activity***

Although the N551K mutant showed a significant increase in cell viability in the presence of H_2O_2, the kinase activity and GTP binding ability was similar to the wildtype suggesting that the protection did not involve the kinase pathway. Other functional aspects will need to be considered before being able to identify the pathway in which it confers protection. It could be possible that the N551K may be acting through a different pathway such as through other interacting partners. Recent studies are beginning to reveal binding partners of LRRK2 such as Rab32 that is involved in a

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variety of events such as autophagy, mitochondrial dynamics and inflammation (Waschbusch et al., 2014). Rab32 has been shown to bind within the 267 to 552 amino acid of LRRK2 suggesting that N551K may alter its binding capability hence modifying the downstream effect on the cell.

(b) ***R1398H effect on LRRK2 kinase and GTPase activity***

The R1398H is located within the conserved Switch II motif that is responsible for the hydrolysis of GTP. Before the GTP hydrolysis activity of the mutations is measured, the GTP-binding ability was first determined as hydrolysis will not occur if there is no GTP-binding ability. Our preliminary result with the full length LRRK2 showed that all the mutations had similar binding abilities to GTP including the kinase dead mutant. Both the ROC-COR GST mutants and full length LRRK2 mutants showed similar results suggesting that the presence of other domains of LRRK2 does not contribute to the GTP binding ability. We also observed that R1398H showed a significant decrease in kinase activity (compared to G2019S) as measured by MBP phosphorylation, suggesting that the decrease is not due to impaired GTP binding of R1398H as it had similar binding ability with the other mutants. The GTP binding of G2019S results concur with published findings (Biosa et al., 2012; West et al., 2007). The increase in kinase activity of G2019S is likely due to its location on the activation loop of the kinase.

A decreased kinase activity potentially reduces activation of downstream pathways that induces cell death, hence resulting in higher cell viability as seen in R1398H. As it is still debatable if autophosphorylation occurs physiologically (Greggio et al., 2008), the kinase activity observed through MBP phosphorylation is a better indicator of LRRK2 kinase activity.

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6.5 The way forward in PD genetics

6.5.1 Predictive testing

Most studies focused on the identification of causal gene or genes that cause susceptibility in order to be able to aid in disease diagnosis or treatment by characterising possible drug targets. To this end, a panel of biomarkers or diagnostic genetic tests would be essential to predict their response to these drugs. Currently in Malaysia and other parts of the world, diagnosing PD – especially LOPD is largely through clinical investigations. To offer genetic diagnosis for this disease at this stage would be very challenging as it is a heterogenetic and multifactorial disease.

Even with EOPD which is likely to be inherited, only 30% have been linked to mutations in genes. It is possible to a certain degree, to perform genetic testing for some EOPD cases. For LOPD however, the situation is much more complicated as there are many other contributing factors such as environmental factors as well as exposures to infectious agents which play a role in triggering the disease, regardless of the genetic background of an individual.

With the advent of better genetic techniques and tools, it may be easier to mine the genome for loci or epigenetic changes that contribute to the development of PD. An example of genetic susceptibility study that leads to the better understanding of possible interacting proteins in the disease mechanism is seen in the work performed by MacLeod and colleagues (MacLeod et al., 2013). They showed that deficiency of Rab7L1 protein in rodent brains, a gene that is within the *PARK16* locus (identified by Satake et al., 2009 through a GWAS) mimicked degeneration observed in familial PD mutants form of *LRRK2*. The Rab7L1 protein has been shown to be important in maintaining trans-golgi network and involved in vesicular trafficking (Wang et al., 2014) suggesting that errors in this pathway can contribute to the development of PD.

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6.5.2 Identification of novel PD-linked loci unique to our population

Our data suggests that different populations have different genetic susceptibility, including Malaysia. The identification of novel loci that are unique to our population can be achieved by collaborating with neighbouring countries to form an Asian consortium. Singapore is a suitable candidate as they are genetically similar and essentially the same origin as Malaysia. Continuous efforts to increase the sample size of PD patients in Malaysia will go on in hopes to be able to conduct our own GWAS studies to identify new loci.

6.5.3 Next generation sequencing for PD patients

Alternatively, the way forward could be to perform next generation sequencing (NGS) on our samples to uncover any copy number variations (CNV) associated with our cohort, as indicated by Toft and colleagues who highlighted the role of CNV in PD (Toft & Ross, 2010). An established example of CNV that gives rise to PD is the α-synuclein gene whereby patients have shown to have duplication or triplication in this gene (Chartier-Harlin et al., 2004; Singleton et al., 2003). It will be interesting to find out if CNVs are more generally associated in LOPD.

In our EOPD patients, whole exome sequencing will be helpful to identify genes and mutations that are responsible for EOPD since the cause of 70% of the EOPD cases is still not known. Whole exome sequencing is helpful when studying EOPD cases as the mutations are likely to be inherited and unaffected family members can be used to segregate the mutation. NGS is also helpful in identifying gene susceptibility in LOPD. Zimprich and colleagues were able to discover a mutation in the *VPS35* gene that is associated in LOPD through this technique (Zimprich et al., 2011).

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6.6 Future work for functional studies

(a) ***GTP hydrolysis***

The exact mechanism in which R1398H confers a protective mechanism is still unclear although in our hands we observe a decreased kinase activity as seen with decreased MBP phosphorylation. There are a few possible areas that can be explored to better understand the mechanism. One of the immediate areas is to look at the GTP hydrolysis of the LRRK2-R1398H mutation to identify if hydrolysis is affected and if the decrease in kinase activity is due to the GTP hydrolysis.

(b) ***Kinase activity using LRRK2 specific substrates***

Another aspect that can be explored is to repeat the kinase activity using specific substrate such as LRRKtide, peroxiredoxin 3 or the newly identified LRRK2 substrate, Rab5b (Yun et al., 2015) can be used to confirm the kinase activity of the mutation since the autophosphorylation at T1503 appears to be increased for the R1398H mutation. Apart from that, LRRK2 has been shown to exist in dimers and autophosphorylation occurs within dimer molecules *in vitro* (Greggio et al., 2008). The ROC domain of LRRK2 is responsible for its ability to form dimer. As R1398H is within this domain, another area that can be explored is the effect of R1398H on LRRK2's ability to form dimer. The mutation may alter the protein's ability to form dimer and hence modify its autophosphorylation activity or binding partners.

(c) ***Synapse formation***

A different aspect to study the mechanism in which R1398H confers protection is to study the synapse and formation of neurites. LRRK2 has been shown to be involved in the neurite development through its interaction with actin (Meixner et al., 2011). LRRK2 interacts with actin through its WD40 domain and its kinase function as described in Chapter 1, Section 1.5.1 and 1.5.7. R1398H mutation may also be

interacting with actin through the decrease kinase activity in a way that it favours neurite formation, making it less likely to compromise under stress. Apart from that, LRRK2 have also shown to modulate synaptic vesicle trafficking and R1441C knock-in mice showed impairment of dopamine D2 receptor resulting in faulty dopamine neurotransmission suggesting other possible pathways in which R1398H could be affecting (Piccoli et al., 2011; Tong et al., 2009).

(d) ***Reversing the pathogenicity of risk mutations***

We found that around 0.4% of the PD cases carried both the G2385R risk mutation and R1398H protective mutation. It is interesting to establish how the two mutations with contrasting effect modulate each another. An aspect that would be interesting to find out is whether R1398H is able to 'delay' the neurodegenerative effects of G2385R. This can be done by co-expressing the R1398H and G2385R mutations and examining whether the cells are able to withstand cellular stress better.

(e) ***In vivo model & therapeutic challenges***

Apart from the cell based experiments discussed above, *in vivo* models (in both non-human primate model and human iPSC) can be carried out. Non-human primate models such as Drosophila and mice carrying the R1398H mutation can be used to check if the mutation is able to show rescue the effect of G2019S (better climbing scores in Drosophila or resistance to toxin treatment). An example of this is seen in Drosophila where *PARKIN* was able to rescue G2019S effect (Ng et al., 2009). The use of *in vivo* model is also beneficial as it can be used as a platform for drug screening.

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6.7 Lessons learnt

LRRK2 is a very large protein, making it a challenging protein to work with. Many other groups have also encountered similar problems when working with LRRK2 (personal communications with Professor Tan Eng King's and Associate Professor Lim Kah Leong's group). The large size of the gene makes it difficult for transfection and the expression of the protein. The use of pEGFP vector and optimisation of transfection reagents helped with the expression of the protein. HEK-293T cells were easier to transfect and had better expression therefore HEK-293T cells were used for the kinase assay and the full length GTP-binding assay. Neuronal cell lines producing dopamine were used for the cell viability to simulate the dopaminergic neurons in PD brains. Of the two neuronal cell lines we tried using, SH-SY5Y cells had better transfection efficiency and expression compared to SK-N-SH. We acknowledge that there are a few limitations to studying LRRK2 *in vitro*. Firstly the overexpression of the LRRK2 mutations may differ between the different mutants. The exact overexpression of the each LRRK2 mutation may differ in different sets of experiments due to different transfection efficiency. To overcome this limitation, the experiments were repeated at least three times and had at least duplicates for each mutation studied in each experiment conducted. Apart from that, the substrate level in the kinase reaction *in vitro* is kept constant throughout the experiment. This scenario does not best mimic the physiological system where substrates are carefully regulated by other molecules within the cell. The presence of the substrate in a constant and saturating manner may mask or alter the true function of LRRK2 within the cell.

6.8 Concluding remarks

The work presented in this thesis provides for the first time insights to the genetic susceptibility relevant to the Malaysian PD context and showed new data on the functional evidence of the protective mutation that is present in Malaysia.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

iii. List of publications

Gopalai, A. A., Ahmad-Annuar, A., Li, H. H., Zhao, Y., Lim, S. Y., Tan, A. H., . . . Shanthi, V. (2016). PARK16 is associated with PD in the Malaysian population. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*.

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iv. Papers presented

Gopalai, A. A., Chua, L. L., Lim, S. Y., Zhao, Y., Tan, E.K., Ahmad-Annuar, A. LRRK2 N551K and R1398H variants: a genetic and functional study. Human Genome Meeting 2015, Kuala Lumpur, Malaysia, 14-17th March 2015.

Gopalai, A. A., Lim, S. Y., Zhao, Y., Tan, E.K., Ahmad-Annuar, A. Assessing the genetics of PD in Malaysia. 5th International Neuroscience Symposium, Kuala Lumpur, Malaysia, 27th Sept 2014.

Gopalai, A. A., Lim, S. Y., Zhao, Y., Tan, E.K., Ahmad-Annuar, A. Analysis of N551K and R1398H LRRK2 variants in an Asian cohort. 17th International Congress of Parkinson's Disease and Movement Disorder, Sydney, Australia, 14-18th June 2013.

Gopalai, A. A., Lim, S. Y., Zhao, Y., Tan, E.K., Ahmad-Annuar, A. Is PARK16 a protective variant in Asians. Joint Conference of HGM 2013 and 21st International Congress of Genetics, Singapore, 13-18th Apr 2013.

Gopalai, A. A., Lim, S. Y., Zhao, Y., Tan, E.K., Ahmad-Annuar, A. Analysis of N551K and R1398H LRRK2 variants in a Malay cohort. 2nd AMM-AMS-HKAM Tripartite Congress & 47th Singapore-Malaysia Congress of Medicine, Singapore, 23-24th Aug 2013.

Gopalai, A. A., Lim, S. Y., Zhao, Y., Tan, E.K., Ahmad-Annuar, A. HLA-DRA locus – a protective variant in Malaysian Parkinson's disease patients. 10th Asia-Pacific Conference on Human Genetics, 6-8th Dec 2012.

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APPENDIX

Appendix A: List of variants and the primers used to determine the error rate

SNP	Primer sequence (5' to 3')	Annealing temperature (^{o}C)	Product size (bp)
rs3129882	F- CATTATTCCCTTGGTGTTGGTT R- GGGTTTTAAACTCTGGGATCAA	60	500
rs947211	F- GGTTGTCACATTTGCCTCCT R- TGTCTGTGTTGGAAGGGAACT	59	196
rs823128	F- AAATTAGGCCTCCCCACAAC R- TGTTAGGCCTGAGGTTAGGA	59	226
rs823156	F- CATTCATGTGCAGGAAGGTG R- TCTTCTTTTGGGGACTGGTG	59	225
rs11240572	F- CCCAATCTTAGTCAAAGGCATTA R- CAGAGGGAGTGCTGGAAGAG	59	246
rs16856139	F- AGGTGGTGAGTGGCTCAGAT R- TGAAAGCCCTCAAAGACCTC	59	248
rs11248051	F- AGGATTCAGCTGTGGCTTGT R- GTGGGATGCACCTAACGACT	59	241
rs1564282	F- CTGGGAGTCCACTCCTTCTG R- CAGGTGGTTTTGGGGATTTA	59	209
GRN, rs5848	F- GGGATGGCAGCTTGTAATGT R- CAGCTGCTGTGAGGGACAG	59	216
G2385R (rs34778348)	F- TGCAGCTTTCAGTGATTCCA R- GGCAGAAAGGAAGAAAAATCC	59	233
R1628P (rs33949390)	F- TGAGCAAAGAGACATAAAATGCTT R- ATTGGCAAAGCAATCTGGAA	58	242
A419V (rs34594498)	F- GTAAGTGGAGGTGGCATGAA R- CCATATGTCAATACATCTCTACAC	55	390
N551K (rs7308720)	F- TAGGCATGCCAGAAGAATCC R- AACCACATGACTTCCTCCTATCA	60	244
R1398H (rs7133914)	F- GGTCAATCCTAGTAAAAACCCAGA R- TTCATGGCATCAACTTCAGC	60	275

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Appendix B: Primers to determine the *LRRK2* sequence in the expression vectors

Position	Sequence
LRRK2 607 F	CAT GTG CTG CAT TGT TTA CA
LRRK2 1010 R*	TCA TGA TCA AAA ATG GGC CC
LRRK2 1403 F	GGA TAT AAT GGC AGC AGT GGT C
LRRK2 1942 F	GGA TTT CAG ACA CAA TCT TAG C
LRRK2 2620 F	GAA TGG ACC TTT ATT CCT GAC
LRRK2 3303 F	CCT CAC TGA TGT GGT AGA G
LRRK2 3900 F	GGG ATC TTC CTT TGG ATG AAC TGC
LRRK2 4227 F	GAC GCA GCG AGC ATT GTA C
LRRK2 4794 F	GGA ACC CAA GTG GCT TTG
LRRK2 5265 F	GAC AAT CAT CCA GAG AGT TTC
LRRK2 5523 F*	CAA GGC TCA CCA TTC CAA TAT CTC AG
LRRK2 5795 F	GAT ATC TTT GCT GGC AGC
LRRK2 6285 F	GTT AAA GAA TAT GGT TGT GC
LRRK2 6886 F	GTC AGT ACT CCA TTG ATG TG
LRRK2 7277 F	GGA TAG GAA CTG GAG GAG G

***are primers used to ensure ROC-COR GST construct has in-frame start and stop codon**

Appendix C: The mean ages of control and PD cases of the cohort used for the respective loci. SD – standard deviation.

Loci	Control mean age (±SD)	PD mean age (±SD)	*p*-value
HLA-DRA	61.6 ± 9.56	61.0 ± 11.1	0.05
PARK16	59.3 ± 9.5	57.3 ± 11.7	0.0014
GAK	59.8 ± 9.6	58.2 ± 11.5	0.0035
GRN	59.8 ± 9.6	58.1 ± 11.4	0.0020

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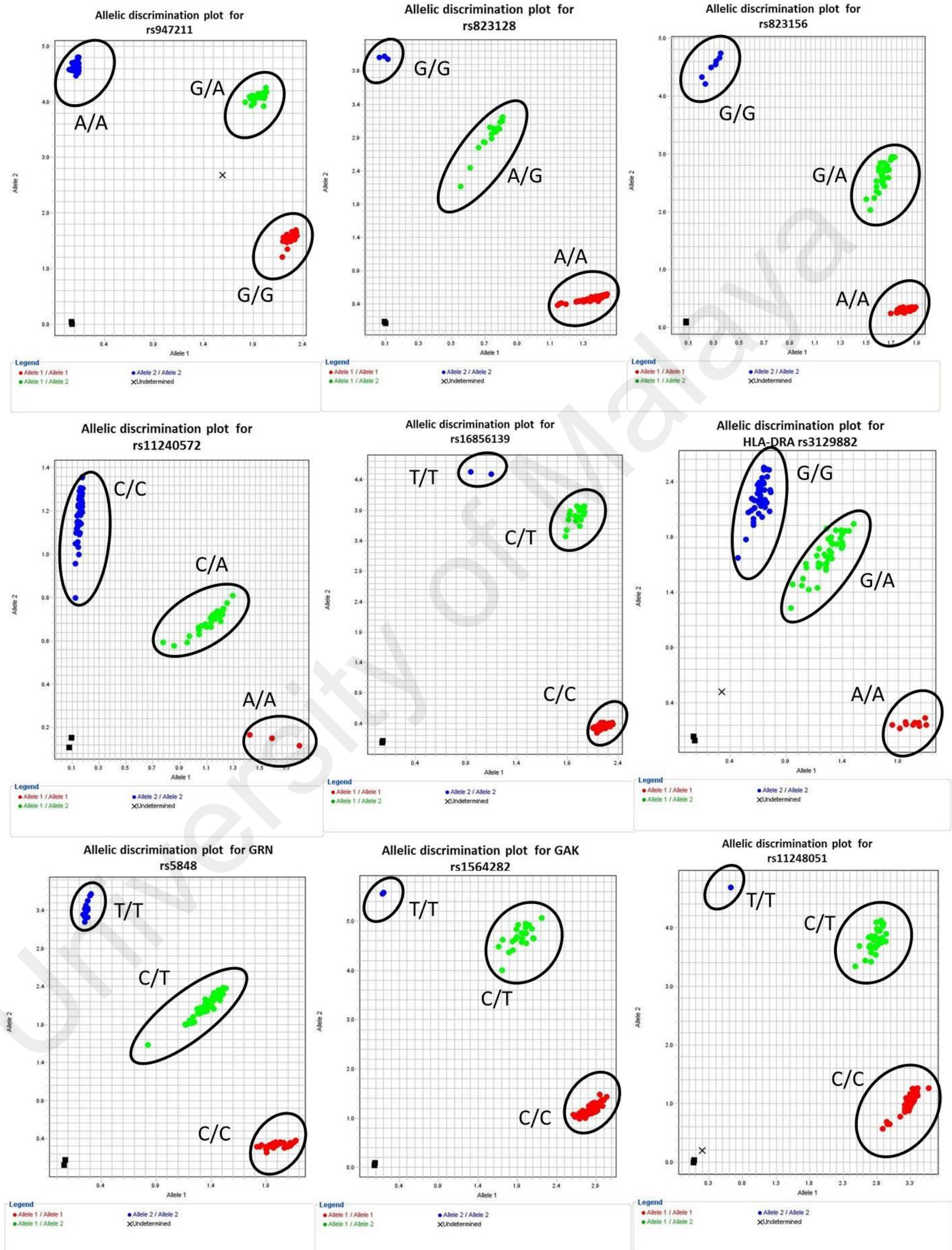
Appendix D: Representative plots of the allelic discrimination assays carried out for the selected loci considered in Chapter 3



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Appendix E: Complete *PARK16* genotyping data, divided by race

Genotype Malay	Controls	PD cases	Odds ratio (OR)
rs947211			
G/G	38	86	Reference
A/G	63	107	0.70 (0.42, 1.17)
A/A	12	47	0.57 (0.31, 1.05)
Odds ratio			0.66 (0.41, 1.06)
p-value			0.0810
rs823128			
A/A	99	177	Reference
A/G	31	59	1.06 (0.64, 1.76)
G/G	3	4	0.59 (0.11, 3.09)
rs823156			
A/A	78	137	Reference
A/G	44	90	1.09 (0.69, 1.74)
G/G	10	13	0.64 (0.26, 1.60)
rs11240572			
C/C	88	164	Reference
A/C	41	66	0.86 (0.54, 1.39)
A/A	4	10	1.27 (0.37, 4.33)
rs16856139			
C/C	95	171	Reference
C/T	38	64	0.87 (0.54, 1.41)
T/T	0	5	-
Chinese			
rs947211			
G/G	92	189	Reference
A/G	123	229	0.91 (0.65, 1.27)
A/A	66	65	0.47 (0.31, 0.73)
Odds ratio			**0.50 (0.34, 0.74)**
p-value			**0.0004**
rs823128			
A/A	222	377	Reference
A/G	54	101	1.09 (0.75, 1.59)
G/G	5	4	0.48 (0.13, 1.81)
rs823156			
A/A	182	337	Reference
A/G	87	132	0.85 (0.61, 1.18)
G/G	12	16	0.68 (0.31, 1.51)
rs11240572			
C/C	195	354	Reference
A/C	75	122	0.91 (0.65, 1.29)
A/A	11	8	**0.37 (0.14, 0.97)**
Odds ratio			**0.38 (0.14, 0.99)**
p-value			**0.0443**
rs16856139			
C/C	209	370	Reference
C/T	66	107	0.87 (0.61, 1.25)
T/T	5	7	0.81 (0.25, 2.61)
Indian			
rs947211			
G/G	34	28	Reference
A/G	45	54	1.47 (0.75, 2.87)

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A/A	14	18	1.17 (0.46, 2.99)
Odds ratio			1.39 (0.73, 2.64)
p-value			0.3083
rs823128			
A/A	67	67	Reference
A/G	20	30	1.50 (0.74, 3.03)
G/G	2	3	1.84 (0.29, 11.79)
Odds ratio			1.53 (0.78, 3.01)
p-value			0.2133
rs823156			
A/A	44	46	Reference
A/G	36	42	0.95 (0.49, 1.82)
G/G	10	12	1.28 (0.48, 3.43)
rs11240572			
C/C	69	65	Reference
A/C	21	32	1.63 (0.81, 3.28)
A/A	3	3	1.40 (0.27, 7.34)
rs16856139			
C/C	74	68	Reference
C/T	17	27	1.79 (0.85, 3.75)
T/T	2	5	3.58 (0.65, 19.74)
Odds ratio			1.83 (1.02, 3.29)
p-value			0.0381

Appendix F: Demographic of PD and control samples screened in the respective variants

Variants	Subjects	Number of males in brackets			
		Malay	Chinese	Indian	Total
LRRK2 G2385R	PD	200 (122)	394 (214)	101 (60)	695 (396)
	Control	136 (67)	277 (141)	94 (44)	507 (252)
LRRK2 A419V	PD	122 (77)	223 (115)	59 (40)	404 (232)
	Control	110 (53)	236 (116)	80 (35)	426 (204)
LRRK2 R1628P	PD	200 (122)	396 (215)	102 (60)	698 (397)
	Control	136 (67)	278 (141)	96 (44)	510 (252)
LRRK2 N551K	PD	168 (107)	279 (149)	76 (51)	523 (307)
	Control	133 (67)	269 (134)	89 (44)	491 (245)
LRRK2 R1398H	PD	168 (107)	279 (149)	76 (51)	523 (307)
	Control	133 (67)	269 (134)	89 (44)	491 (245)
HLA-DRA locus (MY)	PD	145 (95)	243 (132)	74 (42)	462 (269)
	Control	134 (68)	269 (132)	90 (43)	493 (243)
HLA-DRA locus (SG)	PD	-	394 (224)	11 (6)	405 (230)
	Control	3 (1)	406 (224)	11 (8)	420 (233)
PARK16 locus	PD	240 (145)	485 (258)	100 (69)	825 (472)
	Control	133 (67)	281 (139)	93 (44)	507 (250)
GAK locus	PD	298 (176)	592 (317)	136 (82)	1,026 (575)
	Control	155 (72)	279 (141)	90 (44)	524(257)
GRN rs5848	PD	292 (173)	586 (314)	135 (82)	1,013 (569)
	Control	157 (72)	275 (141)	90 (44)	522 (257)

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Appendix G: Summary of the Asian published data on *PARK16*

Study	Population (N)	rs947211 (A/G)	rs11240572 (A/C)	rs16856139 (T/C)	rs823156 (G/A)	rs708730 (G/A)	rs823128 (G/A)	rs823122 (C/T)
			The values in italics indicate MAF of controls vs PD					
Satake 2009	Japanese (2,011 PD; 18,381 controls)	*0.48 vs 0.43* p= 1.15 × 10^{-4} OR 1.23 **RISK**	*0.16 vs 0.13* p= 1.66 × 10^{-4} OR 1.34 **RISK**	*0.14 vs 0.10* p= 2.55 x 10^{-6} OR 1.5 **RISK**	*0.17 vs 0.13* p= 1.20 × 10^{-5} OR 1.40 **RISK**	*0.18 vs 0.14* p= 2.60 × 10^{-5} OR 1.37 **RISK**	*0.14 vs 0.10* p= 2.09 × 10^{-5} OR 1.43 **RISK**	*0.14 vs 0.10* p= 7.98 × 10^{-5} OR 1.39 **RISK**
Vilarino-Guell 2010	Taiwanese Chinese (348 PD; 403 controls)	**Not indicated**	**Not indicated**	**Not indicated**	**Not indicated**	**Not indicated**	*0.151 vs 0.117* p=0.015 OR 1.46 (modeled over the major allele) **PROTECTIVE**	**Not indicated**
Tan 2010	Singaporean Chinese (433 PD; 916 controls)	*0.42 vs 0.39* p= 0.1250 OR 0.88 **No association**	*0.18 vs 0.15* p= 0.0135 OR 0.75 **PROTECTIVE**	*0.14 vs 0.12* p= 0.1447 OR 0.83 **No association**	*0.22 vs 0.18* p= 0.0161 OR 0.77 **PROTECTIVE**	In LD with rs823156 **Not screened**	*0.15 vs 0.12* p= 0.0316 OR 0.76 **PROTECTIVE**	In LD with rs823128 **Not screened**
Chang 2011	Chinese (Mainland China) (636 PD; 510 controls)	Not in HWE *0.41vs 0.380*	*0.173 vs 0.057* *p=0.303* **No association**	*0.123 vs 0.092* p=0.0192 OR 0.73 Additive model **PROTECTIVE**	*0.206 vs 0.161* p=0.007 OR 0.73 Additive model **PROTECTIVE**	**Not screened**	*0.133 vs 0.103* p=0.0327 OR 0.73 Dominant model **PROTECTIVE**	**Not screened**
Yan 2011	Eastern Chinese (226 PD; 230 controls)	*0.465 vs 0.431* p= 0.305 OR 0.872 **No association**	*0.174 vs 0.115* p= 0.012 OR 0.618 **PROTECTIVE**	*0.172 vs 0.099* p= 0.001 OR 0.533 **PROTECTIVE**	*0.224 vs 0.186* p= 0.154 OR 0.791 **No association**	*0.228 vs 0.193* p= 0.185 OR 0.806 **No association**	*0.161vs 0.155* p= 0.804 OR 0.956 **No association**	*0.161vs 0.137* p= 0.315 OR 0.829 **No association**
Chang 2013	Taiwan (497 PD; 500 controls)	*0.43 vs 0.40* p=0.181 OR 0.89 **No association**	*0.164 vs 0.147* p=0.293 OR 0.88 **No association**	**Not screened**	*0.205 vs 0.178* p=0.127 OR 0.84 **No association**	**Not screened**	*0.13 vs 0.13* p=0.726 **No association**	**Not screened**

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Chang 2013 (Merged with Yan and Tan)	(1,731 PD; 1,574 controls)	*0.43 vs 0.40* p=0.047 OR 0.90 **PROTECTIVE**	*0.177 vs 0.14* p<0.001 OR 0.76 **PROTECTIVE**	**Not screened**	*0.214 vs 0.17* p<0.001 OR 0.77 **PROTECTIVE**	**Not screened**	*0.14 vs 0.12* p=0.010 OR 0.83 **PROTECTIVE**	**Not screened**
Current study	Malaysian (730 PD; 414 controls)	*0.461 vs 0.387* p=0.0003 OR 0.57 **PROTECTIVE**	*0.176 vs 0.155* **No association**	*0.138 vs 0.135* **No association**	*0.212 vs 0.193* **No association**	**Not screened**	*0.122 vs 0.122* **No association**	**Not screened**
Meta-analysis	Asian (2,876 PD; 2,374 controls)	*0.381 vs 0.423* p=0.0001 OR 0.71 **PROTECTIVE**	*0.176 vs 0.149* p=0.0002 OR 0.79 **PROTECTIVE**	*0.131 vs 0.117* p=0.0949 OR 0.86 **PROTECTIVE**	*0.213 vs 0.179* p=0.0001 OR 0.79 **PROTECTIVE**	**Not applicable**	*0.141 vs 0.120* p=0.0155 OR 0.84 **PROTECTIVE**	**Not applicable**

*MAF = minor allelic frequency; OR= odds ratio; the first nucleotide is the alternate allele; annotated here is what was reported in the original Satake paper, although we note that the MAF values for patient and controls may have been erroneously exchanged.

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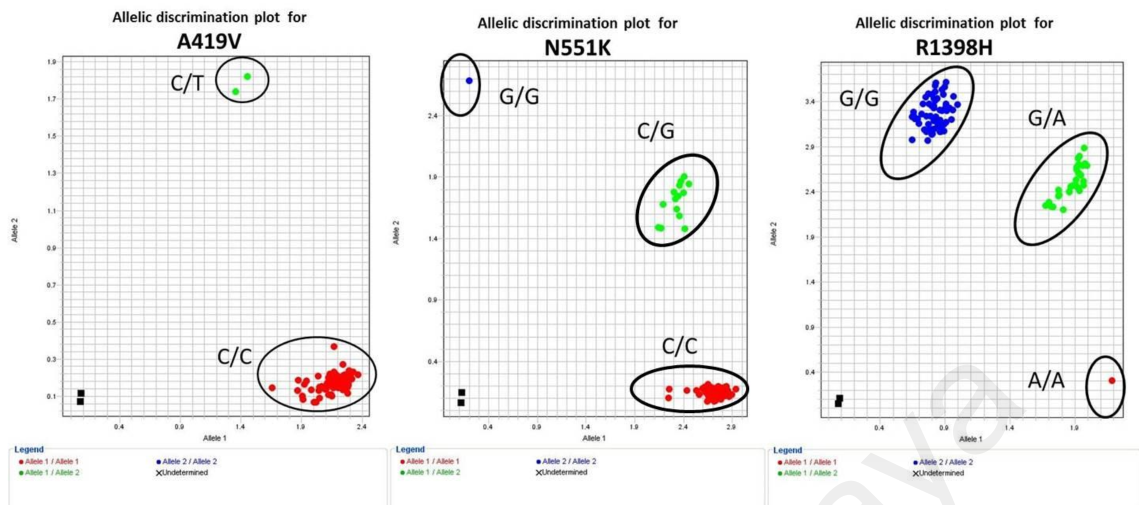
Appendix H: Analysis of *GAK* rs11248051 with our cohort shows no significant association

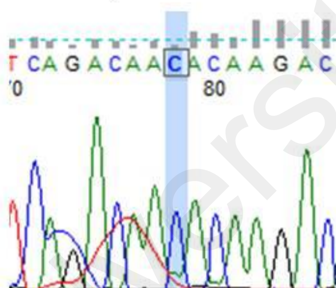
	PD cases			**Control**		
Genotype	**Malay**	**Chinese**	**Indian**	**Malay**	**Chinese**	**Indian**
Wildtype (C/C)	192	459	77	99	223	55
Heterozygous mutant (C/T)	96	121	52	48	52	32
Homozygous mutant (T/T)	8	12	6	6	5	4
Allelic frequency (%)						
Wildtype (C)	81.1	87.8	76.3	80.4	88.9	78.0
Mutant (T)	18.9	12.2	23.7	19.6	11.1	22.0

Appendix I: Representative allelic discrimination assay plots carried out for the LRRK2 mutations



Appendix J: Electropherogram from rs1564282, A419V, N551K and R1398H samples to determine the error rate

NC000004.12:g.858525 C>T, rs1564282

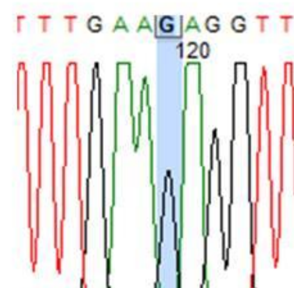


Wildtype rs1564282

c.1256 C>T, A419V

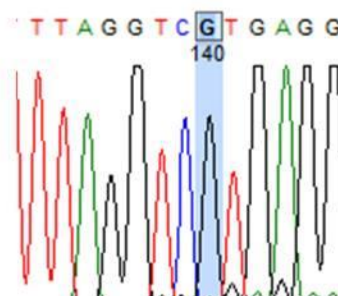
Heterozygous A419V (reverse sequence)

c.1653 C>G, N551K

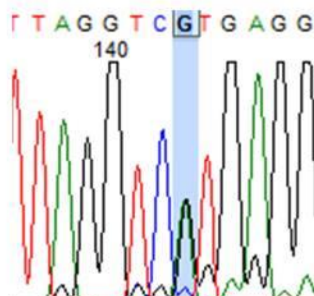


Homozygous N551K

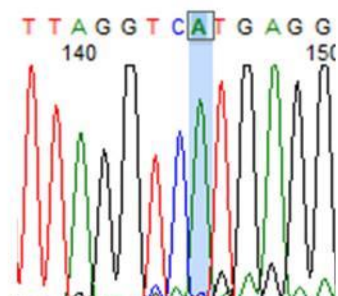
c.4193 G>A, R1398H



Wildtype R1398H



Heterozygous R1398H



Homozygous R1398H

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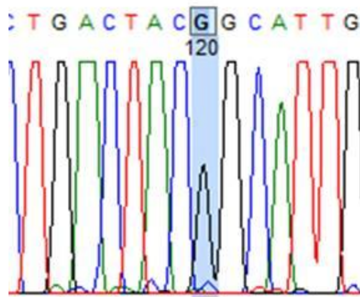
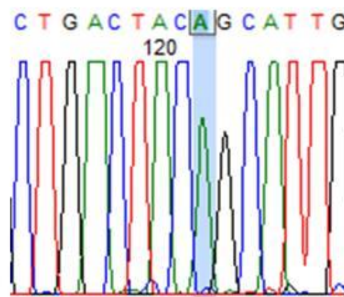
Appendix K: Electropherogram from the wildtype, G2019S and R1398H constructs to ensure the constructs were carrying the right mutation

c.6055 G>A, **G2019S**
Glycine to Serine
(**G**GC to **A**GC)

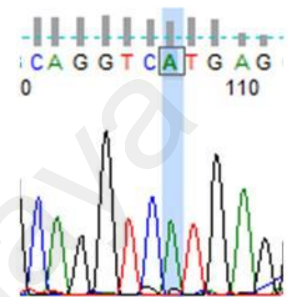
c.4193 G>A, **R1398H**
Arginine to histidine
(C**G**T to C**A**T)



Wildtype construct at position G2019S



G2019S construct carrying the G2019S mutation



R1398H construct carrying the R1398H mutation

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Short Report

Association of HLA locus variant in Parkinson's disease

Zhao Y, Gopalai AA, Ahmad-Annuar A, Teng EWL, Prakash KM, Tan LCS, Au W-L, Li H-H, Lim S-Y, Lim SK, Chong YB, Tan LP, Ibrahim NM, Tan E-K. Association of HLA locus variant in Parkinson's disease. Clin Genet 2012. © John Wiley & Sons A/S. Published by Blackwell Publishing Ltd, 2012

A variant (rs3129882) in the genome-wide association study (GWAS)-linked variant [in the human leukocyte antigen (HLA) gene region] has been reported to associate with an increased risk of Parkinson's disease (PD) in Caucasian population. Studies among Chinese are limited. To address this, we analysed rs3129882 in a total of 1312 subjects of Chinese ethnicity from independent Asian centers comprising of 675 controls and 637 PD cases. The rs3129882 variant was associated with a decreased risk in our ethnic Chinese PD patients. Logistic regression analysis taking into consideration variables of age, gender and race showed that allele A reduced the risk of PD via a dominant model [odds ratio (OR) = 0.77, 95% confidence interval (CI) = 0.62, 0.96, p = 0.018]. As HLA is a highly polymorphic region, it is possible that ethnic-specific effect or environmental agents may modulate the effect of this GWAS-linked locus in influencing the risk of PD.

Conflict of interest

The authors have no conflict of interest to declare.

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Key words: Chinese – gene – Parkinson's disease – polymorphism

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Parkinson's disease (PD) is a progressive neurodegenerative disease which frequently leads to neurological disability. There have been a number of genes that have been associated with familial and sporadic PD, including *LRRK2*, *PINK1*, *Parkin/Park2* (1). A recent genome-wide association study (GWAS) identified another possible genetic marker that is associated with PD among Caucasians, and this was particularly strong for men and for late-onset PD (2). This GWAS-linked single nucleotide polymorphism (SNP) is located in the human leukocyte antigen (HLA) gene on chromosome 6p21.3. The SNP rs3129882 in intron 1 of HLA-DRA has been shown to be most robustly associated with PD. HLA-DR is a major histocompatibility complex which has a role in immune responses and inflammation. The identification of rs3129882 highlights the links between PD and human immunity and inflammation, and potentially opens up a new avenue for therapeutic intervention.

Original Article

Lack of Association between the *LRRK2* A419V Variant and Asian Parkinson's Disease

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Abstract

Introduction: The G2385R and R1628P *LRRK2* gene variants have been associated with an increased risk of Parkinson's disease (PD) in the Asian population. Recently, a new *LRRK2* gene variant, A419V, was reported to be a third risk variant for PD in Asian patients. Our objective was to investigate this finding in our cohort of Asian subjects. Materials and Methods: Eight hundred and twenty-eight subjects (404 PD patients, and 424 age and gender-matched control subjects without neurological disorders) were recruited. Genotyping was done by Taqman® allelic discrimination assay on an Applied Biosystems 7500 Fast Real-Time PCR machine. Results: The heterozygous A419V genotype was found in only 1 patient with PD, compared to 3 in the control group (0.4% vs 1.3%), giving an odds ratio of 0.35 (95% confidence interval (CI), 0.01 to 3.79; *P* = 0.624). Conclusion: A419V is not an important *LRRK2* risk variant in our Asian cohort of patients with PD. Our data are further supported by a literature review which showed that 4 out of 6 published studies reported a negative association of this variant in PD.

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Key words: Asian, A419V, Genetics, *LRRK2*, Parkinson's disease

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Introduction

Recent studies have linked certain single nucleotide polymorphisms (SNPs) in the *LRRK2* gene with familial and sporadic Parkinson's disease (PD). Interestingly, there appear to be important population differences in the contribution of these SNPs to the risk of PD occurrence. The G2019S variant is very common amongst Ashkenazi Jews and African Arabs with PD,[1,2] but it is undetectable in Asian populations.[3,4] On the other hand, in Asians and in particular, the Han Chinese, the G2385R and R1628P variants have been consistently identified as important risk factors.[5-7]

Recently, Ross and colleagues[8] reported the *LRRK2* A419V variant (position c.1256 C>T, rs34594498) to be a third common risk variant in Asian patients with PD, with an odds ratio (OR) of 7.51 in the Taiwan Han Chinese, 2.21 (Koreans), and 1.26 (Japanese). In contrast, Di Fonzo and colleagues[9] found a low frequency (below 1%) of A419V in Han Chinese PD patients from Taiwan, with no significant difference between PD patients. Similarly, an Asian multicentre study reported this variant to be monomorphic in their cohort of PD patients.[7] More recently, Wu et al,[10] in 2012 and Wu-Chou et al,[11] in 2013 found that there was no association with A419V in Chinese PD patients in a multicentre study in Taiwan and Singapore. These discrepant findings prompted us to investigate if A419V is

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Research Article

LRRK2 G2385R and R1628P Mutations Are Associated with an Increased Risk of Parkinson's Disease in the Malaysian Population

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The *LRRK2* gene has been associated with both familial and sporadic forms of Parkinson's disease (PD). The G2019S variant is commonly found in North African Arab and Caucasian PD patients, but this locus is monomorphic in Asians. The G2385R and R1628P variants are associated with a higher risk of developing PD in certain Asian populations but have not been studied in the Malaysian population. Therefore, we screened the G2385R and R1628P variants in 1,202 Malaysian subjects consisting of 695 cases and 507 controls. The G2385R and R1628P variants were associated with a 2.2-fold ($P = 0.019$) and 1.2-fold ($P = 0.054$) increased risk of PD, respectively. Our data concur with other reported findings in Chinese, Taiwanese, Singaporean, and Korean studies.

1. Introduction

Parkinson's disease (PD) is an age-related illness, and, as populations age, the proportion of people with this neurodegenerative disease will continue to rise. It is projected that, by the year 2030, 9.3 million individuals above the age of 50 will suffer from PD and these cases will be concentrated outside the western world [1]. Studies have implicated exposure to environmental toxins and trauma as aetiological factors for PD [2]. Genetic variations also play a role, especially in cases where there is a family history of PD, which account for around 10–20% of all PD cases [3]. However, studies have shown that even late-onset sporadic PD may also have a genetic contribution [4].

One of the genes commonly implicated in both familial and sporadic PD is the leucine-rich repeat kinase 2 (*LRRK2*) gene. Several variants of *LRRK2* such as R1441C, G2019S, and I2020T have been well established as risk factors for PD [3]. Interestingly, there appear to be population-specific variants in LRRK2; for example, the G2019S variant is prevalent among the Ashkenazi Jews and North African Arabs

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART B
Neuropsychiatric Genetics

PARK16 Is Associated With PD in the Malaysian Population

Aroma Agape Gopalai,[1] Azlina Ahmad-Annuar,[1*] Hui-Hua Li,[2] Yi Zhao,[3] Shen-Yang Lim,[4] Ai Huey Tan,[4] Thien Thein Lim,[5] Gaik Bee Eow,[6] Puvanarajah Santhi,[7] Viswanathan Shanthi,[7] Mohamed Ibrahim Norlinah,[8] Zariah Abdul Aziz,[9] Soo Kun Lim,[4] Chong Tin Tan,[4] and Eng-King Tan[10,11]

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PARK16 was identified as a risk factor for Parkinson's disease in a Japanese cohort; however, subsequent studies in the other populations including the Chinese, European, Caucasian, and Chilean have shown a protective role instead. To investigate this locus in our Malaysian cohort, 1,144 individuals were screened for five SNPs in the _PARK16_ locus and logistic regression analysis showed that the A allele of the rs947211 SNP reduced the risk of developing PD via a recessive model (Odds ratio 0.57, _P_-value 0.0003). Pooled analysis with other Asian studies showed that A allele of the rs947211 SNP decreased the risk of developing PD via a recessive model (Odds ratio 0.71, _P_-value 0.0001). In addition, when meta-analysis was performed with other Asian population, three SNPs (rs823128, rs823156, and rs11240572) reduced risk of developing PD via a dominant model.

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Key words: ***PARK16*; Parkinson's disease; Malaysia; genetics; meta-analysis**

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INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease, characterized by the loss of dopaminergic neurons in the pars compacta of the substantia nigra. Although several causative genes or susceptibility loci have been associated with familial and sporadic forms of PD, these loci account for a small

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