Neurology Asia 2020; 25(1) : 39 – 46

Glucocerebrosidase genetic variants in Malays with early and late-onset Parkinson's disease

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Abstract

Background: Mutations in glucocerebrosidase (*GBA*) have been associated with the risk of developing Parkinson's disease (PD) in different ethnic populations. The prevalence of *GBA* mutations among Malay PD patients is unknown. Thus, the aim of this study was to determine the frequency of *GBA* mutations among Malay PD patients, focusing on early (EOPD) and late-onset (LOPD) patients. *Methods:* EOPD (n = 50) and LOPD (n = 50) patients along with 50 ethnically and age-matched control were recruited. The *GBA* exons of these patients were sequenced using the Ion Torrent PGM™ System. *Results:* Five heterozygous mutations exclusive to EOPD patients were identified; c.-203A>G, p.S146L, p.R159Q, p.L483P and p.L483R+c.-145G>A. In LOPD patients, c.543C>T(p.(F181=)), c.28-10C>A and p.R202Q were identified in which this p.R202Q was also present in a control subject. In addition, c.259C>A(p.(R87=)) and c.-145G>A were identified in two control subjects. In summary, we observed *GBA* mutations in 8% and 6% of Malay PD cases and control subject, respectively. The prevalence of *GBA* mutations was higher in EOPD (10%) than LOPD (6%). However, these differences were not statistically significant; [PD vs. controls: OR = 1.36, 95%CI 0.35-5.38, $p = 0.752$] and [EOPD vs. LOPD: OR = 1.74, 95%CI 0.39-7.71, $p = 0.715$].
Conclusion: We identified five exclusive heterozygous *GBA* mutations in EOPD patients which might predict the increase susceptibility of Malays to develop PD at young age. These findings could add knowledge into the existing evidences linking genetic alterations in *GBA* and PD.

Keywords: Glucocerebrosidase, Malay, Parkinson's disease (PD), EOPD, LOPD

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease characterised by bradykinesia, tremor, rigidity and postural instability.[1] Pathologically, there is progressive degeneration of dopaminergic neurons within the substantia nigra pars compacta with consequent depletion of dopamine in their striatal projections.[1] The current treatments for PD are asymptomatic and the administered therapies do not present any disease-modifying effect.[2]

The cause of PD is still unknown, but several aspects have been recognised and linked to the PD development which include age, gender, environmental factors and genetic alterations.[1] Genetically, mutations in *SNCA, LRKK2*,and *VPS35* could lead to the development dominantly-inherited PD.[1] In addition, autosomal recessive PD could be due to the mutations in *Parkin*, *PINK1*, and *DJ-1*.[1] Genome wide-association studies (GWAS) and meta-analyses on PD have revealed several susceptibility loci that include *GBA, ADH1C, ATXN2,MAPT* and *GLUD2*.[1] The role of these genes and the identified susceptibility loci in the development and progression of PD warrant further investigations. These may provide better insights into the aetiology and pathogenesis of PD and may pave the way for the development of novel and efficient therapeutic strategies for PD.[3]

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GBA encodes for beta-glucocerebrosidase, a lysosomal enzyme that catalyses the breakdown of glucocerebroside, a component of cell membrane, to glucose after the cells die. This gene had been previously associated with Gaucher disease (GD) which is a lysosomal storage disorder.[3] Recent studies have shown that *GBA* mutations are associated with PD and the frequencies differ within ethic groups.[4-13] To date, more than 300 potentially-to-be-pathogenic *GBA* mutations have been identified in PD[14] and the most common are the p.N370S and p.L483P.[4-13] It has been proposed that impaired *GBA* function either due to gain-of or loss-of function leads to the development and progression of PD; nevertheless the exact mechanism on how *GBA* mutations contribute to the pathogenesis of this disease are yet to be fully understood.[14] Moreover, the presence of specific *GBA* mutations are linked with more aggressive phenotype such as a greater degree of cognitive dysfunction.[15]

In Malaysia, there were approximately 19,000 PD cases estimated in 2016, an increase of 26.4% from 1990 after age standardization.[16] The number of PD cases are expected to rise globally in the coming years due to increase in life expectancy and the contribution of genetic and/or environmental factors that promote PD pathogenesis.[16] The Malaysian population is comprised of several ethnic groups in which the Malays are the largest ethnic group (68%) followed by Chinese (23.0%) and Indian (6.9%).[17] To date, the prevalence rate of PD in each Malaysian ethnic group is unknown. However, study on Singaporean PD population found that there was no significant difference in the prevalence rates of PD between Chinese, Malays and Indians in Singapore.[18]

Due to the increase number of PD cases among the Malaysian population, there were studies conducted recently investigating the frequency of genetic alterations in two PD-associated genes, *LRRK2*[18] and *PARK16*[19] among the Malaysian population. This study aim to identify the SNPs in *GBA* gene in the Malay PD patients. In addition, we would like to find whether there were any differences in the *GBA* mutations profile between the Malay EOPD and LOPD cases.

METHODS

Samples collection

The study protocol was approved by the Universiti Kebangsaan Malaysia (UKM) Ethics Committee (FF-279-2011) and written informed consent was obtained from all participants. This case-control study involved 50 early-onset PD (EOPD), 50 late-onset PD (LOPD) patients and 50 non-neurological controls. The controls were selected based on questionnaires (age, ethnicity and health history) and no clinical examinations were performed. Meanwhile, the patients were diagnosed by neurologists based on the UKPDS Brain Bank Criteria. Samples were collected from two hospitals, UKM Medical Centre (UKMMC) and Universiti Malaya Medical Centre (UMMC). EOPD and LOPD were defined as age at PD onset ≤50 years and ≥60 years, respectively.

DNA isolation and polymerase chain reaction (PCR) for GBA exons

In total, 4 mL blood sample was collected from the participants and DNA was extracted using salt extraction method. DNA integrity and concentration were determined using Nanodrop (Thermo Fisher Scientific, USA). PCR was performed using Platinum® PCR SuperMix High Fidelity (Thermo Fisher Scientific, USA), AmpliTaq Gold® 360 Master Mix (Thermo Fisher Scientific, USA) and three sets of primer pairs based on sequences published by Lesage *et al*.[20]

Library preparation

The Ion Plus Fragment Library Kit (Thermo Fisher Scientific, USA) was used to prepare the libraries. Briefly, 100 ng of pooled PCR products from the same sample was fragmented using Covaris S2 (Covaris, USA). The libraries were prepared according to the protocols described by the manufacturer. The libraries were quantitated using Agilent High Sensitivity DNA Kit (Agilent Technologies, USA) to determine the quality and concentration of the libraries prepared. Each of the library was diluted and mixed in equimolar amount of 20 pM for template preparation.

Template preparation, PCR emulsion, Ion Personal Genome Machine (PGM) sequencing and data analysis

Template-positive Ion Sphere™ Particles for multiplexed barcoded libraries were prepared on the Ion OneTouch™. Enrichment process was performed using the Ion One Touch™ ES. Six or fifty barcoded samples were multiplexed on the Ion 314™ or Ion 316™ chip, respectively with a target coverage of 200. Sequencing was performed on the Ion PGM™ sequencer using the Ion PGM™ 200 sequencing kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The Torrent Suite software was

used to parse barcoded reads, align reads to the reference genome hg19 and generate run metrics. Mutations were identified using Torrent Variant Caller plug-in and target coverage was evaluated with Coverage Analysis plug-in. Pathogenic predictions of mutations were performed using ANNOVAR[21] PROVEAN Genome Variants[22], FATHMM-MKL[23] and MutationTester[24] software tools.

Statistical Analysis

Fisher's exact test was used to analyse the differences in the frequency of *GBA* mutations between PD patients and controls; and EOPD and LOPD. The *p* values was calculated using the Easy Fisher Exact Test Calculator from Social Science Statistics.[25] Statistical significance was set at $p<0.05$. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated using the MedCalc odds ratio calculator.[26]

RESULTS

Demographic data of the study participants

A total of 150 Malay subjects comprising of 50 EOPD, 50 LOPD patients and 50 controls (25 young and 25 elderly) similar in age and sex were recruited in this study. The mean ± SD ages at onset of the EOPD patients and young controls were 43.32 ± 6.59 years (25-50 years) and 41.48 ± 7.91 years (24-52 years), respectively, comprising of 53.3% men and 46.7% women. The mean ± SD ages at onset of the LOPD patients and elderly controls were 65.76 ± 4.04 years (60-74 years) and 66.32 ± 5.38 years (58-77 years), respectively, comprising of 52.0% men and 48% women.

Non-synonymous and synonymous GBA mutations identified using targeted-sequencing

Upon the *GBA* targeted-sequencing in these recruited subjects, we discovered that 6% (n = 6/100) of the PD cases had *GBA* mutations in the exonic regions. In addition, we also found genetic alterations in the control subjects (n = 2/50, 4%). Interestingly, when comparing the *GBA* mutations frequency between the EOPD and LOPD patients, we discovered that higher frequency of mutations in EOPD (n = 4/50, 8.0%) compared to LOPD cases (n = 2/50, 4.0%) (Table 1). However, the differences between these groups were not statistically significant; i) PD vs. control: OR = 1.53, 95%CI 0.30-7.88, $p = 0.719$ and ii) EOPD vs. LOPD: OR = 2.09, 95%CI 0.37-11.95, $p = 0.678$. These findings remained statistically insignificant even after the exclusion of synonymous *GBA* mutations for both comparisons; i) PD vs. control: OR = 2.58, 95%CI 0.29-22.69, $p = 0.664$ and ii) EOPD vs. LOPD: OR = 4.26, 95%CI 0.46-39.55, $p = 0.362$.

In specific, we uncovered seven heterozygous mutations in the *GBA* exonic regions of these recruited subjects. Out of these seven mutations, five were non-synonymous whereas the other two were synonymous mutations. The mutations are in exon 3 (c.259C>A(p.(R87=)), exon 4 (p.S146L), exon 5 (p.R159Q and c.543C>T(p.(F181=))), exon 6 (p.R202Q) and in exon 10 (p.L483P and p.L483R). The position of these mutations is depicted in Fig. 1. As listed in Table 2, all of these exonic mutations were identified in PD patients except for the synonymous c.259C>A(p.(R87=)) which was only found in a young control subject (48 years). In addition, the p.R202Q, identified in one LOPD case, was also found in an elderly control subject (69 years). All mutations except the aforementioned p.R202Q were predicted to be pathogenic by most of the pathogenicity prediction softwares (Table 3).

Potentially pathogenic mutations in GBA non-coding regions

We identified nine mutations in the *GBA* non-coding regions of these subjects.Three mutations were predicted to be damaging; i) the intronic c.28-10C>A, ii) the 5' gene flanking region c.-203A>G, and iii) the 5' untranslated region (UTR) c.-145G>A (Table 3).The c.-203A>G variant was found in an EOPD patient whereas the c.28-10C>A was found in a LOPD patient. In addition, c.-145G>A was found in 2 subjects; an EOPD patient that has p.L483R and in an elderly control (62 years) (Table 3).

In summary, genetic alterations in both *GBA* coding and non-coding sequences were identified, predicted to be damaging in the sequenced samples (n = 11/150, 7.33%). The prevalence of *GBA* mutations in all PD cases was 8% (n = 8/100) and 6% (n = 6/100) in the control subjects, PD vs. control: OR = 1.36, 95%CI 0.35-5.38, $p = 0.752$. Furthermore, the *GBA* mutations frequency was higher in EOPD patients (n = 5/50, 10%) as compared to the LOPD cases (n = 3/50, 6%), EOPD vs. LOPD: OR = 1.74, 95%CI 0.39-7.71, $p = 0.715$). These observed differences between these two comparisons failed to reach statistical significance.

Table 1: Total cases and frequency of Malay PD patients and controls with *GBA* mutations, OR (95%CI) and *p* value in PD vs. controls as well as EOPD vs. LOPD.

	Total cases		Percentage (%)	OR (95%CI)	*p* value
	All	With *GBA* mutations			
Potentially pathogenic *GBA* mutations					
Normal	50	3	6		
All PD cases	100	8	8	1.36 (0.35-5.38)	0.752
EOPD	50	5	10		
LOPD	50	3	6	1.74 (0.39-7.71)	0.715
Exonic *GBA* mutations					
Normal	50	2	4		
All PD cases	100	6	6	1.53 (0.30-7.88)	0.719
EOPD	50	4	8		
LOPD	50	2	4	2.09 (0.37-11.95)	0.678
Non-synonymous *GBA* mutations					
Normal	50	1	2		
All PD cases	100	5	5	2.58 (0.29-22.69)	0.664
EOPD	50	4	8		
LOPD	50	1	2	4.26 (0.46-39.55)	0.362

Table 2: Potentially pathogenic *GBA* mutations carrier in the Malay PD patients and controls

GBA mutations	refSNP	PD (age at onset)		Control (age)		No. of carriers
		EOPD (n = 50)	LOPD (n = 50)	Young (n = 25)	Elderly (n = 25)	
p.S146L	rs758447515	1 (50 years)	-	-	-	1
p.R159Q	rs79653797	1 (44 years)	-	-	-	1
p.R202Q	rs398123531	-	1 (67 years)	-	1 (69 years)	2
p.L483P	rs421016	1 (43 years)	-	-	-	1
p.L483R + c.-145G>A	rs421016 + rs371157845	1 (50 years)	-	-	-	1
c.259C>A(p.(R87=))	rs1141814	-	-	1 (48 years)	-	1
c.543C>T(p.(F181=))	rs563689350	-	1 (61 years)	-	-	1
c.28-10G>A	rs778920100	-	1 (70 years)	-	-	1
c.-203A>G	rs188978150	1 (50 years)	-	-	-	1
c.-145G>A	rs371157845	-	-	-	1 (62 years)	1
No. of carriers for each subgroup		**5**	**3**	**1**	**2**	
Total of all carriers		**8**		**3**		**11**

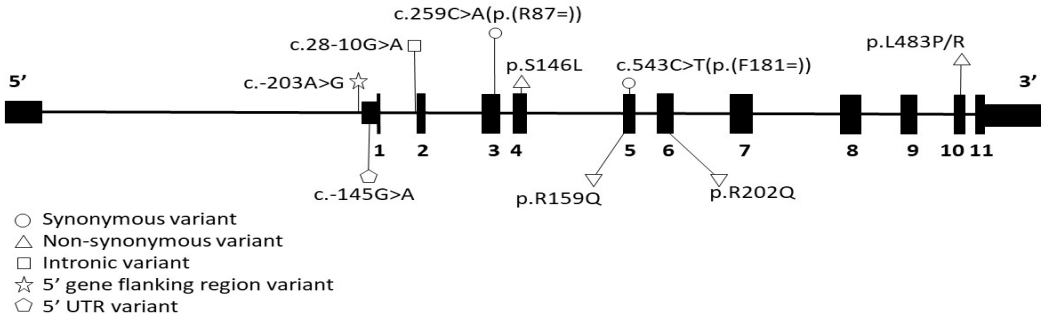

Figure 1. Position of potentially pathogenic GBA mutations identified in the Malay PD patients and controls.

DISCUSSION

The frequency of potentially-to-be-pathogenic *GBA* mutations in Malay PD patients our cohort was 8%. This was within the range of observed *GBA* mutations in other studied Asian populations which was between 3.2-9.4%.[5,9-12] Of note, the prevalence of *GBA* mutations in the Malay PD population was relatively similar to those observed in the Eastern China population (8.7%).[5] However, we also observed that the frequency of potentially-to-be-pathogenic *GBA* mutations in the control subjects was 6% which was higher compared to controls of other Asian populations studies (0.0-1.5%).[5,9-12] This finding was rather surprising as the controls did not present any known PD clinical manifestations. Also, the controls were recruited based on questionnaires without prior neurological evaluation and other relevance clinical examinations. However, in this regards, it would be interesting to monitor these control subjects closely in order to determine whether they might eventually develop PD in the future.

Interestingly, we discovered that the EOPD patients had higher frequency of *GBA* mutations as compared to the LOPD patients in which some of these mutations were exclusive and only found in the EPOD patients (Table 2). These observations seemed to suggest that harbouring several specific SNPs in *GBA*, p.S146L, p.R159Q, p.L483P and c.-203A>G, might increase the risk of developing EOPD among Malay populations. Nevertheless, in support of our recent findings, studies in several other Asian populations have also found that patients with *GBA* mutations had the predisposition to develop PD at a relatively young age compared to patients without the *GBA* mutations.[9-12] Contradictory to these reports, Mao *et al*.[7] and Sun *et al*.[8] found that genetic alterations in *GBA* did not modify the PD age of onset in Chinese population from the Mainland China. The reason behind this opposing observation remains unknown, but one possible explanation is that different genetic alterations in *GBA* could affect the functions of glucocerebrosidase enzyme differently, which in turn could lead to diverse PD symptoms and clinical manifestations.

Overall, the potentially-to-be-pathogenic heterozygous *GBA* mutations, seven in the exonic regions and three in the non-coding regions were identified. Five of the seven *GBA* exonic mutations were non-synonymous: p.S146L, p.R159Q, p.R202Q, p.L483P and p.L483R. p.L483P is one of the most common *GBA* mutations found in PD cases[6,13] in which the p.L483P carriers have a 11.68X risk of developing PD.[13] In Asia populations, the p.L483P variant was identified in the Japanese.[6,12], Thai[10], Korean[11] and also Chinese PD populations.[5-9] p.R202Q was reported only in Korean[11] and Chinese PD populations from Eastern China.[5] One LOPD patient and one elderly control subject harboured this p.R202Q variant. Furthermore, our mutation prediction analysis revealed that this mutation was non-pathogenic. The other three identified pathogenic non-synonymous *GBA* mutations, p.S146L, p.R159Q and p.L483R, were novel and not observed in other PD studies, but were reported in GD cases.[27]

The five non-synonymous exonic *GBA* mutations identified are likely to produce destabilized and misfolded glucocerebrosidase protein. SNP effect database using FoldX Forecast[28] predicted that the stability of glucocerebrosidase enzyme is severely disrupted upon p.L483R amino acid change and moderately disrupted upon p.S146L, p.R159Q and p.L483P amino acid changes.Consistent with the prediction of this alteration to be non-pathogenic, p.R202Q amino acid change only showed slight reduction in the glucocerebrosidase enzyme stability.[28] Moreover, p.R159Q may affect catalytic activity of glucocerebrosidase enzyme because the arginine residue is located in the proximity of glucocerebrosidase catalytic sites, p.E379 and p.E274.[29]

Table 3: Pathogenicity prediction for *GBA* mutations identified in Malay PD patients and controls using MutationTester, FATHMM MKL, PROVEAN Genome Variants and ANNOVAR softwares

GBA gene mutations			Pathogenicity prediction							
			Mutation Tester	FATHMM MKL		PROVEAN Genome Variants		ANNOVAR		
NC_000001.10	NC_000001.10 GBA_v004)	NC_000001.10 (GBA_i004)		Coding	Non-coding	PROVEAN	SIFT	Poly phen2	Mutation Assessor	Meta SVM
Non-synoymous										
g.155209424G>A	c.437C>T	p.S146L	N	D	D	D	D	D	L	D
g.155208420C>T	c.476G>A	p.R159Q	A	N	D	D	D	D	H	D
g.155208081C>T	c.605G>A	p.R202Q	D	N	D	N	T	B	L	D
g.155205043A>G	c.1187T>C	p.L483P	A	D	D	D	D	P	M	D
g.155205043A>C	c.1448T>G	p.L483R	D	D	D	D	D	D	M	D
Synonymous										
g.155209725G>T	c.259C>A	p.(=)	D	D	D	-	-	-	-	-
g.155208353G>A	c.543C>T	p.(=)	D	D	D	-	-	-	-	-
Intron										
g.155210518G>T	c.28-10C>A	p.(=)	D	N	D	-	-	-	-	-
5' UTR										
g.155211048C>T	c.-145G>A	p.(=)	N	N	D	-	-	-	-	-
5' gene flanking region										
g.155211106T>C	c.-203A>G	p.(=)	N	N	D	-	-	-	-	-

MutationTester = A: Disease causing automatic, D: Disease causing, N: Polymorphism
FATHMM MKL & PROVEAN = D: Deleterious, N: Neutral
SIFT = D: Damaging, T: Tolerated
Polyphen2 = D: Probably damaging, P: Possibly damaging, B: Benign
MutationAssessor = H: High, M: Medium, L: Low. H/M means functional and L means non-functional
metaSVM = D: Deleterious, T: Tolerated

Genome wide association studies (GWAS) have revealed the importance and association of synonymous gene mutations with the development of diseases such as neurological disorders.[30] Non-synonymous and synonymous mutations have similar degree of probability to be associated with diseases, at 1.26% and 1.46%, respectively.[30] Thus, we assessed the frequency of synonymous *GBA* mutations and identified two potentially pathogenic synonymous mutations: c.543C>T(p.F181(=)) and c.259C>A(p.R87(=)).[23] These mutations might affect pre-mRNA splicing process. SKIPPY [31] and Human Splicing Finder[32] predicted that c.543C>T(p.F181(=)) causes loss of one ESE site and replaces with two new ESS site. However, c.259C>A(p.R87(=)) result in the loss of 3 ESE sites [31,32] These ESR changes disrupt ESE/ESS density[31] and might result in the formation of dysfunctional glucocerebrosidase enzyme that would eventually lead to PD. Therefore based on this prediction, we postulated that the young control subject with c.259C>A(p.R87(=)) might probably at risk of getting EOPD in which we would be interested in monitoring this subject closely.

Many studies have demonstrated the equal importance of mutations in the coding and non-coding sequences in disease pathogenesis.[33] Thus, we also analysed the non-coding regions and identified three potentially pathogenic non-coding *GBA* mutations; i) one was in intron (c.28-10C>A), ii) one was in 5' gene flanking region (c.-203A>G) and iii) one was in 5' UTR (c.-145G>A). These mutations are present in a highly conserved region.[23] Therefore, the effect of genetic alterations in this region is likely to be more severe than in less conserved regions. In addition, mutation prediction analysis revealed that the c.-203A>G and c.-145G>A variants are likely to interfere with the binding of transcription factors at the *GBA* proximal regulatory region.[23] In contrast, c.28-10C>A may affect pre-mRNA splicing due to the location of this variant that is 50 base pairs away from the exon-intron junction.[34]

In conclusion, we have identified seven potentially pathogenic heterozygous *GBA* exonic mutations. Five were non-synonymous, p.R120Q, p.S146L, p.R202Q, p.L483P and p.L483R, and two were synonymous mutations, c.543C>T(p.F181(=)) and c.259C>A(p.R87(=)). Three non-coding *GBA* mutations were identified which were the c.28-10C>A, c.-203A>G and c.-145G>A. It is also important to highlight that we identified five *GBA* mutations that were exclusive to the EOPD patients. These mutations might predict the risk of developing PD at a young age among the Malay ethnic group. Nonetheless, our findings were based on a small study cohort and a larger sample size is needed to further validate these findings.

ACKNOWLEDGEMENTS

We thank UKM for the Dana Lonjakan Perdana. We also would like to thanks Alex Teoh View Hune and Janice Khor from the Thermo Fisher Scientific for their guidance on the targeted-sequencing technique and data analysis.

DISCLOSURE

Financial support: This work was funded by Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor, Malaysia [UKM-DLP-2011-048].

Conflicts of interest: None

Ethics: The study protocol was approved by the Universiti Kebangsaan Malaysia (UKM) Ethics Committee (FF-279-2011).

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