

Mitochondrial Respiratory Chain Enzymatic Activities on Skin Fibroblasts in Patients With Mutant Glucocerebrosidase and PARK2 Genes

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Abstract

Background: Impairments of mitochondrial respiratory chain have been observed in skin fibroblast of patients with sporadic Parkinson's disease (PD) and PARK2 mutations. This study assesses enzymatic activity of mitochondrial respiratory chain in skin fibroblasts of Thai PD patients with glucocerebrosidase (GBA) and PARK2 mutations.

Methods: Skin fibroblasts were obtained from 14 individuals including four mutant GBA alleles, three mutant PARK2 alleles, and seven PD patients without mutations. Five age-matched healthy controls were recruited. Activities of complex I - V were assessed using enzyme-linked immunosorbent assay, and normalized with citrate synthase activity. All measurements were performed in triplicate.

Results: Complex V activity seemed to be lower in PD patients than controls. Among patients, activities of complexes I, II, IV and V seemed to be lower in the mutant group than those without mutation. Among patients with mutations, the mutant GBA group showed slightly lower activities of complexes II, IV, and V than the mutant PARK2 group.

Conclusions: A skin fibroblast culture may be a useful tool to investigate how GBA mutation leads to impairment of the energetic metabolism in patients with PD.

Keywords: Respiratory chain; Mitochondria; Fibroblast; Parkinson's disease; Parkin; GBA

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease whose characteristic clinical signs include muscle rigidity, slowness of movement, rest tremors, and postural instability. While most PD cases are sporadic, mutations of several genes have been identified as a causative factor for rare familial PD. PARK2 (Parkin) mutations are known to be the most common cause of familial or early-onset PD [1]. Compound heterozygous abnormalities in the PARK2 gene have been found in skin fibroblasts isolated from patients with middle age-onset familial parkinsonism [2]. Skin fibroblasts from patients with PARK2 mutations have also demonstrated a distinct and unique mRNA expression pattern of key genes in neurodegeneration, which are different from those found in idiopathic PD and other mutations [3]. The association between PD and mutations in the acid β -glucocerebrosidase (GBA) gene has been reported [4-9]. Mechanisms, by which loss-of-function mutations of GBA lead to clinical phenotypes similar to sporadic PD, still remain elusive. The possible mechanism is that the GBA mutation compromises lysosomal protein degradation and leads to accumulation of α -synuclein, which results in neurotoxicity [10, 11]. The reduction in the GBA activity has been observed in skin fibroblasts of PD patient with GBA mutation [12].

Functional and morphological impairments of mitochondrial respiratory chain have been observed in skin fibroblast of sporadic PD patients and PD patients with PARK2 mutations, such as lower mitochondrial complex I and V activities and adenosine triphosphate (ATP) production [13-15]. GBA inhibition can cause decreased adenosine diphosphate (ADP) phosphorylation, reduced mitochondrial membrane potential and increased free radical formation in the SH-SH5Y human neuroblastomal cell line [16]. Here, we investigate the enzymatic activity of mitochondrial respiratory chain in skin fibroblast cultures of Thai PD patients with PARK2 and GBA mutations, in comparison to those of controls and non-mutant PD patients.

Materials and Methods

Ethics statement

The study was approved by the Ethical Clearance Committee

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Table 1. Details of PCR, Mismatched PCR and Restriction Fragment Length Polymorphisms

Mutation	Primer sequence	PCR product size (bp)	Restriction enzyme	Restriction position	Restriction product size (bp)
C1309delG	GBA-F9-11: AAC CAT GAT TCC CTA TCT TC GBA-R9-11: ACC ACC TAG AGG GGA AAG TG	1,290	<i>SacII</i>	WT 358 MT none	WT 358, 932 MT 1,290 WT/MT 358, 932, 1,290 WT 72, 401, 473 MT 72, 116, 285, 473 WT/MT 72, 116, 285, 401, 473
IVS10-9_10 GT>AG			<i>Hpy188III</i>	WT 817, 889 MT 817, 889, 1005	WT 28, 32, 69, 88, 141, 183, 524 MT 28, 32, 69, 88, 183, 665 WT/MT 28, 32, 69, 88, 141, 183, 524, 665
IVS2+1G>A	GBA-F2: CTT CTC TTC ATC TAA TGA CCC TGA GBA-R2: CCA CCG AGC TGT AGC C	1,065	<i>HphI</i>	WT 524, 665, 848, 880, 908, 977 MT 665, 848, 880, 908, 977	WT 44, 116 MT 160 WT/MT 44, 116, 160
N386K	GBA-N386K-F: CTG GAC CGA CTG GAA CCT TGC CCT GTA (T is a mismatch nucleotide) GBA-N386K-R: 5'-GTG CCC GCC CTC CAC TCA CCT	160	<i>RsaI</i>	WT 116 MT none	
MIT	Parkin_1_E1_F: ACG TAA GAT TGC TGG GCC TGA AG Parkin_1_E1_R: GGC ACT TTG GCC CCG TCA TTG AC	392	<i>NlaIII</i>	WT 224, 267 MT 224	WT 43, 126, 223 MT 169, 223 WT/MT 43, 126, 169, 223

bp: base pairs; WT: wide-type; MT: mutant-type.

on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathodi Hospital, Mahidol University (ID 03-54-22). All participants provided both verbal and written informed consent prior to the enrollments.

Patients and controls

The authors had recent works on a comprehensive genetic analysis of GBA [17] and PARK2 genes (unpublished data), and screening for p.R1628P, p.G2019S and p.G2385R variants of the leucine-rich repeat kinase 2 gene (LRRK2) in a large Thai PD cohort [18]. Here, we recruited the PD patients from the previous cohort for further studying on a mitochondrial respiratory chain function associated with a heterozygous allele of GBA and PARK2 mutations. Fourteen patients diagnosed with PD and five healthy age-matched controls were enrolled in this study. The patient and control groups were evaluated in the Neurology Department of the same University Hospital. All PD patients had onset of disease before the age of 50 years, and all fulfilled the UK Parkinson’s Disease Brain Bank criteria [19]. Four patients carried heterozygous GBA point mutations, two had heterozygous deletion of exon 8 of PARK2, one had a heterozygous PARK2 point mutation, and seven remaining patients had no mutations in the genes studied (Table 1). Details of the identification of GBA variants have been described in our previous publication, which can distinguish functional GBA from its pseudogene [17]. To our knowledge, all but p. IVS2+1g>A mutations of the GBA were identified in only Thai PD patients [17]. The PARK2 point mutation, p.MIT, is a novel mutation identified in 0.8% of the Thai PD cohort (4/513), and absent in over 400 control subjects. In contrast, a heterozygous deletion of exon 8 of PARK2 was detected in both PD patients (1.2%) and control group (2%), suggesting that it may not play a pathogenic role on risk of developing PD. Controls were recruited from patients presenting with other medical conditions and had no signs of parkinsonism and were analyzed for the absence of GBA and PARK2 mutations.

Skin fibroblast cultures

Skins were obtained from the dorsal region of the inner upper arm of each PD patient or control. The biopsy specimens were maintained in tubes with Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Gaithersburg, MD, USA), stored at 4 °C and processed within 4 h in laminar flow. Fibroblast harvesting was done by explant, isolating the dermis from the epidermis with scalpels and scissors. Then dermis specimens were fragmented in small pieces. These fragments were laid onto the surface of Petri dishes containing high glucose-DMEM, penicillin (100 UI/mL), streptomycin (100 mg/dL), L-glutamine (4 mM) and supplemented with heat-inactivated fetal calf serum. The cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Fibroblasts were grown to confluence, harvested by trypsinization at 37 °C, washed with culture medium, resuspended with phosphate buffer, and then sonicated to obtain the cell homogenate. In all assays, fibroblast passage

Table 2. Phenotypic and Genotypic Data of Parkinson's Disease Patients and Controls

	Code	Gender	Age (years)	Causative gene	Mutation
Patients	1	Male	51	None	-
	2	Male	57	None	-
	3	Male	47	None	-
	4	Male	37	None	-
	5	Male	45	None	-
	6	Male	46	None	-
	7	Female	53	None	-
	8	Male	56	<i>PARK2</i>	exon 8 deletion
	9	Female	43	<i>PARK2</i>	p.Met1Thr
	10	Female	41	<i>PARK2</i>	Exon 8 deletion
	11	Male	56	<i>GBA</i>	IVS11-9,10 gt>ag
	12	Female	57	<i>GBA</i>	c.1309delG
	13	Female	41	<i>GBA</i>	p.Asp386Lys
	14	Female	49	<i>GBA</i>	IVS2+1g>A
Mean ± SD			48.5 ± 6.7		
Controls	1	Male	53	None	-
	2	Female	45	None	-
	3	Female	59	None	-
	4	Female	46	None	-
	5	Female	45	None	-
Mean ± SD			49.6 ± 6.2		

SD: standard deviation.

numbers were no greater than 10.

Confirmation of the genotypes in fibroblasts

DNA was extracted from fibroblasts using a standard phenol-chloroform method. All point mutations were confirmed by using both direct sequencing of the interested exons, and appropriated PCR-RFLPs methods (Table 1). Direct sequencing of all exons and exon-intron boundaries of GBA and PARK2 genes was performed in all DNA samples of the control subjects in order to exclude the presence of any point variants in the studied genes. PARK2 exon rearrangement was assessed in all DNA samples by using both semi-quantitative fluorescently labeled multiplex PCR and denaturing high-performance liquid chromatography (DHPLC) as prior protocols described [20, 21].

Assessment of mitochondrial respiratory chain function

Activities of respiratory chain enzymes (complex I, NADH dehydrogenase; complex II, succinate-coenzyme Q reductase; complex III, cytochrome c reductase; complex IV, cytochrome c oxidase; and complex V, ATP synthase) and citrate synthase were determined using enzyme activity microplate assay

kits (catalog no. ab109721, ab109908, ab124537, ab109909, ab109714, and ab119692; Abcam, Cambridge, MA, USA) in whole cell lysates at an immunocapture based manner, according to the manufacturer's instructions. The amount of protein was determined using a BCA protein assay. The enzyme activity was expressed as the change in absorbance per minute (mOD/min) per amount of sample loaded into the microplate well. The sample rate was compared with the rate of the control sample and with the rate of the background to get the relative enzyme activity. The enzyme activity was determined by reading the optical density using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The rate was determined by calculating the slope between two points within the linear region. To correct for mitochondrial volume, complex I - V activities were normalized with the activity of citrate synthase, an exclusive marker of the mitochondrial matrix. All measurements were performed in triplicate and in three independent runs per sample.

Statistical analysis

Results of enzymatic activities were expressed as mean ± standard error of measurement (SEM) and scatter plots. Statistical analysis was done by the GraphPad Prism 5 Software for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Table 3. Enzymatic Activities (Mean \pm SEM) of Complexes I - V Corrected by Citrate Synthase Activity

	Control (N = 5)	Patients				
		All patients (N = 14)	No mutation (N = 7)	All mutations (N = 7)	<i>PARK2</i> (N = 3)	<i>GBA</i> (N = 4)
Complex I ($/\times 10^3$)	8.92 \pm 0.55	10.78 \pm 0.45	11.48 \pm 0.50	10.09 \pm 0.68	9.44 \pm 0.71	10.57 \pm 1.10
Complex II ($/\times 10^3$)	107.9 \pm 11.57	117.4 \pm 7.45	122.1 \pm 13.77	112.6 \pm 6.40	125.70 \pm 6.25	102.70 \pm 6.90
Complex III ($/\times 10^2$)	53.11 \pm 3.69	51.69 \pm 3.00	48.88 \pm 2.60	54.50 \pm 5.43	47.42 \pm 11.66	59.81 \pm 3.64
Complex IV ($/\times 10^3$)	27.15 \pm 2.28	29.30 \pm 1.31	31.61 \pm 2.04	26.99 \pm 1.21	27.61 \pm 1.35	26.52 \pm 2.01
Complex V ($/\times 10$)	34.13 \pm 2.39	32.95 \pm 2.06	35.08 \pm 3.96	30.83 \pm 1.08	31.63 \pm 2.41	30.23 \pm 0.94
Citrate synthase (mOD/min)	13.32 \pm 0.19	12.66 \pm 0.13	12.46 \pm 0.14	12.87 \pm 0.21	12.97 \pm 0.30	12.79 \pm 0.31

SEM: standard error of measurement.

The Student's two-sample *t*-test was used for comparisons between patients and controls, or between mutant and non-mutant groups. A *P* less than 0.05 was considered statistically significant.

Results

Phenotypic and genotypic data of PD patients and controls are summarized in Table 2. Patients (mean age \pm SD, 48.5 \pm 6.7 years) included seven without *PARK2* and *GBA* mutations, three with *PARK2* mutation, and four with *GBA* mutation. Five age-matched controls had a mean age \pm SD of 49.6 \pm 6.2 years.

Enzymatic activities of mitochondrial respiratory chain, complex I - V, for patients and controls are shown in Table 3. All of the enzymatic activities were corrected with citrate synthase activity. Scatter plots of the level of enzymatic activities are shown in Figure 1. As shown, complex V activity seemed to be lower in PD patients than controls. Among patients, activities of complexes I, II, IV and V seemed to be lower in the mutant group than those without mutation. Among patients with mutations, the mutant *GBA* group showed slightly lower activities of complexes II, IV, and V than the mutant *PARK2* group.

Discussion

In this study, we investigate whether enzymatic activities of mitochondrial respiratory chain complexes I - V are affected in skin fibroblast cultures of Thai PD patients with *GBA* and heterozygous *PARK2* mutations. The effect of single heterozygous mutations in *PARK2* and *GBA* is still unclear. Although we for the first time report enzymatic activities of mitochondrial respiratory chain complexes I - V in skin fibroblasts of *GBA*-mutant patients, the slightly decreased activities of complexes II, IV, and especially V, compared with controls, need to be confirmed in a larger scale of patients. Complex V is involved in the ATP synthesis, and is very crucial in the energetic metabolism of the cells. Impairment of mitochondrial functions caused by *GBA* inhibition has been demonstrated in the human dopaminergic cell line [16], and the finding of

mitochondrial dysfunction in skin fibroblasts of *GBA*-mutant patients, if confirmed, would facilitate research on the mechanism of *GBA* mutation-related PD.

Our findings on activities of mitochondrial respiratory chain complexes in patients with *PARK2* mutation (N = 3) are similar to those of Grunewald et al (N = 6) [13], showing no significant differences in complexes I, II + III, and IV activities, compared with controls. However, Grunewald et al measured ATP synthesis rates and intracellular ATP concentrations, and revealed a significant reduction of both parameters compared to controls, suggesting an existence of abnormal mitochondrial function in skin fibroblasts of PD patients with *PARK2* mutation.

Del Hoyo et al reported insignificant differences of complexes I - IV activities between PD patients (N = 20) and control groups, in skin fibroblast cultures, which is similar to our results on non-mutant PD patients (N = 7) [15]. However, a decreased activity of complex V was observed in del Hoyo et al's work. Compared PD patients with and without *GBA* or *PARK2* mutation, our results showed no significant differences of complexes I - V activities, although a slightly decreased activity was observed for complexes IV and V in the mutation group.

The discrepancies of the results of complexes I - V activities among skin fibroblast studies in PD patients may be related to the difference in methods used in assessing the mitochondrial respiratory chain complexes, the presence or absence of PD-related mutations, types of mutation, types of samples (whole cell extract or mitochondrial extract), and sample size. However, evidence that mutations of PD-related genes can cause functional and morphological abnormalities of mitochondria in skin fibroblasts has been reported, such as for *PARK2*, and *PINK1* [13, 22, 23].

Conclusion

A mild decrease in some mitochondrial respiratory chain complexes, especially complex V, was detectable in skin fibroblasts of patients with *GBA* mutation. However, the measurement of steady-state total ATP present in fibroblasts is required to support this finding. Another point of note, although with a negative result, is related to the fact that this is the first report

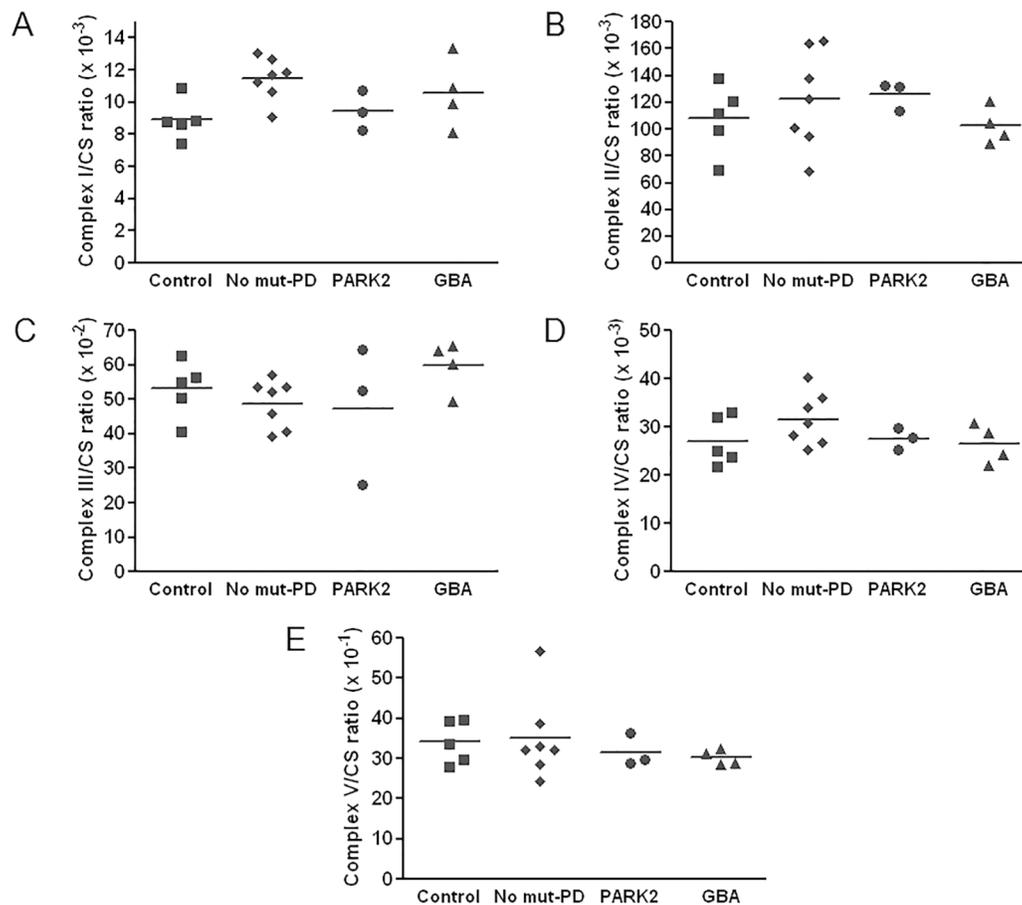


Figure 1. Scatter plots of values of enzymatic activities of complexes I - V in Parkinson's disease (PD) patients and in controls. All respiratory chain enzyme activities are normalized to the activity of citrate synthase (CS). Horizontal bars represent the mean values.

on respiratory chain enzymatic activities in skin fibroblasts of GBA-mutant individuals. Further validation in a larger patient population is required, using a variety of analyzes for mitochondrial functions. Target specificity of mutant GBA on respiratory complexes also remains to be investigated. The present results suggest that a skin fibroblast culture may be a useful tool to investigate the mechanisms underlying how GBA mutation leads to impairment of the energetic metabolism of the cells of peripheral tissues in patients with PD.

Disclosure

This research is under the research framework of Mahidol University.

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Conflicts of Interest

All authors declare no conflict of interest.

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