สัญญาเลขที่ RSA5480019

รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษาหาปัจจัยเสี่ยงทางพันธุกรรมที่เป็นสาเหตุของการเกิดโรคพาร์กินสัน

โดย

รองศาสตราจารย์ ดร.นพ. ธีรธร พูลเกษ

คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

มิถุนายน ๒๕๕๗

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

และคณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

กิตติกรรมประกาศ

ผมขออุทิศคุณงามความดีทั้งหมดที่พึงจะเกิดขึ้นจากงานวิจัยชุดนี้ให้แก่คุณยายจำเนียร แสงอินและอาจารย์ปรีดา พัวประดิษฐ์ที่เคารพรักยิ่ง

ผมขอขอบพระคุณผู้ป่วย ผู้เข้าร่วมโครงการและครอบครัวทุกท่าน ที่เสียสละและให้ความร่วมมือกับโครงการวิจัย

ผมขอขอบพระคุณอาจารย์ของผมทุกท่านโดยเฉพาะ อาจารย์อรรถสิทธิ์ อาจารย์ประเสริฐ อาจารย์ปรีดา อาจารย์รวิพรรณและพี่สุพจน์ที่กรุณาสอนผมทั้งด้านกระบวนการคิด วิชาการ จรรยาแพทย์และเป็นตัวอย่างที่ดีในการดำรงชีวิตในฐานะแพทย์และมนุษย์

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ผมขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัยที่กรุณาให้ทุนวิจัยแก่โครงการวิจัย

ผมขอขอบพระคุณคณะแพทยศาสตร์รพ.รามาธิบดี มหาวิทยาลัยที่มอบทุนพัฒนาศักยภาพอาจารย์แพทย์ และให้การสนับการดำเนินการวิจัยในทุกๆด้าน

ผมขอขอบพระคุณอาจารย์ปิยมิตร ศรีธราและอาจารย์ สุพจน์ ตุลยาเดชานนท์ ที่ให้ใช้เงินทุนในความดูแลของท่านทั้งสองในการทำวิจัยของโครงการนี้

ตลอดจนขอขอบคุณผู้ปริจาคและครอบครัวทุกท่านที่สนับสนุนทุนวิจัยประสาทพันธุศาสตร์ มูลนิธิรามาธิบดี ที่ใช้เป็นเงินสนับสนุนในโครงการ

สุดท้ายนี้ผมขอขอบคุณภรรยาของผม คุณหมอรัชดาวรรณ และลูกสาวทั้งสอง แป้งและปรินที่ให้ความรักเป็นกำลังใจให้และคอยให้การสนับสนุนอยู่เสมอมา

บทคัดย่อ

รหัสโครงการ: RSA5480019

ชื่อโครงการ: การศึกษาหาปัจจัยเสี่ยงทางพันธุกรรมที่เป็นสาเหตุของการเกิดโรคพาร์กินสัน

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วัตถุประสงค์ โรคพาร์กินสัน (Parkinson's disease) เป็นโรคสมองเสื่อมที่มีปัจจัยทางสิ่งแวดล้อมและพันธุกรรมเป็นปัจจัยเสี่ยงสำคัญที่ทำให้เกิดโรคพาร์กินสัน มีการค้นพบว่าตัวแปรทางพันธุกรรมต่างๆในยีนจำนวนมากเป็นสาเหตุและปัจจัยเสี่ยงของโรคพาร์กินสัน คณะผู้วิจัยได้ทำการศึกษานี้เพื่อหาปัจจัยเสี่ยงทางพันธุกรรมที่เกี่ยวข้องกับการเกิดโรคพาร์กินสันในผู้ป่วยไทย
วิธีทดลอง ทำ genotyping ด้วยวิธีที่เหมาะสมในยีน leucine-rich repeat kinase 2 (*LRRK2*), glucocerebrosidase (*GBA1*) และ *parkin* หาความสัมพันธ์กับการเกิดโรคพาร์กินสันและลักษณะทางคลินิกของโรคพาร์กินสัน
ผลการทดลอง การศึกษากลุ่มผู้ป่วยโรคพาร์กินสันขนาดใหญ่จำนวน 485 รายและกลุ่มควบคุมจำนวน 480 ราย การศึกษา *LRRK2* พบว่า p.R1628P เป็นปัจจัยเสี่ยงที่พบบ่อยที่สุด (11% ในกลุ่มผู้ป่วยและ 6% ในกลุ่มควบคุม; OR=1.81, 95%CI=1.10-2.97) การศึกษา *GBA1* พบว่ามีการกลายยีน (mutation) ในยีน *GBA1* จำนวน 6 ตำแหน่ง (4 ตำแหน่งเป็นการกลายยีนที่ค้นพบใหม่) ในผู้ป่วยจำนวน 24 ราย การศึกษาครอบครัวของผู้ป่วย 2 ราย ผู้ป่วยมีโอกาสแสดงอาการก่อนอายุ 50 ปีได้สูงและสุดท้าย การศึกษา *parkin* พบว่ามี exon rearrangements ของยีน parkin ในผู้ป่วย 9 รายที่มีการขาดหาย (deletion) ของ exon และพบ point mutations 5 ตำแหน่ง (3 ตำแหน่งเป็นการกลายยีนที่ค้นพบใหม่) ในผู้ป่วยจำนวน 11 ราย ผู้ป่วยมักแสดงอาการก่อนอายุ 50 ปีเหมือนผู้ป่วยที่มี *GBA1* mutation
สรุปและวิจารณ์ผลการทดลอง มากกว่า 18% ของผู้ป่วยโรคพาร์กินสันชาวไทยมีปัจจัยเสี่ยงทางพันธุกรรมอย่างมีนัยสำคัญ ลักษณะทางคลินิกของผู้ป่วยที่มีการกลายยีนต่างๆมีความเฉพาะที่แสดงความรุนแรงของโรคและการตอบสนองต่อยาต่างจากผู้ป่วยโรคพาร์กินสันอื่นๆ
ข้อเสนอแนะสำหรับงานวิจัยในอนาคต การศึกษาต่อไปในยีนอื่นๆที่เกี่ยวข้องกับการเกิดโรคพาร์กินสันจะช่วยทำให้เราได้ข้อมูลที่สำคัญเกี่ยวกับตัวแปรที่เป็นปัจจัยเสี่ยงของโรคพาร์กินสันซึ่งจะนำไปสู่การพัฒนาสร้าง biomarker ของโรคพาร์กินสันที่มีประสิทธิภาพต่อไปในอนาคต

Keywords: Parkinson's disease; glucocerebrosidase (*GBA1, GBA*); leucine rich kinase 2 (*LRRK2*); parkin (*PARK2*)

Abstract

Project Code: RSA5480019

Project Title: Study of genetic factors as risk of developing Parkinson's disease

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Project Period: 15 Jun 2011 to 14 Jun 2014

Objectives Parkinson's disease (PD) is heterogeneous neurodegenerative disorders associated with various genetic and environmental factors. Several susceptible genes relate to risk of developing PD have been established. The study aims to investigate the genetic factors of PD in Thai patients in order to develop a genetic biomarker panel specifically for Thai PD in the future. This genetic data is essential for providing early neuroprotective therapy, when it is available.

Methods PD patients and control subjects were comprehensively reviewed. Leucine-rich repeat kinase 2 (*LRRK2*), glucocerebrosidase (*GBA1*) and *parkin* were genotyped using appropriated methods. Phenotype-genotype correlations were determined.

Results The study enrolled 485 PD patients and 480 control subjects. Regarding *LRRK2* study, p.R1628P was the most common risk variants in Thai patients (OR=1.81, 95%CI=1.10-2.97). Regarding *GBA1* study, we identified 4 novel point mutations and 2 reported mutations in 24 patients. Regarding *parkin* study, both exon deletions and point mutations were observed in a total of 20 patients. PD patients carrying mutations in those 3 genes had earlier age at onset and more rapidly progressive course than non-carriers. *GBA1* and *parkin* mutations appeared to exhibit slightly earlier age at onset compared to *LRRK2*.

Discussion Over 18% of the Thai PD patients studied had mutations or variants in these 3 susceptible genes. Most mutations were unique to Thais or Asians. Clinical characteristics associated with each studied genes were observed.

Suggestions Further study on other known susceptible genes related to PD will be benefit for helping us setting up a reliable predictive test as a biomarker for Thai PD patients. This tool is likely to be essential for effective neuroprotective therapy of PD in the future.

Keywords: Parkinson's disease; glucocerebrosidase (*GBA1, GBA*); leucine rich kinase 2 (*LRRK2*); parkin (*PARK2*)

โครงการ : การศึกษาหาปัจจัยเสี่ยงทางพันธุกรรมที่เป็นสาเหตุของการเกิดโรคพาร์กินสัน

บทนำ

โรคพาร์คินสัน (Parkinson's disease) เป็นโรคสมองเสื่อมที่พบบ่อยที่สุดโรคหนึ่งประมาณว่ามีผู้ป่วยเกือบ 1 แสนรายในประเทศไทย นอกจากค่ายารักษาที่มีราคาแพงมาก ในระยะท้ายของโรคผู้ป่วยเกิดความทุพพลภาพจนต้องอาศัยผู้ดูแลเป็นภาระแก่ครอบครัวในการดูแล โรคพาร์กินสันนี้ในขณะที่ผู้ป่วยเริ่มต้นมีอาการของโรค แต่เซลล์สมองในส่วนของ substantia nigra กลับมีการตายเกิดขึ้นไปแล้วถึง 50% ดังนั้นการรักษาเพื่อหวังผลในการชะลอการตายของเซลล์อย่างมีประสิทธิภาพจึงควรเริ่มรักษาให้เร็วที่สุดหรือก่อนเกิดการตายของเซลล์สมอง ดังนั้นการให้การวินิจฉัยโรคให้เร็วที่สุดก่อนที่ผู้ป่วยจะเริ่มมีอาการจึงมีความสำคัญต่อการพัฒนาการรักษาโรคพาร์กินสันอย่างมาก

คณะผู้วิจัยได้ทำการศึกษาหา risk alleles และ mutations ต่างๆจาก candidate genes ของโรคพาร์คินสันเพื่อที่จะเป็นเครื่องมือในการใช้วินิจฉัยหรือคาดคะเนความเสี่ยงที่จะเกิดโรคพาร์คินสันก่อนผู้ป่วยแสดงอาการได้

วิธีการทดลอง

งานด้านพันธุศาสตร์

1. เก็บตัวอย่างเลือดและข้อมูลทางคลินิกของผู้ป่วยรวม (485 ราย) และกลุ่มควบคุม (480 ราย) และสร้างฐานข้อมูล
2. การศึกษายีน *Parkin* (*PARK2*) ในผู้ป่วยโรคพาร์กินสัน

2.1 ศึกษา large-scale rearrangements ของ parkin gene ด้วยวิธี multiplex PCR ร่วมกับ Genescan และ การวิเคราะห์ DHPLC ทำการวิเคราะห์ 2 วิธีเพื่อใช้ยืนยันผลให้ถูกต้องแม่นยำได้มาตรฐานสูง และเพิ่มความน่าเชื่อถือของงานวิจัย

2.2 Direct sequencing ในกลุ่มผู้ป่วยเพื่อหา point mutations ต่างๆ และ screening mutations ที่พบทั้งหมดในกลุ่มควบคุมด้วยวิธี RFLP

3. การศึกษายีน glucocerebrosidase (*GBA*1) ด้วยวิธี direct sequencing และ screening mutations ที่พบทั้งหมดในกลุ่มตัวอย่างทั้งหมด
4. การศึกษายีน *LRRK2* – การศึกษา common variant คือ p.R1628P ในกลุ่มตัวอย่างทั้ง
5. โครงการเพาะเลี้ยงเซลล์ตัวอ่อนผิวหนังของผู้ป่วยโรคพาร์กินสัน (ที่มี mutation ในยีน *parkin* และ *GBA*1) ร่วมกับคณะวิทยาศาสตร์ ม.มหิดลและสถาบันวิจัยจุฬาภรณ์ ได้ดำเนินการเพาะเลี้ยงเซลล์ผิวหนังของผู้ป่วยโรคพาร์คินสันทั้งหมด 20 ราย ตามเป้าหมาย งานในส่วนห้องปฏิบัติการณ์พันธุศาสตร์

เพื่อวิเคราะห์ DNA และ RNA เพื่อตรวจหา mutation ใน *parkin, GBA1* ในตัวอย่างเลือดและเซลล์ผิวหนังเพาะเลี้ยงได้ดำเนินการเสร็จสิ้นทั้งหมดแล้ว (งานในส่วนที่เหลือเป็นการศึกษาด้านชีวเคมีที่คณะวิทย์ฯและสถาบันวิจัยจุฬาภรณ์_เมื่องานวิจัยเสร็จสิ้นจะส่ง manuscript ให้สกว.อีกครั้ง)

6. การศึกษาภาพถ่ายสมองผู้ป่วยโรคพาร์กินสันด้วย magnetic resonance Imaging (MRI) ดำเนินการแล้วในผู้ป่วย 93 ราย (งานในส่วนวิเคราะห์ข้อมูลทางคลินิกทั้งหมดเสร็จสิ้นแล้ว ที่เหลือเป็นวิเคราะห์ข้อมูลดิบจาก MRI และข้อมูลทางสถิติโดยอาจารย์ทางประสาทรังสีวิทยา_เมื่องานวิจัยเสร็จสิ้นจะส่ง manuscript ให้สกว.อีกครั้ง)

ผลการทดลอง

วิเคราะห์ยีน Parkin เพื่อหา gene copy number variation ด้วยวิธี semi-quantitative fluorescently labeled multiplex PCR และ DHPLC ในผู้ป่วย early-onset Parkinson's disease จำนวน 108 ราย, late-onset Parkinson's disease 119 รายและกลุ่มควบคุม 102 ราย พบ heterozygous exon deletions ในผู้ป่วย 8 รายและ homozygous exon deletion ในผู้ป่วย 1 ราย และการศึกษา direct sequencing ในผู้ป่วย early-onset Parkinson's disease จำนวน 108 ราย, late-onset Parkinson's disease 119 รายและกลุ่มควบคุม 102 ราย พบว่ามี 5 point mutations (3 mutations เป็น novel mutations) แล้ว screen mutations ทั้งหมดที่พบด้วยวิธี RFLP ในผู้ป่วยและกลุ่มควบคุมที่เหลือทั้งหมด รวมแล้วพบ *parkin* point mutations ในผู้ป่วย 11 ราย สรุปรวมพบผู้ป่วยที่มี *parkin* mutations ทั้งหมด 20 รายจากผู้ป่วย 485 ราย ในจำนวนนี้มี 2 รายเป็น homozygous mutations ที่เหลือเป็น heterozygous mutations

หมายเหตุ โครงการนี้อยู่ระหว่างวิเคราะห์ข้อมูลทางสถิติและเขียนบทความ โดยวางแผนส่งบทความให้วารสาร **BMC Neurology (impact factor = 2.56)** เมื่อได้รับตีพิมพ์แล้วจะส่ง manuscript ให้สกว.อีกครั้ง

วิเคราะห์ยีน GBA ด้วยวิธี direct sequencing ในผู้ป่วย early-onset Parkinson's disease จำนวน 108 ราย, late-onset Parkinson's disease 100 ราย พบ heterozygous point mutations 6 mutations (4 new mutations: 2 missense, 1 frameshift, 1 splice-site mutation และ 2 previously reported mutations) ในผู้ป่วย 17 ราย ได้วิเคราะห์ RNA ใน new splice-site mutation ที่พบและ screen mutations ที่พบในคนไข้ (277 ราย) และกลุ่มควบคุม (420 ราย) ที่เหลือทั้งหมดด้วยวิธี RFLP สรุปรวมแล้วพบ *GBA* point mutations ในผู้ป่วย 24 ราย จากผู้ป่วย 485 ราย

รายละเอียดของโครงการดังในภาคผนวก ในต้นฉบับ manuscript ที่ส่งเพื่อตีพิมพ์ในวารสาร **Parkinsonism and Related Disorders (impact factor = 3.274)** ได้รับการตอบรับตีพิมพ์ (16 มิย. 2557) แล้วจะส่งต้นฉบับให้สกว.อีกครั้ง

วิเคราะห์ *LRRK2* p.R1628P variant ในผู้ร่วมวิจัยทั้งหมด ได้คำนวน sample size ควรจะใช้กลุ่มตัวอย่างกลุ่มละ 472 ราย (ศึกษาจริงมากกว่า 480 คนต่อกลุ่ม) พบว่ายืนยันความสัมพันธ์ของ risk

variant นี้กับการเกิดโรคพาร์กินสัน และได้วิเคราะห์ความสัมพันธ์กับลักษณะทางคลินิกไว้ เป็นรายงานที่มีผู้ป่วยโรคพาร์กินสันที่เป็นพาหะของ *LRRK2* p.R1628P มากที่สุดที่เคยรายงานลักษณะทางคลินิก

รายละเอียดของโครงการดังในภาคผนวก ในต้นฉบับ manuscript ที่ส่งเพื่อตีพิมพ์ในวารสาร **Parkinsonism and Related Disorders (impact factor = 3.274)** อยู่ในระหว่างพิจารณา revised manuscript (minor changes) เมื่อได้รับตีพิมพ์แล้วจะส่งต้นฉบับให้สกว.อีกครั้ง

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 **Pulkes T**, Choubtum L, Chitphuk S, Thakkinstian A, Pongpakdee S, Kulkantrakorn K, Hanchaiphiboolkul S, Tiamkao S, Boonkongchuen P. Glucocerebrosidase mutations in Thai patients with Parkinson's disease. Parkinsonism and Related Disorders 2014 (in press)

1.2 **Pulkes T**, Papsing C, Thakkinstian A, Pongpakdee S, Kulkantrakorn K, Hanchaiphiboolkul S, Tiamkao S, Boonkongchuen P. Confirmation of the association between LRRK2 R1628P variant and susceptibility to Parkinson's disease in the Thai population. Parkinsonism and Related Disorders 2014 (under review_revised version)

1.3 **Pulkes T**, Papsing C, Taweewongsounton A, Arj-ong Vallipakorn S, Pongpakdee S, Kulkantrakorn K, Hanchaiphiboolkul S, Tiamkao S, Boonkongchuen P. Parkin mutations in Thai patients with Parkinson's disease. BMC Neurology 2014 (manuscript preparation)

2. การนำผลงานวิจัยไปใช้ประโยชน์

เชิงวิชาการ

- ได้เผยแพร่ข้อมูลงานวิจัยทั้งในการประชุมระดับชาติและระดับนานาชาติ อีกทั้งได้เตรียมบทความเพื่อตีพิมพ์ในวารสารระดับนานาชาติ 3 เรื่อง โดย 1 บทความได้รับการตอบรับตีพิมพ์ (16 มิย. 2557) อีก 1 บทความอยู่ระหว่างการพิจารณาขั้นสุดท้าย (minor revision) และอีก 1 บทความอยู่ระหว่างการเขียนบทความ
- มีการสร้างนักวิจัยใหม่ในทีมเป็นนักวิทยาศาสตร์ 3 คน มีการใช้เทคนิกที่ทางห้องปฏิบัติการไม่เคยใช้มาก่อน ได้แก่ DHPLC, Fluoresent multiplex PCR, Quantitative multiplex real-time PCR, RNA study ซึ่งขณะนี้นักวิทยาศสตร์ใหม่ในทีมเหล่านี้ได้เริ่มโครงการที่แต่ละคนรับผิดชอบเป็น first author และผู้รับทุนเป็น principal investigator ได้แก่ โครงการศึกษาโรค Charcot-Marie-Tooth, Spinocerebellar ataxias และ CADASIL syndrome
- มีการใช้ข้อมูลไปพัฒนาการเรียนการสอนระดับแพทย์เฉพาะทางภายในคณะแพทย์

ภาคผนวก

Manuscripts

Your Submission

ees.parkreldis.0.29ee80.bb978ed0@eesmail.elsevier.com on behalf of scalne@mail.ubc.ca
Sent: Monday, June 16, 2014 4:17 AM
To: Teeratorn Pulkes

Ms. Ref. No.: PARKRELDIS-D-14-00265R2
Title: Glucocerebrosidase mutations in Thai patients with Parkinson's disease
Parkinsonism & Related Disorders

Dear Dr. Pulkes,

I am pleased to inform you that your paper "Glucocerebrosidase mutations in Thai patients with Parkinson's disease" has been accepted for publication in Parkinsonism & Related Disorders.

Thank you for submitting your work to this journal.

With kind regards,

Zbigniew K Wszolek, M.D.
Editor-in-Chief
Parkinsonism & Related Disorders

Elsevier Editorial System(tm) for Parkinsonism & Related Disorders
Manuscript Draft

Manuscript Number: PARKRELDIS-D-14-00265R2

Title: Glucocerebrosidase mutations in Thai patients with Parkinson's disease

Article Type: Full Length Article

Keywords: Glucocerebrosidase; beta-glucosidase; early-onset Parkinson's disease; familial Parkinson's disease.

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Abstract: Background: GBA mutations are an important risk factor in developing Parkinson's disease (PD) worldwide. The study aimed to determine the frequency and clinical characteristics of GBA mutations in a Thai PD cohort of 480 patients and 395 control subjects.
Methods: Direct sequencing of GBA was performed in all early-onset PD patients (EOPD: n=108) and 100 PD patients with age at onset over 50 years (AAO>50y-PD). The study subsequently screened all identified mutations in the remaining AAO>50y-PD patients and all control subjects. Predictive factors associated with risk of developing PD were analyzed. Comparisons of clinical characteristics of PD patients with and without GBA mutations were also carried out.
Results: Heterozygous GBA mutations were identified in 24 patients (5%) and 2 controls (0.5%). Seven identified GBA point mutations comprised p.L444P, p.N386K, p.P428S, IVS2+1G>A, IVS9+3G>C, IVS10-9_10GT>AG and c.1309delG, of which five mutations were novel. Multiple logistic regression analysis revealed that GBA mutations were more frequent in EOPD than AAO>50y-PD groups (OR=4.64, P<0.022). Patients with GBA mutations had mean age at onset (43.1±10.2, mean±standard deviation) earlier than patients without GBA mutations (54.4±13.9, P=0.002). The patients with GBA mutations also had a more rapid progressive course, in which they were more likely to have higher Hoehn and Yahr staging (OR=4.20, P=0.006) and slightly lower means of Schwab-England ADL score [74.1±17.1 vs 81.0±18.08 (OR=0.98, 95%CI=0.96-1.01, P=0.162)].
Conclusion: GBA mutations are an important risk of PD in the Thai population. Patients having the mutations are likely to have early onset and may exhibit more rapid motor progression.

Author Declaration

Parkinsonism & Related Disorders is committed to proper scientific conduct and the protection of animal and human research subjects. Submission of this manuscript implies compliance with the following ethical requirements. Please affirm that you are representing all of the authors in stating compliance with these policies by checking the box at the end of this section.

1. Studies with human subjects must have been conducted in accordance with the Declaration of Helsinki. All persons must have provided informed consent prior to being included in the study.

2. Studies with animal subjects must have been conducted in accordance with the Guide for the Care and Use of Laboratory Subjects as adopted by the US National Institutes of Health and/or according to the requirements of all applicable local, national and international standards.

3. Protocols with animal or human subjects must have been approved by the relevant local committee(s) charged with ensuring subject protection. Studies that entail pain or distress will be assessed in terms of the balance between the distress inflicted and the likelihood of benefit.

4. The authors declare that the manuscript is original, that it is not being considered for publication elsewhere, and that it will not be submitted elsewhere while still under consideration for Parkinsonism & Related Disorders or after it has been accepted by Parkinsonism & Related Disorders.

5. All authors have seen and approved the manuscript in the form submitted to the journal. The authors declare that they have conformed to the highest standards of ethical conduct in the submission of accurate data and that they acknowledge the work of others when applicable.

6. All sources of financial support for the work have been declared in the Acknowledgements section of the manuscript. Any additional conflicts of interest must also be declared. Please include declarations of any consultancy or research funding received from relevant companies from three years prior to performance of the research until the time of manuscript submission. If the research is supported by internal funds, that should be stated as well.

To indicate compliance with the preceding declaration and that you have obtained agreement from all of the authors of this paper to declare their compliance as well, please place an x here: _x_

In cases of uncertainty please contact an editor for advice.

***Cover Letter**

13rd June 2014

Dear Professor Wszolek,

Re: "Glucocerebrosidase mutations in Thai patients with Parkinson's disease"

Thank you very much for reviewing and a valuable suggestion for the above manuscript. We have revised the manuscript responding to the issues addressed by the reviewer. I would be most grateful if you would consider the revised manuscript for publication as 'Full-length Article' in Parkinsonism & Related Disorders. All authors have read the revised manuscript.

Thank you for considering our work for publication.

Yours sincerely,
Asso. Prof. Teeratorn Pulkes MD, PhD (London)
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Response to reviewers

We would like to thank the reviewer for the careful reading of our manuscript and thoughtful comments and suggestions. We will respond to the comments on a point by point basis. We have included the reviewer's comments in regular font and our responses in bold type. We also highlighted all the changes in the manuscript in red color.

Reviewer's comments:

The authors have addressed my major concerns and I have only a few minor comments.

1. Introduction Page 1

On Line 31 the authors state that Parkinsonism is a major clinical manifestation of Gaucher's disease, I think this inaccurate and should be clarified.

This point is appreciated. We changed the sentence to "Parkinsonism has been increasingly observed as one of the neurological manifestation in neuropathic Gaucher's disease."

2. On Line 58 The authors state GBA mutations account for 3-9% in PD patients but the word account is not correct as the GBA mutations are merely a risk factor, this should be reworded.

This point is appreciated. We changed the sentence to "Overall, the heterozygous GBA mutations were identified as risk factors of PD in about 3-9% of the patients."

The word 'whose' should be deleted in the first line of the Discussion.

This point is appreciated. We deleted the word "whose" in that sentence.

Highlights

- This is the first study on *GBA* mutations in Parkinson's disease (PD) in Thailand.
- The study was relatively large population including 480 patients and 395 control subjects.
- The study enrolled elderly controls in order to decrease a possibility to develop PD later.
- The study described two previous described and five novel *GBA* mutations in 24 PD patients.
- Heterozygous *GBA* mutations associated with early-onset PD, typical L-dopa responsive, but more rapid clinical course.

Glucocerebrosidase mutations in Thai patients with Parkinson's disease

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Word counts = 3,000 words, Abstract = 250 words, 4 tables, 2 supplementary tables

Abstract

Background: *GBA* mutations are an important risk factor in developing Parkinson's disease (PD) worldwide. The study aimed to determine the frequency and clinical characteristics of *GBA* mutations in a Thai PD cohort of 480 patients and 395 control subjects.

Methods: Direct sequencing of *GBA* was performed in all early-onset PD patients (EOPD: n=108) and 100 PD patients with age at onset over 50 years (AAO>50y-PD). The study subsequently screened all identified mutations in the remaining AAO>50y-PD patients and all control subjects. Predictive factors associated with risk of developing PD were analyzed. Comparisons of clinical characteristics of PD patients with and without *GBA* mutations were also carried out.

Results: Heterozygous *GBA* mutations were identified in 24 patients (5%) and 2 controls (0.5%). Seven identified *GBA* point mutations comprised p.L444P, p.N386K, p.P428S, IVS2+1G>A, IVS9+3G>C, IVS10-9_10GT>AG and c.1309delG, of which five mutations were novel. Multiple logistic regression analysis revealed that *GBA* mutations were more frequent in EOPD than AAO>50y-PD groups (OR=4.64, $P<0.022$). Patients with *GBA* mutations had mean age at onset (43.1±10.2, mean±standard deviation) earlier than patients without *GBA* mutations (54.4±13.9, $P=0.002$). The patients with *GBA* mutations also had a more rapid progressive course, in which they were more likely to have higher Hoehn and Yahr staging (OR=4.20, $P=0.006$) and slightly lower means of Schwab-England ADL score [74.1±17.1 vs 81.0±18.08 (OR=0.98, 95%CI=0.96-1.01, $P=0.162$)].

Conclusion: *GBA* mutations are an important risk of PD in the Thai population. Patients having the mutations are likely to have early onset and may exhibit more rapid motor progression.

Keywords: Glucocerebrosidase; beta-glucosidase; early-onset Parkinson’s disease; familial Parkinson's disease

Introduction

Glucocerebrosidase (GBA) is an important lysosomal enzyme responsible for hydrolyzing the ß-glucosidic linkage of glucosylceramide in the plasma membrane into glucosyl and ceramide. Deficiency of the enzyme activity causes the most common inherited lysosomal storage disease, Gaucher's disease. Human *GBA* gene is located on chromosome 1q21. Importantly, 16kb downstream to the *GBA* functional gene locates *GBA* pseudogene, in which about 96% of exons share homologous sequence to the functional *GBA* gene [1]. To date, hundreds of mutations including several complex recombinant alleles consisting of two or more mutations have been described in association with Gaucher's disease. Thus, frequent recombination events between the functional *GBA* gene and the highly homologous *GBA* pseudogene may explain the high number of recombinant alleles, and mutations identified in Gaucher's disease [2].

Parkinsonism has been increasingly observed as one of the neurological manifestation in neuropathic Gaucher's disease [3]. A study on brain samples of patients clinically or pathologically diagnosed with Parkinson's disease (PD) demonstrated that *GBA* mutations were more frequent in PD patients than in the general population [4]. The association between the heterozygous *GBA* mutations and PD was then further supported by a study on the Ashkenazi cohort showing that PD patients had significantly higher odds for carrying the common *GBA* mutations than patients having Alzheimer's disease and healthy subjects [5]. Subsequent studies in various ethnicities including Chinese, Caucasians of multiple origins, and Japanese have consistently confirmed the correlation especially in individuals with early onset [6-10]. *GBA* mutations such as L444P, N370S, R120W and IVS2+1G>A were generally common among multiple ethnicities [8]. However, each population appeared to have its own unique and rare mutations [8, 10, 11]. Overall, the heterozygous *GBA* mutations were identified as risk factors of

PD in about 3-9% of the patients. With the exception of the PD patients of Ashkenazi Jewish origin, in which the common N370S mutation is very prevalent, frequency of the PD patients having the heterozygous *GBA* mutations was much greater (up to 31%) [5].

There is increasing evidence of the importance of *GBA* mutations as one of the major risk factors of PD in various ethnicities, and no epidemiological or association data regarding *GBA* mutations and PD have been described to date in the Thai population. Furthermore, recognition of the specific *GBA* genotypes in association with PD in each specific population may be essential in order to diagnose PD cases at the very early stage of the disease, which in turn is one of the key factors for a successful neuroprotective therapy in the future. Therefore, we conducted the study in order to identify the frequency and the role of *GBA* mutations as the risk factors of developing PD, and their correlations with phenotypes in Thais.

Materials and methods

Patients and control subjects

All consecutive PD cases in the Neurology Clinic at the Ramathibodi Hospital and the Bhumibol Adulyadej Hospital were recruited during May 2008-October 2013. Other collaborating hospitals and institutes enrolled patients in only a one-year period. The research protocol was approved by the ethics committees from all collaborating hospitals. All participants provided both verbal and written informed consent prior to the enrollments. PD was diagnosed by using UK Parkinson's disease Brain Bank Criteria except allowed patients to have family history of PD [12]. Early-onset Parkinson's disease (EOPD) was defined as patients with age at onset≤50 years [13]. Thus, the patients having age at onset over 50 years (AAO>50y) would be categorized as AAO>50y-PD group. Clinical information comprehensively obtained from the patients.

The majority of the Thai population is Thai and Chinese in origin (~90% of the population), and none of the studied participants were ethnically other than Thai or Chinese. Most Chinese migrated to Thailand during the Chinese Civil War and World War II. Regarding the ethnic origins of the participants, they were therefore determined by asking about their pedigrees up to their grandparents.

In order to avoid the control subjects developing PD after the enrollment, the study tried to recruit elderly participants as old as possible, and keep the number of controls similar to the patient group. So controls were recruited by enrolling participants aged ≥65 years old, who have no signs of parkinsonism. Nevertheless, two of the controls developed signs and symptoms of PD afterward (at the age of 71 and 73 years), and they were excluded from the study. Medical conditions of the control subjects included hypertension, type 2 diabetes mellitus, dyslipidemia, cerebrovascular disease, epilepsy, hemifacial spasm, polymyositis, trigeminal neuralgia, cancers of breast, and colon, atrial fibrillation, coronary artery disease, chronic kidney disease, asthma, degenerative bone and joint diseases, depression and no underlying disease.

Sequencing analysis

Genomic DNA was extracted from peripheral blood leukocytes by phenol-chloroform method or using QIAGEN DNA purification kit (QIAGEN, CA, USA). All 11 exons and exon-intron boundaries of *GBA* were sequencing on both strands in all EOPD patients (n=108), and 100 patients of AAO>50y-PD group by using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, CA, USA) as previously described methods in order to avoid amplification of *GBA* pseudogene [14]. The PCR products were then loaded on the 3730XL DNA Analyzer and analyzed with the Sequence Analysis software v3.0 (Applied Biosystems, CA, USA).

Prediction of splice-site scores were analyzed by using NNSPLICE version 0.9 (http://www.fruitfly.org/seq_tools/splice.html) [15].

Screening for identified GBA mutations

The remaining samples of AAO>50y-PD group (n=274) and all of the control samples (n=396) were subsequently screened for all putative mutations identified from the sequencing analyses. Appropriated PCR, mismatch-PCR and restriction fragment length polymorphisms (RFLPs) were designed to screen these seven mutations (supplement table). All samples, which RFLP results suggested harbored the mutations, would then be confirmed the existence of mutations by direct sequencing.

Statistical analysis

Data was described by means, standard deviations (SD), and frequencies (%) for continuous and categorical data, respectively. Characteristic features of the participants among EOPD, AAO>50y-PD and control groups were compared using analysis of variances and chi-square tests for continuous and categorical data, respectively.

In order to evaluate the predictive factors for PD, analysis of all participants together, in which the methods of *GBA* genotyping were performed by different techniques, might result in some bias since individuals who were analyzed by only RFLPs might miss identifying some other unknown variants. Therefore a logistic regression model was applied to assess whether *GBA* mutations and other factors were associated with PD by comparisons between EOPD vs AAO>50y-PD groups (data from *GBA* sequencing), and AAO>50y-PD vs control groups (data from RFLPs). Odds ratio (OR) along with 95% confidence interval (95%CI) for each factor was then estimated by exponential of coefficient. A goodness of fit of the logistic model was subsequently assessed using Hosmer-Lemeshow goodness of fit test.

Clinical characteristics of patients carrying *GBA* mutations and non-carriers were compared using unpaired t test and Fisher exact test, where it was appropriated. All analyses were performed using STATA version 13.0 (Stata Corp. TX, USA). *P* value <0.05 was considered as statistically significant.

Results

Demographic data of the patient cohort and the control subjects are shown in table 1. We enrolled 108 (22.4%) Thai patients having EOPD, 374 (77.6%) patients having AAO>50y and 396 control subjects. Family history of PD was more frequent in the patients with EOPD (14.8%), and AAO>50y-PD (9.4%) than the control subjects (0.8%). Thai ethnicity was more common in PD than in the control group (*P*=0.009). The presence of the heterozygous *GBA* mutations was also most frequent in EOPD than AAO>50y-PD and control groups (*P*<0.001).

Identified GBA mutations

Sequencing analysis identified seven putative *GBA* mutations consisting of two common mutations (L444P and IVS2+1G>A) and five novel putative mutations (N386K, P428S, IVS9+3G>C, IVS10-9_10GT>AG and c.1309delG) in 17 out of 208 PD patients (table 2). All patients harbored heterozygous mutations. By RFLPs screening, further 7 out of 272 AAO>50y-PD patients (2.6%) and only 2 out of the 395 controls (0.5%) harbored those mutations. Of the 16 patients who carried L444P mutations, 10 had lone L444P mutation, 4 had complex alleles RecA456P (L444P and A456P), and 2 had complex alleles Rec*Nci*I (L444P, A456P and V460V).

The novel c.1309delG mutation in exon 9 was predicted to result in valine substitution by serine at the codon 437, and premature stop codon at the codon 443 leading to translation into abnormal shorter protein product (442 instead of normal 536 amino acids). The novel N386K

and P428S missense mutations were in the highly evolutionarily conserved position of the GBA protein among different species. Both novel splice-site mutations, IVS9+3G>C and IVS10-9_10GT>AG, were calculated to have low splice-site scores [0.79 (normal score = 0.99) and 0.54 (normal score = 0.94), respectively] implying that they might result in frame shifts, and translate to truncated protein products.

Other identified variants were considered to be polymorphisms. These variants included intron variants c.762-180A>G (rs762488), c.762-257C>T (rs2009578), c.1388+141A>G (rs28373017), c.1389-101C>T (rs426516), and two newly identified missense variants c.271A>T (S91C), and c.1093G>A (E365K). Both novel variants had similarly high minor allele frequencies in both patient and control groups.

Multivariate logistic regression analysis of predictive factors associated with the occurrence of PD

Multivariate logistic regression models were designed to investigate the predictive factors in association with PD by dividing data into 2 models (table 3). Heterozygous *GBA* mutations were associated with increased risk of developing early onset (OR=4.64, *P*=0.022). The *GBA* mutations were shown to be more prevalent in AAO>50y-PD compared to control groups under univariate analysis; however the association was not demonstrated in multivariate analysis. Male, Thai ethnic and family history of PD were all shown to be predictive factors of PD in both patient groups. Smoking appeared to be a protective factor of PD as previously described.

The study also analyzed the predictive factors of PD by using all data together (supplement table 2). Overall, the heterozygous *GBA* mutations were more prevalent in PD patients than control subjects (OR = 10.44, 95%CI=2.45-44.47, *P*<0.001). Multivariate analysis

revealed that *GBA* mutations were a predictive factor of risk of developing PD compared to control subjects especially in EOPD patients (OR=21.33, *P*<0.001).

Comparison of the clinical manifestations between PD patients with and without GBA mutations

Comparisons of clinical characteristics between PD patients having *GBA* mutations and the patients without *GBA* mutation are demonstrated in table 4. PD patients having *GBA* mutations tended to have age at onset before 50 years old (OR=5.53, 95%CI=2.38-12.83, P<0.001), and mean age at onset (43.1±10.2) was significantly earlier than PD patients without *GBA* mutations (54.4±13.9;*P*=0.002). Furthermore, the clinical staging score appeared to be worst in the carriers of *GBA* mutations than non-carriers, while patients in both groups had similar disease duration, and duration of L-dopa treatment (*P*>0.05). Patients having *GBA* mutation also had L-dopa induced dyskinesia more often than the other group without *GBA* mutation (*P*=0.006). However, clinical signs of PD were undistinguishable between both groups reflecting that the PD patients having *GBA* mutations all had typical clinical presentations of PD with excellence response to L-dopa. Family history of PD tended to be more frequent in carriers of *GBA* mutation than non-carriers (24% vs 9%), although it was not significant.

Discussion

Twenty-four out of the 482 PD patients harbored heterozygous *GBA* mutations, ~~whose~~ their frequency was clearly greater than in control subjects (2/396;*P*<0.0001). This data strongly supports the contention that heterozygous *GBA* mutations are important risk factors of PD in Thais. The most common mutation was L444P accounting for 58% of all cases carrying heterozygous *GBA* mutant alleles. The L444P mutation is likely to originate from various founders because it was associated with three different recombinant alleles. Recent haplotype

analysis of Thai patients with Gaucher's disease also showed that at least four different haplotypes were observed in those L444P carriers [16]. The L444P mutation has been shown to be highly prevalent in other East Asian ethnic groups and Caucasians worldwide. Unlike the well-known N370S mutation, the most common *GBA* mutation in Ashkenazi Jews and Caucasians of various origins, were apparently absent in Thais and other East Asian populations [5, 6, 8, 9, 17-19]. IVS2+1G>A mutation previously associated with Gaucher's disease, and it was also described in Gaucher's disease of Korean origin, but not previously in other East Asian ethnic groups including Thai, Chinese and Japanese suggesting the rarity of the IVS2+1G>A in East Asian populations [3, 16, 20]. Furthermore, to our knowledge, this is the first time evidence has been provided that IVS2+1G>A is associated with PD. Other common mutations including R120W, D409H, and R496C/H, which were described in PD patients of East Asian populations, were not observed in the Thai study [6, 7, 9, 18, 20]. Five novel *GBA* mutations were identified in the study. In addition to the data of two other known and four unique *GBA* mutations earlier described in Thai patients having Gaucher's disease [3, 16] reveals how widely genetic heterogeneity of *GBA* in association with PD, and Gaucher's disease, which may result from high rate of recombination between functional *GBA* and its pseudogene [1].

Although, the PD patients having *GBA* mutations exhibited typical characteristics of PD, they appeared to have earlier onset and develop worst clinical scores than PD patients without *GBA* mutation. The findings of early age at onset of PD [5-8, 21], and poorer motor progression [17, 19, 22, 23] in the PD patients with *GBA* mutations are comparable with previous studies on the other ethnic groups. In the study, all identified mutations in PD patients with AAO>50y were also carried by EOPD patients. In contrast, some mutations identified in EOPD patients were absent in the AAO>50y-PD group. These data emphasized the significance of the *GBA*

mutations particularly in EOPD patients [11, 17]. However, some studies did not observe the association of *GBA* mutations with earlier age at onset [13, 19, 24, 25]. The reasons for differences in the observations among various studies may be due to factors such as different methods in participant selection, or categorized group of patients, a small sample sizes, frequencies of *GBA* mutations among different ethnics, methodologies for genetic screening, mutations in other susceptible genes apart from *GBA* gene, and different severity of defects by the different *GBA* mutations, for example in Gaucher's disease, homozygous L444P mutation is almost invariably associated with nervous system disorders, whereas N370S mutation is less related to the brain disease [26].

Most studies suggested that PD patients with *GBA* mutations had typical L-dopa responsive PD. It is difficult to clinically distinguish PD patients with *GBA* mutations from the patients without *GBA* mutation. Apart from age at onset and more progressive motor outcome, family history of PD might give some clue suggesting the underlying genetic mutations [19, 27, 28]. The study revealed that PD patients having *GBA* mutations had a tendency to have a higher frequency of positive family history than in the patients without *GBA* mutation, and non-PD subjects consistent with previous reports [27]. In fact, this finding is not so surprising since *GBA* mutations are predicted to be inherited by autosomal dominant fashion with age-dependent incomplete penetrance [23, 27, 29]. Recent studies demonstrated that PD patients with *GBA* mutations tended to develop earlier cognitive decline, dementia and psychosis more often than patients without *GBA* mutation [23, 27]. Furthermore, *GBA* mutations were recently shown to be a risk factor of Lewy body disease [30]. Therefore, neurotoxicity results from *GBA* mutations appear to closely relate to the occurrence of Lewy body in the nervous system. Unfortunately, the study was not designed to investigate the neuropsychiatric aspect. Only 14 of the 24 patients

with *GBA* mutations have still been followed up at the Ramathibodi Hospital. So far, two patients, aged 49 and 66 years old, had marked visual hallucination apparently related to dosage of L-dopa (one also have cognitive decline). Both of them have suffered from PD for 9 years, and they have experienced hallucination for a few years. Three patients (21%) had recent memory impairment or dementia. They are currently 66, 77 and 90 year old with the disease duration of 4 to 15 years. Although, cognitive impairment may be relatively common in this small group of patients, all of these patients appeared to develop dementia at the elderly period. Age or other factors may also influence the risk of cognitive impairment in these patients. This issue merits a further study in a larger sample size.

The study had some limitations. Firstly, the study was undertaken in referral hospitals, and university-based hospitals, thus participants might not represent the general Thai population. Secondly, it was not an age-matched case-control study. So some identified disease-associated factors including sex and ethnic origins may have incorrectly shown significant correlations resulting from selection bias (table 1). However, it should logically not influence the significance of such factors as family history of PD and the presence of *GBA* mutations. The other important pitfall as mentioned in the previous section is that a large proportion of the AAO>50y-PD group and all control subjects were analyzed by RFLPs, thus the study might underestimate the presence of rare *GBA* mutations or variants due to a limitation of funding. However, the error of the detection of other rare *GBA* mutations should be minimal. It is very likely that L444P is by far the most prevalent *GBA* mutation in the Thai population regarding the study and the others [3, 16]. The L444P mutation was still very rare in the Thai control subjects (1/395). Finally, although the study was a relatively large cohort, the numbers of patients with *GBA* mutations

were still rather small. This cohort may not be large enough to have a power to demonstrate some clinical correlations with the mutation such as motor complications.

In conclusion, this is the first study providing evidence of *GBA* mutations as an important risk of PD in Thailand. Although L444P mutation is common in Thai PD patients having *GBA* mutations, over 40% of cases associated with other rare mutations. Therefore the strategy to identify *GBA* mutations in patients at risk is not only to screen specific mutations in a certain population by gathering data from a large ethnic-matched cohort, but it may be necessary to perform sequencing of the whole exons and exon-intron boundaries of *GBA* especially in EOPD and familial PD.

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Conflict of interest:

All authors declare no conflict of interest.

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Glucocerebrosidase mutations in Thai patients with Parkinson's disease

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Abstract

Background: *GBA* mutations are an important risk factor in developing Parkinson's disease (PD) worldwide. The study aimed to determine the frequency and clinical characteristics of *GBA* mutations in a Thai PD cohort of 480 patients and 395 control subjects.

Methods: Direct sequencing of *GBA* was performed in all early-onset PD patients (EOPD: n=108) and 100 PD patients with age at onset over 50 years (AAO>50y-PD). The study subsequently screened all identified mutations in the remaining AAO>50y-PD patients and all control subjects. Predictive factors associated with risk of developing PD were analyzed. Comparisons of clinical characteristics of PD patients with and without *GBA* mutations were also carried out.

Results: Heterozygous *GBA* mutations were identified in 24 patients (5%) and 2 controls (0.5%). Seven identified *GBA* point mutations comprised p.L444P, p.N386K, p.P428S, IVS2+1G>A, IVS9+3G>C, IVS10-9_10GT>AG and c.1309delG, of which five mutations were novel. Multiple logistic regression analysis revealed that *GBA* mutations were more frequent in EOPD than AAO>50y-PD groups (OR=4.64, $P<0.022$). Patients with *GBA* mutations had mean age at onset (43.1±10.2, mean±standard deviation) earlier than patients without *GBA* mutations (54.4±13.9, $P=0.002$). The patients with *GBA* mutations also had a more rapid progressive course, in which they were more likely to have higher Hoehn and Yahr staging (OR=4.20, $P=0.006$) and slightly lower means of Schwab-England ADL score [74.1±17.1 vs 81.0±18.08 (OR=0.98, 95%CI=0.96-1.01, $P=0.162$)].

Conclusion: *GBA* mutations are an important risk of PD in the Thai population. Patients having the mutations are likely to have early onset and may exhibit more rapid motor progression.

Keywords: Glucocerebrosidase; beta-glucosidase; early-onset Parkinson’s disease; familial Parkinson's disease

Introduction

Glucocerebrosidase (GBA) is an important lysosomal enzyme responsible for hydrolyzing the ß-glucosidic linkage of glucosylceramide in the plasma membrane into glucosyl and ceramide. Deficiency of the enzyme activity causes the most common inherited lysosomal storage disease, Gaucher's disease. Human *GBA* gene is located on chromosome 1q21. Importantly, 16kb downstream to the *GBA* functional gene locates *GBA* pseudogene, in which about 96% of exons share homologous sequence to the functional *GBA* gene [1]. To date, hundreds of mutations including several complex recombinant alleles consisting of two or more mutations have been described in association with Gaucher's disease. Thus, frequent recombination events between the functional *GBA* gene and the highly homologous *GBA* pseudogene may explain the high number of recombinant alleles, and mutations identified in Gaucher's disease [2].

Parkinsonism has been increasingly observed as one of the neurological manifestation in neuropathic Gaucher's disease [3]. A study on brain samples of patients clinically or pathologically diagnosed with Parkinson's disease (PD) demonstrated that *GBA* mutations were more frequent in PD patients than in the general population [4]. The association between the heterozygous *GBA* mutations and PD was then further supported by a study on the Ashkenazi cohort showing that PD patients had significantly higher odds for carrying the common *GBA* mutations than patients having Alzheimer's disease and healthy subjects [5]. Subsequent studies in various ethnicities including Chinese, Caucasians of multiple origins, and Japanese have consistently confirmed the correlation especially in individuals with early onset [6-10]. *GBA* mutations such as L444P, N370S, R120W and IVS2+1G>A were generally common among multiple ethnicities [8]. However, each population appeared to have its own unique and rare mutations [8, 10, 11]. Overall, the heterozygous *GBA* mutations were identified as risk factors of

PD in about 3-9% of the patients. With the exception of the PD patients of Ashkenazi Jewish origin, in which the common N370S mutation is very prevalent, frequency of the PD patients having the heterozygous *GBA* mutations was much greater (up to 31%) [5].

There is increasing evidence of the importance of *GBA* mutations as one of the major risk factors of PD in various ethnicities, and no epidemiological or association data regarding *GBA* mutations and PD have been described to date in the Thai population. Furthermore, recognition of the specific *GBA* genotypes in association with PD in each specific population may be essential in order to diagnose PD cases at the very early stage of the disease, which in turn is one of the key factors for a successful neuroprotective therapy in the future. Therefore, we conducted the study in order to identify the frequency and the role of *GBA* mutations as the risk factors of developing PD, and their correlations with phenotypes in Thais.

Materials and methods

Patients and control subjects

All consecutive PD cases in the Neurology Clinic at the Ramathibodi Hospital and the Bhumibol Adulyadej Hospital were recruited during May 2008-October 2013. Other collaborating hospitals and institutes enrolled patients in only a one-year period. The research protocol was approved by the ethics committees from all collaborating hospitals. All participants provided both verbal and written informed consent prior to the enrollments. PD was diagnosed by using UK Parkinson's disease Brain Bank Criteria except allowed patients to have family history of PD [12]. Early-onset Parkinson's disease (EOPD) was defined as patients with age at onset≤50 years [13]. Thus, the patients having age at onset over 50 years (AAO>50y) would be categorized as AAO>50y-PD group. Clinical information comprehensively obtained from the patients.

The majority of the Thai population is Thai and Chinese in origin (~90% of the population), and none of the studied participants were ethnically other than Thai or Chinese. Most Chinese migrated to Thailand during the Chinese Civil War and World War II. Regarding the ethnic origins of the participants, they were therefore determined by asking about their pedigrees up to their grandparents.

In order to avoid the control subjects developing PD after the enrollment, the study tried to recruit elderly participants as old as possible, and keep the number of controls similar to the patient group. So controls were recruited by enrolling participants aged ≥65 years old, who have no signs of parkinsonism. Nevertheless, two of the controls developed signs and symptoms of PD afterward (at the age of 71 and 73 years), and they were excluded from the study. Medical conditions of the control subjects included hypertension, type 2 diabetes mellitus, dyslipidemia, cerebrovascular disease, epilepsy, hemifacial spasm, polymyositis, trigeminal neuralgia, cancers of breast, and colon, atrial fibrillation, coronary artery disease, chronic kidney disease, asthma, degenerative bone and joint diseases, depression and no underlying disease.

Sequencing analysis

Genomic DNA was extracted from peripheral blood leukocytes by phenol-chloroform method or using QIAGEN DNA purification kit (QIAGEN, CA, USA). All 11 exons and exon-intron boundaries of *GBA* were sequencing on both strands in all EOPD patients (n=108), and 100 patients of AAO>50y-PD group by using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, CA, USA) as previously described methods in order to avoid amplification of *GBA* pseudogene [14]. The PCR products were then loaded on the 3730XL DNA Analyzer and analyzed with the Sequence Analysis software v3.0 (Applied Biosystems, CA, USA).

Prediction of splice-site scores were analyzed by using NNSPLICE version 0.9 (http://www.fruitfly.org/seq_tools/splice.html) [15].

Screening for identified GBA mutations

The remaining samples of AAO>50y-PD group (n=274) and all of the control samples (n=396) were subsequently screened for all putative mutations identified from the sequencing analyses. Appropriated PCR, mismatch-PCR and restriction fragment length polymorphisms (RFLPs) were designed to screen these seven mutations (supplement table). All samples, which RFLP results suggested harbored the mutations, would then be confirmed the existence of mutations by direct sequencing.

Statistical analysis

Data was described by means, standard deviations (SD), and frequencies (%) for continuous and categorical data, respectively. Characteristic features of the participants among EOPD, AAO>50y-PD and control groups were compared using analysis of variances and chi-square tests for continuous and categorical data, respectively.

In order to evaluate the predictive factors for PD, analysis of all participants together, in which the methods of *GBA* genotyping were performed by different techniques, might result in some bias since individuals who were analyzed by only RFLPs might miss identifying some other unknown variants. Therefore a logistic regression model was applied to assess whether *GBA* mutations and other factors were associated with PD by comparisons between EOPD vs AAO>50y-PD groups (data from *GBA* sequencing), and AAO>50y-PD vs control groups (data from RFLPs). Odds ratio (OR) along with 95% confidence interval (95%CI) for each factor was then estimated by exponential of coefficient. A goodness of fit of the logistic model was subsequently assessed using Hosmer-Lemeshow goodness of fit test.

Clinical characteristics of patients carrying *GBA* mutations and non-carriers were compared using unpaired t test and Fisher exact test, where it was appropriated. All analyses were performed using STATA version 13.0 (Stata Corp. TX, USA). *P* value <0.05 was considered as statistically significant.

Results

Demographic data of the patient cohort and the control subjects are shown in table 1. We enrolled 108 (22.4%) Thai patients having EOPD, 374 (77.6%) patients having AAO>50y and 396 control subjects. Family history of PD was more frequent in the patients with EOPD (14.8%), and AAO>50y-PD (9.4%) than the control subjects (0.8%). Thai ethnicity was more common in PD than in the control group (*P*=0.009). The presence of the heterozygous *GBA* mutations was also most frequent in EOPD than AAO>50y-PD and control groups (*P*<0.001).

Identified GBA mutations

Sequencing analysis identified seven putative *GBA* mutations consisting of two common mutations (L444P and IVS2+1G>A) and five novel putative mutations (N386K, P428S, IVS9+3G>C, IVS10-9_10GT>AG and c.1309delG) in 17 out of 208 PD patients (table 2). All patients harbored heterozygous mutations. By RFLPs screening, further 7 out of 272 AAO>50y-PD patients (2.6%) and only 2 out of the 395 controls (0.5%) harbored those mutations. Of the 16 patients who carried L444P mutations, 10 had lone L444P mutation, 4 had complex alleles RecA456P (L444P and A456P), and 2 had complex alleles Rec*Nci*I (L444P, A456P and V460V).

The novel c.1309delG mutation in exon 9 was predicted to result in valine substitution by serine at the codon 437, and premature stop codon at the codon 443 leading to translation into abnormal shorter protein product (442 instead of normal 536 amino acids). The novel N386K

and P428S missense mutations were in the highly evolutionarily conserved position of the GBA protein among different species. Both novel splice-site mutations, IVS9+3G>C and IVS10-9_10GT>AG, were calculated to have low splice-site scores [0.79 (normal score = 0.99) and 0.54 (normal score = 0.94), respectively] implying that they might result in frame shifts, and translate to truncated protein products.

Other identified variants were considered to be polymorphisms. These variants included intron variants c.762-180A>G (rs762488), c.762-257C>T (rs2009578), c.1388+141A>G (rs28373017), c.1389-101C>T (rs426516), and two newly identified missense variants c.271A>T (S91C), and c.1093G>A (E365K). Both novel variants had similarly high minor allele frequencies in both patient and control groups.

Multivariate logistic regression analysis of predictive factors associated with the occurrence of PD

Multivariate logistic regression models were designed to investigate the predictive factors in association with PD by dividing data into 2 models (table 3). Heterozygous *GBA* mutations were associated with increased risk of developing early onset (OR=4.64, *P*=0.022). The *GBA* mutations were shown to be more prevalent in AAO>50y-PD compared to control groups under univariate analysis; however the association was not demonstrated in multivariate analysis. Male, Thai ethnic and family history of PD were all shown to be predictive factors of PD in both patient groups. Smoking appeared to be a protective factor of PD as previously described.

The study also analyzed the predictive factors of PD by using all data together (supplement table 2). Overall, the heterozygous *GBA* mutations were more prevalent in PD patients than control subjects (OR = 10.44, 95%CI=2.45-44.47, *P*<0.001). Multivariate analysis

revealed that *GBA* mutations were a predictive factor of risk of developing PD compared to control subjects especially in EOPD patients (OR=21.33, $P<0.001$).

Comparison of the clinical manifestations between PD patients with and without GBA mutations

Comparisons of clinical characteristics between PD patients having *GBA* mutations and the patients without *GBA* mutation are demonstrated in table 4. PD patients having *GBA* mutations tended to have age at onset before 50 years old (OR=5.53, 95%CI=2.38-12.83, P<0.001), and mean age at onset (43.1±10.2) was significantly earlier than PD patients without *GBA* mutations (54.4±13.9;$P=0.002$). Furthermore, the clinical staging score appeared to be worst in the carriers of *GBA* mutations than non-carriers, while patients in both groups had similar disease duration, and duration of L-dopa treatment ($P>0.05$). Patients having *GBA* mutation also had L-dopa induced dyskinesia more often than the other group without *GBA* mutation ($P=0.006$). However, clinical signs of PD were undistinguishable between both groups reflecting that the PD patients having *GBA* mutations all had typical clinical presentations of PD with excellence response to L-dopa. Family history of PD tended to be more frequent in carriers of *GBA* mutation than non-carriers (24% vs 9%), although it was not significant.

Discussion

Twenty-four out of the 482 PD patients harbored heterozygous *GBA* mutations, their frequency was clearly greater than in control subjects (2/396;$P<0.0001$). This data strongly supports the contention that heterozygous *GBA* mutations are important risk factors of PD in Thais. The most common mutation was L444P accounting for 58% of all cases carrying heterozygous *GBA* mutant alleles. The L444P mutation is likely to originate from various founders because it was associated with three different recombinant alleles. Recent haplotype analysis of Thai patients

with Gaucher's disease also showed that at least four different haplotypes were observed in those L444P carriers [16]. The L444P mutation has been shown to be highly prevalent in other East Asian ethnic groups and Caucasians worldwide. Unlike the well-known N370S mutation, the most common *GBA* mutation in Ashkenazi Jews and Caucasians of various origins, were apparently absent in Thais and other East Asian populations [5, 6, 8, 9, 17-19]. IVS2+1G>A mutation previously associated with Gaucher's disease, and it was also described in Gaucher's disease of Korean origin, but not previously in other East Asian ethnic groups including Thai, Chinese and Japanese suggesting the rarity of the IVS2+1G>A in East Asian populations [3, 16, 20]. Furthermore, to our knowledge, this is the first time evidence has been provided that IVS2+1G>A is associated with PD. Other common mutations including R120W, D409H, and R496C/H, which were described in PD patients of East Asian populations, were not observed in the Thai study [6, 7, 9, 18, 20]. Five novel *GBA* mutations were identified in the study. In addition to the data of two other known and four unique *GBA* mutations earlier described in Thai patients having Gaucher's disease [3, 16] reveals how widely genetic heterogeneity of *GBA* in association with PD, and Gaucher's disease, which may result from high rate of recombination between functional *GBA* and its pseudogene [1].

Although, the PD patients having *GBA* mutations exhibited typical characteristics of PD, they appeared to have earlier onset and develop worst clinical scores than PD patients without *GBA* mutation. The findings of early age at onset of PD [5-8, 21], and poorer motor progression [17, 19, 22, 23] in the PD patients with *GBA* mutations are comparable with previous studies on the other ethnic groups. In the study, all identified mutations in PD patients with AAO>50y were also carried by EOPD patients. In contrast, some mutations identified in EOPD patients were absent in the AAO>50y-PD group. These data emphasized the significance of the *GBA*

mutations particularly in EOPD patients [11, 17]. However, some studies did not observe the association of *GBA* mutations with earlier age at onset [13, 19, 24, 25]. The reasons for differences in the observations among various studies may be due to factors such as different methods in participant selection, or categorized group of patients, a small sample sizes, frequencies of *GBA* mutations among different ethnics, methodologies for genetic screening, mutations in other susceptible genes apart from *GBA* gene, and different severity of defects by the different *GBA* mutations, for example in Gaucher's disease, homozygous L444P mutation is almost invariably associated with nervous system disorders, whereas N370S mutation is less related to the brain disease [26].

Most studies suggested that PD patients with *GBA* mutations had typical L-dopa responsive PD. It is difficult to clinically distinguish PD patients with *GBA* mutations from the patients without *GBA* mutation. Apart from age at onset and more progressive motor outcome, family history of PD might give some clue suggesting the underlying genetic mutations [19, 27, 28]. The study revealed that PD patients having *GBA* mutations had a tendency to have a higher frequency of positive family history than in the patients without *GBA* mutation, and non-PD subjects consistent with previous reports [27]. In fact, this finding is not so surprising since *GBA* mutations are predicted to be inherited by autosomal dominant fashion with age-dependent incomplete penetrance [23, 27, 29]. Recent studies demonstrated that PD patients with *GBA* mutations tended to develop earlier cognitive decline, dementia and psychosis more often than patients without *GBA* mutation [23, 27]. Furthermore, *GBA* mutations were recently shown to be a risk factor of Lewy body disease [30]. Therefore, neurotoxicity results from *GBA* mutations appear to closely relate to the occurrence of Lewy body in the nervous system. Unfortunately, the study was not designed to investigate the neuropsychiatric aspect. Only 14 of the 24 patients

with *GBA* mutations have still been followed up at the Ramathibodi Hospital. So far, two patients, aged 49 and 66 years old, had marked visual hallucination apparently related to dosage of L-dopa (one also have cognitive decline). Both of them have suffered from PD for 9 years, and they have experienced hallucination for a few years. Three patients (21%) had recent memory impairment or dementia. They are currently 66, 77 and 90 year old with the disease duration of 4 to 15 years. Although, cognitive impairment may be relatively common in this small group of patients, all of these patients appeared to develop dementia at the elderly period. Age or other factors may also influence the risk of cognitive impairment in these patients. This issue merits a further study in a larger sample size.

The study had some limitations. Firstly, the study was undertaken in referral hospitals, and university-based hospitals, thus participants might not represent the general Thai population. Secondly, it was not an age-matched case-control study. So some identified disease-associated factors including sex and ethnic origins may have incorrectly shown significant correlations resulting from selection bias (table 1). However, it should logically not influence the significance of such factors as family history of PD and the presence of *GBA* mutations. The other important pitfall as mentioned in the previous section is that a large proportion of the AAO>50y-PD group and all control subjects were analyzed by RFLPs, thus the study might underestimate the presence of rare *GBA* mutations or variants due to a limitation of funding. However, the error of the detection of other rare *GBA* mutations should be minimal. It is very likely that L444P is by far the most prevalent *GBA* mutation in the Thai population regarding the study and the others [3, 16]. The L444P mutation was still very rare in the Thai control subjects (1/395). Finally, although the study was a relatively large cohort, the numbers of patients with *GBA* mutations

were still rather small. This cohort may not be large enough to have a power to demonstrate some clinical correlations with the mutation such as motor complications.

In conclusion, this is the first study providing evidence of *GBA* mutations as an important risk of PD in Thailand. Although L444P mutation is common in Thai PD patients having *GBA* mutations, over 40% of cases associated with other rare mutations. Therefore the strategy to identify *GBA* mutations in patients at risk is not only to screen specific mutations in a certain population by gathering data from a large ethnic-matched cohort, but it may be necessary to perform sequencing of the whole exons and exon-intron boundaries of *GBA* especially in EOPD and familial PD.

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Conflict of interest:

All authors declare no conflict of interest.

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Table(s)

Table 1 describes characteristics of subjects between Parkinson's disease groups

Characteristics	**EOPD (%) n=108**		**AAO>50y-PD (%) n=372**		**Control (%) n=395**		***P* value**
Age, year, mean (SD)	42.0	(7.1)	64.8	(8.1)	71.7	(7.6)	<0.001*
Sex, no (%)							
Male	56	(51.9)	202	(54.3)	153	(38.7)	<0.001*
Female	52	(48.1)	170	(45.7)	242	(61.3)	
Ethnicity, N (%)							
Thai	80	(74.1)	253	(68.0)	231	(58.8)	
Chinese	13	(11.1)	50	(13.4)	57	(14.5)	0.007*
Thai-Chinese	15	(14.8)	69	(18.6)	105	(27.0)	
Family history, N (%)							
Yes	16	(14.9)	35	(9.4)	3	(0.8)	<0.001*
No	92	(85.1)	337	(90.6)	392	(99.2)	
Smoking, N (%)							
Yes	14	(13.0)	59	(15.9)	75	(19.0)	0.230
No	94	(87.0)	313	(84.1)	320	(80.0)	
GBA mutation							
Yes	14	(13.0)	10	(2.7)	2	(0.5)	<0.001*
No	94	(87.0)	362	(97.3)	393	(99.5)	

Abbreviations as follow: PD = Parkinson's disease; EOPD = early-onset Parkinson's disease; AAO>50y-PD = Parkinson's disease with age at onset over 50 years; *GBA* = glucocerebrosidase gene; N = number

*Parameters are statistically difference among different groups ($P < 0.05$).

Table 2 Identified *GBA* mutations

Nucleotide substitution[a]	Protein[b]	Alelle name[c]	Location	EOPD		AAO>50y-PD		All PD		Controls	
				N	%	N	%	N	%	N	%
Known mutations											
c.1448T>C	p.Leu483Pro	L444P	Exon 10	8	7.4	7	1.9	15	3.1	1	0.3
IVS2+1G>A	skipping of exon 2		Intron 2	1	0.9	0	0	1	0.2	0	0
Novel mutations											
c.1275C>A	p.Asn425Lys	N386K	Exon 9	1	0.9	0	0	1	0.2	0	0
c.1399C>T	p.Pro467Ser	P428S	Exon 10	1	0.9	1	0.3	2	0.4	1	0.3
c.1309delG	premature stop at codon 443	V398fsX404	Exon 9	1	0.9	0	0	1	0.2	0	0
IVS9+3G>C	possible truncated protein		Intron 9	1	0.9	0	0	1	0.2	0	0
IVS10-9_10 GT>AG	possible truncated protein		Intron 10	1	0.9	2	0.5	3	0.6	0	0
Total				14	12.8	10	2.7	24	5.0	2	0.5

a cDNA sequence numbering is referred from GenBank reference sequence NM_001005741.2

b Protein names are based on the primary translation protein including 39-residue signal peptide.

c Allele names use the common published nomenclature, which are not include the 39-residue signal peptide.

Abbreviations as follow: PD = Parkinson's disease; EOPD = early-onset Parkinson's disease; AAO>50y-PD = Parkinson's disease with age at onset over 50 years

Table 3 Logistic regression analysis of factors associated with EOPD and PD with age at onset after 50 years.

Disease groups	Factors	Univariate analysis			Multivariate analysis		
		OR	***P***	**95%CI**	**OR**	***P***	**95%CI**
Sequencing data **EOPD (108) vs AAO>50y-PD (100)**	*GBA* mutation	4.82	0.016*	1.34-17.30	4.64	0.022*	1.25-17.16
	Male vs Female	0.66	0.141	0.38-1.15	0.67	0.200	0.37-1.23
	Thai vs Thai-Chinese	1.45	0.230	0.79-2.68	2.35	0.034*	1.06-5.18
	Chinese vs Thai-Chinese	1.20	0.671	0.52-2.73	1.88	0.246	0.65-5.48
	Family History vs none	2.72	0.045*	1.02-7.27	2.58	0.071	0.92-7.21
	Smoking vs non-smoking	0.60	0.173	0.28-1.26	0.65	0.303	0.29-1.47
RFLPs data **AAO>50y-PD (272) vs control (395)**	*GBA* mutation	5.19	0.041*	1.07-25.17	2.88	0.240	0.49-16.89
	Male vs Female	1.67	0.001*	1.22-2.28	2.38	<0.001*	1.66-3.43
	Thai vs Thai-Chinese	1.81	0.003*	1.22-2.68	1.74	0.009*	1.15-2.62
	Chinese vs Thai-Chinese	1.45	0.176	0.85-2.48	1.20	0.522	0.68-2.13
	Family History vs none	15.63	<0.001*	4.72-51.87	16.30	<0.001*	4.79-55.37
	Smoking vs non-smoking	0.70	0.104	0.46-1.07	0.46	0.002*	0.28-0.75

Abbreviations as follow: PD = Parkinson's disease; EOPD = early-onset Parkinson's disease; AAO>50y-PD = Parkinson's disease with age at onset over 50 years; *GBA* = glucocerebrosidase gene

*Parameters are statistically difference among different groups ($P < 0.05$).

Table 4 Association between *GBA* mutations and clinical characteristics of PD.

Clinical characteristics	*GBA* mutation		*P*	OR	95%CI
	Yes n = 17 (%)	No n = 191 (%)			
Age at onset (mean±SD)	43.1±10.2	54.4±13.9	0.002*	0.94	0.90-0.98
Disease duration (mean±SD)	7.4±4.6	6.1±4.7	0.283	1.05	0.96-1.15
Family history	4 (24)	18 (9)	0.088	2.96	0.87-10.02
Bradykinesia	17 (100)	189 (99)	1.000	-	-
Rigidity	16 (94)	187 (98)	0.350	0.34	0.04-3.25
Rest tremor	15 (88)	172 (90)	0.684	0.83	0.16-3.90
Postural instability	6 (35)	46 (24)	0.379	1.71	0.60-4.90
Unilateral onset	16 (94)	178 (93)	1.000	1.17	0.14-9.51
Persistent asymmetry	13 (76)	123 (64)	0.428	1.80	0.56-5.74
Progressive disorder	16 (94)	169 (88)	0.700	2.08	0.26-16.48
≥10-year duration	4 (24)	27 (14)	0.291	1.87	0.57-6.15
Excellence response to L-dopa	13 (76)	137 (72)	0.785	1.28	0.40-4.10
Dopa-responsive ≥5 years	11 (65)	73 (38)	0.040*	2.96	1.05-8.36
Hoehn and Yahr staging ≥ 3	11 (64)	58 (30)	0.006*	4.20	1.48-11.91
Schwab-England ADL score (mean±SD)	74.4±17.1	81.0±18.08	0.162	0.98	0.96-1.01
Duration of L-dopa treatment (months;median (range))	60 (0-204)	48 (0-240)	0.133	1.00	0.99-1.02
Wearing-off/on-off	8 (47)	82 (43)	0.801	1.18	0.44-3.19
Freezing	2 (12)	21 (11)	1.000	1.08	0.23-5.05
Dopa-induced Dyskinesia	7 (41)	24 (13)	0.006*	4.87	1.69-14.00

Abbreviations as follow: PD = Parkinson's disease; GBA = glucocerebrosidase gene; n = number; OR = odd ratio; 95%CI = 95% confidence interval; SD = standard deviation; ADL = activities of daily living

*Parameters are statistically difference among different groups (P < 0.05).

Optional E-Only Supplementary Files

Click here to download Optional E-Only Supplementary Files: Revised supplement table_GBA in Thai PD.docx

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Manuscript Draft

Manuscript Number: PARKRELDIS-D-14-00306R1

Title: Confirmation of the association between LRRK2 R1628P variant and susceptibility to Parkinson's disease in the Thai population

Article Type: Short Communication

Keywords: LRRK2; R1628P; Parkinson's disease; Early-onset Parkinson's disease

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Abstract: Objective: LRRK2 p.R1628P (c.4883G>C) is associated with Parkinson's disease (PD) in Chinese and Thais. However, some studies in other East Asian ethnic groups did not observe this association. Carriers of p.R1628P are about 3-5% Chinese and Thais. In contrast, Japanese, Koreans and Malays are much less prevalent (0-<1%). The contradictory results may be caused by insufficient sample sizes especially studies in ethnic groups with low prevalence, which, theoretically need a much larger sample size. We conducted a case-control Thai PD study with appropriate size in order to support the role of p.R1628P related to susceptibility to PD.
Methods: Estimated total sample size of 958 Thai subjects was needed. 485 PD patients and 480 controls were recruited. The p.R1628P was screened by RFLP and confirmed by direct sequencing. Clinical characteristics were compared between PD patients with and without p.R1628P.
Results: 54 PD patients (11%) and 29 control subjects (6%) carried p.R1628P. Multiple logistic regression analysis showed that GC and CC genotypes were significantly higher in PD patients than in controls (OR=1.81, 95%CI=1.10-2.97). The PD patients carrying p.R1628P had earlier age at onset (56±13 vs 60±12; P=0.021) and a more rapidly progressive course (P<0.001) than the patients carrying wild-type nucleotide.
Conclusions: We confirm the association between p.R1628P and risk of developing PD in the appropriated sample-sized cohort. Certain LRRK2 variants appear to be generally distributed among East Asians, however, in widely different frequencies. In order to study role of such variants in PD, it should be carefully estimated the appropriate sample size.

Author Declaration

Parkinsonism & Related Disorders is committed to proper scientific conduct and the protection of animal and human research subjects. Submission of this manuscript implies compliance with the following ethical requirements. Please affirm that you are representing all of the authors in stating compliance with these policies by checking the box at the end of this section.

1. Studies with human subjects must have been conducted in accordance with the Declaration of Helsinki. All persons must have provided informed consent prior to being included in the study.

2. Studies with animal subjects must have been conducted in accordance with the Guide for the Care and Use of Laboratory Subjects as adopted by the US National Institutes of Health and/or according to the requirements of all applicable local, national and international standards.

3. Protocols with animal or human subjects must have been approved by the relevant local committee(s) charged with ensuring subject protection. Studies that entail pain or distress will be assessed in terms of the balance between the distress inflicted and the likelihood of benefit.

4. The authors declare that the manuscript is original, that it is not being considered for publication elsewhere, and that it will not be submitted elsewhere while still under consideration for Parkinsonism & Related Disorders or after it has been accepted by Parkinsonism & Related Disorders.

5. All authors have seen and approved the manuscript in the form submitted to the journal. The authors declare that they have conformed to the highest standards of ethical conduct in the submission of accurate data and that they acknowledge the work of others when applicable.

6. All sources of financial support for the work have been declared in the Acknowledgements section of the manuscript. Any additional conflicts of interest must also be declared. Please include declarations of any consultancy or research funding received from relevant companies from three years prior to performance of the research until the time of manuscript submission. If the research is supported by internal funds, that should be stated as well.

To indicate compliance with the preceding declaration and that you have obtained agreement from all of the authors of this paper to declare their compliance as well, please place an x here: _x_

In cases of uncertainty please contact an editor for advice.

***Cover Letter**

7th June 2014

Dear Professor Tan,

Re: "Confirmation of the association between LRRK2 R1628P variant and susceptibility to Parkinson's disease"

Thank you very much for reviewing and a valuable suggestion for the above manuscript. We have revised the manuscript responding to the issues addressed by the reviewers. I would be most grateful if you would consider the revised manuscript for publication as 'Short Communications' in Parkinsonism & Related Disorders. All authors have read the revised manuscript.

Thank you for considering our work for publication.

Yours sincerely,
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Response to reviewers

We would like to thank the reviewer for the careful reading of our manuscript and thoughtful comments and suggestions. We will respond to the comments on a point by point basis. We have included the reviewer's comments in regular font and our responses in bold type. We also highlighted all the changes in the manuscript in red color.

Reviewer's comments:

Reviewer #1: This manuscript is a replication study focused on LRRK2 p.R1628P variant and development of Parkinson's disease (PD) using large cohort of Thai subjects. In addition, the authors examined the relationship between the clinical course of PD and this variant. The authors found LRRK2 p.1628P variant has a risk for developing PD among Thai patients. Interestingly, patients with this variant were earlier onset and progression of PD compared with non-carrier. I think this paper will be highly interesting to the field. Data and methodology are reasonable and the data quality is high.

Reviewer #2: The article of Pulkes et al. investigates the frequency of the LRRK2 R1628P variant in Thai subjects (485 patients with PD and 480 controls). This study follows on from their previous publication on the frequency of this variant in 154 patients with PD compared to 156 controls, and the relative frequencies and association are confirmed. In essence the study has the same findings as the previous smaller study but with eth larger sample size it allows a better comparison of the clinical features of affected carriers of R1628P versus non-carriers. I have only a couple of minor questions.

1) The authors should clarify if the 310 samples in the earlier study are also included within the 965 samples included in the present study.

We appreciated this point. All patients in the study were newly enrolled, and this point was not clearly stated in the manuscript. So we added the sentence as follow: All participants did not take part in our first study.[6]

2) Have previous studies of LRRK2 R1628P or G2385R carriers examined the clinical phenotype of carriers versus non-carriers, and if so how do the results compare?

We appreciated this point. Unfortunately we did not record details of clinical information in the first study. A large number of the patients of the Thammasat University in the first study were also lost to follow up because of the severe flooding in the Central part of Thailand in the year 2011 (the Thammasat university hospital had to close for several months during that period). The flooding also damaged most of the medical records there. Therefore we could not get the complete clinical data of the patients enrolled to the first study, and did not perform clinical analysis of the patients from the first study.

Editorial comment:

Please amend title to indicate confirmation of the risk variant in the Thai population.

This point is appreciated. We changed the title to "Confirmation of the association between *LRRK2* R1628P variant and susceptibility to Parkinson's disease in the Thai population"

Highlights

- We address the important of sample size and selected population studied in association study.
- We demonstrate an association between LRRK2 R1628P and Parkinson's disease in non-Chinese.
- We describe the clinical characteristic of a large group of PD patients carrying R1628P.
- The haplotype of *LRRK2* has no protective effect for PD in contrast to Taiwanese data.

Confirmation of the association between *LRRK2* R1628P variant and susceptibility to Parkinson's disease in the Thai population

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Short title: *LRRK2* R1628P associated with Parkinson's disease

Word counts: main text = 1,667 words, Abstract = 250 words, 2 tables, 12 references

Abstract

Objective: *LRRK2* p.R1628P (c.4883G>C) is associated with Parkinson's disease (PD) in Chinese and Thais. However, some studies in other East Asian ethnic groups did not observe this association. Carriers of p.R1628P are about 3-5% Chinese and Thais. In contrast, Japanese, Koreans and Malays are much less prevalent (0-<1%). The contradictory results may be caused by insufficient sample sizes especially studies in ethnic groups with low prevalence, which, theoretically need a much larger sample size. We conducted a case-control Thai PD study with appropriate size in order to support the role of p.R1628P related to susceptibility to PD.

Methods: Estimated total sample size of 958 Thai subjects was needed. 485 PD patients and 480 controls were recruited. The p.R1628P was screened by RFLP and confirmed by direct sequencing. Clinical characteristics were compared between PD patients with and without p.R1628P.

Results: 54 PD patients (11%) and 29 control subjects (6%) carried p.R1628P. Multiple logistic regression analysis showed that GC and CC genotypes were significantly higher in PD patients than in controls (OR=1.81, 95%CI=1.10-2.97). The PD patients carrying p.R1628P had earlier age at onset (56±13 vs 60±12; P=0.021) and a more rapidly progressive course (P<0.001) than the patients carrying wild-type nucleotide.

Conclusions: We confirm the association between p.R1628P and risk of developing PD in the appropriated sample-sized cohort. Certain *LRRK2* variants appear to be generally distributed among East Asians, however, in widely different frequencies. In order to study role of such variants in PD, it should be carefully estimated the appropriate sample size.

Keywords: LRRK2; R1628P; Parkinson's disease; Early-onset Parkinson's disease

Introduction

Various mutations and risk variants of *Leucine-rich repeat kinase 2* gene (*LRRK2*) (NM_198578.3) are commonly associated with familial and sporadic Parkinson's disease (PD) worldwide. Specific *LRRK2* variants often share only one or a few common founders causing carriers of each specific *LRRK2* variant to be distributed as a low minor alelle in only particular population [1]. P.G2385R (c.7153G>R; rs34778348) and p.R1628P (c.4883G>C; rs33949390) are the most common variants susceptible for developing to PD in East Asian populations accounting for 3-5% of the population [2-5]. Unlike the p.G2385R, the p.R1628P is not widely distributed in East Asia, it is highly prevalent in only individuals of Chinese and Thai ethnicities [5, 6], while almost absent in Japanese, Koreans and Malays [7-9]. The association between the p.G2385R and risk of developing PD were consistently replicated; however, there were some contrary results regarding p.R1628P. Two studies on PD patients of non-Chinese Asian origins showed no association between p.R1628P and PD [8, 9]. Subsequently, a recent large multicenter study on *LRRK2* exonic variants in Caucasian, East Asian and Arab-Berber cohorts confirmed only p.G2385R as the susceptible risk variant of PD, but the association with p.R1628P was not identified in the population studied [10]. Nevertheless, the study enrolled Asian individuals of Japanese, Korean and Taiwanese origins, in which Japanese and Koreans were the majority of the cohort (~70%). Similar to the previous data, the study found that p.R1628P was extremely rare in the participants of these two ethnic groups [10]. Thus, the findings of no association of p.R1628P and PD might result from an insufficient power of the studies that comprised a very low prevalence of p.R1628P in the studied populations, or too small sample sizes.

Our group previously described the association between p.R1628P and PD in a small cohort of Thai individuals, in which the study was only non-Chinese cohort observing the association [6]. The study showed that Thais had a relatively high prevalent of carriers of p.R1628P similar to Chinese (>3%). Moreover, all Thai carriers of p.R1628P had the same haplotype of *LRRK2* as Chinese suggesting the founder effect [6]. However, there were some contradictory data concerning p.R1628P as risk of developing PD [10], and our preliminary study had inadequate sample size [6]. Therefore, we conducted the study in order to ratify the role of the p.R1628P variant on susceptibility of PD in an appropriate sample-size, case-control study of Thai PD patients.

Materials and methods

Participants

The study recruited all PD cases in the Neurology Clinics at five collaborating hospitals and institute during May 2008-October 2013. All participants did not take part in our first study.[6] The research protocol was approved by the ethics committees from all collaborating hospitals and institute. All participants provided both verbal and written informed consent prior to the enrollments. PD was diagnosed by using UK Parkinson's disease Brain Bank Criteria except the study allowed patients to have a family history of PD [11]. Clinical features were comprehensively recorded. Control subjects were recruited by enrolling participants aged ≥65 years old, with no signs of parkinsonism.

Genetic analysis

Genomic DNA was extracted from peripheral blood samples by using QIAGEN DNA purification kit (QIAGEN, CA, USA) or phenol-chloroform method. Genotyping of the *LRRK2* c.4883G>C (p.R1628P) was carried out by mismatch PCR and RFLP and all the samples with

positive RFLP result were subjected to direct sequencing of exon 34 (p.R1628P, rs33949390; p.S1647T, rs11564148), and exon 49 (M2397T, rs3761863) by using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, CA, USA) as previously described [6]. The PCR products were then loaded on the 3730XL DNA Analyzer and analyzed with the Sequence Analysis software v3.0 (Applied Biosystems, CA, USA).

Sample size estimation

A sample size was estimated by setting type I, type II errors, and detected odds ratio (OR) of 5%, 20%, and 2, respectively. Frequency of LRRK2 minor C allele in Thais was 0.028 [6]. A total sample size for Fisher's exact test of 958 (479 for each group) was therefore required.

Statistical analysis

Data was described by means, standard deviations (SD), and frequencies (%) for continuous and categorical data, respectively. Demographic data and clinical characteristics of the PD patients carrying p.R1628P and without p.R1628P were compared by analysis of variances and chi-square tests for continuous and categorical data, respectively. A logistic regression model was applied to assess factors associated with PD. OR along with 95% confidence interval (95%CI) for each factor was then estimated by exponential of coefficient. A goodness of fit of the logistic model was subsequently assessed using Hosmer-Lemeshow goodness of fit test. All analyses were performed using STATA version 13.0. *P* value <0.05 was considered as statistically significant.

Results

485 PD patients and 480 control subjects were enrolled on the study. Mean age of the PD group (65±12, mean±SD) was less than the control group (71±7; *P*<0.001). Male to female ratio was significantly different between the two groups [P<0.001; PD = 46:54 (female: male); control

= 61:39]. Family history of PD was significantly more frequent in PD than control groups (11% vs 1%; *P*<0.001).

Frequencies of the p.R1628P variant

The study identified the p.R1628P in 54 PD patients (11%) and 29 control subjects (6%). All but one participant carrying c.4883G>C allele were heterozygous in both groups, and all carriers of the variant had S1647T-M2397T haplotype similar to our previous study [6]. The genotypes GC and CC were significantly more common in the PD group than in controls (OR=2.02, 95%CI=1.25-3.25, *P*=0.004). The C variant allele was also more frequent in the PD patients than controls (OR=1.86; 95%CI=1.18-2.93; P=0.006).

Multivariate logistic regression analysis of predictive factors associated with the occurrence of PD

A multivariate logistic regression model was performed to evaluate whether the p.R1628P was an independent factor associated with PD. Logistic regression analysis was demonstrated in table 1. The p.R1628P was associated with PD (OR=1.81, 95%CI=1.10-2.97). Male, ethnically Thai and a family history of PD were all identified to be risk factors of PD.

Comparison of the clinical manifestations between PD patients carrying LRRK2 p.R1628P and without the variant

Comparisons of clinical characteristics between PD patients having p.R1628P and the patients without p.R1628P are shown in table 2. PD patients carrying p.R1628P having a mean age at onset of 56.0±13.0 was significantly earlier than PD patients without p.R1628P (60.1±12.2; *P*<0.021) although patients in both groups had similar disease duration, and duration of L-dopa treatment. The Hoehn and Yahr staging, and the Schwab-England ADL score was poorer in the PD patient with p.R1628P than the patients without the variant (*P*=0.001 for both

clinical scores). Other clinical features of PD patients carrying p.R1628P and frequency of the positive family history of PD were not statistically different from other PD patients.

Discussion

The study strongly confirms that *LRRK2* p.R1628P play an important role as a risk of susceptibility to PD with OR of 1.8. The observed OR was similar to the studies of Han Chinese [1, 5]. So far, Thai has been the only non-Chinese population, in which the association was demonstrated. As mention earlier, the negative studies were likely to result from inadequate sample sizes in relation to a paucity of the variant in the population studied. Interestingly, Wu *et al*. recently described that a *LRRK2* p.S1647T-p.M2397T haplotype appeared to have a protective effect against PD in a large Taiwanese cohort [12]. Although the protective haplotype was not able to counteract with genetic effect on the PD risk of the p.G2385R, it seemed to decrease in risk of developing PD of the other carriers (OR=0.8, 95%CI=0.65-0.97) including carriers of the p.R1628P [12]. Our finding was quite different; all Thai carriers of the p.R1628P had p.S1647T-p.M2397T haplotype, and they were still more susceptible to develop PD than the non-carrier group. Both the Taiwanese and this study were likely to have adequate sample size. Therefore, other environmental or genetic factors possibly influence the genetic effect of the p.R1628P in these different populations. If the genetic factor is involved, *trans*-element rather than *cis*-element is more likely to alter the p.R1628P genetic effect since in all available data regarding the p.R1628P haplotype to date, all carriers are likely to have the same *LRRK2* haplotype [5, 6]. The other point is that *LRRK2* mutations such as p.G2019S are genetically transmitted with highly age-dependent penetrance [1]. Thus, a long-term study of the asymptomatic carriers of p.R1628P may be helpful to clarify this particular point.

The PD patients having p.R1628P had classical phenotype of PD (Table 2), although they had rather earlier onset than non-carriers. In contrast, most previous studies, in which the PD patients having *LRRK2* risk variant generally exhibited similar age at onset to the other PD patients. This study also observed that those PD patients with the p.R1628P developed slightly more rapid progressive course and the patients had slightly poorer activity daily living status than the non-carrier group. This finding needs to be elucidated since several other genetic factors may have influenced our study. If all or most susceptible genetic factors have been identified in the cohort, the genotype-phenotype correlation will be more reliable.

The study had some limitations. Firstly, the studied subjects might not represent the Thai PD population, since all collaborating hospitals were tertiary medical centers. Secondly, the study was designed to reduce a possibility that asymptomatic carriers had developed PD after the enrollment. So control subjects were selected over 65 years of age. Nonetheless, two control subjects developed PD after the enrollment and they were excluded from the study. Regarding this point, female sex and Thai ethnic groups may be incorrectly observed as having significant correlations resulting from selection bias. However, the association between the *LRRK2* genotype and risk of PD should rationally not be influenced by the bias. On the other hand, the relatively older age of the control subjects might in turn become a robust point of the study.

In conclusion, the study strongly supports that the *LRRK2* p.R1628P increases the risk of developing PD. The Thai PD patients carrying the p.R1628P had slightly earlier age at onset and poorer disease progression compared to the non-carriers.

Acknowledgement:

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acquisition of funding. We thank to Mr. Maurice M. Broughton for his help with English editing. This study was supported by the Thailand Research Fund (RSA5480019), the Research Potential Development grant of the Ramathibodi Hospital, Mahidol University and the Neurogenetics Fund (3001180) of Ramathibodi Hospital Foundation. The research protocol was approved by the ethic committees of Ramathibodi Hospital, Mahidol University (ID 03-53-18); Khon Kaen University (HE531154); Thammasat University (MTU-IM-9-CR046-046/53); Prasat Neurological Institute (54001); and Bhumibol Adulyadej Hospital.

Appendix: author roles

1. Teeratorn Pulkes: (1) Research project: A. Conception, B. Organization, C. Execution; (2) Statistical Analysis: Review and Critique; (3) Manuscript Preparation: A. Writing of the first draft
2. Chutima Papsing: (1) Research project: C. Execution (genetic analysis); (3) Manuscript Preparation: Read and Approve
3. Ammarin Thakkinstian: (2) Statistical Analysis: A. Design, B. Execution; (3) Manuscript Preparation: Read and Approve

4-7. Sunsanee Pongpakdee, Kongkiat Kulkantrakorn, Suchat Hanchaiphiboolkul, and Somsak Tiamkao: (1) Research project: C. Execution (collected patients' data and samples); (3) Manuscript Preparation: Read and Approve

8. Pairoj Boonkongchuen: (1) Research project: C. Execution (collected patients' data and samples); (3) Manuscript Preparation: Read and Approve

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Confirmation of the association between *LRRK2* R1628P variant and susceptibility to Parkinson's disease in the Thai population

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Abstract

Objective: *LRRK2* p.R1628P (c.4883G>C) is associated with Parkinson's disease (PD) in Chinese and Thais. However, some studies in other East Asian ethnic groups did not observe this association. Carriers of p.R1628P are about 3-5% Chinese and Thais. In contrast, Japanese, Koreans and Malays are much less prevalent (0-<1%). The contradictory results may be caused by insufficient sample sizes especially studies in ethnic groups with low prevalence, which, theoretically need a much larger sample size. We conducted a case-control Thai PD study with appropriate size in order to support the role of p.R1628P related to susceptibility to PD.

Methods: Estimated total sample size of 958 Thai subjects was needed. 485 PD patients and 480 controls were recruited. The p.R1628P was screened by RFLP and confirmed by direct sequencing. Clinical characteristics were compared between PD patients with and without p.R1628P.

Results: 54 PD patients (11%) and 29 control subjects (6%) carried p.R1628P. Multiple logistic regression analysis showed that GC and CC genotypes were significantly higher in PD patients than in controls (OR=1.81, 95%CI=1.10-2.97). The PD patients carrying p.R1628P had earlier age at onset (56±13 vs 60±12; P=0.021) and a more rapidly progressive course (P<0.001) than the patients carrying wild-type nucleotide.

Conclusions: We confirm the association between p.R1628P and risk of developing PD in the appropriated sample-sized cohort. Certain *LRRK2* variants appear to be generally distributed among East Asians, however, in widely different frequencies. In order to study role of such variants in PD, it should be carefully estimated the appropriate sample size.

Keywords: LRRK2; R1628P; Parkinson's disease; Early-onset Parkinson's disease

Introduction

Various mutations and risk variants of *Leucine-rich repeat kinase 2* gene (*LRRK2*) (NM_198578.3) are commonly associated with familial and sporadic Parkinson's disease (PD) worldwide. Specific *LRRK2* variants often share only one or a few common founders causing carriers of each specific *LRRK2* variant to be distributed as a low minor alelle in only particular population [1]. P.G2385R (c.7153G>R; rs34778348) and p.R1628P (c.4883G>C; rs33949390) are the most common variants susceptible for developing to PD in East Asian populations accounting for 3-5% of the population [2-5]. Unlike the p.G2385R, the p.R1628P is not widely distributed in East Asia, it is highly prevalent in only individuals of Chinese and Thai ethnicities [5, 6], while almost absent in Japanese, Koreans and Malays [7-9]. The association between the p.G2385R and risk of developing PD were consistently replicated; however, there were some contrary results regarding p.R1628P. Two studies on PD patients of non-Chinese Asian origins showed no association between p.R1628P and PD [8, 9]. Subsequently, a recent large multicenter study on *LRRK2* exonic variants in Caucasian, East Asian and Arab-Berber cohorts confirmed only p.G2385R as the susceptible risk variant of PD, but the association with p.R1628P was not identified in the population studied [10]. Nevertheless, the study enrolled Asian individuals of Japanese, Korean and Taiwanese origins, in which Japanese and Koreans were the majority of the cohort (~70%). Similar to the previous data, the study found that p.R1628P was extremely rare in the participants of these two ethnic groups [10]. Thus, the findings of no association of p.R1628P and PD might result from an insufficient power of the studies that comprised a very low prevalence of p.R1628P in the studied populations, or too small sample sizes.

Our group previously described the association between p.R1628P and PD in a small cohort of Thai individuals, in which the study was only non-Chinese cohort observing the association [6]. The study showed that Thais had a relatively high prevalent of carriers of p.R1628P similar to Chinese (>3%). Moreover, all Thai carriers of p.R1628P had the same haplotype of *LRRK2* as Chinese suggesting the founder effect [6]. However, there were some contradictory data concerning p.R1628P as risk of developing PD [10], and our preliminary study had inadequate sample size [6]. Therefore, we conducted the study in order to ratify the role of the p.R1628P variant on susceptibility of PD in an appropriate sample-size, case-control study of Thai PD patients.

Materials and methods

Participants

The study recruited all PD cases in the Neurology Clinics at five collaborating hospitals and institute during May 2008-October 2013. All participants did not take part in our first study.[6] The research protocol was approved by the ethics committees from all collaborating hospitals and institute. All participants provided both verbal and written informed consent prior to the enrollments. PD was diagnosed by using UK Parkinson's disease Brain Bank Criteria except the study allowed patients to have a family history of PD [11]. Clinical features were comprehensively recorded. Control subjects were recruited by enrolling participants aged ≥65 years old, with no signs of parkinsonism.

Genetic analysis

Genomic DNA was extracted from peripheral blood samples by using QIAGEN DNA purification kit (QIAGEN, CA, USA) or phenol-chloroform method. Genotyping of the *LRRK2* c.4883G>C (p.R1628P) was carried out by mismatch PCR and RFLP and all the samples with

positive RFLP result were subjected to direct sequencing of exon 34 (p.R1628P, rs33949390; p.S1647T, rs11564148), and exon 49 (M2397T, rs3761863) by using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, CA, USA) as previously described [6]. The PCR products were then loaded on the 3730XL DNA Analyzer and analyzed with the Sequence Analysis software v3.0 (Applied Biosystems, CA, USA).

Sample size estimation

A sample size was estimated by setting type I, type II errors, and detected odds ratio (OR) of 5%, 20%, and 2, respectively. Frequency of LRRK2 minor C allele in Thais was 0.028 [6]. A total sample size for Fisher's exact test of 958 (479 for each group) was therefore required.

Statistical analysis

Data was described by means, standard deviations (SD), and frequencies (%) for continuous and categorical data, respectively. Demographic data and clinical characteristics of the PD patients carrying p.R1628P and without p.R1628P were compared by analysis of variances and chi-square tests for continuous and categorical data, respectively. A logistic regression model was applied to assess factors associated with PD. OR along with 95% confidence interval (95%CI) for each factor was then estimated by exponential of coefficient. A goodness of fit of the logistic model was subsequently assessed using Hosmer-Lemeshow goodness of fit test. All analyses were performed using STATA version 13.0. *P* value <0.05 was considered as statistically significant.

Results

485 PD patients and 480 control subjects were enrolled on the study. Mean age of the PD group (65±12, mean±SD) was less than the control group (71±7; *P*<0.001). Male to female ratio was significantly different between the two groups [P<0.001; PD = 46:54 (female: male); control

= 61:39]. Family history of PD was significantly more frequent in PD than control groups (11% vs 1%; *P*<0.001).

Frequencies of the p.R1628P variant

The study identified the p.R1628P in 54 PD patients (11%) and 29 control subjects (6%). All but one participant carrying c.4883G>C allele were heterozygous in both groups, and all carriers of the variant had S1647T-M2397T haplotype similar to our previous study [6]. The genotypes GC and CC were significantly more common in the PD group than in controls (OR=2.02, 95%CI=1.25-3.25, *P*=0.004). The C variant allele was also more frequent in the PD patients than controls (OR=1.86; 95%CI=1.18-2.93; P=0.006).

Multivariate logistic regression analysis of predictive factors associated with the occurrence of PD

A multivariate logistic regression model was performed to evaluate whether the p.R1628P was an independent factor associated with PD. Logistic regression analysis was demonstrated in table 1. The p.R1628P was associated with PD (OR=1.81, 95%CI=1.10-2.97). Male, ethnically Thai and a family history of PD were all identified to be risk factors of PD.

Comparison of the clinical manifestations between PD patients carrying LRRK2 p.R1628P and without the variant

Comparisons of clinical characteristics between PD patients having p.R1628P and the patients without p.R1628P are shown in table 2. PD patients carrying p.R1628P having a mean age at onset of 56.0±13.0 was significantly earlier than PD patients without p.R1628P (60.1±12.2; *P*<0.021) although patients in both groups had similar disease duration, and duration of L-dopa treatment. The Hoehn and Yahr staging, and the Schwab-England ADL score was poorer in the PD patient with p.R1628P than the patients without the variant (*P*=0.001 for both

clinical scores). Other clinical features of PD patients carrying p.R1628P and frequency of the positive family history of PD were not statistically different from other PD patients.

Discussion

The study strongly confirms that *LRRK2* p.R1628P play an important role as a risk of susceptibility to PD with OR of 1.8. The observed OR was similar to the studies of Han Chinese [1, 5]. So far, Thai has been the only non-Chinese population, in which the association was demonstrated. As mention earlier, the negative studies were likely to result from inadequate sample sizes in relation to a paucity of the variant in the population studied. Interestingly, Wu *et al*. recently described that a *LRRK2* p.S1647T-p.M2397T haplotype appeared to have a protective effect against PD in a large Taiwanese cohort [12]. Although the protective haplotype was not able to counteract with genetic effect on the PD risk of the p.G2385R, it seemed to decrease in risk of developing PD of the other carriers (OR=0.8, 95%CI=0.65-0.97) including carriers of the p.R1628P [12]. Our finding was quite different; all Thai carriers of the p.R1628P had p.S1647T-p.M2397T haplotype, and they were still more susceptible to develop PD than the non-carrier group. Both the Taiwanese and this study were likely to have adequate sample size. Therefore, other environmental or genetic factors possibly influence the genetic effect of the p.R1628P in these different populations. If the genetic factor is involved, *trans*-element rather than *cis*-element is more likely to alter the p.R1628P genetic effect since in all available data regarding the p.R1628P haplotype to date, all carriers are likely to have the same *LRRK2* haplotype [5, 6]. The other point is that *LRRK2* mutations such as p.G2019S are genetically transmitted with highly age-dependent penetrance [1]. Thus, a long-term study of the asymptomatic carriers of p.R1628P may be helpful to clarify this particular point.

The PD patients having p.R1628P had classical phenotype of PD (Table 2), although they had rather earlier onset than non-carriers. In contrast, most previous studies, in which the PD patients having *LRRK2* risk variant generally exhibited similar age at onset to the other PD patients. This study also observed that those PD patients with the p.R1628P developed slightly more rapid progressive course and the patients had slightly poorer activity daily living status than the non-carrier group. This finding needs to be elucidated since several other genetic factors may have influenced our study. If all or most susceptible genetic factors have been identified in the cohort, the genotype-phenotype correlation will be more reliable.

The study had some limitations. Firstly, the studied subjects might not represent the Thai PD population, since all collaborating hospitals were tertiary medical centers. Secondly, the study was designed to reduce a possibility that asymptomatic carriers had developed PD after the enrollment. So control subjects were selected over 65 years of age. Nonetheless, two control subjects developed PD after the enrollment and they were excluded from the study. Regarding this point, female sex and Thai ethnic groups may be incorrectly observed as having significant correlations resulting from selection bias. However, the association between the *LRRK2* genotype and risk of PD should rationally not be influenced by the bias. On the other hand, the relatively older age of the control subjects might in turn become a robust point of the study.

In conclusion, the study strongly supports that the *LRRK2* p.R1628P increases the risk of developing PD. The Thai PD patients carrying the p.R1628P had slightly earlier age at onset and poorer disease progression compared to the non-carriers.

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Appendix: author roles

1. Teeratorn Pulkes: (1) Research project: A. Conception, B. Organization, C. Execution; (2) Statistical Analysis: Review and Critique; (3) Manuscript Preparation: A. Writing of the first draft

2. Chutima Papsing: (1) Research project: C. Execution (genetic analysis); (3) Manuscript Preparation: Read and Approve

3. Ammarin Thakkinstian: (2) Statistical Analysis: A. Design, B. Execution; (3) Manuscript Preparation: Read and Approve

4-7. Sunsanee Pongpakdee, Kongkiat Kulkantrakorn, Suchat Hanchaiphiboolkul, and Somsak Tiamkao: (1) Research project: C. Execution (collected patients' data and samples); (3) Manuscript Preparation: Read and Approve

8. Pairoj Boonkongchuen: (1) Research project: C. Execution (collected patients' data and samples); (3) Manuscript Preparation: Read and Approve

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Table(s)

Table 1. Logistic regression analysis of *LRRK2* genotypes and other factors associated with PD

Factors	Univariate analysis		Multivariate analysis	
	OR	**95%CI**	**OR**	**95%CI**
LRRK2 genotypes				
CC/GC	1.95	1.22-2.12	1.81	1.10-2.97
GG	1			
Male vs Female	1.81	1.40-2.34	1.84	1.41-2.41
Ethnicity				
Thai	1.71	1.25-2.35	1.61	1.16-2.24
Chinese	1.56	1.00-2.43	1.47	0.92-2.34
Thai-Chinese	1			
Family History vs none	19.09	9.52-61.58	20.42	6.29-66.25
Smoking vs non-smoking	0.76	0.53-1.08	-	-

Abbreviations are as follows: PD = Parkinson's disease; *LRRK2* = *Leucine-rich repeat kinase 2* gene; n = number; OR = odd ratio; 95%CI = 95% confidence interval; SD = standard deviation

Table 2. Association between *LRRK2* p.R1628P and clinical characteristics of PD

Clinical characteristics	*LRRK2* R1628P genotype		*P*	OR	95%CI
	Yes n = 54 (%)	No n = 431 (%)			
Age at onset (mean±SD)	56.0±13.0	60.1±12.2	0.021*	-	-
Disease duration (mean±SD)	6.0±4.5	5.6±4.6	0.568	-	-
Family history	7 (13)	45 (10.4)	0.639	1.27	0.55-2.99
Bradykinesia	54 (100)	429 (99.5)	1.000	-	-
Rigidity	52 (96.3)	416 (96.5)	1.000	0.94	0.21-4.22
Rest tremor	47 (87)	388 (90)	0.478	0.74	0.32-1.75
Postural instability	12 (22.2)	105 (24.4)	0.866	0.89	0.45-1.75
Unilateral onset	49 (90.7)	496 (91.9)	0.792	0.87	0.32-2.32
Persistent asymmetry	30 (55.6)	259 (60.1)	0.558	0.83	0.47-1.46
Progressive disorder	48 (88.9)	343 (79.6)	0.142	2.05	0.85-4.95
≥10-year duration	9 (16.7)	53 (12.3)	0.386	1.43	0.66-3.10
Excellence response to L-dopa	37 (68.5)	311 (72.2)	0.631	0.84	0.45-1.55
Dopa-responsive ≥5 years	22 (40.7)	127 (29.5)	0.117	1.65	0.92-2.94
Hoehn and Yahr staging (mean±SD)	3.0±0.5	2.5±0.8	<0.001*	-	-
Schwab-England ADL score (mean±SD)	72.9±18.0	81.9±15.4	<0.001*	-	-
Duration of L-dopa treatment (months; mean±SD)	53.4±49.4	46.4±45.1	0.287	-	-
Wearing-off/on-off	12 (22.2)	92 (21.3)	0.861	0.99	0.49-2.01
Freezing	8 (14.8)	34 (7.9)	0.118	2.03	0.89-4.66
Dopa-induced Dyskinesia	6 (11.1)	46 (10.1)	0.819	1.05	0.42-2.58

Abbreviations are as follows: PD = Parkinson's disease; *LRRK2 = Leucine-rich repeat kinase 2* gene; n = number; OR = odd ratio; 95%CI = 95% confidence interval; SD = standard deviation; ADL = activities of daily living

*Parameters are statistically different among different groups (P < 0.05)