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Published on: 20 Mar 2020 - medRxiv (Cold Spring Harbor Laboratory Press)

Topics: Compound heterozygosity

A comprehensive analysis of dominant and recessive parkinsonism genes in REM sleep behavior disorder

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Counts:

References: 38

Tables: 2

Supplementary tables: 4

Appendixes: 1

Figures: 0

Abstract – words: 201

Article – words: 2489

Title – characters: 100

Search Terms:

1. REM Sleep Behavior Disorder
2. Genetic analysis
3. Parkinson's disease

Study funding:

This work was financially supported by the Michael J. Fox Foundation, the Canadian Consortium on Neurodegeneration in Aging (CCNA), Parkinson Canada the Canada First Research Excellence Fund (CFREF), awarded to McGill University for the Healthy Brains for Healthy Lives (HBHL) program. The Montreal cohort was funded by the Canadian Institutes of Health Research (CIHR) and the W. Garfield Weston Foundation. The Oxford Discovery study is funded by the Monument Trust Discovery Award from Parkinson's UK and supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre based at Oxford University Hospitals NHS Trust and University of Oxford, the NIHR Clinical Research Network and the Dementias and Neurodegenerative Diseases Research Network (DeNDRoN).

Disclosures:

Kheireddin Mufti – Reports no conflict of interest to disclose.

Uladislau Rudakou - Reports no conflict of interests to disclose.

Eric Yu - Reports no conflict of interests to disclose.

Jennifer A. Ruskey – Reports no conflict of interests to disclose.

Farnaz Asayesh – Reports no conflict of interests to disclose.

Sandra B. Laurent – Reports no conflict of interests to disclose.

Dan Spiegelman – Reports no conflict of interests to disclose.

Isabelle Arnulf – Received fees for speaking engagements from UCB pharma, consultancy for Roche, Novartis, and Ono Pharma.

Michele T.M. Hu – Received consultancy fees from Roche and Biogen Pharmaceuticals.

Jacques Y. Montplaisir – Received consultancy fees from Eisai Co.

Jean-François Gagnon – Reports research funding from the Canadian Institutes of Health Research (CIHR).

Alex Desautels – Received grants from Flamel Ireland, Pfized, Biron, Canopy Growth, as well as fees for speaking engagements from Biogen and from consultancy from UCB pharma.

Yves Dauvilliers – Reports no conflict of interests to disclose.

Gian Luigi Gigli – Reports no conflict of interests to disclose.

Mariarosaria Valente – Reports no conflict of interests to disclose.

Francesco Janes - Reports no conflict of interests to disclose.

Birgit Högl – Received consultancy fees from Axovant, benevolent Bio, Takeda, Roche, ono, and received speaker honoraria from Eli Lilly, Mundipharma, UCB, Abbvie, Inspire, Lundbeck.

Ambra Stefani – Reports no conflict of interests to disclose.

Evi Holzknecht – Reports no conflict of interests to disclose.

Karel Sonka – Reports no conflict of interests to disclose.

David Kemlink – Reports no conflict of interests to disclose.

Wolfgang Oertel – Reports no conflict of interests related to the study. He received consultancy or speaker fees from Adamas, Abbvie, Desitin, Novartis and Roche. He has received research

funding from the Deutsche Forschungsgemeinschaft (DFG), EU (Horizon2020), Parkinson Fonds Deutschland, Deutsche Parkinson Vereinigung and Roche Pharma, Basel, Switzerland.

Annette Janzen – Reports no conflict of interests to disclose. She has received research funding from Parkinson Fonds Deutschland.

Giuseppe Plazzi – Reports no conflict of interests to disclose.

Elena Antelmi – Reports no conflict of interests to disclose.

Michela Figorilli – Reports no conflict of interests to disclose.

Monica Puligheddu – Reports no conflict of interests to disclose.

Brit Mollenhauer – Has received honoraria for consultancy from Roche, Biogen, UCB and Sun Pharma Advanced Research Company. BM is member of the executive steering committee of the Parkinson Progression Marker Initiative and PI of the Systemic Synuclein Sampling Study of the Michael J. Fox Foundation for Parkinson's Research and has received research funding from the Deutsche Forschungsgemeinschaft (DFG), EU (Horizon2020), Parkinson Fonds Deutschland, Deutsche Parkinson Vereinigung and the Michael J. Fox Foundation for Parkinson's Research.

Claudia Trenkwalder – Honoraria for lectures from UCB, Grünenthal, Otsuka and consultancy fees from Britannia Pharmaceuticals and Roche.

Friederike Sixel-Döring – Honoraria for lectures from Abbott, Desitin, Grünenthal, Licher MT, STADA Pharm, UCB. Seminar fees from Boston Scientific, Licher MT. Serves on an advisory board for STADA Pharm. No conflict of interest with the presented study.

Valérie Cochen De Cock – Reports no conflict of interests to disclose.

Christelle Charley Monaca – Reports no conflict of interests to disclose.

Anna Heidbreder – Reports no conflict of interests to disclose.

Luigi Ferini-Strambi – Reports no conflict of interests to disclose.

Femke Dijkstra – Reports no conflict of interests to disclose.

Mineke Viaene – Reports no conflict of interests to disclose.

Beatriz Abril – Reports no conflict of interests to disclose.

Bradley F. Boeve – Dr. Boeve has served as an investigator for clinical trials sponsored by Biogen and Alector. He serves on the Scientific Advisory Board of the Tau Consortium. He receives research support from the NIH, the Mayo Clinic Dorothy and Harry T. Mangurian Jr. Lewy Body Dementia Program, the Little Family Foundation, and the LBD Functional Genomics Program.

Ronald B. Postuma – Reports grants and Fonds de la Recherche en Sante, as well as grants from the the Canadian Institute of Health Research, The Parkinson Society of Canada, the WestonGarfield Foundation, the Michael J. Fox Foundation, and the Webster Foundation, as well as personal fees from Takeda, Roche, Teva Neurosciences, Novartis Canada, Biogen, Boehringer Ingelheim, Theranexus, GE HealthCare, Jazz Pharmaceuticals, Abbvie, Jannsen, Otsuko, Phytopharmics, and Inception Sciences.

Guy A. Rouleau – Reports no conflict of interests to disclose.

Ziv Gan-Or – Received consultancy fees from Lysosomal Therapeutics Inc. (LTI), Idorsia, Prevail Therapeutics, Inceptions Sciences (now Ventus), Ono Therapeutics, Denali and Deerfield.

Abstract

Objective: To examine the role of autosomal dominant (AD) and recessive (AR) Parkinsonism genes in the risk of isolated rapid-eye-movement (REM) sleep behavior disorder (iRBD).

Methods: Ten genes implicated in AD and AR Parkinsonism were fully sequenced using targeted next-generation sequencing in 1,039 iRBD patients and 1,852 controls of European ancestry. These include the AR genes *PRKN, DJ-1 (PARK7), PINK1*, *VPS13C, ATP13A2, FBXO7* and *PLA2G6,* and the AD genes *LRRK2, GCH1* and *VPS35*. To examine the role of rare heterozygous variants in these genes, burden test and SKAT-O analyses were performed. The contribution of homozygous and compound heterozygous variants was further examined in the AR genes. Copy number variants (CNVs) in *PRKN* were tested in a subset of samples (n=374) using multiplex ligation-dependent probe amplification followed by analysis of all samples using ExomeDepth.

Results: We found no association between rare heterozygous variants in the tested genes and risk for iRBD. Several homozygous and compound heterozygous carriers were identified with variants of unknown significance, yet there was no overrepresentation in iRBD patients versus controls.

Conclusion: Our results do not support a major role for variants in *PRKN, PARK7, PINK1*, *VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* and *VPS35* in the risk of iRBD.

Glossary

ATP13A2 = Probable cation-transporting ATPase 13A2; **AD** = Autosomal dominant; **AR** = Autosomal recessive; **CNVs** = Copy number variants; ***FBXO7*** = F-box only protein 7; ***GCH1*** = GTP cyclohydrolase I; **iRBD** = Isolated rapid eye movement (REM) sleep behavior disorder; ***LRRK2*** = Leucine-rich repeat kinase 2; **MIPs** = Molecular Inversion Probes; **NGS** = Next generation sequencing; ***PARK7*** = Parkinson disease protein 7; **PD** = Parkinson's disease; ***PINK1*** = PTEN-induced kinase 1; ***PLA2G6*** = 85 kDa calcium-independent phospholipase A2; ***PRKN*** = Parkinson disease protein 2; ***VPS13C*** = Vacuolar protein sorting-associated protein 13C; ***VPS35*** = Vacuolar protein sorting-associated protein 35.

Introduction

Isolated rapid eye movement (REM)-sleep behavior disorder (iRBD) is a prodromal neurodegenerative disease. More than 80% of iRBD patients diagnosed with video-polysomnography (vPSG) will eventually convert to an overt α-synucleinopathy.[1] These include mostly Parkinson's disease (PD) and dementia with Lewy bodies (DLB), and a small minority will convert to multiple system atrophy (MSA).[2]

While not much is known about the genetic background of DLB and MSA, accumulating data from the last two decades have unraveled the role of common and rare genetic variants in PD. Currently, 90 independent risk factors of PD in 78 genetic loci are known, discovered through genome-wide association studies (GWAS).[3] Other, less common genetic variants, have been implicated in familial forms of PD, including autosomal dominant (AD) inherited variants in genes such as *SNCA*, *LRRK2, GCH1* and *VPS35,*[4-6] and autosomal recessive (AR) inherited variants in *PRKN, PINK1* and *PARK7.*[7] Bi-allelic mutations in other genes, including *ATP13A2, VPS13C, FBXO7* and *PLA2G6* may cause AR atypical syndromes with Parkinsonism,[4,8] in some of which α-synucleinopathy has also been reported.[9-11]

The genetic background of iRBD has only been studied in recent years, with studies showing that there is no full genetic overlap between the genetic background of iRBD and that of PD or DLB. For example, *GBA* mutations are associated with risk of iRBD, PD and DLB,[2,12] but pathogenic *LRRK2* mutations seem to be involved only in PD and not in iRBD and DLB.[8,13,14] *MAPT* and *APOE* variants are important risk factors of PD and DLB, respectively,[15,16] but both genes are not associated with iRBD.[15,17] In the *SNCA* locus, there are independent risk variants of PD, DLB and iRBD; specific 3' variants are associated with PD, and other, independent variants at the 5' of *SNCA* are associated with iRBD and DLB.[18] Within the *TMEM175* locus, there are

two independent risk factors of PD, but only one of them, the coding polymorphism p.M393T, has also been associated with iRBD.[19]

Thus far, the role of most of the familial PD genes or genes involved in rare forms of atypical parkinsonism has not been studied in iRBD. Here, since *GBA* and *SNCA* have been studied previously,[2,18] we aimed to thoroughly examine the roles of *PRKN, PINK1*, *PARK7* (*DJ-1*), *VPS13C*, *ATP13A2*, *FBXO7*, *PLA2G6*, *LRRK2*, *GCH1* and *VPS35* in iRBD.

Methods

Population

A total of 1,039 unrelated iRBD patients and 1,852 unrelated controls were included in this study, all of European ancestry (confirmed by principal component analysis of GWAS data). Approximately 81% of the patients were male, the mean reported age at onset (AAO) was 60.1 ± 10.5 years and the average age at diagnosis was 65.3 ± 8.7 years. Data on sex and age were available for 1,032 and 1,004 patients, respectively. Among the controls, about 51% were male, and the mean age at sampling was 52.3 ± 14.3 years, age was not available for nine controls. RBD diagnosis was done with video polysomnography according to the ICSD-2/3 criteria (International Classification of Sleep Disorders, version 2 or 3).[20]

Standard protocol approvals, registrations, and patient consents

All patients signed an informed consent form before entering the study, and the study protocol was approved by the institutional review boards.

Genetic analysis

The coding sequences and 5' and 3' untranslated regions (UTRs) of *PRKN, PINK1*, *DJ-1, VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* and *VPS35* were captured using molecular inversion probes (MIPs) designed as previously described,[21] and the full protocol is available upon request. Details of the MIPs used in the current study are listed in Supplementary Table 1. The library was sequenced on illumina HiSeq 2500\4000 platform at the McGill University and Génome Québec Innovation Centre. Sequencing reads were mapped to the human reference genome (hg19) using the Burrows-Wheeler Aligner.[22] Post-alignment quality control and variant calling were done using the Genome Analysis Toolkit (GATK, v3.8),[23] and

annotation with ANNOVAR.[24] The Frequency of each variant was extracted from the Genome Aggregation Database (GnomAD).[25] We used ClinVar and specific searches on PubMed to examine whether variants that were found in these genes are known or suspected to be pathogenic in PD or atypical parkinsonism.

Quality control

To perform quality control (QC), we used the PLINK software. We excluded variants with: genotyping rate lower than 90%, deviation from Hardy-Weinberg equilibrium set at p=0.001 threshold and when the variant was identified in <25% of the reads for a specific variant. To be included in the analysis, the minimum quality score (QS) was set to 30. Threshold for rate of missingness difference between cases and controls was set at p=0.05, and variants below this threshold were removed. Genotyping rate cut-off for individuals was 90%, and individuals with a lower genotyping rate were excluded. After the QC steps, 1,039 patients and 1,852 controls were included in the analysis. Since we aimed to examine the role of variants that cause monogenic PD, only rare variants (minor allele frequency [MAF]<0.01) were included in the analysis. To ensure that we capture high quality variants, we performed analyses for variants with coverage depth of >30X and variants with >50X.

Data and statistical analysis

We used different approaches to examine the effect of multiple variants on iRBD risk. To examine whether there is a burden of rare (MAF<0.01) heterozygous variants in each of our targeted genes, we used optimized sequence Kernel association test (SKAT-O, R package)[26] and burden tests for different types of variants: all rare variants, potentially functional rare variants (nonsynonymous, frame-shift, stop-gain and splicing), rare loss-of-function variants (frame-shift,

stop-gain and splicing), and rare nonsynonymous variants only. We then examined the association between variants predicted to be pathogenic based on Combined Annotation Dependent Depletion (CADD) score of ≥12.37 (representing the top 2% of potentially deleterious variants) and iRBD. For this analysis, we used burden test (R package SKAT) since the direction of the association was presumed as pathogenic prior to the test. In addition, since copy number variants (CNVs) are frequent in the *PRKN* gene,[27] we included CNVs when we analyzed the association of *PRKN* variants with iRBD. To call CNVs, we first performed multiplex ligation probe amplification (MLPA, the gold standard for CNV detection in *PRKN*) analysis of 374 samples using the SALSA MLPA Probemix P051 Parkinson mix 1 according to the manufacturer's instructions (MRC Holland). Then, using the ExomDepth tool,[28] we determined the ideal parameters for CNV calls using the MIPs data, with sensitivity of 100% and specificity of 97% when compared to the MLPA results. These parameters were subsequently applied to call CNVs from the MIPs data across all iRBD patients and controls. The contribution of homozygous and compound heterozygous variants in all the genes was also examined by comparing the frequencies of the very rare (MAF<0.001) nonsynonymous, splice-site, frame-shift and stop-gain variants between patients and controls. Bonferroni correction for multiple comparisons was applied in all analyses.

Availability of data and materials

Data used for the analysis is available in the supplementary tables. Anonymized raw data can be shared upon request from any qualified investigator.

Results

Quality of coverage

The average coverage of the 10 genes analyzed in this study was >144X for all genes, and the coverage of 8 of the genes was >900X. The per-gene coverage for all 10 genes, although not perfect, is better than the coverage of these specific genes in gnomAD. Supplementary Table 2 details the average coverage and the percentage of nucleotides covered at 20X and 50X for each gene. There were no differences in the coverage across the samples (patients and controls).

Rare homozygous and compound heterozygous variants are not enriched in iRBD patients

To examine whether homozygous or compound heterozygous variants in our genes of interest may cause iRBD, we compared the carrier frequencies of very rare (MAF <0.001) bi-allelic variants between iRBD patients and controls. Three carriers (one patient and two controls) were identified with homozygous variants across all genes. All three carried homozygous non-coding variants that are not likely to cause a disease: one male patient with AAO of 76 years who carried the *PINK1* variant rs181532922, c.*717T>C at the 3' UTR of the gene, one female control recruited at age 72 who carried the *DJ-1* rs7534132, an intronic variant, and one control recruited at the age of 26 who carried the *LRRK2* rs72546315 synonymous (p.H275H) variant.

For the analysis of compound heterozygous carriers, since phasing could not be performed, we considered carriers of two rare variants as compound heterozygous carriers, with two exceptions: 1) when variants were physically close and we could determine their phase based on the sequence reads and 2) if the same combination of very rare variants appeared more than once, we assumed that the variants are likely on the same allele. We found a total of 9

patients and controls, presumably compound heterozygous carriers in the studied genes (Table 1). Three affected and three unaffected carriers of compound heterozygous variants in *VPS13C* were identified, with no overrepresentation in iRBD patients (Fisher test, p=1).

Rare heterozygous variants are not enriched in any of the studied genes

In order to further study the role of rare (MAF<0.01) heterozygous variants, we performed SKAT-O and burden tests, repeated twice for variants detected at coverage depth of >30X and variants detected at >50X (see methods). All rare heterozygous variants identified in each gene are detailed in supplementary table 3. We performed SKAT-O and burden tests at 5 different levels: all rare variants, all potentially functional variants (nonsynonymous, splice site, frame-shift and stop-gain), loss-of-function variants (frame-shift, stop-gain and splicing), nonsynonymous variants only, and variants with CADD score ≥12.37 (Table 2). The Bonferroni corrected p value for statistical significance was set on p<0.001. We found no statistically significant association between iRBD and any of the variant types in any of the genes, suggesting that these genes either have no role in iRBD or have a minor role that we could not detect with this sample size. The nominal association between *PARK7* and iRBD in the SKAT-O analysis of rare functional variants is driven by the nonsynonymous variant rs71653622 (p.A179T) which was ~10 times more frequent in iRBD patients (0.003074) compared to controls (0.000277), but not statistically significant (p=0.09, see supplementary table 3). We did not identify any iRBD patient with known biallelic pathogenic variants in *PARK7, PINK1, VPS13C* and *ATP13A2,* or heterozygous pathogenic variants in *LRRK2, GCH1* and *VPS35*. Two controls were found with the pathogenic *LRRK2* p.G2019S variant. We identified 9 (0.86%) iRBD patients and 13 (0.70%, p=0.65) controls who were heterozygous carriers of the potentially pathogenic variant p.R275W

in *PRKN*, and two additional controls with the *PRKN* p.T240M pathogenic variant. One patient and one control with the pathogenic variant p.R299C in *FBXO7* were also found.

Analysis of copy number variants in *PRKN*

We further examined the association between deletions and duplications in *PRKN* and risk for iRBD. Using ExomeDepth, 7 patients (0.7 %) and 17 controls (0.9%, p=0.53) were found to carry CNVs in *PRKN*, and none of the patients found to have an additional nonsynonymous variant. Therefore, there were no homozygous or compound heterozygous carriers of rare *PRKN* variants among the iRBD patients. Supplementary table 4 lists all the CNVs found in our cohort.

Discussion

The present study provides the first large-scale, full sequencing analysis to examine the possible role of the dominant and recessive parkinsonism genes *PRKN, PARK7, PINK1*, *VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* and *VPS35* in iRBD. We did not find evidence for association of any of these genes with iRBD. In the recessive genes, there was no over-representation of carriers of homozygous or compound heterozygous variants in iRBD patients, and no single patient with bi-allelic pathogenic variants. In the dominant genes, we did not find any known pathogenic variants in these genes, and SKAT-O and burden analyses did not identify burden of rare heterozygous variants in any of these 10 genes.

Whether heterozygous carriage of mutations in recessive PD or atypical parkinsonism related genes is a risk factor for PD is still controversial.[29] *PRKN*-associated PD is characterized by pure nigral degeneration without α-synuclein accumulation,[30] and reports on synucleinopathy and Lewy bodies in *PINK1*-associated PD are inconclusive, as some studies identified Lewy bodies while others did not.[31,32] Since iRBD is a prodromal synucleinopathy, it is not surprising that we did not identify bi-allelic mutations or burden of heterozygous variants in any of these genes. Of note, 380 (36.5%) of the iRBD cohort had a self-reported AAO <50 years. In the case of iRBD, reported AAO may be especially unreliable, as patients may have had RBD symptoms long before they were noticed by themselves or their bed partners. Therefore, the true percentage of iRBD patients with AAO <50 is likely higher, yet none of the known genes involved in early onset PD seems to be involved in early onset iRBD.

Recently, we have shown that the *SNCA* locus is important in RBD, yet with different and distinct variants that are associated with risk of PD.[18] In the same study, *SNCA* was fully sequenced and no known PD-causing variants were found in iRBD patients. We and others have

previously reported that pathogenic *LRRK2* variants were not identified in smaller cohorts of iRBD,[14] which was further confirmed in the current study. In addition, several studies of PD patients with and without RBD have shown reduced prevalence of RBD [33-36] or reduced scores in RBD questionnaires among *LRRK2* mutation carriers. *VPS35* mutations have not been identified in iRBD in the current study, although pathogenic *VPS35* mutations are generally rare.[37,38] Altogether, these results provide no evidence that known, well-validated familial gene mutations involved in PD (including *SNCA*, *LRRK2*, *VPS35*, *PRKN*, *PINK1* and *PARK7*) are also involved in iRBD. *GBA* is the only gene in which strong risk variants associated with PD are also associated with iRBD.[2]

Our study has some limitations. While being the largest genetic study of iRBD to date, it may still be underpowered to detect rare variants in familial PD-related genes. Therefore, our study does not completely rule out the possibility that variants in these genes may lead to iRBD in very rare cases. Another potential limitation of the study design is the earlier age and the different sex distribution in the control population, and the fact that they have not been tested for iRBD. However, since iRBD is not common, found in about 1% of the population,[1] age would have a minimal or no effect on the results. The differences in sex ratios are less likely to have an effect, since in AD and AR Mendelian diseases, the risk is typically similar for men and women.

To conclude, the lack of association between different PD and Parkinsonism genes may suggest that either iRBD is an entity more affected by environmental factors, or that there are other, yet undetected genes that may be involved in iRBD. To examine these possibilities, larger studies that include carefully collected epidemiological data and more extensive genetic data such as whole-exome or whole-genome sequencing will be required. Our study also suggests that screening for variants in the tested genes will have a very low yield.

Acknowledgment

This work was supported by the Michael J. Fox Foundation, the Canadian Consortium on Neurodegeneration in Aging (CCNA), Parkinson Canada and the Canada First Research Excellence Fund (CFREF), with funds awarded to McGill University for the Healthy Brains for Healthy Lives (HBHL) program. The Montreal cohort was funded by the Canadian Institutes of Health Research (CIHR) and the W. Garfield Weston Foundation. The Oxford Discovery study is funded by the Monument Trust Discovery Award from Parkinson's UK and supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre based at Oxford University Hospitals NHS Trust and University of Oxford, the NIHR Clinical Research Network and the Dementias and Neurodegenerative Diseases Research Network (DeNDRoN). JFG holds a Canada Research Chair in Cognitive Decline in Pathological Aging. WO is Hertie Senior Research Professor, supported by the Hertie Foundation. EAF holds a Canada Research Chair (Tier 1) in Parkinson disease. GAR holds a Canada Research Chair in Genetics of the Nervous System and the Wilder Pen field Chair in Neurosciences. ZGO is supported by the Fonds de recherche du Québec–Santé Chercheur-Boursier award and is a Parkinson Canada New Investigator awardee. We thank D. Rochefort, H. Catoire, and V. Zaharieva for their assistance.

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Tables

Table 1. Summary of all samples carrying two nonsynonymous variants detected in the present study

Gene	Sample	Sex	AAS	dbSNP	Allele*	Substitution	F_A	F_C	gnomAD ALL	gnomAD NFE
PRKN	C	M	46	rs137853054	G/A	p.T212M	0	0.0005504	0.0004	0.0003
				rs9456735	G/T	p.M192L	0	0.001101	0.0043	0.0003
PINK1	C	M	57	rs370906995	C/T	p.T257I	0	0.0002756	7.02E-05	0.0001
				rs372280083	C/G	p.L268V	0	0.0002756	9.34E-05	0.0001
VPS13C	A	M	75	15:62165489	C/A	p.D3469Y	0.0005092	0	-	-
				15:62204039	A/C	p.E2862D	0.0005139	0	-	-
VPS13C	C	F	60	rs746819519	C/T	p.G3172D	0	0.001096	1.76E-05	0.00003753
				rs202056315	A/C	p.V2235G	0	0.0002744	4.06E-05	0.00001793
VPS13C	C	M	30	rs780081183	G/C	p.A2368P	0	0.0002738	1.24E-05	0.00002724
				15:62302740	C/G	p.E271D	0	0.0002738	-	-
VPS13C	C	M	52	rs767080349	A/G	p.M2344T	0	0.0002738	1.87E-05	0.0000187
				rs370832130	C/T	p.M1416V	0	0.0002738	0.0001	0.0001
VPS13C	A	M	64	rs760460320	G/C	p.D1496H	0.0005081	0	1.75E-05	0.00002803
				rs765303583	G/C	p.Q660E	0.0005081	0	0	0
VPS13C	A	M	59	rs141515062	T/A	p.S522T	0.001016	0	0.0002	0.0004
				rs376219715	C/T	p.Y365C	0.001016	0	1.63E-05	0.00003598
LRRK2	C	M	63	rs886344692	A/T	p.R1282S	0	0.000275	1.63E-05	2.69E-05
				rs202179802	A/G	p.T2310A	0	0.000275	4.47E-05	7.17E-05

Abbreviations: A = Affected; C = Control; M = Male; F = Female; AAS Age at sampling; dbSNP = Single nucleotide polymorphism database; *Allele = Reference allele/mutant allele; F_A = Frequency in affected; F_C = Frequency in controls; gnomAD ALL= Exome allele frequency in all populations; gnomAD NFE = Exome allele frequency in non-Finnish European.

DOC	Gene	All rare (p value)		Rare functional (p value)		Rare LOF (p value)		Rare NS (p value)		Rare CADD (p value)	
		SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden
30x	Recessive genes										
	PRKN	0.4316	0.484	0.388	0.240	NV	NV	0.508	0.331	1	0.889
	PARK7	0.104	0.254	0.008	0.369	0.175	0.174	0.005	0.005	NV	NV
	PINK1	0.703	0.505	0.117	0.605	NV	NV	0.117	0.605	0.124	0.494
	Recessive (atypical) genes										
	ATP13A2	0.543	0.383	0.379	0.227	NV	NV	0.379	0.227	0.201	0.121
	FBXO7	0.525	0.562	0.266	0.140	0.163	0.252	0.327	0.160	0.228	0.279
	PLA2G6	0.325	0.859	0.222	0.663	0.260	0.193	0.243	0.948	0.196	0.688
	VPS13C	0.018	0.047	0.334	0.206	0.237	0.137	0.343	0.207	0.468	0.834
	Dominant genes										
	GCH1	0.361	0.217	0.730	0.804	0.730	0.804	NV	NV	NV	NV
	LRRK2	0.601	0.827	0.578	0.888	0.134	0.199	0.590	0.966	0.610	0.871
	VPS35	0.159	0.111	0.161	0.247	0.382	0.522	0.161	0.247	0.434	0.807
50x	Recessive genes										
	PRKN	0.085	0.084	0.452	0.609	NV	NV	0.452	0.609	0.771	0.564
	PARK7	0.180	0.288	0.017	0.436	NV	NV	0.010	0.010	NV	NV
	PINK1	0.572	0.546	0.050	0.133	NV	NV	0.050	0.133	0.050	0.133
	Recessive (atypical) genes										
	ATP13A2	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
	FBXO7	0.618	0.624	0.209	0.125	0.331	0.613	0.256	0.148	0.540	0.309
	PLA2G6	0.528	0.853	0.360	0.680	0.680	0.452	0.360	0.680	0.680	0.452
	VPS13C	0.101	0.055	0.073	0.038	0.777	0.971	0.149	0.082	0.332	0.227
	Dominant genes										
	GCH1	0.901	0.817	0.734	0.760	0.734	0.760	NV	NV	NV	NV
	LRRK2	0.030	0.019	0.279	0.173	0.062	0.088	0.525	0.377	0.527	0.365
	VPS35	0.453	0.549	NV	NV	NV	NV	NV	NV	NV	NV

Table 2. Summary of results from burden analyses of rare heterozygous variants

Abbreviations: DOC = Depth of coverage; CADD = Combined annotation dependent depletion; NS = Nonsynonymous; LOF = Loss of function; SKAT-O = Optimized sequence kernel association test; SKAT = Kernel association test; NV = No variants were found for this filter.

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