Research Article

Mutations in *GBA*, *SNCA*, and *VPS35* are not associated with Alzheimer's disease in a Chinese population: a case-control study

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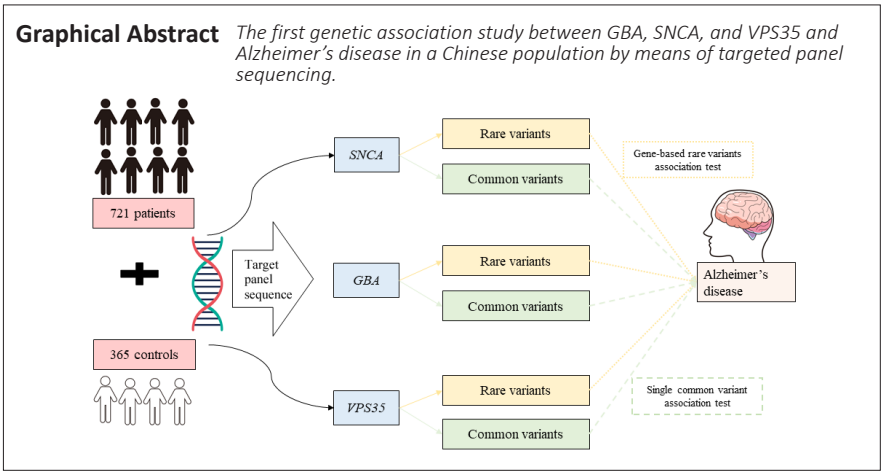
Graphical Abstract *The first genetic association study between GBA, SNCA, and VPS35 and Alzheimer's disease in a Chinese population by means of targeted panel sequencing.*



Abstract

SNCA, *GBA*, and *VPS35* are three common genes associated with Parkinson's disease. Previous studies have shown that these three genes may be associated with Alzheimer's disease (AD). However, it is unclear whether these genes increase the risk of AD in Chinese populations. In this study, we used a targeted gene sequencing panel to screen all the exon regions and the nearby sequences of *GBA*, *SNCA*, and *VPS35* in a cohort including 721 AD patients and 365 healthy controls from China. The results revealed that neither common variants nor rare variants of these three genes were associated with AD in a Chinese population. These findings suggest that the mutations in *GBA*, *SNCA*, and *VPS35* are not likely to play an important role in the genetic susceptibility to AD in Chinese populations. The study was approved by the Ethics Committee of Xiangya Hospital, Central South University, China on March 9, 2016 (approval No. 201603198).

Key Words: Alzheimer's disease; Chinese population; common variants; *GBA*; Parkinson's disease; rare variants; *SNCA*; *VPS35*

Chinese Library Classification No. R446.1; R741; Q344+.12

Introduction

Clinically, Alzheimer's disease (AD) is characterized by episodic memory decline, executive dysfunction, and difficulty with daily life activities. The neuropathological features of AD are amyloid plaques of accumulated amyloid-β (Aβ) and neurofibrillary tangles formed by hyperphosphorylated tau protein (Hane et al., 2017; Kozlov et al., 2017; Wang et al., 2020). The etiology of AD is multifactorial and complex; mutations in the genes encoding amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) are the main causes of familial early-onset AD [age at onset (AAO) ≤ 65 years], while the convergence of genetic and environmental factors in aging is the primary drive for sporadic late-onset AD (AAO > 65 years) (Lane et al., 2018). Among multiple genetic risk factors for sporadic AD, apolipoprotein E (*APOE*) is the single biggest risk gene; the APOE ε4 allele shows a strong association with increased risk for AD (Lane et al., 2018; Endres, 2021). To date, genetic approaches have identified more than 50 AD-related genes/loci, shedding new light on the pathogenesis of AD (Sims et al., 2020).

Parkinson's disease (PD) is the most common neurodegenerative movement disorder with pathological

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aggregations of α-synuclein (α-syn) in Lewy bodies (LBs) and Lewy neurites (Trinh and Farrer, 2013; Kalia and Lang, 2015; Seguella et al., 2020). Although AD and PD are clinically distinct diseases with different pathological hallmarks, the pathological features and clinical symptoms of AD can also appear in PD patients, and vice versa (Zhu et al., 2017). Being age-related neurodegenerative disorders, they share overlapping pathological mechanisms and genetic background (Xie et al., 2014; Sanchez-Mut et al., 2016; Tan et al., 2019). Genes like *APOE*, *MAPT*, *PON1*, *GSTO*, and *NEDD9* have been found to affect the risk for these two diseases (Zhu et al., 2017; Dunn et al., 2019); one study has discussed the roles that two PD-related genes termed *PINK1* and *PARKIN* may play in AD (Quinn et al., 2020), which indicates that there are more potential genetic factors to be discovered. The synuclein alpha gene (*SNCA*), encoding α-syn, which is the key component of inclusions in PD, is the first gene reported to be associated with inherited PD (Kalia and Lang, 2015; Brás et al., 2021). Both missense variants and copy number variants of *SNCA* have been shown to cause PD (Brás et al., 2021). Regarding the association between *SNCA* and AD, two *SNCA* single nucleotide polymorphisms (rs3857059 and rs2583988) have been demonstrated to increase the risk for LB pathology in AD subjects, which may exert effects via interaction with leucine-rich repeat kinase2 (*LRRK2*) (Linnertz et al., 2014). Wang et al. (2016) have further applied PCR-restriction fragment length polymorphism to examine the association between three *SNCA* single nucleotide polymorphisms and AD in 98 AD cases and 105 age-matched controls. They found that rs10516846 GG was excessively represented in the AD group compared with the control group, highlighting the association between *SNCA* and AD.

Mutations in the glucocerebrosidase gene (*GBA*), encoding the lysosomal enzyme glucocerebrosidase, are the most common genetic cause of PD (Sidransky and Lopez, 2012). Although rare, *GBA* variants were observed in patients with pure AD with a frequency of 3.7% (Sklerov et al., 2017), suggesting there may be an association between *GBA* and AD. However, Tsuang and colleagues (Tsuang et al., 2012) concluded that *GBA* is not a susceptibility gene in AD, even though subjects presenting with LB disease (LBD) with high-level concomitant AD pathology were more likely to carry mutations than controls. Considering the controversy regarding the association between *GBA* and AD, it is necessary to conduct a study to verify these findings.

The vacuolar protein sorting 35 homolog gene (*VPS35*) was identified as a novel genetic cause of autosomal dominant PD by exome sequencing in 2011 (Vilariño-Güell et al., 2011; Zimprich et al., 2011). There is evidence indicating that VPS35 protein is not only involved in the neuropathology of AD, but it also plays a direct role in the development of an AD-like phenotype (Wen et al., 2011; Deng et al., 2013; Li et al., 2020). On the basis of the observations above, we speculated that it could be meaningful to examine the *GBA*, *SNCA*, and *VPS35* genes in an AD population to identify novel loci implicated in AD. Moreover, few studies have investigated these three genes in Chinese patients with AD. Therefore, we conducted a variant screening study using a targeted gene sequencing panel to examine whether these three common PD-related genes are associated with AD risk in a Chinese Han population.

Participants and Methods

Study subjects

This prospective case-control study recruited 721 Chinese Han patients with AD (40.87% male; mean AAO 65.80 ± 10.91 years) from Xiangya Hospital, Central South University, between January 1, 2016 and December 31, 2019. Each patient was thoroughly examined and evaluated by two experienced neurologists and was diagnosed as probable AD on the basis of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria (McKhann et al., 1984). Patients with other neurological diseases were excluded. Among the 721 AD patients, no patients carried pathogenic mutations in *APP*, *PSEN1*, or *PSEN2*. Additionally, 365 unrelated individuals (from communities near Xiangya Hospital) matched for age, gender, and ethnical origin without any AD-related symptoms or other neurological disorders were recruited as normal controls [NCs; 47.95% male; mean age 70.65 ± 5.35 years], and the cognitive examination using the Mini-Mental State Examination showed their cognitive functions were normal. The Ethics Committee of Xiangya Hospital, Central South University approved the study on March 9, 2016 (No. 201603198; **Additional file 1**). All participants or the legal guardians voluntarily agreed to participate in this study, and all of them provided written informed consent forms (**Additional file 2**). This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for protocol reporting (**Additional file 3**) and *Declaration of Helsinki*. Demographic data [age, gender, education, Mini-Mental State Examination (Jiang et al., 2021)] of the participants were collected.

Genetic testing

Genomic DNA from peripheral blood leukocytes was isolated according to standard procedures as previously described (Jiao et al., 2014; Zhang et al., 2020). The DNA quality and quantity were assessed by both NanoDrop spectrophotometer 2000 (Thermo Scientific, Wilmington, DE, USA) and Qubit Fluorometer 3 (Life Technologies, Carlsbad, CA, USA). We designed a targeted panel including *GBA*, *SNCA*, and *VPS35*, and used a targeted gene sequencing panel to screen all exon regions and the nearby sequences of these three genes in each subject. As previously reported (Xiao et al., 2020), the extracted DNA was sheared into fragments using Bioruptor Pico (Diagenode, Seraing, Belgium), and fragments were restricted to around 200 bp using Qseq100 DNA Analyzer (Bioptic Inc., New Taipei City, Taiwan, China). The prepared libraries and target enrichment were obtained using SureSelectXT Reagent kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions. The resulting libraries were sequenced on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using paired-end 150-bp sequencing. The resulting data were trimmed to remove low-quality bases and adapter contamination using Fastp (version 0.18.0). They were then aligned to the human genome reference sequence GRCh37/hg19 using the BWA software (version 0.7.15, http://bio-bwa.sourceforge.net/) (Li and Durbin, 2010), duplicates were marked using Picard (version 2.18.7, https://github.com/broadinstitute/picard), and variant calling was performed with Genome Analysis Toolkit 4 (GATK) HaplotypeCaller (version 3.2, https://github.com/broadinstitute/gatk/) (McKenna et al., 2010). ANNOVAR4 (https://annovar.openbioinformatics.org/) (Wang et al., 2010) was used to annotate genomic variants. The mean depth of coverage per individual was 641.6×, and the average sample coverage was 99.94%. Furthermore, among these samples, 98.76% of the bases were covered > 20×, and 97.51% of the bases were covered > 30×. Additionally, we determined the APOE genotype in each subject using PCR amplification and sequenced all PCR products on an ABI 3730xl DNA analyzer (Applied Biosystems, Waltham, MA, USA). Sequencher software (http://www.genecodes.com/) was applied to analyze the DNA sequences. We used SIFT (http://provean.jcvi.org/index.php) (Ng and Henikoff, 2001), PolyPhen-2 (Adzhubei et al., 2010), and other online software to predict the pathogenicity of nonsynonymous variants in the three

genes mentioned above. Among the software used, ReVe is a novel computational method proposed by our team in which rare missense variants with ReVe > 0.7 are considered pathogenic (Li et al., 2018).

Statistical analysis

Continuous variables are presented as mean ± standard deviation (SD). We used the Mann-Whitney *U* test to analyze the differences in age and education and in the Mini-Mental State Examination. We used the chi-square test to perform comparisons of gender and the allele frequency distribution of *APOE* between AD patients and control individuals using SPSS (version 25.0, https://www.ibm.com/products/spss-statistics). We used PLINK 1.9 (http://zzz.bwh.havard.edu/plink/index.shtml) (Purcell et al., 2007) to exclude variants with a genotyping rate < 80% and Hardy-Weinberg $P < 0.001$, which means deviation from the Hardy-Weinberg equilibrium. Then, we divided all remaining variants into two parts: common variants [0.01 ≤ minor allele frequency (MAF) ≤ 0.5] and rare variants (0 < MAF < 0.01), according to the MAF in the controls. The single common variant association test was executed between the AD and control groups using PLINK 1.9. Additionally, we used PLINK 1.9 to adjust age, sex, and *APOE* ε4 status (*APOE* ε4+ and *APOE* ε4–) for each common variant. For rare variants, we combined them and studied the entire effect of each gene on AD through the sequence kernel association test-optimal (SKAT-O) (Lee et al., 2012), where three related variates (age, sex, and *APOE* ε4 status) were controlled. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Participant characteristics

In total, 1086 Han Chinese participants comprising 721 AD patients and 365 healthy controls were recruited in this study. Among the 721 cases, 310 were early-onset AD patients (42.26% male; mean AAO 55.49 ± 6.02 years) and 411 were late-onset AD patients (39.17% male; mean AAO 73.94 ± 5.93 years). The mean AAO of all AD patients was (65.80 ± 10.91) years, with a mean disease course of 3.34 ± 2.53 years. The Mini-Mental State Examination scores showed a statistically significant difference between these two groups ($P < 0.001$); the mean scores of the AD and control groups were 10.96 and 27.79, respectively. The percentage of APOE ε4 carriers was significantly higher in the AD cases (43.27%) compared with the controls (19.72%), which is consistent with a previous study (Farrer et al., 1997).

Common variant association test

We screened all the *SNCA*, *GBA*, and *VPS35* exon regions and their nearby sequences in all individuals. After weeding out variants whose genotyping rate was less than 80%, which also deviated from the Hardy-Weinberg equilibrium, we identified 12 common variants including eight *SNCA* variants, two *GBA* variants, and two *VPS35* variants (**Table 1**). We performed the single common variant association test on each common variant between AD cases and NCs using PLINK 1.9, but none of these 12 common variants reached statistical significance before adjustments. Furthermore, we corrected for gender, age, and *APOE* ε4 status, but all *P*-values were still higher than 0.05.

Gene-based rare variants association test

After weeding out variants whose genotyping rate was less than 80%, which also deviated from the Hardy–Weinberg equilibrium, 117 rare variants (0 < MAF < 0.01) remained, comprising 38 *SNCA* variants, 28 *GBA* variants, and 51 *VPS35* variants. We applied SKAT-O to compare the cumulative burden of the rare *SNCA*, *GBA*, and *VPS35* variants between AD cases and control participants, but no statistical difference was found. As **Table 2** shows, among the 38 rare *SNCA* variants, 20 were only identified in AD patients and 9 were only found in control individuals. Compared with the healthy controls, the frequency of carriers of rare variants was not significantly higher in AD cases (corrected SKAT-O $P = 0.33$). Of the 28 rare *GBA* variants (**Table 3**), 67.86% of the variants ($n = 19$) were only found in AD patients and 14.49% ($n = 4$) were only found in controls. The *P*-value in SKAT-O after correction was 0.56, suggesting no significant association between rare GBA variants and AD in our cohort. Of the 51 rare variants identified in *VPS35* (**Table 4**), which were mostly located in untranslated regions, 32 were only found in AD patients. No statistical difference in the cumulative effect of all 51 variants was observed between the AD and control groups, with a corrected *P*-value of 0.38.

Additionally, we further analyzed the effects of ultra-rare variants (MAF < 0.001) on AD; however, the SKAT-O results failed to show an association between these three genes and AD (**Additional Table 1**). Considering rare pathogenic variants may affect AD, we performed an additional SKAT-O that included only rare pathogenic variants, which were predicted as loss of function or ReVe > 0.7, however no significant difference was found between the two groups (**Additional Table 2**).

Table 1 | **Association analysis of common variants of *GBA*, *SNCA*, and *VPS35* genes**

					MAF				
Gene	**Position**	**Ref**	**Alt**	**dbSNP**	**AD**	**NC**	***P* value**	***P'***	**OR (95% CI)**
SNCA	Chr4:90645671	T	A	rs1045722	0.477	0.479	0.9279	0.9791	0.991 (0.823–1.195)
	Chr4:90645674	C	T	rs3857053	0.477	0.485	0.7176	0.7665	0.966 (0.801–1.165)
	Chr4:90646886	G	A	rs356165	0.427	0.392	0.1403	0.1855	1.155 (0.954–1.398)
	Chr4:90757941	T	–	rs1412247618	0.185	0.213	0.1291	0.1598	0.838 (0.668–1.053)
	Chr4:90757947	T	A	rs2583986	0.015	0.014	0.7961	0.8601	1.106 (0.515–2.376)
	Chr4:90757948	T	A	rs2245804	0.339	0.345	0.7992	0.8563	0.975 (0.802–1.185)
	Chr4:90758225	G	C	rs555070398	0.014	0.011	0.5587	0.4164	1.281 (0.558–2.940)
	Chr4:90758361	C	T	rs372025454	0.01	0.013	0.5833	0.9011	0.790 (0.340–1.835)
GBA	Chr1:155214473	A	G	rs12034326	0.282	0.267	0.4554	0.4652	1.082 (0.879–1.332)
	Chr1:155214576	–	GA	rs1571981318	0.012	0.015	0.5403	0.4638	0.786 (0.363–1.702)
VPS35	Chr16:46693140	G	A	rs76259065	0.067	0.06	0.5744	0.4928	1.113 (0.766–1.616)
	Chr16:46693303	T	A	rs79050797	0.104	0.103	0.9532	0.7324	1.009 (0.748–1.359)

AD: Alzheimer's disease; Alt: alternate allele; Chr: chromosome; CI: confidence interval; dbSNP: dbSNP137 (https://www.ncbi.nlm.nih.gov/snp/); GBA: glucocerebrosidase; MAF: minor allele frequency; NC: normal control; OR: odds ratio; *P'*: *P*-value after the adjustment of age, gender, and APOE ε4 status; Ref: reference allele; SNCA: synuclein alpha; VPS35: vacuolar protein sorting 35 homolog.

Table 2 | **Rare variants of SNCA gene in AD patients and normal controls**

Position	dbSNP	Mutation regions	cDNA change	AA change	MAF gnomAD	Number		Functional predictions: damaging/total
						AD	NC	
Chr4:90645280	–	3′UTR	c.*2499G>T	–	–	1	0	–
Chr4:90645296	–	3′UTR	c.*2483G>A	–	–	0	1	–
Chr4:90645337	–	3′UTR	c.*2442G>T	–	–	1	0	–
Chr4:90645349	–	3′UTR	c.*2430C>A	–	9.70E-05	1	0	–
Chr4:90645392	–	3′UTR	c.*2387C>T	–	–	1	0	–
Chr4:90645430	–	3′UTR	c.*2349C>G	–	3.20E-05	0	3	–
Chr4:90645502	rs375815714	3′UTR	c.*2277A>C	–	6.50E-05	6	0	–
Chr4:90645552	rs551886776	3′UTR	c.*2227C>A	–	2.00E-04	4	1	–
Chr4:90645576	rs566899780	3′UTR	c.*2203G>A	–	3.20E-05	1	0	–
Chr4:90645673	rs555697933	3′UTR	c.*2106G>A	–	5.00E-04	9	2	–
Chr4:90645883	rs186189862	3′UTR	c.*1896C>T	–	4.80E-03	0	1	–
Chr4:90646311	–	3′UTR	c.*1468T>C	–	–	1	0	–
Chr4:90646313	–	3′UTR	c.*1466G>T	–	3.30E-05	0	1	–
Chr4:90646469	–	3′UTR	c.*1309_*1310insT	–	–	2	0	–
Chr4:90646469	rs777296100	3′UTR	c.*1309_*1310insTT	–	–	27	21	–
Chr4:90646472	–	3′UTR	c.*1306_*1307insCTTT	–	–	1	2	–
Chr4:90646473	–	3′UTR	c.*1305_*1306insGTTT	–	–	1	2	–
Chr4:90646477	–	3′UTR	c.*1301_*1302insCTTTT	–	–	2	0	–
Chr4:90646493	–	3′UTR	c.*1286C>T	–	1.80E-03	2	0	–
Chr4:90646501	–	3′UTR	c.*1277_*1278insCT	–	2.00E-04	3	0	–
Chr4:90646556	–	3′UTR	c.*1223A>G	–	–	1	0	–
Chr4:90646601	–	3′UTR	c.*1177_*1178insA	–	–	0	1	–
Chr4:90646686	–	3′UTR	c.*1092_*1093insAA	–	4.00E-04	4	1	–
Chr4:90646790	rs954649153	3′UTR	c.*989C>T	–	3.30E-05	0	1	–
Chr4:90646795	–	3′UTR	c.*984delT	–	–	0	1	–
Chr4:90646858	rs148246747	3′UTR	c.*921G>A	–	3.20E-05	4	2	–
Chr4:90646885	rs192179063	3′UTR	c.*894G>A	–	3.00E-04	13	4	–
Chr4:90647186	–	3′UTR	c.*593C>T		3.20E-05	1	0	–
Chr4:90647276	–	3′UTR	c.*503A>G		–	1	0	–
Chr4:90647277	rs560621582	3′UTR	c.*502G>A		4.00E-04	2	0	–
Chr4:90647315	rs183204610	3′UTR	c.*464C>A		6.50E-05	1	0	–
Chr4:90647374	rs985546471	3′UTR	c.*405C>T		2.00E-04	1	2	–
Chr4:90647505	–	3′UTR	c.*274T>G		–	1	0	–
Chr4:90647531	–	3′UTR	c.*248C>T		–	0	1	–
Chr4:90647662	rs184023281	3′UTR	c.*117G>A		6.50E-05	1	0	–
Chr4:90650354	rs191055637	Exon	c.381G>A	p.M127I	3.20E-05	1	0	14/24
Chr4:90650386	rs145138372	Exon	c.349C>T	p.P117S	–	1	0	6/24
Chr4:90650392	–	Exon	c.343G>A	p.D115N	–	0	1	6/24
Carriers (*n*)						95	48	
Frequency (%)						13.18	13.15	
SKAT-O	*P* = 0.33 (adjusted by age, gender, and APOE ε4)							

Twenty-four online software were used including: SIFT, PolyPhen2-HDIV, PolyPhen2-HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, VEST3, MetaSVM, MetaLR, M-CAP, CADD, DANN, fathmm-MKL, Eigen, GenoCanyon, fitCons, GERP++, phyloP, phastCons, SiPhy, REVEL, and ReVe; damaging/total is the ratio of online software that predicted the variant as damaging among the 24 online software. Transcript NM_001146055 was used for SNCA variants nomenclature. AA: Amino acid; AD: Alzheimer's disease; APOE: apolipoprotein E; cDNA: complementary deoxyribonucleic acid; dbSNP: dbSNP137 (https://www.ncbi.nlm.nih.gov/snp/); MAF gnomAD: the minor allele frequency of variants in the genome aggregation database; NC: normal control; SKAT-O: sequence kernel association test-optimal; SNCA: synuclein alpha; 3′UTR: 3′ untranslated region.

Discussion

Though AD and PD have markedly different clinical and pathological features, being the two most common neurodegenerative disorders, they have shared mechanisms in the development of neurodegeneration (Xie et al., 2014; Ferencz and Gerritsen, 2015; Dai et al., 2020), indicating the significance of verifying PD-related genetic risk factors in populations with AD and *vice versa*. We presented a comprehensive analysis of the association between AD risk and three common PD-related genes termed *GBA*, *SNCA*, and *VPS35* in a Chinese cohort including 721 AD patients and 365 controls. To our knowledge, it is the first reported study that investigated the association of *GBA*, *SNCA*, and *VPS35* variants with AD in Chinese patients using a targeted gene sequencing panel. However, no nominally significant associations were identified between these three PD-related genes and AD.

Mutations in the synuclein family play an important role in PD, which is not surprising because synucleins are the main marked pathology of PD (Ferencz and Gerritsen, 2015). It has been reported that about half of the individuals with AD have enough LB pathology to be considered to have a secondary diagnosis of LBD (Azar et al., 2020). Furthermore, single nucleotide polymorphisms in SNCA play a role in LB pathology in AD subjects (Linnertz et al., 2014). However, no significant association between *SNCA* and AD was observed in our study, which is consistent with Zhu et al. (2017), who concluded that the *SNCA* variant was unlikely to play important roles in the genetic susceptibility to late-onset AD in a northern

Table 3 | **Rare variants of GBA gene in AD patients and normal controls**

Position	dbSNP	Mutation regions	cDNA change	AA change	MAF gnomAD	Number		Functional predictions: damaging/total
						AD	NC	
Chr1:155204900	rs552938719	Intron	–	–	3.30E-05	3	2	–
Chr1:155204901	rs577529715	Intron	–	–	3.30E-05	4	1	–
Chr1:155204979	rs750970574	Intron	–	–	–	1	0	–
Chr1:155204994	rs1135675	Exon	c.1497G>C	p.V499V	3.00E-04	2	0	–
Chr1:155205043	rs421016	Exon	c.1448T>C	p.L483P	7.00E-04	0	1	15/24
Chr1:155205498	–	Exon	c.1362C>T	p.P454P	-	1	0	–
Chr1:155205513	rs750193229	Exon	c.1347G>A	p.T449T	3.20E-05	2	1	–
Chr1:155205548	–	Exon	c.1312G>A	p.D438N	–	1	0	18/24
Chr1:155206051	rs773947710	Exon	c.1209C>T	p.S403S	–	1	0	–
Chr1:155206101	rs765182863	Exon	c.1159T>G	p.W387G	–	1	0	11/24
Chr1:155206170	rs121908305	Exon	c.1090G>A	p.G364R	–	1	0	11/24
Chr1:155207193	rs747591577	Exon	c.938A>G	p.H313R	–	1	0	7/24
Chr1:155207383	–	Intron	–	–	–	0	1	–
Chr1:155207387	rs140335079	Intron	–	–	–	2	0	–
Chr1:155208081	rs398123531	Exon	c.605G>A	p.R202Q	1.00E-04	4	2	8/24
Chr1:155209678	–	Exon	c.306A>G	p.T102T	–	0	1	–
Chr1:155209716	–	Exon	c.268C>T	p.L90L	–	0	1	–
Chr1:155210467	–	Exon	c.69C>G	p.G23G	–	1	0	–
Chr1:155210478	rs143187997	Exon	c.58A>G	p.I20V	3.20E-05	1	0	4/24
Chr1:155210969	–	5′UTR	c.-66G>A	–	–	1	0	–
Chr1:155211040	rs567284407	5′UTR	c.-137G>A	–	–	1	0	–
Chr1:155211079	rs534311114	Intron	–	–	6.50E-05	1	0	–
Chr1:155210969	–	5′UTR	c.-66G>A	–	–	1	0	–
Chr1:155211040	rs567284407	5′UTR	c.-137G>A	–	–	1	0	–
Chr1:155211079	rs534311114	Intron	–	–	6.50E-05	1	0	–
Chr1:155214308	–	5′UTR	–	–	–	1	0	–
Chr1:155214332	–	5′UTR	–	–	–	1	0	–
Chr1:155214398	–	5′UTR	–	–	9.70E-05	1	2	–
Carriers (*n*)						35	12	
Frequency (%)						4.85	3.29	
SKAT-O	$P = 0.56$ (adjusted by age, gender, and APOE ε4)							

The 24 online software used included: SIFT, PolyPhen2-HDIV, PolyPhen2-HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, VEST3, MetaSVM, MetaLR, M-CAP, CADD, DANN, fathmm-MKL, Eigen, GenoCanyon, fitCons, GERP++, phyloP, phastCons, SiPhy, REVEL, ReVe; and damaging/total is the ratio of online software that predict the variant as damaging among the 24 online software. Transcript NM_000157 was used for GBA variants nomenclature. AA: Amino acid; AD: Alzheimer's disease; APOE: apolipoprotein E; cDNA: complementary deoxyribonucleic acid; dbSNP: dbSNP137 (https://www.ncbi.nlm.nih.gov/snp/); GBA: glucocerebrosidase; MAF gnomAD: the minor allele frequency of variants in the genome aggregation database; NC: normal control; SKAT-O: sequence kernel association test-optimal; 5′UTR: 5′ untranslated region.

Han Chinese population. In contrast, Yoshino and colleagues (Yoshino et al., 2016) have found that the *SNCA* mRNA expression was significantly elevated in peripheral leukocytes from AD patients. Recent studies have further demonstrated that the cerebrospinal fluid α-syn concentration was significantly higher in AD compared with PD, dementia with LB (Wang et al., 2015), and healthy controls (Wang et al., 2016). Another study has revealed that soluble α-syn was involved in the pathophysiology of AD and may be a better predictor of cognitive impairment associated with AD than soluble Aβ and tau levels (Larson et al., 2012). We thus speculated that the α-syn effects on AD mainly result from gene expression and interactions with other genes and proteins (Twohig and Nielsen, 2019), rather than from *SNCA* variants, though further confirmations are needed.

GBA mutations were initially discovered to be associated with PD through clinical observations, and subsequent studies further identified the association between *GBA* and other diseases, including Gaucher's disease, dementia with LB, and multiple system atrophy (Gan-Or et al., 2018). Compared with non-carriers, patients carrying *GBA* mutations tend to have increased risk of cognitive impairment, psychosis, depression, and rapid eye movement sleep behavior disorder; however, the underlying mechanism is unclear (Creese et al., 2018; Gan-Or et al., 2018). It has been estimated that there are about 300 mutations and gene re-arrangements in *GBA* with different effects on the enzymatic activity of glucocerebrosidase (Gan-Or et al., 2018). Because *GBA* mutations show association with reduced cerebrospinal fluid levels of total α-syn in patients with PD (Lerche et al., 2020) and dementia with LB (Lerche et al., 2019), the cross talk between glucocerebrosidase and α-syn is a potential target for therapy of LBD (Blandini et al., 2019). However, the difference in frequencies of *GBA* mutations between the AD and control groups in our cohort did not reach statistical significance, which is consistent with a study by Tsuang et al. (2012) in which the entire *GBA* coding region was screened, but GBA was not identified as a susceptibility gene in pure AD. Therefore, although the association between *GBA* and LBD has been confirmed, whether *GBA* variants increase the predisposition to AD needs further investigation.

Dysfunction of the endosomal-lysosomal network has gained increased attention in the field of neurodegenerative disorders including AD (Choy et al., 2012; Vagnozzi et al., 2019). *VPS35*, located at 16q11.2, encodes VPS35 protein, one of the major components of the retromer complex (Deng et al., 2013; Li et al., 2020). As a main protein involved in endosomal protein sorting, VPS35 plays a role in the suppression of AD neuropathology by inhibiting β-secretase (BACE1) activity and Aβ production (Wen et al., 2011),

Table 4 | **Rare variants of VPS35 gene in AD patients and normal controls**

Position	dbSNP	Mutation regions	cDNA change (NM_018206)	AA change	MAF gnomAD	Number		Functional predictions: damaging/total
						AD	NC	
Chr16:46691879	rs925652164	3′UTR	c.*2505C>T	–	6.50E-05	1	0	–
Chr16:46691918	rs193290216	3′UTR	c.*2466T>C	–	1.00E-04	5	4	–
Chr16:46691997	–	3′UTR	c.*2387T>C	–	6.50E-05	0	1	–
Chr16:46692152	–	3′UTR	c.*2232A>G	–	–	2	0	–
Chr16:46692330	rs200961009	3′UTR	c.*2053_*2054insT	–	4.70E-03	1	1	–
Chr16:46692533	–	3′UTR	c.*1851A>G	–	–	1	0	–
Chr16:46692681	–	3′UTR	c.*1703delA	–	3.20E-05	1	0	–
Chr16:46692771	rs568752537	3′UTR	c.*1612_*1613insC	–	1.00E-03	0	1	–
Chr16:46692816	–	3′UTR	c.*1567_*1568insAA	–	–	1	0	–
Chr16:46692888	rs892792235	3′UTR	c.*1496A>T	–	–	2	1	–
Chr16:46692995	–	3′UTR	c.*1389G>A	–	–	0	1	–
Chr16:46693004	rs545490118	3′UTR	c.*1380C>T	–	3.20E-05	1	0	–
Chr16:46693075	–	3′UTR	c.*1309_*1282delTCTTGAGGGCAGAGGCCCCAGTTCCTCT	–	–	1	0	–
Chr16:46693086	–	3′UTR	c.*1298C>G	–	–	1	0	–
Chr16:46693086	–	3′UTR	c.*1298delC	–	–	1	0	–
Chr16:46693105	–	3′UTR	c.*1279T>G	–	–	1	0	–
Chr16:46693127	rs931658990	3′UTR	c.*1257G>A	–	6.50E-05	1	0	–
Chr16:46693279	rs145628294	3′UTR	c.*1105A>G	–	–	1	3	–
Chr16:46693418	–	3′UTR	c.*966T>C	–	–	1	0	–
Chr16:46693522	–	3′UTR	c.*862_*861delTA	–	–	0	1	–
Chr16:46693546	–	3′UTR	c.*837_*838insT	–	–	1	0	–
Chr16:46693621	rs34926621	3′UTR	c.*762_*763insTT	–	2.00E-04	3	2	–
Chr16:46693817	–	3′UTR	c.*567T>C	–	–	0	1	–
Chr16:46693839	–	3′UTR	c.*545G>A	–	3.20E-05	1	0	–
Chr16:46693873	–	3′UTR	c.*511delA	–	3.20E-05	0	1	–
Chr16:46693901	–	3′UTR	c.*483C>G	–	–	0	1	–
Chr16:46693980	–	3′UTR	c.*403_*404insGA	–	–	1	0	–
Chr16:46694038	–	3′UTR	c.*346G>C	–	–	1	0	–
Chr16:46694095	–	3′UTR	c.*289C>G	–	–	2	0	–
Chr16:46694100	rs879053227	3′UTR	c.*284_*281delCACA	–	2.00E-04	1	2	–
Chr16:46694100	rs879039152	3′UTR	c.*284_*283delCA	–	–	2	0	–
Chr16:46694101	rs878915318	3′UTR	c.*283_*281delCAC	–	1.80E-03	6	5	–
Chr16:46694101	–	3′UTR	c.*282_*283insAAAAAA	–	–	1	2	–
Chr16:46694104	rs886052006	3′UTR	c.*279_*280insCAAA	–	2.00E-04	1	0	–
Chr16:46694107	rs886052008	3′UTR	c.*276_*277insCAA	–	4.10E-05	2	0	–
Chr16:46694108	rs886052010	3′UTR	c.*275_*276insCAA	–	8.10E-05	1	1	–
Chr16:46694110	–	3′UTR	c.*273_*274insCAA	–	3.00E-03	2	0	–
Chr16:46694237	rs759782658	3′UTR	c.*147C>T	–	–	1	0	–
Chr16:46694245	–	3′UTR	c.*139A>T	–	–	1	0	–
Chr16:46694330	rs527800239	3′UTR	c.*54T>A	–	6.50E-05	1	0	–
Chr16:46695785	–	Intron	–	–	–	1	0	–
Chr16:46696916	rs755145722	Exon	c.1806C>T	p.V602V	–	1	0	–
Chr16:46696946	rs770819201	Exon	c.1776G>C	p.G592G	v	2	0	–
Chr16:46697081	–	Intron	–	–	–	1	0	–
Chr16:46706261	rs530665086	Exon	c.1284T>C	p.F428F	1.00E-04	1	2	–
Chr16:46708460	–	Intron	–	–	–	1	0	–
Chr16:46708588	rs757051230	Intron	–	–	9.80E-05	4	1	–
Chr16:46716039	rs193077277	Exon	c.151G>A	p.G51S	5.00E-04	1	1	8/24
Chr16:46717515	rs774604183	Exon	c.7A>G	p.T3A	–	1	0	6/24
Chr16:46723055	rs763096994	5′UTR	c.-10G>T	–	–	1	0	–
Chr16:46723075	rs746704070	5′UTR	c.-30G>A	–	3.20E-05	1	0	–
Carriers (*n*)						65	32	
Frequency (%)						9.02	8.77	
SKAT-O	*P* = 0.38 (adjusted by age, gender, and APOE ε4)							

Functional predictions using 24 online software including: SIFT, PolyPhen2-HDIV, PolyPhen2-HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, VEST3, MetaSVM, MetaLR, M-CAP, CADD, DANN, fathmm-MKL, Eigen, GenoCanyon, fitCons, GERP++, phyloP, phastCons, SiPhy, REVEL, and ReVe; damaging/total is the ratio of online software that predict the variant as damaging among the 24 online software. Transcript NM_018206 was used for VPS35 variant nomenclature. AA: Amino acid; AD: Alzheimer's disease; APOE: apolipoprotein E; cDNA: complementary deoxyribonucleic acid; dbSNP: dbSNP137 (https://www.ncbi.nlm.nih.gov/snp/); MAF gnomAD: the minor allele frequency of variants in the genome aggregation database; NC: normal control; SKAT-O: sequence kernel association test-optimal; 3′UTR: 3′ untranslated region; 5′UTR: 5′ untranslated region. VPS35: vacuolar protein sorting 35 homolog.

which has been further confirmed by Bhalla et al. (2012), who observed that *VPS35* deficiency was associated with increased levels of Aβ. Additionally, VPS35 is significantly reduced in primary tauopathy like progressive supranuclear palsy and Picks' disease, and is considered as a new potential target for therapy of human tauopathies (Vagnozzi et al., 2019). However, Vardarajan et al. (2012) have analyzed the association between AD and 15 genes related to retromer function in a case-control study recruiting 8309 Caucasian AD cases and 7366 normal individuals, among which, four genes showed significant association, whereas no significant difference in *VPS35* variants was observed between the AD cases and the controls. Taken together with our results, we infer that currently there is no significant evidence proving that *VPS35* variants are associated with the genetic susceptibility to AD.

It should be noted that there were several limitations in the present study. First, the number of participants was relatively small, which to some degree may contribute to the negative results. Second, all patients were clinically diagnosed with AD, but because we did not have access to histopathological evidence in this study, the enrolled patients fulfilling clinical criteria were labeled as probable AD with relatively high specificity. Additionally, the study subjects were mostly from southern China and all the patients were from one center; therefore, further studies on subjects with different ethnicities from different centers are warranted.

In summary, to our knowledge, this is the first reported study that investigated the association between Chinese AD cases and variants of the *GBA*, *SNCA*, and *VPS35* genes using a targeted gene sequencing panel. Our results suggest that these genes do not play important roles in the genetic susceptibility to AD in the Chinese population. Because of the relatively small number of eligible participants in our study, further studies are needed to assess the association between *GBA*, *SNCA*, and *VPS35* and AD. Additionally, further studies involving more genes, genetic interactions, mRNA expression, and other gene products (e.g., proteins and other RNA types) in larger cohorts with different ethnicities are essential to identify more shared mechanisms between AD and PD.

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Reporting statement: *This study followed the STrengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidance for protocol reporting.*
Biostatistics statement: *The statistical methods of this study were reviewed by the epidemiologists of Central South University.*

Data sharing statement: *All related data can be accessed by contacting the corresponding author via jbin0911@163.com.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Additional files:

Additional file 1: *Ethics approval document (Chinese).*
Additional file 2: *Informed consent form (Chinese).*
Additional file 3: *STROBE checklist.*
Additional Table 1: *Ultra-rare variants (MAF < 0.001) in gene-based SKAT-O test.*
Additional Table 2: *Rare pathogenic variants (MAF < 0.01, LoF or ReVe > 0.7) in gene-based SKAT-O test.*

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STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation	
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	Page 1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Page 1
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Page 1-2
Objectives	3	State specific objectives, including any prespecified hypotheses	Page 1-2
Methods			
Study design	4	Present key elements of study design early in the paper	Page 2-3
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Page 2
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	Page 2-3
		(*b*) For matched studies, give matching criteria and the number of controls per case	Page 3
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Page 3
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Page 3
Bias	9	Describe any efforts to address potential sources of bias	Page 3
Study size	10	Explain how the study size was arrived at	Page 3
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Page 4
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	Page 4
		(*b*) Describe any methods used to examine subgroups and interactions	Page 4
		(*c*) Explain how missing data were addressed	NA
		(*d*) If applicable, explain how matching of cases and controls was addressed	NA
		(*e*) Describe any sensitivity analyses	NA
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Page 4
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Page 4
		(b) Indicate number of participants with missing data for each variable of interest	NA
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	Page 4
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Page 4-5
		(*b*) Report category boundaries when continuous variables were categorized	Page 4
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Page 5
Discussion			
Key results	18	Summarise key results with reference to study objectives	Page 5-7
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	Page 5-7
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Page 5-7
Generalisability	21	Discuss the generalisability (external validity) of the study results	Page 5-7
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	#Page 1

"#Page 1" is included in the file named "Title PageFirst Page File", others are in the file named "Blinded Article file"

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Additional Table 1 Ultra-rare variants (MAF < 0.001) in gene-based SKAT-O test

Gene	Number of variants	P-value	P’
SNCA	32	0.57	0.73
GBA	28	0.31	0.25
VPS35	38	0.21	0.20

APOE: Apolipoprotein E; *GBA*: glucocerebrosidase; MAF: minor allele frequency; P’: P-value after the adjustment of age, gender, and *APOE* ε4 status; SKAT-O: sequence kernel association test-optimal; *SNCA*: synuclein alpha; *VPS35*: vacuolar protein sorting 35 homolog.

Additional Table 2 Rare pathogenic variants (MAF < 0.01, LoF or ReVe > 0.7) in gene-based SKAT-O test

Gene	Number of variants	P-value	P’
SNCA	2	0.43	0.48
GBA	4	0.66	0.57
VPS35	1	0.62	0.90

APOE: Apolipoprotein E; *GBA*: glucocerebrosidase; MAF: minor allele frequency; LoF: loss of function; P’: P-value after the adjustment of age, gender, and *APOE* ε4 status; SKAT-O: sequence kernel association test-optimal; *SNCA*: synuclein alpha; *VPS35*: vacuolar protein sorting 35 homolog.