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Short Communication

Potential Novel Genes for Late-Onset Alzheimer's Disease in East-Asian Descent Identified by *APOE*-Stratified Genome-Wide Association Study

Sarang Kang[a,b,1], Jungsoo Gim[a,b,c,d,1,*], Jiwoon Lee[d], Tamil Iniyan Gunasekaran[a,d], Kyu Yeong Choi[a], Jang Jae Lee[a], Eun Hyun Seo[a,e], Pan-Woo Ko[f], Ji Yeon Chung[a,g], Seong-Min Choi[a,h], Young Min Lee[i], Jee Hyang Jeong[j], Kyung Won Park[k], Min Kyung Song[a,l], Ho-Won Lee[f], Ki Woong Kim[m], Seong Hye Choi[n], Dong Young Lee[o], Sang Yun Kim[p], Hoowon Kim[a,g], Byeong C. Kim[a,h], Takeshi Ikeuchi[q] and Kun Ho Lee[a,b,c,d,r,*]

[a]*Gwangju Alzheimer's & Related Dementias Cohort Research Center, Chosun University, Gwangju, Republic of Korea*
[b]*Department of Integrative Biological Sciences, Chosun University, Gwangju, Republic of Korea*
[c]*Neurozen Inc., Seoul, Republic of Korea*
[d]*Department of Biomedical Science, Chosun University, Gwangju, Republic of Korea*
[e]*Department of Premedical Science, Chosun University College of Medicine, Gwangju, Republic of Korea*
[f]*Department of Neurology, Kyungpook National University School of Medicine, Daegu, Republic of Korea*
[g]*Department of Neurology, Chosun University Hospital, Gwangju, Republic of Korea*
[h]*Department of Neurology, Chonnam National University Medical School, Gwangju, Republic of Korea*
[i]*Department of Psychiatry, Pusan National University School of Medicine, Busan, Republic of Korea*
[j]*Department of Neurology, Ewha Womans University Mokdong Hospital, Ewha Womans University School of Medicine, Seoul, Republic of Korea*
[k]*Department of Neurology, Donga University College of Medicine, Busan, Republic of Korea*
[l]*Chonnam National University Gwangju 2nd Geriatric Hospital, Gwangju, Republic of Korea*
[m]*Department of Neuropsychiatry, Seoul National University Bundang Hospital, Seongnam, Republic of Korea*
[n]*Department of Neurology, Inha University School of Medicine, Incheon, Republic of Korea*
[o]*Department of Neuropsychiatry, Seoul National University Hospital, Seoul, Republic of Korea*
[p]*Department of Neurology, Seoul National University Bundang Hospital, Seongnam, Republic of Korea*
[q]*Department of Molecular Genetics, Brain Research Institute, Niigata University, Niigata, Japan*
[r]*Korea Brain Research Institute, Daegu, Republic of Korea*

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[1]These authors contributed equally to this work.

*Correspondence to: Jungsoo Gim, Department of Biomedical Science, Chosun University, Gwangju 61452, Republic of Korea. E-mail: jgim@chosun.ac.kr and Kun Ho Lee, Department of Biomedical Science, Chosun University, Gwangju 61452, Republic of Korea. Tel.: +82 62 230 6246; Fax: +82 62 230 7791; E-mail: leekho@chosun.ac.kr.

Abstract. The present study reports two novel genome-wide significant loci for late-onset Alzheimer's disease (LOAD) identified from *APOE* ε4 non-carrier subjects of East Asian origin. A genome-wide association study of Alzheimer's disease was performed in 2,291 Korean seniors in the discovery phase, from the Gwangju Alzheimer's and Related Dementias (GARD) cohort study. The study was replicated in a Japanese cohort of 1,956 subjects that suggested two novel susceptible SNPs in two genes: *LRIG1* and *CACNA1A*. This study demonstrates that the discovery of AD-associated variants is feasible in non-European ethnic groups using samples comprising fewer subjects from the more homogeneous genetic background.

Keywords: Alzheimer's disease, *APOE*, genome-wide association study, late-onset Alzheimer's disease, stratified genome analysis

INTRODUCTION

As a genetic component that has been consistently identified as an important antecedent factor in Alzheimer's disease (AD), Apolipoprotein E (*APOE*) is the most prevalent risk factor of the disease [1, 2]. The effect of the *APOE* genotype, however, varies greatly among different ethnicities [3]. For instance, the ε4 allele of *APOE*, which is the primary genetic risk factor for AD, is absent among Arabs from the northern Israeli community residing in Wadi Ara village, even though the prevalence of dementia is approximately twice than that reported in Europeans [4]. Since the identification of the only locus in *APOE* using a sample consisting of 1,086 individuals in 2007 [5], genome-wide association studies (GWASs) have increased the sample size to improve the statistical power in identifying the missing causal variants for late-onset AD (LOAD) [6, 7]. The latest and largest GWAS includes 1,126,563 cases and controls (including 364,859 proxies) [8]. The variants and associated genes from these GWASs were mostly identified in individuals of European ancestry and found enriched in various biological pathways, including immune responses and endocytosis. These studies, thus suggest the genetic heterogeneity or the complex nature of the LOAD.

Genetic studies in diverse populations have increased our understanding of the genetic architecture of LOAD [9–14]. Studies involving subjects from population with non-European ancestry might be useful in the discovery of variants that are rare or absent, thus showing a smaller effect size in European ancestry [15]. Notably, the discovery of AD-susceptible loci that display allelic heterogeneity among diverse population, has been accomplished using samples from a more homogeneous non-European genetic background with several thousand or much fewer subjects [11, 12].

The East Asian population with Korean ethnicity is a homogeneous population that maintains a distinct genetic profile with a high prevalence of AD among seniors (65 years and older). In the present study, we leverage this distinct genetic architecture of Korean population to discover AD-associated variants. For this purpose, we first performed a GWAS and a *post-hoc* analysis by performing the *APOE* stratification of Koreans using the Korea Biobank SNP Array (referred to as Korean Chip) designed for Koreans [16]. The findings were further replicated by combining genotype level data using the findings from another East Asian population [12].

MATERIALS AND METHODS

Study sample

We analyzed the genotype level data of 2,291 subjects (1,119 AD cases and 1,172 controls) for the discovery stage, and 1,956 subjects (980 AD cases and 976 controls) for the replication stage (Table 1). Participants for the discovery stage were sampled from the Gwangju Alzheimer's & Related Dementias (GARD) cohort, a longitudinal single-center study designed to develop clinical, imaging, genetic, and other biomarkers for early detection and tracking of dementia (mostly AD) from local senior citizens aged 60 years or older in Gwangju, Republic of Korea (Supplementary Material). The study protocol was approved by the Institutional Review Board of Chosun University Hospital, Gwangju, Republic of Korea (CHOSUN 2013-12-018-070). Written informed consents were obtained from all the volunteers or authorized guardians for cognitively impaired subjects before participation.

Clinical evaluation was performed through brain imaging of the subjects using the Seoul Neuropsychological Screening Battery (SNSB) [17] test and 3T MRI (Skyra, Siemens Healthineers Ltd., Seoul, Republic of Korea) single scanner. ^{18}F-Florbetaben (FBB) amyloid-beta positron emission tomography (PET) was performed for those who agreed. All

Table 1
Summary of demographic information for each group in GWAS analyses

	Discovery ($n = 2,291$)		p	Replication ($n = 1,956$)		p
	CN	AD		CN	AD	
All samples, n	1,172	1,119		976	980	
Female, n (%)	661 (56.4)	715 (63.9)	<0.001	564 (57.8)	702 (71.6)	<0.001
Age at exam, m (s.d.)	76.03 (8.9)	74.60 (8.9)	<0.001	76.94 (5.9)	72.99 (4.3)	<0.001
APOE ε4 non-carrier, n	976	621		815	435	
Female, n (%)	544 (55.7)	393 (63.3)	0.003	470 (57.7)	31 (71.3)	<0.001
Age, m (s.d.)	76.10 (8.9)	75.43 (9.1)	0.029	77.11 (5.9)	73.42 (4.3)	<0.001
APOE ε4 carrier	196	498		161	545	
Female, n (%)	117 (59.7)	322 (64.7)	0.222	94 (58.4)	392 (68.3)	0.001
Age, m (s.d.)	95.45 (8.9)	73.32 (9.0)	<0.001	76.06 (5.9)	72.65 (4.3)	<0.001

CN, cognitive normal; AD, Alzheimer's disease; s.d., standard deviation.

diagnoses were evaluated by dementia specialists in neurology and psychiatry at Chosun University Hospital and Chonnam National University Hospital, Gwangju, Republic of Korea. Cognitively normal (CN) subjects exhibited no evidence of neurological disorders and impairment in cognitive function or routine activities. Excluded subjects included those with less than three years of education, a history of brain disease, and poor mental health in addition to those who were on related medications, consuming high levels of alcohol, or diagnosed with depression. All cases were at least 60 years old and fulfilled the NINCDS-ADRDA criteria for AD [18]. The healthy controls were at least 70 years old and either judged to be cognitively normal or did not meet the pathological criteria. The same inclusion criteria of age used in the discovery stage (case over 60 years and control over 70 years) was identically applied in the replication study. Demographic information about the samples used in the replication analysis is summarized in Table 1. Further details about the replication cohort can be found in the study by Miyashita et al. [12].

Genotyping, imputation, and gene mapping

Extensive quality control measures used in the standard protocol [19] were employed and subjects with a low genotyping success rate (95%), heterozygosity outliers, and those that presented cryptic relatedness were excluded. The study population heterogeneity was assessed by means of principal component analysis (PCA) and multidimensional scaling (MDS) and the subjects that did not meet the criteria were eliminated before proceeding with further analyses. Subjects with SNPs with a minor allele frequency < 1%, a genotyping success rate < 95%, or a deviation of the genotype distribution from Hardy-Weinberg equilibrium in the control group ($p < 1 \times 10^{-6}$) were also excluded. To increase the genotyping coverage between the two datasets, missing genotypes and SNPs were imputed using pre-phased reference haplotypes from the Haplotype Reference Consortium (HRC) panel version 1.1 for each dataset. All the imputed SNPs were subjected to the same quality criteria described above in addition to the requirement of imputation quality with an info score > 0.5, resulting in 35,685,761 and 39,044,005 SNPs for discovery and replication analysis, respectively. Functional mapping of SNPs to genes was conducted using a gene annotation file from Affymetrix [16] and the SNPnexus web tool [20].

Statistical analysis

Power calculations for our genome-wide study were performed using Genetic Association Study (GAS) Power Calculator [21]. Demographic information with the statistical significance was determined by *t*-test or Chi-square test. A set of GWAS analyses for AD status were performed in three models of a two-stage framework (discovery and replication): 1) all samples; 2) *APOE* ε4 carriers; and 3) *APOE* ε4 non-carriers. The GWAS was performed separately in each model for both the discovery and replication stages. The association of genotype dosages of each SNP in the additive component with the AD case-control status was estimated using logistic regression analysis adjusting for age, sex, and first four principal components using PLINK and EIGENSTRAT software [22–24]. Only the SNPs that attained a suggestively significant association level ($p < 5 \times 10^{-5}$) were tested in the replication stage using a Japanese dataset. A cohort of 4,247 total subjects was assembled for meta-analysis and SNP effect estimates along with their standard errors (SEs) were

combined by a fixed effect model with inverse variance weighted method using the METAL software [25]. The SNP heritability, which is defined as the fraction of the phenotypic variance explained by additive effects of a given set of genetic variants (or SNPs), was estimated using the GCTA software [26, 27].

Gene expression analysis

The mRNA and protein expression in normal human tissues were reviewed via using data generated by Genotype-Tissue Expression (GTEx, release v8) (https://gtexportal.org/home/) [28] and Human Protein Atlas (HPA) (http://www.proteinatlas.org/) [29, 30], and Functional Annotation of Mammalian Genomes 5 (FANTOM5) [31]. We evaluated the genotype-specific expression of rs2280575 via expression quantitative trait loci (eQTL) analysis through the GTEx. In addition, differential expression (DE) analysis of *CACNA1A* and *LRIG1* was performed using a publicly available expression dataset (GEO accession number: GSE118553) [32]. The dataset consists of various brain tissues from 167 AD cases (including both asymptomatic and dementia stage) and 100 CN controls. We used a linear regression model adjusting for age and sex to examine the differences among disease status.

RESULTS

Genome-wide inference for AD status

For discovery, we first conducted GWAS with the GARD sample, which included 2,291 subjects and identified 54 genome-wide significant ($p < 5 \times 10^{-8}$) SNPs across three genes namely *APOE*, *PVRL2*, and *TOMM40* (genomic inflation = 1.03), which were found to be associated with LOAD in previous studies (Table 2, Supplementary Figure 1A, B). The heritability of LOAD was estimated without these genes using a liability-threshold model [27, 33] with the set of SNPs excluding the loci annotated in these genes; and a relatively large estimate (0.566 ± 0.152) was obtained. In addition, a portion of SNPs in other previously known genes, such as *ABCA7* and *BIN1*, was replicated at a genome-wide suggestive level ($p < 5 \times 10^{-5}$) (Supplementary Figure 1A). These results suggest that unreported additional common variants with smaller effects (or possibly rarer variants with larger effects) may be identified through a *post-hoc* analysis with a less stringent statistical significance. Furthermore, the rs11218343 SNP of *SORL1*, which was previously identified in a Japanese cohort [12], has been confirmed in this study through replication analysis with a significance of $p = 5.9 \times 10^{-4}$.

We carried out *APOE*-stratified GWAS using subgroups of subjects on the basis of *APOE* ε4 carrier status. A total of 219 variants (annotated in 61 genes) and 306 variants (annotated in 82 genes) were identified as ε4 carriers (genomic inflation = 1.02) and non-carriers (genomic inflation = 1.03) with statistical significance respectively. Relaxing statistical significance might result in increases false discoveries. Accordingly, an independent cohort dataset was incorporated in our study for replication. For valid novel discovery, a meta-analysis was performed with the replication dataset in which previous GWAS signals were filtered. This analysis resulted in the examination of 7 out of 61 and 15 out of 82 genes for *APOE* ε4 carriers and non-carriers, respectively. Considering the effect direction and statistical significance of the variants in both the discovery and replication datasets, *CACNA1A* (OR = 1.726, lead SNP rs189753894) and *LRIG1* (OR = 0.539, lead SNP rs2280575) SNPs from the ε4 non-carrier association result were selected for further functional annotation (Table 2). Additionally, we evaluated the expected power of the ε4 non-carrier findings in our discovery study. Given the prevalence of dementia at 0.087 and a SNP in LD (D' = 1) with a risk allele frequency 0.082, the power was estimated at 85% with significant association at $p = 5 \times 10^{-5}$ under an additive model with strong effect size of OR 1.8.

Functional annotation of CACNA1A and LRIG1

Tissue specificity analysis with publicly available databases (GTEx, FANTOM5, and HPA) shows that *CACNA1A* is expressed at both the transcript and protein levels in brain tissues, particularly in the cerebellum (Supplementary Figure 1C, Fig. 1A). The *CACNA1A* gene plays an important role in neuronal cell death and codes subunits of neuronal calcium channels, which are involved in the neuronal cell death and amyloid-beta toxicity [34, 35].

Expression levels of *LRIG1* were high in the cerebral cortex at 74.1 protein-coding transcripts per million (pTPM) in HPA while 16.0 *LRIG1* pTPM were reported by GTEx (Supplementary Figure 1D). In the FANTOM5 dataset, the RNA expression levels of *LRIG1* were higher in the brain tissues than in other tissues, especially for the hippocampal formation, which was found to be 179.9 scaled-tags per

Table 2
Top-ranked genome-wide association results in the Korean discovery sample ($p < 5 \times 10^{-5}$) and their replication in Japanese and meta-analyses

Gene	Chr	Lead SNP (type)*	BP	m/M	MAF	Discovery		Replication		Meta-analysis		Previously reported GWAS			
						OR (95% CI)	*p*	OR (95% CI)	*p*	OR	*p*	GENE (SNP)	*P*	ref	Alt Allele Frq†
All samples (CN/AD)						(1,172/1,119)		(976/980)							
APOE	19	rs429358 (m)	45411941	C/T	0.169	3.637 (3.03–4.37)	3.74×10^{-43}	2.608 (2.04–3.33)	1.89×10^{-14}	3.227	5.90×10^{-55}	rs429358	1.4×10^{-546}	[9]	0.036
TOMM40	19	rs10119 (3')	45406673	A/G	0.180	3.378 (2.83–4.03)	9.58×10^{-42}	2.230 (1.88–2.65)	7.49×10^{-20}	2.731	1.59×10^{-57}	rs10119	1.2×10^{-342}	[46]	0.279
PVRL2	19	rs12972156 (i)	45387459	G/C	0.151	2.942 (2.45–3.54)	1.52×10^{-30}	2.656 (2.06–3.43)	5.42×10^{-14}	2.841	8.30×10^{-43}	PVRL2 (rs412776)	2.2×10^{-21}	[9]	0.001
APOC1	19	rs10414043 (g)	45415713	A/G	0.143	3.082 (2.55–3.73)	3.92×10^{-31}	2.629 (2.02–3.43)	8.76×10^{-13}	2.920	4.29×10^{-42}	rs10414043	1.2×10^{-522}	[46]	0.109
APOE ε4 non-carrier (CN/AD)						(976/621)		(815/435)							
CACNA1A	19	rs189753894 (i)	13624489	A/C	0.082	1.726 (1.34–2.23)	2.68×10^{-5}	1.900 (1.35–2.67)	2.22×10^{-4}	1.787	$\mathbf{2.49 \times 10^{-8}}$	–	–	–	0.007
LRIG1	3	rs2280575 (i)	66542863	G/A	0.092	0.539 (0.41–0.71)	1.15×10^{-5}	0.551 (0.40–0.76)	3.46×10^{-4}	0.544	$\mathbf{1.51 \times 10^{-8}}$	–	–	–	0.267
APOE ε4 carrier (CN/AD)						(196/498)		(161/545)							

*m, missense; I, intronic; g, intergenic; 3', 3' UTR; †SNP alternative allele frequencies of Europeans reported in the allele frequency aggregator (ALFA) [47]. Chr, chromosome; BP, base-pair position; m/M, minor allele/major allele; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; ref, reference.

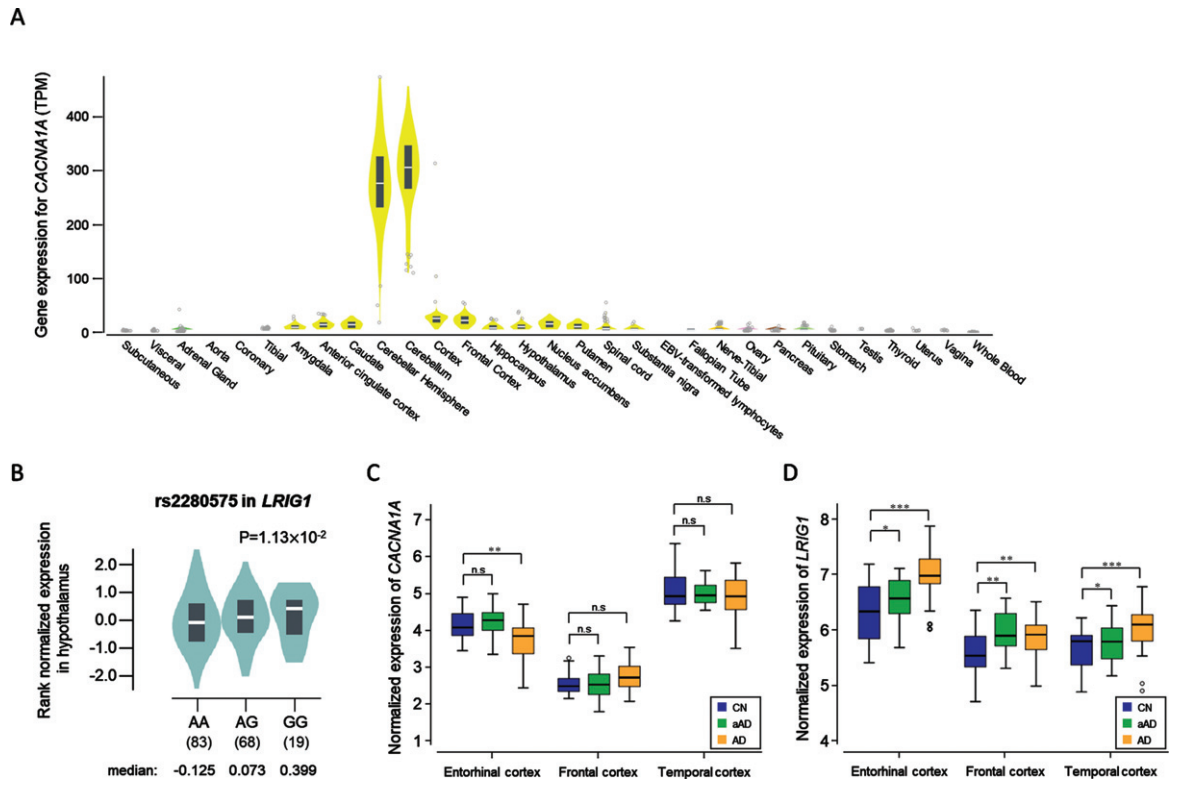

Fig. 1. The expression level of two genes that show significant differences by tissue, genotype, and diagnosis. A) The *CACNA1A* is expressed at a high level in the cerebellum (median TPM: 304.8) and in the cerebellar hemisphere (median TPM: 275.0). B) Violin plot showing the effect of the eQTL rs2280575 on *LRIG1* expression in the hypothalamus ($p = 1.13 \times 10^{-2}$). C) The *CACNA1A* gene showed significant difference in expression between CN and AD in the entorhinal cortex ($p = 0.0019$). D) The *LRIG1* gene showed significant differences in expression between the pairs among CN, aAD, and AD in the entorhinal cortex, frontal cortex, and temporal cortex. The significance was labeled above the box plots ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). CN, cognitive normal; AD, Alzheimer's disease dementia; aAD, asymptomatic AD; n.s, not significant.

million. eQTL analysis with lead SNP rs2280575 in *LRIG1* showed significant results in the hypothalamus (Fig. 1B). The association between *LRIG1* and the hippocampus was evaluated using *in vitro* and *in vivo* analyses from the previous study by Alsina et al. [36].

From the DE analysis with a public dataset (GSE 118553), the gene expression level of *CACNA1A* presented a significant difference ($p = 0.0019$) only in the entorhinal cortex between CN and AD (Fig. 1C), while the expression of *LRIG1* is significantly increased even in cases of asymptomatic stage AD (denoted aAD) for all three tissues analyzed (Fig. 1D).

DISCUSSION

In the present study, we have conducted genome-wide analysis with a Korean sample to identify AD-associated loci. Our study validated the association between AD status and *APOE* locus in chromosome 19 with very strong genome-wide significance. In addition to *APOE*, *SORL1* has also been identified as a potential risk factor for AD with highly significant association in certain East Asian population [12, 37]. However, this highly significant association of *SORL1* with AD is greatly influenced by the presence or absence of ε4 allele of *APOE*. To mask the effect of *APOE* and identify novel candidate loci in East Asian population, we further carried out an *APOE* ε4-stratified GWAS with two independent East Asian ethnic populations. Our data identified *CACAN1A* (lead SNP rs189753894) and *LRIG1* (lead SNP rs2280575) among *APOE* ε4 non-carriers as novel candidate loci associated with AD.

From the functional annotation and literature survey, we found that the two genes play an essential role in neurobiological function. *CACNA1A* is a candidate risk gene located on chromosome 19. However,

it is not likely to be influenced by the *APOE* signal because *CACNA1A* is located far (more than 32 Mb) from the *APOE* locus and is identified with *APOE* non-carriers. *CACNA1A,* a subunit of the neuronal calcium channel, is predominantly and specifically expressed in brain tissue and is found to be involved in neuronal cell death and toxicity of amyloid-beta, suggesting a plausible risk gene for AD [34, 35]. The genetic function of *LRIG1* is associated with the dendritic formation of neurons as well as affecting the hippocampus neural cell functions [36]. Abnormal expression and dysfunction of *LRIG1* lead to dendritic abnormalities, which are involved in morphogenesis of hippocampal dendrites by brain-derived neurotrophic factor (BDNF) signaling. BDNF plays a major role in the growth, development, and survival of neurons. It is also known to be involved in regulating synaptic plasticity and synaptogenesis for learning and memory in the adult brain [38–40]. BDNF is expressed in low levels in patients with AD [41]. Several studies have shown that high expression of BDNF could slow down cognitive decline in the elderly and in patients with AD [38, 42–44].

This study paved the way for discovery of the novel loci in *CACNA1A* and *LRIG1* genes that could not be revealed in large-scale GWASs using Caucasian subjects. The respective 0.082 and 0.007 allele frequencies of rs18973894 SNP of *CACNA1A* in Korean and European populations indicate that there is a significant difference in the association of this SNP with AD among these populations. A meta-analysis with a far larger sample size of Caucasians from Alzheimer's Disease Sequencing Project (ADSP) in NIAGADS demonstrated significant genome-wide difference in the signals for AD risk (3:66455703 in *LRIG1* and 19:13395952 in *CACNA1A* showed $p = 6.2 \times 10^{-12}$ and $p = 3.8 \times 10^{-27}$, respectively) of both genes [45].

There are certain limitations to this study that need to be considered. The sample size in this study is relatively smaller than those in other GWASs for AD. Also, this study used only Korean and Japanese population and does not include other East Asian ethnic groups. However, the findings of this study are promising due to the high reliability of accurate diagnostic information based on pathophysiology available in the GARD as well as Japanese datasets. Another limitation of this study is that the results are based on the imputed SNPs. Although the genotype imputation is likely to be inaccurate for the rare variants, the top findings in this work are common variants. Moreover, we have also conducted a replication analysis with an independent sample to improve the accuracy of these findings.

Although the previous studies about the function of *CACNA1A* and *LRIG1* suggest their role in the development of LOAD, a conclusion whether each gene has an impact on the progression of dementia or protecting neurons from degeneration requires further biological validation with an AD model system. We also acknowledge that the conclusions drawn in this study are based on the analysis of two East Asian populations (Korean and Japanese), and therefore, one is required to be cautious during the interpretation of the results to conclude whether our findings are specific to the East Asian or Korean and Japanese populations. A further replication analysis or meta- or mega-analysis with a larger sample size of other East Asian individuals is strongly recommended to conclusively arrive at such an interpretation.

In conclusion, we validated a portion of previously reported LOAD-associated genes, identified from mostly the Caucasian subjects, in the Korean population. We have also identified and replicated East Asian-specific novel loci in *CACNA1A* and *LRIG1* through a *post-hoc* GWAS with *APOE* stratification. Our findings thus, provide an improved understanding about the complex genetic signatures associated with LOAD.

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SUPPLEMENTARY MATERIAL

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