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Validating GWAS-Identified Risk Loci for Alzheimer's Disease in Han Chinese Populations

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Abstract In recent years, genome-wide association studies (GWASs) have identified many novel susceptible genes/loci for Alzheimer's disease (AD). However, most of these studies were conducted in European and populations of European origin, and limited studies have been performed in Han Chinese. In this study, we genotyped 14 single-nucleotide polymorphisms (SNPs) in eight GWAS-reported AD risk genes in 1509 individuals comprising two independent Han Chinese case-control cohorts. Four SNPs (rs11234495, rs592297, rs676733, and rs3851179) in the *PICALM* gene were significantly associated with late-onset (LO)-AD in populations from Southwest China, whereas SNPs rs744373 (*BIN1*), rs9331942 (*CLU*), and rs670139 (*MS4A4E*) were linked to LO-AD in populations from East China. In the combined Han Chinese population, positive associations were observed between *PICALM*, *CLU*, *MS4A4E* genes, and LO-AD. The association between rs3851179 (*PICALM*), rs744373 (*BIN1*), and AD was further confirmed by meta-analysis of Asian populations. Our study verified the association between *PICALM*, *BIN1*, *CLU*, and *MS4A4E* variants and AD susceptibility in Han Chinese populations. We also discerned some regional differences concerning AD susceptibility SNPs.

Keywords GWAS · Variants · Alzheimer's disease Han Chinese

Hui-Zhen Wang and Rui Bi contributed equally to this study.

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Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease, which mainly leads to memory loss in the elderly population over 65 years old. Extracellular senile plaques (SP), which were mainly composed of amyloid β (Aβ) peptide and intracellular neurofibrillary tangles (NFT) formed by hyperphosphorylated tau protein, are the hallmarks observed in AD brain [1]. It has been widely accepted that AD can be divided into early-onset (EO-AD, <65 years) and late-onset Alzheimer's disease (LO-AD, ≥65 years).

Genetic linkage studies have identified rare mutations in the amyloid precursor protein (*APP*), presenilin 1 and presenilin 2 (*PSEN1* and *PSEN2*) genes in early-onset familial Alzheimer's disease (EO-FAD) [2]. Most of these mutations are inherited in an autosomal dominant manner [3, 4], and can lead to an increased ratio of Aβ42/Aβ40 or Aβ aggregation [5, 6]. However, the etiology of LO-AD is extremely intricate and influenced by complicated interactions between genetic components and environmental risk factors. The *ε4* allele of the apolipoprotein E gene (*APOE*) has been established unequivocally as a susceptible gene for LO-AD, which confers 4–15-fold risk to AD in *ε4* allele carriers than in noncarriers [2]. In recent years, several genome-wide association studies (GWAS) were performed to identify risk gene/loci for LO-AD, and ten novel AD risk genes (*BIN1*, *CLU*, *ABCA7*, *CR1*, *PICALM*, *MS4A6A*, *CD33*, *MS4A4E*, *CD2AP*, and *EPHA1*) were identified [7–11]. Some of these genes were repeatedly identified as AD risk genes in subsequent independent studies [12–14]. In 2011, two large GWASs of European origin populations identified another five potential AD risk genes (*ABCA7*, *MS4A6A*/*MS4A4E*, *CD33*, *CD2AP*, and *EPHA1*); meanwhile, these two GWASs successfully replicated the association between those previously reported loci (*CLU*, *CR1*, *PICALM*, and *BIN1*) and LO-AD [7, 8]. Collectively, these risk genes can explain around 50 % heritability of LO-AD and are involved in pathways of production, degradation, and clearance of Aβ, cholesterol metabolism, immunity, and cellular signaling [2].

It should be noted that these large-scale GWASs about AD were conducted in European and populations of European origin. Several case-control studies had been carried out to validate the potential association of these genes with AD in different populations [12, 13, 15, 16]. Recently, Tan et al. [17] assessed the association of GWAS-linked genes/loci with LO-AD in a Han Chinese population from North China, and they confirmed that the *MS4A6A* and *CD33* genes were associated with LO-AD.

In this study, we aimed to test whether these GWAS-associated AD risk genes confer genetic susceptibility to AD in different Han Chinese populations from Southwest and East China. We screened 14 single-nucleotide polymorphisms (SNPs) of the GWAS-associated AD risk genes in two independent sample sets and verified the association between several loci of those GWAS-reported genes and AD in Han Chinese populations.

Materials and Methods

Subjects

Two independent samples from Southwest China and East China were recruited and analyzed in this study. The cohort from Southwest China was collected at the Mental Health Center of West China Hospital, which was composed of 333 unrelated sporadic LO-AD patients and 334 cognitively healthy controls. The cohort from East China was composed of 416 unrelated sporadic LO-AD patients and 426 cognitively healthy individuals, which was recruited from the Shanghai Mental Health Center and Tongde Hospital of Zhejiang Province. Around 67 % of the AD patient and control samples had been genotyped for the *LRRK2* genetic polymorphisms in our previous study [18]. All participants were of Han Chinese origin. Patients were independently diagnosed by two psychiatrists according to the criteria of DSM-IV and NINCDS-ADRDA [19]. The healthy controls were confirmed as cognitively intact and neurologically normal. All patients were identified as sporadic LO-AD since none of their first-degree relatives had dementia. Written informed consents following the principles of the Declaration of Helsinki were obtained from each participant or guardian. This study was approved by the institutional review board of the Kunming Institute of Zoology, Chinese Academy of Sciences.

SNP Selection and Genotyping

A total of 14 SNPs, including 8 GWAS-reported variants (*BIN1*, rs744373; *CLU*, rs11136000; *ABCA7*, rs3764650; *PICALM*, rs3851179; *MS4A6A*, rs610932; *CD33*, rs3865444; *MS4A4E*, rs670139; *CD2AP*, rs9349407) and 6 tag SNPs (*BIN1*, rs1060743; *CLU*, rs9331942; *ABCA7*, rs3752237; *PICALM*, rs11234495, rs592297, and rs676733), were selected according to the linkage disequilibrium (LD) pattern of the respective genes based on data from HapMap (HapMap, http://hapmap.ncbi.nlm.nih.gov/, phase 3, CHB) and 1000 Genomes (http://www.broadinstitute.org/mpg/snap/). These tag SNPs had a r^2 value >0.8 with the other SNPs and/or were located in different LD blocks with the GWAS hit SNPs. For those genes that span a relatively short genomic region (*MS4A6A*, *CD33*, *MS4A4E*), or tag SNPs that were located in the same LD block with the GWAS hit SNPs (*CD2AP*), we did not analyze tag SNPs but only the GWAS hit SNPs. The detailed information of each SNP is shown in Table 1. We genotyped different alleles of the *APOE* gene,

Table 1 Detailed information of 14 SNPs in eight GWAS-linked genes

Gene	dbSNP rs no.[a]	Allele	MAF[b]	Chr.	Position	Annotation	Source[c]
BIN1	**rs744373**	C/T	0.33	2	127611085	5′ near region	GWAS
	rs1060743	C/T	0.39	2	127542003	Synonymous	Tag SNP
CLU	**rs11136000**	C/T	0.21	8	27520436	Intron 3	GWAS
	rs9331942	C/T	0.34	8	27511031	3′ near region	Tag SNP
ABCA7	**rs3764650**	G/T	0.27	19	997520	Intron 12	GWAS
	rs3752237	A/G	0.16	19	998161	Synonymous	Tag SNP
PICALM	**rs3851179**	A/G	0.47	11	85546288	5′ near region	GWAS
	rs11234495	C/T	0.41	11	85353082	3′ near region	Tag SNP
	rs592297	C/T	0.41	11	85403585	Synonymous	Reported
	rs676733	C/T	0.46	11	85411658	Intron 3	Tag SNP
MS4A6A	**rs610932**	A/C	0.34	11	59695883	3′ near region	GWAS
CD33	**rs3865444**	G/T	0.18	19	56419774	5′ near region	GWAS
MS4A4E	**rs670139**	A/C	0.42	11	59728371	5′ near region	GWAS
CD2AP	**rs9349407**	C/G	0.12	6	47561337	Intron 1	GWAS

[a] SNPs in bold were distilled from previous GWAS reports, and other SNPs were selected according to linkage disequilibrium blocks of the respective gene based on HapMap (http://hapmap.ncbi.nlm.nih.gov/) CHB data set and 1000 Genomes (http://www.broadinstitute.org/mpg/snap/)

[b] Minor allele frequency in CHB data set was obtained from 1000 Genomes database (http://asia.ensembl.org/)

[c] The SNPs marked with "GWAS" were obtained from Alzgene (http://www.alzgene.org/TopResults.asp)

which was a well-established risk gene for LO-AD [20, 21], using the same approach described in our previous study [18].

All these 14 SNPs were genotyped by using the SNaPshot assay, which comprising a multiplex PCR and a subsequent single-base extension process, according to the detailed step-by-step procedure in our previous studies [18, 22]. Briefly, multiplex PCR was carried out in a volume of 8-μL reaction solution containing 20–50 ng genomic DNA, 0.4 mM dNTPs, 0.2–0.5 μM of each primer (Supplementary Table S1), 2.0 mM $MgCl_2$, and 1.0 U of FastStart Taq DNA Polymerase (Roche Applied Science). The amplification program was composed of a pre-denaturation cycle at 94 °C for 2 min; 40 amplification cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and ended with an incubation cycle at 4 °C. Multiplex PCR products were cleaned up at 72 °C for 40 min with 1.0 U of shrimp alkaline phosphatase (SAP) and 0.5 U of Exonuclease I (TaKaRa Biotechnology Co. Ltd., Dalian, China), followed by a final incubation at 96 °C for 10 min to inactivate the enzyme. The single-base extension was conducted in a total of 10-μL reaction solution containing 4-μL multiplex PCR products, 5-μL SNaPshot Multiplex Ready Reaction Mix, and 0.4–0.8-μM pooled SNP-specific oligonucleotide primers (Supplementary Table S1) according to the protocol of the ABI PRISM® SNaPshot® Multiplex Kit (Applied Biosystems). The single-base extension program contained 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. The products were then purified by SAP (1.0 U) at 37 °C for 40 min, followed by a heat inactivation at 75 °C for 20 min. A mixture of 4-μL purified products and 9 μL Hi-Di™ formamide was analyzed by the ABI PRISM™ 3730xl DNA analyzer (Applied Biosystems) at the Kunming Biodiversity Large-Apparatus Regional Center, Kunming Institute of Zoology. The GeneMarker software (http://www.softgenetics.com/GeneMarker.html) was employed to read the genotyping results..

Statistical Analysis and Meta-Analysis

The power analysis was calculated using the Quanto software [23]. The frequency of allele, genotype, and haplotype was analyzed using the PLINK software [24]. Binary logistic regression under the general model was performed by SPSS 16.0 (SPSS Inc., Chicago, Illinois) to assess the association between these SNPs and LO-AD, with an adjustment of *APOE ε4* status (*APOE* $\varepsilon4^+$ or *APOE* $\varepsilon4^-$). A P value <0.05 was regarded as statistically significant. When considering Bonferroni correction for multiple testing, a cutoff of $P<0.0035$ (0.05/14) was set as statistically significant. The deviation from the Hardy-Weinberg equilibrium of each SNP was calculated for both the control and case populations by using the PLINK software [24]; SNPs with a P value less than 0.001 were regarded as departure from the Hardy-Weinberg equilibrium. LD structures of the *BIN1*, *CLU*, *PICALM*, and *ABCA7* genes were reconstructed using Haploview software version 4.2 [25]. Haplotypes were reconstructed by using the PHASE 2.0 program [26]. All the above analyses were performed for the two independent cohorts separately. Considering the fact that these individuals are all of Han Chinese origin and a combined sample may increase the power of the statistical test [27], we pooled all the samples together to investigate

the association between these GWAS hit SNPs and tag SNPs and AD risk even though there existed some potential genetic differences between populations from Southwest China and East China. In addition, we investigated whether these SNPs affect messenger RNA (mRNA) expression level of relevant genes in different tissues in the GTEx database (http://www.gtexportal.org/home/) [28].

We performed meta-analysis for SNPs rs3851179, rs3764650, rs11136000, rs744373, rs610932, rs3865444, and rs9331942 with AD by using the RevMan 5.2 software (http://www.cochrane.org/revman) based on data from this study and reported Asian populations (Supplementary Table S2). Those SNPs with no available data in Asian populations were not considered in the meta-analysis.

Results

Statistical Power and LD Pattern of Analyzed SNPs

As the minor allele frequency (MAF) of all SNPs in this study ranged from 11.9 to 46.4 %, assuming a false positive rate controlled as 0.05, the power to detect the odds ratio (OR) value as 1.5 for risk allele was expected to be from 72.8 to 95.5 % based on the samples from Southwest China. However, when we considered the OR value as 1.2, the power was much reduced, with a value range from 20.0 to 38.2 %.

Total genotyping call rate of all individuals was 99.8 %. The allele and genotype counts for each SNP were shown in Supplementary Table S3. All 14 SNPs analyzed in this study were in Hardy-Weinberg equilibrium in both AD patients and controls (Supplementary Table S3). We validated the genotyping results of SNaPshot assay by direct sequencing 5 % of total samples and obtained consistent results. The ε4 allele of the *APOE* gene showed a significant association with AD risk in both cohorts and in the combined Han Chinese sample (Supplementary Table S4). The LD patterns of these SNPs in the *BIN1* (rs1060743-rs744373), *CLU* (rs9331942-rs11136000), *PICALM* (rs11234495-rs592297-rs676733-rs3851179), and *ABCA7* (rs3764650-rs3752237) genes were similar between AD patients and controls (Fig. 1).

SNPs in the *PICALM* Gene Were Associated with LO-AD in Han Chinese

Genotype frequencies of four SNPs of the *PICALM* gene were significantly different between the case and control populations from Southwest China (rs11234495, $P=0.013$; rs592297, $P=0.035$; rs676733, $P=0.0004$; rs3851179, $P=0.001$), and two of them also showed a significant difference at the allelic level (rs11234495, $P=0.016$, OR=0.767, 95 % confidence interval (CI) 0.618–0.951; rs676733, $P=0.030$, OR=0.782, 95 % CI 0.626–0.976) (Table 2). We failed to discern any positive association of these SNPs with LO-AD in samples from East China. When we combined these two cohorts together, the results remained to be significant (Table 2). Logistic regression analysis revealed that these SNPs were significantly associated with LO-AD in populations from Southwest China and the combined sample after adjustment for the *APOE4* status (Table 2). However, after Bonferroni correction for multiple testing, only SNPs rs676733 and rs3851179 remained to be significantly associated with AD (Table 2) in the cohort from Southwest China. The inconsistence of association between the two independent cohorts might simply be an issue of low power of the study and/or potential region difference.

Haplotype construction based on the four SNPs of the *PICALM* gene revealed that haplotype TTTG (SNP order: rs11234495-rs592297-rs676733-rs3851179) increased the risk of AD in the Southwest China cohort ($P=0.043$, OR=1.256, 95 % CI 1.013–1.558), haplotype CTCA increased the risk of AD in the East China cohort ($P=0.023$, OR=1.620, 95 % CI 1.070–2.454), and haplotype CCCA decreased the risk of AD in the combined population ($P=0.039$, OR=0.848, 95 % CI 0.726–0.991) (Table 3). However, all haplotype associations did not survive the Bonferroni correction for multiple testing, possibly due to the low power of this study.

Intriguingly, the GWAS hit variant rs3851179 and tag SNP rs592297 of the *PICALM* gene were correlated to its mRNA level in brain cingulate cortex (rs3851179, $P=0.01$; rs592297, $P=0.01$) according to expression data in the GTEx database (http://www.broadinstitute.org/gtex/), suggesting that these variants might be functional.

SNP rs3851179 of the *PICALM* Gene Confers Susceptibility to LO-AD in Asian Populations

SNP rs3851179 (*PICALM*) has been reported to be a susceptibility locus for AD in European populations [9–11], whereas recent studies in different Asian populations had controversial results. In three reported Han Chinese studies, rs3851179 was not associated with AD [15, 29, 30], but in one Japanese population, it showed a weak association [31].

To evaluate the association between rs3851179 and AD, we performed a meta-analysis of 7739 individuals from Asian populations, which were composed of the above-mentioned four validation studies and the two cohorts analyzed in this study (Supplementary Table S2). Results of the Cochran's Q test indicated no significant heterogeneity among these populations. The allele test ($Z=2.97$, $P=0.003$, OR=0.90, 95 % CI 0.84–0.96) indicated a significant association between rs3851179 and LO-AD (Fig. 2a).

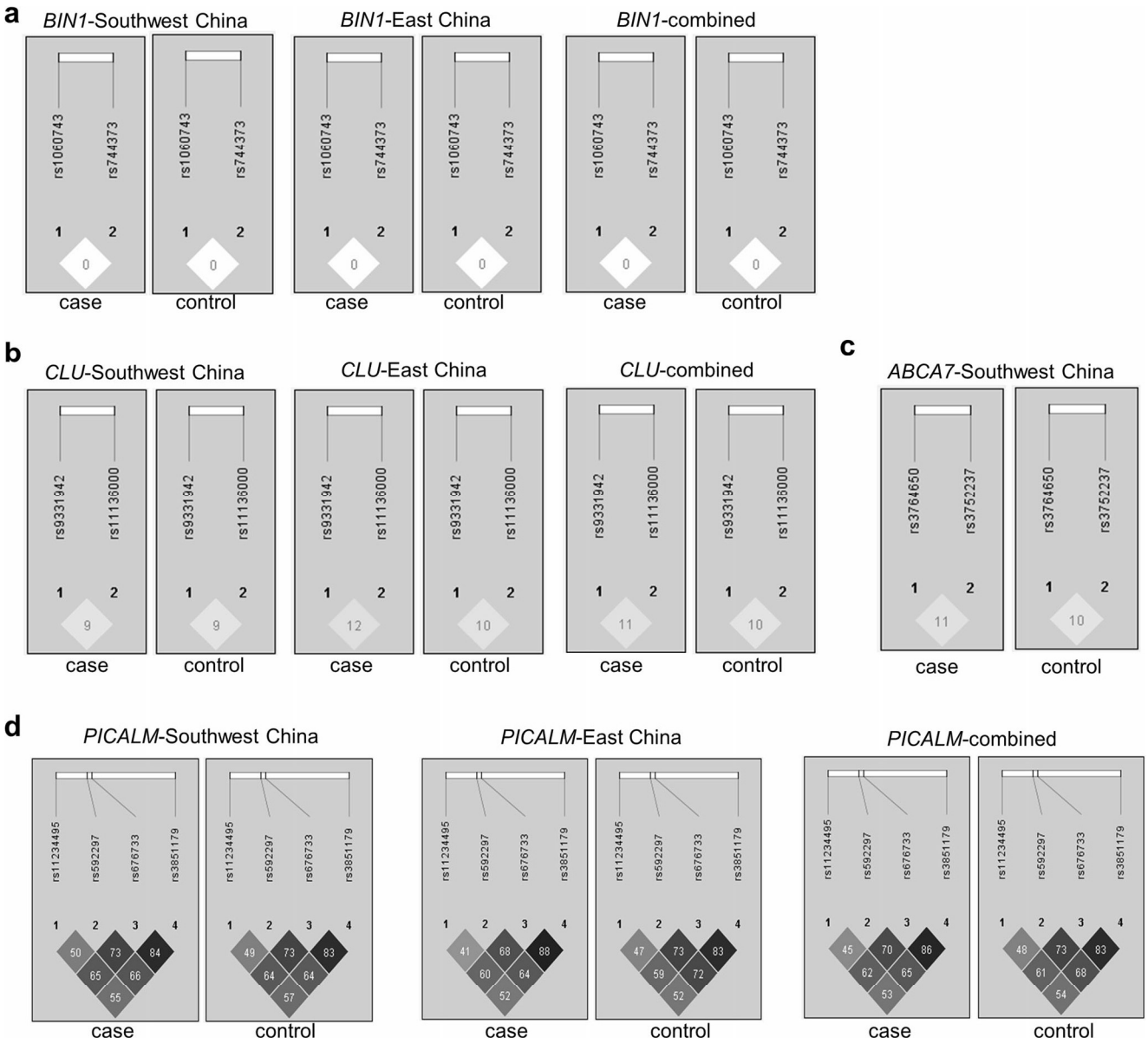

Fig. 1 Linkage disequilibrium (LD) patterns of the *BIN1* (**a**), *CLU* (**b**), *ABCA7* (**c**), and *PICALM* (**d**) genes in AD cases and controls from Southwest China, East China, and combined population. The *value in each square* refers to $r^2 \times 100$

SNPs of the *BIN1* (rs744373), *CLU* (rs9331942), *MS4A4E* (rs670139), and *CD2AP* (rs9349407) Genes Were Associated to LO-AD in Han Chinese

In Han Chinese cohort from East China, SNPs rs744373 (*BIN1*, $P=0.038$), rs9331942 (*CLU*, $P=0.013$), and rs670139 (*MS4A4E*, $P=0.022$) were significantly different between cases and controls at the genotypic level. SNP rs744373 (*BIN1*, $P=0.026$, OR=1.256, 95 % CI 1.028–1.535) and rs9349407 (*CD2AP*, $P=0.037$, OR=1.256, 95 % CI 1.013–1.558) showed a significant difference at the allelic level (Table 2) in the East China cohort and the combined sample, respectively. However, we failed to discern any association between these SNPs (rs744373, rs9331942, rs670139, and rs9349407) and AD in Han Chinese from Southwest China. Note that a positive association of SNPs rs9331942 and rs670139 with LO-AD was observed in the combined Han Chinese populations both before and after adjustment for *APOE4* status. Nonetheless, all these associations were no longer significant after Bonferroni correction for multiple testing.

Haplotype analysis showed that there was no significant difference of haplotype frequency of *BIN1* and *CLU* SNPs between AD cases and controls in both cohorts and the

Table 2 Association between SNPs in GWAS-linked genes and AD in Han Chinese

Gene	SNP ID	Populations	MAF (case/control)	OR (95 % CI)	*P* value (allelic)[a]	*P* value (genotypic)[a]	*P* value trend[a]	Adjusted *P* value[b]
BIN1	rs1060743	Southwest	0.456/0.464	0.969 (0.781–1.202)	0.777	0.692	0.777	0.557
		East	0.445/0.413	1.138 (0.938–1.380)	0.191	0.238	0.184	0.166
		Combined	0.450/0.436	1.060 (0.918–1.224)	0.428	0.715	0.425	0.667
	rs744373	Southwest	0.366/0.361	1.024 (0.820–1.281)	0.832	0.884	0.838	0.874
		East	0.380/0.328	1.256 (1.028–1.535)	0.026	0.038	0.023	0.038
		Combined	0.374/0.342	1.147 (0.989–1.332)	0.071	0.164	0.073	0.187
CLU	rs11136000	Southwest	0.215/0.196	1.125 (0.862–1.468)	0.385	0.280	0.399	0.209
		East	0.210/0.185	1.170 (0.920–1.487)	0.200	0.442	0.207	0.304
		Combined	0.213/0.190	1.150 (0.962–1.374)	0.124	0.185	0.133	0.107
	rs9331942	Southwest	0.297/0.310	0.942 (0.746–1.190)	0.617	0.331	0.622	0.297
		East	0.325/0.356	0.871 (0.711–1.065)	0.178	0.013	0.177	0.017
		Combined	0.312/0.336	0.900 (0.773–1.048)	0.175	0.006	0.177	0.005
PICALM	rs3851179	Southwest	0.377/0.422	0.828 (0.665–1.031)	0.091	0.001[d]	0.085	0.002[d]
		East	0.371/0.385	0.943 (0.774–1.148)	0.557	0.716	0.549	0.771
		Combined	0.374/0.401	0.890 (0.769–1.031)	0.119	0.011	0.112	0.024
	rs11234495	Southwest	0.453/0.520	0.767 (0.618–0.951)	0.016	0.013	0.016	0.019
		East	0.494/0.500	0.976 (0.806–1.182)	0.804	0.139	0.805	0.145
		Combined	0.483/0.509	0.901 (0.781–1.040)	0.153	0.011	0.155	0.019
	rs592297	Southwest	0.298/0.347	0.798 (0.633–1.005)	0.055	0.035	0.055	0.048
		East	0.298/0.331	0.856 (0.696–1.052)	0.140	0.249	0.126	0.268
		Combined	0.298/0.338	0.830 (0.711–0.968)	0.017	0.014	0.015	0.018
	rs676733	Southwest	0.353/0.411	0.782 (0.626–0.976)	0.030	0.0004[d]	0.028	0.002[d]
		East	0.383/0.375	1.036 (0.850–1.262)	0.730	0.891	0.725	0.901
		Combined	0.370/0.391	0.914 (0.789–1.060)	0.234	0.027	0.227	0.066
MS4A6A	rs610932	Southwest	0.374/0.377	0.984 (0.788–1.229)	0.888	0.486	0.887	0.695
		East	0.368/0.365	1.012 (0.830–1.234)	0.906	0.960	0.901	0.820
		Combined	0.370/0.370	1.000 (0.862–1.159)	0.997	0.827	0.997	0.973
CD33	rs3865444	Southwest	0.200/0.189	1.073 (0.818–1.408)	0.609	0.831	0.616	0.617
		East	0.208/0.201	1.042 (0.823–1.321)	0.731	0.930	0.732	0.845
		Combined	0.204/0.196	1.055 (0.883–1.262)	0.554	0.837	0.558	0.667
MS4A4E	rs670139	Southwest	0.438/0.423	1.063 (0.856–1.321)	0.580	0.269	0.585	0.318
		East	0.393/0.419	0.897 (0.738–1.090)	0.273	0.022	0.274	0.049
		Combined	0.413/0.421	0.968 (0.838–1.119)	0.622	0.012	0.664	0.019
CD2AP	rs9349407	Southwest	0.146/0.121	1.235 (0.900–1.696)	0.190	0.327	0.191	0.338
		East	0.135/0.109	1.273 (0.950–1.707)	0.106	0.134	0.105	0.091
		Combined	0.140/0.115	1.256 (1.013–1.558)	0.037	0.108	0.037	0.058
ABCA7[c]	rs3764650	Southwest	0.332/0.311	1.104 (0.877–1.390)	0.401	0.703	0.404	0.939
	rs3752237	Southwest	0.184/0.182	1.014 (0.768–1.339)	0.923	0.630	0.924	0.639

MAF minor allele frequency

[a] *P* value was calculated by PLINK software [24]

[b] Binary logistic regression analysis was performed to assess the association of these GWAS-linked genes with AD, with an adjustment for *APOE4* status

[c] SNPs rs3764650 and rs3752237 were not genotyped in samples from East China

[d] The association of rs3851179 and rs676733 with AD remained significant after Bonferroni correction for multiple testing, in which $P<0.0035$ (0.05/14) was set as statistically significant

combined population (Table 3). Notably, SNPs rs744373 (*BIN1*, $P=0.009$) and rs670139 (*MS4A4E*, $P=0.03$) were found to be significantly associated with their mRNA levels in pituitary, and rs670139 showed a weak association with

Table 3 Haplotype frequencies of SNPs in the *BIN1*, *CLU*, *PICALM*, and *ABCA7* genes in 333 AD cases and 334 healthy controls from Southwest China and in 416 AD cases and 426 normal controls from East China

Haplotype[a]	Southwest China		*P* value	OR (95 % CI)	East China		*P* value	OR (95 % CI)	Combined		*P* value	OR (95 % CI)
	Case	Control			Case	Control			Case	Control		
rs1060743-rs744373 (*BIN1*)												
CC	151	151	1.000	1.004 (0.777–1.297)	198	172	0.078	1.235 (0.980–1.556)	349	323	0.189	1.126 (0.948–1.336)
TC	93	90	0.812	1.042 (0.763–1.424)	117	107	0.389	1.139 (0.860–1.510)	210	197	0.424	1.095 (0.888–1.349)
CT	152	159	0.700	0.947 (0.734–1.220)	172	180	0.857	0.973 (0.769–1.231)	324	339	0.660	0.961 (0.809–1.142)
TT	270	268	0.911	1.018 (0.818–1.267)	345	393	0.056	0.827 (0.682–1.003)	615	661	0.185	0.905 (0.783–1.046)
rs9331942-rs11136000 (*CLU*)												
TT	143	130	0.378	1.132 (0.867–1.477)	174	156	0.197	1.180 (0.927–1.501)	317	286	0.111	1.158 (0.969–1.385)
CC	196	206	0.592	0.935 (0.740–1.182)	269	301	0.198	0.875 (0.715–1.071)	465	507	0.185	0.899 (0.772–1.048)
TC	325	331	0.785	0.970 (0.783–1.203)	388	393	0.845	1.021 (0.843–1.236)	713	724	1.000	0.999 (0.866–1.152)
Rare[b]	2	1			1	2			3	3		
rs11234495-rs592297-rs676733-rs3851179 (*PICALM*)												
CCCA	190	221	0.075	0.807 (0.640–1.019)	240	265	0.338	0.898 (0.729–1.106)	430	486	0.039	0.848 (0.726–0.991)
CTCA	37	42	0.643	0.877 (0.560–1.383)	60	39	0.023	1.620 (1.070–2.454)	97	81	0.216	1.221 (0.901–1.656)
TTTA	20	15	0.398	1.348 (0.684–2.656)	10	15	0.422	0.679 (0.303–1.520)	30	30	1.000	1.008 (0.605–1.681)
CTTG	65	69	0.785	0.939 (0.657–1.342)	103	98	0.599	1.087 (0.810–1.460)	168	167	0.908	1.016 (0.809–1.275)
TTTG	344	307	0.043	1.256 (1.013–1.558)	404	411	0.922	1.013 (0.837–1.226)	748	718	0.202	1.100 (0.954–1.269)
Rare[b]	10	14			15	24			25	38		
rs3764650-rs3752237 (*ABCA7*)												
GG	220	207	0.446	1.099 (0.873–1.383)	–	–	–	–	–	–	–	–
TA	124	123	0.944	1.014 (0.769–1.336)	–	–	–	–	–	–	–	–
TG	322	338	0.412	0.914 (0.737–1.133)	–	–	–	–	–	–	–	–

[a] The haplotype analysis was performed by using PHASE 2.0 program [26]

[b] Haplotypes with a frequency less than 3 % were pooled together

mRNA level of the *MS4A4E* gene in brain cingulate cortex (P=0.07) based on the GTEx database.

Meta-Analysis of the *ABCA7*, *CLU*, *BIN1*, *MS4A6A*, and *CD33* Genes with LO-AD in Asian Populations

Meta-analysis of SNPs rs3764650 (*ABCA7*), rs11136000 (*CLU*), rs9331942 (*CLU*), rs744373 (*BIN1*), rs610932 (*MS4A6A*), and rs3865444 (*CD33*) by using the reported data from Asian populations and data from this study revealed that rs744373 (*BIN1*) and rs11136000 (*CLU*) were significantly associated with LO-AD risk in Asian populations (rs744373: Z=3.22, P=0.001, OR=1.14, 95 % CI 1.05–1.24; rs11136000: Z=2.05, P=0.04, OR=0.93, 95 % CI 0.87–1.00) (Fig. 2b, c), while no significant association was observed for other SNPs (Fig. 2d–g). It should be mentioned that the association pattern of the two *CLU* SNPs were different in our samples and in the result of meta-analysis: rs9331942 was not associated with AD in the meta-analysis but was positively associated with AD in Han Chinese, whereas rs11136000 had a reverse association pattern with rs9331942.

Discussion

GWAS is a burgeoning method to seek novel susceptibility genes/loci for AD. Recently, several GWASs had identified many novel AD susceptibility genes/loci in European and populations of European origin [7–11]. Independent replication studies in different populations are important for interpreting the case-and-effect relationship and the mechanism of genetic susceptibility.

In order to investigate whether the reported AD susceptibility loci confer risk to AD in Han Chinese populations, we screened GWAS hit and tag SNPs in eight GWAS-reported AD risk genes in two independent Han Chinese sample sets. Albeit the sample size of our sample was modest, we were able to validate the association of three SNPs (rs744373 of *BIN1*, rs3851179 of *PICALM*, and rs670139 of *MS4A4E*) with LO-AD in our Han Chinese samples, and the effect direction of these AD risk SNPs was as reported in previous GWAS [7, 8, 10, 11]. SNP rs9349407 of *CD2AP* was marginally associated with LO-AD in population from East China, with similar effect direction as reported in previous studies [7, 8]. The GWAS hit SNP rs11136000 in

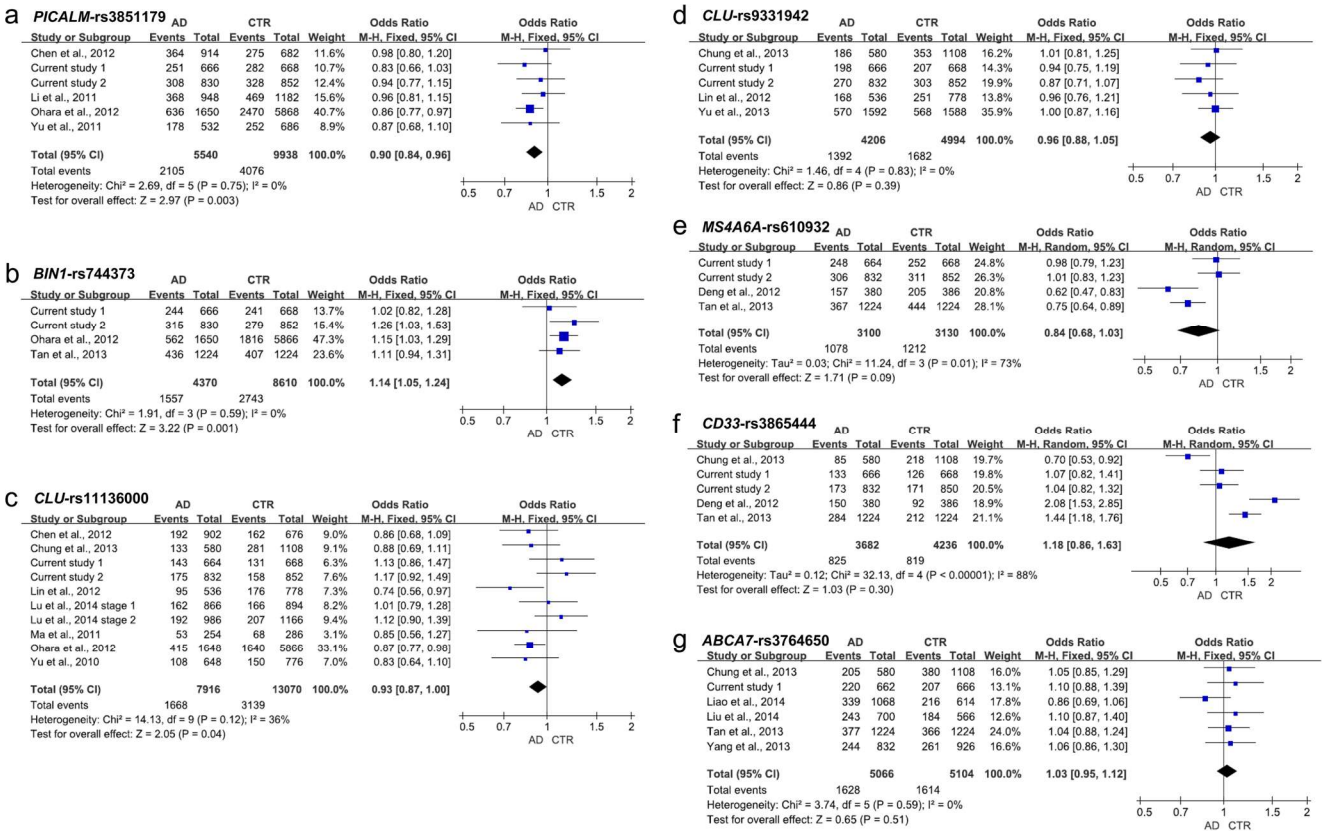

Fig. 2 Meta-analysis of SNPs rs3851179, rs744373, rs11136000, rs9331942, rs610932, rs3865444, and rs3764650 with AD in Asian populations. Allele tests of SNPs rs3851179 (**a**), rs744373 (**b**), rs11136000 (**c**), rs9331942 (**d**), rs610932 (**e**), rs3865444 (**f**), and rs3764650 (**g**) were performed by using the RevMan 5.2 software (http://www.cochrane.org/revman). The detailed information of the reported data from Asian populations is listed in the Supplementary Table S2

the *CLU* gene showed no association with AD in our study, but we observed a positive association of another SNP rs9331942 in this gene with LO-AD.

Of note, the selected *PICALM* tag SNPs in this study (rs11234495, rs592297, and rs676733) were all associated with LO-AD in our Han Chinese population from Southwest China and the combined population, indicating an important role of the *PICALM* gene in AD. Moreover, the association of rs3851179 and rs676733 of the *PICALM* gene with AD risk in the cohort from Southwest China (rs3851179, P=0.002; rs676733, P=0.002, with an adjustment of the *APOE ε4* status) remained to be significant even after Bonferroni correction.

The *PICALM* gene encodes phosphatidylinositol-binding clathrin assembly protein (PICALM), which is a key component in clathrin-mediated endocytosis [32]. As APP must be internalized into cells through endocytosis before being cleaved to Aβ [33], PICALM may play a key role in the Aβ production and release and influence Aβ levels. In this study, all four *PICALM* SNPs (rs3851179, rs11234495, rs592297, and rs676733) were significantly associated with LO-AD. Several haplotypes determined by these SNPs also conferred AD risk. Note that this association did not survive the Bonferroni correction, which can be explained by the limited sample size of this study. The GWAS hit SNP rs3851179 (which is located at 88.5-kb upstream of *PICALM*) and tag SNPs rs11234495 and rs676733 (both are within the intron region) may be linked to some functional variants conferring the etiology of AD. SNP rs592297 is a synonymous variant in exon 5 that may potentially influence exon splicing, which was first reported to be associated with LO-AD in a large-scale GWAS, but the association did not reach statistical significance at the genome level (2×10^{-7}) [11]. Furthermore, according to the GTEx database, SNPs rs3851179 and rs592297 were associated with mRNA expression level of the *PICALM* gene in brain cingulate cortex, which was identified to be involved in learning and memory process [34]. Though the association between rs3851179 and LO-AD has been widely identified in European populations [9–13] and was validated in this study, several replication studies produced inconsistent results in different populations [12, 30, 35]. Meta-analysis of rs3851179 based on the data of this study and previously reported studies [15, 29–31] demonstrated that this SNP indeed conferred susceptibility to LO-AD in Asians, which was in accordance with a recent report by Liu and colleagues [36]. Our result and previous reports supported a notion that the *PICALM* gene was a common susceptibility gene for AD in different populations.

Gene expression data in the GTEx database indicated that the GWAS-associated SNPs in the *BIN1* (rs744373) and *MS4A4E* (rs670139) genes may affect AD risk through their influence on gene expression. SNP rs744373 of *BIN1* was confirmed to be associated with AD risk in different ethnic backgrounds including East Asian and Caucasian populations according to a meta-analysis based on several validation studies [37]. In this study, we also observed an association between rs744373 and AD risk. Furthermore, meta-analysis for rs744373 in Asian populations supported its role in AD. The *BIN1* mRNA expression level was found to be significantly associated with different genotypes of rs744373 in pituitary region by re-analyzing expression data from the GTEx database, which was in line with previous evidence that variant rs59335482 (rs744373 is tightly linked to this SNP) could mediate AD risk by increasing cerebral expression of *BIN1* [38]. In addition, we successfully replicated the association between rs670139 and LO-AD in this study. SNP rs670139 lies within an intergenic region between the *MS4A4E* and *MS4A6A* genes [7]. The gene expression data of the GTEx database indicated a potential association between rs670139 and *MS4A4E* mRNA level in both cingulate cortex and pituitary region. One recent study also demonstrated that rs670139 was associated with AD patient's Braak tangle and Braak plaque score [39]. There may be some other functional variants that were tightly linked with rs670139 and affected the expression and function of nearby genes [40]. SNPs rs9331942 in the 3′ UTR of *CLU* and rs9349407 in the 5′ UTR of *CD2AP* showed an association with AD in the combined Han Chinese sample, which may participate in the transcriptional regulation of the related genes. Further experimental assay should be performed to solidify our speculations.

Failure in replicating GWAS results is a common issue in genetic studies of complex disease, which is influenced by genetic heterogeneity and environmental risk factors [41]. For instance, we recently failed to validate the GWAS hit risk loci for schizophrenia in Han Chinese, albeit these susceptibility loci were initially identified in the same ethnic populations [42]. We encountered similar conditions in this study, in which the association of certain GWAS hit SNPs with AD was inconsistent between the two case-control cohorts from Southwest China and East China (Table 2). The discrepancy may be attributed to several reasons. First, the weak statistical power due to relatively small sample size may account for the inconsistent results in different populations. Second, we only considered the GWAS top hit SNP in each locus and one to four tag SNPs for some of the top hit genes. As the GWAS hit SNPs are most probably not the functional variants, but themselves tag SNPs of the functional variants (in populations from European ancestry). Those top hit SNPs might not correctly tag the functional variants in Han Chinese because the LD pattern differs among populations. Third, the gene-gene and gene-environment interactions may also influence the effect of risk allele between different populations. Genetic heterogeneity of AD may imply more and more risk genes for this disease, as exemplified by the most recent meta-analysis that identified 11 new susceptibility loci [43].

In summary, we verified the association between the *PICALM*, *CLU*, *MS4A4E*, *BIN1* genes and LO-AD in Han Chinese populations, whereas the association of the remaining GWAS hit genes with AD was not validated in our populations. Particularly, meta-analysis revealed that the *PICALM*, *BIN1*, and *CLU* genes were significantly associated with AD risk in Asian populations. One limitation of this study is that we lacked demographical data, such as age, sex, and education year of subjects for all individuals, which disabled us from retrieving more information in the association analyses. Further independent validating studies and essential functional assays are needed to solidify the current conclusions and to characterize the putative role of these genes concerning the production, transport, release, and clearance of Aβ from brain into blood and their participation in innate immunity in the central nervous system.

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Conflict of Interest The authors declare that they have no conflict of interest

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Supplementary Materials

Validating GWAS-identified risk loci for Alzheimer's disease in Han Chinese populations

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Table S1. Primer information of 14 SNPs in 8 GWAS-linked genes

Gene	SNP ID	Primer (5'-3') [a]
BIN1	rs744373	F: AAATTAGGCCTCGCTGGT
		R: AAGTCCTGGTCTGAAAGCC
		E: g$(atcg)_{13}$CACCAGGGACAGGCAGGTCTGAGGC
	rs1060743	F: TGGTGGACTACGACAGTGC
		R: TCCAGCCCTTACCTTGGC
		E: g$(atcg)_{8}$GGCACCACTACGAGTCCCTTCAAAC
CLU	rs11136000	F: TTTCTATTGCAACCATGCCT
		R: ATTCTTACAGAATTATTGGGTCAAGT
		E: atcgACCAAAGCCACACCAGCTATCAAAA
	rs9331942	F: AAAGTTAAGGAGAAGAATCAGTAAGTGTA
		R: ATAATTTGGACTTCTGGGCAC
		E: cg$(atcg)_{4}$GTGTACTTGGTATTATAATGCATAA
ABCA7	rs3764650	F: CTCTGTTGGGAACCTTCCT
		R: AGAGTCCCCTGCTCCTCC
		E: $(atcg)_{7}$CGGTCCAGGCTGCGAACTTTGCACC
	rs3752237	F: AATCCAGGAGCTGCACCC
		R: AGAACAGGAAGACCACGC
		E: CG$(atcg)_{9}$GCTCCCGTTGCCTCTCACAGCTGGG
PICALM	rs3851179	F: AATACTATTACCCGCTTCATAGGG
		R: AGTGTCAGCAGTCAACACACC
		E: $(atcg)_{12}$GCAAACAATACACACTTCAGTAAAT
	rs11234495	F: ATTGGCAAAATCAACCTCTTAA
		R: ATTAGATTCAGTTCCATCTTTCTACAA
		E: cg$(atcg)_{11}$AGAAAAATGGATTGTACTCCCTTTG
	rs592297	F: AAAACTTGAGGTTAAAAATTCTCATG
		R: AACACAGAAAAACTCCTAAAAACTGT
		E: cg$(atcg)_{9}$CATTAAAATCAAGAAGTGCATCCAT
	rs676733	F: TATGTGCCAACCTTATAAAAAGTATAAA
		R: TCATGATGAAACAAAATCTTAGAATG
		E: cg$(atcg)_{7}$TACTGTGTAATATAAGAAATGGCAA
MS4A6A	rs610932	F: TTCCCAGAAACATTTCCCA
		R: AAAGTTGTGTCCTTTGCTTCAC
		E: g$(atcg)_{3}$AAATGTTTCCCAGAAAACTAGACAG
CD33	rs3865444	F: AACTGTTTACACCAGGGCTG
		R: TCACACGGACCCTATAGAATC
		E: $(atcg)_{2}$GAGTCGCAGCCTCACCTAGATCCAT
MS4A4E	rs670139	F: CAAGTGAAGGATCAGACCCTAG
		R: ATTGAAGGATTTTGCCCAG
		E: TTTGCATCTCCAAGTCAAAGTTTAC
CD2AP	rs9349407	F: AGTAAGCTAAGGTTAATTTTGAAAG
		R: GAGTCAGTGAGTGGTGAGCAAAT
		E: tcg$(atcg)_{10}$AAAATCTATAGTAGTGTATACTAAT
APOE	APOE-112-158	F: ACAAATCGGAACTGGAGGAA
		R: GGCCAGGGAGCCCACAGT
		E-112: act$(gact)_{3}$GCTGGGCGCGGACATGGAGGACGTG
		E-158: t$(gact)_{5}$CCGCGATGCCGATGACCTGCAGAAG

[a] In the "$(gact)_n$", n means repeats of "gact". F: forward primer; R: reverse primer; E: extension primer.

Table S2. Information of the reported Asian populations included in the meta-analysis

SNP.ID	Population	Ethnicity	No. of cases	No. of controls	Reference [a]
rs3851179	Chinese	East Asian	474	591	Li et al. [1]
	Chinese	East Asian	266	343	Yu et al. [2]
	Chinese	East Asian	457	341	Chen et al. [3]
	Japanese	East Asian	825	2934	Ohara et al. [4]
rs3764650	Taiwan	East Asian	534	307	Liao et al. [5]
	Chinese	East Asian	416	463	Yang et al. [6]
	Chinese	East Asian	350	283	Liu et al. [7]
	Chinese	East Asian	612	612	Tan et al. [8]
	Korean	East Asian	290	554	Chung et al. [9]
rs11136000	Chinese	East Asian	926	1030	Lu et al. [10]
	Chinese	East Asian	324	388	Yu et al. [11]
	Chinese	East Asian	127	143	Ma et al. [12]
	Chinese	East Asian	451	338	Chen et al. [3]
	Chinese	East Asian	268	389	Lin et al. [13]
	Korean	East Asian	290	554	Chung et al. [9]
	Japanese	East Asian	824	2933	Ohara et al. [4]
rs744373	Chinese	East Asian	612	612	Tan et al. [8]
	Japanese	East Asian	825	2933	Ohara et al. [4]
rs610932	Chinese	East Asian	612	612	Tan et al. [8]
	Chinese	East Asian	190	193	Deng et al. [14]
rs3865444	Chinese	East Asian	612	612	Tan et al. [8]
	Chinese	East Asian	190	193	Deng et al. [14]
	Korean	East Asian	290	554	Chung et al. [9]
rs9331942	Chinese	East Asian	268	389	Lin et al. [13]
	Chinese	East Asian	796	794	Yu et al. [15]
	Korean	East Asian	290	554	Chung et al. [9]

[a] We searched PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) with key words to retrieve the reported studies in Asian populations. Taken SNP rs3851179 as an example, we searched PubMed with "*PICALM*" and "Alzheimer's disease", and identified three studies in Chinese populations and one study in a Japanese population met our criteria. A total of 2770 LO-AD patients and 4969 healthy controls from these reported populations and the two cohorts analyzed in this study were included in the meta-analysis.

Table S3. Genotypes of 14 SNPs in 8 GWAS-linked genes in Han Chinese with and without Alzheimer's disease

SNP ID	Populations	Allele	Number of samples		Genotype	Number of samples		HWE *P*-value (Case/Control)
			Case	Control		Case	Control	
rs1060743	Southwest China	C/T	303/361	310/358	CC/CT/TT	67/169/96	75/160/99	0.660/0.510
	East China		370/462	352/500		83/204/129	66/220/140	0.921/0.195
	Combined		673/823	662/858		150/373/225	141/380/239	0.883/0.659
rs744373	Southwest China	C/T	244/422	241/427	CC/CT/TT	52/140/141	48/145/141	0.098/0.287
	East China		315/515	279/573		52/211/152	46/187/193	0.119/1.000
	Combined		559/937	520/1000		104/351/293	94/332/334	1.000/0.421
rs11136000	Southwest China	T/C	143/521	131/537	TT/CT/CC	22/99/211	13/105/216	0.035/1.000
	East China		175/657	158/694		21/133/262	16/126/284	0.460/0.632
	Combined		318/1178	289/1231		43/232/473	29/231/500	0.049/0.724
rs9331942	Southwest China	C/T	198/468	207/461	CC/CT/TT	35/128/170	30/147/157	0.150/0.701
	East China		270/562	303/549		52/166/198	45/213/168	0.073/0.072
	Combined		468/1030	510/1010		87/294/368	75/360/325	0.021/0.103
rs3851179	Southwest China	A/G	251/415	282/386	AA/AG/GG	54/143/136	46/190/98	0.129/0.003
	East China		308/522	328/524		55/198/162	57/214/155	0.752/0.222
	Combined		559/937	610/910		109/341/298	103/404/253	0.482/0.004
rs11234495	Southwest China	C/T	300/362	346/320	CC/CT/TT	73/154/104	84/178/71	0.269/0.228
	East China		408/418	424/424		109/190/114	100/224/100	0.115/0.285
	Combined		718/770	770/744		187/344/213	184/402/171	0.047/0.095
rs592297	Southwest China	C/T	197/465	231/435	CC/CT/TT	34/129/168	35/161/137	0.236/0.276
	East China		246/580	281/567		33/180/200	38/205/181	0.480/0.079
	Combined		443/1045	512/1002		67/309/368	73/366/318	0.861/0.035
rs676733	Southwest China	C/T	233/427	273/391	CC/CT/TT	49/135/146	44/185/103	0.070/0.007
	East China		315/507	318/530		58/199/154	55/208/161	0.677/0.407
	Combined		548/934	591/921		107/334/300	99/393/264	0.386/0.014
rs610932	Southwest China	A/C	248/416	252/416	AA/AC/CC	49/150/133	43/166/125	0.558/0.351
	East China		306/526	311/541		45/216/155	47/217/162	0.020/0.047
	Combined		554/942	563/957		94/366/288	90/383/287	0.210/0.029
rs3865444	Southwest China	T/G	133/533	126/542	TT/GT/GG	16/101/216	13/100/221	0.390/0.720
	East China		173/659	171/679		18/137/261	18/135/272	1.000/0.764
	Combined		306/1192	297/1221		34/238/477	31/235/493	0.574/0.645
rs670139	Southwest China	A/C	292/374	282/384	AA/AC/CC	71/150/112	57/168/108	0.120/0.576
	East China		326/504	357/495		73/180/162	66/225/135	0.065/0.091
	Combined		618/878	639/879		144/330/274	123/393/243	0.016/0.101
rs9349407	Southwest China	C/G	97/569	81/587	CC/CG/GG	6/85/242	6/69/259	0.826/0.604
	East China		112/718	93/759		9/94/312	3/87/336	0.528/0.453
	Combined		209/1287	174/1346		15/179/554	9/156/595	0.879/0.859
rs3764650	Southwest China	G/T	220/442	207/459	GG/GT/TT	37/146/148	33/141/159	0.902/0.800
rs3752237	Southwest China	A/G	122/542	121/545	AA/AG/GG	14/94/224	10/101/222	0.358/0.854

Table S4. Genotyping results of the *APOE* gene.

Haplotype [a]	Isoform	Southwest China		*P*-value	OR (95%CI)	East China		*P*-value	OR (95%CI)	Combined		*P*-value	OR (95%CI)
		Case [b]	Control [b]			Case [b]	Control			Case	Control		
CC	ε4	85	60	0.053	1.414(0.996-2.008)	187	79	6.109×10^{-14}	2.846(2.144-3.777)	272	139	1.572×10^{-12}	2.166(1.741-2.696)
TC	ε3	505	513			585	699			1090	1212		
TT	ε2	58	49			58	74			116	123		
Total		648	622			830	852			1478	1474		
	ε4+	79	55	0.041	1.501(1.020-2.208)	161	74	5.044×10^{-12}	3.015(2.191-4.148)	240	129	2.977×10^{-11}	2.267(1.775-2.895)
	ε4-	245	256			254	352			499	608		
	Total [b]	324	311			415	426			739	737		

[a] The order of variants for each haplotype: the first nucleotide of the codon that coded the 112th and 158th amino acid of the APOE protein, respectively.

[b] Nine patients and 23 control individuals from Southwest China and one patient from East China failed to be genotyped.

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