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Neprilysin Confers Genetic Susceptibility to Alzheimer's Disease in Han Chinese

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Abstract Alzheimer's disease (AD) is a progressive neurodegenerative disease, with increasing incidence all over the world. Amyloid-β (Aβ) was considered to be the original cause to AD, and many reported pathogenic or risk genes for AD were located in the Aβ generation and degradation pathways. Neprilysin (NEP), insulin-degrading enzyme (IDE), and matrix metalloprotease-9 (MMP-9) are the most important Aβ-degrading proteases. Accumulating genetic evidence suggested that single nucleotide polymorphisms (SNPs) of these genes confer susceptibility to AD in Caucasian populations. In this study, we screened eight SNPs within these three Aβ-degrading protease genes in 1475 individuals of two independent Han Chinese case-control cohorts. SNP rs1816558 of *NEP* was found to be significantly associated with AD after adjustment for *ε4* allele of the apolipoprotein E gene (*APOEε4*) and the Bonferroni correction. The remaining variants were not associated with risk of AD in Han Chinese sample set. Further data mining revealed that messenger RNA (mRNA) level of *NEP* substantially increased during the development of AD and was positively correlated with *APP* expression. The combined results indicated that *NEP* confers genetic susceptibility to AD in Han Chinese populations.

Keywords Alzheimer's disease · Neprilysin · Aβ-degrading enzyme · Chinese · Genetic susceptibility

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

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Abbreviations

AD	Alzheimer's disease
Aβ	β amyloid
NEP	Neprilysin
IDE	Insulin-degrading enzyme
MMP-9	Matrix metalloprotease-9
SNP	Single nucleotide polymorphisms
APOE	Apolipoprotein E gene
EO-AD	Early-onset Alzheimer's disease
LO-AD	Late-onset Alzheimer's disease
FAD	Familial AD
SAD	Sporadic AD
APP	Amyloid precursor protein
PSEN1	Presenilin 1
PSEN2	Presenilin 2
EO-FAD	Early-onset familial AD
GWAS	Genome-wide association study
MME	Membrane metallo-endopeptidase
DAPPLE	Disease association protein-protein link evaluator

Introduction

Alzheimer's disease (AD) is the most prevalent progressive neurodegenerative disease characterized by severe memory deficit and neuronal loss in the elderly populations over 65 years old. Extracellular senile plaques and intracellular neurofibrillary tangles are the main pathological features observed in AD brains [1, 2]. It has been widely accepted that AD can be divided into early-onset (<65 years) (EO-AD, which is inherited in an autosomal dominant model) and late-onset (≥65 years) (LO-AD, with no consistent mode of inheritance). In addition to aging, family history is the second greatest risk factor for AD, and AD can also be divided into familial AD (FAD, which presents only 5 %) and sporadic AD (SAD, which accounts for 90–95 % AD) according to the presence and absence of family history, respectively [3].

Genetic studies have identified rare mutations in the amyloid precursor protein (*APP*), and presenilin 1 and presenilin2 (*PSEN1* and *PSEN2*) genes guaranteed early-onset familial AD (EO-FAD) [4–6], but the etiology of LO-AD has not been fully uncovered yet. The pathogenesis of LO-AD was complicated and influenced by genetic components and environmental risk factors. Up to date, only *ε4* allele of the apolipoprotein E gene (*APOE*) was unequivocally established as a risk gene for LO-AD [7]. Recently, several genome-wide association studies (GWASs) conducted in Caucasian population identified 10 genes/loci that were associated with LO-AD [8–12], and these GWAS-reported genes were almost involved in production, degradation, and clearance of amyloid-β (Aβ), cholesterol metabolism, immunity, and cellular signaling [3]. In our previous studies, we could confirm that some of these GWAS-associated genes/loci as genetic risk factors for AD in Han Chinese populations [13, 14].

Following ectodomain shedding by β-secretase, proteolytic cleavages within the transmembrane region of the APP protein catalyzed by γ-secretase generated Aβ peptides of variable length, mainly Aβ40 and Aβ42. It has been widely accepted that Aβ acts as an initiating factor for AD, and many previous studies supported that the balance between processing of APP to generate Aβ and the clearance/degrading of Aβ was indispensable in the central nervous system. Once the equilibrium was disrupted by complicated factors, such as rare mutations in the *APP*, *PSEN1* and *PSEN2* genes [4–6], the abnormal generation and secretion of Aβ emerged and finally led to senile plaque deposition. The proteolytic degradation mechanism is thus of primary significance for clearance of Aβ. Neprilysin (NEP) has been found to be a primary Aβ-degrading enzyme [15]. There were also other candidates of Aβ-degrading enzyme, including insulin-degrading enzyme (IDE) and matrix metalloprotease-9 (MMP-9) [16–19]. Notably, these three Aβ-degrading proteases were considered to play important roles in the degradation of Aβ and in the regulation of cerebral Aβ levels [20, 21].

NEP, also known as membrane metallo-endopeptidase (MME), is a type II metalloproteinase exclusively expressed in neurons, whose active site towards the lumen or extracellular side of membranes [22]. Increasing evidence suggested that *NEP* was a potential gene for AD. The human *NEP* gene is located in chromosome 3q25.1-q25.2, which is near the putative linkage region of 3q23-q24 observed in familial AD [23]. NEP has been found to degrade both monomeric and oligomeric forms of Aβ and to inhibit the formation of Aβ deposition [21]. Experiments on *NEP*-deficient mice demonstrated an obviously increased level of Aβ in a gene dose-dependent manner [15]. In addition, the messenger RNA (mRNA) and protein levels of *NEP* were correlated with levels of plaque density in postmortem samples of AD patients [24, 25]. There were several genetic association studies to discern the association of *NEP* polymorphisms to AD risk, but the results were controversial [26–32].

IDE is located in chromosome 10q23.33, within a region linked to both type 2 diabetes [33] and AD [34]. IDE was originally recognized as the major proteolysis enzyme for insulin [35] and was the first identified Aβ-degrading proteases [18]. Kurochkin and colleagues demonstrated that purified rat IDE can degrade synthetic Aβ42 efficiently in vitro [18]. Several genetic association studies focusing on *IDE* single nucleotide polymorphisms (SNPs) have been carried out to investigate the relationship between *IDE* and AD, and produced both positive [36–42] and negative results [43–46], which suggested that more studies should be carried out to clarify its association with AD.

MMP-9 plays a vital role in the degradation of Aβ, which could cleave the Leu34-Met35 chemical bond within the transmembrane domain of the Aβ peptide [19]. It was demonstrated that MMP-9 was actively engaged in the neuronal injury in many neurodegenerative disorders [47]. A possible relationship between *MMP-9* polymorphisms and AD risk has been reported [48], but more genetic and functional assay is needed to further define the association of *MMP-9* with AD.

In this study, we attempted to explore the potential association between the SNPs of the three Aβ-degrading proteases and AD genetic risk in two Han Chinese populations from East and Southwest China, respectively. Gene expression analyses and protein-protein interaction assays were performed to comprehensively estimate the association of these genes with AD. Our results showed that *NEP* conferred genetic susceptibility to AD in Han Chinese.

Materials and Methods

Subjects

Two independent cohorts were recruited and analyzed in this study. The initial discovery sample from East China was

composed of 382 unrelated sporadic LO-AD patients and 426 cognitively healthy individuals, which were recruited from the Shanghai Mental Health Center and Tongde Hospital of Zhejiang Province. The validation samples from Southwest China were composed of 333 unrelated sporadic LO-AD patients and 334 cognitively healthy controls, which were collected at the Mental Health Center of West China Hospital. All of these AD patient and control samples had been genotyped to evaluate whether these GWAS-hit AD risk genes/variants contribute the susceptibility to AD in our recent study [13]. All participants were of Han Chinese descendants. The AD patients were independently diagnosed by two psychiatrists according to the criteria of DSM-IV and NINCDSADRDA [49]. The healthy participants were confirmed as cognitively intact and neurologically normal. All AD subjects were identified as sporadic late-onset pattern, as none of their first-degree relatives had dementia. Written informed consents in compliance to the principles of the Declaration of Helsinki were obtained from each participant or guardian. The current study was approved by the institutional review board of the Kunming Institute of Zoology.

SNP Selection and Genotyping

A total of eight SNPs (four SNPs within *NEP*: rs989692, rs2196521, rs1816558, and rs701109; two SNPs within *IDE*: rs2149632 and rs6583817; two SNPs within *MMP-9*: rs17576 and rs2274756) were selected according to the following criteria: (1) SNPs/non-synonymous mutations which can lead to the amino acid change according to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), (2) SNPs that can alter the expression level of the given gene according to the expression quantitative trait locus (eQTL) analysis (http://caprica.genetics.kcl.ac.uk/BRAINEAC/), (3) SNPs are capable of tagging more SNPs based on the linkage disequilibrium (LD) pattern in HapMap CHB data set (http://www.hapmap.ncbi.nlm.nih.gov), (4) risk SNPs reported in previous studies that were deposited in the Alzgene database (http://www.alzgene.org/) and in publications indexed in the ISI Webs of Knowledge (http://apps.webofknowledge.com), (5) and all eligible SNPs should have a minor allele frequency (MAF) >5 % in HapMap CHB dataset (http://hapmap.ncbi.nlm.nih.gov) and dbSNP dataset (http://www.ncbi.nlm.nih.gov/SNP/). The detailed information of each SNP is shown in Table 1.

Genomic DNA of all subjects was extracted from peripheral blood using the AxyPrep™ Blood Genomic DNA Miniprep Kit (Axygen, USA) according to the manufacturer's instruction. All these eight SNPs were genotyped by the SNaPshot assay following the procedure described in our previous studies [13, 50–52]. Primers for PCR amplification and single-base extension were listed in supplementary Table S1. The GeneMarker software (http://www.softgenetics.com/GeneMarker.html) was used to read the genotyping results.

Table 1 Detailed information of eight SNPs in three Aβ-degrading enzyme genes

Gene	dbSNPrs#	Allele	MAF[a]	Chr.	Position	Annotation
NEP	rs989692	C/T	0.220	3	156284059	Near gene-5′
	rs2196521	A/G	0.107	3	156295332	Near gene-5′
	rs1816558	A/G	0.392	3	156326301	Intron 5
	rs701109	C/T	0.375	3	156381101	3′-UTR
IDE	rs2149632	C/T	0.357	10	94222227	Intron 17
	rs6583817	C/T	0.274	10	94237227	Intron 12
MMP-9	rs17576	A/G	0.268	20	44073632	p.Q279R
	rs2274756	A/G	0.143	20	44076518	p.R668Q

[a] Minor allele frequency in CHB (Han Chinese in Beijing, China) was obtained from HapMap (http://hapmap.ncbi.nlm.nih.gov/)

Meta-analysis

We performed a meta-analysis for SNP rs1816558 of the *NEP* gene based on a total of 18491 LO-AD patients and 39076 healthy controls from four previous studies and the current study. The reported data includes (1) a meta-analysis consisted of 17008 AD patients and 37154 controls from 4 GWAS samples of European ancestry analyzed by International Genomics of Alzheimer's Project (IGAP) [8], (2) 390 AD patients and 468 controls from Finnish population [53], (3) 297 Caucasian AD patients and 296 matched controls [32], and (4) 85 AD patients and 399 controls from Swedish population [29]. Meta-analysis was performed by using the RevMan 5.2 software (http://www.cochrane.org/revman) under both random-effect and fixed-effect model.

Statistical Analysis

Power value was estimated using the Quanto software [54]. The deviation from the Hardy-Weinberg equilibrium (HWE) was calculated using the PLINK software [55]. SNPs with a P value less than 0.001 were considered as departure from the HWE. The PLINK software was also used to estimate the frequency of allele, genotype, and haplotype of all SNPs. A P value <0.05 was regarded as statistically significant. For Bonferroni correction, a cutoff P value <0.00625 (0.05/8) was set as statistically significant. Binary logistic regression under the general model was conducted with SPSS 16.0 (SPSS Inc., Chicago, Illinois) to assess the association of these SNPs with the risk of AD, with an adjustment for *APOE ε4* status (*APOE ε4*$^+$, *APOE ε4*$^-$). LD patterns of the *NEP*, *IDE*, and *MMP-9* genes were reconstructed using the Haploview software version 4.2 [56]. All the mentioned analyses were carried out in the two independent cohorts separately and in the combined Han Chinese populations.

In order to investigate the association between gene expression level and AD, we further performed data mining using the gene expression datasets GSE29652 [57] and GSE1297 [58] (http://www.ncbi.nlm.nih.gov/sites/GDSbrowser) in

Gene Expression Omnibus database. Additionally, protein-protein interaction assessment was conducted using the Disease Association Protein-Protein Link Evaluator (DAPPLE) tool (http://www.broadinstitute.org/mpg/dapple/dappleTMP.php) [59] to evaluate whether these Aβ-degrading proteases connected to the recognized AD-associated proteins. The AD top genes analyzed in the protein-protein interaction assay were listed in supplementary Table S2.

Results

Association of rs1816558 of *NEP*, but Not Other SNPs, with AD in Han Chinese Populations

Assuming a false positive rate being controlled as 0.05, for the MAF ranged from 0.1 to 0.4, the statistical power to detect the odds ratio (OR) value as 1.5 for risk allele was expected to be from 90.2 % to 95.3 % for the combined Han Chinese cohort in this study. All the eight analyzed SNPs in the current study were in Hardy-Weinberg equilibrium both for AD patients and controls (Table 2). The LD patterns of these variants in the *NEP* (rs989692–rs2196521–rs1816558–rs701109), *IDE* (rs2149632–rs6583817), and *MMP-9* (rs17576 - rs2274756) genes were similar between AD patients and controls in both cohorts and the combined samples (Fig. 1).

Among the eight SNPs, frequency of rs1816558 was significantly different between AD case and control subjects from East China both at allelic ($P=5.223\times10^{-5}$, OR [95 % CI]=1.510 [1.236–1.844]) and genotypic levels ($P=0.0002$) (Table 2). This association trend was validated in another independent Han Chinese population from Southwest China, though did not reach a statistically

Table 2 Allele and genotype frequencies of eight SNPs in three Aβ-degrading enzyme genes in 382 AD cases and 426 controls from East China, and 333 AD cases and 334 controls from Southwest China and in the combined samples

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]	HWE *P* value (case/control)[a]
NEP	rs989692	East China	0.217/0.213	1.022 (0.805–1.296)	0.861	0.206	0.860	0.490	0.364/0.149
		Southwest China	0.248/0.201	1.318 (1.018–1.706)	0.036	0.088	0.038	0.340	1.000/0.393
		Combined	0.231/0.208	1.150 (0.965–1.369)	0.117	0.207	0.117	0.496	0.529/0.659
	rs2196521	East China	0.105/0.097	1.096 (0.792–1.516)	0.581	0.852	0.575	0.907	0.784/0.784
		Southwest China	0.110/0.099	1.123 (0.790–1.596)	0.518	0.785	0.511	0.790	0.781/0.756
		Combined	0.107/0.098	1.109 (0.874–1.408)	0.394	0.685	0.387	0.851	0.555/0.533
	rs1816558[c]	East China	0.459/0.360	1.510 (1.236–1.844)	5.223×10^{-5}	0.0002	4.512×10^{-5}	0.001	0.837/0.461
		Southwest China	0.405/0.386	1.081 (0.868–1.347)	0.488	0.309	0.482	0.713	0.172/0.644
		Combined	0.434/0.372	1.296 (1.118 –1.503)	0.00057	0.002	0.0005	0.014	0.321/0.816
	rs701109	East China	0.346/0.366	0.918 (0.748–1.127)	0.415	0.399	0.412	0.537	0.572/0.348
		Southwest China	0.348/0.334	1.065 (0.849–1.335)	0.588	0.420	0.589	0.542	0/471/0.389
		Combined	0.347/0.352	0.980 (0.842–1.140)	0.791	0.965	0.790	0.960	0.934/0.937
IDE	rs2149632	East China	0.302/0.296	1.026 (0.829–1.270)	0.814	0.503	0.817	0.549	0.144/1.000
		Southwest China	0.348/0.326	1.101 (0.877–1.382)	0.406	0.697	0.398	0.835	0.631/0.618
		Combined	0.323/0.310	1.065 (0.913–1.244)	0.426	0.498	0.427	0.638	0.442/0.672
	rs6583817	East China	0.146/0.156	0.919 (0.699–1.208)	0.546	0.286	0.547	0.318	0.219/0.464
		Southwest China	0.171/0.159	1.095 (0.820–1.464)	0.538	0.614	0.538	0.790	0.564/0.684
		Combined	0.158/0.157	1.001 (0.820–1.220)	0.995	0.277	0.995	0.291	0.206/0.340
MMP-9	rs17576	East China	0.273/0.276	0.969 (0.778–1.205)	0.775	0.955	0.772	0.915	0.606/0.632
		Southwest China	0.280/0.263	1.088 (0.854–1.385)	0.495	0.785	0.488	0.991	0.683/0.672
		Combined	0.276/0.272	1.020 (0.867–1.199)	0.811	0.971	0.808	0.990	0.454/0.465
	rs2274756	East China	0.273/0.279	1.052 (0.778–1.422)	0.743	0.410	0.747	0.604	0.145/1.000
		Southwest China	0.280/0.263	0.951 (0.691–1.310)	0.760	0.946	0.763	0.826	0.626/0.630
		Combined	0.276/0.272	1.006 (0.807–1.252)	0.961	0.708	0.961	0.889	0.167/0.866

MAF minor allele frequency

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

[b] Binary logistic regression analysis was performed to assess the association, with an adjustment for the APOE4 status

[c] The association of rs1816558 with AD remained to be significant after Bonferroni correction for multiple testing, and a *P* value <0.00625 (0.05/8) was set as statistically significant

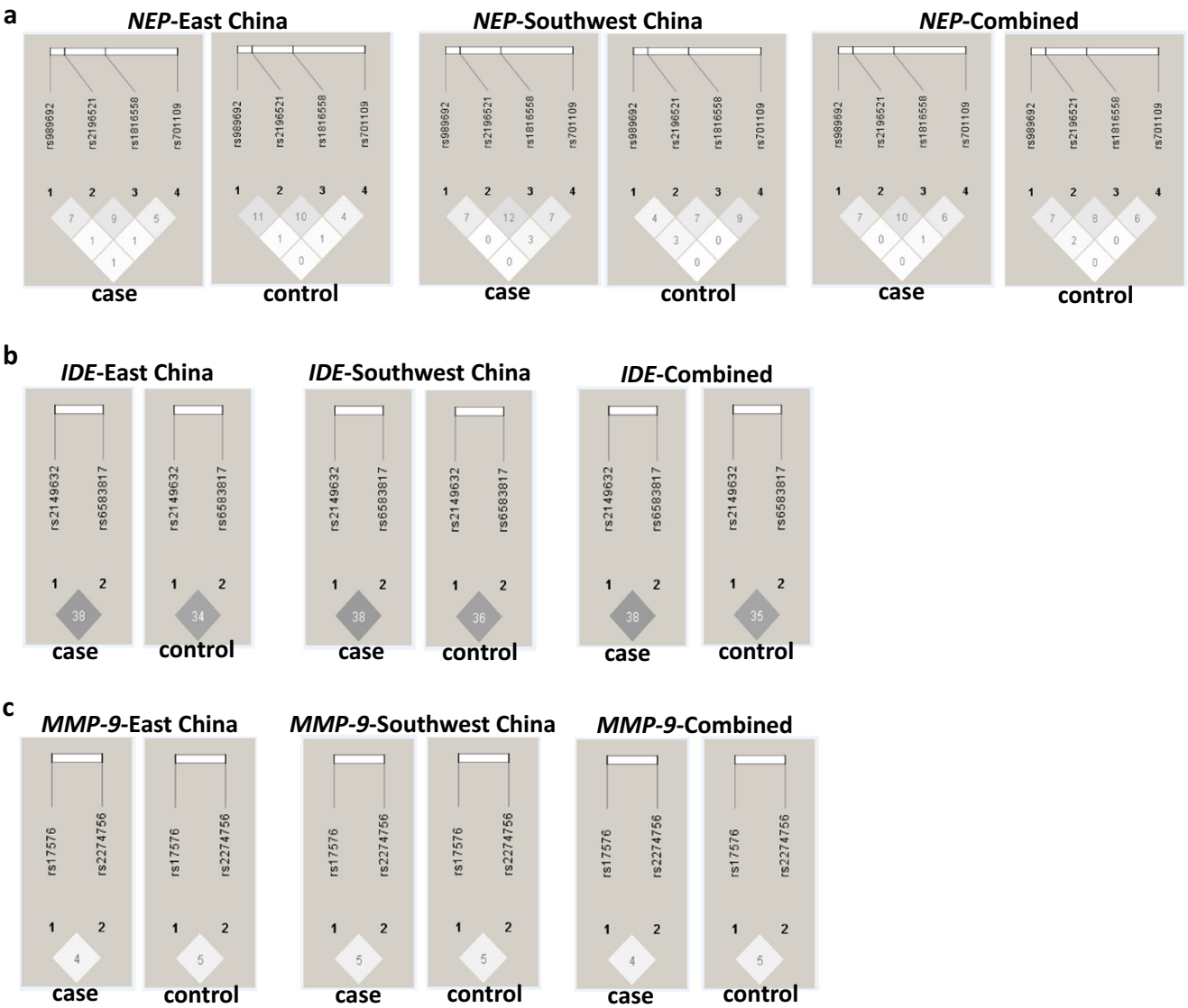

Fig. 1 Linkage disequilibrium (LD) patterns of the NEP (**a**), IDE (**b**), and MMP-9 (**c**) genes in AD cases and controls from East China, Southwest China, and in combined population. The value in each square refers to $r^2 \times 100$

significant level. When we pooled the two Han Chinese cohorts together as a combined case and control samples, we also observed a significant association between rs1816558 and AD risk in the combined populations (allele P=0.00057, OR [95 % CI]=1.296[1.118–1.503]; genotype P=0.002) (Table 2). The association remained to be significant after adjustment with *APOE4* status and after Bonferroni correction for multiple testing. None of the other seven SNPs showed a positive association with AD at the allelic, genotypic, and haplotypic levels after Bonferroni correction for multiple testing (Tables 2 and 3).

Meta-analysis for SNP rs1816558 based on a total of 18491 LO-AD patients and 39076 healthy controls from our current study and previous studies [53, 32, 29, 8] showed a significant association of rs1816558 with AD under the fixed-effect model (P=0.03, OR [95 % CI]=1.04 [1.00–1.07]) (Fig. 2a). However, considering the significant heterogeneity among the analyzed populations (I^2=69 %), we preferred to consider the result under random-effect model, in which no significant association was observed for rs1816558 with AD (P=0.38, OR [95 % CI]=1.06 [0.93–1.21]) (Fig. 2b).

PPI Interaction and Expression of *NEP* During AD

Protein-protein interaction analysis using DAPPLE analysis showed that NEP, which is also known as MME, was physically connected with APP protein in the network (Fig. 3a). Analysis of the gene expression profiles of GSE29652 [57], which includes expression data of astrocytes representing different Braak stages of AD, revealed that the *NEP* mRNA level was obviously increased during the development of AD in astrocytes (Fig. 3b). Furthermore, *NEP* mRNA level was

Table 3 Haplotype frequencies of SNPs of the NEP, IDE, and MMP-9 genes in 382 AD cases and 426 controls from East China and in 333 AD cases and 334 controls from Southwest China and in the combined samples

Haplotype	East China		*P* value	Southwest China		*P* value	Combined		*P* value
	Case	Control		Case	Control		Case	Control	
rs989692-rs2196521-rs1816558-rs701109 (NEP)									
TCCA	0.029	0.032	0.763	0.045	0.029	0.123	0.036	0.031	0.462
CCCA	0.019	0.016	0.629	0.013	0.010	0.716	0.017	0.014	0.493
TTCA	0.036	0.025	0.229	0.016	0.018	0.772	0.028	0.024	0.504
CTCA	0.130	0.111	0.254	0.133	0.139	0.755	0.132	0.124	0.547
TTTA	0.032	0.042	0.272	0.034	0.023	0.250	0.032	0.033	0.969
CTTA	0.101	0.140	0.016	0.105	0.112	0.678	0.104	0.129	0.031
TCCG	0.025	0.020	0.542	0.013	0.010	0.564	0.021	0.016	0.343
CCCG	0.021	0.014	0.230	0.025	0.029	0.658	0.022	0.020	0.721
TTCG	0.036	0.024	0.188	0.041	0.050	0.439	0.037	0.034	0.671
CTCG	0.168	0.123	0.011	0120	0.103	0.319	0.147	0.115	0.012
CCTG	–	–	–	0.007	0.015	0.179	–	–	–
TTTG	0.057	0.063	0.566	0.097	0.068	0.048	0.076	0.067	0.365
CTTG	0.347	0.388	0.087	0.350	0.393	0.100	0.349	0393	0.017
rs2149632-rs6583817 (IDE)									
TA	0.144	0.147	0.878	0.171	0.156	0.459	0.157	0.151	0.673
TG	0.159	0.153	0.773	0.176	0.171	0.826	0.166	0.161	0.688
CG	0.697	0.700	0.912	0.653	0.673	0.449	0.677	0.688	0.518
rs15756-rs2274756 (MMP-9)									
GA	0.120	0.116	0.787	0.127	0.132	0.776	0.123	0.123	0.977
AG	0.271	0.280	0.675	0.280	0.263	0.495	0.275	0.273	0884
GG	0.609	0.604	0.838	0.593	0.605	0.671	0.602	0.604	0.879

positively correlated with *APP* mRNA level in the hippocampus according to the gene expression profiles of GSE1297 [58] (Fig. 3c). All these data mining results further supported that NEP may play important roles in the pathogenesis of AD.

Discussion

Genetic evidence and functional characterization studies have showed that the pathogenic mutations in the well-known *APP*,

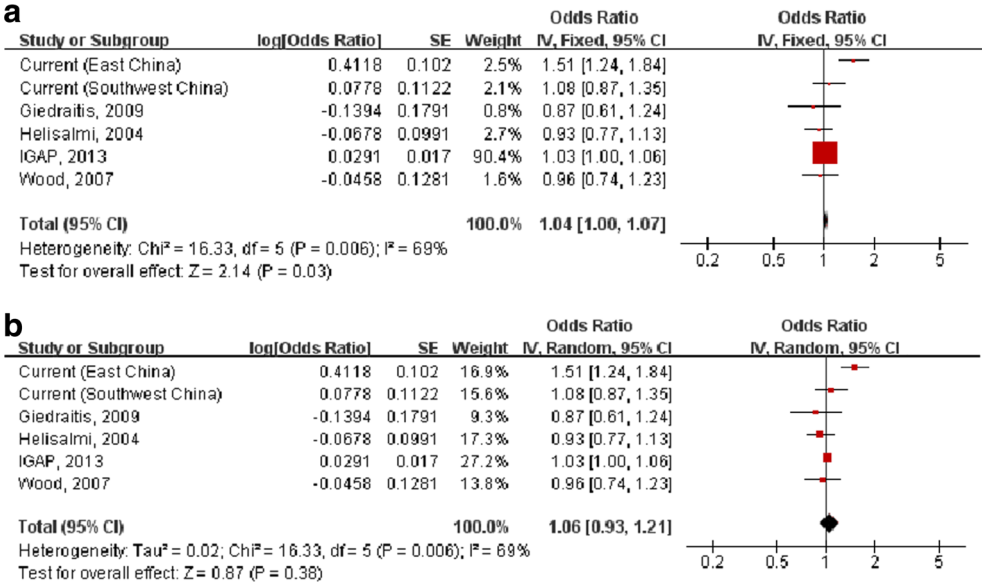

Fig. 2 Meta-analysis of the association between rs1816558 and AD. A total of 18491 AD patients and 39076 controls were included in the analysis, which were taken from four previous studies [53, 32, 29, 8] and the two cohorts analyzed in the current study under fixed-effect model (**a**) and random-effect model (**b**)

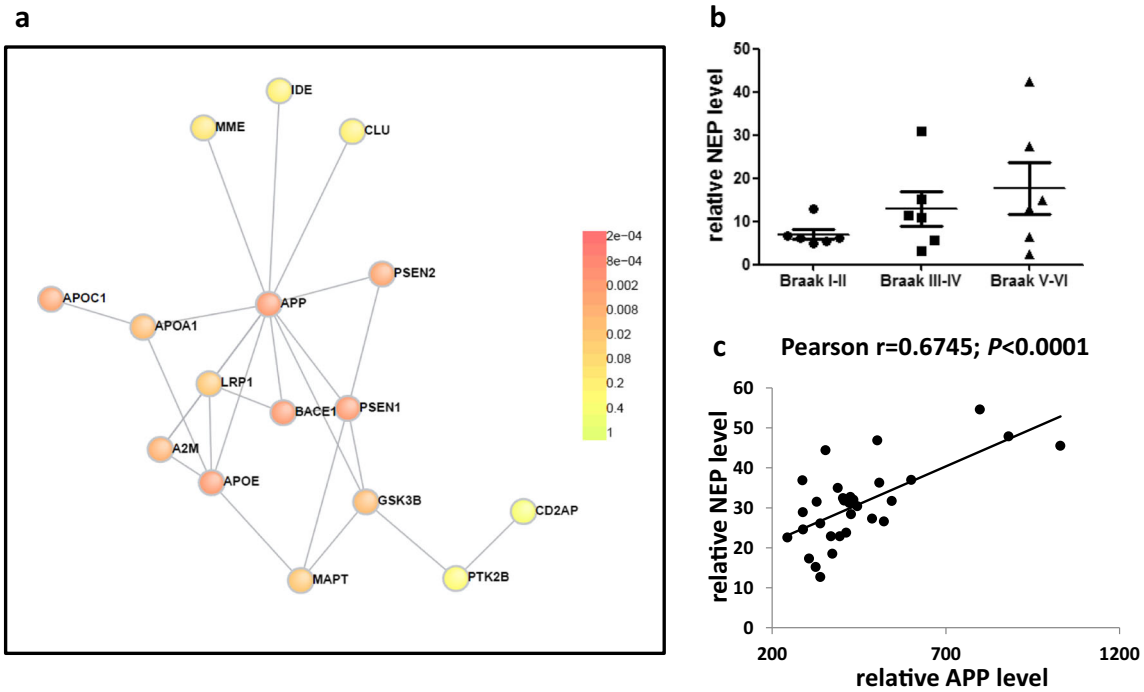

Fig. 3 Gene expression and protein-protein interaction analyses. **a** Interaction networks among proteins encoded by the AD top genes and NEP (also known as MME) using DAPPLE (http://www.broadinstitute.org/mpg/dapple/dappleTMP.php). *Colors* indicate significance of participation in the PPI network. **b** The NEP mRNA level increased at different stages of AD development. The expression profile GSE29652 in Gene Expression Omnibus database was reanalyzed here (http://www.ncbi.nlm.nih.gov/sites/GDSbrowser). **c** The NEP mRNA level was positively correlated with the APP mRNA level. The expression profile GSE1297 in Gene Expression Omnibus database was used for the analysis

PSEN1, and *PSEN2* genes strongly contribute to the pathogenesis of the EO-FAD, which supported the notion that the process of Aβ exerts a pivotal role in etiology and development of AD [3]. *NEP*, *IDE*, and *MMP-9* were capable of degrading Aβ in vitro or in vivo [15–21], but the available genetic data of these critical enzymes in the pathophysiology of AD have not reached a consistent result. Moreover, most of these reported studies were performed in Caucasian populations [26, 27, 29, 32, 53], and there is a need to test it in the world's largest ethnic population—Han Chinese.

Aiming to investigate whether these Aβ-degrading protease genes confer risk to AD in Han Chinese populations, we screened eight SNPs in the *NEP*, *IDE*, and *MMP-9* genes in two independent Han Chinese case-control cohorts and the combined samples. Albeit the sample size of our study was modest, we had relatively sufficient power for the analysis. We found that SNP rs1816558 of *NEP* was significantly associated with AD both at the allelic and genotypic levels in populations from East China and in the combined samples, and this association could survive the Bonferroni correction. Meta-analysis with a total of 18491 LO-AD patients and 39076 healthy controls from our current study and previously reported data [53, 32, 29, 8] also indicated a significant association of rs1816558 with AD under the fixed-effect model; though the association became weak when analyzed under random-effect model, the effect direction of rs1816558 remained the same. We also checked the eQTL (http://caprica.genetics.kcl.ac.uk/BRAINEAC/) information for rs1816558 but found no signal for this SNP.

To date, several variants in the *NEP* gene have been identified to be associated with AD and have been replicated by subsequent studies in different ethnic populations [26, 30–32, 53]. Helisalmi et al. screened rs989692, rs2196521, rs1816558, and other four SNPs in the *NEP* gene in Finnish population; they showed that rs989692 had significantly different allelic and genotypic distributions between AD and control subjects, and haplotype analysis also showed an obvious association with AD [53]. SNP rs1816558, which is a tag SNP for *NEP* and significantly linked to AD risk in our study, showed no association with AD in Finnish population [53], suggesting a population-specific pattern. SNP rs701109 (3′-UTR 159C/T) was found to be associated with AD in an age-dependent manner, and the C/C genotype was a risk factor independent of *APOE ε4* allele in a Spanish population [26]. However, these associations could not be replicated in the follow-up studies in European populations [27, 29, 32, 60] and in Chinese populations [28, 31], as well as in our current study. The different genetic structure of different populations in these previous studies and in our current study, which could be indicated by the meta-analysis of rs1816558 that showed a significant heterogeneity of the analyzed populations (I^2=69 %), may account

for the inconsistent results. It is possible that the real functional variants may be linked to different SNPs in different populations, which resulted in different AD risk SNPs in different populations.

NEP is a 97-kDa protease, ubiquitously expressed in many tissues, including the brain. In the brain, NEP is expressed at the presynaptic and postsynaptic membranes and plays a crucial role in neuropeptide signaling [21, 25] and catabolism of Aβ. The mRNA and protein levels of *NEP* were found to be significantly reduced in regions with senile plaques and in the hippocampus of AD brains [24], whereas other studies produced inconsistent findings that no remarkable alteration for NEP level was observed in AD brains [60]. We performed data-mining to investigate the alteration of *NEP* expression during different stages of AD. The mRNA level of *NEP* was evidently increased during the development of AD and was significantly correlated with the *APP* level. Protein-protein interaction analysis revealed that NEP could interact with APP directly. Therefore, it seemed that NEP actively participates in clearance of Aβ during the process of AD, and different alleles in the *NEP* gene may link to altered function of NEP protein and thus confer genetic susceptibility to AD.

In short, we screened common SNPs in three Aβ-degrading proteases, and identified SNP rs1816558 in the *NEP* gene was significantly associated with AD risk in Han Chinese populations. Gene expression data and protein-protein interaction assay further supported the association between *NEP* and AD. Future genetic studies in independent Asian populations and functional assay are necessary to further explore the role of *NEP* in the pathogenesis of AD.

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Conflict of Interest There are no actual or potential conflicts of interest.

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Supplementary Materials

Table S1. Primer information for genotyping the 8 SNPs in 3 Aβ degrading enzyme genes using the SNaPshot assay

Gene	SNP ID	Primer(5'-3')[a]
NEP	rs989692	F: ACCATTTTCAGAATGTTGTCTAAC
		R: ATGAACACATGAAGTAGTTTATTTTAGTC
		E:$(atcg)_{10}$AGTTAATAGATACTGGTATCAGTAG
	rs2196521	F: ATTGAATACCAAGGATCTAGTCATTC
		R: ATCAGGGACACCTTAGTTTGTAAA
		E: g$(atcg)_6$ATGCAGACCTCTGACTTACTTACTT
	rs1816558	F: TGAGCCATTAGGATCATCAAA
		R: TTTTCTTACAGAAAGTAAGGAACAGC
		E: AACACCTTTCCCCAATATCTAATAA
	rs701109	F: AAATGATTGACTTGATAGACCTTAGAC
		R: TTAACAGAGAGGGCACCATC
		E: g(atcg) CTTCCTCCCTCCACACCCTTTCTAG
IDE	rs2149632	F: AAAGCATATTAACTGTCCTGATAAAAA
		R: TTATTTTGTTGATTGCCTCAGG
		E:cg$(atcg)_7$TACCTGTTCTAGTAAGTGATTTTTA
	rs6583817	F: AAATATTAAGTGCACAAATGTCACA
		R: TATATGAGTGCAAAATCTTTGCTAGTA
		E:g$(atcg)_{11}$TGCTAGTACTAGAAAGACTAACTCA
MMP-9	rs17576	F: TTCTCCCCCTTTCCCACA
		R: AGATGAATGGAAACTGGCAG
		E: cg$(atcg)_{13}$CTCCTCGCCCCAGGACTCTACACCC
	rs2274756	F: GTGGACCGGATGTTCCCC
		R: TTAGTGTGGTGTCTCACGAAG
		E: cg$(atcg)_{13}$GACACGCACGACGTCTTCCAGTACC

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

Table S2. AD top genes for protein-protein interaction analysis

AD top genes [a]				
A2M	*CASS4*	*GAB2*	*MTHFR*	*SORTL1*
ABCA7	*CD2AP*	*GSK3B*	*PICALM*	*TNF*
ACE	*CD33*	*HLA-DRB1*	*PPARG*	*TOMM40*
APOA1	*CDH23*	*HLA-DRB5*	*PSEN1*	*TREM2*
APOC1	*CELF1*	*IDE*	*PSEN2*	*ZCWPW1*
APOE	*CLU*	*INPP5D*	*PTK2B*	
APP	*CR1*	*LRP1*	*RHBDF2*	
BACE1	*DIP2A*	*MAPT*	*RPL13*	
BCHE	*DSG2*	*MEF2C*	*SERPINF2*	
BDNF	*EPHA1*	*MME8*	*SLC24A4*	
BIN1	*FERMT2*	*MS4A6A*	*SORL1*	

[a] The AD top genes were summarized according to AlzGene database [1] and one recent review [2]

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