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Female-specific effect of the *BDNF* gene on Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common neurodegenerative disease influenced by genetic and environmental factors. Brain-derived neurotrophic factor (*BDNF*) plays an important role in the progression of AD, but the genetic association between *BDNF* and AD remains controversial. In this study, we aimed to explore the potential association between genetic variants in *BDNF* and AD in Han Chinese and to investigate whether the association is affected by gender. A 3-stage study was conducted to evaluate the genetic association between *BDNF* and AD. Data mining of the reported expression data, brain-imaging data, and biomarker data in AD patients was also performed to further validate the results. We found a female-specific genetic association of rs6265 with AD and a gender-related messenger RNA expression of *BDNF* in brain tissues of AD patients. In addition, we observed a clear female-specific risk trend for the effect of rs6265 on AD endophenotypes. Our results clarified the available controversies regarding the role of rs6265 in AD and indicated that *BDNF* may be a female-specific risk gene for AD.

1. Introduction

Alzheimer's disease (AD) is a brain disease characterized by a progressive dementia that occurs in middle or late life (Querfurth and LaFerla, 2010). The key pathologic changes are increased levels of amyloid-β (Aβ) peptide in the form of extracellular senile plaques and hyper-phosphorylated tau as the intracellular neurofibrillary tangles in AD brain tissues (Reitz et al., 2011). Nevertheless, the exact neuropathological etiology of AD has not been fully understood. The occurrence of AD is multifactorial and is mainly affected by genetic and environmental factors (Alzheimer's Association, 2011; Blennow et al., 2006). Incidence of AD has a gender-specific pattern, with a higher rate in women (Mielke et al., 2014; Vina and Lloret, 2010), and this can be interpreted by sex difference in metabolism of the brain in males and females (Zhao et al., 2016).

Brain-derived neurotrophic factor (*BDNF*) is a member of the neurotrophic factor family (Maisonpierre et al., 1991), which is synthesized in basal forebrain and plays a key role in central nervous system (Fahnestock et al., 2002). *BDNF* and its receptor TrkB can facilitate memory formation and long-term potentiation (Kemppainen et al., 2012; Rex et al., 2007). Decrease of the BDNF-TrkB signaling results in a declined spatial memory (Minichiello, 2009), whereas overexpression of the full-length TrkB enhances learning and memory (Koponen et al., 2004). *BDNF* was found to be associated with neuropsychiatric (Angelucci et al., 2005; Autry and Monteggia, 2012; Li et al., 2016) and neurodegenerative (Zuccato and Cattaneo, 2009) disorders and plays an important role in AD progression (Doi et al., 2013; Rohe et al., 2009). The expression level of *BDNF* is lower in AD patients than that in healthy controls (Connor et al., 1997; Hock et al., 2000; Peng et al., 2005), and similar results were also found in AD animal models (Francis et al., 2012; Meng et al., 2013; Naert and Rivest, 2012). Previous studies have

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[1] Data used in preparation of this article were partly obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of the ADNI and/or provided data but did not participate in the analysis or the writing of this report. A complete listing of the ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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showed that *BDNF* contributes a protective effect against the neurotoxicity of Aβ peptide by activating the TrkB and ERK1/2 signaling (Doi et al., 2013) and repairs the damage induced by Aβ peptide (Rohe et al., 2009). Because of the active role of *BDNF* in neuronal survival and brain functioning, many studies have been performed to investigate the genetic association between single nucleotide polymorphism (SNP) of *BDNF* and AD. Significant association between rs6265 and AD was reported in several studies (Feher et al., 2009; Fukumoto et al., 2010; Huang et al., 2007; Matsushita et al., 2005; Tsai et al., 2006; Ventriglia et al., 2002), but most of the previous studies showed no significant association (Akatsu et al., 2006; Bagnoli et al., 2004; Bian et al., 2005; Bodner et al., 2005; Boiocchi et al., 2013; Borroni et al., 2012; Combarros et al., 2004; Cozza et al., 2008; Desai et al., 2005; Forero et al., 2006; Giedraitis et al., 2009; He et al., 2007; Lee et al., 2005; Li et al., 2005, 2008; Nishimura et al., 2005; Pivac et al., 2011; Reiman et al., 2007; Saarela et al., 2006; Sonali et al., 2013; Vepsalainen et al., 2005; Vieira et al., 2015; Yu et al., 2008; Zhang et al., 2006). Taking account of these controversial results, a meta-analysis and further investigation in independent populations are essential to clarify this important issue.

BDNF showed a sex-specific influence on Parkinson's disease (Foltynie et al., 2005), and the expression of *BDNF* is regulated by estrogen (Scharfman and MacLusky, 2006; Solum and Handa, 2002). Considering the fact that the incidence of AD is higher in females than that in males (Mielke et al., 2014; Vina and Lloret, 2010), we hypothesized that the association of *BDNF* with AD may be gender-specific, as reported by some previous studies (Chen et al., 2014; Fukumoto et al., 2010; Lin et al., 2014), and this may be the reason for the inconsistent association results in the literatures. This study is thus designed to explore the potential association between genetic variants in the *BDNF* gene and AD in Han Chinese and to investigate whether the association is affected by gender. A 3-stage study was conducted to evaluate the genetic association between *BDNF* and AD. Data mining of the reported expression data, brain-imaging data, and biomarker data in AD patients were also performed to further validate our results.

2. Materials and methods

2.1. Subjects

Two independent Han Chinese cohorts were recruited and used for the stage 1 study and stage 2 study, respectively. Stage 1 study had 382 unrelated sporadic late-onset AD (LO-AD) patients (45.8% men) and 426 cognitively healthy individuals (29% men), which were collected from East China. Stage 2 study had 333 unrelated sporadic LO-AD patients (37.2% men) and 334 healthy controls (48.3% men), which were recruited from Southwest China as the validation sample. The AD patients in these 2 cohorts were diagnosed according to the criteria of DSM-IV and NINCDSADRDA (McKhann et al., 1984). Individuals with normal cognitive state were included as controls. Most of these subjects had been described and were analyzed for other AD-related genes in our previous studies (Bi et al., 2014, 2015; Wang et al., 2016a,b; Zhang et al., 2016). Ethical standards enacted in the Declaration of Helsinki were obeyed in this study, and written informed consents were obtained from each participant or their guardians. This study was approved by the institutional review board of the Kunming Institute of Zoology.

2.2. Genotyping and association analysis

Genomic DNA of all subjects was extracted from peripheral blood using the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen, USA). Two SNPs (rs6265 [p.V66M] and rs12291063) of *BDNF* were selected and detected by the SNaPshot assay following the procedure described in our previous studies (Bi et al., 2014; Wang et al., 2016a; Zhang et al., 2016). Primers for genotyping rs6265 and rs12291063 were listed in Supplementary Table S1. Genotyping results were read using the GeneMarker software (Holland and Parson, 2011). The PLINK software (Purcell et al., 2007) was used to calculate the Hardy-Weinberg equilibrium (HWE) and to estimate the frequencies of allele and genotype of the 2 SNPs. A p-value <0.05 was regarded as departure from the HWE in the HWE test, and a p-value <0.05 was set as significantly different for the comparison of allele and genotype frequencies in cases and controls.

2.3. Stage 3 meta-analysis

To further validate the results, we performed a meta-analysis for rs6265 of *BDNF* as the stage 3 analysis, based on a total of 26,958 LO-AD patients and 46,941 healthy controls from previous studies (Akatsu et al., 2006; Bagnoli et al., 2004; Bian et al., 2005; Bodner et al., 2005; Boiocchi et al., 2013; Borroni et al., 2012; Combarros et al., 2004; Cozza et al., 2008; Desai et al., 2005; Feher et al., 2009; Forero et al., 2006; Fukumoto et al., 2010; Giedraitis et al., 2009; He et al., 2007; Huang et al., 2007; Lambert et al., 2013; Lee et al., 2005; Li et al., 2005, 2008; Matsushita et al., 2005; Nishimura et al., 2005; Pivac et al., 2011; Reiman et al., 2007; Saarela et al., 2006; Sonali et al., 2013; Tsai et al., 2006; Ventriglia et al., 2002; Vepsalainen et al., 2005; Vieira et al., 2015; Yu et al., 2008; Zhang et al., 2006) and our new data in this study. The previous studies were identified through a search of PubMed and Web of Science using the following terms "(Val66Met OR rs6265) AND (AD OR Alzheimer's disease)", we retrieved genotype data of 9238 AD cases and 8579 controls from 30 independent case-control studies. The data of rs6265 from the International Genomics of Alzheimer's Project (Lambert et al., 2013), which contains 17,008 AD cases and 37,154 controls, were also extracted and included in this analysis. As a result, a total of 26,958 AD cases and 46,941 controls were analyzed (Supplementary Table S2) (Akatsu et al., 2006; Bagnoli et al., 2004; Bian et al., 2005; Bodner et al., 2005; Boiocchi et al., 2013; Borroni et al., 2012; Combarros et al., 2004; Cozza et al., 2008; Desai et al., 2005; Feher et al., 2009; Forero et al., 2006; Fukumoto et al., 2010; Giedraitis et al., 2009; He et al., 2007; Huang et al., 2007; Lambert et al., 2013; Lee et al., 2005; Li et al., 2005, 2008; Matsushita et al., 2005; Nishimura et al., 2005; Pivac et al., 2011; Reiman et al., 2007; Saarela et al., 2006; Sonali et al., 2013; Tsai et al., 2006; Ventriglia et al., 2002; Vepsalainen et al., 2005; Vieira et al., 2015; Yu et al., 2008; Zhang et al., 2006). For gender-specific analysis, 3737 AD cases and 3744 controls for females and 2101 AD cases and 2477 controls for males from 16 previous studies with gender information (Akatsu et al., 2006; Bian et al., 2005; Boiocchi et al., 2013; Combarros et al., 2004; Desai et al., 2005; Forero et al., 2006; Fukumoto et al., 2010; He et al., 2007; Lee et al., 2005; Li et al., 2005; Matsushita et al., 2005; Nishimura et al., 2005; Pivac et al., 2011; Saarela et al., 2006; Tsai et al., 2006; Yu et al., 2008) and the current Han Chinese data were considered (Supplementary Table S3). Meta-analysis was performed using the STATA version 13 (Stata Corporation, College Station, TX, USA) under the random-effect model if there was a significant heterogeneity (I^2 >50%) or under the fixed-effect model when I^2 <50%.

2.4. Reappraisal of BDNF expression alteration during aging and AD

We analyzed the expression level of *BDNF* messenger RNA (mRNA) in brain tissues of AD patients and controls using the gene expression data set GSE36980 (Hokama et al., 2014) in Gene

Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). We further validate this result in another 9 data sets [GSE5281 (Liang et al., 2007), GSE15222 (Webster et al., 2009), GSE12685 (Williams et al., 2009), GSE37263 (Tan et al., 2010), GSE28146 (Blalock et al., 2011), GSE26972 (Berson et al., 2012), GSE48350 (Blair et al., 2013), GSE29378 (Miller et al., 2013), and GSE26927 (Durrenberger et al., 2015)] included in Gene Expression Omnibus database. *BDNF* expression data from these 9 data sets were normalized and merged by Combat in R package sva (Leek et al., 2012). Furthermore, data set GSE1572 (Lu et al., 2004) was used to analyze the mRNA expression pattern of *BDNF* in aging process.

2.5. Detecting the effects of BDNF SNPs on AD endophenotypes

To further evaluate the (female-specific) effect of *BDNF* on AD endophenotypes, we retrieved clinical and imaging data, as well as genotyping data from the Alzheimer's Disease Neuroimaging Initiative (ADNI, http://adni.loni.usc.edu/) project (Weiner et al., 2010). Effects of *BDNF* rs6265 on AD-related endophenotypes, such as whole brain atrophy rate, cerebrospinal fluid (CSF) Aβ and tau level, and Mini-Mental State Examination (MMSE) scores were measured by linear regression model using the PLINK software (Purcell et al., 2007), with the "–assoc -qt-means" command. One-way analysis of variance and repeated measures multivariate analysis of variance were performed for the longitudinal analysis of the score of Alzheimer's disease assessment scale (ADAS) using SPSS 16.0 (SPSS Inc, Chicago, IL, USA). Data plotting was generated by the GraphPad PRISM software (GraphPad Software, Inc, La Jolla, CA, USA) with means, SEM, and N.

3. Results

3.1. Genetic association between BDNF and AD in females but not in males

There were no deviations from HWE in controls for both SNPs (Supplementary Table S4). The distribution frequencies of alleles and genotypes of the 2 SNPs were listed in Table 1 and Supplementary Table S5. No association of rs12291063 with AD was observed (Supplementary Table S5). SNP rs6265 showed a significant association with AD at the genotypic level ($p = 0.006$) in the stage 1 sample, and the genotype effect was more significant under the dominant model ($p = 0.003$; Supplementary Table S6). Compared with carriers with the homozygous genotype (GG) of rs6265 major allele, carriers with genotypes AA+AG had a high risk for AD (odds ratio [OR] = 1.612; 95% confidence interval [CI]: 1.171–2.220; Supplementary Table S6). When males and females were analyzed separately, the significant association of rs6265 with AD was only found in females at the allelic ($p = 0.031$ for allele A, OR = 1.326, 95% CI: 1.026, 1.714) and genotypic ($p = 0.015$) levels (Table 1). Consistently, the genotypic effect was more significant ($p = 0.004$; OR = 1.883, 95% CI: 1.221–2.904) under the dominant model in females (Supplementary Table S6). This female-specific association was evaluated in the stage 2 sample, but we found no association in this sample, irrespective of gender (Table 1). Considering previous reports that the *APOE*-ε4 risk for AD was greater in females (Altmann et al., 2014; Riedel et al., 2016), we investigated whether there was an interaction between *APOE* and *BDNF*. Association between *BDNF* and AD was analyzed in 4 groups considering both *APOE*-ε4 and gender status (Supplementary Table S7). No significant female-specific association was observed with or without *APOE*-ε4, indicating that there might be no interaction between *APOE* and *BDNF*, and the effect of *BDNF* in females might be independent of *APOE*-ε4.

Considering the limitation of the relatively small sample size and the failure of validation in the stage 2 sample, we performed a stage 3 validation using a meta-analysis of rs6265 with AD based on a total of 26,958 LO-AD patients and 46,941 healthy controls from this study and previous studies (Akatsu et al., 2006; Bagnoli et al., 2004; Bian et al., 2005; Bodner et al., 2005; Boiocchi et al., 2013; Borroni et al., 2012; Combarros et al., 2004; Cozza et al., 2008; Desai et al., 2005; Feher et al., 2009; Forero et al., 2006; Fukumoto et al., 2010; Giedraitis et al., 2009; He et al., 2007; Huang et al., 2007; Lambert et al., 2013; Lee et al., 2005; Li et al., 2005, 2008; Matsushita et al., 2005; Nishimura et al., 2005; Pivac et al., 2011; Reiman et al., 2007; Saarela et al., 2006; Sonali et al., 2013; Tsai et al., 2006; Ventriglia et al., 2002; Vepsalainen et al., 2005; Vieira et al., 2015; Yu et al., 2008; Zhang et al., 2006) (Supplementary Table S2). We used the sensitivity analysis to evaluate the influence of each study on the overall pattern and found no significant change of the result after excluding the respective study (Supplementary Fig. S1). No publication bias was observed after being assessed by the Begg's and Egger's tests (Supplementary Fig. S2), suggesting that the inclusion of these studies in the meta-analysis is proper. Consistent with the pattern in the stage 2 Han Chinese under study, there was no significant

Table 1
Allele and genotype frequencies of rs6265 in *BDNF* in the 3-stage samples

SNP ID	Gender	Allele		*p*-value	OR	95% CI	Genotype		*p*-value
		AD	Control				AD	Control	
Stage 1: 381 AD cases versus 424 controls									
rs6265 (A/G)	All	383/379	391/457	0.096	1.181	0.971–1.437	84/215/82	97/197/130	**0.006**[a]
	Male	164/182	113/121	0.833	0.965	0.692–1.345	33/98/42	31/51/35	0.087
	Female	208/188	262/314	**0.031**	1.326	1.026–1.714	48/112/38	63/136/89	**0.015**[a]
Stage 2: 331 AD cases versus 334 controls									
rs6265 (A/G)	All	328/334	352/316	0.251	1.134	0.915–1.407	73/182/76	86/180/68	0.471
	Male	123/115	168/150	0.788	0.955	0.682–1.337	29/65/25	45/78/36	0.642
	Female	193/207	179/161	0.233	0.839	0.628–1.120	41/111/48	40/99/31	0.38
Stage 3: 26,958 AD cases versus 46,941 controls									
rs6265 (A/G)	All[b]	5523/14,377	5506/14,068	0.713	1.014	0.941–1.093	—	—	—
	Male	1437/2765	1680/3274	0.298	0.953	0.869–1.044	—	—	—
	Female	2381/5093	2236/5252	**0.001**	1.13	1.050–1.217	—	—	—

p-values less than 0.05 were marked in bold.
Key: CI, confidence interval; OR, odds ratio.
[a] Global genotypic association *p* value. Note that genotype of rs6265 showed a more significant association under the dominant model in both combined samples and female-only samples (Supplementary Table S6).
[b] The data from the IGAP were not included because we could not obtain the detailed allele count data and gender information. Detailed results of the stage 3 meta-analysis could be found in Fig. 1A.

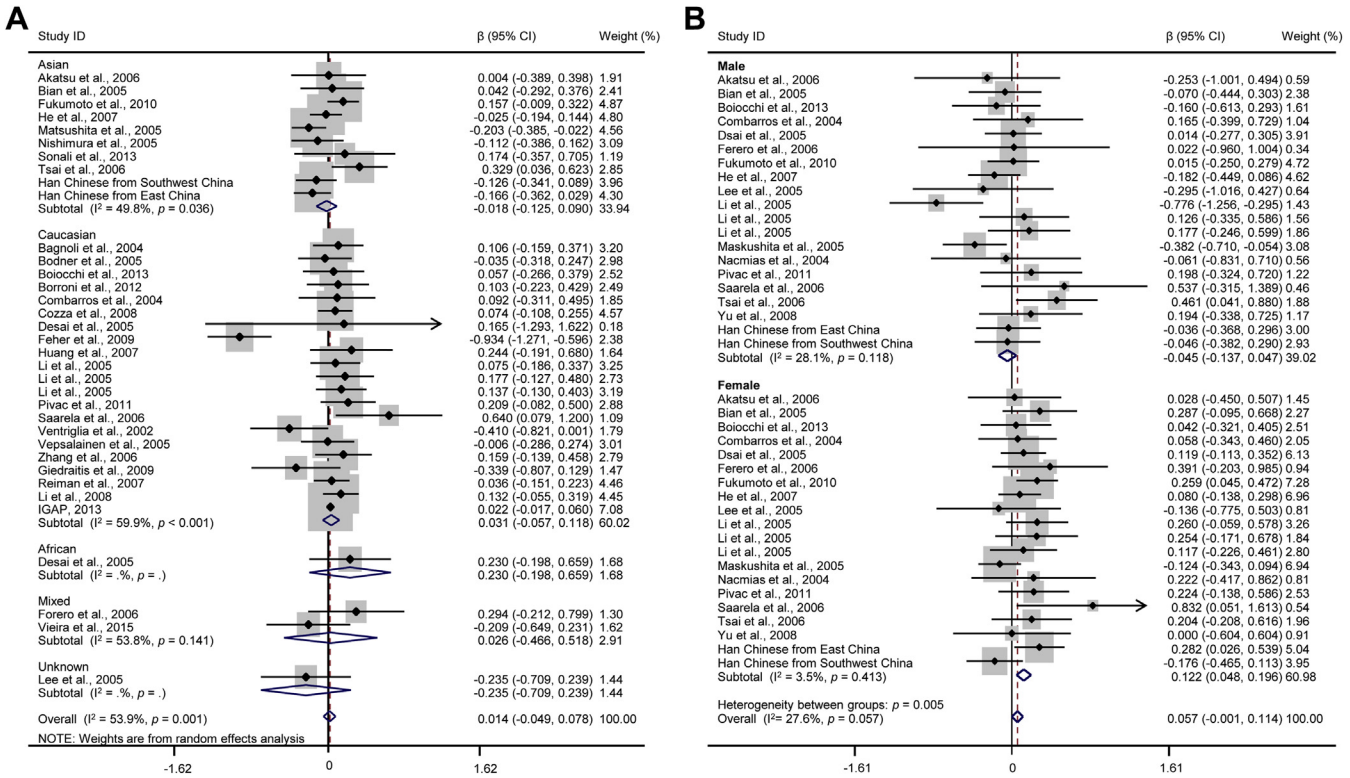

Fig. 1. Forest plots of the meta-analysis between rs6265 and AD under the allelic model. (A) Overall populations ($p = 0.663$ for meta-analysis) or subpopulations ($P_{Asian} = 0.749$, $P_{Caucasian} = 0.492$ for meta-analysis); (B) Male ($p = 0.298$ for meta-analysis) and female ($p = 0.001$ for meta-analysis) subgroups. Abbreviations: β, lnOR; CI, confidence interval; OR, odds ratio; I^2, heterogeneity, with corresponding *p*-values measured by the chi-square test, the I^2 and relative *p* value were not available for subgroups "African" and "unknown", which only contained one study.

association of rs6265 with AD at the allelic level ($p = 0.663$) in the overall samples or subpopulations (e.g., Asian and western European) under the random-effect model ($I^2 = 53.9\%$; Fig. 1A). However, after stratification by gender, a significant association of rs6265 with AD was found in females at the allelic level ($p = 0.001$; Table 1) under the fixed-effect model ($I^2 = 3.5\%$; Fig. 1B).

3.2. The level of BDNF mRNA was significantly decreased in brain tissues of AD patients, especially in females

Considering the fact that the secretion of *BDNF* was affected by rs6265 (Egan et al., 2003; Kennedy et al., 2015), we further analyzed the mRNA expression levels of *BDNF* in brain tissues (including temporal cortex, frontal cortex and hippocampus) of AD patients and controls using the reported data GSE36980 (Hokama et al., 2014). The mRNA expression level of *BDNF* was significantly decreased in temporal cortex and frontal cortex of AD patients (Fig. 2A and B). When males and females were analyzed separately, significantly decreased *BDNF* mRNA levels were observed in temporal cortex and frontal cortex in females, but not in males (Fig. 2A and B). For hippocampus, there was no difference of *BDNF* mRNA level between AD patients and controls (Fig. 2C).

To further validate the aforementioned result, another 9 data sets [GSE5281 (Liang et al., 2007), GSE15222 (Webster et al., 2009), GSE12685 (Williams et al., 2009), GSE37263 (Tan et al., 2010), GSE28146 (Blalock et al., 2011), GSE26972 (Berson et al., 2012), GSE48350 (Blair et al., 2013), GSE29378 (Miller et al., 2013), and GSE26927 (Durrenberger et al., 2015)] in Gene Expression Omnibus database were normalized and merged. Significantly decreased mRNA level of *BDNF* was found in frontal cortex, temporal cortex, and hippocampus in AD patients, with similar patterns in both males and females (Fig. 2D–F). However, for entorhinal cortex, which was heavily affected in AD patients (Harris et al., 2010; Khan et al., 2014), a significantly decreased *BDNF* mRNA level was only found in females, and the mRNA level of *BDNF* was significantly lower in female patients than that in male patients (Fig. 2G). In aging process as demonstrated by re-analyzing the GSE1572 data (Lu et al., 2004), the mRNA level of *BDNF* presented a decreased tendency in frontal cortex of females (Fig. 3A), whereas a reverse trend was observed in males (Fig. 3B). These results suggested that *BDNF* may be a female-specific risk gene for AD.

3.3. Female-specific effect of BDNF SNP rs6265 on AD endophenotypes

We used the clinical, genetic, and biomarker data from the ADNI project (http://adni.loni.usc.edu/) (Weiner et al., 2010) to investigate the potential role of *BDNF* SNP rs6265 on AD endophenotypes. We analyzed the effect of *BDNF* rs6265 on Mini-Mental State Examination but found no significant difference between males and females (Supplementary Fig. S3). Then, we examined the longitudinal change of ADAS score, which measured cognitive decline, in both female and male patients (including mild cognitive impairment and AD, N = 213) with or without the risk allele. As time progressed from the baseline (time of data collection) to 36 months, a significant increase of ADAS score was observed in both female and male patients, and the trend of increase was more remarkable in females than that in males (Fig. 4A), especially in carriers with risk allele A (Fig. 4B). We further determined the effects over time by comparing female to male directly, stratified by genotype information (male A-carrier [AA+AG], male GG, female A-carrier

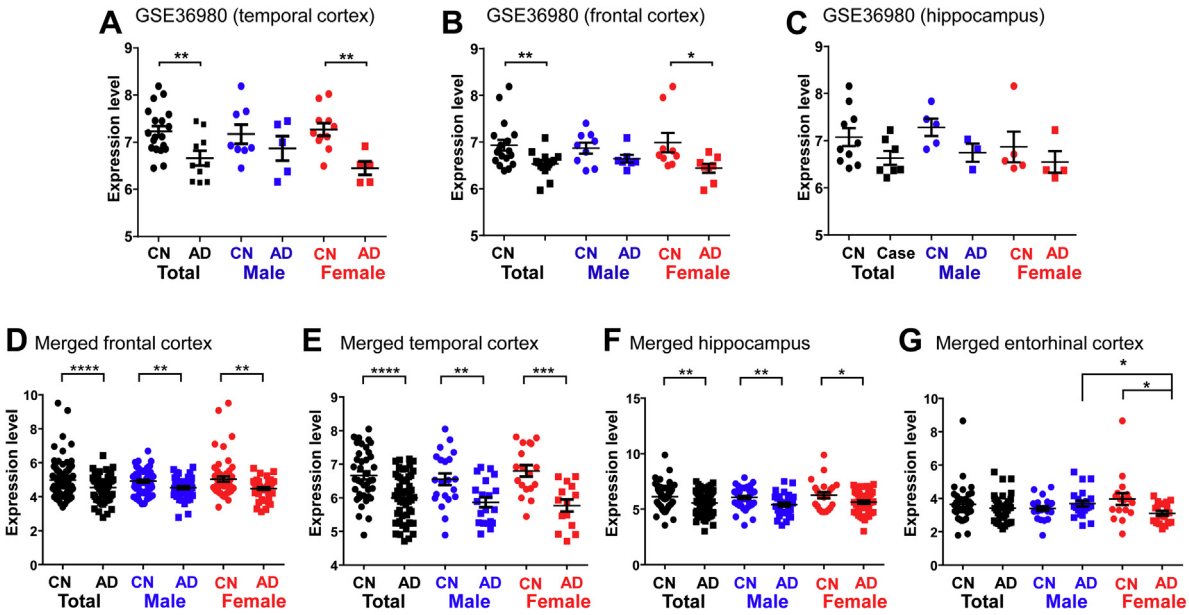

Fig. 2. Messenger RNA (mRNA) level of *BDNF* in different brain tissues in AD patients and healthy controls (CN). The GSE36980 (probe 7947230) (Hokama et al., 2014) from Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) was used to analyze the levels of *BDNF* mRNA in (A) temporal cortex, (B) frontal cortex, and (C) hippocampus in AD patients relative to healthy controls (CN). To further validate the pattern observed in GSE36980, we retrieved more AD-related brain expression data and renormalized and merged as a larger data sets to analyze the mRNA expression of *BDNF* in (D) frontal cortex: GSE5281 (Liang et al., 2007), GSE12685 (Williams et al., 2009), GSE36980 (Hokama et al., 2014), GSE15222 (Webster et al., 2009) and GSE48350 (Blair et al., 2013); (E) temporal cortex: GSE37263 (Tan et al., 2010), GSE36980 (Hokama et al., 2014), GSE15222 (Webster et al., 2009) and GSE5281 (Liang et al., 2007); (F) hippocampus: GSE48350 (Blair et al., 2013), GSE36980 (Hokama et al., 2014), GSE29378 (Miller et al., 2013), GSE28146 (Blalock et al., 2011), and GSE5281 (Liang et al., 2007); (G) entorhinal cortex: (GSE5281 (Liang et al., 2007), GSE26927 (Durrenberger et al., 2015), GSE26972 (Berson et al., 2012), and GSE48350 (Blair et al., 2013). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, 2-tailed Student t-test.

[AA+AG], and female GG) of each subject. The overall pattern of the longitudinal change of cognition in female carriers with risk allele A had a significant difference compared with male carriers with the same risk allele ($p = 0.004$), whereas there was no significant difference between females and males with GG genotype ($p > 0.05$; Fig. 4C). We observed significant differences between female and male carriers with risk allele A at the 24th ($p = 0.019$) and the 36th ($p = 0.002$) months (Fig. 4C). The relative score of ADAS difference between AA+AG carriers and GG carriers in female group was not significant at these time points including baseline, 12th month and 24th month, but it reached a significant level at the 36th month ($p = 0.04$, Fig. 4C). When we further divided A-carriers into AA carriers and AG carriers, we observed a similar result of significant increase of longitudinal change of ADAS in females with AA or AG genotypes compared to males with the corresponding genotypes; and importantly, females with AA genotype showed the greatest longitudinal change, albeit the effect of female AA carrier was based on only 1 patient (Fig. S4).

Furthermore, carriers with risk allele A of rs6265 had a lower CSF $A\beta_{42}$ level (Fig. 4D) and a higher CSF tau level (Fig. 4E), as well as a higher brain atrophy rate (Fig. 4F) in females. Although the difference was not statistically significant partially due to limited sample size, a clear risk trend and consistent pattern was observed in females, whereas there was obviously no such a trend in males (Fig. 4D–F). These observations added more support to the female-specific effect of *BDNF* on AD.

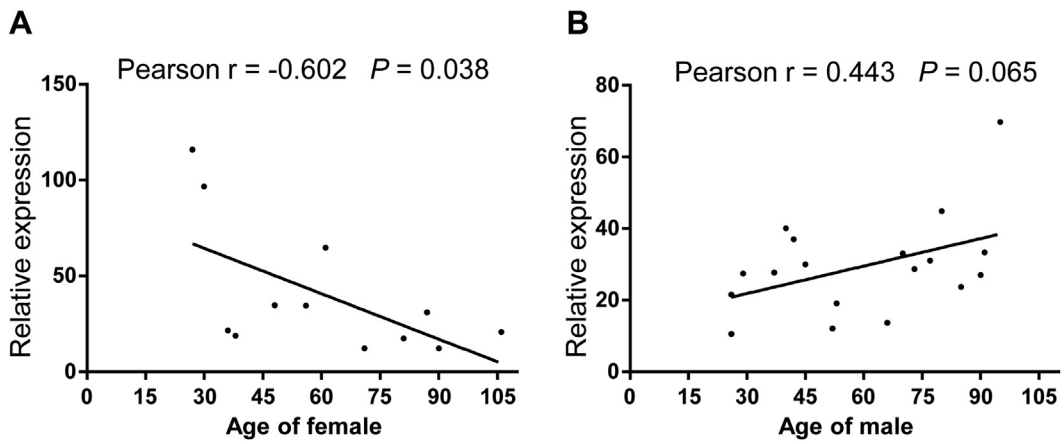

Fig. 3. The expression pattern of *BDNF* mRNA in frontal cortex of (A) females (N = 12) and (B) males (N = 18) in aging process. Data set GSE1572 (probe 1088_at) (Lu et al., 2004) was obtained from Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). Pearson correlation analysis was performed using the GraphPad PRISM software (GraphPad Software, Inc, La Jolla, CA, USA).

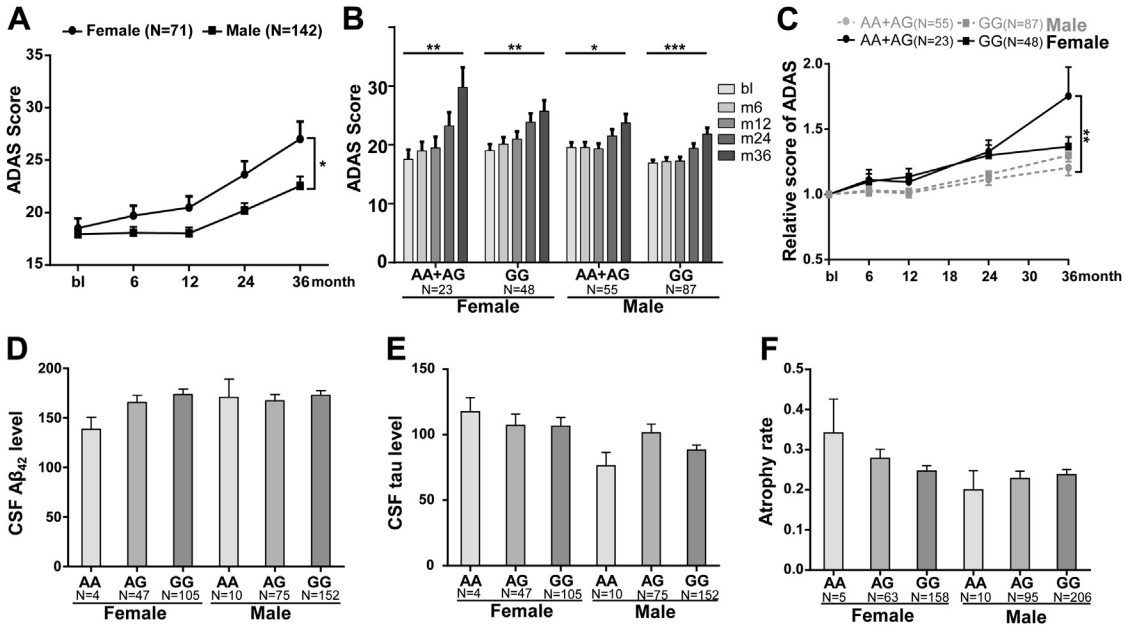

Fig. 4. Effect of rs6265 on AD endophenotypes. (A) The Alzheimer's disease assessment scale (ADAS) scores were significantly increased in both female and male patients from the baseline (bl) to 36 months (m36). The trend of increase was more remarkable in females than that in males. Repeated-measures multivariate analysis of variance (ANOVA) was performed to show the difference between female and male patients. (B) Longitudinal change of the ADAS scores in female and male patients with different genotypes of rs6265. One-way ANOVA was performed to quantify the difference within each group. (C) Cognitive decline pattern in carriers with risk allele A showed a significant difference between females and males. The relative score of ADAS was calculated by normalizing the original ADAS score at each longitudinal point with the score at baseline (relative score of ADAS at baseline = 1). Shown was the result of repeated-measures multivariate ANOVA. (D–F) A clear risk trend was observed in (D) CSF $A\beta_{42}$ level, (E) CSF tau level, and (F) brain atrophy rate in females, but not in males. The genetic and biomarker data were obtained from the ADNI project (Weiner et al., 2010) and were analyzed by the PLINK software using the linear regression model (Purcell et al., 2007). One-way ANOVA was also performed to measure the effect of SNP rs6265 on AD-related biomarkers. Data plotting was generated by GraphPad PRISM with means, SEM, and N. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

4. Discussion

BDNF is a neurotrophic factor and has a protective effect against the neurotoxicity of Aβ peptide (Doi et al., 2013). SNP rs6265 in *BDNF* causes a valine (Val) to methionine (Met) substitution in the 5′ proregion of human BDNF protein (Ventriglia et al., 2002). The substitution decreases the secretion of *BDNF* (Chen et al., 2004; Egan et al., 2003), resulting in memory loss and reduced hippocampal volume (Bueller et al., 2006; Lim et al., 2014). Given the key role of *BDNF* in neuron function, many studies have been performed to investigate the genetic association between the *BDNF* gene and AD (Fukumoto et al., 2010; Lin et al., 2014), but the results had not reached an agreement. In the present study, we found a significant genetic association between rs6265 of *BDNF* and AD in females in Han Chinese population. Meta-analysis of cases and controls from the present study and previous reports (Akatsu et al., 2006; Bian et al., 2005; Boiocchi et al., 2013; Combarros et al., 2004; Desai et al., 2005; Forero et al., 2006; Fukumoto et al., 2010; He et al., 2007; Lee et al., 2005; Li et al., 2005; Matsushita et al., 2005; Nishimura et al., 2005; Pivac et al., 2011; Saarela et al., 2006; Tsai et al., 2006; Yu et al., 2008) also showed a significant association between rs6265 and AD in females but not in males. The female-specific effect was also observed in AD-related endophenotypes from the ADNI project (http://adni.loni.usc.edu/) (Weiner et al., 2010) (Fig. 4). Furthermore, we observed the gender-related mRNA expression of *BDNF* in brain tissues of AD patients (Figs. 2 and 3). Our results were in general consistent with the previous meta-analysis studies (Chen et al., 2014; Fukumoto et al., 2010; Lin et al., 2014), in which a significant association between rs6265 and AD in females, but not in males, was described. Taken together, all these results suggested that *BDNF* might be a female-specific risk gene for AD. Although we observed a positive association of rs6265 with AD in females, it should be received with caution as the previous large genome-wide association studies did not report this female-specific effect, which might be partially caused by mixed population structure. Considering the heterogeneity between males and females as identified in our meta-analysis (Fig. 1), sex-specific effects might be concealed in the combined large samples. More focused studies with sex-specific subjects are needed to further confirm our results.

The mRNA expression level of *BDNF* was found to be regulated by estrogen (Gibbs, 1999; Scharfman and MacLusky, 2006; Simpkins et al., 1997; Solum and Handa, 2002). Downregulation of *BDNF* mRNA levels was observed in the hippocampus of rat after neonatal gonadectomy, and a reversed effect was found after the injection of estrogen (Solum and Handa, 2002). Estrogen regulates *BDNF* mRNA expression in a dose-dependent manner in *in vitro* assay, with no effect at a very low dose (Solum and Handa, 2002). Estrogen rapidly upregulates *BDNF* mRNA in the cerebral cortex and the olfactory bulb of ovariectomized animals (Sohrabji et al., 1995). The relative levels of *BDNF* mRNA and protein in specific regions of the brain were significantly affected by hormone replacement (Gibbs, 1999). In menopausal women, estradiol falls to an undetectable level with aging (Pluchino et al., 2013). Meanwhile, decreased level of *BDNF* mRNA in frontal cortex of female was also observed in aging process (Fig. 3A). All these lines of evidence indicated a potential positive correlation between *BDNF* and estrogen, and the gender-specific effect of rs6265 on AD may be caused by the interaction between *BDNF* and estrogen. Thus, estrogen supplement in the elderly females might protect them against the risk of AD.

Although we presented comprehensive analyses indicating a female-specific effect of *BDNF* on AD, there are some limitations in this study. First, only 2 SNPs in *BDNF* were selected and genotyped,

and the sample size of the 2-stage Han Chinese cohorts was relatively small. Second, articles published not in English were excluded in the stage 3 meta-analysis. Third, we only performed a re-analysis of the available expression data to discern the *BDNF* mRNA expression pattern. Cellular assays and animal model experiments are essential to confirm the association. Studies with a larger sample set, together with gender and detailed clinical information, are also needed to further validate the genetic association between *BDNF* and AD.

In summary, we observed the gender-related alteration of *BDNF* mRNA expression in brain tissues and a positive genetic association of rs6265 in *BDNF* with AD in females. There was a clear female-specific risk trend for the effect of rs6265 on AD endophenotypes. These results clarified the available controversies regarding the role of rs6265 in AD and indicated that *BDNF* might be a female-specific risk gene for AD.

Disclosure statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2016.12.023.

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Supplementary materials

Table S1. Primers information for genotyping the 2 SNPs in *BDNF* by using the SNaPshot assay.

Gene	SNP ID	Primer (5'-3')
BDNF	rs6265	F: AAGGCAGGTTCAAGAGGC
		R: ACTTGACTACTGAGCATCACCC
		E: ATCATTGGCTGACACTTTCGAACAC
	rs12291063	F: TCACTCAAGGCAGATTTCTTATTT
		R: TAATATTTCATTGTTGTTGTTGCTG
		E: GATCGACAGATCCAGCTCTCTCCTACCAAA

F, forward primer; R, reverse primer; E, extension primer.

Table S2. Meta-analysis of 31 published studies and this study.

References	Year	Country	Ethnicity	β	ES	*P*-value
(Akatsu, et al., 2006)	2006	Japan	Asian	0.0042	0.2008	0.983
(Desai, et al., 2005)	2005	USA	African	0.2303	0.2187	0.292
(Bagnoli, et al., 2004)	2004	Italy	Caucasian	0.1058	0.1353	0.434
(Bian, et al., 2005)	2005	China	Asian	0.0419	0.1704	0.806
(Bodner, et al., 2005)	2005	USA	Caucasian	-0.0352	0.1440	0.807
(Boiocchi, et al., 2013)	2013	Italy	Caucasian	0.0565	0.1647	0.731
(Borroni, et al., 2012)	2012	Italy	Caucasian	0.1030	0.1666	0.536
(Combarros, et al., 2004)	2004	Spain	Caucasian	0.0921	0.2056	0.654
(Cozza, et al., 2008)	2008	Italy	Caucasian	0.0735	0.0924	0.426
(Desai, et al., 2005)	2005	USA	Caucasian	0.1645	0.7436	0.825
(Feher, et al., 2009)	2009	Hungary	Caucasian	-0.9337	0.1721	0.000
(Forero, et al., 2006)	2006	Colombia	Mixed	0.2938	0.2578	0.255
(Fukumoto, et al., 2010)	2010	Japan	Asian	0.1568	0.0844	0.063
(He, et al., 2007)	2007	China	Asian	-0.0248	0.0861	0.774
(Huang, et al., 2007)	2007	USA	Caucasian	0.2442	0.2221	0.272
(Lee, et al., 2005)	2005	USA	Unknown	-0.2350	0.2420	0.331
(Li, et al., 2005)	2005	England	Caucasian	0.0755	0.1334	0.572
(Li, et al., 2005)	2005	USA	Caucasian	0.1765	0.1548	0.254
(Li, et al., 2005)	2005	USA	Caucasian	0.1369	0.1359	0.314
(Matsushita, et al., 2005)	2005	Japan	Asian	-0.2035	0.0927	0.028
(Nishimura, et al., 2005)	2005	Japan	Asian	-0.1123	0.1399	0.422
(Pivac, et al., 2011)	2011	Croatia	Caucasian	0.2090	0.1483	0.159
(Saarela, et al., 2006)	2006	Finland	Caucasian	0.6396	0.2860	0.025
(Sonali, et al., 2013)	2013	India	Asian	0.1738	0.2709	0.521
(Tsai, et al., 2006)	2006	China	Asian	0.3295	0.1496	0.028
(Ventriglia, et al., 2002)	2002	Italy	Caucasian	-0.4100	0.2098	0.051
(Vepsalainen, et al., 2005)	2005	Finland	Caucasian	-0.0060	0.1428	0.967
(Vieira, et al., 2015)	2015	Brazilian	Mixed	-0.2092	0.2244	0.351
(Zhang, et al., 2006)	2006	USA	Caucasian	0.1595	0.1523	0.295
Han Chinese from Southwest China	this study	China	Asian	-0.1260	0.1098	0.251
Han Chinese from East China	this study	China	Asian	-0.1665	0.1000	0.096
(Giedraitis, et al., 2009)	2009	Sweden	Caucasian	-0.3392	0.2389	0.156
(Reiman, et al., 2007)	2007	USA	Caucasian	0.0363	0.0953	0.704
(Li, et al., 2008)	2008	Canada	Caucasian	0.1322	0.0956	0.167
(Lambert, et al., 2013)	2013	Mixed	Caucasian	0.0215	0.0198	0.278

Note: *P*-value, *P*-value at the allelic level. β, lnOR; ES, standard error of lnOR.

Table S3. Meta-analysis of 16 published studies with gender information and this study.

References	Year	Ethnicity	Male			Female		
			β	ES	*P*-value	β	ES	*P*-value
(Akatsu, et al., 2006)	2006	Asian	-0.2535	0.3815	0.506	0.0282	0.2442	0.908
(Bian, et al., 2005)	2005	Asian	-0.0705	0.1903	0.711	0.2868	0.1947	0.141
(Boiocchi, et al., 2013)	2013	Caucasian	-0.1601	0.2312	0.489	0.0419	0.1853	0.821
(Combarros, et al., 2004)	2004	Caucasian	0.1653	0.2879	0.566	0.0583	0.205	0.776
(Desai, et al., 2005)	2005	Caucasian	0.0142	0.1486	0.924	0.1192	0.1186	0.315
(Forero, et al., 2006)	2006	Mix	0.0217	0.5009	0.965	0.3914	0.303	0.197
(Fukumoto, et al., 2010)	2010	Asian	0.0146	0.1351	0.914	0.2588	0.1089	0.018
(He, et al., 2007)	2007	Asian	-0.1815	0.1366	0.184	0.08	0.1113	0.472
(Lee, et al., 2005)	2005	Unknown	-0.2948	0.3682	0.423	-0.1361	0.3261	0.676
(Li, et al., 2005)	2005	Caucasian	-0.7758	0.2452	0.002	0.2595	0.1627	0.111
(Li, et al., 2005)	2005	Caucasian	0.1255	0.235	0.593	0.2538	0.2167	0.242
(Li, et al., 2005)	2005	Caucasian	0.1766	0.2155	0.413	0.1174	0.1754	0.503
(Matsushita, et al., 2005)	2005	Asian	-0.3821	0.1673	0.022	-0.1242	0.1115	0.266
(Nishimura, et al., 2005)	2005	Caucasian	-0.0606	0.393	0.877	0.2221	0.3263	0.496
(Pivac, et al., 2011)	2011	Caucasian	0.1983	0.2664	0.457	0.2242	0.1846	0.225
(Saarela, et al., 2006)	2006	Caucasian	0.5371	0.4348	0.217	0.8317	0.3985	0.037
(Tsai, et al., 2006)	2006	Asian	0.4607	0.2141	0.031	0.2039	0.21	0.332
(Yu, et al., 2008)	2008	Asian	0.1936	0.271	0.475	0	0.308	1
Han Chinese from East China	this study	Asian	-0.0357	0.1694	0.833	0.2821	0.1309	0.031
Han Chinese from Southwest China	this study	Asian	-0.0461	0.1720	0.789	-0.1760	0.1477	0.233

Note: *P*-value, *P*-value at the allelic level. β, lnOR; ES, standard error of lnOR.

Table S4. Hardy-Weinberg equilibrium (HWE) test of the two SNPs in *BDNF* in control samples from East China and Southwest China.

Gene	SNP ID	Population	HWE *P*-value
BDNF	rs6265	East China	0.204
		Southwest China	0.155
	rs12291063	East China	0.197
		Southwest China	0.835

Table S5. Allele and genotype frequencies of rs12291063 in *BDNF* in 382 AD patients and 426 healthy controls from Eastern China and 333 AD patients and 334 healthy controls from Southwest China.

SNP ID	Gender	Allele		*P*-value	OR (95% CI)	Genotype		*P*-value [a]
		AD	Control			AD	Control	
Stage 1: 381 AD cases versus 424 controls								
rs12291063 (C/T)	All	120/638	155/693	0.194	0.841 (0.648-1.092)	7/106/266	10/135/279	0.404
	Male	51/291	35/199	0.988	0.997 (0.625-1.589)	2/47/122	1/33/83	0.961
	Female	65/331	110/466	0.285	0.832 (0.594-1.166)	5/55/138	7/96/185	0.428
Stage 2: 333 AD cases versus 334 controls								
rs12291063 (C/T)	All	117/545	103/563	0.279	1.173 (0.878-1.568)	8/101/222	7/89/237	0.52
	Male	42/196	47/271	0.362	1.236 (0.784-1.947)	3/36/80	3/41/115	0.648
	Female	72/328	54/286	0.445	1.163 (0.790-1.712)	5/62/133	4/46/120	0.697

OR, odds ratio; CI, confidence interval.

[a] Global genotypic association *P*-value.

Table S6. Genotypic associations of rs6265 and rs12291063 with AD under the genotypic, dominant and recessive models in Stage 1 and Stage 2 samples.

Gender	SNP ID	Genotype	AD	Control	*P*-value [a]	OR (95% CI)
Stage 1: 381 AD cases versus 424 controls						
All	rs6265					
	Genotypic	AA/AG/GG	84/215/82	97/197/130	**0.006**	-
	Dominant	AA+AG/GG	299/82	294/130	**0.003**	**1.612 (1.171-2.220)**
	Recessive	AA/AG+GG	84/297	97/327	0.778	0.953 (0.684-1.328)
	rs12291063					
	Genotypic	CC/CT/TT	7/106/266	10/135/279	0.404	-
	Dominant	CC+CT/TT	113/266	145/279	0.184	0.817 (0.607-1.101)
	Recessive	CC/CT+TT	7/372	10/414	0.615	0.779 (0.294-2.067)
Male	rs6265					
	Genotypic	AA/AG/GG	33/98/42	31/51/35	0.087	-
	Dominant	AA+AG/GG	131/42	82/35	0.286	1.331 (0.786-2.254)
	Recessive	AA/AG+GG	33/140	31/86	0.135	0.654 (0.374-1.144)
	rs12291063					
	Genotypic	CC/CT/TT	2/47/122	1/33/83	0.961	-
	Dominant	CC+CT/TT	49/122	34/83	0.941	0.980 (0.584-1.647)
	Recessive	CC/CT+TT	2/169	1/116	0.796	1.373 (0.123-15.317)
Female	rs6265					
	Genotypic	AA/AG/GG	48/112/38	63/136/89	**0.015**	**-**
	Dominant	AA+AG/GG	160/38	199/89	**0.004**	**1.883 (1.221-2.904)**
	Recessive	AA/AG+GG	48/150	63/225	0.541	1.143 (0.744-1.754)
	rs12291063					
	Genotypic	CC/CT/TT	5/55/138	7/96/185	0.428	-
	Dominant	CC+CT/TT	60/138	103/185	0.210	0.781 (0.530-1.150)
	Recessive	CC/CT+TT	5/193	7/281	0.947	1.040 (0.325-3.325)
Stage 2: 331 AD cases versus 334 controls						
All	rs6265					
	Genotypic	AA/AG/GG	73/182/76	86/180/68	0.471	-
	Dominant	AA+AG/GG	255/76	266/68	0.415	0.858 (0.593-1.241)
	Recessive	AA/AG+GG	73/258	86/248	0.264	0.816 (0.571-1.166)
	rs12291063					
	Genotypic	CC/CT/TT	8/101/222	7/89/237	0.520	-
	Dominant	CC+CT/TT	109/222	96/237	0.253	1.212 (0.872-1.686)
	Recessive	CC/CT+TT	8/323	7/326	0.785	1.153 (0.413-3.218)
Male	rs6265					
	Genotypic	AA/AG/GG	29/65/25	45/78/36	0.642	-
	Dominant	AA+AG/GG	94/25	123/36	0.745	1.100 (0.618-1.959)

	Recessive	AA/AG+GG	29/90	45/114	0.463	0.816 (0.475-1.404)
	rs12291063					
	Genotypic	CC/CT/TT	3/36/80	3/41/115	0.648	-
	Dominant	CC+CT/TT	39/80	44/115	0.358	1.274 (0.760-2.137)
	Recessive	CC/CT+TT	3/116	3/156	0.719	1.345 (0.267-6.784)
Female	rs6265					
	Genotypic	AA/AG/GG	41/111/48	40/99/31	0.380	-
	Dominant	AA+AG/GG	152/48	139/31	0.177	0.706 (0.425-1.172)
	Recessive	AA/AG+GG	41/159	40/130	0.483	0.838 (0.512-1.373)
	rs12291063					
	Genotypic	CC/CT/TT	5/62/133	4/46/120	0.697	-
	Dominant	CC+CT/TT	67/133	50/120	0.399	1.209 (0.777-1.880)
	Recessive	CC/CT+TT	5/195	4/166	0.927	1.064 (0.281-4.027)

OR, odds ratio; CI, confidence interval.

[a] *P*-values less than 0.05 were marked in bold.

Table S7. Association between *BDNF* and AD grouped by *APOE*-ε4 and gender status.

SNP	APOE4±Male/Female	Allele		*P*-value [a]	OR	95% CI
		AD	control			
Stage 1: Han Chinese from East China						
rs6265 (A/G)	APOE4+/Male	54/66	28/24	0.286	0.701	0.365-1.348
	APOE4+/Female	96/80	38/52	0.057	1.642	0.983-2.742
	APOE4-/Male	108/114	85/97	0.697	1.081	0.730-1.601
	APOE4-/Female	112/108	224/262	0.235	1.213	0.882-1.669
rs12291063 (C/T)	APOE4+/Male	16/102	8/42	0.679	0.824	0.328-2.070
	APOE4+/Female	28/148	16/74	0.698	0.875	0.446-1.718
	APOE4-/Male	35/185	27/157	0.732	1.100	0.638-1.898
	APOE4-/Female	37/183	94/392	0.424	0.843	0.555-1.282
Stage 2: Han Chinese from Southwest China						
rs6265 (A/G)	APOE4+/Male	35/29	30/18	0.407	0.724	0.337-1.555
	APOE4+/Female	19/23	54/52	0.531	0.796	0.388-1.630
	APOE4-/Male	105/117	97/91	0.386	1.188	0.805-1.752
	APOE4-/Female	144/128	136/152	0.176	1.257	0.902-1.752
rs12291063 (C/T)	APOE4+/Male	10/54	6/42	0.640	1.296	0.436-3.854
	APOE4+/Female	9/33	16/90	0.354	1.534	0.618-3.807
	APOE4-/Male	34/188	36/152	0.304	0.764	0.456-1.278
	APOE4-/Female	41/231	55/233	0.207	0.752	0.483-1.172

OR, odds ratio; CI, confidence interval.

[a] *P*-values were calculated by using the Chi-square test.

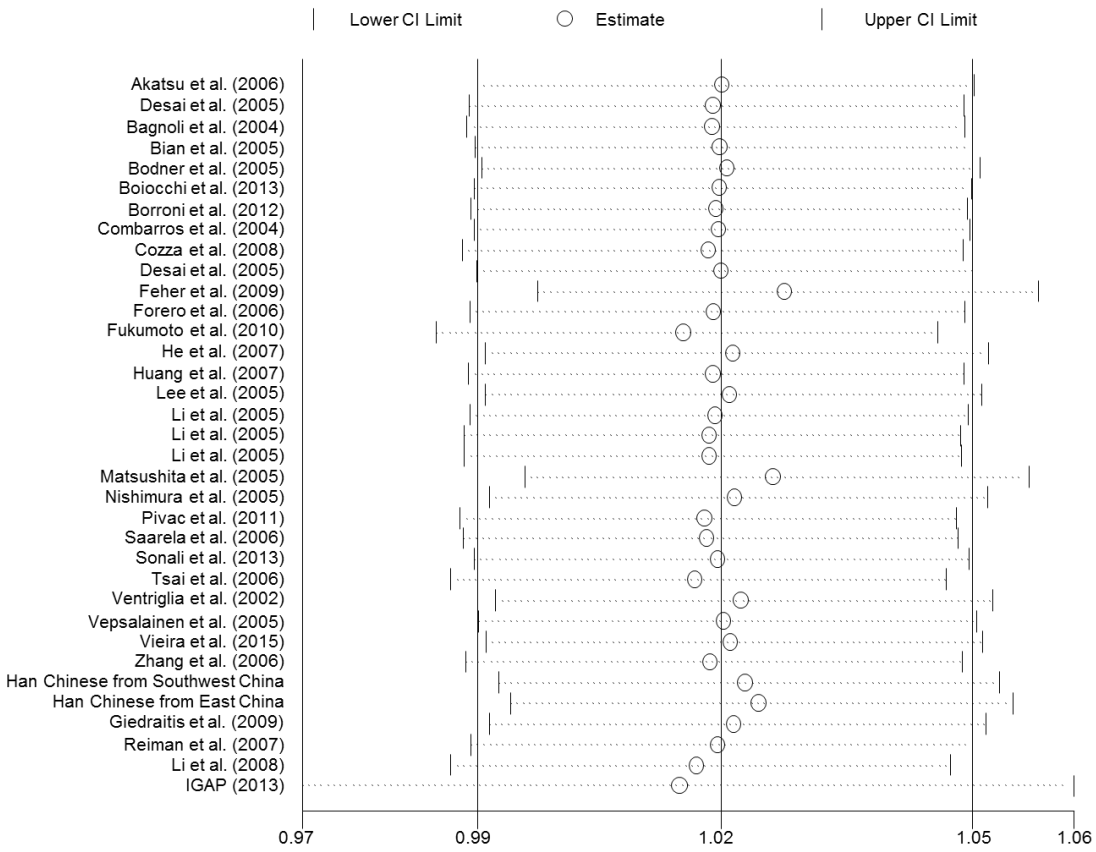

Figure S1. Sensitivity analysis of the summary odds ratio about the association between rs6265 and AD. A total of 31 genetic association studies and our new data in this study were analyzed (Supplementary Table S2); Shown are results with the named study being omitted. Estimate, odds ratio; CI, 95% confidence interval.

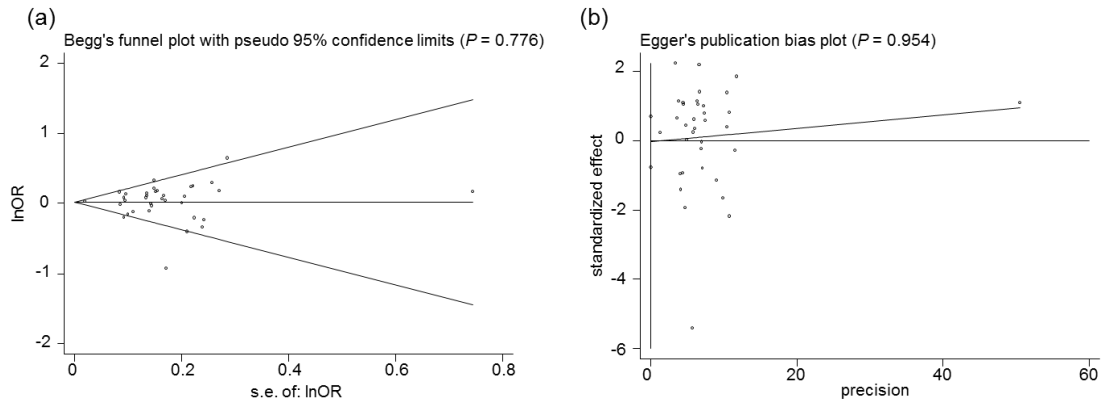

Figure S2. Funnel plots under the allelic model showed no asymmetric trend after being assessed by (a) the Begg's (P = 0.776) and (b) Egger's (P = 0.954) tests. A total of 31 genetic association studies and our new data in this study were considered (Supplementary Table S2). OR, odds ratio; se, standard error.

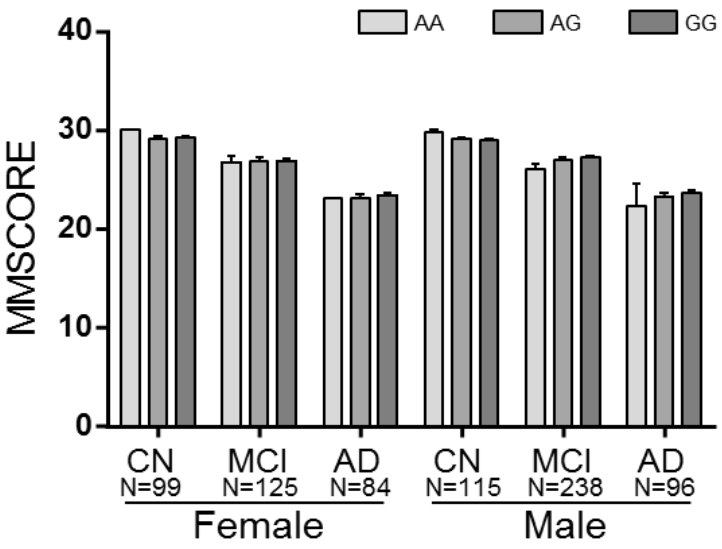

Figure S3. Effect of rs6265 on Mini Mental State Examination scores of males and females. Data was retrieved from ADNI project (http://adni.loni.usc.edu/) (Weiner, et al., 2010) and was analyzed by the PLINK software using the linear regression model (Purcell, et al., 2007). Data plotting was generated by the GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA, USA) with means, SEM, and N. MMSCORE, Mini Mental State Examination (MMSE) score; CN, Control; MCI, Mild cognition impairment; AD, Alzheimer's disease.

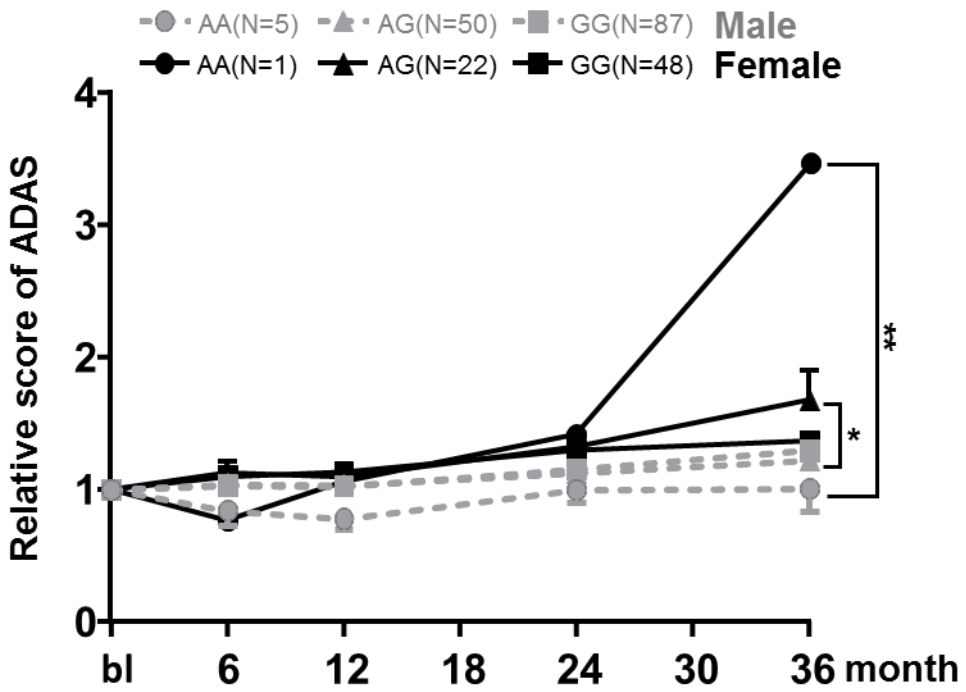

Figure S4. Effect of rs6265 on longitudinal change of ADAS in patients from baseline (bl) to 36 months. As time progresses, female carriers with AA (P= 0.033) or AG (P = 0.017) in patients showed a significantly different pattern compared with males with the same genotypes. The relative score of ADAS was calculated by normalizing the original ADAS score at each longitudinal point with the score at baseline (relative score of ADAS at baseline = 1). Repeated measures multivariate analysis of variance was performed using SPSS 16.0 (SPSS Inc., Chicago, Illinois). Data plotting was generated by GraphPad PRISM software. * $P < 0.05$; ** $P < 0.01$.

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