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CFH Variants Affect Structural and Functional Brain Changes and Genetic Risk of Alzheimer's Disease

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The immune response is highly active in Alzheimer's disease (AD). Identification of genetic risk contributed by immune genes to AD may provide essential insight for the prognosis, diagnosis, and treatment of this neurodegenerative disease. In this study, we performed a genetic screening for AD-related top immune genes identified in Europeans in a Chinese cohort, followed by a multiple-stage study focusing on *Complement Factor H* (*CFH*) gene. Effects of the risk SNPs on AD-related neuroimaging endophenotypes were evaluated through magnetic resonance imaging scan, and the effects on AD cerebrospinal fluid biomarkers (CSF) and *CFH* expression changes were measured in aged and AD brain tissues and AD cellular models. Our results showed that the AD-associated top immune genes reported in Europeans (*CR1*, *CD33*, *CLU,* and *TREML2*) have weak effects in Chinese, whereas *CFH* showed strong effects. In particular, rs1061170 ($P_{meta} = 5.0 \times 10^{-4}$) and rs800292 ($P_{meta} = 1.3 \times 10^{-5}$) showed robust associations with AD, which were confirmed in multiple world-wide sample sets (4317 cases and 16 795 controls). Rs1061170 ($P = 2.5 \times 10^{-3}$) and rs800292 ($P = 4.7 \times 10^{-4}$) risk-allele carriers have an increased entorhinal thickness in their young age and a higher atrophy rate as the disease progresses. Rs800292 risk-allele carriers have higher CSF tau and Aβ levels and severe cognitive decline. *CFH* expression level, which was affected by the risk-alleles, was increased in AD brains and cellular models. These comprehensive analyses suggested that CFH is an important immune factor in AD and affects multiple pathological changes in early life and during disease progress.

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INTRODUCTION

Late-onset Alzheimer's disease (AD, OMIM 104300, 104310) is the most common neurodegenerative disorder and leads to a progressive cognitive decline and dementia in the elderly (Alzheimer's Association, 2013; Querfurth and LaFerla, 2010). The major histological features of the disease include the presence of neurofibrillary tangles, extracellular amyloid β peptide (Aβ) deposition, synaptic dysfunction, and loss of neuronal integrity (Querfurth and LaFerla, 2010). The underlying cause of the disease is unclear in most cases, but numerous genetic alterations have been identified as being associated with Alzheimer's risk (Bertram *et al*, 2007; Karch and Goate, 2015; Lambert *et al*, 2013). Immune-related genes, especially complement genes such as complement receptor 1 (*CR1*) and clusterin (*CLU*; Bertram *et al*, 2007), have been identified as the top AD susceptibility genes

in European populations or of European origin, and the complement system has been reported to be involved in the initiation and development of AD (Crehan *et al*, 2012).

The complement regulator, Complement Factor H (*CFH*, OMIM 134370), has a key role in inhibiting complement activation and inflammation. *CFH* was recognized as the major genetic risk factor for age-related macular degeneration (AMD; Klein *et al*, 2005), which is another age-related neurodegenerative disease and shares similar risk factors and pathological features with AD (Sivak, 2013). CFH protein was suggested to be a potential top serum biomarker for AD (Hye *et al*, 2006, 2014; Thambisetty *et al*, 2008). However, the involvement of CFH in AD is contentious.

We performed a genetic screening in a Han Chinese cohort with AD for five immune genes (*CR1*, *CR2*, *CLU*, *CD33*, and *TREML2*) that were identified as the top AD susceptibility genes for Europeans (Bertram *et al*, 2007; Lambert *et al*, 2013). After the screening, a multiple-stage genetic association study focusing on the *CFH* gene was performed. We aimed to answer two key questions: (1) Do genetic variants in these immune genes, especially *CFH*, confer risk to AD in Han Chinese? and (2) How does CFH function in AD? The involvement of CFH in functional and structural brain changes, as well as AD biomarker (cerebrospinal fluid (CSF) tau and Aβ levels) alterations, were explored using data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) project (Weiner *et al*, 2010). Moreover, the effect of AD-related *CFH* SNPs on morphological changes of hippocampus and entorhinal cortex, which were recognized as the most and the first affected regions of the brain with AD (Harris *et al*, 2010; Khan *et al*, 2014), respectively, was measured in healthy young adults at genetic risk by magnetic resonance imaging (MRI) scan. The effects of *CFH* genotypes and expression changes were analyzed in aged and AD brain tissues and in AD cellular models. Our collective data indicated that *CFH* is an important AD susceptibility gene and may affect the structure and function of the brain and alter the immune response as the disease progresses.

MATERIALS AND METHODS

Subjects

A two-stage cohort of 2041 Han Chinese with and without AD was analyzed. In stage 1, 380 patients (AD1, 45.8% men, mean age 76.5 ± 9.6 years, mean onset age 70.9 ± 9.7 years) and 475 healthy individuals from the general populations (PC1) were recruited from East China. In stage 2, we recruited 345 patients (AD2), 337 healthy individuals from the general populations (PC2), and 504 healthy longevity individuals (LC, age 93 ± 2.6 years; as another control) from Southwest China. Most of these AD patients had been analyzed for other risk loci in our recent studies (Bi *et al*, 2014, 2015; Wang *et al*, 2014). In brief, patients were diagnosed following the DSM-IV and the NINCDS-ADRDA criteria independently by at least two senior clinicians. The healthy controls were confirmed to have normal cognitive ability. Informed consents conforming to the tenets of the Declaration of Helsinki were obtained from all participants, or the supervisors of patients, after being given a complete description of the study. The institutional review board of the Kunming Institute of Zoology, Chinese Academy of Sciences, approved this study.

To confirm the results of the two-stage study, we performed jointed comparisons with multiple world-wide sample sets. Additional cohorts from East and Southwest China: 2460 individuals from Shanghai, 1549 individuals from Sichuan Province, and 2751 individuals from Yunnan Province (Zhang *et al*, 2014), which were enrolled for other genetic association analyses, were included in this analysis to enlarge the population controls. All of these subjects were collected from the general populations with normal cognitive ability and no history of dementia. Individuals with genotype data of rs800292 and rs1061170 available were included in our jointed comparison. Genetic data from ADNI (http://adni.loni.usc.edu/; Weiner *et al*, 2010) were also retrieved for re-analysis. Subjects with available genotype data from all stages of the ADNI 1/GO/2 were included in our analyses. These samples contain 760 individuals in the ADNI1 cohort (180 probable AD patients, 363 mild cognitive impairment (MCI) patients, and 214 cognitively normal aging controls) and 430 individuals in the ADNI GO/2 cohort (29 probable AD patients, 275 MCI patients, and 126 cognitively normal aging controls). Because of the limited sample size of probable AD, AD and MCI participants in these two cohorts were pooled as the patients' group. Previously reported data regarding the association of rs1061170 with AD (Hamilton *et al*, 2007; Le Fur *et al*, 2010; Proitsi *et al*, 2012; Zetterberg *et al*, 2008) were re-analyzed together with the data from our current samples. In total, 719 patients and 6217 population controls from China, and 845 patients and 345 controls of European origin were analyzed for rs800292; 713 patients and 6747 controls from China and 3604 patients and 10 048 controls of European origin were analyzed for rs1061170.

SNP Genotyping and Association Analysis

We genotyped 17 SNPs of five immune genes (*CR1*, *CR2*, *CLU*, *CD33*, and *TREML2*) that were identified as the top Alzheimer's susceptibility genes in Europeans (Bertram *et al*, 2007; Lambert *et al*, 2013) and 11 SNPs of the *CFH* gene in our stage 1 cohort from East China for the preliminary screening. Previously reported genome-wide association study (GWAS) top hits, tagging SNPs and potentially functional SNPs of these genes were selected for genotyping. The selection criteria and details for selected SNPs were described in the Supplementary Methods and Supplementary Table 1. *APOE* ε4 was determined as previously described (Bi *et al*, 2014, 2015; Wang *et al*, 2014).

Association analysis was carried out using PLINK (Purcell *et al*, 2007). Allelic (Table 1) and genotypic (Supplementary Table 2) comparisons with 2 d.f. genotypic, Cochran-Armitage trend, dominant, and recessive models were conducted for individual SNPs. All available samples from the general populations were pooled as a combined sample for Chinese (termed 'Combined Chinese', Table 2) and Europeans (termed 'Combined Europeans', Table 2), respectively. Comparison of the genotype counts between the combined case and control populations was estimated by the χ^2 test. Meta-analysis for the association of *CFH* SNPs with AD in the two combined sample sets was performed by using Review manager (RevMan 5.2, http://tech.cochrane.org/rev

Table 1 Association of *CFH* Variants with AD in Han Chinese Populations ($N = 20411$)

SNP	Allele	Stage 1 (SH:AD1 *vs* PC1)				Stage 2-1 (SC:AD2 *vs* PC2)				Stage 2-2 (SC:AD2 *vs* LC)			Combined (AD1+AD2 *vs* PC1+PC2)				
		F_{AD1}	F_{PC1}	OR (95%CI)	P_A	F_{AD2}	F_{PC2}	OR (95%CI)	P_A	F_{LC}	OR (95%CI)	P_A	F_{AD}	F_{PC}	OR (95%CI)	P_A	$P_{Corrected}$
rs800292	T/C	0.362	0.402	0.844 (0.693–1.028)	0.092	0.376	0.441	0.764 (0.615–0.949)	*0.015*	0.426	0.811 (0.664–0.989)	*0.038*	0.369	0.418	0.812 (0.702–0.940)	*5.1 × 10^{-3}*	*0.019*
rs1061170	C/T	0.066	0.039	1.749 (1.130–2.705)	*0.011*	0.073	0.043	1.749 (1.091–2.805)	*0.019*	0.037	2.047 (1.320–3.174)	*0.001*	0.069	0.041	1.759 (1.277–2.422)	*4.7 × 10^{-4}*	*0.005*
rs10801555	A/G	0.044	0.040	1.098 (0.682–1.769)	0.699	0.057	0.043	1.345 (0.822–2.201)	0.237	0.052	1.105 (0.721–1.693)	0.648	0.050	0.041	1.225 (0.871–1.722)	0.242	0.409
rs10922096	T/C	0.122	0.128	0.943 (0.706–1.260)	0.692	0.109	0.110	0.990 (0.704–1.393)	0.954	0.115	0.942 (0.691–1.284)	0.705	0.116	0.121	0.954 (0.765–1.189)	0.674	0.823
rs2019727	T/A	0.073	0.092	0.781 (0.549–1.110)	0.167	0.051	0.071	0.703 (0.449–1.102)	0.123	0.060	0.847 (0.552–1.300)	0.446	0.063	0.083	0.736 (0.558–0.971)	0.030	0.065
rs10733086	A/T	0.057	0.060	0.948 (0.630–1.425)	0.796	0.069	0.064	1.086 (0.708–1.666)	0.705	0.070	0.986 (0.672–1.446)	0.940	0.063	0.062	1.019 (0.759–1.367)	0.900	0.900
rs10737680	C/A	0.395	0.430	0.868 (0.715–1.055)	0.154	0.452	0.435	1.071 (0.865–1.327)	0.528	0.433	1.078 (0.887–1.311)	0.449	0.422	0.432	0.962 (0.833–1.110)	0.595	0.817
rs1410996	T/C	0.391	0.428	0.858 (0.706–1.042)	0.122	0.431	0.433	0.992 (0.800–1.230)	0.942	0.433	0.993 (0.816–1.208)	0.940	0.410	0.430	0.921 (0.797–1.063)	0.260	0.409
rs11582939	C/T	0.443	0.494	0.816 (0.673–0.988)	*0.037*	0.461	0.510	0.819 (0.662–1.013)	0.066	0.496	0.867 (0.714–1.054)	0.152	0.489	0.492	0.988 (0.857–1.138)	0.863	0.900
rs426736	T/C	0.532	0.458	1.345 (1.110–1.629)	*0.002*	0.494	0.503	0.965 (0.780–1.194)	0.745	0.489	1.021 (0.840–1.240)	0.836	0.520	0.474	1.199 (1.040–1.382)	*0.012*	*0.034*

Abbreviations: AD1, patients with AD of stage 1 from Shanghai (SH); AD2, patients with AD of stage 2 from Sichuan (SC); CI, confidence interval; F_x, minor allele frequency of the 'x' population; LC, longevity controls of stage 2 from Sichuan; OR, odds ratio; P_A, allelic association *P*-value; $P_{Corrected}$, adjusted *P*-value (combined P_A) for multiple testing by Benjamini-Hochberg FDR (FDR_BH) control algorithm; PC1, population controls of stage 1 from Shanghai; PC2, population controls of stage 2 from Sichuan. The *P*-values less than 0.05 were marked in italic. Results of genotypic associations and genotype counts distribution were shown in Supplementary Table 2.

man), with the Cochran–Mantel–Haenszel method under a fixed effect. The heterogeneity was measured by the I^2 index (Higgins and Thompson, 2002; Table 2).

The genetic associations were explored further by estimating the significance of SNP–SNP interaction using the multifactor-dimensionality reduction (MDR) method (Ritchie *et al*, 2001) or the '—epistasis' command in PLINK (Purcell *et al*, 2007).

Neuroimaging Analysis for the Effects of CFH Variants on Structural and Functional Brain Changes

We recruited 360 healthy young adults (age 19.4 ± 1.1 years; 51.7% men) to study the effects of the Alzheimer's risk *CFH* SNPs on morphological changes of the brain. These samples were described in our previous study and were effective to identify risk alleles affecting brain structure variations (Li *et al*, 2015; Zhang *et al*, 2015). MRI scans were performed on a MR750 3.0 Tesla magnetic resonance scanner (GE Healthcare, detailed parameters in Supplementary Methods). The protocol was approved by the Ethics Committee of School of Life Science and Technology at University of Electronic Science and Technology of China.

First, we performed a whole-brain voxel-based morphometry analysis for volume and density of the gray matter. Second, we detected the effect of the AD-risk *CFH* SNPs on total intracranial volume (ICV) and hippocampus volume changes. Finally, we tested the effect of the AD-risk *CFH* SNPs on the thickness of the entorhinal cortex. To test the effect of *CFH* genotypes on brain morphological changes, we applied a general linear regression model adjusted for gender, age, education year, and ICV. To correct for multiple comparisons for the entorhinal cortex, the statistical significance level was set as $P < 0.005$ (0.05/10 [5 SNPs × 2 hemisphere], Bonferroni correction). Details regarding the imaging process and statistics were described in Supplementary Methods.

Detecting the Effects of AD-Risk SNPs on AD Endophenotypes

To confirm our results and investigate further the role of CFH in AD pathogenesis, we obtained genetic, neuroimaging, and biomarker data from the ADNI project (http://adni.loni.usc.edu/; Weiner *et al*, 2010). Effect of the top AD-risk *CFH* SNP (only rs800292 was available) on AD endophenotypes, eg, CSF tau and Aβ levels, cognitive score, entorhinal regional atrophy rate, and entorhinal volume, was analyzed using PLINK (Purcell *et al*, 2007).

Expression Quantitative Trait Loci (eQTL) Analysis

To investigate the effect of *CFH* variants on *CFH* mRNA expression level, we performed eQTL analysis in 10 brain regions of 134 neuropathologically normal individuals. Details were shown in Supplementary File and the brain eQTL database (http://www.braineac.org/; Ramasamy *et al*, 2014). The eQTL effect of the *CFH* variants was validated by using the Genotype-Tissue Expression project (GTEx, http://www.gtexportal.org/home/) database, which provides a comprehensive atlas of gene expression and regulation across multiple human tissues (The GTEx

Table 2 Validating the Associations of rs800292 and rs1061170 with AD in Enlarged Sample Sets

SNP	Population	Case		Control		*P*-value	OR
		N	MAF	*N*	MAF		
rs800292	Shanghai[a]	377	0.362	1917	0.410	1.6×10^{-2}	0.82
	Sichuan[b]	342	0.376	1549	0.419	3.7×10^{-2}	0.84
	Combined[c]	719	0.369	3466	0.414	2.0×10^{-3}	0.83
	Combined Chinese[d]	719	0.369	6217	0.415	7.0×10^{-4}	0.82
	ADNI_1[e]	543	0.236	214	0.304	6.0×10^{-3}	0.71
	ADNI_GO2[e]	302	0.207	126	0.246	2.1×10^{-1}	0.78
	European (ADNI)	845	0.225	340	0.282	3.0×10^{-3}	0.74
	Meta-analysis[f]	1564	—	6557	—	1.3×10^{-5}	0.80
rs1061170	Shanghai[a]	377	0.066	2460	0.055	2.2×10^{-1}	1.21
	Sichuan[b]	336	0.073	1542	0.043	3.0×10^{-3}	1.65
	Combined[c]	713	0.069	4002	0.051	6.0×10^{-3}	1.38
	Combined Chinese[d]	713	0.069	6747	0.057	5.5×10^{-2}	1.24
	European (Zetterberg *et al*, 2008)	800	0.427	1265	0.394	3.9×10^{-2}	1.14
	European (Le Fur *et al*, 2010)	701	0.357	6990	0.361	7.7×10^{-1}	0.98
	European (Proitsi *et al*, 2012)	2103	0.385	1793	0.375	3.4×10^{-1}	1.05
	Combined Europeans[g]	3604	0.389	10048	0.368	1.0×10^{-3}	1.09
	Meta-analysis[h]	4317	—	16795	—	5.0×10^{-4}	1.10

Abbreviation: MAF, minor allele frequency.
[a]Enlarged general population controls including the case-matched controls and available general individuals with normal cognitive ability from Shanghai (author's unpublished data).
[b]Enlarged controls including the case-matched controls and other available general individuals with normal cognitive ability from Sichuan (author's unpublished data).
[c]Samples combining both Shanghai and Sichuan subjects.
[d]Chinese samples combining Shanghai, Sichuan, and Yunnan subjects with available genotype data as the general population control. For rs800292: heterogeneity among combined Chinese populations, $\chi^2 = 0.03$, d.f. = 1 ($P = 0.86$), $I^2 = 0\%$, overall meta-analysis effect $Z = 3.21$ ($P = 0.001$). For rs1061170: heterogeneity among combined Chinese populations, $\chi^2 = 2.34$, d.f. = 1 ($P = 0.13$), $I^2 = 57\%$, overall meta-analysis effect $Z = 3.05$ ($P = 0.002$).
[e]Data retrieved from the ADNI (Alzheimer's Disease Neuroimaging Initiative) project. For rs800292: heterogeneity among combined European populations, $\chi^2 = 0.32$, d.f. = 1 ($P = 0.57$), $I^2 = 0\%$, overall meta-analysis effect $Z = 2.95$ ($P = 0.003$); genotype data of rs1061170 is not available in the ADNI subjects.
[f]Meta-analysis for rs800292 in combined Chinese and Europeans; heterogeneity: $\chi^2 = 0.91$, d.f. = 1 ($P = 0.34$), $I^2 = 0\%$; overall meta-analysis effect $Z = 4.32$ ($P < 0.0001$).
[g]All three available European sample sets were pooled together as a combined European population, with the original genotype counts measured by χ^2 test; heterogeneity among populations, $\chi^2 = 3.04$, d.f. = 2 ($P = 0.22$), $I^2 = 34\%$, overall meta-analysis effect $Z = 1.48$ ($P = 0.14$).
[h]Meta-analysis for rs1061170 in combined Chinese and Europeans, heterogeneity: $\chi^2 = 0.93$, d.f. = 1 ($P = 0.34$), $I^2 = 0\%$, overall effect $Z = 3.49$ ($P = 0.0005$). The *P*-values less than 0.05 were marked in italic.

Consortium, 2013). The tibial nerve tissue ($n = 102$) was used in the analysis; the other brain tissues, such as cortex or hippocampus, had a sample size less than 30 and was not considered (http://www.gtexportal.org/home/). For the effect of the *CFH* variants on CFH protein level, we used an earlier GWAS data of plasma CFH levels (Ansari *et al*, 2013).

CFH mRNA Expression Alterations in Aged and AD Brains and AD Cellular Models

A total of 49 hippocampal samples of *Rattus norvegicus* at 5 age points (3, 6, 9, 12, and 23 months, GSE9990; Kadish *et al*, 2009) and 30 postmortem frontal cortex of normal individuals at 26–106 years of age (GSE1572; Lu *et al*, 2004) were used to assess *CFH* mRNA expression changes during brain aging. Expression data of 272 human postmortem dorsolateral prefrontal cortex (DLPFC) of normal subjects across the lifespan from the BrainCloud (http://braincloud.jhmi.edu/; Colantuoni *et al*, 2011) were also included to investigate the expression pattern of *CFH* with aging. In all, 22 hippocampal samples from postmortems showing AD at different stages of severity (GSE1297; Blalock *et al*, 2004), and entorhinal cortex neurons containing neurofibrillary tangles from 10 mid-stage patients (GSE4757; Dunckley *et al*, 2006) were used to assess *CFH* mRNA expression changes during disease processing. Expression differences between groups were measured by Student's *t*-test using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, USA). The expression data were retrieved through the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/sites/GDSbrowser). Correlation analysis was performed for the mRNA expression of *CFH* and *APP* in hippocampus of AD patients (GSE1297; Blalock *et al*, 2004).

U251 cells (a glioma cell line) with $A\beta_{1-42}$ treatment or with stable overexpression of APP mutant (APP^{Mut}, APP-p.M671L) and PSEN1 mutant ($PSEN1^{Mut}$, PSEN1-p.M139V/M146L/H163R) were used as AD cellular models to test *CFH* expression changes in response to these stimuli. Quantitative real-time PCR was used to determine the relative mRNA level of the *CFH* gene in the AD cellular models. Detailed information was shown in the Supplementary Materials.

RESULTS

Genetic Screening of the AD-Related Immune Genes and CFH in Han Chinese Patients with AD

SNPs within the five AD-related top immune genes (*CR1*, *CR2*, *CLU*, *CD33*, and *TREML2*) identified in Europeans showed no association with AD in our stage 1 Chinese samples (Supplementary Table 1). We observed positive associations of *CFH* SNPs rs426736 (OR = 1.345, allelic $P = 2.4 \times 10^{-3}$, genotypic $P = 1.2 \times 10^{-2}$) and rs1061170 (p.Y402H, OR = 1.749, allelic $P = 1.1 \times 10^{-2}$, genotypic $P = 1.3 \times 10^{-2}$) with AD, whereas rs800292 (p.V62I) and rs11582939 showed a marginal significance (Table 1 and Supplementary Table 2) in our stage 1 samples.

We replicated the association of *CFH* with AD in stage 2 cohort. Associations of both rs1061170 (OR = 1.749, allelic $P = 1.9 \times 10^{-2}$, genotypic $P = 3.4 \times 10^{-2}$) and rs800292 (OR = 0.764, allelic $P = 1.5 \times 10^{-2}$, genotypic $P = 6.5 \times 10^{-4}$) with AD were confirmed. When the cases were compared with the healthy longevity controls (>90 years old), rs1061170 (OR = 2.047, allelic $P = 1.1 \times 10^{-3}$, genotypic $P = 9.3 \times 10^{-4}$) showed a much stronger association with AD, and the association of rs800292 (OR = 0.811, allelic $P = 0.038$, genotypic $P = 3.0 \times 10^{-3}$) with AD remained significant. We combined the two independent samples and found that rs1061170 (OR = 1.759, $P_{\mathrm{FDR\_BH}} = 5.2 \times 10^{-3}$) and rs800292 (OR = 0.812, $P_{\mathrm{FDR\_BH}} = 1.9 \times 10^{-2}$) showed strong associations even after correcting for multiple testing (false discovery rate (Benjamini Hochberg), FDR_BH). No SNP–SNP interaction among *CFH* variants and between *CFH* and *APOE* SNPs (rs429358 and rs7412 that defining the $\epsilon 4$ status) was observed, suggesting that *CFH* was involved in AD independently of *APOE*.

Validating the Association of CFH with AD in the Enlarged Sample Sets

We validated the association of the most robust *CFH* SNPs rs800292 and rs1061170 with AD in enlarged world-wide sample sets (Table 2). Compared with the pooled larger population controls ($n = 3466$) from East and Southwest China, the association of rs800292 with AD remained robust ($P = 2.0 \times 10^{-3}$). When all the population controls ($n = 6217$) were considered, the association was even stronger ($P = 7.0 \times 10^{-4}$). Analysis of the ADNI data showed that rs800292 was also associated with AD in Europeans (845 cases *vs* 340 controls; $P = 3.0 \times 10^{-3}$). Meta-analysis combining all Chinese and European samples (1564 cases *vs* 6557 controls), which would increase the statistical power and had no significant study heterogeneity (cf. the footnote of Table 2), further validated the association of rs800292 with AD ($P_{meta} = 1.3 \times 10^{-5}$, OR = 0.80).

Similarly, we validated the association of rs1061170 with AD in combined population controls ($n = 4002$) from East and Southwest China ($P = 6.0 \times 10^{-3}$), but this effect turned out to be marginally significant ($P = 5.5 \times 10^{-2}$) when all Han Chinese controls ($n = 6747$) were considered. Combined analysis of previously reported data showed a positive association of rs1061170 with AD in Europeans (3604 cases *vs* 10048 controls; $P = 1.0 \times 10^{-3}$). When all combined Han Chinese and European samples (4317 cases *vs* 16795 controls) were used for meta-analysis, we observed a significant association between rs1061170 and AD ($P_{meta} = 5.0 \times 10^{-4}$, OR = 1.10; Table 2).

Effects of the AD-Risk CFH SNPs on Structural Brain Changes in Young Adults

We detected the effects of the AD-risk *CFH* SNPs on morphological changes of the brain in young individuals using structural MRI scan. The AD-related *CFH* SNPs had no apparent effect on estimated total ICV (Supplementary Figure 2) and hippocampal volume (Figure 1 and Supplementary Table 3) in our pilot screening. However, all suggestive AD-associated *CFH* SNPs showed a trend of association with the entorhinal thickness (Supplementary Table 4). Both rs1061170 ($P = 2.5 \times 10^{-3}$) and rs800292 ($P = 4.7 \times 10^{-4}$) were significantly associated with the entorhinal thickness, especially for the right entorhinal cortex (Figure 1). Intriguingly, the AD-risk allele carriers have increased thickness of the entorhinal cortex in the right hemispheres (Figure 1) in their early age. It was reported that trans-synaptic progression of Aβ-induced cortex dysfunction and cortical spread was driven and initiated from the entorhinal cortex in preclinical Alzheimer's disease (Harris *et al*, 2010; Khan *et al*, 2014). Interference of the entorhinal cortex may contribute to the development of AD.

Effects of the AD-Risk CFH SNPs on AD Endophenotypes and CFH Expression

Our MRI scan analyses showed that individuals at risk of AD had a thicker entorhinal cortex in early life, suggesting a potential compensatory effect. Indeed, we observed a decreased volume of the entorhinal cortex in AD patients with risk allele of rs800292 (Figure 2g, h), indicating a higher atrophy rate of risk allele carriers as confirmed in our regional atrophy rate analysis (Figure 2i). In addition, risk allele carriers of rs800292 showed a marginally significant ($P < 0.05$) decrease of cognitive score (Figure 2j), and increase of CSF tau (Figure 2k) and Aβ (Figure 2l) levels. These observations added more support for the contribution of *CFH* variants to AD susceptibility and development. Note that we also found a positive association of *CFH* variant with MCI patients using the ANDI data (Supplementary Table 5), which suggested that an analysis for the association between AD stage and *CFH* genotype might be rewarding.

Besides their effects on AD neuroimaging and biomarker endophenotypes, the risk alleles of rs800292 and rs1061170 were associated with lower *CFH* mRNA level (Figure 2). In particular, the *CFH* mRNA level was significantly decreased in the inferior olivary nucleus (MEDU, $P < 0.01$) and occipital cortex (OCTX, $P < 0.05$) in carriers with the risk allele C of rs1061170 (Figure 2a–c). The most significant genotype-affected *CFH* mRNA changes were observed for an exon-specific probe 2373392, which showed strong associations in all 10 brain regions (aveALL, $P < 0.001$, Figure 2d) and hippocampus (HIPP, $P < 0.01$, Figure 2e). In addition, carriers of rs800292 risk allele showed a significantly decreased *CFH* mRNA expression level in MEDU (Figure 2f). The significant decrease of *CFH* mRNA level associated with rs800292 and rs1061170 risk alleles could be validated in the tibial nerve tissues using the GTEx data (Supplementary Figure 3). Note that our results were in

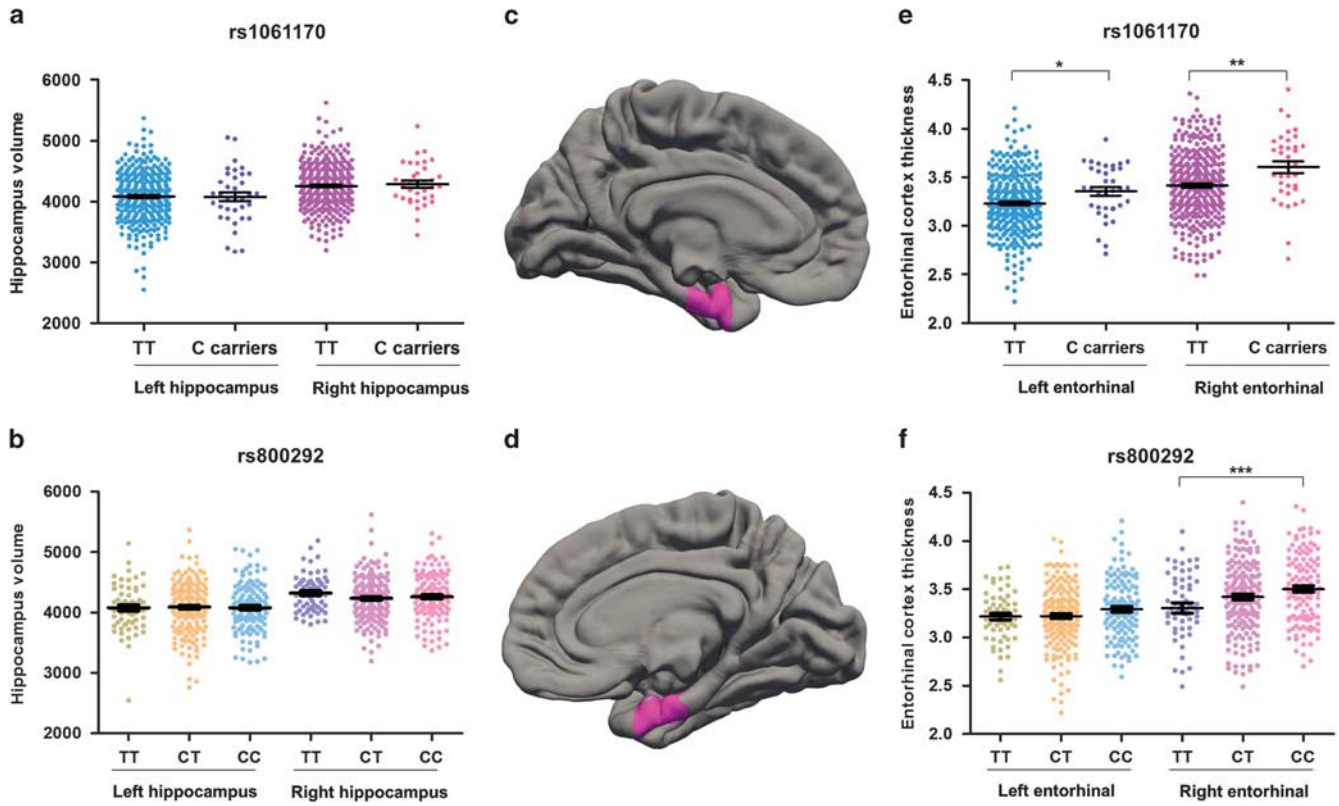

Figure 1 Risk allele carriers have similar hippocampal volume and thicker entorhinal cortex compared with non-risk allele carriers at young age. Regression analysis was conducted to detect the associations of rs1061170 (Y402H) and rs800292 (V62I) with bilateral hippocampal volume (a and b) and entorhinal cortical thickness (e and f). The left entorhinal cortex (c) and the right entorhinal cortex (d) were labeled in FreeSurfer. *$P<0.05$, ** $P<0.01$, ***$P<0.001$, linear regression analyses. Data represent mean ± SEM.

agreement with a reported GWAS study showing a significant association of lower serum CFH protein level with the AD-risk allele of rs1061170 (Ansari *et al*, 2013).

CFH mRNA Expression in Aged and Alzheimer's Brain Tissues and Cellular Assays

As we have observed a decrease of CFH expression levels in risk allele carriers, we further evaluated the alteration of *CFH* mRNA expression in aged and AD brains and AD cellular models. There was an increase of *Cfh* mRNA level with age in the rat hippocampus (Figure 3a). A similar pattern of upregulated *CFH* mRNA during aging was observed in human frontal cortex samples from 30 normal individuals of age 26–106 years (Figure 3b). The significant increase of *CFH* expression level with aging was confirmed in human prefrontal cortex using the BrainCloud data (Supplementary Figure 4). Moreover, *CFH* mRNA expression level increased in hippocampus as the severity of the disease worsened (Figure 3c). We also observed an increase of *CFH* mRNA level in entorhinal neurons containing neurofibrillary tangles compared with normal neurons, although the increase was not significant. The expression of C3, the central component of the complement system, was strongly elevated in tangled entorhinal neurons (Figure 3d). Considering its anti-inflammatory role, the increase of CFH may be the result of aging and balanced CFH level may have a protective effect on aging.

In $A\beta_{1-42}$-treated U251 cells, we observed a significant increase of *CFH* mRNA level (Figure 3f). Expression level of *CFH* was also increased in cells with stable overexpression of APP mutant (APP^{Mut}, APP-p.M671L) and PSEN1 mutant ($PSEN1^{Mut}$, PSEN1-p.M139V/M146L/H163R; Figure 3h). Consistent with the results of cellular assays, *CFH* mRNA level was positively correlated with *APP* mRNA level in hippocampus of AD patients (Pearson $R^2=0.41$, $P=0.007$; Figure 3e). This significant correlation disappeared in control sample or patient–control combined sample (Supplementary Figure 5). Taken together, our results indicated a protective role of increased CFH level in brain aging and AD development, whereas the *CFH* risk alleles were associated with lower CFH level, resulting in an insufficient protection of this immune regulator.

DISCUSSION

Increased activity of the complement system has been reported to be involved in the initiation and development of AD (Crehan *et al*, 2012). Immune genes, especially complement genes, were identified as the top Alzheimer's susceptibility genes in Europeans (Bertram *et al*, 2007; Lambert *et al*, 2013). However, our analysis showed that these genes (*CR1*, *CLU*, *CD33*, and *TREML2*) had very weak effects in Han Chinese. Intriguingly, we found that *CFH*, the most important genetic factor for AMD (Klein *et al*, 2005),

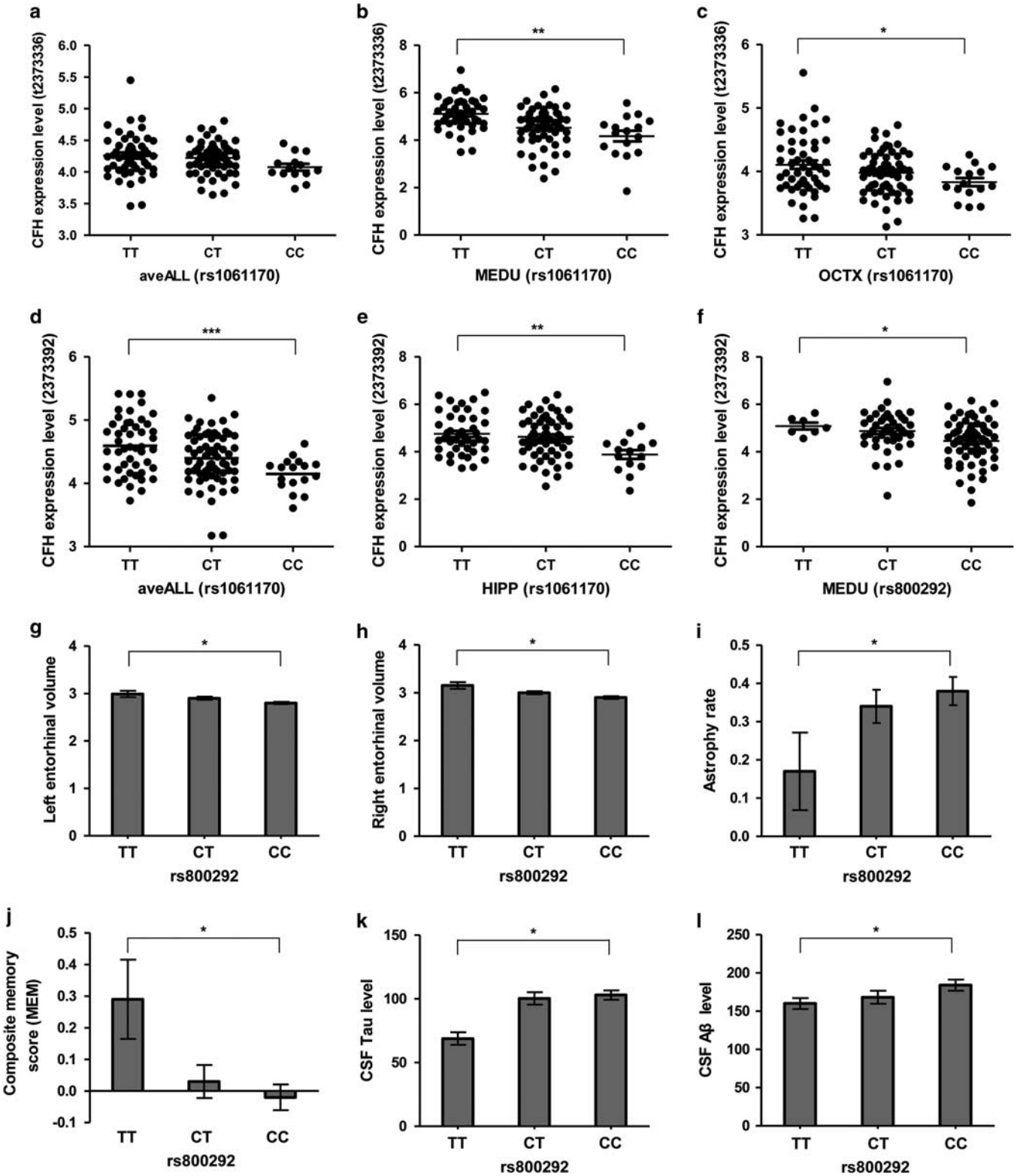

Figure 2 Effects of AD-risk *CFH* SNPs on *CFH* expression and AD endophenotypes. eQTL effect of AD-risk *CFH* SNPs (rs1061170 and rs800292) on *CFH* mRNA expression level was investigated in brain tissues using Affymetrix Human Exon 1.0 ST Array data from the UK Brain Expression Consortium (UKBEC; Ramasamy *et al*, 2014). We retrieved the genotyping and expression data from the UKBEC web server (http://www.braineac.org/; Ramasamy *et al*, 2014). Affymetrix ID t2373336 (a–c), *CFH* transcript probe; Affymetrix ID 2373392 (d–f), *CFH* exon-specific probe (chr1: 196712667–196712698). aveALL, average expression level among the 10 available brain regions; MEDU, the inferior olivary nucleus (sub-dissected from the medulla); OCTX, occipital cortex; HIPP, hippocampus. The potential effects of AD-risk *CFH* SNP rs800292 on AD-related endophenotypes, eg, entorhinal volume (g–h), entorhinal regional atrophy rate (i), composite memory score (MEM, j), and CSF tau (k) and Aβ (l) levels, were analyzed using data retrieved from the ADNI project (http://adni.loni.usc.edu/; Weiner *et al*, 2010). Data represent mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, one-way ANOVA for eQTL analysis; linear regression analyses for SNP rs800292 on AD-related endophenotypes.

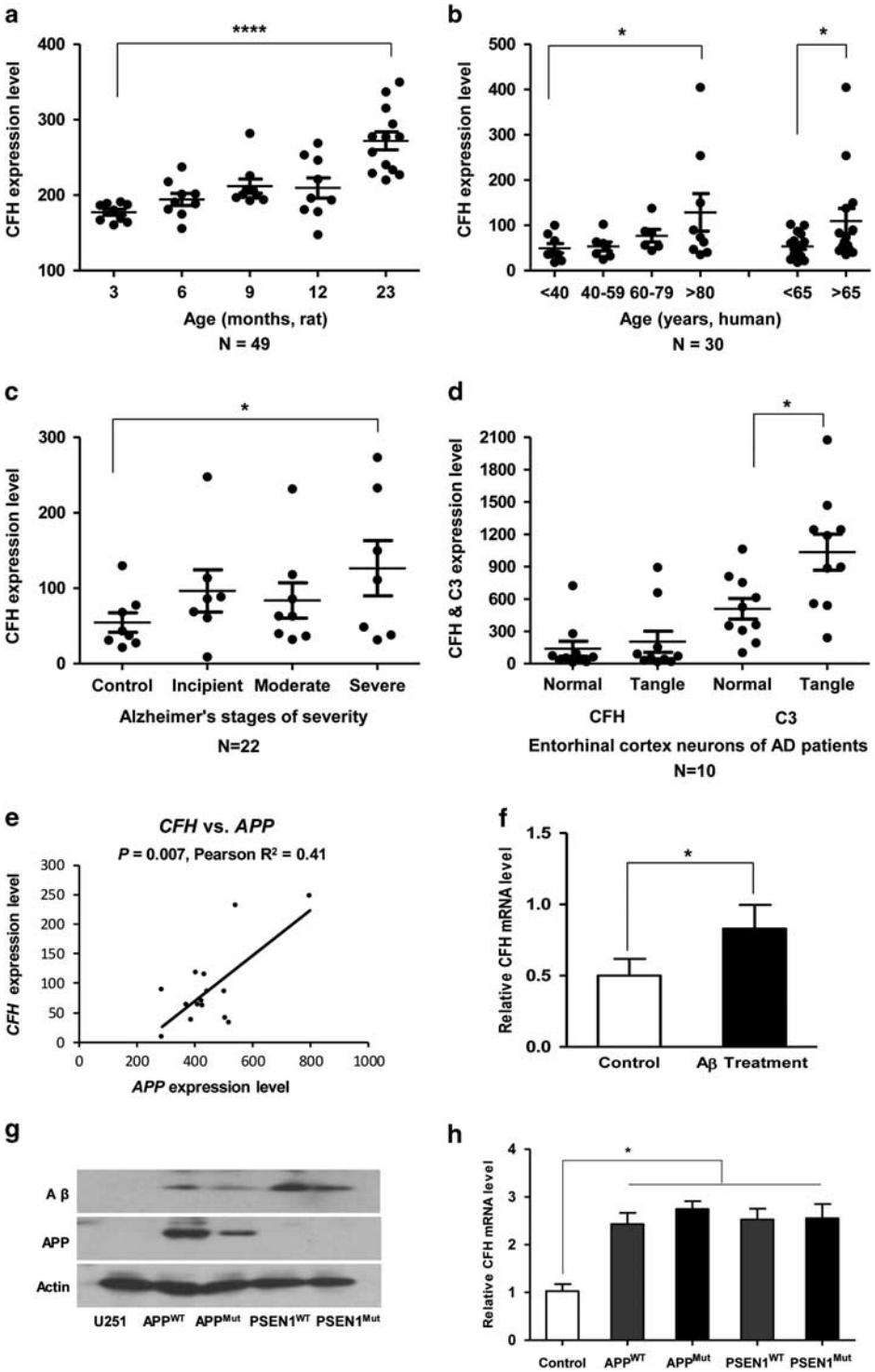

Figure 3 *CFH* expression in aged and AD brains and cellular assays. (a) *Cfh* mRNA expression changes in the hippocampus from 49 *Rattus norvegicus* across the adult lifespan. (b) *CFH* mRNA expression of the postmortem frontal cortex from 30 normal individuals from 26 to 106 years of age. (c) *CFH* mRNA expression in brain hippocampus from 22 postmortem subjects with AD at different stages of severity. (d) *CFH* and *C3* mRNA expression levels in entorhinal cortex neurons containing neurofibrillary tangles were increased relative to those of normal neurons from the same brain region in 10 mid-stage AD patients. (e) Correlation between *CFH* mRNA level (213800_at) and *APP* mRNA level (probe 211277_x_at) in hippocampus of AD patients ($N = 15$) with incipient and moderate stages of severity. (f) Increase of *CFH* mRNA expression in U251 cells with $A\beta_{1-42}$ treatment. (g) The APP and Aβ levels in cells with stable overexpression of APP (wild-type (APP^{wt}) and APP-p.M671L mutant (APP^{Mut})) and PSEN1 (wild-type ($PSEN1^{wt}$) and PSEN1-p.M139V/M146L/H163R ($PSEN1^{Mut}$). (h) *CFH* mRNA expression level was increased in cells with stable overexpression of APP and PSEN1. Data represent mean ± SEM. $^{*}P < 0.05$, $^{****}P < 0.0001$, Student's *t*-test.

acts as an important AD susceptibility gene in Han Chinese patients and has multiple roles in AD pathology.

CFH Variants are Associated with Brain Changes and Confer Alzheimer's Susceptibility

By a comprehensive analysis of the *CFH* SNPs in Han Chinese with and without AD, and a meta-analysis of world-wide published data, we found that several SNPs, especially rs1061170 (a well-known causal risk SNP for AMD; Klein *et al*, 2005) and rs800292, showed robust associations with AD (Table 1). This result clarified the previous conflicting observations (Hamilton *et al*, 2007; Le Fur *et al*, 2010; Proitsi *et al*, 2012; Zetterberg *et al*, 2008). It is to be noted the risk allele C of rs1061170 presents with a marked regional distribution (7% in the East Asian Ancestry population, 28% in the African Ancestry population, 41% in the European Ancestry population; data from the 1000 genome (http://www.1000genomes.org; Abecasis *et al*, 2012) and this might account for the different patterns of association between different populations. The higher risk allele frequency in Europeans might interpret partially the higher prevalence of AMD (Wong *et al*, 2014) and AD (cf. Alzheimer's Disease International, World Alzheimer Report 2009: The Global Prevalence of Dementia, http://www.alz.co.uk/research/world-report-2009) in Europeans than in Asians, although the effect size of the risk allele was smaller in Europeans than in Asians.

Intriguingly, our neuroimaging analysis showed that the Alzheimer's risk alleles were associated with an increased right entorhinal thickness in young adults (Figure 1). The brain immune cell glia, the most abundant cells in brain, was previously reported to contribute to half of brain volume changes and would be overactive in neuro-inflammation (DiBattista *et al*, 2014). It is thus possible that the increase in entorhinal cortex thickness might be due to a deficit in *CFH* risk allele carriers to control neuro-inflammation in the brain, as CFH serves an anti-inflammatory component. In addition, it has been reported that increased entorhinal cortex volume during the brain development could indicate a deficit in neural efficiency (DiBattista *et al*, 2014; Gogtay *et al*, 2004). Although the molecular and cellular mechanisms responsible for the increased entorhinal thickness in young *CFH* risk allele carriers remained to be elucidated, this result is not unexpected as the entorhinal cortex has an essential role in AD (Khan *et al*, 2014). There are further lines of evidence supporting an enhanced entorhinal structure or activity in healthy adults with young age and a higher atrophy rate as the disease progresses for those risk allele carriers. For instance, healthy *APOE* ε4 carriers showed a thicker right entorhinal cortex as compared with the left hemisphere (Donix *et al*, 2013) and a thinner left entorhinal cortex in *APOE* ε4 carriers than in non-carriers could be identified in children and adolescents (Shaw *et al*, 2007). Meanwhile, *APOE* ε4 may lead to an increased activity but greater atrophy in right hemisphere in healthy young subjects (O'Dwyer *et al*, 2012). The Alzheimer's risk *BDNF* genotype (Val/Val of Val66Met) carriers had a thicker entorhinal thickness in early adult life and a higher rate of entorhinal atrophy in elderly (Voineskos *et al*, 2011). These observations indicated that young healthy individuals at risk may have altered entorhinal thickness and improved brain activity. It might reflect a compensatory hypothesis (Filippini *et al*, 2009) wherein disease risk individuals appear to require additional effort to achieve comparable performance levels to overcome potential preclinical neural dysfunction.

Indeed, our data showed that AD-related *CFH* variant rs800292 not only altered the brain structure (eg, entorhinal cortex) in early life, but also affected the atrophy rate of the entorhinal cortex, CSF tau and Aβ levels, and memory decline as the disease progresses (Figure 2). These results suggest that *CFH* is actively involved in the onset and development of AD by promoting structural and functional brain changes. These observations indicated a role of immune genes in neuroimaging alterations in early age.

Alteration of CFH Expression is Involved in AD

Consistent with previous reports that CFH protein has the potential to be a biomarker for AD (Hye *et al*, 2006, 2014) and the above genetic association results, we found that *CFH* mRNA level in the hippocampus increases with age, suggesting an active role of CFH in the brain aging process (Figure 3). Moreover, there was a positive correlation between *CFH* mRNA level and severity of AD (and APP mRNA level) in brain tissues, and this result was consistent with the previous finding of increased CFH protein in the AD brain (Honda *et al*, 2000; Strohmeyer *et al*, 2000, 2002). Note that there are some controversies regarding serum CFH level in AD: an increase of CFH level was observed in the serum of AD patients (Hye *et al*, 2006, 2014); however, serum CFH level was reported to be significantly down-regulated in patients with AD and mild cognitive impairment (Gezen-Ak *et al*, 2013). The exact reason for this discrepancy remains unknown. Based on our results, we speculate that higher brain CFH levels may be related to AD development, supported by the observed increase of *CFH* mRNA levels in our cellular assays in response to $A\beta_{1-42}$ treatment or with stable overexpression of APP mutant and PSEN1 mutant (Figure 3).

Implications of CFH in the Pathogenesis of AD

Recent studies have demonstrated that the risk allele 402H (allele C of rs1061170) interacts less well (compared with 402Y (allele T)) with the binding sites in CFH ligands within the macula, resulting in complement activation and inflammation that may contribute to the accumulation of drusen, thus leading to the initiation and progression of AMD (Clark *et al*, 2010). There may be a similar mechanism by which Y402H may contribute to progression of AD. It is now known that Aβ plaques and local inflammation are central to the pathogenesis of AD (Kamer, 2010; Wyss-Coray, 2006). In addition to the anti-inflammation role, CFH acts as an extracellular matrix component and interacts with a wide selection of ligands (Ferreira *et al*, 2010), such as the C-reactive protein (Strang *et al*, 2012), heparin (Bergamaschini *et al*, 2009), zinc (Suh *et al*, 2000), and sialic acid (Patel *et al*, 2006). All these ligands may be involved in the accumulation of senile plaque in the AD brain. It has been reported that the risk allele 402H presented a reduced affinity to these ligands (Ormsby *et al*, 2008). Hence, patients harboring risk allele 402H might bind less CFH in amyloid

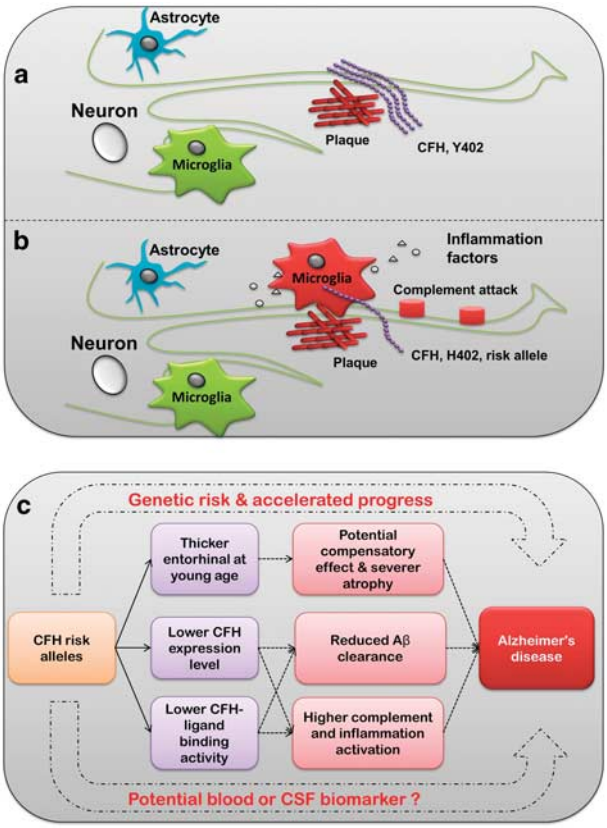

Figure 4 A simplified schematic profile for modeling the effect of CFH in AD. CFH acts as an extracellular matrix component and interacts with a wide range of ligands. The Alzheimer's risk allele 402H (rs1061170 C) was associated with lower CFH expression level and activity, thus presents a reduced affinity for these ligands. Patients harboring the risk allele 402H (b) may bind less CFH in the Aβ plaque compared with the wild-type (a), resulting in impaired regulation of complement activation (eg, membrane attack complex) and chronic local inflammation (inflammatory recruitment) in 402H carriers, which finally contributes to the accumulation of deposits and neuron loss during the development of AD. (c) Summary of the potential role of CFH in AD pathogenesis.

plaques, resulting in an impaired regulation of complement activation and local inflammation that may contribute to the accumulation of deposits and neuron loss in the development of AD (Figure 4). Furthermore, patients harboring the risk allele 402H might have decreased CFH levels in their brains according to our eQTL analysis. With impaired CFH levels and activity, extracellular deposition in the nidus may lead to inappropriate complement activation and thus contribute to the progression of clinical disease (Figure 4). Plaques in the AD brain, drusen in the eyes of patients with AMD, drusen-like deposits in the kidney of patients with kidney diseases, and even in the arteries of patients with atherosclerosis may also be the result of such processes. Further *in vivo* experiments using AD animal models are warranted to confirm our speculation.

It is worth mentioning that *CFH* was identified as the most important genetic factor for AMD (Klein *et al*, 2005) and its SNPs associated with AD were found to be risk SNPs for AMD; are we really bringing these two diseases, which share similar pathological characteristics and environmental risk factors (Keenan *et al*, 2014; Sivak, 2013), much closer based on this study? Significant cognitive impairment and subsequent occurrence of AD in AMD patients have been reported (Baker *et al*, 2009; Kaarniranta *et al*, 2011; Pham *et al*, 2006; Sivak, 2013; Woo *et al*, 2012), although the conclusion remains controversial (Kaarniranta *et al*, 2011; Keenan *et al*, 2014). The sequential occurrence of brain and retinal damage needs to be clarified. Our current observations added more evidence to the notion that AMD and AD, to some extent, share some common pathological features such as chronic oxidative stress and inflammation, active complement involvement, and intra- and extracellular deposits (Kaarniranta *et al*, 2011). These two diseases likely represent two related but distinct parallel amyloidopathies that might benefit from common targeted therapeutic approaches.

The presence of the *CFH* risk alleles in AD and AMD poses an evolutionary paradox during human evolution, as the diseases may have negative effects on fitness, but the risk alleles have not been eliminated by natural selection and persist within global populations. Why the deleterious allele 402H (rs1061170 C) was retained in populations with a relatively high frequency? We performed a positive selection analysis on the *CFH* region to look for an evolutionary explanation of this phenomenon. Two online tools for detecting positive selection in human genome were used: Haplotter (Voight *et al*, 2006; using the HapMap data) and CMS viewer (http://www.broadinstitute.org/mpg/cmsviewer/, using the 1000 genome data; Supplementary Figure 6). Positive selection was observed in a region next to the *CFH* gene cluster in African and Asian populations, which contains the *ASPM* (abnormal spindle-like microcephaly associated) gene. This gene controls brain development and was reported to have evolved rapidly in human (Zhang, 2003). However, it is the *CFH* region itself that showed evidence of positive selection in the European (CEU) population, consistent with the fact that the CEU population also has a higher prevalence of AD and AMD. These observations indicate an evolutionary imprint on this region that may affect AD. The risk allele 402H may provide an advantageous effect against pathogens, which can evade complement attack by recruitment of CFH (Ferreira *et al*, 2010). Therefore, it is possible that the derived 402H allele was retained during evolution to limit immune evasion by potential pathogens. Because of this trade-off effect, the retained allele may contribute a deleterious effect on common diseases (such as AMD, AD, uremia, and atherosis), which commonly affect the elderly in our modern world.

In summary, our results showed that *CFH* may contribute to AD development by affecting neuroimaging endophenotypes and biomarkers as well as immune response. Most of all, *CFH* affects structural change of the entorhinal cortex in early life and atrophy rate during AD progression, indicating a multifaceted role of immune regulators. Population-based longitudinal analyses focusing on neuroimaging (eg, memory task-based functional MRI), biomarker indicators, and AD Braak stage progression in risk allele carriers might provide more support and benefit clinical research and applications. The biological implication of CFH in AD needs further characterization.

FUNDING AND DISCLOSURE

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