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FULL LENGTH ARTICLE

Genetic profiles of familial late-onset Alzheimer's disease in China: The Shanghai FLOAD study

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Abstract Compared with early-onset familial AD (FAD), the heritability of most familial late-onset Alzheimer's disease (FLOAD) cases still remains unclear. However, there are few reported genetic profiles of FLOAD to date. In the present study, targeted sequencing of selected candidate genes was conducted for each of 90 probands with FLOAD and 101 unrelated matched normal controls among Chinese Han population. Results show a significantly lower rate of mutation in APP and PSENs, and *APOE* ϵ4 genetic risk is higher for FLOAD. Among the Chinese FLOAD population, the most frequent variant was *CR1* rs116806486 [5.6%, 95% CI (1.8%, 12.5%)], followed by coding variants of *TREM2* (4.4%, 95%CI (1.2%, 10.9%)) and novel mutations of *ACE* [3.3%, 95%CI (0.7%, 9.4%)]. Next, we found that novel pathogenic mutations in

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[1] Some data used in preparation of this article were 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 ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of 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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ACE including frame-shift and nonsense mutations were in association with FLOAD regardless of *APOE* ϵ4 status. Evidence from the Alzheimer's disease Neuroimaging Initiative (ADNI) database also supported this finding in different ethnicities. Results of *in vitro* analysis suggest that frame-shift and nonsense mutations in *ACE* may be involved in LOAD through decreased ACE protein levels without affecting direct processing of APP.

Introduction

The most common form of dementia, Alzheimer's disease (AD), is divided into early-onset AD (EOAD) and late-onset AD (LOAD) according to the age at onset, with 60 or 65 years as the boundary, usually. In addition to memory impairment, atypical clinical manifestations such as visual space disorder, apraxia, dyscalculia, aphasia, and executive dysfunction are more common in the early stage of EOAD.[1,2] Simultaneously, AD can also be separated into familial AD (FAD) and sporadic AD in terms of family history. Interestingly, genetic backgrounds differ between familial EOAD and familial LOAD (FLOAD). Mutations in genes encoding amyloid precursor protein (APP [OMIM: 104760]), presenilin 1 (PSEN1 [OMIM: 104311]) and presenilin 2 (PSEN2 [OMIM: 600759]), which all lead to Aβ overproduction, account for about 10%—20% of familial AD, especially familial EOAD.[3—5] Meanwhile, the Apolipoprotein E (APOE [OMIM: 107741]) ϵ4 allele is the most powerful genetic risk factor identified for LOAD.[6,7] Increased risks for AD are estimated to 2—4 folds with a single ϵ4 allele, whereas 8—16 folds with two ϵ4 alleles.[8,9] Unfortunately, the majority of heritable risk for FAD remains an open question.

In recent years, whole-genome/whole-exome sequencing technology and data analysis based on large sample sizes have found a series of coding mutations in *ADAM10*[OMIM: 602192],[10] *TREM2*[OMIM: 605086],[11,12] and *PLD3*[OMIM: 615698][13] increase the risk of AD. Genome-wide association studies (GWAS) have discovered more than 20 risk genes for AD,[14,15] involving immunity, metabolism, endocytosis, APP and tau metabolism, and other pathways. Generally, GWAS can only identify correlations between common variants with lower impact on risk for AD, but cannot determine rare coding variants with high pathogenicity. While, some studies choose FAD as the research object, the results are partly consistent with studies of sporadic AD. *ADAM10* and *PLD3* (mentioned above) were also found in LOAD pedigrees. *BIN1* [OMIM: 601248], *CLU* [OMIM: 185430][16]; *CR1* [OMIM: 120620], *PICALM* [OMIM: 104760], *APOE*, *ADAM10*, *ACE* [OMIM: 106180][17]; *PTK2B* [OMIM: 601212][18]; and *TREM2*[19] have also been verified in GWAS analysis of LOAD populations with family history.

However, previous studies of FAD and disease-causing gene mutations mainly focus on familial EOAD and *APP*, *PSEN1*, along with *PSEN2*. Less is known regarding FLOAD. Based on previous studies mentioned above, we selected 13 candidate genes (*APP*, *PSEN1*, *PSEN2*, *ADAM10*, *TREM2*, *PLD3*, *BIN1*, *CLU*, *CR1*, *PICALM*, *APOE*, *ACE*, and *PTK2B*) which were detected not only in sporadic LOAD cohorts, but also verified in FAD cohorts, and we further performed target enrichment sequencing in the Shanghai FLOAD cohort to reveal the genetic profiles of FLOAD in Chinese population.

Material and methods

Participants and clinical neuropsychological assessments

Probands of unrelated LOAD families were recruited from the Memory Clinic in Ruijin Hospital affiliated with Shanghai Jiaotong University School of Medicine and Huashan Hospital affiliated with Fudan University, respectively. All individuals were diagnosed with probable AD dementia[20] according to recommendations of the National Institute on Aging-Alzheimer's Association workgroups (NIA-AA). Additionally, AD-diagnoses met the following inclusion criteria: (1) persons of Chinese Han ethnicity, (2) $>$ 60 years old, (3) with a family history, i.e., at least one first-degree relative suffering from dementia with a probable cause of LOAD. Individuals who had difficulty cooperating with neuropsychological assessments due to severe visual or hearing impairments were excluded. Unrelated elderly individuals with normal cognition who did not possess a family history of dementia were selected from an urban community in Shanghai as the control cohort. Scales of Mini-mental State Examination (MMSE) and Clinical Dementia Rating (CDR) cognitive assessments were performed for each participant. Montreal Cognitive Assessment (MoCA) and Addenbrooke's Cognitive Examination (ACE-III) were used in the probands if necessary. This study was approved by the Ethics Committee of Ruijin Hospital affiliated to Shanghai Jiaotong University School of Medicine and informed consent was obtained from all participants.

Target enrichment sequencing and mutation analysis

We extracted DNA from about 3 ml of peripheral blood samples, which were collected from each participant. After quality examination of each sample, a total of 451 targets of the 13 candidate genes were enriched based on multiplex polymerase chain reactions (Genesky Biotechnologies Inc, Shanghai, China). High-throughput sequencing was performed on an Illumina Hiseq (Illumina, CA, USA). Clean reads were aligned to the reference genome by means of software employing the BWA algorithm[21] after quality control of raw data. The GATK[22] standard procedure was adopted to correct original alignment results and detect

single nucleotide variants (SNVs) and insertions or deletions (InDels). All SNV/InDel positions were annotated by ANNOVAR[23] to assess variant frequency, gene function, pathogenicity prediction, etc. The population database mainly referred to the Genome Aggregation Database (gnomAD) and hazard prediction referred to evidence from online tools (SIFT, POLYPHEN, Mutation Taster, and CADD). Missense variants of *APP*, *PSEN1*, *PSEN2*, and *TREM2* were also checked on the AlzForum database (https://www.alzforum.org/mutations) and defined as a novel mutation if neither recorded in AlzForum nor peer-reviewed publications.

Variants meeting one of the following conditions were selected out for Sanger sequencing verification: (1) defined as 'pathogenic' or 'likely pathogenic' according to the guidelines issued by the American College of Medical Genetics (ACMG)[24]; (2) non-synonymous variants of *APP*, *PSEN1*, *PSEN2*, *ADAM10*, and *TREM2*; (3) defined as rare variants by gnomAD (minor allele frequency <0.01) and carried by more than one index patient while not detected in controls; and/or (4) were reported in association with AD in previous studies. Primer information can be found in supplemental materials (Table S1).

SNP linkage analysis was performed by Plink under different heritability models. The Haploview program was used for analysis of Linkage Disequilibrium (LD) with the aim to identify haplotype blocks with significant association.

ADNI database and related bioinformatics analysis

The Alzheimer's Disease Neuroimaging Initiative (ADNI) is a multisite longitudinal study, which aims to track the progression of AD with clinical, imaging, genetic, and biospecimen biomarkers, to validate biomarkers for use in AD clinical treatment trials. ADNI has had a global impact due to standardized protocols and its open data-sharing policy. Based on our findings, the Linux awk command was used to extract sequencing information for the *ACE* gene from sequencing of Chromosome 17. Gene annotation of extracted variants was performed by means of an online tool, wANNOVAR (wannovar.wglab.org).[23,25] Corresponding clinical and imaging data (including information of family history) were collected according to the identifiers for selected individuals.

Plasmid construction and cell culture, transfection

Wild type and mutant *ACE* plasmids were constructed by Sangon Biotech (Shanghai), respectively named ACE^{WT}, *ACE* p.L1024fs and, for the truncating mutation, *ACE* p.1024X. An additional plasmid encoded the mutant Swedish APP (APP^{Sw}), and the empty vector pcDNA3.1 was used as negative control. Human neuroblastoma SH-SY5Y cells were cultured into 6-well plates coated with poly-lysine and maintained in DMEM with 10% fetal bovine serum and penicillin-streptomycin (Gibco) in a 5% CO_2 incubator at 37°C. After cell density reached 70% confluence, co-transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For each well, 1250 ng APP^{Sw} plasmid DNA with 1250 ng ACE^{WT}/ACE p.L1024fs/ACE p.L1024X plasmid DNA (Sangon Biotech) was mixed with Lipofectamine 2000, which was then added to the cells. 72 h after co-transfection, the culture media and cells were collected for further analysis.

Western blot

After harvesting, cells were lysed with protein extraction reagent (with added Halt protease inhibitor cocktail, EDTA-Free) (ThermoFisher), and were subsequently centrifuged at 14,000 *g* for 10 min. Total protein was determined using the BCA Protein Assay Reagent (ThermoFisher). A total of 20 μg of denatured protein was loaded onto 10% SDS-PAGE gels. Once proteins were separated by electrophoresis, they were transferred to PVDF membranes (Immobilon-P^{SQ}). After blocking in 5% nonfat milk for 2 h, the membranes were incubated with primary antibody (1:1000) overnight at 4°C. Then, membranes were washed with TBST for 3 times (10 min each) and incubated with species-matched peroxidase-conjugated secondary antibody (1:1000) for 2 h. The protein band was visualized by ECL (ThermoFisher). Images were captured, and band intensities were quantified using an Odyssey Image Station (LI-COR). The primary antibodies used in this study included anti-ACE antibody (Invitrogen, PA5-83080), anti-APP antibody (Abcam, ab32136) and anti-β-actin antibody (Sigma, A5441). The secondary antibodies included horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Beyotime, A0216 or A0208).

Enzyme-linked immunosorbent assay (ELISA)

After collection 72 h following transfection, cell culture media was centrifugated at 300 *g* for 10 min to remove cellular debris. ACE was detected using sandwich ELISA kits (Multi Sciences) according to the manufacturer's protocol. Plates were read at 450 nm on a Synergy MX plate reader (Bio-Tek).

Statistical analysis

Demographic analysis was conducted using SPSS Statistics v22.0 and independent-sample *t* tests and Chi-square tests were used for measurements and numeric/integer count data, respectively. In SNP analysis, different models were calculated, including an Allele Model, a Dominant Model, a Recessive Model and a Genotype Model, as well as application of the Chi-square test, Fisher's exact test, and logistic regression analysis. FDR correction by the Benjamini-Hochberg (BH) adjustment and also Bonferroni adjusted *p* values were calculated. Logistic regression analysis was also applied to haplotype analysis. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated. The Clopper-Pearson Method was applied in 95% CI for mutant rates of genes. Results of *in vitro* experiments were analyzed by GraphPad Prism 7 and analysis of

variance (ANOVA) was used when comparing more than two groups. Statistical significance was defined as $P < 0.05$.

Results

Demographic characteristics and SNP analysis

A total of 90 probands from LOAD families and 101 cognitively normal elderly persons were recruited as participants of this study. The two cohorts were matched in age and gender. 66.7% of patients carried at least one *APOE* ϵ4 allele, whereas only 13.9% of normal controls did (Table 1). Heterozygous ϵ4 was estimated to confer risk for FLOAD with an OR of 12.08 (95% CI: 5.68−25.69), while homozygotes of ϵ4 had an approximative risk (OR = 14.50, 95% CI: 3.01−69.97). *APOE* ϵ4 allele frequency in the FLOAD cohort was significantly higher than in the control cohort (38.9% *vs.* 7.9%, OR = 8.31, 95%CI ranged from 4.29 to 16.08, $P < 0.001$). SNP association analysis indicated that only *APOE* rs429358 was linked to FLOAD when taking the adjusted *p* value into consideration (Fig. S1). On the other hand, several haplotypes within *APP* and *BIN1* were found to confer genetic risk for AD in addition to *APOE* ϵ4 (Fig. S2, Table S2).

Genetic profiles of FLOAD

The most frequent variant was *CR1* rs116806486 (p. T173A), which was harbored by five (5.6%, 5/90, 95% CI ranged from 1.8% to 12.5%) probands simultaneously. Followed by variants of *TREM2*, detected in four (4.4%, 4/90, 95% CI ranged from 1.2% to 10.9%) index patients. The individual carrier of both p.A130V and p.H157Y SNPs had an earlier onset age and more aggressive cognitive decline. Moreover, p.H157Y was identified in a second proband. The novel *TREM2* variant (NM_001271821: exon4: c.496G>A: p.V166M) was predicted to be deleterious by more than two predictive algorithms. A few missense variants within *ACE* were detected in both cohorts, which may superficially suggest no conferrence of risk for AD. However, three 'pathogenic' or 'likely pathogenic' mutations of *ACE* (*ACE* p.W343X, *ACE* p. D441fs, *ACE* p. L1024fs) graded according to ACMG guidelines were found in probands from different pedigrees (3.3%, 3/90, 95% CI ranged from 0.7% to 9.4%) (Table 2).

One rare coding variant of *PLD3* (NM_001031696: exon7: c.489C>G: p.I163M) and a novel variant in *ADAM10* (NM_001320570: exon13: c.1748A>G: p.Q583R) were also detected in our AD cohort. There was only one *PSEN2* mutation in an individual with AD phenotype, p.H169N. Three other coding variants of *PSEN2* were carried by cognitive normal controls, including 2 novel variants (p.T128A and p.E322G) and one reported variant (p.G34S). No missense mutations were detected in *APP* nor *PSEN1* in our two cohorts, in addition to 7 synonymous and non-coding region (UTR) mutations (Table S3).

Because the mutations of *ACE* in the AD cohort are novel and account for the third highest frequency (3.3%), we next focused on revealing the phenotype and related function(s) of *ACE* mutation.

Table 1 Demographics and APOE genotypes.

Group		AD ($n = 90$)	Control ($n = 101$)	*p* value
Age		74.03 ± 7.03	73.04 ± 8.31	0.373
Gender	Male	40 (44.4%)	40 (39.6%)	0.499
	Female	50 (55.6%)	61 (60.4%)	
MMSE		17.70 ± 8.57	29.10 ± 0.82	0.000
APOE	ϵ4−/−	30 (33.3%)	87 (86.1%)	0.000
	ϵ4+/−	50 (55.6%)	12 (11.9%)	
	ϵ4+/+	10 (11.1%)	2 (2.0%)	

Frameshifting and stop-gain mutations of *ACE*

We noticed that the 'pathogenic' and 'likely pathogenic' mutations based on ACMG guidelines were all located in *ACE*. No other frame-shift or stop-gain variant was detected. Since AD was the only common phenotype, we questioned whether these mutations were associated with AD risk. These mutations (*ACE* p.W343X, *ACE* p. D441fs, *ACE* p. L1024fs) were predicted to have deleterious effects on protein function(s) of the angiotensin I-converting enzyme, which is encoded by the *ACE* gene. Sequence length shortened as a result of altered downstream amino acid sequence and premature termination (Fig. 1A). However, co-segregation analysis had not been realized due to the lack of samples from affected relatives (Most of them had passed away due to the proband's advanced onset age, which is necessarily distinct from the affected relatives of EOAD of a relatively young age.) (Fig. 1B). Neuroimaging showed typical features of AD, such as medial temporal lobe atrophy (MTA, Fig. 1C), hypometabolism (Fig. 1D), and most importantly, abnormal Aβ deposition (Fig. 2A). As depicted, the patient with *ACE* p.L1024fs (Patient 2) exhibited more extensive Aβ plaques formation than the patient with *ACE* p. D441fs (Patient 1), which is reflected by a wider area of red color.

With the aim to ascertain whether the mutation altering the length of the ACE protein-coding sequence is a likely genetic risk factor of AD, we extracted relative genetic, clinical and imaging results from the ADNI database. One non-frameshifting insertion (*ACE* p.L18_L19insPL) and three frameshifting variants (p.R149Lfs*53; p.D1058Yfs*15; p.S1238Pfs*118) were annotated by wANNOVAR. The non-frameshifting insertion variant was excluded from further analysis as some normal carriers showed negative Aβ deposition according to PET imaging. The corresponding three patients who harbored frameshifted variants of *ACE* not only progressed to dementia, but also presented with positive Aβ deposition (Fig. 2B−D). Detailed information can be found in Table 3 and the patients' identifiers from ADNI are listed. Unfortunately, information of family history was absent due to mismatched identifiers (The three identifiers were absent in the file that recorded details of family history).

To summarize, a total of 6 patients who carried *ACE* frame-shift or stop-gain mutations were in their 70s, and manifested with LOAD independent of gender and *APOE* ϵ4 status. These results suggest that frame-shift or stop-gain mutations in *ACE* might play a role in LOAD with evidence of abnormal Aβ deposition featured by AD.

Table 2 Mutation profiles and detailed information.

Gene	Exon	position	Ref/Alt	variant	Number of carriers	gnomAD_exome	gnomAD_gnome	Pathogenicity prediction				Referrence
								SIFT	PolyPhen	MutationTaster	CADD	
CR1 NM_000573	5	rs116806486	A/G	p.T173A	5	0.00158534	0.0053	T	B	N	0.001	Ma X.Y. et al (2014)
TREM2 NM_018965	3	rs2234255	C/T	p.H157Y	2	0.00490461	0.001	D	D	N	23.1	Jiang T. et al (2016)
TREM2 NM_018965	2	rs145080901	C/T	p.A105V	1	0.0000636274	0.00006372	D	D	N	24.2	Jin et al (2015)
TREM2 NM_001271821	4	rs199795809	G/A	p.V166M	1	0.000234658	0.0002	D	B	D	11.75	Not reported
TREM2 NM_018965	2	rs201280312	C/T	p.A130V	1	0.00000799853	NF	T	P	N	10.98	Jiao et al (2014)
ACE NM_000789	8	—	C/-	p.D441fs	1	0.00000398168	NF	NA	NA	NA	NA	Not reported
ACE NM_000789	20	—	CT/-	p.L1024fs	1	NF	0.00003184	NA	NA	NA	NA	Not reported
ACE NM_000789	7	rs200225958	G/A	p.W343X	1	0.00000796045	NF	NA	NA	A	36	Not reported
PSEN2 NM_000447	7	rs533813519	C/A	p.H169N	1	0.00048	0.0002	D	B	D	31	Shi et al (2015)
ADAM10 NM_001320570	13	rs141701612	A/G	p.Q583R	1	0.000103427	0.0006	T	B	N	8.753	Not reported
PLD3 NM_001031696	7	rs200529365	C/G	p.I163M	1	0.000229557	0.00006373	D	P	N	22.7	Tan M.S. et al (2018)

Note: SIFT (D = damaging, T = tolerated); PolyPhen (B = benign, P = possibly damaging, D = probably damaging); Mutation Taster (A = disease_causing_automatic, D = disease_causing, N = polymorphism, P = polymorphism_automatic); NF = not found; NA = not available.

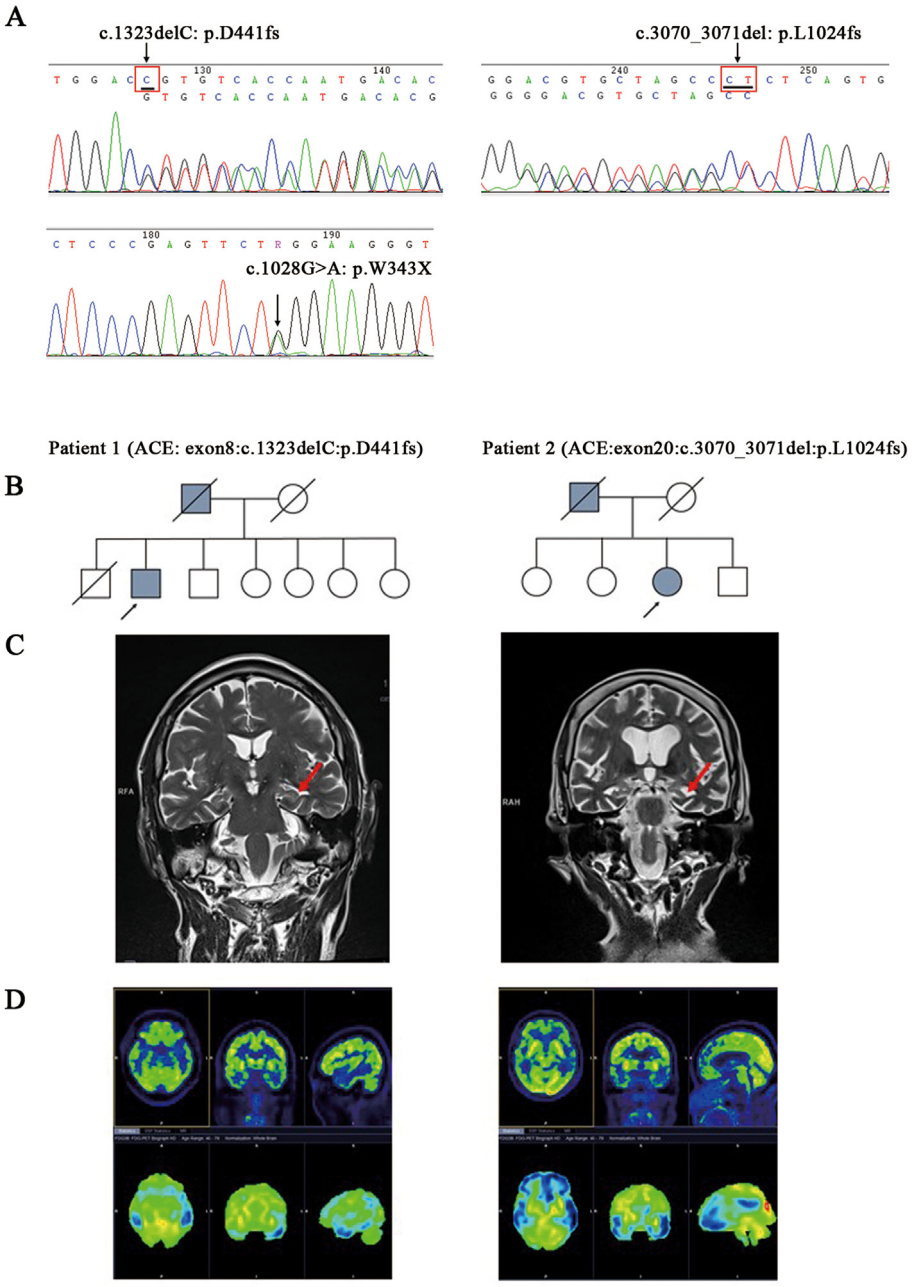

Figure 1 Sanger Sequencing and clinical details of the index patients harboring *ACE* pathogenic mutations. **(A)** Verification of Sanger Sequencing for ACE frameshifting and stop-gain mutations. **(B—D)** The other index patient who carried ACE p.W343X was lost to follow-up. Patient 1: 74 years old, male, APOE $\epsilon 2/\epsilon 3$, MMSE 23/30 (education: 12 years); Patient 2: 74 years old, female, APOE $\epsilon 3/\epsilon 4$, MMSE 11/30 (education: 6 years). **(B)** Pedigrees with the index patient labeled by a black arrow. **(C)** Slices of coronal magnetic resonance imaging demonstrating hippocampal atrophy (red arrow). Patient 1: MTA = 3; Patient 2: MTA = 4. **(D)** Image of 18F-FDG PET shows reduced metabolism, especially in parietal, temporal, and frontal lobes.

ACE mutation decreases ACE expression without influencing APP *in vitro*

As the significantly elevated Aβ deposition shown in the patient with *ACE* p.L1024fs, we further investigated whether this mutation influenced APP processing. In addition, we also constructed another truncating mutant plasmid (*ACE* p.L1024X) to compare to the frame-shifted construct and determine whether there could be a gain-of-function from the shift of reading frames. Membrane-localized ACE protein was significantly decreased after transfection with *ACE* p.L1024fs compared to ACE^{WT} (Fig. 3A, B), and was consistent with the decrease in secreted ACE in the cell culture medium (Fig. 3D). A similar tendency was also observed with transfection of the other construct for *ACE*, p.L1024X. Consistently, the expression of secreted ACE in samples transfected with *ACE* p.L1024fs was even lower compared to *ACE* p.L1024X (Fig. 3D), indicating that the frame-shift

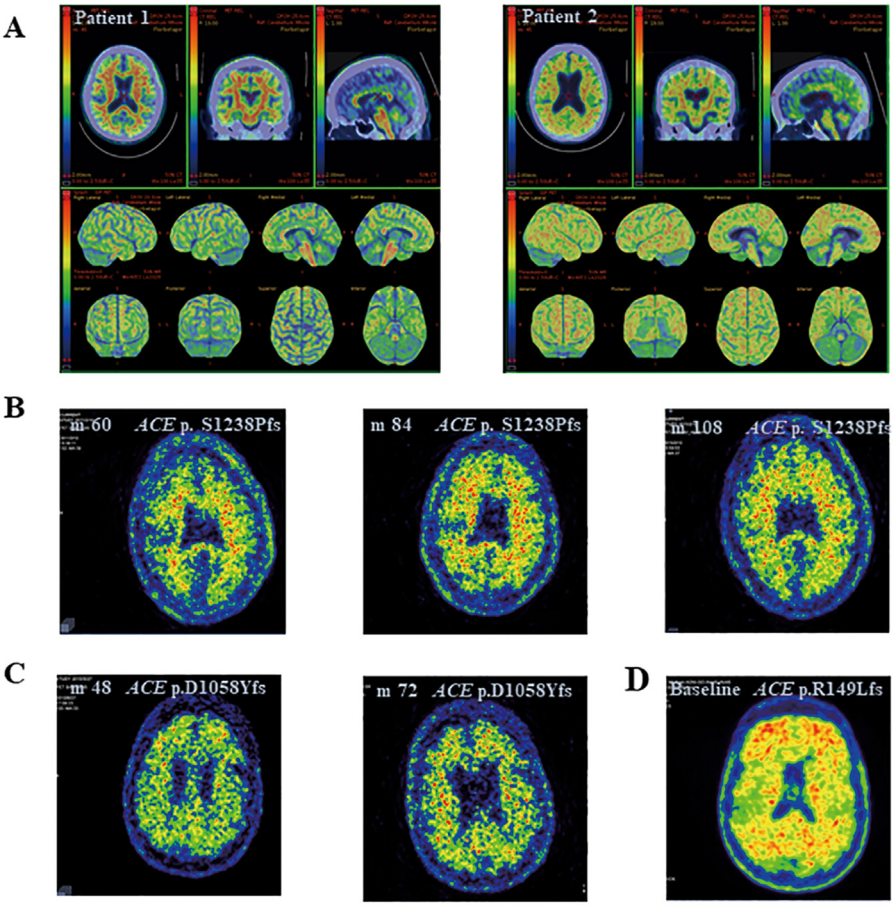

Figure 2 18F-AV-45 (18F-Florbetapir) PET imagings of patients with *ACE* frameshifting mutations. **(A)** Intracranial Aβ deposition for Patient 1 and Patient 2 respectively: Transverse section, Coronal section, Medial sagittal section and 3-dimensional reconstruction models; **(B—D)** Intracranial Aβ deposition for patients from ADNI and "m" represented follow-up months after baseline. As time progressed, more Aβ deposited in the patients with *ACE* p. S1238Pfs (B) and *ACE* p.D1058Yfs(C). The last patient who harbored *ACE* p.R149Lfs had abundant Aβ deposition at baseline (D). Warm color represents an increased uptake, which indicates abnormal Aβ deposition.

mutation might have a more deleterious effect than the nonsense mutation at the same site. However, expression levels of full-length APP remained unchanged among the cells transfected with ACE^{WT}, *ACE* p.L1024fs, *ACE* p.L1024X and negative control (Fig. 3A, C). These results suggest that *ACE* p.L1024fs lowers expression of both membrane-

Table 3 Clinical information of patients carried *ACE* frame-shifting or stop-gain mutations from our study and ADNI.

ADNI PTID	Bases variant	*ACE* mutation	Age (BL)	gender	RACE	diagnosis at baseline	AAO	duration to dementia (years)	*APOE* e4	AV-45-PET	MTA scale[1]
—	c.1323delC	p.D441fs	74	male	yellow	mild AD	72	2	0/0	(+)	3
—	c.3070_3071delCT	p.L1024fs	74	female	yellow	moderate AD	70	3	1/0	(+++)	4
—	c.1028G>A	p.W343X	71	male	yellow	moderate AD	NA	NA	1/0	NA	NA
068_S_2316	c.441_442insAGCTT	p.R149Lfs*53	74.3	female	white	early MCI	NA	1	1/0	(+++)	4
023_S_0887	c.3168_3181del	p.D1058Yfs*15	73.7	female	white	late MCI	NA	1.5	1/0	(++)	4
023_S_0061	c.3712delT	p.S1238Pfs*118	77	female	black	CN	79	6	0/0	(+++)	4

Note: AAO = age at onset; MTA = medial temporallobe atrophy;
[1] Scores based on the latest MRI.

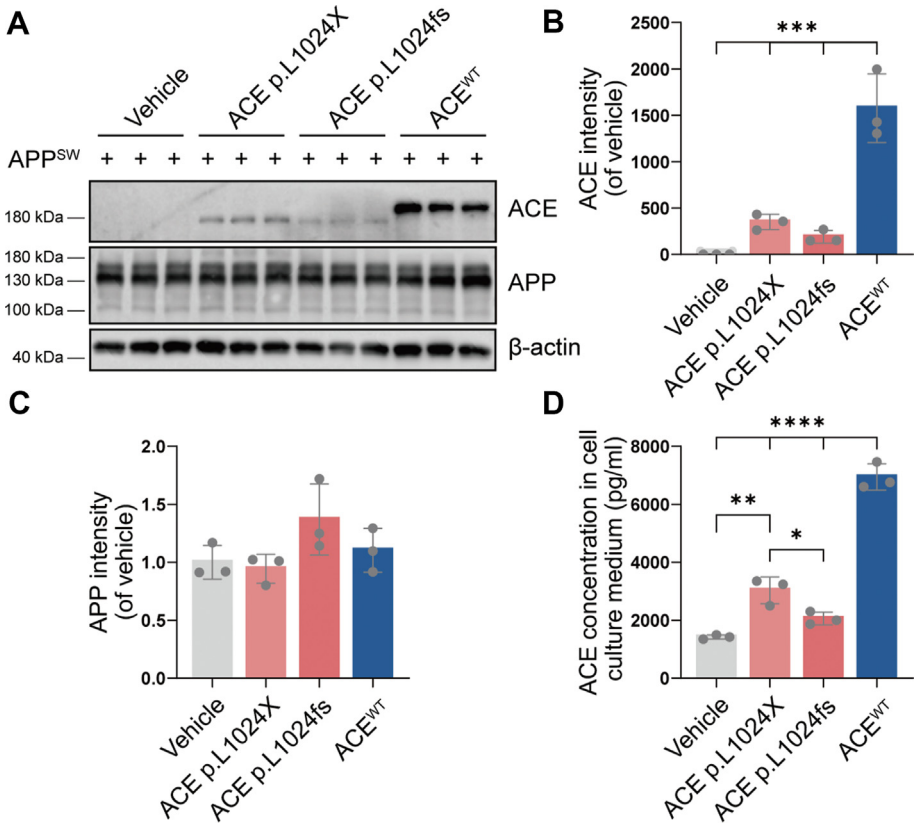

Figure 3 Membrane-localized and secreted ACE change consistently, but expression of APP remains unchanged. **(A)** Expression of ACE and APP in SH-SY5Y cells co-transfected with APP^{Sw} and ACE^{WT}/ACE p.L1024fs/ACE p.L1024X. **(B, C)** quantification of ACE and APP expression, respectively. **(D)** quantification of the secreted ACE in culture medium by ELISA after co-transfection.

localized and secreted ACE, but has no direct impact on APP processing.

Discussion

This study is the first exploration of genetic screening for familial late-onset Alzheimer's Disease (FLOAD) to our knowledge. The frequency (66.7%) of *APOE* ϵ4 allele carriers in our FLOAD cohort is higher compared to that of sporadic AD in a prior Asian cohort (41.88%, 95%CI ranging from 38.48% to 45.27%)[26] and that of the Chinese Familial Alzheimer's Disease Network (CFAN) cohort (51.37%).[4]

The frequency (1.1%, 1/90) of *APP*, *PSEN1*, and *PSEN2* missense mutations in our FLOAD cohort is significantly lower compared to previous studies of different ethnic populations. The major reason might be that only FLOAD was included in our study whereas a considerable proportion of familial EOAD were recruited in other studies. The only *PSEN2* mutation (*PSEN2* p.H169N) in the present case cohort was first discovered in a Chinese patient once before, and this individual was also phenotyped with FLOAD and amyloid deposition.[27] *PSEN2* (p.G34S) was reported in Dutch LOAD patients and not observed in 283 healthy controls with unchanged Aβ42/Aβ40 ratio at the cellular level (unpublished data).[28] In our cohorts, G34S was detected in two controls, both of whom have no complaints about memory nor any cognitive decline. G34S seems benign rather than pathogenic based on these results. Similarly, the other two novel variants (p.T128A and p.E322G) we found might be polymorphisms according to the algorithm proposed by Guerreiro and his colleagues.[29]

TREM2 and *CR1*, encoding triggering receptor expressed on myeloid cells 2 and complement receptor 1 protein, respectively, are involved in neuroinflammation and immune response related pathways.[30,31] A total of 10% of FLOAD probands carried variants of *CR1* rs116806486 (5/90) or *TREM2* (4/90) in our study, making the two genes notable among the candidates. On the one hand, the missense variant *CR1* rs116806486 (p. T173A) was confirmed to be associated with LOAD in northern Han Chinese cohorts with an OR of 3.21 (95%CI: from 1.37 to 7.54).[32] Our study find that the frequency of *CR1* rs116806486 is higher in FLOAD than sporadic LOAD (5.6% versus 1.9%). On the other hand, few missense mutations of *TREM2* have been reported to confer genetic risk for LOAD, with one being R47H.[11,12,33] However, R47H-mediated increase in the risk for AD may be race-specific. This risk locus has only been reported in European and North American populations, but not in Asian populations.[34] Another *TREM2* variant (p.H157Y) has been detected in Chinese populations and conferred considerable risk of LOAD with an OR of 11.01 (95% CI ranged from 1.38 to 88.05).[35] This position, histidine 157, is a cleavage site of TREM2. The p.H157Y variant results in enhanced shedding of TREM2, thus lowering TREM2-dependent phagocytosis.[36] Frequency of p.H157Y is higher in our FLOAD cohort (2.2%, 2/90) than that of previous sporadic

cases (0.8%, 8/988).[35] It seems that H157Y increasing the risk of LOAD is unique to the Chinese population, especially in FLOAD. The proband harboring two TREM2 variants shows an earlier onset age and more aggressive progression, indicating that genetic risks may have an additive effect. These results highlight the role of complement-mediated and microglia-related innate immunity in LOAD.

Most importantly, this study is the first to find correlation between novel *ACE* frameshifting or nonsense mutations and AD. All six patients (three from our FLOAD cohorts, three from ADNI) were diagnosed with AD or onset in their seventies. *ACE* encodes the angiotensin I-converting enzyme (ACE), which converts angiotensin I to angiotensin II. It is a key component of the renin-angiotensin system (RAS), and is crucial in modulating blood pressure. Numerous preclinical and clinical studies have provided evidence supporting an association between ACE and AD, in addition to *ACE* identified as a risk gene by GWAS. On the one hand, increased ACE was found in AD brains, along with angiotensin II and AT1 receptor.[37] On the other hand, ACE protein level and activity in CSF and serum were decreased in AD patients compared to controls.[38] Results of clinical studies were contradictory. Some interpretations were that ACE inhibitors provided protection against AD onset and cognitive decline,[39—42] whereas others have found the opposite.[43—46] *In vitro* studies showed that ACE is involved in Aβ degradation,[47] and the active center might be the N-terminal domain rather than the C-terminal domain.[48] Research within the past 12 years has indicated a potential AD treatment target could be ACE and has led to a proposed Angiotensin hypothesis of AD.[49,50] In a human APP transgenic mouse model, an effective dose of ACE inhibitor was found to be sufficient to promote formation of amyloid plaques. Moreover, heterozygous *ACE* deletion (APP/$ACE^{+/-}$) led to a decline in ACE expression and accelerated Aβ42 deposition.[45] However, the mechanistic role of ACE and related pathways in the pathogenesis of AD remains undetermined. In the present study, our *in vitro* experiments suggest that frame-shift and nonsense *ACE* mutations resulted in decreased ACE protein levels. By contrast, recent research identified *ACE* rs4980 (R1279Q) as a penetrant mutation in AD and demonstrated elevated ACE levels, ACE activity and other RAS components in knock-in mice models, accelerating neurodegeneration and neuroinflammation independent of Aβ pathology.[51] Although these results seem inconsistent, it is consistent with a role for either decreased or elevated ACE playing a role in AD risk through different mechanisms. In addition, ACE2, another RAS regulator which proteolytically activates angiotensin, was found to decrease amyloid pathology and provide a protective effect on cognitive decline.[52] Taken together, ACE and related RAS signaling are critical in AD with still incompletely understood, but intersecting, molecular pathways.

Limitations of this study include: (1) The probands recruited for this study are clinically diagnosed without a pathological diagnosis. (2) It is also a possibility that cognitively normal controls developed dementia in later years, and this has not been ruled out. (3) Furthermore, the absence of relatives' DNA samples makes it impossible to explore co-segregation of variants with AD. (4) Due to target sequencing, negative results in other probands do not mean that there are no mutations in the undetected genes.

Conclusions

In conclusion, we found genetic heterogeneity of FLOAD. Among the patients with FLOAD in China, *CR1* rs116806486 is the most frequent coding variant, followed by missense mutations in *TREM2* and pathogenic mutations in *ACE*. Combined with verification of data in the ADNI database, our study supports a hypothesis that frame-shift and nonsense mutations in *ACE* might contribute to the genetic etiology of LOAD. The imbalance of RAS caused by decreased ACE expression could be involved in pathogenic mechanism(s) of AD which will require further investigation to elucidate.

Conflict of interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.05.001.

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