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Rare Genetic Variants of the *Transthyretin* Gene Are Associated with Alzheimer's Disease in Han Chinese

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Abstract Alzheimer's disease (AD) is the most prevalent form of dementia in the world. The neuropathological characteristics of AD patients are the accumulation of extracellular plaques of β-amyloid (Aβ) and intracellular hyperphosphorylated tau protein. Transthyretin (TTR) may alleviate AD symptom by reducing Aβ concentration in the brain. There were reports for a decreased TTR level in both AD brain and blood. However, there is still no robust evidence to support the genetic association of the *TTR* gene with AD. In this study, we aimed to investigate the potential association of *TTR* variation with AD by directly sequencing the whole exons and the promoter region of the *TTR* gene in 529 AD patients and 334 healthy controls from Han Chinese population. We found no association between *TTR* common variants and AD but observed an enrichment of *TTR* rare variants in AD patients relative to controls. Further in silico prediction analysis and functional assessment at the cellular level identified four potentially pathogenic rare variants in AD patients. In particular, variant c.-239C>A could potentially downregulate the *TTR* promoter activity; c.200+4A>G might influence the constitutive splicing of *TTR* mRNA; c.148G>A (p.V50M) and c.332C>T (p.A111V) would change the structure of TTR and decrease its Aβ-binding ability. Our results provided direct genetic evidence to support the active involvement of TTR in AD.

Keywords Alzheimer's disease · TTR · Rare variant · Genetic association · Aβ-binding ability

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Introduction

Alzheimer's disease (AD), a deteriorating and currently incurable neurodegenerative disease leading to severe memory loss, is now seriously threatening the lives of the elder population [1]. Since the first AD patient was described more than 100 years ago, the pathogenic mechanism of AD has not been sufficiently understood [2]. Extracellular senile plaques of Aβ peptide [3] and intracellular neurofibrillary tangles (NFTs) of the hyperphosphorylated tau protein were neuropathological hallmarks of AD [4, 5].

Hitherto, many hypotheses have been raised to explain the pathogenesis of AD, and the "amyloid cascade hypothesis" is the most favorite one [6]. According to this hypothesis, the abnormal concentration of Aβ resulted from the imbalance between Aβ production, clearance, and degradation, would finally leads to AD [6, 7]. Concordantly with this hypothesis, mutations in the *APP*, *PSEN1*, and *PSEN2* genes have been reported to be associated with the increased generation and accumulation of Aβ in familiar early-onset AD. Recent

genome-wide association studies (GWAS) and candidate gene association studies have identified and confirmed many susceptibility genes for AD, in particular for sporadic late-onset AD patients [8–11]. Furthermore, rare variants in the *TREM2* and *PLD3* genes were found to be associated with late-onset AD (LO-AD) [12–15]. It is of note that some of these AD-related genes were responsible for the imbalance of Aβ production and degradation.

Previous studies have also identified many proteins to interact with Aβ and affect its clearance and degradation [5], such as transthyretin (TTR) [16]. The human *TTR* gene contains four exons and encodes a 55 kDa protein, which is mainly synthesized in the liver and choroids plexus, and serves as a transporter of thyroid and retinol in blood stream and cerebrospinal fluid (CSF) [17]. TTR could bind Aβ and function against the generation of Aβ fibril [18, 19]. Several studies have demonstrated a decreased level of TTR protein in CSF and plasma of AD patients, albeit the fact that the underpinning has not been clearly illuminated [20–23]. TTR variants, like p.V30M (also named p.V50M when the 20-aa leading peptide was included) and p.L55P (named p.L75P with the leading peptide), would vitiate the Aβ-binding ability of TTR protein [19]. Recently, distinctive phospho-tau aggregates were observed subjacent to the subpial TTR amyloid deposits in a dementia patient with *TTR* rare variant Y69H (p.Y89H) [24].

In this study, we aimed to investigate the association between the *TTR* gene and LO-AD in Han Chinese population. We sequenced all exons of the *TTR* gene and nearby region in 529 AD patients and 334 healthy controls to identify rare and common genetic variants. Functional assessment was performed to further explore the potential role of rare variants identified in AD patients. Our results showed that rare *TTR* variants might confer risk to AD.

Materials and Methods

Subjects

A total of 529 AD patients and 334 matched normal controls were recruited from the Shanghai Mental Health Center, Tongde Hospital of Zhejiang Province, and the Second Xiangya Hospital of Central South University. The majority of these samples (around 80 %) have been analyzed for the previously reported GWAS hits and other AD-related genes in our recent studies [9, 11, 25]. All participants were of Han Chinese origin. Patients were all diagnosed according to the criteria of DSM-IV and NINCDS-ADRDA [26], and healthy controls were confirmed to be cognitively and neurologically healthy.

Mutation Analysis of the *TTR* Gene

Genomic DNA was isolated from the peripheral blood by using the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA). Four pairs of primers were designed to amplify and sequence all four exons of the *TTR* gene following our previously reported method [27]. The promoter region was amplified and sequenced by using primer pair Promoter-F/Promoter-R: 5′ AAGATTTGGTTCTCTGTATTTCAGG 3′/5′ TGGGGCTTTTATACTCACTTCTC 3′. The BigDye Terminator v3.1 Cycle Sequencing Kit (ABI, New York, NY) and the 3730 DNA Analyzer (Applied Biosystems) were used to sequence the *TTR* promoter and exon regions. Sequencing was performed at the Kunming Biodiversity Large-Apparatus Regional Center, Kunming Institute of Zoology. Sequence variants were scored relative to reference sequence of the *TTR* gene (GenBank accession number NG_009490.1), and the 20-aa leading peptide of TTR was included for numbering the variants.

Variants with a minor allele frequency (MAF) greater than 1 % were regarded as common variants and variants with a MAF less than 1 % were regarded as rare variants. The power analysis was performed by using the Quanto software [28]. The identified variants were subjected to the following analyses:

1. Allele and genotype frequencies of the variant were compared between AD cases and controls by using the χ^2 test.
2. Variants identified in our subjects were searched in the available databases, including dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP), the Exome Aggregation Consortium (ExAC: http://exac.broadinstitute.org), the International Genomics of Alzheimer's Project (IGAP) Summary Statistics (http://web.pasteur-lille.fr/en/recherche/u744/igap/igap_download.php) [29], and the Combined Annotation Dependent Depletion (CADD) databases [30], to help with the prediction of their putative pathogenicity.
3. Evolutionary conservation analysis of the *TTR* mutant was evaluated by comparing to nine vertebrate species (*Homo sapiens*, GenBank accession number NP_000362.1; *Pan troglodytes*, NP_001009137; *Gorilla*, *gorilla*, *gorilla* XP_004059345; *Mus musculus*, AAH24702; *Rattus norvegicus*, NP_036813; *Bos taurus*, NP_776392; *Ovis aries*, NP_001009800; *Sus scrofa*, NP_999377; *Gallus gallus*, NP_990666).
4. The PROMO software (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to predict whether variants in the *TTR* promoter region could potentially affect the transcriptional factor binding efficiency [31, 32].

5. The potential pathogenicity of nonsynonymous variants were predicted by using five prediction algorithms, including PolyPhen2 HumDiv and HumVar [33, 34], LRT [35], MutationTaster [36], and SIFT [37, 38].
6. The RaptorX (http://raptorx.uchicago.edu/) [39] was used to predict whether the nonsynonymous variants could change the TTR protein structure.
7. The Spliceport software (http://spliceport.cbcb.umd.edu/) [40] was used to predict whether the variants residing in the spanning region of exon and intron would influence the splicing of the TTR mRNA.
8. We referred to the webserver MicroRNA.org (http://www.microrna.org/microrna/getMirnaForm.do) [41] to predict whether variants in the *TTR* 3′ UTR would influence microRNA binding.

Dual Luciferase Reporter Assay

The promoter region of the *TTR* gene (~2 kb) was amplified from AD patients with different variants and was inserted into the pGL3-Basic luciferase reporter vector (Promega) within the *Nhe*I restriction site. Luciferase reporter assay was performed in 293T cells and HeLa cells. The pGL3 vectors with wild-type and mutant *TTR* promoter inserts were cotransfected with TK vector in a molar ratio of 10:1 by using the Lipofectamine 2000 transfection system (Thermo). 293T cells were lysed at 24 h after transfection and luciferase activities were measured by using the Dual Luciferase Reporter Assay Kit (Promega). HeLa cells were harvested at 48 h after transfection. All experiments were conducted in triplicate. Two-tailed Student's *t* test was performed to quantify the statistical difference between two groups by using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). A p value <0.05 was considered as statistically significant.

Overexpression of the *TTR* Variants

The coding region of the *TTR* gene was cloned into FLAG-tagged pcDNA3.1(−) vector between *Xho*I and *BamH*I sites. Point mutations were introduced into the wild-type *TTR* sequence, to obtain two *TTR* nonsynonymous variants (TTR-p.V50M and TTR-p.A111V). The U251 cells with stable expression of mutant *APP* gene ($U251^{APP\ K670N/M671L}$) as described in our recent study [10] were cultured in RPMI 1640 medium supplemented with 10 % FBS (Gibco) at 37 °C in 5 % CO_2. A total of 10 μg vectors were transfected into 1×10^6 $U251^{APP\ K670N/M671L}$ cells by electroporation following the manufacturer's instruction (NEPA GENE).

Expression of the *APP* gene was induced by adding 1 μg/mL doxycycline (Sigma) into culture medium for 72 h after the transfection of *TTR* overexpression vectors. Supernatant and cell lysates were collected to determine the relative level of Aβ and TTR, respectively. The cellular protein and culture supernatant were subjected to 12 % SDS-PAGE and were transferred onto the polyvinylidene fluoride (PVDF) membranes (Roche). The membranes were blocked with 5 % BSA in Tris-buffered saline (TBS) containing 0.1 % Tween 20 (TBST) at room temperature for 2 h. The membranes were then incubated with the following primary antibodies overnight at 4 °C (mouse anti-FLAG (1:5000; Abmart) to detect the wild-type TTR-Flag and its mutants; mouse anti β-actin (1:100,000; Enogene) to detect human β-actin; rabbit anti-Aβ (1:1000; Cell Signaling Technology) to detect Aβ). PVDF membranes were washed three times with TBST for 5 min each and were then incubated with the corresponding secondary antibody (1:10,000; KPL, USA) for 1 h at room temperature. The proteins were detected using the enhanced chemiluminescence (ECL) reagents (Millipore). ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to analyze the relative level of each protein, and the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was used to compare the Aβ level between different groups by using two-tailed Student's *t* test.

Results

TTR Rare Variants Are Enriched in Han Chinese Patients with AD

A total of 9 common variants (Table 1) and 16 rare variants (Table 2; Supplementary Fig. S1) in the *TTR* gene were identified in 863 Han Chinese (529 AD patients and 334 matched healthy controls). The MAF of these common variants ranged from 1.9 to 7.8 %, assuming a false positive rate controlled as 0.05, and the power to detect the odds ratio (OR) value as 2.0 for risk allele was expected to be from 54.8 to 95.5 %.

None of the identified common variants showed an association with AD in the Han Chinese cohort under study. Considering the limited sample size and relatively low statistical power of this cohort, we performed further comparison with the data from the ExAC East Asian population (http://exac.broadinstitute.org) and confirmed the lack of association of common *TTR* SNPs with AD after Bonferroni correction. We also checked the common variants in the −10-kb to +10-kb region of the *TTR* gene from the IGAP database, which contains the meta-analysis results of the GWAS data of 17,008 AD patients and 37,154 controls [29]. Our results showed that none of 47 common variants in the *TTR* gene region were associated with AD (Supplementary Table S1).

Table 1 *TTR* common variations in 529 AD patients and 334 normal controls from Han Chinese and in the East Asians of the ExAC dataset

Variants[a]	ID in dbSNP	Function	AD case, $n = 529$	AD control, $n = 334$	p value[b]	East Asians in ExAC	p value[b]
c.69+22C>G	rs191045778	Intron	25/1058	22/668	0.28795	228/8648	0.68264
c.69+66C>G	rs145765195	Intron	17/1058	10/668	1.00000	–	–
c.69+103A>G	rs9304103	Intron	26/1058	15/668	0.87168	–	–
c.201-76T>A	rs59882235	Intron	33/1058	15/668	0.29782	–	–
c.337-47G>A	rs140686255	Intron	21/1058	15/668	0.73153	116/8176	0.17447
c.337-18G>C	rs36204272	Intron	78/1058	57/668	0.40782	619/8600	0.95024
c.360C>T	rs150127220	Synonymous	24/1058	15/668	1.00000	300/8636	0.03719
c.417G>A	rs2276382	Synonymous	18/1058	6/668	0.20706	160/8644	0.80897
c.*3_*11del	rs143948820	Del in 3'UTR	34/1058	16/668	0.37781	227/8634	0.26802

– no data available

[a] Variants with minor allele frequency (MAF) >0.01 were regarded as common variants. Asterisk means that the variant is located in 3′UTR region

[b] p Value, Fisher's exact test

Taken together, common variants in the *TTR* gene might not affect the genetic risk of AD.

Among the 16 rare variants, 10 variants were only observed in AD patients, while 4 variants were only identified in healthy controls. Two variants were identified in both AD cases and controls (Table 2). There seems to be more carriers with rare *TTR* variant in AD patients (16/529, 3.0 %) than in control population (8/334, 2.4 %), indicating the tendency for an enrichment of rare *TTR* variants in AD patients (Fig. 1) albeit the overall difference was not statistically different ($p = 0.676$). Frequency of the APOEε4 carriers was higher in AD cases than in normal controls, although we found no significant association between *TTR* rare variants and APOEε4 +/− genotype (Supplementary Fig. S2).

Table 2 *TTR* rare variants in 529 AD patients and 334 normal controls from Han Chinese and in ExAC dataset

Variants[a]	ID in dbSNP	Function	AD case, $n = 529$	AD control, $n = 334$	p value[b]	East Asian in ExAC	p value[b]	Global frequency in ExAC	p value[b]	TFBS[c]	PHRED[d]
c.-352G>del	rs546102028	Promoter	0/1058	1/668	0.387	–	–	–	–	1	0.19
c.-307G>C	–	Promoter	0/1058	1/668	0.387	–	–	–	–	2	1.802
c.-239C>A	–	Promoter	1/1058	0/668	1.000	–	–	–	–	9	6.505
c.-31C>T	–	5′UTR	1/1058	0/668	1.000	–	–	–	–	18	10.08
c.69+24T>C	–	Intron	1/1058	0/668	1.000	–	–	–	–	5	2.637
c.148G>A	rs28933979	Missense	1/1058	0/668	1.000	–	–	18/121,404	0.152	NA	25.3
c.200+4A>G	rs751512499	Splicing	2/1058	0/668	1.000	3/8654	0.09482	3/121,408	0.0007	NA	9.724
c.332C>T	–	Missense	1/1058	0/668	1.000	–	–	–	–	NA	12.08
c.337-48C>T	rs756480693	Intron	1/1058	0/668	1.000	2/8066	0.30912	3/113,276	0.03650	NA	1.538
c.371G>A	rs121918095	Missense	3/1058	2/668	1.000	77/8650	0.04463	79/121,336	0.03448	NA	0.011
c.*30C>T	rs747775821	3′UTR	1/1058	0/668	1.000	9/8564	1.0000	10/120,586	0.09162	NA	10.58
c.*34G>A	–	3′UTR	0/1058	1/668	0.387	–	–	–	–	NA	11.64
c*101T>C	–	3′UTR	0/1058	1/668	0.387	–	–	–	–	NA	4.573
c.*137G>A	–	3′UTR	1/1058	0/668	1.000	–	–	–	–	NA	7.709
c.*261C>T	rs62093482	3′UTR	1/1058	0/668	1.000	–	–	–	–	NA	6.25
c.*262G>T	–	3′UTR	2/1058	2/668	0.643	–	–	–	–	NA	1.412

– no data available

[a] Variants with MAF <0.01 were considered as rare variants. Asterisk means that the variant is located in 3′UTR region of the *TTR* gene

[b] p Value, Fisher's exact test

[c] TFBS: indicates the number of different overlapping ChIP transcription factor binding sites [30]

[d] The PHRED-like scaled CADD-score: a score greater than 10 indicates that the variant belongs to the 10 % most deleterious substitutions in the human genome; a score greater than 20 indicates the 1 % most deleterious variants [30]

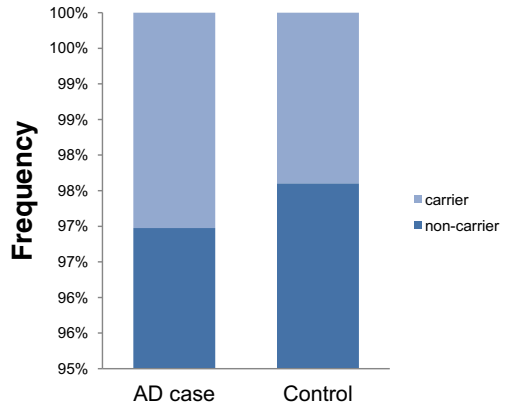

Fig. 1 Different distribution frequency of the rare *TTR* variants in Han Chinese patients with or without AD

Variants c.148G>A (p.V50M) and c.332C>T (p.A111V) May Change the TTR Protein Structure and Decrease Its Aβ-Binding Ability

There were two rare missense variant, c.148G>A (p.V50M) and c.332C>T (p.A111V), identified in AD patients but were absent in controls, and no missense variant specific for the control population was identified. Evolutionary conservation analysis of nine vertebrate species showed that both missense variants (p.V50M and p.A111V) occurred at the highly conserved positions (Fig. 2a, b). Three-dimensional structure prediction showed that these two variants could potentially affect the TTR protein structure (Fig. 2c, d). Furthermore, these two variants were predicted to belong to the 1–10 % most deleterious mutations according to the CADD score (Table 2). Pathogenicity prediction analysis by using five reported algorithms suggested that variants c.148G>A was probably pathogenic; variant c.332C>T was predicted to be benign in four of the five algorithms (Table 3).

We further performed functional assessment of these two missense mutations by overexpressing the wild-type and mutant TTR protein in U251$^{APP\ K670N/M671L}$ cells [10] (Fig. 3). The Aβ concentration in culture supernatant was decreased in cells overexpressing TTR protein, indicating that TTR protein may play an active role in Aβ clearance. In contrast, cells overexpressing *TTR* mutants showed an upregulated Aβ level in culture supernatant when compared to cells overexpressing wild-type TTR protein. Though the difference was not statistically significant, the increased Aβ level in cells overexpressing *TTR* mutant relative to cells overexpressing wild-type TTR implied that variants p.V50M and p.A111V had an inferior ability for Aβ clearance, possibly by affecting the binding ability of TTR protein with Aβ (Fig. 3).

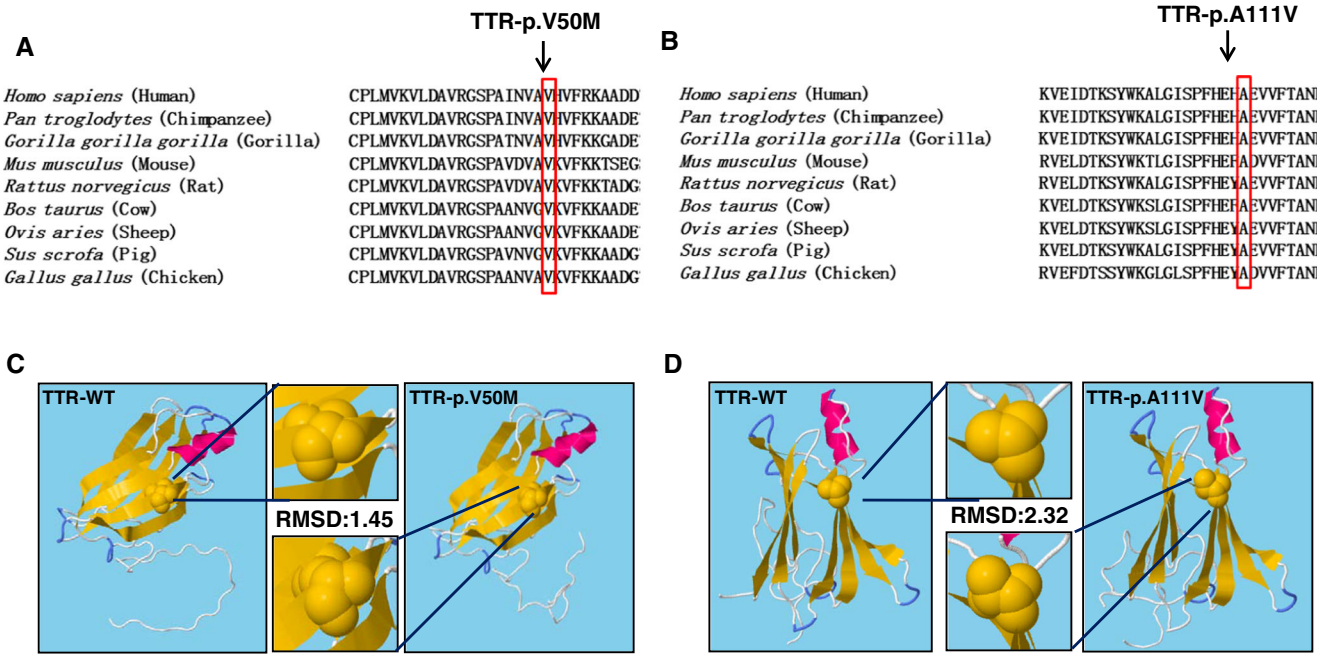

Fig. 2 Evolutionary conservation analysis and secondary structure modeling of the TTR protein. **a**, **b** Evolutionary analysis of the 50th and 111st residues in the TTR protein. TTR protein sequences of nine vertebrate species (*Homo sapiens*, GenBank accession number NP_000362.1; *Pan troglodytes*, NP_001009137; *Gorilla*, *gorilla*, *gorilla*, XP_004059345; *Mus musculus*, AAH24702; *Rattus norvegicus*, NP_036813; *Bos taurus*, NP_776392; *Ovis aries*, NP_001009800; *Sus scrofa*, NP_999377; *Gallus gallus*, NP_990666) were retrieved from GenBank for comparison. **c**, **d** Secondary structure modeling of the wild-type TTR and variants p.V50M and p.A111V. The modeling was performed by using RaptorX (http://raptorx.uchicago.edu/) [39]. *RMSD,* root mean square deviation, which means the average distance between the atoms of the wild-type and mutant TTR proteins

Table 3 Prediction of the potential pathogenicity of nonsynonymous variants by using five algorithms

Mutation	Function	SIFT (score)[a]	Polyphen2 HDIV (score)[b]	Polyphen2 HVAR (score)[c]	LRT (score)[d]	MutationTaster (score)[e]
c.148G>A	p.V50M	D (0.03)	D (0.999)	D (0.969)	D (0)	A (1)
c.332C>T	p.A111V	T (0.75)	B (0.201)	B (0.049)	N (0)	D (1)

[a] A SIFT score ≤ 0.05 was regarded as deleterious (D), and a score value >0.05 was regarded as tolerated (T) [37, 38]

[b] Polyphen2 HDIV score ≥ 0.957, probably damaging (D); 0.453 < Polyphen2 HDIV score < 0.956, possibly damaging (P); Polyphen2 HDIV score ≤ 0.452, benign (B) [33, 34]

[c] Polyphen2 HVAR score ≥ 0.909, probably damaging (D); 0.447 < Polyphen2 HVAR score < 0.909, possibly damaging (P); Polyphen2 HVAR score ≤ 0.446, benign (B) [33, 34]

[d] D, deleterious; N, neutral [35]

[e] A, disease causing automatic; D, disease causing [36]

Variant c.200+4A>G Confers Genetic Risk to AD and May Affect the Constitutive Splicing of *TTR* mRNA

Variant c.200+4A>G, which lies in the splicing region of exon2 and intron2, was identified in two independent AD patients but was absent in the controls. We further investigated the frequency of this variant in the ExAC populations, which contain the exome sequencing data of 60,706 subjects across the world (including 4327 individuals from East Asian; ExAC: http://exac.broadinstitute.org) (Table 2). Results showed that variant c.200+4A>G was significantly associated with AD risk, and the association could survive Bonferroni correction (Table 2). According to the prediction by Spliceport (http://spliceport.cbcb.umd.edu/), this variant would cause a significantly decreased splicing score from 1.17086 (allele A) to 0.526152 (allele G), indicating that c.200+4A>G may influence the constitutive splicing of *TTR* pre-mRNA.

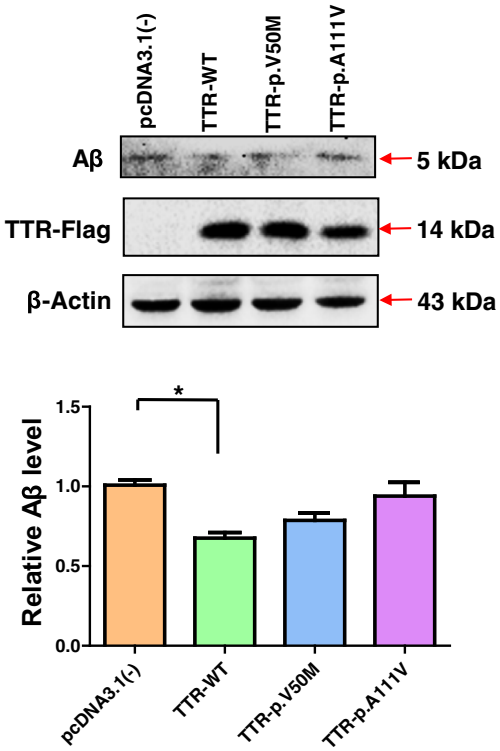

Fig. 3 Nonsynonymous *TTR* variants c.148G>A (p.V50M) and c.332C>T (p.A111V) had a decreased Aβ-binding ability. The U251$^{APP\ K670N/M671L}$ cells [10] were transfected with wild-type or *TTR* mutants (p.V50M and p.A111V). Seventy-two hours after transfection, culture supernatant was collected to determine the relative level of Aβ and cell lysate was collected to determine the expression of TTR-flag. The relative Aβ level between different groups was compared by using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Asterisk means a p value <0.05; two-tailed Student's t test

Variant c.-239C>A in the *TTR* Promoter Region May Affect Its Expression

A total of five rare variants in the promoter and UTR region were identified only in AD patients. One of the 3′ UTR variants (c.*30C>T) showed a high CCAD score of 10.58, indicating a potentially deleterious role of this variant (Table 2). However, according to the microRNA binding prediction analysis (http://www.microrna.org/microrna/getMirnaForm.do) [41], none of the 3′UTR variants resides in the microRNA binding sites. CCAD prediction of the variants in the promoter and 5′UTR region revealed that c.-239C>A and c.-31C>T had a high possibility to change the transcript factor binding sites (Table 2). Transcript factor binding prediction using the PROMO software (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) [31, 32] showed that these two variants might have different effects: variant c.-239C>A would influence the binding sites of transcript factors like C/EBPdelta, MYB2, POU1F1a, while c.-31C>T had no effect on the binding of transcript factors. We further validated this prediction result by using the luciferase reporter assay. Comparing to the wild-type promoter, promoter insert carrying variant c.-239C>A had a decreased level of relative luciferase activity and the difference was statistically different (p = 0.0004, 24 h after transfection in 293T cells; p = 0.0027, 48 h after

transfection in HeLa cells). Consistent with the program-affiliated prediction, promoter insert with variant c.-31C>T did not affect the promoter activity (Fig. 4).

Discussion

Previous studies have showed that TTR plays an important role in AD [16, 19], and TTR can bind to all forms of soluble Aβ [18, 19, 42]. Significantly decreased TTR concentration has been observed in CSF of AD patients [20, 22, 23]. The relatively low level of TTR in CSF was said to be AD-specific comparing to other dementia types, such as fronto-temporal dementia and dementia with Lewy bodies [43]. Concordantly, the plasma TTR levels were significantly decreased in AD cases when compared with control individuals [21]. Although these results indicated that TTR played an important role in AD pathogenesis by interacting with Aβ, and decreased TTR level might be a risk factor and a biomarker for AD, positive genetic association of the *TTR* gene with AD was observed in only a few of previous studies [44]. Relatively small sample size and inefficient genotyping in the previous studies [45] may account for the negative result.

In this study, we analyzed all exons and the promoter region of the *TTR* gene in 529 Han Chinese AD patients and 334 normal controls. Considering the limited sample size in this study, we also took the available public resource data, including IGAP data (http://web.pasteur-lille.fr/en/recherche/u744/igap/igap_download.php) [29] and ExAC data (http://raptorx.uchicago.edu/), for comparison. Consistent with previous studies [44–46], we found no association of *TTR* common SNPs with AD in both our samples and the East Asian samples of the ExAC data (Table 2). The allele frequency of rare variants showed no significant difference between the case and control groups in our cohort (Table 2). We further analyzed the missense and splicing *TTR* mutations in another 152 in-house controls and still found no significant association (Supplementary Table S2). However, we observed an enrichment of rare variants in the *TTR* gene in AD patients. This result suggested that rare variants, but not common variants in the *TTR* gene, confer genetic risk to AD. In line with our findings, there was a report during the review of our manuscript showing that the rare coding variants, but not the common coding variants in APP-Aβ metabolism genes (including *TTR*), would be the main hits for sporadic AD [44]. By using in silico program-affiliated prediction analysis and functional assay at the cellular level, we identified four potentially pathogenic rare variants (c.-239C>A, c.200+4A>G, c.148G>A (p.V50M), and c.332C>T (p.A111V)) in AD patients.

Variant c.-239C>A in the promoter region was predicted to affect the binding of transcriptional factor. This prediction was proved by the luciferase assay, as promoter with allele c.-239A has a much lower luciferase activity than that of promoter with c.-239C. This result is consistent with the reduced TTR level in AD patients [20, 21]. It is possible that variant c.-239C>A conferred risk to AD through downregulating the expression level of the *TTR* gene. Similarly, mutation that affected the exon splicing may also influence the expression level of TTR. This speculation was confirmed by the observation of a splicing variant c.200+4A>G in two unrelated AD patients. Indeed, the frequency of c.200+4A>G in AD patients was significantly higher than in 60,706 subjects from the world population in the

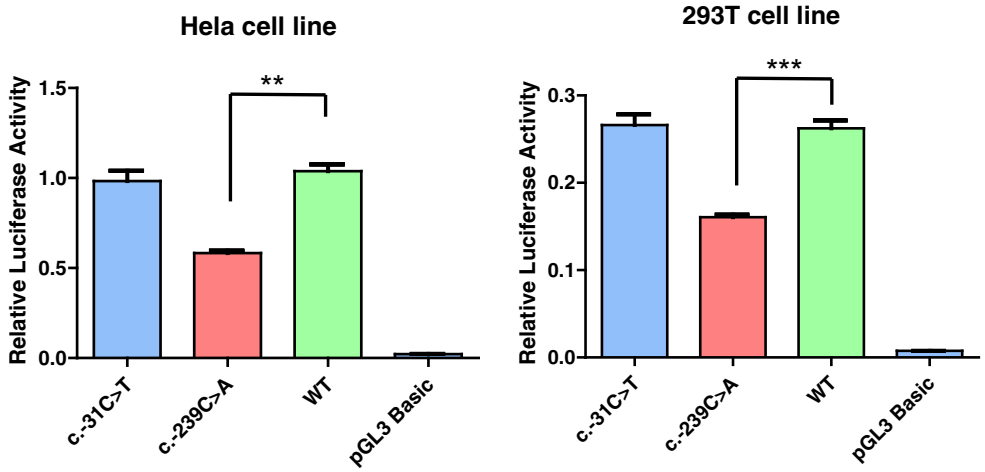

Fig. 4 Luciferase reporter assay of the *TTR* promoter with variants c.-239C>A and c.-31C>T. The *TTR* promoter sequences with wild-type or variants c.-239C>A and c.-31C>T were cloned in to the pGL3-Basic vector. Considering different transfection efficiencies for different cell lines, we used two cell lines (HeLa and 293T) to check whether the pattern was consistent. HeLa cells were cotransfected with the TK vector and the pGL3 vectors for 48 h before the harvest. 293T cells were transfected using the same way and harvested at 24 h after the transfection. Though different luciferase activities were observed for HeLa cells and 293T cells (which might be caused by different transfection time of the luciferase reporter vectors), we found a consistent trend of decreased luciferase activity in cells transfected with the promoter containing c.-239C>A. Two-tailed Student's *t* test was performed to quantify the statistical difference of relative luciferase activities between two groups. $^{**}p < 0.01$; $^{***}p < 0.001$

ExAC dataset. Note that this comparison should be received with caution as we lacked the related information regarding the population sources and disease status.

Among the two rare missense variants (c.148G>A (p.V50M) and c.332C>T (p.A111V)) in AD patients that were predicted to change highly conserved amino acid and TTR protein structure, p.V50M was previously reported to be a pathogenic mutation for familial amyloid polyneuropathy—a disease that was mainly caused by the abnormal accumulation of amyloid protein [47], similar to the abnormal deposits of β-amyloid (Aβ) in AD. Mutation p.V50M could affect the binding ability of TTR with Aβ [19]. Therefore, it is possible that p.V50M leads to AD by affecting the Aβ production and clearance. Our cellular experiments supported this speculation: overexpression of TTR-p.V50M and TTR-p.A111V mutants had resulted in a higher Aβ level in culture supernatant than that of wild-type TTR. These results provided direct genetic evidence to support the important role of TTR in AD.

Our study had raised several unresolved questions: why the rare pathogenic *TTR* variants were retained in the population and enriched in AD patients? What is the exact role of abnormal TTR level in AD and the underpinning of the regulation? Can TTR be considered as a therapeutic target for future prevention of AD? Evidently, more studies should be carried out to answer these questions.

In conclusion, we found no evidence for an association between *TTR* common variation and AD but observed an enrichment of rare variants in the *TTR* gene in AD patients. We further provided convincing evidence that some of the rare variants are potentially pathogenic. Future studies with independent sample sets and comprehensive functional assessment will be essential to further define the role of TTR in the development of AD.

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Compliance with Ethical Standards Written informed consents following the principles of the Declaration of Helsinki were obtained from each participant or guardian. This study was approved by the institutional review board of the Kunming Institute of Zoology, Chinese Academy of Sciences.

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

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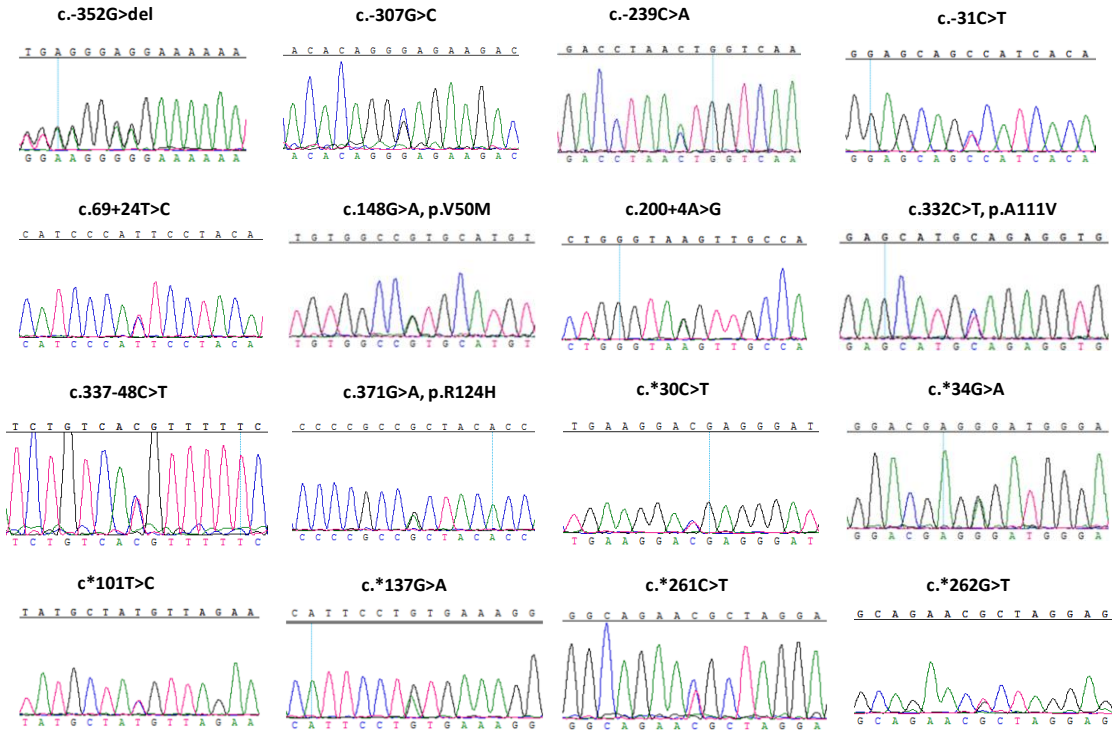

Figure S1. Sequencing electrophoregrams of the rare *TTR* variants identified in AD patients and healthy controls. Variants c.-239C>A, c.-31C>T, c.69+24T>C, c.148G>A (p.V50M), c.200+4A>G, c.332C>T (p.A111V), c.337-48C>T, c.*30C>T, c.*137G>A and c.*261C>T were only identified in AD patients; variants c.-352G>del, c.-307G>C, c.*34G>A and c*101T>C were only found in controls; c.*262G>T and c.371G>A (p.R124H) were identified in both groups. The numbering of *TTR* variant was scored relative to NG_009490.1.

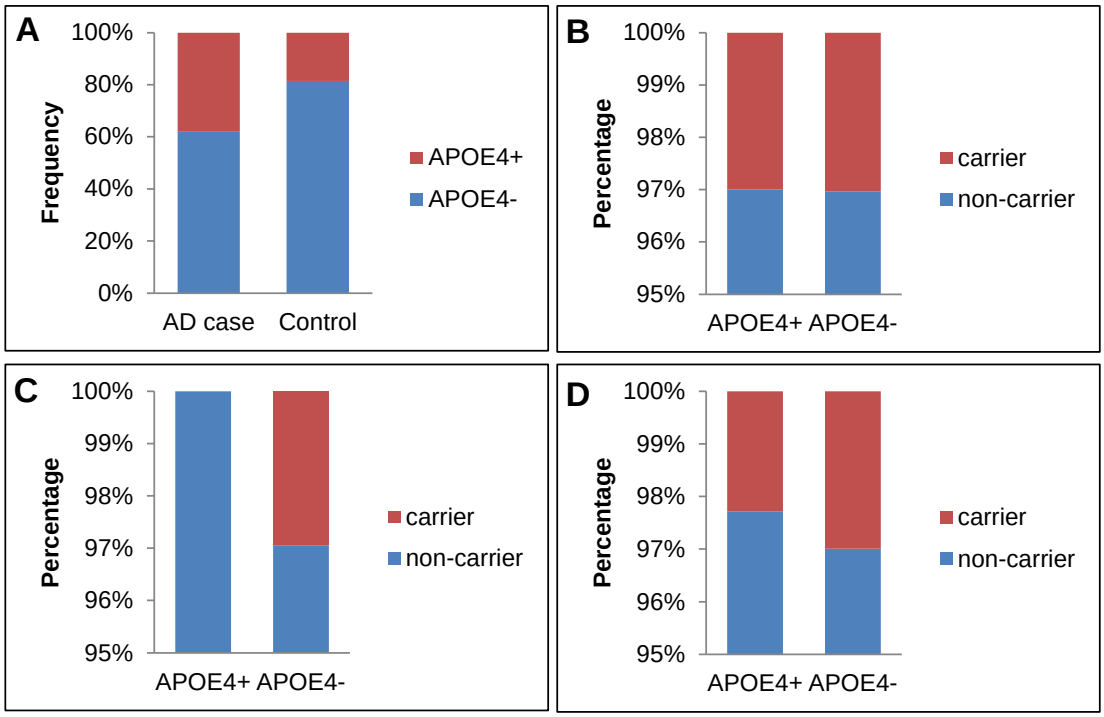

Figure S2. Distribution frequencies of *APOEε4* +/- genotype and *TTR* rare variants. (A) *APOEε4* allele frequencies in AD and control groups. (B) Percentage of *TTR* rare variant carriers in AD patients with *APOEε4* or without *APOEε4*. (C) Percentage of *TTR* rare variant carriers in normal controls with *APOEε4* or without *APOEε4*. (D) Percentage of *TTR* rare variant carriers in all the samples with *APOEε4* or without *APOEε4*.

Online Supplementary Materials

Table S1. Common variants in the *TTR* gene (spanning the -10 kb ~ +10 kb region of the *TTR* gene) from the IGAP data set.

Chr	Location	SNP	Allele	Beta	SE	*P*-value
18	29162219	rs1791185	T>C	0.0307	0.0385	0.4247
18	29162660	rs79715330	G>A	0.0364	0.08	0.649
18	29163607	rs140467514	T>C	0.0528	0.1192	0.6576
18	29164147	rs12962216	T>C	0.0183	0.0263	0.4875
18	29165531	rs113035076	A>T	0.0667	0.0667	0.3175
18	29165918	rs17740847	T>C	0.0201	0.0253	0.428
18	29167064	rs875120	A>C	0.0307	0.0387	0.4278
18	29167091	rs875119	C>T	0.0113	0.0396	0.7755
18	29167669	rs76431866	A>G	-0.0022	0.0611	0.9716
18	29167905	rs1667244	G>A	0.0215	0.0162	0.1862
18	29168467	rs72922938	T>C	-0.0916	0.0994	0.3567
18	29168735	rs111395060	G>A	0.0314	0.0387	0.4164
18	29169825	rs3764479	G>A	0.0194	0.0166	0.241
18	29169871	18:29169871	C>T	-0.0351	0.1109	0.7516
18	29169933	rs13381522	T>C	0.0202	0.041	0.6225
18	29170483	rs3764478	T>G	0.0213	0.0255	0.405
18	29170698	rs72922940	G>A	-0.0087	0.0246	0.7233
18	29170709	rs3764477	A>G	0.0281	0.0388	0.4692
18	29170730	rs58616646	T>C	0.0266	0.0388	0.4932
18	29172476	rs723744	T>G	0.0195	0.0165	0.2376
18	29172865	rs1800458	A>G	0.008	0.0292	0.7835
18	29173680	rs1080093	G>C	0.0132	0.0157	0.3988
18	29173784	rs72922947	A>G	0.0173	0.0769	0.8221
18	29173795	rs1080094	G>A	0.0136	0.0157	0.3867
18	29176460	rs3764476	A>C	0.0107	0.0161	0.5038
18	29176873	rs7235277	C>G	0.0092	0.0162	0.5688
18	29176971	rs3794884	G>T	0.0206	0.0167	0.2172
18	29178513	rs36204272	C>G	0.0241	0.0389	0.5356
18	29179040	rs1791228	T>C	0.0133	0.0155	0.3922
18	29179228	rs75032823	A>G	0.0261	0.0267	0.3279
18	29180481	rs113289164	A>G	0.0232	0.0387	0.5489
18	29182352	rs1791229	G>T	0.0141	0.0158	0.3699
18	29183587	rs4799583	C>A	0.0126	0.0157	0.4241
18	29183812	rs1473342	C>T	0.0104	0.0161	0.5184
18	29184503	rs140136831	C>T	-0.0298	0.1145	0.7946
18	29184618	rs1900880	T>A	0.0235	0.0388	0.5456
18	29185268	rs1791201	A>G	0.0135	0.0158	0.392
18	29186128	rs1667254	T>C	0.0211	0.0165	0.202
18	29186781	rs17740990	G>C	0.0235	0.0388	0.5448

18	29187166	rs1611949	G>C	0.0151	0.0157	0.3371
18	29187227	rs112749152	C>T	0.0236	0.0388	0.5437
18	29187279	rs1667255	C>A	0.0158	0.0157	0.3155
18	29187573	rs1791200	T>C	0.0227	0.0166	0.1713
18	29187741	rs1791199	C>A	0.0154	0.0158	0.3305
18	29187889	rs1791198	C>G	0.0145	0.0159	0.3616
18	29188781	rs1667257	G>A	0.013	0.0162	0.4197
18	29188955	rs72922962	C>T	0.0249	0.0783	0.7507

Note - IGAP data source: http://web.pasteur-lille.fr/en/recherche/u744/igap/igap_download.php

Table S2. Missense and splicing variations of the *TTR* gene in 529 AD patients and 486 controls.

Variants	ID in dbSNP	Function	case n=529	control n=486 [a]	*P*-value [b]
c.148G>A	rs28933979	missense	1/1058	0/972	1.000
c.200+4A>G	rs751512499	splicing	2/1058	0/972	0.501
c.332C>T	-	missense	1/1058	0/972	1.000
c.371G>A	rs121918095	missense	3/1058	4/972	0.716

[a] The control cohorts including 334 matched normal controls and 152 in-house controls without neurologic disorders.

[b] *P*-value, Fisher's exact test.